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Effect of gibberellins on growth and biochemical constituents in *Chlorella minutissima* (Trebouxiophyceae)

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ABSTRACT

A hormonal network regulates growth processes and stress responses in vascular plants. There is evidence for a similar hormonal network in microalgae. This study investigated the effect of exogenous gibberellins (GAs) on *Chlorella minutissima* Fott et Nováková growth and biochemical composition. Two bioactive GAs i.e. GA_3 and GA_4 were applied at 10^{-8}–10^{-5} M. Growth was monitored until cultures were harvested on day 7 when in an exponential growth phase. Primary metabolites (protein, chlorophyll and carotenoids) were quantified and endogenous GAs and phenolic acids were identified and quantified. GA_3 had little beneficial effect on growth in *C. minutissima* while GA_4 was inhibitory. GA application had little effect on the protein, chlorophyll and total carotenoid content. Analysis of the GA content suggested that GA_3 was not readily taken up by the cells while GA_4 was absorbed but not further metabolised. This high accumulation of GA_4 could account for its inhibitory effect. Three phenolics acids were detected in *C. minutissima* i.e. *p*-hydroxybenzoic acid > salicylic acid > protocatechuic acid. Their concentrations were not affected by GA treatments or GA-type. The physiological role of GAs in microalgae is still unclear and further studies are required to gain clearer insight into uptake rates, metabolism and function.

1. Introduction

Phytohormones control most aspects of growth and development in vascular plants and modulate physiological processes in response to environmental stimuli where rapid responses are required to survive abiotic and biotic stresses. This is achieved via a complex signaling network where the levels and sensitivity of each hormone is modulated by other hormones and external stimuli. Phytohormones are also present in algae with auxins, cytokinins (CKs), gibberellins (GAs), abscisic acid (ABA) and brassinosteroids (BRs) detected in both wild collected seaweeds (Gupta et al., 2011; Stirk et al., 2003, 2014a; Yokoya et al., 2010) and axenic cultured microalgae (Bajguz, 2009; Jirásková et al., 2009; Lu et al., 2014; Stirk et al., 2013a, 2013b, 2014b). They are physiologically active with evidence that a functional phytohormonal signaling system operates, responding to both external stimuli and phytohormone levels. For example, changes in the levels of endogenous CKs and auxins correlated to the growth phases of *Scenedesmus obliquus*, suggesting a role in cell division (Žižková et al., 2017). In actively growing *Chlorella minutissima* cultures, there was an increase in auxin and CK content over time and a decrease in BR and GA content. In contrast, cultures maintained in the dark had little cell growth and no change in the auxin, CK and GA content but had an increase in ABA and a decrease in BR content (Stirk et al., 2014b). In *Nannochloropsis oceanica*, nitrogen-depletion activated the ABA biosynthetic pathway and antagonistic transcription of CK biosynthetic genes, resulting in elevated ABA levels and a decrease in active CK levels (Lu et al., 2014).

Many GAs have been found in vascular plants but only a few show certain biological activity including GA_1, GA_3, GA_4, GA_5, GA_6 and GA_7 (Yamaguchi, 2008). The other GAs are either biosynthetic intermediates or deactivation products of bioactive GAs. There are two main biosynthetic pathways described in vascular plants arising from the common *ent*-kaurene precursor giving rise to GA_{12}. This is converted to the bioactive GA_4 via an oxidation pathway or to GA_1 via oxidation and hydroxylation processes. This hydroxylated pathway also incorporates other minor pathways to synthesize GA_6 and GA_3 via GA_5. Deactivation of bioactive GAs is an important step in regulating the levels of bioactive GAs

Abbreviations: ABA, abscisic acid; BA, benzyl adenine; BRs, brassinosteroids; Chl, chlorophyll; CK, cytokinin; DW, dry weight; FAME, fatty acid methyl ester; GA, gibberellin; IAA, indole-3-acetic acid; iP, isopentenyladenine; k, relative growth constant; MACC, Mosonmagyaróvár Algal Culture Collection; *tZ*, *trans*-zeatin; UHPLC/MS/MS, ultra-high performance liquid chromatography–tandem mass spectrometry.

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and it is provided mainly through 2β-hydroxylation (Urbanová et al., 2011; Yamaguchi, 2008).

Gibberellins were quantified in 24 axenic microalgae strains from the Chlorophyceae and Trebouxiophyceae and 1 strain each from the Ulvophyceae and divergent Streptophyta clades. Each of these four-day-old cultures contained between 18 and 20 GAs, including bioactive, intermediate and deactivation forms (Stirk et al., 2013b). A similar GA profile was described in the kelp *Ecklonia maxima* (Stirk et al., 2014a). While no homologous sequences have yet been found for enzymes catalyzing the early stages of GA biosynthesis in algae, some protein sequences and homologous enzymes involved in the later stages of GA biosynthesis have been identified, suggesting that the biosynthetic pathway in algae may be similar to that in vascular plants (Kiseleva et al., 2012). Based on the GA profiles in the 24 microalgae strains, it was speculated that the main biosynthetic pathway in microalgae is the 13-hydroxylated pathway leading to the formation of GA_6 while the 13-non-hydroxylated pathway producing GA_4 is a subsidiary pathway (Stirk et al., 2013b).

Exogenous application of specific phytohormones alter the growth rate and biochemical constituents in microalgae in a dose-dependent manner, generally being stimulatory at lower concentrations and inhibitory at higher concentrations (Bajguz and Piotrowska-Niczyporuk, 2014; Piotrowska-Niczyporuk and Bajguz, 2014). While there are numerous positive reports on the effects of exogenously applied phytohormones, these mainly focus on auxins, CKs and stress hormones such as ABA and BRs. For example, in a series of experiments with *Chlorella vulgaris*, exogenous BRs increased the level of endogenous ABA in heat stressed cultures (Bajguz, 2009) and exogenous CKs stimulated BR accumulation (Bajguz and Piotrowska-Niczyporuk, 2014). These elevated phytohormone levels improved the growth rate and increased primary metabolite accumulation (soluble proteins, chlorophyll, carotenoid and monosaccharides; Piotrowska-Niczyporuk and Bajguz, 2014). Methyl jasmonate and GA_3 applied separately to the unicellular microalgae *Haematococcus pluvialis* acted as signal molecules to increase the content of the secondary carotenoid astaxanthin with strong antioxidant activity by altering the transcription of three β-carotene ketolase genes (Lu et al., 2010). Brassinosteroids, auxin and ABA stimulated *Scenedesmus quadricauda* growth, pigment content and fatty acid accumulation (Kozlova et al., 2017). Medium supplemented with IAA, GA_3, kinetin and the plant stimulant 1-triacontanol stimulated growth in *Chlamydomonas reinhardtii* and ABA was slightly inhibitory. All treatments increased the protein and chlorophyll content but had no effect on the fatty acid methyl ester (FAME) content apart from ABA which increased the FAME yield (Park et al., 2013).

Secondary metabolites play an integral role in a plant's response to environmental stimuli, providing protection and defense mechanisms against abiotic and biotic stress. Gibberellins are an integral part of a network regulating perception to environmental signals in vascular plants. They are involved in light perception such as the phytochrome system during seed germination, photomorphogenesis of etiolation and stem elongation and photoperiod regulated processes such as leaf expansion and flower and seed development (Yamaguchi, 2008). Many cold-response genes are correlated to GA-regulated genes indicating the role of GA in regulating cold temperature signals. There is also a relationship between GA and ABA regulating protection mechanisms in response to abiotic stress (Yamaguchi, 2008).

Phenolics are the most widespread group of secondary metabolites, comprising of structurally diverse aromatic compounds. They are grouped into various classes based on their carbon skeleton e.g. phenolic acids, simple phenols, flavonoids, phlorotannins and condensed tannins (Balasundram et al., 2006; Cheynier et al., 2013). Phenolics have a wide range of physiological effects linked to their ability to generate free radicals, thus modifying radical-mediated oxidative processes often caused by environmental stress (Balasundram et al., 2006; Cheynier et al., 2013). Phenolics have a similar role in microalgae (Balasundram et al., 2006; Freile-Pelegrín and Robledo, 2013). For example, exposure of *Scenedesmus quadricauda* to heavy metal stress by the addition of copper to the media, stimulated the production of phenolic acids (Kováčik et al., 2010). However, there are only a few examples where the effect of phytohormones on the synthesis of secondary metabolites has been investigated. For example, GA_3 application in the cyanobacterium *Microcystis aeruginosa* increased growth as well as improving nitrogen absorption, protein and pigment accumulation and the content of the toxin microcystin (Pan et al., 2008).

Earlier studies on the effect of GAs on microalgae growth mainly used GA_3. As more recent studies have indicated that both the 13-non-hydroxylated and 13-hydroxylated pathways may occur in microalgae, the aim of the present study was to investigate the effect of bioactive GAs from the 13-hydroxylated pathway (GA_3) and 13-non-hydroxylated pathway (GA_4) on growth and metabolite content in the microalga *Chlorella minutissima*. Endogenous GAs were also quantified as an indication of how the GAs were assimilated into the cells.

2. Materials and methods

2.1. Experimental design

Axenic *Chlorella minutissima* MACC-452 (Mosonmagyaróvár Algal Culture Collection, Hungary) Fott et Nováková was maintained on agar slants made with Tamiya Nutrient Medium (Kuznjecov and Vladimirova, 1964) with 10 g/l agar (Oxoid Bacteriological Agar No. 1) in continuous low light (5 μmol/m^2/s) at 25 ± 2 °C. The media was sterilized by autoclaving at 121 °C, 103 kPa for 20 min and left to cool overnight. All transfers were carried out under sterile conditions. Suspension cultures were initiated by inoculating two flasks containing 250 ml liquid Tamiya nutrient medium; pH 6.10 ± 0.05. The cultures were grown at 25 ± 2 °C in a 14:10 h light:dark photoperiod, illuminated from below with 140 ± 21 μmol/m^2/s and aerated with sterile, humidified air.

After 7 days, the suspension culture from one flask was used to inoculate the experimental flasks with 10 mg/l DW starting biomass. Each flask contained 250 ml modified Tamiya medium where the nitrogen concentration was reduced from 700 mg/l (100% N) to 70 mg/L (10% N). GA (either GA_3 or GA_4; Sigma) was added at four concentrations, namely 10^{-8}–10^{-5} M GA as well as a control. There were four replicates per treatment. Each experiment ran for 7 days in the growth conditions described above. To monitor growth, 2 ml samples were removed on day 0, 2, 5 and 7 and the optical density measured at 550 nm (Varian Cary 50 UV–Visible Spectrophotometer, Australia). The relative growth constant (k) was calculated as $k = (\log_{OD7} - \log_{OD0})/t$ where t = days.

On day 7, the four replicates from each treatment were combined. An aliquot was removed for dry weight and chlorophyll and total carotenoid measurements. The remaining biomass was centrifuged at 5000*g* for 10 min at 20 °C (Beckman Coulter Avanti J-E). The resulting pellet was rinsed with distilled water, centrifuged and then lyophilized (Bench Top Pro, VirTis SP Scientific). The dried biomass was stored at −70 °C until analysis. The experiments were run three times for the GA_3 treatments and four times for the GA_4 treatments.

2.2. Endogenous gibberellin content

Gibberellin content was quantified using the method described earlier by Urbanová et al. (2013). The capacity of this approach is simultaneous determination of 20 GAs. Briefly 7 mg DW was extracted overnight in 1 ml 80% acetonitrile containing 5% formic acid and 19 internal gibberellin standards. Extracts were centrifuged at 36,670 *g* for 10 min at 4 °C and the supernatant further purified using mixed mode SPE cartridges (Waters, Milford, USA). Samples were analysed by ultra-high performance liquid chromatography–tandem mass spectrometry (UHPLC/MS/MS; Micromass, Manchester, UK) with GAs detected using multiple-reaction monitoring mode of the transition of the precursor ion $[M–H]^-$ to the appropriate product ion. The data were

acquired using Masslynx 4.1 software (Waters, Milford, USA) and the standard isotope-dilution method used to quantify GA levels in the samples. Each biological sample was analysed using three independent technical replicates.

2.3. Dry weight determination

Samples (6 ml) were filtered through Whatman GF/C filters (25 mm diameter) that had been dried at 110 °C for 30 min, cooled in a desiccator at room temperature for 30 min and weighed. Following filtration, the algae-loaded filters were dried at 110 °C for 90 min, cooled and weighed. The biomass of the suspension was calculated as mg DW/l.

2.4. Pigment determination

Samples (6 ml) were centrifuged at 5000 *g* at 20 °C for 10 min. The resulting pellet was suspended in 1.0 ml acetone and transferred to a tube containing 50 ceramic beads (1.4 mm diameter; Omni International). The centrifuge vial was rinsed with 0.5 ml acetone and added to the tube. The microalgae cells were disrupted using a cell disruptor (processing power 120 *g*; speed 4.4 m/s) for 180 s (Bead Ruptor 4; Omni International) and left in the dark for 10 min. The extract was transferred to a falcon tube and centrifuged at 1250 rpm for 5 min at 24 °C (Univapo 150H, UniEquip, Germany) and the supernatant recovered. The remaining cell debris and ceramic beads were suspended in 1.5 ml acetone and further disrupted as described above. The two supernatant fractions were combined, made up to 5 ml with acetone and absorbance measured at 470, 645 and 662 nm. Chlorophyll *a* (chl *a*), chl *b* and total carotenoids were calculated based on equations described by Lichtenthaler (1987).

2.5. Quantification of total soluble protein content

The total soluble protein content was determined using the Bradford method (Bradford, 1976). Samples (10–15 mg DW) were suspended in 1 ml 0.2 M sodium phosphate buffer (pH 7.02) and 50 ceramic beads (1.4 mm diameter) added. Cells were disrupted using a cell disruptor (120 *g* processing power; speed 4.4 m/s) for 360 s (Bead Ruptor 4, Omni) and then sonicated for 30 min. Thereafter, samples were centrifuged for 20 min at 48 *g* (microcentrifuge Sigma-201 M). Duplicate samples were prepared where supernatant (50 μl) was added to 1.5 ml Bradford Reagent, gently mixed and left at room temperature for at least 5 min. Absorbance at 595 nm (Jenway Genova Plus) was measured within 30 min. Total soluble protein concentrations (mg protein/mg DW) were calculated from a standard curve prepared using bovine serum albumin.

2.6. Phenolic quantification

Phenolic acids were determined by UHPLC/MS/MS method described earlier (Gruz et al., 2008). In brief, deuterium-labelled internal standards of 4-hydroxybenzoic (2,3,5,6-D4) and salicylic (3,4,5,6-D4) acids were added to extraction solvent prior to homogenization of microalgae material in an oscillation ball mill. The supernatant was filtered through a 0.45 μm Nylon membrane filter (Alltech, Breda, Netherlands) and subsequently analysed by ACQUITY Ultra Performance LC™ (UPLC) system (Waters, Milford, MA, USA) linked to a Micromass Quattro micro™ API benchtop triple quadrupole mass spectrometer (Waters MS Technologies, Manchester, UK).

2.7. Statistical analysis

Each treatment consisted of four replicates and the experiments were carried out three times for the GA_3 treatments and four times for the GA_4 treatments. The optical density was determined for each replicate during the course of the experiment and the relative growth constant (k) calculated (GA_3 n = 12; GA_4 n = 16). The four replicate flasks for each treatment were combined on day 7 for metabolite analysis (GA_3 n = 3; GA_4 n = 4). One-way ANOVA followed by the post hoc Tukey test were used to detect significant differences ($P < .05$) between treatments (SigmaPlot v.13).

3. Results

3.1. Growth

All cultures had a short lag phase following inoculation (day 0–day 2) and then remained in an exponential growth phase for the remainder of the experiment (Fig. 1). While GA treatments did not significantly affect the relative growth rate (k) when compared to the control (Table 1), higher GA_3 concentrations (10^{-5}–10^{-6} M) enhanced growth while lower GA_3 and GA_4 concentrations (10^{-7}–10^{-8} M) were slightly inhibitory (Fig. 1) with a significant difference recorded between the 10^{-7} M and 10^{-6} M GA_3 treatments (Table 1).

3.2. Gibberellin content

In total, 16 GAs were detected in the various samples harvested on day 7, although many occurred in very low concentrations (Table 2). GA_{51} was the predominate GA present in all samples. In the GA_3-treated cultures, only addition of 10^{-5} M GA_3 on day 0 caused a slight increase in the endogenous GA_3 content by day 7. However, other biologically active GAs within the 13-hydroxylated pathway, namely GA_6 and GA_1 increased in a dose-dependent manner. There was little change in the GAs associated with the 13-non-hydroxylated pathway (Table 2). In the GA_4-treated cultures, there was a large dose-dependent increase in endogenous GA_4 but with no change in the concentration of the other GAs associated with the 13-non-hydroxylated pathway. However, both GA_6 and GA_1 increased in response to GA_4 treatments (Table 2).

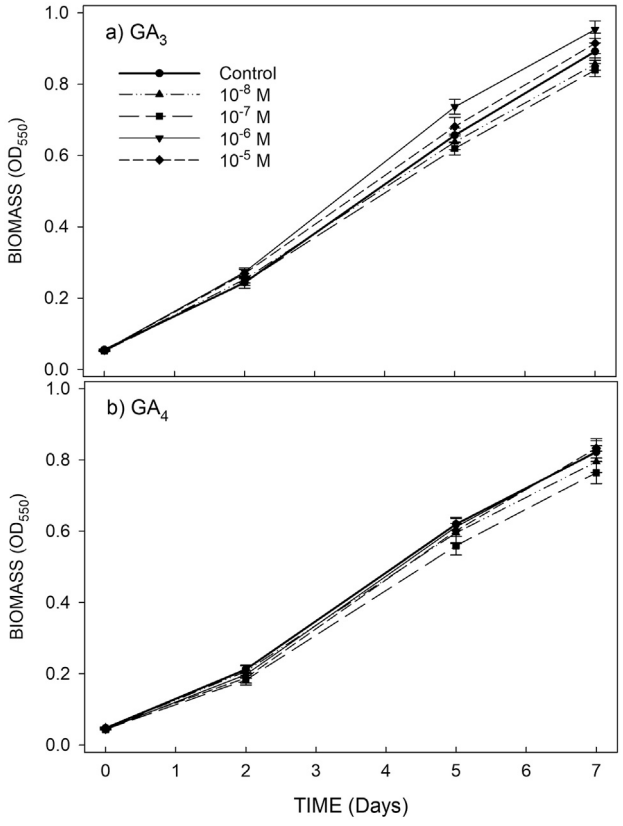

Fig. 1. Growth of *Chlorella minutissima* MACC-452 over 7 days when treated with a) GA_3 and b) GA_4. Results are shown as mean ± SE (GA_3 n = 12; GA_4 n = 16).

Table 1
Relative growth rate (k) over 7 days and endogenous GA and phenolic acid content on day 7 for *Chlorella minutissima* MACC-452 treated with exogenous GA. Growth data are presented as mean ± SE (GA_3 n = 12; GA_4 n = 16). Different letters indicate significant differences (P < .05).

Treatment	Relative growth rate	
GA	k	
	GA_3	GA_4
Control	0.128 ± 0.01^{ab}	0.103 ± 0.008^{a}
10^{-8} M	0.113 ± 0.009^{ab}	0.088 ± 0.016^{a}
10^{-7} M	0.107 ± 0.011^{b}	0.074 ± 0.018^{a}
10^{-6} M	0.160 ± 0.012^{a}	0.108 ± 0.016^{a}
10^{-5} M	0.141 ± 0.014^{ab}	0.106 ± 0.015^{a}
P	0.023	0.681

Although not significantly different due to the large variation in the samples, the total GA content was higher in the GA_4 treated samples compared to the GA_3 treated samples, mainly due to the large increase in GA_4 (Table 2).

3.3. Pigment content

Although not significant, GA_3 treatments had a positive effect on the pigment content in 7-day old *C. minutissima* cultures compared to the control with cultures exposed to lower GA concentrations (10^{-7}–10^{-8} M) having the highest pigment content and decreasing when exposed to higher GA concentrations (10^{-5}–10^{-6} M; Fig. 2a). GA_4 treatments had no effect on the pigment content in *C minutissima* (Fig. 2b).

3.4. Soluble proteins

GA_3 treatment had no effect on the total soluble protein content in *C. minutissima* harvested on day 7. Although not significant, there was a dose-dependent increase in total soluble proteins in GA_4 treated cultures with 10^{-6} M GA_4 treated cultures having the highest protein content (Fig. 3).

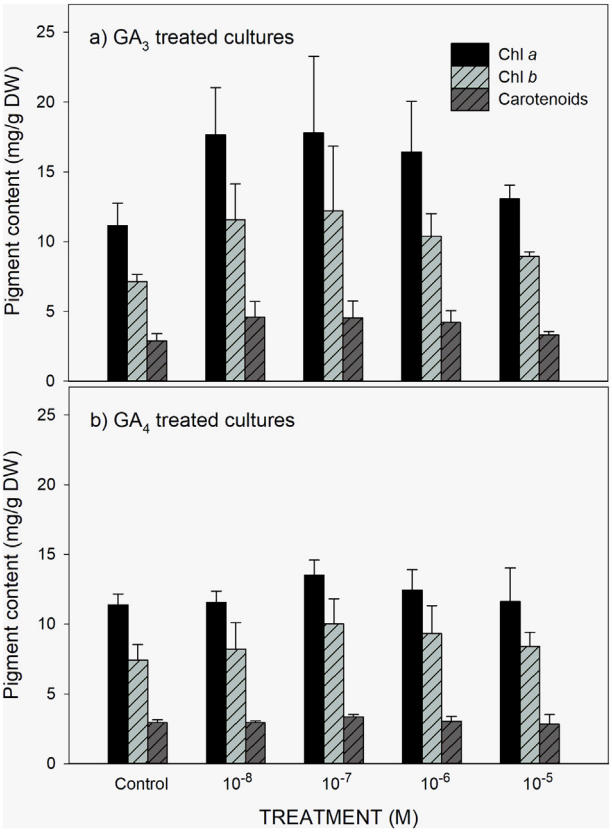

Fig. 2. Effect of a) GA_3 and b) GA_4 treatments on chlorophyll and total carotenoid content in *Chlorella minutissima* MACC-452 harvested on day 7. Results are shown as mean ± SE (GA_3 n = 3; GA_4 n = 4).

3.5. Phenolic content

Three hydroxybenzoic acids were detected in *C. minutissima*. The predominant phenolic acid was *p*-hydroxybenzoic acid with

Table 2
Endogenous GA content (pg/mg DW) in 7-day old *Chlorella minutissima* MACC-452 after treatment with GA_3 (n = 3) and GA_4 (n = 4). Results are shown as mean ± SE. Biologically active GAs are highlighted in bold. LOD = below limit of detection.

GA	Treatment									
	GA_3					GA_4				
	Control	10^{-8} M	10^{-7} M	10^{-6} M	10^{-5} M	Control	10^{-8} M	10^{-7} M	10^{-6} M	10^{-5} M
	13-non-hydroxylated pathway									
GA_{12}	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD
GA_{15}	0.14 ± 0.06	0.24 ± 0.19	0.20 ± 0.16	LOD	LOD	LOD	LOD	0.09 ± 0.08	LOD	LOD
GA_{24}	LOD	LOD	0.69 ± 0.56	0.61 ± 0.50	LOD	LOD	LOD	LOD	LOD	LOD
GA_{13}	LOD	0.04 ± 0.03	LOD	0.05 ± 0.04	0.04 ± 0.04	0.05 ± 0.04	0.04 ± 0.04	0.08 ± 0.04	0.05 ± 0.04	0.05 ± 0.01
GA_9	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD
GA_{51}	15.83 ± 0.65	13.47 ± 6.49	4.25 ± 3.47	2.17 ± 1.77	8.73 ± 3.57	18.29 ± 2.36	12.58 ± 5.43	17.90 ± 4.15	8.97 ± 3.13	14.21 ± 6.47
GA_7	0.04 ± 0.03	0.06 ± 0.02	LOD	LOD	LOD	LOD	LOD	0.18 ± 0.17	LOD	0.15 ± 0.09
GA_4	**0.23 ± 0.19**	**LOD**	**LOD**	**LOD**	**0.53 ± 0.43**	**LOD**	**LOD**	**0.28 ± 0.07**	**12.71 ± 10.51**	**18.68 ± 3.28**
GA_{34}	0.05 ± 0.04	0.09 ± 0.03	0.08 ± 0.04	LOD	0.04 ± 0.03	0.11 ± 0.03	0.07 ± 0.01	0.01 ± 0.01	0.07 ± 0.02	0.11 ± 0.04
	13-hydroxylated pathway									
GA_{53}	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD	LOD
GA_{44}	0.41 ± 0.17	LOD	0.25 ± 0.20	0.23 ± 0.19	LOD	0.26 ± 0.25	0.36 ± 0.34	LOD	LOD	0.26 ± 0.24
GA_{19}	LOD	LOD	LOD	0.09 ± 0.07	LOD	LOD	LOD	LOD	0.19 ± 0.09	LOD
GA_{20}	0.15 ± 0.12	0.07 ± 0.06	0.09 ± 0.07	LOD	LOD	LOD	LOD	0.14 ± 0.07	0.07 ± 0.02	LOD
GA_{29}	0.03 ± 0.02	LOD	0.38 ± 0.31	0.03 ± 0.03	0.04 ± 0.03	0.22 ± 0.09	0.04 ± 0.01	0.08 ± 0.07	0.05 ± 0.05	0.07 ± 0.07
GA_5	LOD	0.03 ± 0.02	LOD	LOD	LOD	LOD	0.04 ± 0.04	LOD	LOD	LOD
GA_3	**0.03 ± 0.02**	**0.03 ± 0.02**	**0.05 ± 0.02**	**0.07 ± 0.04**	**0.38 ± 0.01**	**0.08 ± 0.02**	**0.08 ± 0.03**	**0.04 ± 0.02**	**0.07 ± 0.03**	**0.06 ± 0.04**
GA_6	**0.22 ± 0.01**	**0.29 ± 0.03**	**0.47 ± 0.09**	**0.35 ± 0.15**	**0.50 ± 0.10**	**0.42 ± 0.12**	**0.37 ± 0.16**	**0.29 ± 0.08**	**0.78 ± 0.35**	**0.77 ± 0.16**
GA_1	**LOD**	**0.15 ± 0.12**	**0.24 ± 0.10**	**0.54 ± 0.16**	**1.08 ± 0.25**	**0.30 ± 0.15**	**0.28 ± 0.18**	**0.68 ± 0.37**	**0.78 ± 0.36**	**0.58 ± 0.16**
GA_8	0.11 ± 0.03	0.15 ± 0.04	0.44 ± 0.14	0.20 ± 0.03	0.21 ± 0.05	0.19 ± 0.06	0.14 ± 0.02	0.14 ± 0.02	0.17 ± 0.07	0.12 ± 0.04
Total	17.24 ± 1.04	14.60 ± 6.51	7.12 ± 2.81	4.35 ± 2.26	11.53 ± 3.05	19.91 ± 2.30	13.99 ± 5.37	19.90 ± 4.61	23.89 ± 10.87	35.03 ± 4.78

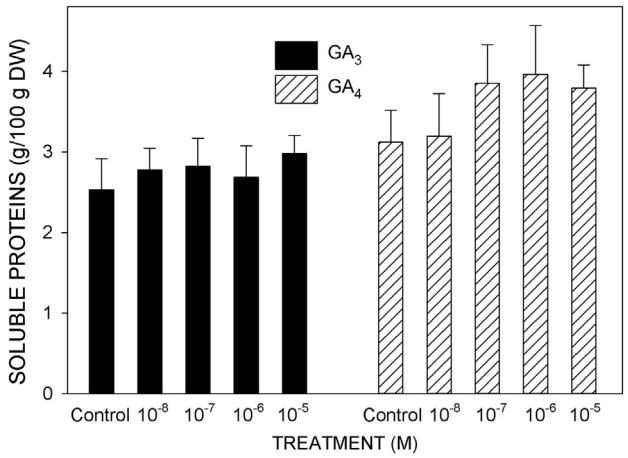

Fig. 3. Effect of GA_3 and GA_4 treatments on the total soluble protein content in *Chlorella minutissima* MACC-452 harvested on day 7. Results are shown as mean ± SE (GA_3 n = 3; GA_4 n = 4).

concentrations ranging from 668 to 1182 ng/g DW. Salicylic acid and protocatechuic acid occurred in low amounts ranging from 57 to 150 ng/g DW and 15–51 ng/g DW respectively (Fig. 4). Although not statistically different, the control cultures generally had a higher total phenolic acid content compared to the GA-treated cultures (Fig. 4).

4. Discussion

The 16 GAs detected in *C. minutissima* in the present study were similar to those detected in the 24 microalgae strains previously analyzed (Stirk et al., 2013b). However, the total GA content was substantially lower in the present 7-day-old *C. minutissima* cultures which can be explained by the culture age. When endogenous hormones were monitored in *C. minutissima* cultures, there was a decrease in GAs over 48 h (Stirk et al., 2014b). Although the total GA content was much lower in the 7-day-old cultures in the present study, the GA profiles were similar with the inactive GA_{51} (a product of GA_4 precursor deactivation by 2β-hydroxylation) being the predominant GA detected and GA_6 being the predominant active GA (Stirk et al., 2014b).

In the present study, application of GA_3 had no effect on the endogenous GA_3 concentration but there was a dose-dependent increase in the levels of GA_6, GA_1 and the end inactive product GA_8 (Table 2). GA_3 treatment had no effect on GAs associated with the 13-non-hydroxylated pathway. These results suggest that exogenous GA_3 is not readily taken up by *C. minutissima*. In contrast, application of GA_4 resulted in a very large dose-dependent increase in the endogenous GA_4 content but had little effect on the other GAs associated with the 13-non-hydroxylated pathway. There was a small increase in the bioactive GAs e.g. GA_6 and GA_1 in the 13-hydroxylated pathway (Table 2).These results suggest that while GA_4 is taken up by the cells, it is not metabolized or converted to other GAs. The effects of exogenously applied phytohormones may be moderate due to the precise homeostasis mechanisms controlling hormone levels via biosynthesis, conjugation and degradation (Lu et al., 2014). In vascular plants, levels of bioactive GAs are regulated via a feedback mechanism where bioactive GAs reduce their own accumulation via negative feedback on GA biosynthesis and positive feed-forward on GA deactivation (O'Neill et al., 2010). Application of bioactive GAs downregulate the GA biosynthesis genes and upregulate the deactivation genes (Yamaguchi, 2008).

Previous studies with *Chlorella vulgaris* recorded the greatest increases in the biochemical profiles 72 h after auxin application after which the stimulatory effect decreased (Piotrowska-Niczyporuk and Bajguz, 2014) whereas the largest response to exogenous CK application was recorded on day 6 (Piotrowska and Czerpak, 2009). Additional studies need to be carried out where the microalgae cultures are analyzed at shorter time intervals after GA_3 application to gain clearer insight into uptake rates and metabolism. In addition, GA biosynthetic precursors should also be considered rather than bioactive end products.

In the present study, exogenous application of GA_3 and GA_4 had no significant effect on the growth of *C. minutissima* although higher GA_3 concentrations slightly enhanced growth while lower GA_3 and GA_4 concentrations were slightly inhibitory (Fig. 1). The lack of a positive growth response to GA_3 treatment may be explained by the GA profiles which suggest that GA_3 was not taken up from the media. The elevated endogenous GA_4 content may account for the slight inhibitory effect of the GA_4 treatments on *C. minutissima* growth (Fig. 1b). In the previously analyzed 24 microalgae cultures, the general trend was for the slower growing strains to have a higher gibberellin content and faster growing strains to have a lower gibberellin content (Stirk et al., 2013b). This muted response was similar to previous reports where the effects of exogenous GA on microalgae are variable with the elicited growth response depending on species, GA-type and concentration with *Chlorella* cultures often showing no or inhibitory responses. For example, GAs had no effect on the growth of synchronous *Chlorella fusca* cultures (Lien et al., 1971); 10–100 ppm GA_3 had no effect on four *Chlorella* species while higher concentrations (200 ppm) were inhibitory (Evans and Sorokin, 1971); GA_3 and GA_7 elicited a positive growth response with *Senedesmus quadricauda* and *Dictyosphaerium pulchellum* being the most responsive to GA treatments while *Chlorella vulgaris* was the least affected (Burkiewicz, 1987). When GA_3 was applied to *Chlorella vulgaris* cultures in their early stationary phase (day 22), there was a further gradual increase cell density and delayed senescence. However, there was no effect on lipid content and fatty acid composition (Jusoh et al., 2019). In addition, in comparison of growth responses elicited with other groups of plant hormones, GAs are generally less active. Growth (cell number) was stimulated in *Euglena gracilis* with ABA

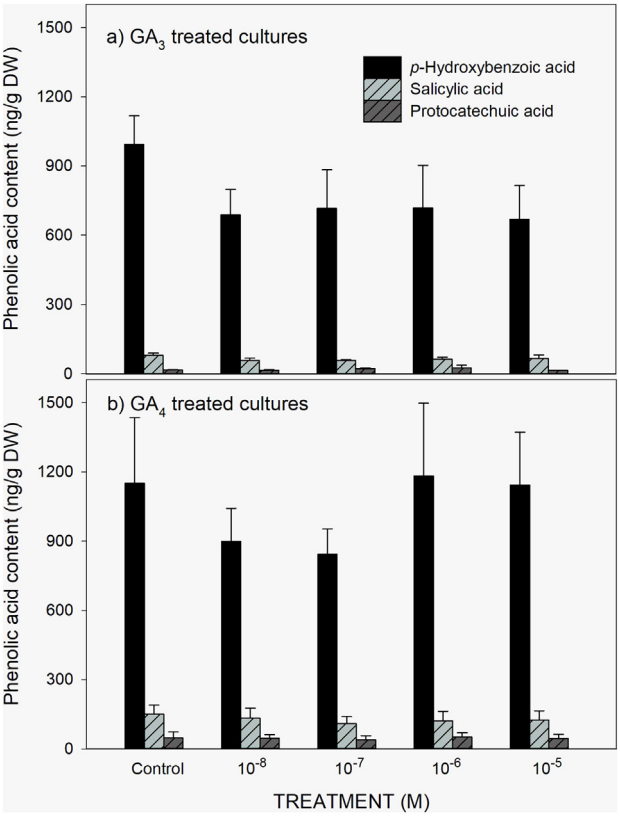

Fig. 4. Effect of a) GA_3 and b) GA_4 treatments on phenolic acid content in *Chlorella minutissima* MACC-452 harvested on day 7. Results are shown as mean ± SE (GA_3 n = 3; GA_4 n = 4).

being the most stimulatory and GA_3 having the least effect (ABA > indole-3-acetic acid (IAA) > benzyl adenine (BA) > *trans*-zeatin (*t*Z) > isopentenyladenine (iP) > GA_3; Noble et al., 2014); when conchocelis filaments of three species of *Porphyra* were treated with kinetin, IAA and GA_3 and grown in a range of temperatures, irradiance and photoperiods, kinetin and IAA elicited stronger growth-stimulating effects compared to GA_3 under all environmental variables (Lin and Stekoll, 2007).

Generally, protein content correlates to metabolic activity and accumulates in actively growing cultures with high metabolic and mitotic activity (Park et al., 2013) and high chlorophyll content increases the accumulation of other biochemical constituents. In the present study, exogenous application of GA_3 and GA_4 had no significant effect on the soluble protein, chlorophyll and total carotenoid content in *C. minutissima*. The results support the suggestion that GA_3 was not taken up by the cultures and that GA_4, while taken up, was not metabolized.

Phenolics are synthesized via different pathways. Hydroxybenzoic acids (C6–C1 carbon skeleton) are synthesized directly from intermediates of the shikimate pathway and hydroxcinnamic acids (C6–C3 carbon skeleton) are synthesized via the phenyl propanoid pathway. In addition, the acetate/malonate pathway synthesizes simple phenols (Caretto et al., 2015; Cheynier et al., 2013). In algae, phenolic acid synthesis is limited to the shikimate pathway, only producing phenylalanine and tyrosine as precursors. Unlike bryophytes and vascular plants, post-tyrosine chemistry based on cinnamic acid is not present in algae (Caretto et al., 2015; Cheynier et al., 2013). The greater phenolic diversity in vascular plants is linked to the colonization of land and the increased number of associated environmental stresses (Caretto et al., 2015; Cheynier et al., 2013). While there are numerous reports on total phenolic content in microalgae, these are mostly based on colorimetric tests such as the Folin–Ciocalteu method. There are only a few reports where the phenolic acids in microalgae were positively identified. In the present study, the phenolic compounds detected in *C. minutissima* were three hydroxybenzoic acids, namely *p*-hydroxybenzoic acid, salicylic acid and protocatechuic acid (Fig. 4) which are synthesized via the shikimate pathway. Similarly, only a few phenolic acids were detected in other microalgae – gallic acid was detected in *Nostoc commune* (Cyanophyceae), protocatechuic acid was detected in *Ankistrodesmus* sp. (Chlorophyceae), *Spirogyra* sp. (Zygnematophyceae) contained gallic acid and *Euglena cantabrica* (Euglenophyceae) had the highest and most diverse phenolic profile, dominated by gallic acid > protocatechuic with low concentrations of catechin, chlorogenic acid and epicatechin (Jerez-Martel et al., 2017).

Plants maintain a balance between constitutive and induced defense compounds in order to ensure optimum protection and metabolic plasticity to survive changing environmental conditions (Caretto et al., 2015). The production of secondary metabolites is energetically expensive and requires a balance in the allocation of resources between primary metabolites for growth and secondary metabolites for defense. In vascular plants, there is a negative correlation between growth rates and phenolic content with low productivity species generally having a higher phenolic content than high-productivity species – if growth is limited, resources can be allocated to secondary metabolite synthesis (Caretto et al., 2015). Gibberellin application had some effect on the phenolic acid content of *C. minutissima* in the present study where cultures treated with high concentrations of GA_4 (10^{-6}–10^{-5} M) had a higher *p*-hydroxybenzoic acid content. This increased phenolic content may be linked to the inhibitory effect of GA_4 on growth and the higher chlorophyll content in the cultures.

Further experiments where cultures are exposed to specific stresses rather than grown in optimal conditions may shed more insight into the role of GAs in microalgae. For example, exogenous GA_3 stimulated the growth and increased cellular biochemical constituents of *Chlorella vulgaris* when exposed to low metal concentrations (Cd and Pb; Falkowska et al., 2011). Cytokinins, followed by GA_3, auxins and polyamines were the most effective in blocking the bioaccumulation of heavy metals (Cd, Cu and Pb) and restoring growth and primary metabolite content in *C. vulgaris* while jasmonic acid stimulated heavy metal bioaccumulation and inhibited growth and metabolite content. They were also involved in anti-stress activity inducing the inhibition of an oxidative burst through the stimulation of non-enzymatic antioxidant metabolites and anti-oxidant enzymatic activity (Piotrowska-Niczyporuk et al., 2012).

In conclusion, application of GA_3 had little beneficial effect on growth and metabolite content in *C. minutissima* while GA_4 was inhibitory to growth. Analysis of the GA content in cultures harvested on day 7 indicated that GA_3 was not readily taken up by the cells while GA_4 was absorbed but not further metabolized. This high accumulation of GA_4 could account for the inhibitory effect. Further studies need to be carried out where the microalgae cultures are analyzed at shorter time intervals after GA application, GA biosynthetic intermediates should applied rather than bioactive end products and cultures stressed in order to gain clearer insight into uptake rates, metabolism and the physiological role of GAs in microalgae.

Author contributions

WA Stirk designed the experiments, ran the growth experiments and was first author on the manuscript; D Tarkowska conducted the gibberellin analysis and editing the manuscript; J Gruz conducted the phenolic analysis and editing the manuscript; M Strnad was involved in the drafting and editing the manuscript; V Ördög contributed to the original concept and editing the manuscript; J van Staden contributed to the original concept and editing the manuscript.

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