Journal of Applied Phycology
https://doi.org/10.1007/s10811-025-03673-z

RESEARCH

Biological complexity in rapid biostimulant screening across multiple seasons

Iida-Maria Rantanen[1] · Janne Isojärvi[2] · Erik Chovancek[1] · Martina Jokel[1] · Sema Sirin[1] · Yagut Allahverdiyeva[1]

Received: 12 May 2025 / Revised: 8 August 2025 / Accepted: 10 September 2025

Abstract

Microalgae are a diverse group of photosynthetic microorganisms and offer a sustainable source of bioactive compounds for next generation plant biostimulants. In this study the biostimulant effect of 15 aqueous microalgal extracts, obtained by maceration and centrifugation, were screened using *Arabidopsis thaliana* root growth assay. Due to significant variations in root growth in treated and untreated (control) seedlings, even under controlled conditions, an integrative statistical strategy, incorporating dimensional reduction approaches, was employed to address this complexity. This analysis revealed significant seasonal variations in root growth, likely sensed by endogenous plants mechanisms, despite controlled growth conditions. By employing statistical methods and accounting for seasonal effects, *Porphyridium purpureum* and *Chlorococcum* sp. were identified as potent strains, consistently stimulating root growth by 15 - 45%. Based on available literature, no previous studies have explicitly addressed this issue in biostimulant assays. The observed circannual rhythm suggests that results from *in vitro* assays may vary significantly depending on the season in which they are conducted, and that future studies should carefully consider this factor. These findings provide valuable insights for optimizing rapid root/germination screening assays and guiding extract selection for evaluating biostimulant potential.

Keywords Microalgae · Biostimulant · Algal extracts · In-vitro assays · Seasonal variation

Introduction

Increasing demand for food, feed, energy, and chemicals as a result of population growth and development necessitates the search for alternative resources. Consequently, agricultural practices are rapidly transitioning towards more sustainable methods, with bio-based products playing a key role in promoting crop growth, soil health, and natural pest control (Donate & Frederico 2019; Velasco-Muñoz et al. 2021).

Plant biostimulants have emerged as an effective innovation to enhance crop resilience against various stressors, stimulate growth and productivity, and enhance nutrient use efficiency (EU, 2019). These biostimulants may include beneficial microorganisms, and a range of natural bioactive compounds, as well as derivatives from both natural and synthetic sources. Among them, microalgae have gained attention as one of the most promising sources of biostimulants (Chiaiese et al. 2018; Brito-Lopez et al. 2025).

Since the 1960 s research has demonstrated that algae can enhance plant nutrition by supplying essential micronutrients and bioactive metabolites that support growth and stress tolerance (Booth 1969; Dmytryk & Chojnacka 2018). In addition, algae-based treatments improve soil structure and fertility by enriching it with macro- and micronutrients, as well as various bioactive compounds (Sharma et al., 2014; Alvarez et al. 2021). Particular attention has been given to algal derivatives such as phytohormones, hormone-like compounds, poly- and oligosaccharides, and phenolic compounds (Mazhar et al. 2013; Gheda and Ahmed, 2015; Behera et al. 2021; Parmar et al. 2023).

The diversity of derivatives from photosynthetic microorganisms is vast, reflecting their extensive natural biodiversity (Chiaiese et al. 2018; Ikram et al. 2022). Although

✉ Sema Sirin
sesiri@utu.fi

✉ Yagut Allahverdiyeva
allahve@utu.fi

[1] Molecular Plant Biology, Department of Life Technologies, University of Turku, 20014 Turku, Finland

[2] Turku PET Centre, Turku University Hospital and University of Turku, 20521 Turku, Finland

Published online: 10 November 2025

the term microalgae is commonly used to broadly describe photosynthetic microorganisms, including prokaryotic cyanobacteria and various eukaryotic taxa such as green algae, euglenoids, and diatoms, it is important to recognize that cyanobacteria and microalgae are phylogenetically distinct, belonging to separate bacterial and eukaryotic lineages, respectively (Occhipinti et al. 2023). For simplicity, both microalgae and cyanobacteria are referred as *microalgae* in this study, unless specified otherwise. Due to their diversity and metabolic flexibility, microalgae are a potent source of high-value metabolites, including proteins, amino acids, enzymes, pigments, polyunsaturated fatty acids, polysaccharides, vitamins, antioxidants, and phytohormones (Parmar et al. 2023). Consequently, microalgae are recognized for their potential to act as growth promoters and to mitigate both biotic and abiotic stress in plants (Ferreira et al. 2023).

In vitro assays have been developed to facilitate and accelerate the rapid screening of algal extracts. These methods typically involve germination or root elongation assays performed under sterile conditions (e.g., in Petri dishes) in a controlled environment, where growth parameters can be precisely modulated and monitored. Extracts are added to either solid or liquid medium at varying concentrations to evaluate dose-response effects. These experimental conditions enable rapid screening using plant models, eliminating the influence of soil and other environmental parameters, including microbial competition (Povero et al. 2016). However, while seasonality is widely acknowledged as a significant factor e.g., in seed germination and crop experiments, its influence on *in vitro* bioassays is often overlooked. Specifically, when *in vitro* assays are performed over extended periods spanning different seasons, the potential impacts of seasonality remain largely unconsidered, due to the assumption that controlled environmental conditions fully mitigate such effects.

Numerous studies have documented the ability of many different organisms to sense seasonal changes, even under constant and highly controlled conditions. Such circannual cycles persist without external time cues across a diverse range of taxa (Helm et al. 2013). In plants, several examples have been reported, including the germination capacity of seeds stored in a dried state, the water-binding capacity of seeds, metabolic and growth changes in duckweed under continuous conditions, and the production of winter shoots in *Spirodela* (Spruyt et al. 1987; Franceschi et al. 2019; Ziegler 2024). Significant seasonal variations occur in oat growth rates (Abidin, 1956). Current research on *Arabidopsis thaliana* focuses primarily on circadian rhythms (Cervela-Cardona et al. 2021) as well as the seasonal changes regulating flowering and their underlying endogenous mechanisms (Freytes et al. 2021). Despite these findings, in plants there are relatively few studies focused on rhythms that are sustained under constant environmental conditions, indicating their endogenous nature.

The primary aim of this study was to evaluate the biostimulant potential of 15 microalgae strains by conducting *A. thaliana* root growth elongation assays using extracts obtained by maceration (Chovancek et al. 2023). During the course of data analysis, variation in *A. thaliana* root growth over time across different seasons was observed. As a result, the scope of the study was expanded to include an investigation of the temporal patterns in root growth, using appropriate statistical modeling approaches, in order to account for temporal factors potentially confounding the primary analysis.

Materials and methods

Growth of microalgal strains and biomass preparation

The 15 microalgal species (Table 1) were acquired from the Norwegian Culture Collection of Algae (NORCCA) and the Microbial Domain Biological Resource Centre (HAMBI) and maintained according to the culture collection instructions. Pre-cultures (50 mL) were inoculated in BG11 medium (pH = 7.5) (Stanier et al., 1971), with seawater and vitamins added when required according to strain specific guidelines. Cultures were maintained at room temperature under continuous light (50 µmol photons m^{-2} s^{-1} photosynthetic photon flux density; PPFD). After four weeks, 2 L fresh BG11 medium was inoculated with pre-cultures to a final concentration of 0.1 ± 0.05 g L^{-1} (n = 3), determined by measuring the dry weight of biomass. The experimental cultures were grown at 21 °C under 200 µmol photons m^{-2} s^{-1}, with a 14/10 h light-dark cycle and continuous bubbling with 1.5% CO_2. Cultures were harvested during the early stationary phase (supplementary information S1) after 22-29 days depending on the strain (supplementary information S2) by centrifugation (6000 ×*g*, 15 min, 18 °C). The resulting biomass was freeze-dried (74 Pa, −53°C) and stored at −20°C. The above procedure was performed twice independently, resulting in two biological replicates, replicate 1 (R1) and replicate 2 (R2). Both cultures were cultivated during the summer season. However, they were initiated in different months and originated from separate pre-cultures and thus, do not originate from the same biomass. Biomass R1 was stored at −20 °C for 14–16 months prior to analysis, while R2 was stored for 15–19 months.

Microalgal extract preparation

The extract was prepared utilizing a short period of mild thermo-maceration (Chovanček et al. 2024). A stock extract

Table 1. List of microalgal species employed in this research. Strains H1-H8 were obtained from HAMBI, while N1-N20 are from NORCCA

Genus/Species	Order	#	Phylum	Culture Collection Catalog #
Nostoc sp.	Nostocales	H1	Cyanobacteria	UHCC0252
Nostoc sp.		H2		UHCC0360
Nostoc sp.		H6		UHCC0268
Anabaena variabilis		H7		PCC7937
Nodularia sp.		H8		HAN37/1
Selenastrum sp.	Sphaeropleales	N1	Green algae, Chlorophyta	K-1877
Scenedesmus sp.		N5		NIVA-CHL99
Tetradesmus obliquus		N6		NIVA-CHL107
Monoraphidium contortum		N7		NIVA-CHL100
Haematococcus lacustris	Chlamydomonadales	N9	Green algae, Chlorophyta	K-0084
Dunaliella tertiolecta		N13		NIVA-CHL26
Dunaliella salina		N20		K-1830
Chlorococcum sp.	Chlorococcales	N3	Green algae, Chlorophyta	NIVA-CHL131
Apatococcus lobatus	Chlorellales	N8	Green algae, Chlorophyta	NIVA-CHL144/1
Porphyridium purpureum	Porphyridiales	N12	Red algae, Rhodophyta	NIVA-1/92

(15 g DW L^{-1}) was prepared by weighing 150 DW mg microalgal biomass, homogenizing it shortly with mortar and pestle, and diluting it to 10 mL deionized water. The mixture of microalgal biomass and water was shaken (120 rpm) for 3 h at +30 °C and then centrifuged (6000 ×*g*, 10 min, 4 °C). The supernatant was collected and sterile-filtered (0.45 µm) before diluting into working concentrations (0.1 g L^{-1}, 0.3 g L^{-1}, and 0.5 g L^{-1}) and applying to the agar-based plates.

A. thaliana root tip elongation assay

To assess the biostimulant potential of the microalgal extracts, a root assay (Chovanček et al. 2023) was used. In this method, the seeds of *Arabidopsis thaliana* (Col-0) were sterilized with chlorine gas and planted on agar plates with ½ MS media (Murashige & Skoog), 1% sucrose, and 0.8% agar. The plates were placed first to +4°C for 48 h for stratification and then vertically in the growth chamber (120 µmol photons m^{-2} s^{-1} PPFD, 16/8h, 23 °C, ca 40% humidity) for 5 days. After this, 15 seedlings with root lengths between 16-20 mm were transferred to agar plates (½ MS plates + 0.8% agar) with algae extract in different concentrations (0.1 g L^{-1}, 0.3 g L^{-1}, and 0.5 g L^{-1}) as treatment groups. For the control group, seedlings were transferred to similar plates without any extract. The seedlings were aligned in one line with 0.7 mm spaces in between, and the position of the seedlings' root tip was marked with a permanent marker. The plates were placed back into the growth chamber. The root tip elongation was measured on the 7th day from RGB images analyzed with ImageJ. The lateral roots were counted on the 5th day from RGB images. For one biological replicate of the microalgae biomass, one *A. thaliana* root assay with 15 plants was performed. Normalized root stimulation (RS) was calculated by comparing the average primary root tip elongation of the treated plants (RE) to the average primary root tip elongation of the control plants (AEC) as follows:

$$RS = (RE/AEC) * 100$$

Assays were performed from July to February, excluding December, due to assessment of multiple strains. A minimum of two assays were conducted per month in a staggered manner, with multiple assays running at different growth stages. The detailed monthly distribution of assays performed is provided in supplementary information 3 (S3).

Statistical analysis

All the statistical analyses were done with RStudio, R version 4.3.1 (2023-06-16) -- "Beagle Scouts". To assess the statistical significance of normalized root stimulation ($p < 0.05$), normality of the data was first analyzed with the Shapiro-Wilk test. As the data did not meet the assumptions of normality, non-parametric analysis was conducted using Kruskal-Wallis test followed by Dunn's post hoc test with Bonferroni correction. This analysis was applied to the normalized root stimulation calculated from a dataset containing root tip elongation results for all plants across different treatment groups (0.1 g L^{-1}, 0.3 g L^{-1} and 0.5 g L^{-1}) in both replicates (R1 and R2), as well as to a subset where the two replicates were combined for the control group and the 0.5 g L^{-1} treatment group.

Dimensionality reduction-based techniques were applied to represent the data in a lower dimensional space with preserving the key information. Among linear

methods, multidimensional scaling (MDS) and principal component approaches were applied. MDS was used to visualize the relationships among samples in a two-dimensional space. For MDS, a dataset containing primary root tip elongation measurements from all plants in the different treatment groups (0.1 g L^{-1}, 0.3 g L^{-1}, and 0.5 g L^{-1}), as well as the control group, with data combined from both replicates (R1 and R2), was used. First, to determine the optimal number of clusters, the Elbow and Silhouette methods were applied using the factoextra package (version 1.0.7). Based on these results, K-means clustering was performed using the base R stats package (version 4.3.1) to assess the distribution of the two biological replicates of microalgae. A distance matrix was calculated using Euclidean distance, and classical MDS (metric MDS) was performed using the cluster (version 2.1.6) and factorextra (version 1.0.7) packages. Ordination plots were generated using the ggpubr package (version 0.6.0) to highlight treatment group clustering. Different clusters were color-coded to facilitate visual interpretation.

Principal Component Analysis (PCA) was conducted to extract the most prominent patterns and simplify the dataset, focusing on key factors affecting root tip elongation while minimizing the impact of seasonality and reducing associated noise. The analysis was conducted using the same primary root tip elongation dataset described above, with PCA performed on both the combined dataset (including data from replicates R1 and R2) and separately for each replicate. The analyses were carried out in R using the FactoMineR (version 2.11) and factoextra packages (version 1.0.7). An eigenvalue analysis was used to select the principal components for further analysis based on the proportion of variance explained. Eigenvalues were calculated by squaring the standard deviations from the PCA results. The percentage of variance explained by each component was then computed, and a bar plot was generated to visualize the distribution of variance. Samples were color-coded by treatment group in the PCA plots, with confidence ellipses added to represent the variability within each treatment group to facilitate visual interpretation of clustering and group separation.

To detect seasonal variation in primary root tip elongation and the number of lateral roots, a time series analysis was performed. The analysis of primary root tip elongation was conducted using the same primary root tip elongation dataset described above, while a separate dataset, containing the number of lateral roots across the same treatment groups, was used for the analysis of lateral root development. Both datasets were analyzed using the ggplot2 package (version 3.5.1) to create boxplots over time (month), with each treatment group color-coded, and LOESS smoothing curves fitted to each treatment to highlight temporal trends. Rectangular background shading and annotations were used to visually distinguish the two biological replicates. To highlight temporal dependencies, the autocorrelation function (ACF) was calculated.

Results

Phenotypic variability in *A. thaliana* root responses between two microalgal biomass replicates

The root elongation response to varying doses of aqueous extracts from 15 microalgal strains were examined using two biological replicates (R1 and R2) per strain. A detailed analysis of primary root elongation showed significantly more pronounced effects (both inhibitory and stimulatory) in R2 than R1 (Fig 1). Most strains in R1 showed some degree of primary root stimulation at the applied concentrations of 0.1 and 0.3 g L^{-1}. Particularly, H2 - *Nostoc* sp., N3 - *Chlorococcum* sp., N5 - *Scenedesmus* sp., N7 - *M. contortum*, and N20 - *D. salina* showed stimulation above 10% in these concentrations. Although three strains (N3 - *Chlorococcum* sp., N8 - *A. lobatus*, and N12 - *P. purpureum*) showed up to 30% stimulation at 0.5 g L^{-1}, only the effect observed for N12 - *P. purpureum* was statistically significant. In contrast, R2 showed statistically significant effects, with strains N3 – *Chlorococcum* sp. and N7 - *M. contortum,* showing distinct stimulation across all tested concentrations (+22-52%). The strongest stimulation was observed with strain N3 – *Chlorococcum* sp. at 0.5 g L^{-1} and N7- *M. contortum* at 0.1 g L^{-1}, both above 50% root elongation. In contrast, cyanobacterial strains (*Nostoc* sp. -strains, *A. variabilis* and *Nodularia* sp.) mostly inhibited root growth, with the inhibitory effects being more statistically pronounced in R2. In R1, the maximum inhibition was 40% with H8 - *Nodularia* sp., while in R2, the maximum inhibition was 61%, observed with H2 - *Nostoc* sp.

Among the studied strains, three are standing out with repeatedly stimulating effects. N3 – *Chlorococcum* sp. consistently stimulated the root growth by +20-50% across both replicates and all concentrations. N7 - *M. contortum* significantly stimulated root elongation (+50%) in R2, but only moderately in R1 (+10%). N12 - *P. purpureum* had a clear stimulating effect on the root growth in R1 (+20%) but only moderate stimulation in the R2 (+5%) at highest concentration. Furthermore, a dose-dependent root stimulation or inhibition was observed in response to increasing concentration of aqueous microalgal extracts. This pattern was particularly noticeable in strain N5 – *Scenedesmus* sp., where the highest stimulation in R1 occurred at 0.1 g L^{-1} (+15%), while in R2 it peaked at 0.3 g L^{-1} (+15 %). These results suggest that the optimal concentration for this extract likely falls between those concentrations.

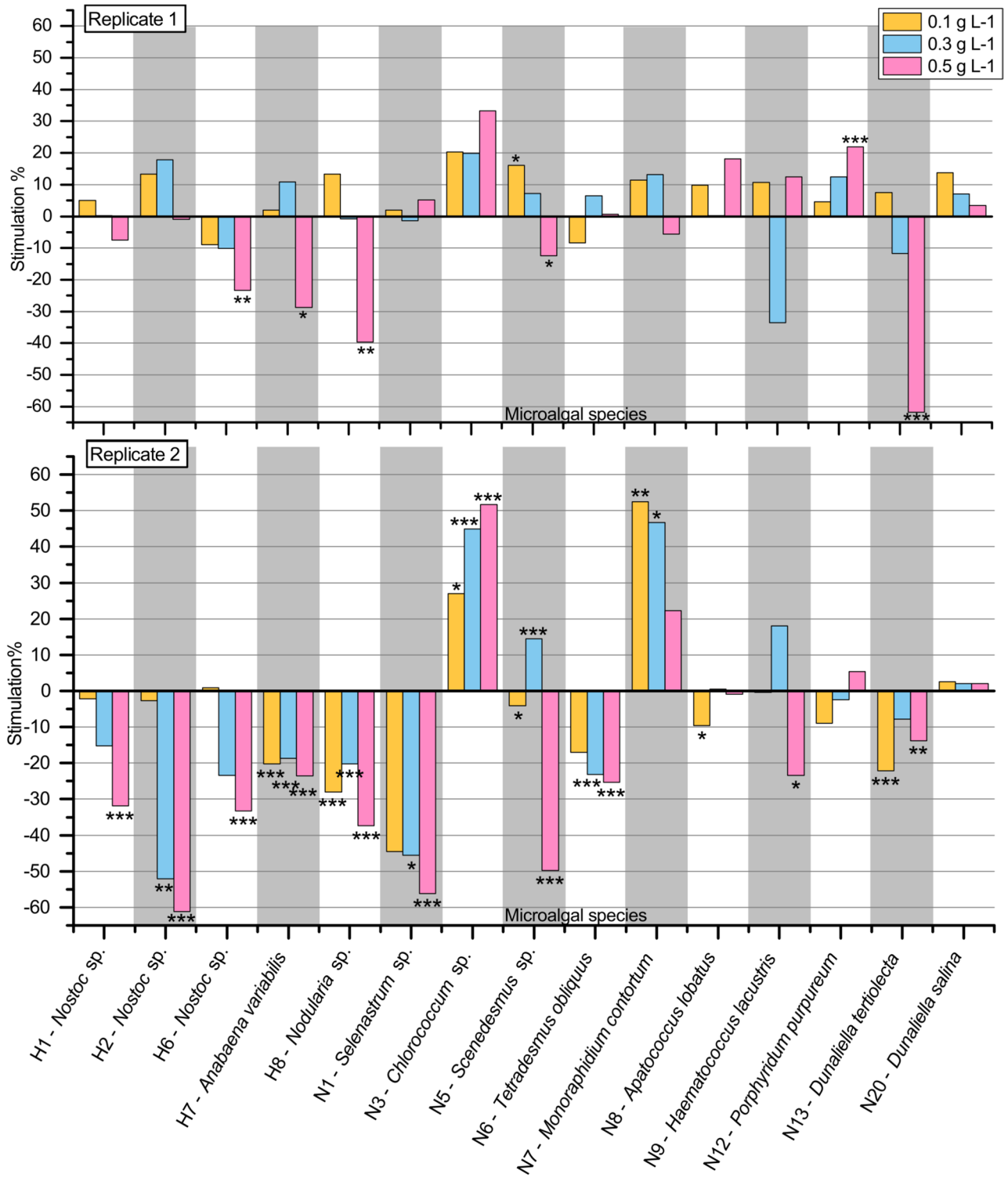

Fig 1. Effect of various microalgal extracts on *A. thaliana* roots. *A. thaliana* root growth response was tested using 15 aqueous microalgal extracts, prepared from two independent cultivations and applied at three concentrations (0.1 g L^{-1}, 0.3 g L^{-1} and 0.5 g L^{-1}). The normalization was carried out by comparing the root tip elongation of the plants treated with microalgal extract to that of control plants and the results were expressed as a percentage. The asterisks indicate the statistical significance after Kruskal-Wallis test and Dunn's test with Bonferroni adjustment (* = p-value < 0.05; ** = p-value < 0.01; *** = p-value < 0.001).

Synchronised performance of *A. thaliana* root elongation within microalgal extract replicates

The root assay results showed consistent trends in stimulatory or inhibitory effects across two independently grown replicates (R1 and R2) of 15 microalgal strains. This consistency prompted a further investigation, whether the observed trends were statistically significant. To better understand the patterns in the data, a multidimensional scaling (MDS) (Fig 2) was used, which enabled reconstructing the data topology and defining similarity matrices within the root assays, thereby clarifying the underlying trends.

Initially, the optimum number of the clusters was determined based on Elbow and Silhouette methods and root tip elongation levels are optimally represented by two clusters (supplementary data S4). Once the optimal number of clusters has been determined, K-means clustering method and MDS were applied to the combined dataset. This dataset included root tip elongation data from the plants treated with different microalgal extracts and their respective controls. After dimensional reduction, the data from two biological replicates showed distinct clustering patterns. Approximately 93% of the R1 samples clustered within Group 2 (yellow), whereas 77% of the R2 samples clustered in Group 1 (blue). This separation suggests a clear difference between R1 and R2, although a slight overlap between clusters likely reflects similar root tip elongation responses in the control plants.

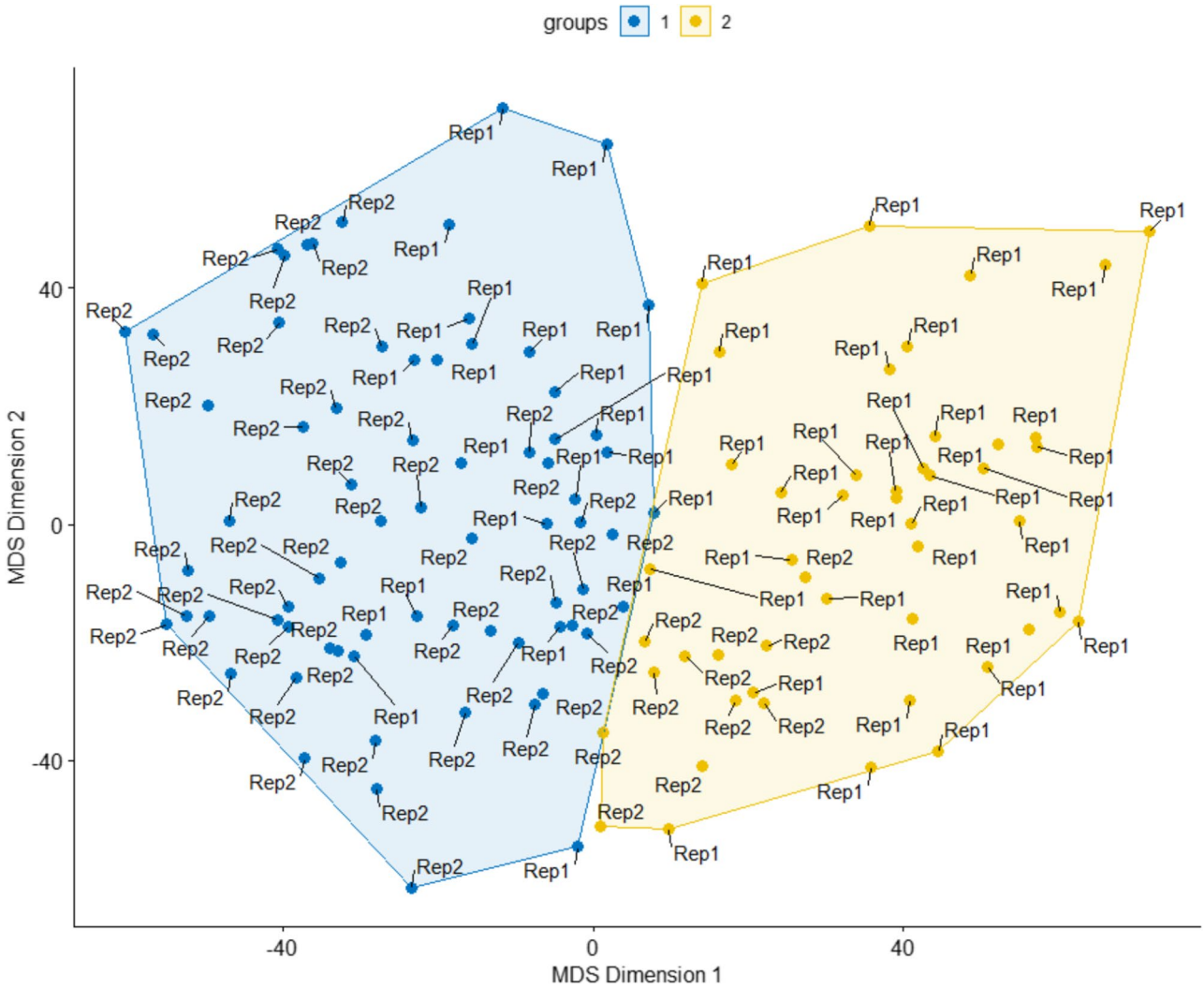

Fig 2. The multidimensional scaling (MDS) plot of *A. thaliana* root tip elongation data. MDS was performed with K-means clustering on a dataset containing plants treated with different microalgal extracts and their respective controls. The optimal number of clusters was determined to be two, based on the Elbow and Silhouette methods. Most samples from R1 fall into the group 2 (yellow) while most samples from R2 fall into the group 1 (blue).

Seasonal variation patterns of *A. thaliana* root length under controlled conditions

After confirming the clear difference between R1 and R2, to examine if the timing of the root assays (screening period) is correlated with the root growth responses, a time series analysis was applied to the dataset from all plants including controls. Figure 3a shows monthly root tip elongation presented as boxplots grouped by applied extract concentrations to visualize potential seasonal dynamics. The red trend lines illustrate overall trends over time. Analysis of root tip elongation boxplots across all treatments and controls, along with the four trendlines representing each treatment and control group, demonstrates that the root tip elongation was more stable during summer months (July, August), with the trendlines providing the clearest illustration of this pattern. This stability is further reflected in the relatively uniform root tip lengths, with only slight variation across measurements. A minor increase in root tip elongation was observed towards September (Fig 3a). The primary root elongation decreased during autumn and winter (October, November, January). As the season shifted to late winter (February), root tip elongation increased once more (Fig 3a). Fig 3b clearly demonstrates this trend, with a seasonal pattern becoming apparent: the first two images (July and August) show a more stable growth period, while the latter two images (November and February) correspond to periods of either significantly reduced or enhanced root tip elongation in control plants. A seasonal pattern, similar to that observed in primary root elongation, was also evident in lateral root development (Fig 3c), supporting the occurrence of circannual growth in *A. thaliana.*

Minor differences in controlled growth conditions (light, temperature, and humidity) of *A. thaliana* seeds were observed between R1 (summer) and R2 (autumn/winter), with R2 showing slightly higher temperatures (< 0.75 °C) and slightly lower humidity (< 5%) (supplementary information S5). However, these variations remained within a narrow, stable range and are unlikely to have influenced *A. thaliana* root growth. The sealed MS media plates provided a stable microclimate, supporting the conclusion that short-term seasonal spikes in root tip elongation observed in September and February were not correlating by external environmental variations.

The root tip elongation assays with microalgal biomass extract from R1 were performed between July and September, whereas the assays with biomass extract from R2 took place during October to February period. This introduces potential seasonal bias into the analysis of biological replicates. However, all microalgal biomass replicates were grown under controlled summer conditions, and harvested at matching growth stages to maximize comparability (supplementary information S1). Therefore, the observed seasonal variation in root elongation is more likely attribute to the *A. thaliana* plants themselves rather than differences in the microalgal biomass.

To assess potential temporal dependencies in the root development data, autocorrelation function (ACF) was calculated for primary root tip elongation and lateral root count (Fig 4). The ACF plot for primary root elongation (Fig 4a) shows statistically significant autocorrelations ($p < 0.05$) at lags 1 to 5, with values exceeding the 95% confidence interval (dotted horizontal line). The gradual decay in autocorrelation indicates a time-dependent structure in the data, and alternating positive and negative peaks suggests the presence of seasonality. A similar trend was observed for lateral root amount (Fig. 4b), with autocorrelation gradually decaying up to lag 7, although the absence of negative values points to a weaker seasonal effect in lateral root formation compared to primary root elongation.

Principal component analysis separates *A. thaliana* root elongation data into distinct clusters

To simplify the dataset, reduce noise and identify underlying growth patterns, given the seasonal variability impacting root tip elongation, a Principal Component Analysis (PCA) was performed on both the combined R1 and R2 datasets and the individual replicates, R1 and R2 separately.

When grouping the combined data of R1 and R2 based on extract concentration (Fig 5a), the different concentrations of microalgal extract treatments formed distinct clusters at a 95% confidence interval. The analysis was focused on the first two principal components (PCs) based on their eigenvalues (supplementary data S6). In this context, PC1 and PC2 combined explained most of the variation in the dataset (28 %). While the 0.1 g L^{-1} and 0.3 g L^{-1} treatments showed overlapping indicating no apparent distinction between the groups based on the principal components. They also partially overlap with the control group, however the cluster associated with the control group remains slightly shifted along PC2 and somewhat distinct suggesting subtle differences.

Separate PCAs were performed for each replicate, R1 and R2 (Fig 5b and 5c), revealing both consistent patterns and some discrepancies between the replicates. Following eigenvalue analysis (supplementary data S6), attention was directed to the first two PCs which explained 26% of the variance in R1, and 30% in R2. A consistent form across both R1 and R2 was noticed as distinct clustering of the 0.5 g L^{-1} treatment, indicating the same subset pattern also in the level of replicates. Another similarity between the PCAs from the separate replicates is the overlapping clusters formed by 0.1 g L^{-1} and 0.3 g L^{-1} treatments, suggesting a similar response pattern at these lower concentrations. However, the replicates differed from each other in the clustering of the control

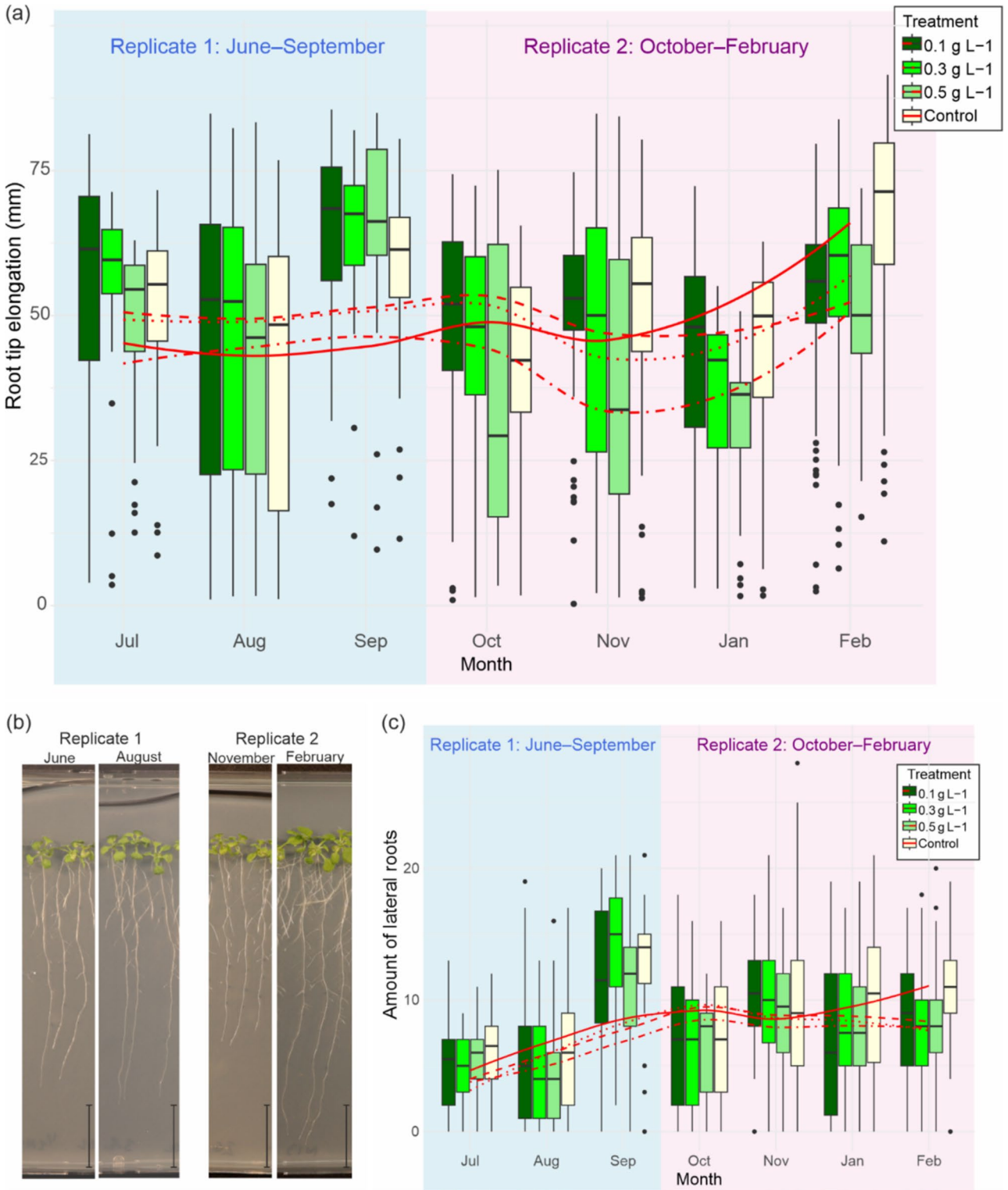

Fig 3. Seasonal dynamics of primary root elongation and amount of lateral roots of *A. thaliana* in response to extracts. (a) Boxplot illustrating seasonal variation in primary root tip elongation (mm) across treatments. Treatment concentrations are color-coded (green shades), with the control in light yellow. LOESS curves (red) show temporal trends. The vertical shaded areas indicate the two replicates (R1 with blue and R2 with purple). **(b)** RGB images of *A. thaliana* control seedlings at day 7. Images of seedlings taken in November and February reflect differences observed over time compared to those taken in July and August. Scale bar = 20 mm. **(c)** Boxplot showing seasonal variation in amount of lateral roots across treatments. The interpretation of the figure follows the same approach as the time series analysis from primary root tip elongation.

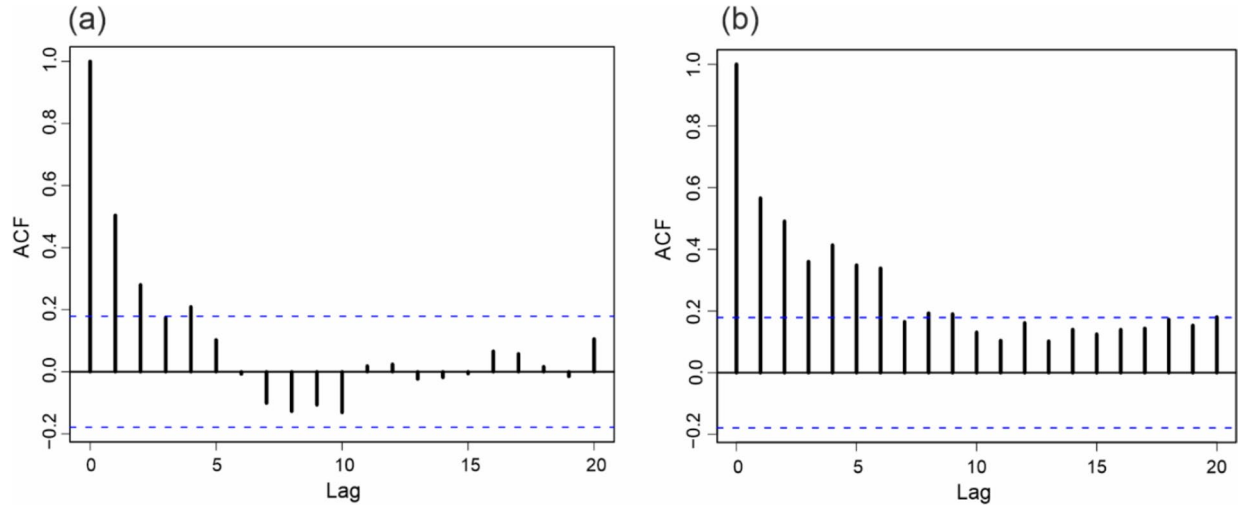

Fig 4. Autocorrelation function (ACF) plot of (a) primary root tip elongation over time and (b) amount of lateral roots over time. The x-axis represents the time lags (number of time steps between observations), and the y-axis shows the autocorrelation values. Significant correlations are indicated by values outside the 95% confidence interval (dashed horizontal line). Both plots reveal a gradual decay in autocorrelation, suggesting a dependence over time in the data. Potential seasonal pattern indicated by alternating positive and negative values is stronger in the plot containing data from primary roots

group. In R2, the control group formed a more distinct and clearly separated cluster, whereas in R1, it overlaps partially with the 0.1 g L^{-1} and 0.3 g L^{-1} treatment clusters. The key difference between R1 and R2 was the spatial separation of all the clusters. R2 showed greater inter-clustering distances, suggesting a potential treatment effect or a more pronounced distinction of the response profiles. This distinction in R2 might be caused by variations in growth seasons, which could have amplified treatment-related differences and led to more distinct data patterns.

This analysis showed that the 0.1 g L^{-1} and 0.3 g L^{-1} treatments cannot be analyzed in a statistically significant manner as they could not be reliably distinguished from the control due to overlapping values. However, the 0.5 g L^{-1} treatment consistently formed a distinct cluster, both in the combined and separate replicate analysis. This clear separation allowed the pooling and further analyzing the biological replicates from 0.5 g L^{-1} treatment even in the presence of seasonal growth variation.

Despite seasonal variation the screening consistently revealed two promising candidates for future biostimulant assessment

As demonstrated by PCA, root tip elongation data from R1 and R2 for the 0.5 g L^{-1} treatment group can be combined despite seasonal variation. The data pooling allows a larger sample size, and thus provides a more robust and generalized view of the effect of treatments. Figure 6 displays the stimulation and/or inhibition observed in the 0.5 g L^{-1} treatment group, calculated as previously described.

All the cyanobacterial strains belonging to the Nostocales order (strains H1 - *Nostoc* sp., H2 - *Nostoc* sp., H6 - *Nostoc* sp., H7 - *A. variabilis* and H8 - *Nodularia* sp.) have a strong plant growth inhibiting effect (from −20% to −40%). Strains in the Sphaeropleales order (N1 - *Selenastrum* sp., N5 - *Scenedesmus* sp., N6 - *T. obliquus* and N7 *M. contortum*) mainly inhibit plant growth (from −12% to −30%), except for one strain, N7 *M. contortum*, which has a slightly stimulating effect (+5%). The strains in Chlamydomonadales order (N9 - *H. lacustris*, N13 - *D. tertiolecta* and N20 - *D. salina*) have similar effects as the strains in the Sphaeropleales order (from +3% to −30%). There is slight stimulation with strains from Chlorellales (N8 - *A. lobatus*, +8%) but the strains that have the strongest plant growth stimulating effect belong to the Porphyridiales (N12 - *P. purpureum* with +15%) and Chlorococcales (N3 - *Chlorococcum* sp. with +45%) orders.

Discussion

Diversity provides options The vast diversity of microalgae presents considerable potential for the development of plant biostimulants that support more sustainable agricultural practices (Ronga et al. 2019). To harness this potential efficiently, rapid screening approaches are essential for evaluating the bioactive properties of diverse strains. In this study, *Chlorococcum* sp. and *P. purpureum* were identified as promising strains for biostimulant formulations, as they consistently showed positive results across both independent experiments (R1 and R2 extracts) and at all tested

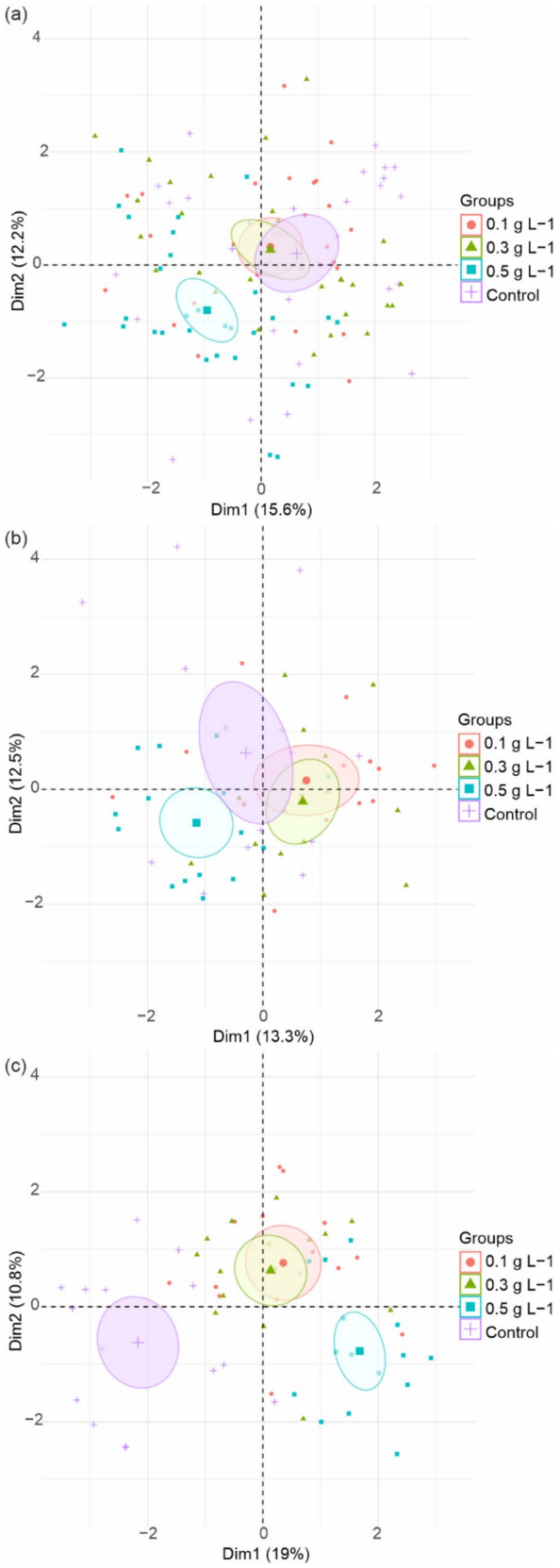

Fig 5. Principal Component Analysis (PCA) of root tip elongation. PCA performed using **(a)** the combined dataset from both replicates, and **(b)** the dataset from R1, and **(c)** R2 separately. Samples are grouped by extract concentration, and ellipses represent 95% confidence intervals for each group. When combining data from both replicates (a) the clusters corresponding to 0.1 g L^{-1} (red dots) and 0.3 g L^{-1} (green triangles) showed partial overlap with each other and with the control group (purple crosses), while the 0.5 g L^{-1} (blue squares) cluster appeared more distinct. A similar clustering pattern was observed in the separate analyses of replicate 1 (b) and replicate 2 (c).

concentrations. While *M. contortum* and *A. lobatus* showed promising trends, statistical validation was hindered by seasonal variability. However, more in-depth analysis may reveal their full biostimulant potential.

Both of the identified top-performing species have previously been reported to display plant biostimulant properties, supporting their potential observed in this study. For example, *C. vacuolatum* improved tomato germination and root length (Kolman et al. 2024) and a *Chlorococcum* sp. extract enhances spinach germination (Rupawalla et al. 2022), likely due to high concentrations of cytokinins and gibberellins, hormones known to promote root cell division and elongation (Ubeda-Tomás et al. 2009; Takahashi et al. 2013). Since *Chlorococcum* sp. (Alhattab et al. 2018) has a relatively resilient cellulose-based cell wall, complete extraction of intracellular compounds is unlikely with the maceration method applied in this study. Unlike harsher methods such as high-pressure homogenization and enzymatic hydrolysis, mild thermo-maceration is a gentle method that avoids mechanical or chemical disruption of microalgae cells. Instead, it primarily yields compounds with high water solubility, such as amino acids, mono- and polysaccharides, phenols, flavonoids, antioxidants and phytohormones (Chovancek et al. 2024; Jamshidi-Kia et al. 2024), all of which have potential biostimulant activity (Ferreira et al. 2023).

Following the positive effects observed for *Chlorococcum* sp., *P. purpureum* has likewise been associated with plant growth promotion, including enhanced *A. thaliana* root tip elongation and increased lettuce yield (Chovancek et al. 2023). Also, extracts from *Porphyridium* sp. have improved tomato growth by increasing root and shoot dry weight, and shoot length (Mutale-Joan et al. 2020; Rachidi et al. 2020). *Porphyridium purpureum* lacks traditional cell wall structure and is instead encapsulated by a complex and robust polysaccharide capsule, which limits extraction to only easily water-soluble molecules during maceration (Keidan et al. 2009). Among these molecules exopolysaccharides (EPS) are a plausible source of the observed biostimulant effect (Medina-Cabrera et al. 2021). Algal polysaccharides support root growth and enhance photosynthesis (Chanda et al. 2019), and are likely

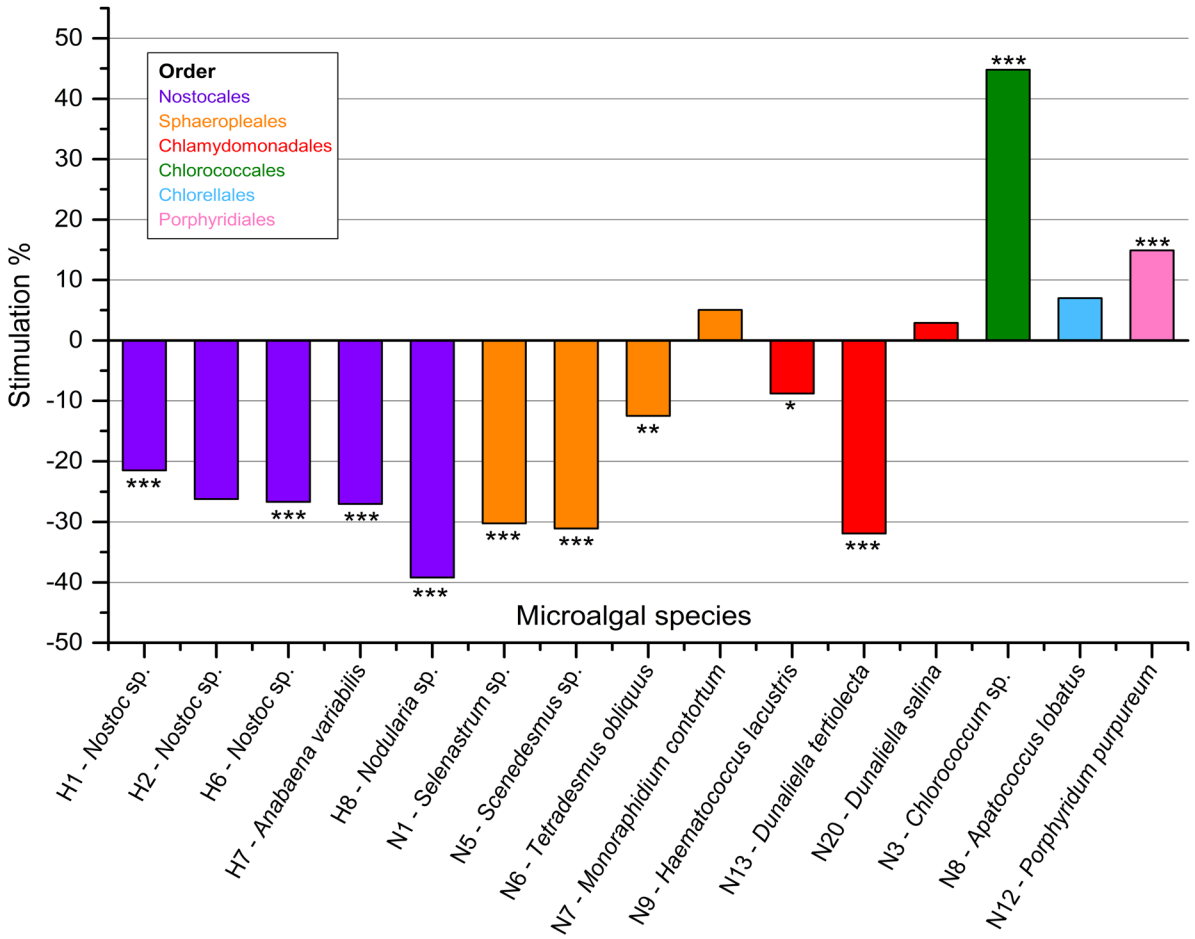

Fig 6. Effects of 0.5 g L^{-1} extract treatment on *A. thaliana* roots based on combined data from both replicates. The normalized effect in root growth is calculated by comparing the root tip elongation of the plants treated with microalgal extract to control plants and reported as percentage. Colours of the bars indicate the order of algae strains. PCA supports the feasibility of data merging, which helps mitigate seasonal effects. The asterisks indicate the statistical significance after Kruskal-Wallis test and Dunn's test with Bonferroni adjustment (* = p-value < 0.05; ** = p-value < 0.01; *** = p-value < 0.001; ns = not significant)

present in macerated extracts due to their solubility in warm water (Caetano et al. 2022). This study also revealed strains that had an inhibiting effect on plant growth. The strongest inhibition was observed with strains belonging to the Nostocales. This finding contrasts with previous research, as Nostocales have been reported to function as potential biostimulants (Silambarasan et al. 2021; Santini et al. 2022).

Accounting for seasonal variability is essential for rapid biostimulant screening in long-term experiments Biological replicates across different seasons often show similar trends but varying values, weakening conclusions and complicating large dataset analysis. This phenomenon was addressed by employing an integrative approach combining multivariate statistical techniques with time series analysis, ultimately revealing an underlying seasonal pattern. This strategy is valuable in long-term studies with multiple independent biological replicates.

Seasonal variation of root growth is linked to environmental factors, nutrient availability, life cycle, and presence of plant hormones (Walter et al. 2009; Jung & McCouch 2013). However, despite maintaining stable growth conditions (light, temperature, and humidity) within a narrow range throughout assays, noticeable differences related to seasonal variations were observed in untreated *A. thaliana* roots. These variations are possibly influenced by an internal circannual clock, independent of external environmental factors. This endogenous mechanism has been well documented in *Arabidopsis*, in other plants species such as *Dendrocalamus strictus*, *Mesembryanthemum nodiflorum*. (Aizoaceae) and some orchids species (Rawal & Thapliyal 2003; Gutterman 2005; Durán-Mendoza et al. 2025); and various organisms (Helm & Lincoln 2017; Lincoln 2019) even in the unicellular protist dinoflagellate *Gonyaulax tamarensis* (Andersen & Keafer 1987). Despite being well-documented, this mechanism can be easily overlooked in

screening approaches conducted in long-term screenings, where statistical tools have shown to be profoundly helpful in deriving relevant data from complex datasets. These results suggest that minimizing seasonal effects requires careful consideration in experimental design, prioritizing experiments during the natural growing season. Additionally, performing all replicates within the same season will mitigate these pronounced seasonal variations, as growing seasons follow a distinct spatiotemporal pattern.

Beyond the observed seasonal pattern, the extended storage time of R2 microalgal biomass, is also an important factor to consider, as it might be potentially contributing to the observed outcomes. Storage of *Chlorella vulgaris* biomass for over a year has been shown to enhance root promotion in mung bean (*Vigna radiata*) due to cell wall degradation and the increased release of bioactive compounds (Stirk et al. 2021). In this study, the freeze-dried biomass R1 was stored for 14 to 16 months, while R2 was stored for a longer period of 15 and 19 months. It is possible that similar degradation processes contributed to the stronger effects seen with R2 extracts.

Data analysis tools facilitate seasonality assessment Exploratory data analysis (EDA) is considered an important preliminary step before formal statistical modeling or hypothesis testing, as it helps uncover patterns, relationships, and potential anomalies within datasets. PCA and MDS, universal tools for EDA, effectively evaluate biological replicates. Replicates coded with the same colors should cluster together along the same dimensions, indicating consistency and reproducibility. Furthermore, time series analysis methods such as ACF plots are important for detecting temporal patterns, including seasonality. The results of this study highlight the usefulness of ACF plots in identifying seasonal effects, suggesting that similar analyses could reveal hidden seasonal trends in other long-term datasets as well. Together, these EDA tools enhance the reliability and interpretation of complex biological experiments.

Conclusion

This study demonstrated that aqueous microalgal extracts from *Chlorococcum* sp. (N3) and *P. purpureum* (N12) at a concentration of 0.5 g L^{-1} significantly enhanced root elongation in *A. thaliana*, while also revealing significant seasonal variations most possibly sensed by plants via endogenous mechanisms even under controlled growth conditions. By acknowledging and addressing this variability, it is possible to improve the robustness and reproducibility of fast screening root/germination assays. Future studies could benefit from applying time series analysis with the autocorrelation function (AFC) when long-term experiments require extended timelines, as this method provides a valuable tool for mitigating seasonal effects and enhancing data reliability.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10811-025-03673-z.

Acknowledgements The authors would like to thank the Norwegian Culture Collection of Algae (NORCCA) at the NIVA (Norway) and the HAMBI Culture Collection at the University of Helsinki (Finland) for providing the algal strains. We also thank Pragya Karmacharya and Joni Vuorinen for their assistance in preparing the microalgal cultivations.

Authors' contributions I.-M.R. performed experiments, curated and analyzed data, and drafted the manuscript. J.I. supervised and validated the bioinformatics analysis; E.C. provided methodological support for screening experiments; M.J. contributed to writing and editing the manuscript; S.S. conceptualized and supervised the research; Y.A. conceived and supervised the research and acquired funding. All authors contributed to the discussion and revision of the manuscript draft. S.S. and Y.A. finalized the paper with input from all authors. All authors read and approved of the published version of the manuscript.

Funding Open Access funding provided by University of Turku (including Turku University Central Hospital). This research was financially supported by the European Union Horizon Europe IA project REALM (grant agreement # 101060991).

Availability of data and material The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Competing interests The authors declare no competing interests.

References

Abidin Z ul (1956) Über die Beeinflussung der Samenkeimung durch den Temperaturwechsel. Z Bot 44:207–220

Alhattab M, Kermanshahi-Pour A, Brooks MS-L (2018) Microalgae disruption techniques for product recovery: influence of cell wall composition. J Appl Phycol 31:61–88

Alvarez AL, Weyers SL, Goemann HM, Peyton BM, Gardner RD (2021) Microalgae, soil and plants: a critical review of microalgae as renewable resources for agriculture. Algal Res 54:102200

Andersen DM, Keafer BA (1987) An endogenous annual clock in the toxic marine dinoflagellate *Gonyaulax tamarensis*. Nature 325:616–617

Behera B, Venkata Supraja K, Paramasivan B (2021) Integrated microalgal biorefinery for the production and application of biostimulants in circular bioeconomy. Bioresour Technol 339:125588

Booth E. (1969) The manufacture and properties of liquid seaweed extracts. In: Margalef R (ed) Proceedings of the 6th International Seaweed Symposium. Subsecretaria de la Marina Mercante, Dirreccion General de Pesca Maritima, Madrid, pp 655–662

Brito-Lopez C, van der Wielen N, Barbosa M, Karlova R (2025) Plant growth–promoting microbes and microalgae-based biostimulants: sustainable strategy for agriculture and abiotic stress resilience. Philos Trans R Soc Lond B 380:20240251

Caetano PA, do C Fernandes AS, Nass PP, Vieira KR, Maróstica MR, Jacob-Lopes E, Zepka L Q (2022) Microalgae-based polysaccharides: Insights on production, applications, analysis, and future challenges. Biocatal Ag Biotech 45:102491–102491

Cervela-Cardona L, Alary B, Mas P (2021) The *Arabidopsis* circadian clock and metabolic energy: a question of time. Front Plant Sci 12:804468

Chanda M, Merghoub N, EL Arroussi H (2019) Microalgae polysaccharides: the new sustainable bioactive products for the development of plant bio-stimulants? World J Microbiol Biotechnol 35:177

Chiaiese P, Corrado G, Colla G, Kyriacou MC, Rouphael Y (2018) Renewable sources of plant biostimulation: Microalgae as a sustainable means to improve crop performance. Front Plant Sci 9:1782

Chovancek E, Poque S, Bayram E, Borhan E, Jokel M, Rantanen I-M, Haznedaroglu BZ, Himanen K, Sirin S, Allahverdiyeva Y (2024) Stepwise processing of *Chlorella sorokiniana* confers plant biostimulant that reduces mineral fertilizer requirements. Bioresour Technol 418:131923

Chovancek E, Salazar J, Sirin S, Allahverdiyeva Y (2023) Microalgae from Nordic collections demonstrate biostimulant effect by enhancing plant growth and photosynthetic performance. Physiol Plant 175:e13911

Dmytryk A, Chojnacka K (2018) Algae as fertilizers, biostimulants, and regulators of plant growth. In: Chojnacka K, Wieczorek P, Schroeder G, Michalak I (eds) Algae Biomass: characteristics and applications. Developments in Applied Phycology, vol 8. Springer, Cham, pp 115–122

Donate PM, Frederico D (2019) Synthesis of new agrochemicals. In: Vaz S (ed) Sustainable Agrochemistry. Springer, Cham, pp 223–273

Durán-Mendoza E, Cornejo-Gallegos M, Martínez-Palacios A, Pedraza-Santos ME, Sánchez-Vargas NM, Valdovinos-Ramírez GA, Hernández-Valencia AS, Chavarrieta-Yáñez JM, Vidal-Lezama E, Mandujano-Sánchez MdC (2025) Circannual clock in *Laelia speciosa* (Orchidaceae) through dormancy vs. germination dynamics of seeds stored under controlled conditions. Plants 14:336

European Union (2019) EUR-Lex - 32019R1009 - EN - EUR-Lex. Eur-Lex.europa.eu. http://data.europa.eu/eli/reg/2019/1009/oj

Ferreira A, Bastos CRV, Marques-dos-Santos C, Acién-Fernández FG, Gouveia L (2023) Algaeculture for agriculture: from past to future. Front Agron 5:1064041

Franceschi CRB, Smidt EC, Vieira LN, Ribas LLF (2019) Storage and in vitro germination of orchids (Orchidaceae) seeds from Atlantic Forest – Brazil. An Acad Bras Cienc 91:e20180439.

Freytes SN, Canelo M, Cerdán PD (2021) Regulation of flowering time: when and where? Curr Opin Plant Biol 63:102049

Gheda SF, Ahmed DA (2015) Improved soil characteristics and wheat germination as influenced by inoculation of *Nostoc kihlmani* and *Anabaena cylindrica*. Rend Lincei 26:121–131

Gutterman Y, Gendler T (2005) Annual rhythm of germination of seeds of *Mesembryanthemum nodiflorum* 32 years after collection. Seed Sci Res 15:249–253

Helm B, Ben-Shlomo R, Sheriff MJ, Hut RA, Foster R, Barnes BM, Dominoni D (2013) Annual rhythms that underlie phenology: biological time-keeping meets environmental change. Proc R Soc Lond B 280:20130016

Helm B, Lincoln GA (2017) Circannual rhythms anticipate the earth's annual periodicity. In: Kumar V (ed) Biological Timekeeping: Clocks, Rhythms and Behaviour. Springer, New Delhi, pp 545–569

Ikram SF, Singh L, Kumar D, Sharma CM (2022) Prospects and constraints in studying the biodiversity of agriculturally important microalgae and cyanobacteria and useful statistical tools. Biodivers Conserv 31:1095–1124

Jamshidi-Kia F, Saeidi K, Lorigooini Z, Samani BH, Barzegar R (2024) Optimization of extraction of liquid extract from microalgae *Chlorella vulgaris* via cavitation-based techniques. J Food Process Eng 47:e14665

Jung JKH, McCouch S (2013) Getting to the roots of it: genetic and hormonal control of root architecture. Front Plant Sci 4:186

Keidan M, Friedlander M, Arad SM (2009) Effect of brefeldin a on cell-wall polysaccharide production in the red microalga *Porphyridium* sp. (Rhodophyta) through its effect on the Golgi apparatus. J Appl Phycol 21:707–717

Kolman MdlA, Miño ML, Sadañoski MA, Zapata PD (2025) Biostimulant potential of two agrochemical tolerant microalgae isolated from subtropical clay soil. J Appl Phycol 37:355–346

Lincoln G (2019) A brief history of circannual time. J Neuroendocrinol 31:e12694

Mazhar S, Cohen JD, Hasnain S (2013) Auxin producing non-heterocystous cyanobacteria and their impact on the growth and endogenous auxin homeostasis of wheat. J Basic Microbiol 53:996–1003

Medina-Cabrera EV, Gansbiller M, Rühmann B, Schmid J, Sieber V (2021) Rheological characterization of *Porphyridium sordidum* and *Porphyridium purpureum* exopolysaccharides. Carbohydr Polym 253:117237–117237

Mutale-Joan C, Redouane B, Najib E, Yassine K, Lyamlouli K, Laila S, Zeroual Y, Hicham EA (2020) Screening of microalgae liquid extracts for their bio stimulant properties on plant growth, nutrient uptake and metabolite profile of *Solanum lycopersicum* L. Sci Rep 10:2820

Occhipinti PS, Russo N, Foti P, Zingale IM, Pino A, Romeo FV, Randazzo CL, Caggia C (2023) Current challenges of microalgae applications: exploiting the potential of non-conventional microalgae species. J Sci Food Agric 104:3823–3833

Parmar P, Kumar R, Neha Y, Srivatsan V (2023) Microalgae as next generation plant growth additives: functions, applications, challenges and circular bioeconomy based solutions. Front Plant Sci 14:1073546

Povero G, Mejia JF, Di Tommaso D, Piaggesi A, Warrior P (2016) A systematic approach to discover and characterize natural plant biostimulants. Front Plant Sci 7:435

Rachidi F, Benhima R, Sbabou L, El Arroussi H (2020) Microalgae polysaccharides bio-stimulating effect on tomato plants: growth and metabolic distribution. Biotechnol Rep 25:e00426

Rawal MMS, Thapliyal RC (2003) Endogenous rhythm in seed germination of *Dendrocalamus strictus*. Seed Sci Technol 31:21–27

Ronga D, Biazzi E, Parati K, Carminati D, Carminati E, Tava A (2019) Microalgal biostimulants and biofertilisers in crop productions. Agronomy 9:192

Rupawalla Z, Shaw L, Ross IL, Schmidt S, Hankamer B, Wolf J (2022) Germination screen for microalgae-generated plant growth biostimulants. Algal Res 66:102784

Santini G, Rodolfi L, Biondi N, Sampietro G, Tredici MR (2022) Effects of cyanobacterial-based biostimulants on plant growth and development: a case study on basil (*Ocimum basilicum* L.). J Appl Phycol 34:2063–2073

Sharma HSS, Fleming C, Selby C, Rao JR, Martin T (2014) Plant biostimulants: a review on the processing of macroalgae and use of extracts for crop management to reduce abiotic and biotic stresses. J Appl Phycol 26:465–490

Silambarasan S, Logeswari P, Sivaramakrishnan R, Kamaraj B, Lan Chi NT, Cornejo P (2021) Cultivation of *Nostoc* sp. LS04 in municipal wastewater for biodiesel production and their deoiled biomass cellular extracts as biostimulants for *Lactuca sativa* growth improvement. Chemosphere 280:130644

Spruyt E, Verbelen Jean-Pierre, De JA (1987) Expression of circaseptan and circannual rhythmicity in the imbibition of dry stored bean seeds. Plant Physiol 84:707–710

Stanier RY, Kunisawa R, Mandel M, Cohen-Bazire G (1971) Purification and properties of unicellular blue-green algae (order Chroococcales) Bacteriol Rev 35:171–205

Stirk WA, Bálint P, Vambe M, Kulkarni MG, van Staden J, Ördög V (2021) Effect of storage on plant biostimulant and bioactive properties of freeze-dried *Chlorella vulgaris* biomass. J Appl Phycol 33:3797–3806

Takahashi N, Kajihara T, Okamura C, Kim Y, Katagiri Y, Okushima Y, Matsunaga S, Hwang I, Umeda M (2013) Cytokinins control endocycle onset by promoting the expression of an APC/C activator in *Arabidopsis* roots. Curr Biol 23:1812–1817

Ubeda-Tomás S, Federici F, Casimiro I, Beemster GTS, Bhalerao R, Swarup R, Doerner P, Haseloff J, Bennett MJ (2009) Gibberellin signaling in the endodermis controls *Arabidopsis* root meristem size. Curr Biol 19:1194–1199

Velasco-Muñoz JF, Mendoza JMF, Aznar-Sánchez JA, Gallego-Schmid A (2021) Circular economy implementation in the agricultural sector: definition, strategies and indicators. Resour Conserv Recycl 170:105618

Walter A, Silk WK, Schurr U (2009) Environmental effects on spatial and temporal patterns of leaf and root growth. Annu Rev Plant Biol 60:279–304

Ziegler P (2024) The developmental cycle of *Spirodela polyrhiza* turions: a model for turion-based duckweed overwintering? Plants 13:2993