# Green microalgae chlorella in the study of the biosynthetic potential of higher plants *in vitro*

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**Abstract.** Biostimulants and biofertilizers based on macro- and microalgae have managed to establish themselves as an actual replacement for toxic substances, demonstrating high efficiency on plants of various taxonomic groups. There is an unique organic product chlorella (*Chlorella vulgaris*), a green microalgae containing more than 650 substances, which creates the prerequisites for its effective use as a substrate for the propagation of cultures *in vitro*. A method has been developed for propagating plants of different taxonomic groups *in vitro*. It has been shown that there is a difference in the perception of chlorella as a source of nutrients depending on the taxonomy group of plants.

## 1 Introduction

Microalgae are microscopic single-celled plants that have high rates of growth and photosynthetic efficiency and relatively low natural resource requirements compared to traditionally grown crops [1].

Now they are becoming more relevant due to the many advantages: food ingredients from them do not contain GMOs, they are organic and reduce the negative impact of the anthropogenic factor on the environment. For the production of products from them, there is no need for deforestation, so these technologies are in line with the current global trend for environmental sustainability. In addition, microalgae production has a low carbon, land and water footprint.

As for the methods of cultivating microalgae, these organisms require the main chemical elements for nutrition (nitrogen, oxygen, hydrogen, potassium, magnesium, sulfur, phosphorus, etc.), water and sunlight for the photosynthesis, however they do not need fertile soil to grow, because they are cultivated in suspension. Most often, systems of open (or semi-open) reservoirs or closed photobioreactors in the form of various installations are used [2].

Chlorella (*Chlorella* sp.) is a green eukaryotic microalgae. The microscopic cell is spherical, 2–10 µm in diameter. This microalgae is one of the most important and promising for biomass production [3].

Chlorella contains a pool of biologically active substances: about 50% protein (including essential amino acids); a complex of essential unsaturated fatty acids (including

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Omega-3); vitamins (A, B1, B2, B3, B5, B6, E) and macro- and microelements. This creates the prerequisites for its commercial production for use in agriculture, medicine, cosmetology and other fields.

Many known species of microalgae are quite demanding in terms of cultivation conditions: the level of illumination, the composition of the nutrient medium, the concentration of carbon dioxide, and mechanical mixing, which greatly hinders their effective cultivation. However, the green single-celled microalgae chlorella has been an object of biotechnology for quite a long period of time and is widely used as a vitamin and mineral supplement for farm animals, poultry and fish, as well as in the production of drugs in the chemical and pharmaceutical industries, which determines the choice of this microalgae as a source of valuable compounds for studying its effect on the biosynthetic potential and morphophysiological parameters of plants.

As a rule, when cultivating cell cultures *in vitro*, various nutrient media differing in mineral composition are used. Moreover, the mineral composition of the nutrient medium may differ for plants of different taxonomic groups. For example, a nutrient medium containing mineral salts according to Murashiga and Skoog's recipes is used for *in vitro* cultivation of agricultural, medicinal, and some berry plants; WPM (Woody Plant Medium) nutrient medium is used for *in vitro* cultivation of tree species [4], and Hamburg or Nitsch nutrient medium is used to obtain haploid crop plants *in vitro* [5]. However, the proposed nutrient media are not optimal for growing *in vitro* cell cultures of some plant species, and their modification is required for use in cell engineering.

Currently, research on the cultivation of isolated plant tissues and cells *in vitro* is searching for alternative nutrient media and organic biostimulants that reduce the cost of the biotechnological process and promote satisfactory growth of cell cultures of higher plants *in vitro*. The use of nutrient media with an organic composition is a potentially commercially effective way to avoid the use of expensive components of nutrient media while maintaining and increasing the biosynthetic potential of cell cultures of higher plants *in vitro* [5,6,7].

An unique organic product is chlorella (*Chlorella vulgaris*), a green microalgae containing more than 650 substances, which creates the prerequisites for its effective use as a substrate for the propagation of cultures *in vitro*.

One study assessed the *in vitro* propagation and establishment of *S. crispa* (*Schomburgkia crispa* Lindley (*Orchidaceae*), an epiphytic species native to the Brazilian Cerrado) in a modified growth medium containing an extract of the microalgae *Chlorella sorokiniana*. Supplementation of WPM (Woody Plant Medium) with microalgae suspended in NPK medium, or as the supernatant resulting from the centrifugation of a culture in NPK medium, was analyzed. The extracts were added to WPM instead of distilled water. The compounds 6-benzylaminopurine (BAP) and indolebutyric acid (IBA) were used as reference in the in vitro multiplication and rooting of *S. crispa*, respectively. Both growth regulators were tested at 0, 2.5, and 5.0 mg $L^{-1}$. During in vitro multiplication of *S. crispa*, WPM supplemented with 5.0 mg $L^{-1}$ BAP favored the formation of more sprouts, whereas WPM containing 2.5 mg $L^{-1}$ IBA supplemented with microalgae extract stimulated *in vitro* rooting. *S. crispa* explants cultivated in medium supplemented with microalgae suspension or the supernatant of *C. sorokiniana* showed growth similar to explants cultivated in WPM alone. Therefore, it is possible to use the microalga *C. sorokiniana* as a supplement and/or alternative to WPM for the *in vitro* cultivation of *S. crispa* [6].

The effect of microalgae *Messastrum gracile* and *Chlorella vulgaris* on the *in vitro* reproduction of *Cattleya labiata* orchids was studied in another research [7]. The aim of this study was to evaluate the effect of two green microalgae (*M. gracile* and *C. vulgaris*) in comparison with plant growth regulators (6-benzylaminopurine, BAP; thidiazuron, TDZ; zeatin, ZEA) on the *in vitro* propagation of *C. labiata*, an endangered orchid, using the thin cell layer (TCL) technique from protocorms. TCL sections were cultivated in MS/2 medium containing *M. gracile* extract (EM) and biomass (BM); *C. vulgaris* extract (EC) and biomass (BC); and BAP, TDZ, and ZEA in different concentrations. Subsequently, the explants were grown in MS/2 medium, with 2 g L−1 of activated charcoal, to induce elongation and roots formation. For acclimatization, plants were transplanted in trays using *Sphagnum sp.* as substrate. TCL explants showed a higher formation of protocorm-like bodies (PLBs) than entire protocorms. Explants cultivated in media supplemented with BM showed a high rate of PLB regeneration (59%) and high mean number of PLBs (4 per explant) and 85% survival after acclimatization of the plants. Supplementation with BAP stimulated similar morphogenic responses to those observed with BM and superior results obtained with ZEA and TDZ. Mass propagation of *C. labiata* plants was successfully achieved using TCL, and it is recommended to supplement the MS/2 medium with 4 g L−1 of BM or 4 mg L−1 of BAP. Microalgae extracts and biomasses are effective alternatives for in vitro propagation of *C. labiata* that can replace plant growth regulators, as they favored the formation of PLBs and plants.

To fully exploit the commercial potential of microalgae biotechnology, bottlenecks in biological, agronomic, economic and technological fields need to be addressed [8]. Recently, many companies in Spain (Agroplasma, AlgaEnergy, Agrialgae, Allgrow and Biorizon biotech), Turkey (Mikroalg Inc. and MCT Tarim Ltd.), USA (AgroValley Inc.), Hungary (Natur Agro) and India (Soley Biotech, Hindustan bioenergy Ltd.) have advanced their research and increased investment in the commercialization of biostimulants and biofertilizers from microalgae. Extracts of microalgae such as *Chlorella*, *Arthrospira*, *Scenedesmus*, *Haematococcus* and *Nannochloropsis* are mainly studied [9-11].

## 2 Methods

The object of the study was a strain of chlorella with a thick cell wall (*C. vulgaris* Beijer), used as a component of a biostimulator for plants. The composition of the biostimulant includes: cultural aquatic medium, microalgae *C. vulgaris*.

We have developed a method for *in vitro* cultivation of plants of different taxonomic groups on a nutrient medium containing a suspension of the microalgae *C. vulgaris*.

Eight variants of nutrient media were created: 1) a suspension of chlorella without the addition of phytohormones, 2) a suspension of chlorella with the addition of 2-(2,4-dichlorophenoxy)acetic acid (2,4-D) at a concentration of 1 mg $L^{-1}$ and 6-benzylaminopurine (BAP) at a concentration of 0.3 mg $L^{-1}$, 3) chlorella suspension with the addition of 1-Naphthaleneacetic acid (NAA) at a concentration of 1 mg $L^{-1}$ and BAP at a concentration of 0.3 mg $L^{-1}$, 4) chlorella suspension with the addition of 3-Indoleacetic acid (IAA) at a concentration of 1 mg $L^{-1}$ and BAP at a concentration of 0.3 mg $L^{-1}$, as well as 5) Murashiga and Skoog's medium without the addition of phytohormones, 6) Murashiga and Skoog's medium with the addition of 2,4-D at a concentration of 1 mg $L^{-1}$ and BAP at a concentration of 0.3 mg $L^{-1}$, 7) Murashiga and Skoog's medium with the addition of NAA at a concentration of 1 mg $L^{-1}$ and BAP at a concentration of 0.3 mg $L^{-1}$, 8) Murashiga and Skoog's medium with the addition of IAA at a concentration 1 mg $L^{-1}$ and BAP at a concentration of 0.3 mg $L^{-1}$. All medium contained sucrose at a concentration of 20 g $L^{-1}$, pH=5.6-5.8.

Chlorella-based nutrient medium as well as Murashiga-Skoog nutrient medium with appropriate amounts of phytohormones were used to grow 5 taxonomic plant species: *Ipomoea batatas* – varietiy Vinnitsky rozovyy, *Mentha ×piperita* – variety Simferopolskaya, *Ocīmum basilīicum*, *Alternanthera reineckii roseafolia* and *Oldenlandia salzmannii* (DC.) Benth. & Hook.f. ex B.D.Jacks. Plants were germinated in plastic containers with a volume of 250 ml in a light room, where the temperature was maintained at 23°C, a 16-hour photoperiod, and illumination with white fluorescent lamps. The results were taken on the 30th day.

The studies were carried out in 5 biological and 5 analytical replicates. Averages of all data were calculated using Microsoft Excel 2013 (Microsoft Corporation, USA). Analysis of variance (ANOVA) was performed using Statistica version 10.0 and means were compared using Fisher's least significant difference (LSD) test at a $p \leq 0.05$ significance level.

## 3 Results

The results of the study of the different taxonomic groups plants microcuttings in the *in vitro* culture allowed us to note the difference in the biometric parameters of them (Table 1).

**Table 1.** Influence of chlorella suspension and Murashiga and Skoog's medium with the various content of plant hormones on the biometric parameters of microcuttings.

| Medium type | Chlorella suspension | | |
|---|---|---|---|
| | Leaf length, cm | Root length, cm | Stem length, cm |
| | *I. batatas* | | |
| 1 | 1,00±0,05 | 0 | 0 |
| 2 | 0 | 0 | 0 |
| 3 | 0 | 0 | 0 |
| 4 | 0 | 0 | 2,01±0,10 |
| 5 | 3,00±0,15 | 27,07±1,35 | 4,02±0,20 |
| 6 | 0 | 0 | 0 |
| 7 | 2,00±0,10 | 14,02±0,70 | 1,52±0,08 |
| 8 | 1,50±0,08 | 8,03±0,40 | 1,03±0,05 |
| | *M. ×piperita* | | |
| 1 | 0,50±0,25 | 1,00±0,05 | 1,01±0,05 |
| 2 | 0,30±0,02 | 1,00±0,05 | 1,02±0,05 |
| 3 | 0,80±0,04 | 2,50±0,13 | 4,08±0,20 |
| 4 | 0,70±0,04 | 1,30±0,07 | 1,51±0,08 |
| 5 | 1,50±0,08 | 5,04±0,25 | 6,03±0,30 |
| 6 | 0,50±0,03 | 0 | 0,81±0,04 |
| 7 | 1,00±0,05 | 6,08±0,30 | 5,02±0,25 |
| 8 | 1,00±0,05 | 6,02±0,30 | 3,00±0,15 |
| | *O. basilīicum* | | |
| 1 | 3,03±0,15 | 1,02±0,05 | 1,00±0,05 |
| 2 | 0,50±0,03 | 0 | 1,00±0,05 |
| 3 | 1,01±0,05 | 2,02±0,10 | 2,11±0,10 |
| 4 | 0,81±0,04 | 0 | 1,50±0,08 |
| 5 | 2,02±0,10 | 5,22±0,25 | 3,21±0,15 |
| 6 | 0 | 0 | 0 |
| 7 | 0 | 0 | 0 |
| 8 | 0 | 0 | 0 |
| | *A. reineckii roseafolia* | | |
| 1 | 3,21±0,15 | 0,11±0,01 | 0,21±0,01 |
| 2 | 2,50±0,13 | 0 | 0,21±0,01 |
| 3 | 2,11±0,10 | 0,52±0,03 | 2,08±0,10 |
| 4 | 2,51±0,13 | 1,54±0,08 | 1,51±0,08 |
| 5 | 2,52±0,13 | 3,09±0,15 | 3,11±0,15 |
| 6 | 0 | 0 | 0 |
| 7 | 2,04±0,10 | 2,06±0,10 | 2,08±0,10 |
| 8 | 1,50±0,08 | 1,51±0,08 | 2,06±0,10 |
| | *O. salzmannii* (DC.) Benth. & Hook.f. ex B.D.Jacks. | | |
| 1 | 0,50±0,03 | 4,51±0,20 | 2,07±0,10 |

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| | | | |
|---|---|---|---|
| 2 | 0,52±0,03 | 7,09±0,35 | 7,04±0,35 |
| 3 | 0,61±0,03 | 5,06±0,25 | 11,22±0,55 |
| 4 | 1,08±0,05 | 4,02±0,20 | 2,52±0,13 |
| 5 | 1,51±0,08 | 9,11±0,45 | 11,08±0,55 |
| 6 | 0,52±0,03 | 0 | 1,02±0,05 |
| 7 | 0,71±0,04 | 3,03±0,15 | 5,32±0,25 |
| 8 | 1,08±0,05 | 5,01±0,25 | 6,54±0,3 |

When using a suspension of chlorella, the yellowing of the *I. batatas* microcuttings and further death of the explants were observed, as can be seen in Figure 1.


**Fig. 1.** Microcuttings of *I. batatas*. From left to right: from the medium 1 to the medium 8.

Similar results were observed in the case of *M. piperita*, however, in this case, the use of 2,4-D at a concentration of 1 mg/L and BAP at a concentration of 0.3 mg/L also resulted in microclone death, that is shown in Figure 2.

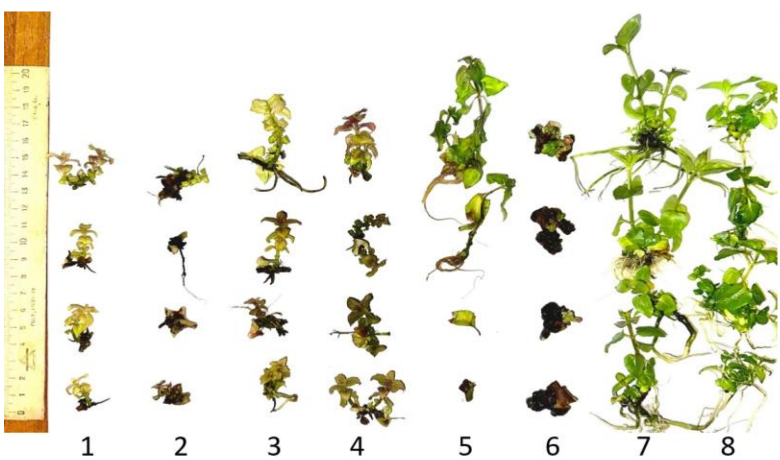


**Fig. 2.** Microcuttings of *M. piperita*. From left to right: from the medium 1 to the medium 8.

The use of nutrient media 7 and 8 led to the stable formation of callus in the *O. basilīicum*, while the use of the same hormones in the same concentrations, but on a chlorella-based medium led to the active formation of roots and aerial parts of plants, but not callus, as can be seen in Figure 3.

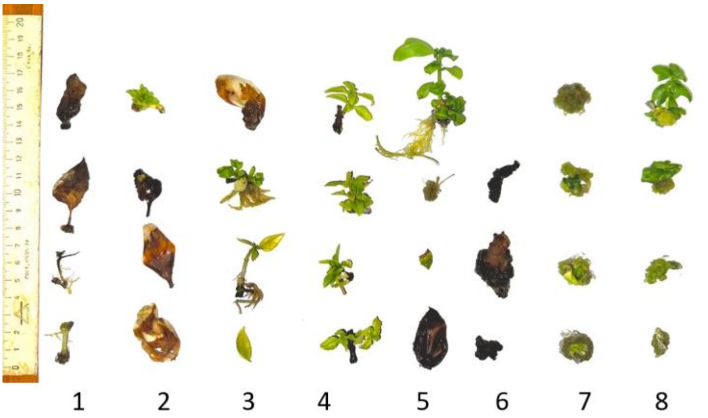


**Fig. 3.** Microcuttings of *O. basilīicum*. From left to right: from the medium 1 to the medium 8.

Alternanthera plants are known for their sensitive response to the environment in which they are grown, manifested by changes in the color of the plant. In this case, the plants grown on chlorella had a pink tint, in contrast to the dark green or red plants grown on Murashiga and Skoog's medium (Figure 4).

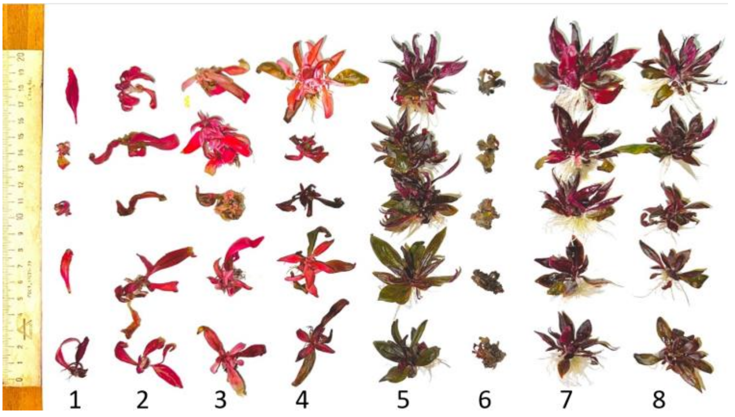


**Fig. 4.** Microcuttings of *A. reineckii roseafolia*. From left to right: from the medium 1 to the medium 8.

The best result was obtained when growing the aquatic plant *O. salzmannii* (DC.) Benth. & Hook.f. ex B.D.Jacks., where we could observe sustained growth of shoots and roots, even more active than in the case of Murashiga and Skoog's medium (compare media 3 and 7 containing similar concentrations of hormones). In addition, the chlorella-based nutrient medium is characterized by an atypical effect of phytohormones on the plant: in contrast to Murashiga and Skoog's medium with the addition of 2,4-D at a concentration of 1 mg/L and BAP at a concentration of 0.3 mg/L, at which callus was formed, the medium a suspension of chlorella with the addition of 2,4-D at a concentration of 1 mg/L and BAP at a concentration of 0.3 mg/L did not form callus, but on the contrary, healthy plants developed with large roots, stems, leaves (Figures 5-6).


**Fig. 5.** Microcuttings of *O. salzmannii*. From left to right: from the medium 1 to the medium 4.


**Fig. 6.** Microcuttings of *O. salzmannii*. From left to right: from the medium 5 to the medium 8.

## 4 Discussions

In the last twenty years, there has been an explosive interest in biotechnology based on the cultivation of photoautotrophic microorganisms - cyanobacteria and microalgae - which are a promising source of environmentally friendly and renewable raw materials. Using atmospheric carbon dioxide and sunlight, these objects are able to synthesize a wide range of unique molecules and accumulate valuable bioproducts in their cells: antioxidants, vitamins, polyunsaturated fatty acids, dyes, polysaccharides, UV protectors. In countries with a warm climate, many large enterprises for the cultivation of microalgae have appeared, for example, Cyanotech, Mera Pharmaceuticals Inc. (Hawaii), Jingzhou Natural Astaxanthin Inc (China), Algaetech International (Malaysia), Parry Nutraceuticals (India), etc. In the Russian Federation, there is currently no mass cultivation of microalgae, although the natural and climatic conditions of the Black Sea coast of the Krasnodar Territory, Crimea, Stavropol Territory, are suitable for organizing such production.

At the same time, microalgae and products of their processing are widely used both in animal husbandry and in crop production in a number of countries, and their consumption in this direction is increasing from year to year. One of the most promising areas is their use in agriculture as biofertilizers and biostimulants. Algae extracts contain a large amount of cytokinins, betaine, algopolyphenols, vitamins, hormones, natural antioxidants and mineral elements (Mg, Ca, B, Mo, etc.) that promote plant growth and development and improve soil fertility. The biomass of microalgae is considered as an organic fertilizer, which compares favorably with traditional species, since it contains neither pathogenic microflora, nor weed residues, nor pests. Microalgae are successfully used to improve soil fertility, to replenish organic matter, which helps to increase crop yields.

The biostimulating effect of a suspension of chlorella was demonstrated during the active growth of *O. salzmannii*, which is most likely explained by the fact that it is an aquatic plant, and *C. vulgaris* is an algae, which allows them to enter into complex mutually beneficial relationships.

## 5 Conclusion

Chlorella is an actual object of study due to its wide use in various areas of the national economy, such as agriculture.

The use of a nutrient medium based on a suspension of chlorella in the study of biometric parameters of different taxonomic groups plants microcuttings showed different results: leaf mass and volume, stem and root length increased in plants of *O. salzmannii*, while in *I. batatas* – varietiy Vinnitsky rozovyy, *M. piperita* – variety Simferopolskaya, *O. basilīicum*, *A. reineckii roseafolia* the growth decreased, and in several cases the yellowing of microcuttings and further death of the explants were observed, which indicates a difference in the perception of chlorella suspension by plants of different taxonomic groups.

## References

1. Q. Hu, M. Sommerfeld, E. Jarvis, M. Ghirardi et al., Plant J., **54(4)**, 621-39 (2008)
2. I. Dudina, E. Kalashnikova, R. Kirakosyan, E3S Web of Conference, **376**, 02026 (2023)
3. M. Yamamoto, I. Kurihara, S. Kawano, Planta, **221(6)**, 766-75 (2005)
4. I.I. Koncevaya, Lesovedenie, **5**, 50-56 (2009)
5. E.A. Kalashnikova, R.N. Kirakosyan, *Kul'tura tkaney i kletok rasteniy* (KnoRus, Moscow, 2023)
6. N.S. Pereira, B.R.R. Ferreira, E.M. Carvalho et al., J. Appl. Phycol., **30**, 2347 (2018)
7. J.R. Corbellini, L.L.F. Ribas, F.R Maia et al, J. Appl. Phycol, **32**, 4013 (2020)
8. V. Barone, A. Baglieri, P. Stevanato, C. Broccanello, G. Bertoldo, M. Bertaggia, M. Cagnin, D. Pizzeghello, V. M. C. Moliterni, G. Mandolino, F. Fornasier, A. Squartini, S. Nardi, G. Concheri, J. Appl. Phycol., **30**, 1061–1071 (2018)
9. F. G. Aci´en, ´ C. Gomez-Serrano, M. M. Morales-Amaral, ´ J. M. Fernandez-Sevilla, E. MolinaGrima, Appl. Microbiol. Biotechnol., **100**, 9013–9022 (2016)
10. J. de S. Castro, M. L. Calijuri, P. P. Assemany, P. R. Cecon, I. R. de Assis, V.J. Ribeiro, Sci. Total Environ., **574**, 1640–1648 (2017)
11. E. A. N. Marks, O. Montero, C. Rad, Sci. Total Environ., **692**, 784–790 (2019)