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# Strategies for enhancing valuable metabolites produced by the soil microalga**

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Abstract. The current knowledge of metabolite accumulation by soil microalgae is not fully recognised. This study provides insights into the production of bio-products and the photosynthetic activity of the soil alga *Vischeria calaminaris.*The cell response to environmental abiotic factors, nitrogen limitation and indole-3-acetic acid (IAA) supplementation under high light intensity to induce the accumulation of high-value metabolites was investigated. The addition of IAA improved cell growth only in the nitrogen-limited treatments. In the nitrogen-limited conditions, the maximum photochemical quenching of PS II decreased over the cultivation period. Additionally, IAA regulated nonphotochemical quenching in the nitrogen-limited treatment, showing an enhanced capacity to actively dissipate excess light energy in low-nitrogen growth conditions. In response to the nitrogen stress, the protein content of the *V. calaminaris* decreased and increased in the IAA supplemented cultures. The results showed that IAA significantly stimulated the content of Chl *a* and carotenoids in both nitrogen treatments tested. The highest lipid content was obtained in the nitrogen-limited variant supplemented with IAA. The lipidomic analysis revealed high content of oleic and palmitoleic acids in *V. calaminaris*. The IAA supplementation in the low-nitrogen growth conditions was suitable for accumulation of high-value monounsaturated fatty acids for biofuel production from *V. calminaris* biomass.

Keywords: microalgae, fatty acids, biodiesel, *Vischeria calaminaris*

## 1. INTRODUCTION

Given their ability to produce intracellular metabolites, *e.g.* lipids and fatty acids, unicellular algae are perceived as organisms with high biotechnological potential. Microalgal fatty acids and pigments are valuable in the production of nutraceuticals (Barbosa *et al.*, 2024). Variability in abiotic conditions has an impact on metabolic pathways that lead to the synthesis and accumulation of specific bioproducts in algal cells. Nitrogen and phosphate deficiency, salinity, and high light intensity are factors that increase the accumulation of lipids in microalgal cells. Nitrogen limitation affects remodelling of intracellular membranes and leads to the accumulation of neutral lipids (Singh *et al.*, 2024). However, conditions that favour the synthesis of lipids slow down the processes of photosynthesis and algal growth. The use of higher light intensities in microalgal cultures has a beneficial effect on nutrient utilisation, resulting in increased growth rates (Grudzinski *et al.*, 2016).

Indolyl-3-acetic acid (IAA, phytohormone) is produced in trace amounts by microorganisms and plants (Malhotra and Srivastava, 2008; Jose *et al*., 2024). It acts as a signalling molecule in algae-bacteria interactions (Lin *et al.*, 2022). IAA occurs naturally in aquatic and soil ecosystems, *e.g*. as part of extracellular polymers (EPS). EPS are secreted by plant growth-promoting bacteria and algae with

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which algae associate in consortia (Perera *et al.*, 2022). IAA is used in algal cultures to regulate microalgal growth and reduce the effects of stress on cells (Krzemińska *et al.*, 2023). Exogenous supplementation of indolyl-3-acetic acid in continuous light conditions has been reported to promote increased growth rates of microalgae, compared to photoperiod conditions (Magierek *et al.*, 2017). Research on unicellular algae has shown that IAA has also a stimulating effect on the processes of biosynthesis of bioactive compounds, including fatty acids and carbohydrates (Krzemińska *et al.*, 2023). Evaluating the effects of exogenous IAA on metabolic processes and photosynthesis in unicellular algae will provide a better understanding of algal-bacterial interactions and microbial biodiversity. It has been reported that, in N-limiting conditions in *Chlorella sorokiniana* culture, IAA increased the expression levels of the RuBisCo and acetyl-CoA carboxylase genes involved in photosynthesis and carbon and lipid metabolism (Babu *et al.*, 2017). Nevertheless, further investigation is required to elucidate the role of indole-3-acetic acid in the metabolism of energy storage compounds in microalgal cells under environmental stress factors (Shah *et al.*, 2022; Lin *et al.*, 2022).

*Eustigmatophyceae* are a diverse class of photosynthetic coccoid microalgae that are primary producers of polyunsaturated fatty acids (PUFAs) and produce high biomass (Kryvenda *et al.*, 2018; Rodolfi *et al.*, 2009). *Eustigmatophyceae* are distinguished by a lack of chlorophyll *c* (Eliáš *et al.*, 2017). Research into the biochemistry of this class of algae has mainly focused on marine species, while terrestrial strains are less commonly studied (Amaral *et al.*, 2020, Sinetova *et al.*, 2021). *Vischeria calaminaris* (previously *Eustigmatos calaminaris*) is a soil strain isolated from an extreme environment – calamine mine spoils (Trzcińska *et al.*, 2014; Krzemińska *et al.*, 2023). The biochemistry of this soil alga is poorly understood. In this regard, this work represents the first investigation of the photochemical traits and cellular metabolite accumulation of *Vischeria calaminaris* in response to simultaneous nitrogen stress and IAA supplementation under high light intensity. The aim of this study was to determine the physiological characteristics and metabolic changes in *Vischeria calaminaris* in order to identify optimal conditions for increasing biomass yield and accumulation of metabolites and biotechnologically important fatty acids.

## 2. EXPERIMENTAL PROCEDURES

### 2.1. Algal species and growth conditions

The axenic microalgal strain of *Vischeria calaminaris* was obtained from the culture collection of autotrophic organisms (CCALA, Czech Republic). Inoculum cultures were grown in BG 11 sterile medium with soil extract (5 ml $L^{-1}$) in 500 mL Erlenmeyer flasks with 250 mL culture volume under continuous illumination of 400 µmol photons $m^{-2}$ $s^{-1}$ photon flux density with continuous shaking at 90 rpm, at 26±1°C, with aeration by sterile air.

### 2.2. Experimental setup

The control culture (C) of *V. calaminaris* cells was grown in 500 mL Erlenmeyer flasks with 250 mL culture volume in BG-11 sterile medium enhanced with soil extract (5 ml $L^{-1}$) in the phototrophic mode. For nitrogen-limited treatments (Nl), the modified BG-11 medium (with soil extract) with reduced $NaNO_3$ control treatment of 75% (0.375 g $L^{-1}$) was used as the experimental medium. $10^{-4}$ M of indole-3-acid (IAA, stock solution of IAA was prepared using 98% ethanol) was added to the control and the nitrogen-limited variant of the medium (Table 1). The cultures were started with the same concentration of cells OD 650 = 0.28. The cells were cultured with continuous shaking at 90 rpm, at 26±1°C, with aeration by sterile air, and in high light conditions: continuous illumination of 400 µmol photons $m^{-2}$ $s^{-1}$ photon flux density white light. The experiments lasted for seventeen days. The experiments were carried out in three replications.

### 2.3. Analytical methods

#### 2.3.1. Determination of algal growth

The specific growth rate was determined spectrophotometrically by measuring the optical density of algal samples at 650 nm (Cary 300/Biomelt spectrophotometer). The specific growth rate was estimated according to Krzemińska *et al.* (2020). The *V. calaminaris* biomass productivity and biomass yield was determined with the gravimetric method. A sample of the culture was filtered through filter paper (Whatman GF/C), dried to constant weight, and weighed. The results were expressed in the form of biomass yield (g $L^{-1}$) and daily biomass productivity (g $L^{-1}$ $day^{-1}$).

#### 2.3.2. Pigment analysis

Pigments were extracted from *V. calaminaris* cells using DMSO at 65°C for 1 h according to Kalinowska *et al.* (2010) and Wellburn *et al*. (1994). Chlorophyll *a* and

**Table 1.** Autotrophic *V. calaminaris* growth conditions

| C | C IAA | Nl | Nl IAA |
|---|---|---|---|
| Control conditions: BG 11 with soil extract (5 ml $L^{-1}$) | BG 11 with soil extract (5 ml $L^{-1}$) supplemented with indole-3-acetic (IAA)$10^{-4}$ M | Nitrogen-limited treatments: BG 11 medium (with soil extract) with reduced $NaNO_3$ content of 0.375 g $L^{-1}$ (25%) | Nitrogen-limited treatments: BG 11 medium (with soil extract) with reduced $NaNO_3$ content of 0.375 g $L^{-1}$ (25%) supplemented with indole-3-acetic (IAA)$10^{-4}$ M |

carotenoid contents were calculated using the equations of Kalinowska *et al.* (2010). The results obtained were converted into μg $mg^{-1}$ DW. The measurements of the pigment content were carried out on the 4th and 9th day.

#### 2.3.3. Protein estimation

Protein content was determined using the spectrophotometric measurements according to Bradford (1976) and Piasecka *et al.* (2022). Prior to the measurements, the cell disruption method based on ultrasound with ethanol as a solvent was applied (Vibra cell 500, Sonics, Poland). A sample was mixed with Bradford reagent and measured at the wavelength of 595 nm using a UV-Vis spectrophotometer. Bovine serum albumin was used to create the standard curve. The results obtained were converted into the protein concentration using the calibration curve.

#### 2.3.4. Photochemical traits

The maximum quantum yield of photosystem II (Fv/Fm) and regulated nonphotochemical quenching Y(NPQ) were measured using Imaging PAM Maxi with an IMAG-K7 CCD camera (Walz GmbH, Germany) at 5th and 9th day of cultivation in multiwell plates filled with 1 ml of the culture. Prior measurements samples were dark adapted for 20 min, during dark adaptation microalgae samples were placed on orbital shaker and before measurements of induction curve mixed for uniform distribution using pipette. Measurements of induction curve were conducted under illumination of blue (450 nm) actinic light at an intensity 56 μmol $m^{-2}$ $s^{-1}$.

#### 2.3.5. Estimation of lipids

Lipids were extracted using a modified Bligh and Dyer method (Krzemińska *et al.*, 2020). Briefly, after centrifugation, the microalgal cultures were mixed with chloroform/methanol extraction solvents (1:2, v/v) and ultrasonicated in an ice bath and then centrifuged. Butylated hydroxytoluene was added as an antioxidant. The chloroform phase was collected and evaporated at 40°C on a vacuum evaporator. After evaporation, the lipid content was determined gravimetrically. The lipid content was expressed in % of dry weight (w/w). Extracted lipids were suspended in 99% n-hexane for further analysis.

#### 2.3.6. Preparation of fatty acid methyl esters

The fatty acid methyl esters (FAME) were prepared by transesterification of lipids with methanol in the presence of catalysts according to Krzemińska *et al.* (2020). FAME samples were obtained by adding 0.5 M KOH-methanol to extracted total lipids and hydrolysed at 85-90°C for 1 h. Then, 10% BF3 in methanol was added to the samples and heated at 100°C for 20 min. The mixture was cooled and 99% n-hexane and a saturated NaCl solution were added. The upper hexane layer was collected and transferred to vials for GC-MS analysis.

#### 2.3.7. GC-MS analysis

A Trace GC Ultra (Thermo Scientific) chromatograph coupled with an ITQ 1100 mass spectrometer (Thermo Scientific) with an Rtx-2330 column (dimensions: 105 m × 0.25 mm × 0.25 μm) was used to analyse the fatty acid profile. Helium at a flow rate of 2.1 ml $min^{-1}$ was the carrier gas. Heptadecanoic acid was used as an internal standard. To identify the fatty acid methyl esters, the FAME MIX 37 standard (CRM 47885 Supelco) was used and the concentration of FAMEs was calculated from the internal standard concentration, the area of the peaks, and the amount of the injected sample (Krzemińska *et al.*, 2023).

#### 2.3.8. Statistical analysis

The statistical analysis was performed using the STATISTICA v. 13 program (TIBCO Software Inc. USA). To determine the impact of the culture conditions on the analysed parameters of the microalgal cells, two-way ANOVA was used at a significance level of $p \leq 0.05$ using the post-hoc Tukey test.

## 3. RESULTS

### 3.1. Growth characteristics of *V. calaminaris*

The availability of nutrients in the culture medium plays a key role in the processes of microalgal growth. The impact of the nitrogen concentration and IAA addition on the specific growth rate and biomass yield are shown in Fig. 1 and Table 2. The data showed that the nitrogen concentrations had an effect on the growth rate. The highest growth rate in each of the culture variants was observed on the third day of growth. The maximum specific growth rate (0.752 $day^{-1}$) was observed for *V. calaminaris* in the control conditions with IAA and the lowest growth rate was recorded in the Nl culture conditions. The addition of the IAA phytohormone under nutrient stress stimulated the growth of *V. calaminaris* more efficiently than the growth in the optimal nutrient conditions. Depending on the nitrogen availability, the strain showed different physiological responses to the phytohormone.

The combination of the phytohormone and nitrogen stress increased biomass productivity, indicating the positive role of IAA in accumulation of the biomass of *V. calaminaris*. The daily biomass productivity in the culture grown in nitrogen limited conditions with the phytohormone was over two-times higher (0.662 g $L^{-1}$ $day^{-1}$) than that without the phytohormone (Table 2).

In contrast, no effect of the phytohormone addition on daily biomass productivity was observed in the control conditions (BG 11). The maximum daily *V. calaminaris* biomass productivity was obtained in the control conditions; it was slightly higher than that in the control conditions with the addition of IAA.

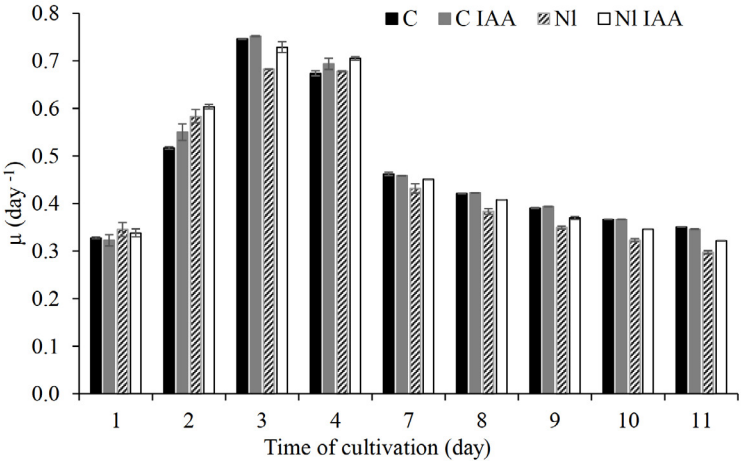


**Fig. 1.** Influence of the growth conditions on the specific growth rate of *V. calaminaris* in the logarithmic phase (0-8 days). C – control conditions (BG11); C IAA – BG11 supplemented with indole-3-acetic (IAA)$10^{-4}$ M; Nl – BG 11 medium with reduced $NaNO_3$ (25%), Nl IAA – BG 11 medium with reduced $NaNO_3$ content (25%) supplemented with indole-3-acetic (IAA)$10^{-4}$ M. The results are presented as the means (9 measurements from three biological replicates ±SD).

**Table 2.** Daily biomass productivity of *V. calaminaris* cultures

| Growth parameter | Growth conditions | | | |
|---|---|---|---|---|
| | C | C IAA | NL | NL IAA |
| Daily biomass productivity (g $L^{-1}$ $day^{-1}$) | 0.800 ± 0.033a | 0.754 ± 0.008a | 0.291 ± 0.020b | 0.662 ± 0.033c |

Data are reported as average ± SD (n= 9), different letters indicate statistical differences at $p \leq 0.01$. Other explanations as in Fig. 1.

The *V. calaminaris* biomass yield at the end of cultivation varied as follows: 13.7 and 13 g $L^{-1}$ for C and C IAA and 5.1 and 11.4 g $L^{-1}$ for the Nl and Nl IAA conditions (Fig. 2).

### 3.2. Characterization of pigments

To understand the effect of IAA on cell metabolism under nutrient stress, the content of chlorophyll *a* and carotenoids was analysed. The content of Chl *a* and carotenoids was significantly stimulated by the addition of the phytohormone in both the nutrient stress and control conditions on the 4th and 10th day of growth. The greatest increase in the chlorophyll *a* and carotenoid levels was observed on the 4th day of cultivation in the simultaneous conditions of nutrient stress and IAA addition (Nl IAA) (Fig. 3a, b). This effect of the IAA addition during nutrient stress on the pigment content showed a similar trend to that exerted on the specific growth rate on the fourth day of cell growth, where the addition of IAA exceeded the inhibitory effect of nutrient stress. The levels of the analysed pigments decreased significantly in response to the nitrogen depletion. The lowest chlorophyll and carotenoid content was found in the nitrogen-limited treatments (Nl).

### 3.3. Photosynthetic activity of *V. calaminaris*

Maximum photochemical quenching of PS II (Fig. 4) was generally lowered in nitrogen limited conditions. The differentiation between treatments of different initial N was increasing with time of from start of culture growth, with N limited being 82-87% of C at 5th day, down to 47-48% of C at 9th day of culture growth. IAA reduced slightly Fv/Fm at 5th day in N limited treatment, however the difference was negligible by the 9th day.

Generally, stress resulting from nitrogen deficiency decreased the potential for dissipation of excessive light energy, which was reflected in higher regulated non-photochemical quenching Y(NPQ) (Fig. 5). This effect was visible between treatments at both the 5th and 9th day of culture growth. However, a substantial general buildup of protective non-photochemical quenching was noted over time, as a response to the decline in photosynthetic capacity. There were negligible effect of IAA on Y(NPQ) in treatments of high N availability, but substantial in N limited treatments, where higher nonphotochemical quenching was noted in IAA supplemented growth medium.

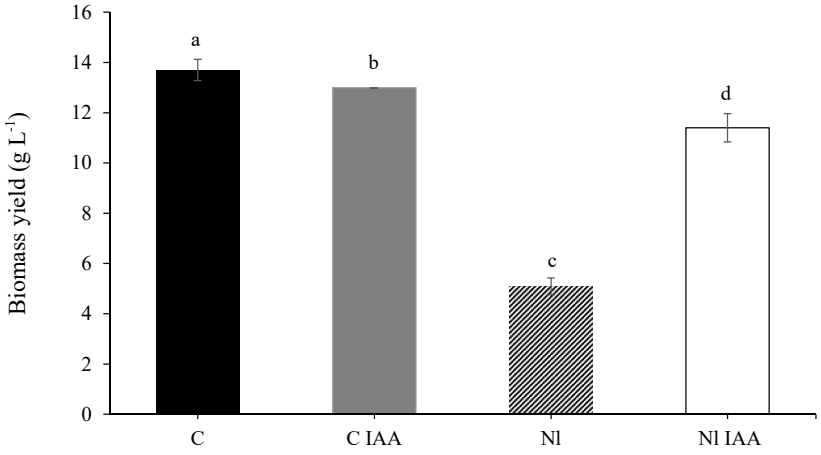


**Fig. 2.** Biomass yield (g $L^{-1}$) of cultures. Data are reported as average ± SD (n= 9). Other explanations as in Fig. 1.

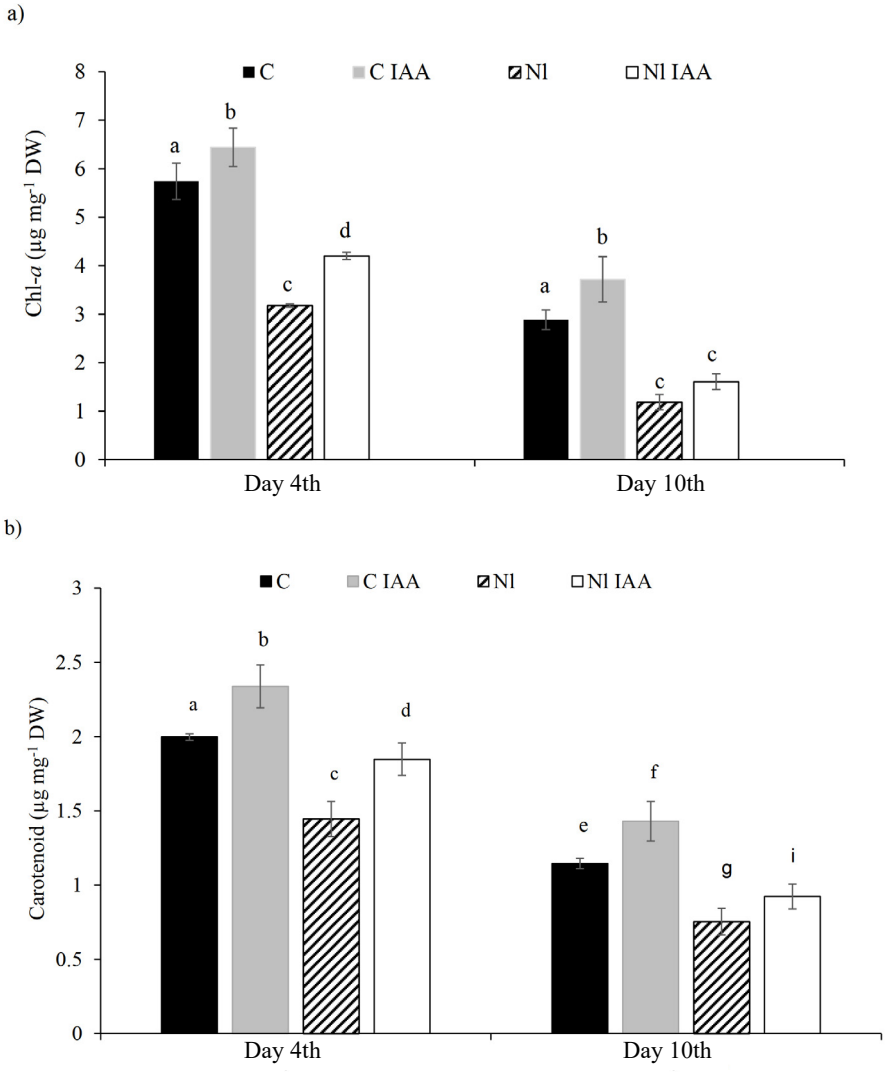


**Fig. 3.** Influence of the growth conditions on chlorophyll *a* a) and carotenoid content b) of *V. calaminaris.* Different letters indicate statistical differences at $p \leq 0.01$. Other explanations as in Fig. 1.

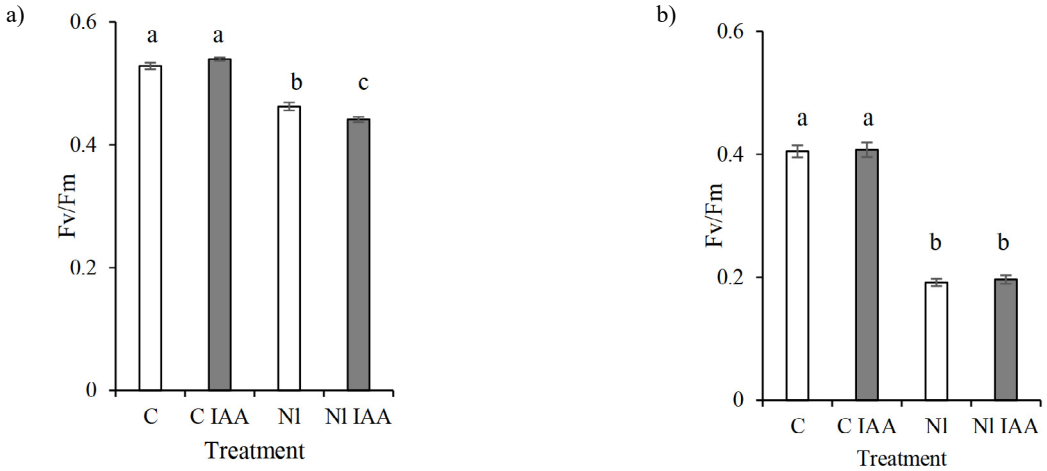


**Fig. 4.** Maximum photochemical quenching of photosystem II (Fv/Fm) at: a) 5th and b) 9th day of culture growth. Means and standard error of means are shown, $n \geq 8$, different letters indicate statistical differences at $p \leq 0.01$. Other explanations as in Fig. 1.

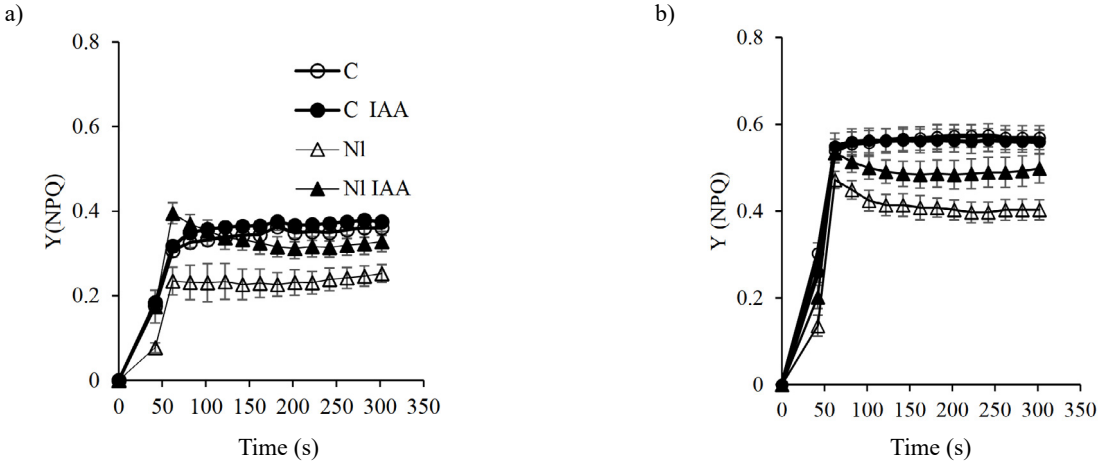


**Fig. 5.** Quantum yield of regulated energy dissipation in photosystem II Y(NPQ) at: a) 5th and b) 9th day of culture growth. Means and standard error of means are shown, $n \geq 4$. Other explanations as in Fig. 1.

### 3.4. Metabolic analysis

#### 3.4.1. Analysis of protein

The effect of the IAA supplementation at different nitrogen concentrations on the protein content in *V. calaminaris* cells is shown in Fig. 6. *V. calaminaris* responded similarly to the IAA supplementation regardless of the nitrogen availability in the growth medium. The exogenous addition of IAA increased the protein content in the microalgal cells. The highest increase in the protein content (a 2-fold increase) was found in the nitrogen-limited variant.

The highest protein content (35.11%) in the *V. calaminaris* cells was observed in cultures grown on the BG 11 medium supplemented with IAA (C IAA). In response to the nitrogen deprivation, the protein content in the *V. calaminaris* cells decreased. The lowest protein content (3.8%) in the *V. calaminaris* cells was found in the nitrogen-deficient conditions.

#### 3.4.2. Lipid accumulation

At the end of the cultivation, the lipid content was determined. In the present study, *V. calaminaris* cultured in the control conditions (C) accumulated 15.25 % lipid content. Higher content of lipids (17.85%) was achieved in the control conditions with the IAA addition. The highest lipid content, *i.e.* 27.40%, was observed in the nitrogen-limited variant supplemented with IAA (Fig. 6).

#### 3.4.3. Fatty acid profiling

The effects of the nitrogen concentrations and the auxin addition on the accumulation and composition of fatty acids were determined at the end of cultivation. The lipidomic analysis revealed that myristic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), and linolenic acid (C18:3) were the major fatty acids (over 1%) (Table 3). The content of fatty acids varied depending on the availability

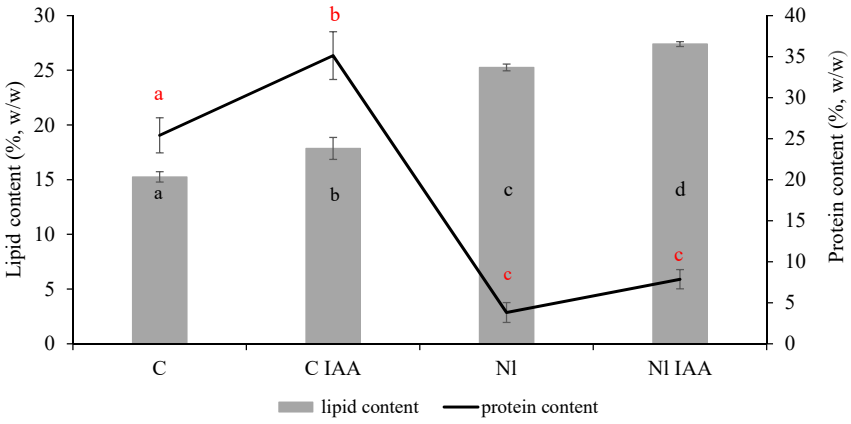


**Fig. 6.** Protein and total lipid content of *V. calaminaris* under different growth conditions. Columns represent total lipid content; black line represents protein content. Data are reported as average ± SD (n = 9). Different letters (red colour for protein content, black colour for lipid content) indicate statistical differences at $p \leq 0.01$. Other explanations as in Fig. 1.

**Table 3.** Fatty acids classes and distribution of *V. calaminaris*

| Distribution of major fatty acids composition (%, of total fatty acids) | Growth conditions | | | |
|---|---|---|---|---|
| | C | C IAA | Nl | Nl IAA |
| C 14:0 | 0.85±0.1 | 0.66±0.06 | 1.29±0.06 | 2.19±0.04 |
| C 16:0 (Palmitic acid) | 24.32±0.66 | 22.96±0.98 | 19.60±0.27 | 17.68±0.63 |
| C 16:1 (Palmitoleic acid) | 20.42±4.90 | 17.83±4.88 | 48.66±4.73 | 55.39±1.43 |
| C 18:0 (Stearic acid) | 2.65±0.05 | 3.00±0.13 | 3.64±0.04 | 1.03±0.18 |
| C 18:1 (Oleic acid) | 38.82±0.23 | 41.13±0.21 | 16.57±2.45 | 21.48±0.81 |
| C 18:2 (Linoleic acid) | 6.02±0.21 | 6.64±1.48 | 6.31±0.64 | 2.47±0.16 |
| 18.3 (Linolenic acid) 3 | 2.55±0.22 | 1.29±0.39 | 1.35±0.55 | 0.66±0.11 |
| Total polyunsaturated acids (PUFA) | 8.41±0.01 | 9.23±1.87 | 6.12±2.72 | 3.45±0.23 |
| Total saturated acids (SFA) | 27.53±0.72 | 26.79±0.09 | 24.53±0.66 | 20.67±0.28 |
| Total monounsaturated acids (MUFA) | 59.24±4.67 | 58.96±4.62 | 62.02±6.25 | 77.91±0.03 |

Data are reported as average ± SD (n = 6). Other explanations as in Fig. 1.

of nitrogen and the presence of the phytohormone in the growth medium. The highest percentages were found for the following acids: oleic acid (21.5-41.1% of the total FAs), palmitoleic acid (17.8-55.4% of the total FAs), and palmitic acid (17.7-24.3% of the total FAs). The study showed significant accumulation of oleic acid in the control and C IAA conditions. High content of oleic acid (over 16%) was also observed in the Nl and Nl IAA conditions.

Palmitoleic acid (C16:1) was accumulated in the nitrogen stress conditions. In the stress-induced microalgal cells, palmitoleic acid production increased twofold compared to the control. The fatty acid profile revealed that the addition of IAA increased the fatty acid content in cells growing under nitrogen stress. The highest content of C16:1 was observed in the nitrogen-depleted conditions and in the presence of IAA. The content of the other main fatty acid – palmitic acid (C16:0) decreased with the N reduction in

the medium and IAA addition. This study showed that the content of polyunsaturated fatty acids in *V. calaminaris* cells was less than 10% of the total acids. The highest concentration of monounsaturated fatty acids (75%) was found in the growth medium with the N reduction and addition of IAA (Nl IAA).

## 4. DISCUSSION

In response to environmental factors, unicellular algae can induce changes in their metabolic activity. Limitation of the availability of a nitrogen source to algal cells in the culture medium is one of the strategies used in algal cultivation to increase lipid biosynthesis. Abiotic stress conditions lead to inhibition of cell growth, which can be caused by the accumulation of an excess of cellular reactive oxygen species (Cointet *et al.*, 2019). Previous research has shown that auxins can influence growth processes, metabolic pathways, and the regulation of oxidative stress responses (Magierek *et al.*, 2017; Gee *et al.*, 2020). Growth of *Desmodesmus komarekii* at similar concentration of IAA and light intensity as in this study was enhanced at 5th day of culture growth, however the bifacial effect decreased with time and at 9th day of algae grown with IAA had similar cell number as control (Chung *et al.*, 2018). On the other hand short effect of IAA (24-72 h) on *Chlorella vulgaris* revealed suppressing effect on algae growth (Piotrowska-Niczyporuk *et al.*, 2014). Much lower doses of IAA were reported to have differential impact on growth of *Picochlorum celery* and *Monoraphidium minutum* 26B-AM cultures. A concentration of 1 μM IAA positively affected both cultures, while an inhibitory effect was observed at 5 μM IAA only in *P. celery* starting from 6th day of growth (Negi *et al.*, 2024).

The current study showed a stimulatory effect of the addition of exogenous IAA on the specific growth rate, biomass yield, and daily biomass productivity in conditions where the nitrogen source in the growth medium was limited by 75%, compared to the control conditions. The available literature reports that the effect of phytohormones on microalgal growth processes depends on the type of phytohormone, its concentration, and growth conditions (Kozlova *et al.*, 2017). As reported in the current study, the addition of the phytohormone to the culture of cells growing under nitrogen limitation eliminated the negative effects of this stress on the specific growth rate (culture days 1-4) and biomass yield (culture day 14) of *V. calaminaris.* The effect of IAA on algal cells also depends on the process of IAA degradation in the aquatic environment (Kozlova *et al.*, 2017). Factors that may affect auxin degradation include long periods of low-intensity white light, oxygen, vitamin B6, and a combination of light with nutrient salts at low pH (Dunlap *et al.*, 1988; Tan *et al.*, 2021). The lack of an effect of the addition of IAA in the control conditions on the *V. calaminaris* growth rate and biomass yield may be related to the light-catalysed gradual destruction of IAA by nitrates (sodium nitrate content in the culture medium 1.5 g $L^{-1}$) through an oxidation process (Dunlap *et al.*, 1988).

The metabolism of autotrophic algae depends on their photosynthetic activity and pigment content: chlorophyll and carotenoids (Vijay *et al*., 2020). In response to the addition of the phytohormone, *V. calaminaris* was able to increase the pigment content (chlorophyll *a* and carotenoids) both in the control and nitrogen stress conditions. Carotenoids are efficient quenchers of excessive light energy in for of heat to avoid photodamage (Accomasso *et al.*, 2024). Carotenoids increases absorbance window and are of importance providing stability of the light harvesting complexes. Increase of accumulation of carotenoids in response to IAA resulted in increased capability to dissipate excessive light energy in response to N stress in Nl IAA in comparison to Nl treatment. Nitrogen deficiency and in consequence N assimilation and transport was shown to be important electron sink in *Synechocystis* sp. PCC 6803 at fluctuating light (Mustila *et al.*, 2021). Periodic changes in light intensity are typical for algae growth conditions in mixed cultures even if light source of constant intensity is used. Differentiated N availability could lead to photoinhibition in N limited growth conditions and the effect was observed to enhance with time and use of available N. It was demonstrated that there is close relationship between the plant ability to evacuate excess electrons accumulated between photosystems and NPQ that was displayed in lower Y(NPQ) in N limited treatments as compared to control (Cardol *et al.*, 2010). While increased carotenoid content in IAA supplemented treatments (Fig. 3) enhanced Y(NPQ) within treatments of specified N availability. Mandal *et al.* (2020) in *G. emersonii* NC-M1 and Yu *et al.* (2018) in *Scenedesmus* SDEC-8 and *Chlorella sorokiniana* SDEC-18 observed that IAA supplementation can reduce oxidative stress resulted from limited N availability through increase in the production of SOD antioxidant enzyme. Here we show that IAA enhanced synthesis of carotenoids in *V. calaminaris* is another potential mechanisms limiting oxidative stress resulting from nitrogen deficiency and related to IAA. Carotenoids are effective scavengers of singlet oxygen that targets unsaturated fatty acids of membrane lipids (Kruk and Szymańska, 2021).

Increased chlorophyll content can increase metabolic processes and the accumulation of intracellular metabolites, *i.e*. sugars and proteins (Stirk and van Staden, 2020). The increased protein content in the *V. calaminaris* cells observed in this study after the application of IAA confirms this information. Vijay *et al.* (2020) observed that supplementation of 100 μM IAA increased chlorophyll *a* and carotenoid content in *Ankistrodesmus falcatus* compared to the control conditions. Studies on the effects of IAA on *Scenedesmus quadricauda* have shown a stimulating effect of this phytohormone on the biosynthesis of pigments (chlorophyll-*a* and total carotenoids), lipids, and

fatty acids (Kozlova *et al.*, 2017). An IAA-induced increase in the chlorophyll content of *Scenedesmus* sp. LX1 in nitrogen-replete conditions was also reported by Dao *et al.* (2018). The protein and pigment levels observed in the present study decreased in the nitrogen stress conditions. Park *et al.* (2013) reported chlorosis and degradation of protein in *Chlamydomonas reinhardtii* in nitrogen depletion conditions.

The application of abiotic stress, including the limitation of the nitrogen source in the medium, is one of the strategies for increasing the synthesis of neutral lipids in algal cells used to produce biofuels (Krzemińska *et al.*, 2023). The main weakness of this solution is the limitation of the cell growth rate, which ultimately reduces biomass production (Stirk and van Staden, 2020). Studies on the effect of IAA on microalgal lipid content are inconsistent. Several reports have indicated a stimulating effect of exogenous IAA supplementation on the lipid synthesis in *Scenedesmus quadricauda* (Liu *et al.*, 2016) and *Chlorella sorokiniana* (Babu *et al.*, 2017). Previous reports suggest that phytohormones lead to an increase in lipid content also in nitrogen-limited conditions by increasing the activity of lipid biosynthesis enzymes (Ajayan *et al.*, 2022). Consistent with these results, the lipid content in *V. calaminaris* in the nitrogen-limited conditions was more than 1.5 times that of the control conditions in this study. In turn, the addition of IAA had a lesser effect on the lipid content, which increased by approximately 2%. Udayan *et al.* (2018) showed that *Nannochloropsis oceanica* cells exposed to 40 ppm IAA almost doubled the lipid content, compared to the control culture. In contrast, IAA supplementation did not significantly change the total lipid content in *Ankistrodesmus falcatus*, regardless of the concentration used (from 0.01 to 100 μM) (Vijay *et al*., 2020). It was found in the current study that the strategy of nitrogen limitation and IAA addition in the high light intensity conditions led to accumulation of palmitoleic acid (55%) in the *V. calaminaris* cells (Table 3). These findings are consistent with the results obtained in a study conducted by Wang *et al.* (2018), where nitrogen deficiency promoted the accumulation of palmitoleic acid (29.17%) in eustigmatophytes. Palmitoleic acid is indicated as a favourable substrate for biodiesel production (Wang *et al.*, 2018; Pinzi *et al.*, 2009). Palmitoleic acid is also classified as a functional fatty acid due to its beneficial properties for human health, *e.g.* protection of the cardiovascular system, reduction of liver inflammation and the level of inflammatory cytokines, and mitigation of insulin resistance (Wang *et al.*, 2018).

It was found in the present study that IAA played a role in enhancing the oleic acid content. The highest content of oleic acid (over 41%) in the *V. calaminaris* cells was observed in the conditions of 100% N and the IAA addition. In turn, the content of oleic acid in the NL fraction of *V. calaminaris* lipids grown in light/dark cycle conditions (18 h/6 h) at lower light intensity than that in the present study (80 mol photons $m^{-2}$ $s^{-1}$, medium BG 11) was only 8% (Krzemińska *et al.*, 2023). These results suggest that the addition of IAA and the light conditions (high light intensity and continuous light) contributed to the increase in the accumulation of oleic acid in the present study. A similar effect of high light intensity on the increase in the oleic acid content was observed in *Chlorella vulgaris*, *Desmodesmus* sp., and *Scenedesmus obliquus* (Nzayisenga *et al.*, 2020). Oleic acids were found to accumulate in response to high light intensity in the cells of the green alga *Chlorella protothecoides* (Krzemińska *et al.*, 2015). In algal cells, PUFA synthesis occurs by converting oleic acid to linoleic acid, which can then be converted to α-linolenic acid (Udayan *et al.*, 2020).

The analysis of the fatty acid profiles revealed low content of polyunsaturated fatty acids in the *V. calaminaris* cells, which was associated with the culture conditions used, *i.e.* continuous light and high light intensity. Consistent with these results, the loss of the ability to synthesise polyunsaturated fatty acids by unicellular algae cultivated at high light intensity and in combination with nitrogen limitation was confirmed by Cointet *et al.* (2019). Polar lipids associated with chloroplast membranes are synthesised in response to low light conditions. Their content was found to decrease with increasing light intensity (Chin *et al.*, 2023). PUFAs are involved in regulation of cell membrane fluidity, and reduction of their level increases membrane rigidity (Krzemińska *et al.*, 2023). The decrease in the content of polyunsaturated fatty acids in the conditions of nutritional stress observed in the present study is in line with the findings in *P. tricornutum* cells (Yang *et al.*, 2014). Nitrogen deficiency results in a decrease in membrane lipids and an increase in TAG levels. The remodelling of membrane lipids results in changes in the fatty acid profile (Zulu *et al.*, 2018). It was found in the current study that monounsaturated fatty acids accounted for 58-77% while saturated fatty acids represented from 20 to 27% of the total fatty acids in *V. calaminaris*. The increase in the level of monounsaturated fatty acids was observed in the conditions of the simultaneous limitation of nitrogen and the addition of IAA.

## 5. CONCLUSIONS

This study provides information on the accumulation of cellular metabolites, photochemical characteristics and changes in the fatty acid profile of a non-model soil alga in response to nitrogen stress and indole-3-acetic acid (IAA) supplementation under high light intensity. The high content of monounsaturated fatty acids under the nitrogen limitation and IAA addition and the high content of palmitoleic acid these conditions indicate that *V. calaminaris* is a strain with biotechnological importance for biodiesel application and nutraceutical production.

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**Conflict of interest statement.** The authors declare no conflicts of interest.

**Data availability.** The datasets used and analysed during the current study available from the corresponding author on reasonable request.

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