MICRO-ALGAE BASED PLANT BIOSTIMULANT AND ITS EFFECT ON WATER STRESSED TOMATO PLANTS

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Abstract: A cascade process was developed for the production of a micro-algae based plant biostimulant. This process is intended to assure a high recovery of the main components of plant biostimulant (phytohormones, betaines, oligosaccharides elicitors, micro-algal protein hydrolysates), without affecting yield of micro-algae lipids extraction. The main steps of the process are: micro-algae cell walls lysis by pressure homogenization and hydrolysis with mixture a lytic enzymes; separation of phytohormones, osmoprotectants, free amino acids and solubles carbohydrates by tangential ultrafiltration; extraction of lipids from ultrafiltration retentate; enzymatic hydrolysis of protein from defatted retentate; mixing protein hydrolisate with ultrafiltrate retentate, resulting the micro-algae based plant biostimulant. The composition of the resulted plant biostimulant dry matter is: 88.26% hydrolyzed algal protein, from which 5.63% is proline, 9.41% carbohydrates, 2.23 others compounds, including 0.023% total betaines and 0.012% cytokinins, kinetin activity equivalents. Micro-algae based biostimulant was tested on water stressed and non-water stressed tomato plants. In non-water stressed plants the biostimulant obtained from micro-algae determined a better development of root length (108.08% control), and an increased leaves number (120.31% control) and of leaves area (105.16% control), comparing to a commercial seaweed extract – 105.98% control, 106.25% control, and, respectively, 104.45%. On leaves number and leaves area the differences are statistically significant, micro-algae based bio-stimulant being more active. These stimulatory effects of both algae based biostimulants compensate the negative influence of water stress. On water stressed tomato plant height and root length decrease by almost 20% (from an average of 58.45 cm on control, well watered and not-treated with biostimulants, to 48.87 cm on water stressed tomato plant and not treated with plant biostimulants) and, respectively, by more than 25% (from 58.82 cm average on control plants, to 39.85 cm on water stressed tomatoes). Application of algae based biostimulant alleviated the effects of water stress on plants root development and reduced by almost 50% the negative influences on plants height. Average production of treated tomato plants (expressed as production per 30 days cycle of flowering and fructification) is similar for both tested macro- and micro- algae based plant biostimulants. Mechanisms of action of the main components of algae plant biostimulants are discussed and further solutions for optimization of micro-algae based biostimulant are envisaged.

Key words: plant biostimulant, micro-algae biomass, mixotrophic culture, water stressed tomatoes

INTRODUCTION

Seaweeds (macro-algae) have been used for the production of plant biostimulants, i.e. of bioproducts which contain substances "whose function, when applied to plants or the rhizosphere, is to stimulate natural processes, to enhance/benefit nutrient uptake, nutrient efficiency, tolerance to abiotic stress, and crop quality". (European Biostimulant Industry Council, EBIC 2014). Macro-algae contain important quantities of plant growth hormones, auxins, abscisic acid, cytokinins, gibberellins (Tarakhovskaya *et al*., 2007). Cytokinins have been proved to be one of the main active ingredients in seaweed extracts used as plant biostimulants. In macro-algae biomass endogenous *cis*-zeatins generally predominated over the *trans*-zeatins (Stirk *et al*., 2003) Commercial seaweed extracts, especially those

made from *Ascophyllum nodosum* and *Ecklonia maxima*, contain between 0.1-1.0 $mg.l^{-1}$ total cytokinins (Craigie, 2011). Beside cytokinins seaweed extracts contain also abscisic acid, gibberellins and brassinosteroids (Stirk *et al*., 2014).

In the macro-algae extracts, obtained through processes which involve release of the cellular content after cell rupture under rapid change in pressure, were identified also important quantities of signaling compounds involved in plant response to biotic and abiotic stresses. Seaweed extracts contain polyamines, mainly putrescine and spermidine (Papenfus *et al*., 2012). Polyamines are ubiquitous active signal factors, required for different processes in plant development and which participate in plant abiotic and biotic stress responses (Kusano *et al*., 2008). Betaines, plant signaling compounds which are not traditionally considered among the classical plant hormones, are also widely distributed in commercial seaweed extract (MacKinnon *et al*., 2010). Betaines sustain cultivated plant response to abiotic stress (Chen and Murata, 2008).

Micro-algae, which could be cultivated, represent a more sustainable alternative to macro-algae, which right now are gathered from sea. Macro-algae breeding, for enhanced biostimulants production, it is not yet an option, but micro-algae strains could be selected for various purposes, including an optimized production of plant biostimulants. Micro-algae have been largely investigated as a practical approach for the production of lipids derived biofuels, with concomitant mitigation of CO_2 from industrial emissions, and for waste water treatments. Micro-algae biomass contains also important quantities of plant hormones and plant stress factors, polyamines and betaines. Stirk *et al*. (2013a) quantified auxins and cytokinins in 24 axenic micro-algae strains from the *Chlorophyceae*, *Trebouxiophyceae*, *Ulvophyceae,* and *Charophyceae* families. The general trend was that *cis*-zeatin types were the predominant cytokinins, similar to macro-algae. Several strains, with total cytokinin concentrations which reach more than 20 $nmoli.g^{-1}$, (corresponding to more than 4 $mg.Kg^{-1}$) could represent a cytokinins source, substituting less sustainable macro-algae bio-resource. In 15 strains from 21 analysed, the auxins content was 2- to 4-fold higher than the cytokinins content. Micro-algae contain also important quantities of gibberellins and brassinosteroids. Slower growing strains of micro-algae (like *Scotiellopsis terrestris* MACC 44) accumulate higher endogenous gibberellins and brassinosteroids than faster growing strains (*Raphidocelis subcapitata* MACC 317 and *Coelastrum excentrica* MACC 504) (Stirk *et al*., 2013b). Polyamines were reported to be produced in mixed cultures of *Chlorella* (Tate *et al*., 2013). Micro-algal strains were shown also to accumulated different zwitterionic metabolites, including glycine betaine (GBT) as response to osmotic stress (Gebser and Pohner, 2013).

There are already reported beneficial effects of micro-algae extracts on stressed cultivated plants. Extracts from *Dunaliella salina* and *Phaeodactylum tricornutum* mitigated salt stress during germination process of bell pepper (Guzmán-Murillo *et al*., 2013), due to a significant reduction in superoxide radical production and of a lower lipid peroxidation. Water extract of micro-algae *Spirulina maxima* and *Chlorella ellipsoida* improve wheat tolerance to salinity and enhanced antioxidant capacity of the whole grains produced by treated plants (El-Baky *et al*., 2010).

In algal biomass the active ingredients, phytohormones and plant stress signal factors, are enclosed inside the cell walls and/or bound to various structures, which significantly reduce their biological activity (Craigie, 2011). For an improved recovery of active ingredients and their release from the structures wherein these are bound, macro-algae biomass are submitted to different processes (recently reviewed by Shekar Sharma *et al*., 2014), including cryo-processing and cell rupture with high pressure treatments. In micro-algae, a higher ratio of cell walls requires more advanced treatments for an enhanced cell rupture. Enzymatic treatments, with a lytic enzymes mixture including proteases, could also produce microalgal protein hydrolisate. Protein hydrolysates are also included among the active ingredients of plant biostimulants (Calvo *et al*., 2014), and their presence into resulted micro-algae plant biostimulants should enhance their biological activity on cultivated plants. Such processes for micro-algae biomass conversion into plant biostimulants were not yet developed.

The objectives of the present work were: (*i*) to develop a process for production of a plant biostimulants from microalgal biomass, obtained from a mixotrophic culture, which should assure also a high yield of microalgal lipids extraction; (*ii*) to characterize the resulted micro-algae based plant biostimulant in terms of its active ingredients; (*iii*) to investigate the effects of plant biostimulants obtained from micro-algae on water stressed tomato. Below are presented and discussed the obtained results, after a short description of the used materials and methods.

MATERIAL AND METHODS

Algal strain. The used algal strain was *Nannochloris* sp. 424-1, deposited under number CCAP 251/10 in the Culture Collection of Algae and Protozoa (CCAP), SAM Research Services Ltd., Scottish Marine Institute, Aryll, UK. The strain was obtained by mutation - selection, after cultivation on media with metronidazole, from a wild strain, and has the ability to grow at light intensities between 100 and 1100 µl $Em^{-2}s^{1}$, with reduced accumulation of anti-oxidants carotenoids. It resists to high concentrations, of 7% and higher, of carbon dioxide in the aeration gases and tolerates high concentrations of salts, particularly $NaHCO_3$ / Na_2CO_3. It presents a high competence of specific ecotechnological niches colonization, due to high rate of growth, between 0.275 and 0.4 OD/day, and a short doubling time, from 1 to 3 days. Strain is characterized by solitary cells or arranged in pairs, this arrangement suggesting a pre-existing cell division. The shape is spherical or sub-spherical, sometimes oval, the polymorphism resulting from both conditions of growth and life cycle. In the aging stages can form a layer of mucilage on cells surface. Cells are small (4-5 mm matures, 1.5-3 mm autospores). Reproduction is either by mitotic division, either through the formation of autospores as a result of two successive divisions inside the cell wall of a parent cell. Debris of parental cellular walls are presents in cell suspensions and are visible on optical microscopy. Cell organization is simple, cellular walls are without visible ornaments; the single chloroplast is parietal and is apparently without pyrenoid. The strain grows well on Zarrouk medium, tolerating large variations of the basic pH, 7.4 - 10. (Oancea *et al*., 2013).

Culturing condition. *Nannochloris* sp. strain 424-1 was grown on an integrated photosynthetic system (Stepan and Velea, 2012). In essence, this photosynthetic system is composed of a central body in the form of open box at the top, where are placed a variable number of photosynthesis cells, parallel linked by a pipeline for feeding with nutritive solutions (nutrient solutions + algal biomass or algal biomass brought to the stage of exponential growth) and a pipeline for gas supply with variable content of carbon dioxide. Over the photobioreactor is set-up the lighting system. This integrated photosynthetic system combines the advantages of open pond, with those of the system with flat plates, and could be considered on the hybrid photobioreactor class. *Nannochloris* sp. strain 424-1, with high growth rates, was considered as being the one with optimal characteristics for cultivation into this original photobioreactor, due to its growth rate and doubling time.

To test the growing of the strain the photosynthesis cells were first loaded with culture medium with 16.8 g/l $NaHCO_3$, or with Zarrouk medium supplemented with glycerol and hydrolysates of algal proteins (mixotrophic media), and freshly prepared inoculum, in ratio 9: 1. The formula of Zarrouk and Zarrouk mixotrophic media are presented in table 1 below. Loading was done to fill the photosynthesis cells, so the feed fluid flows into the photobioreactor, through the overflow fitting. The lighting system was coupled and the gas with carbon dioxide was introduced, circulated by a blower, with an established flow, allowing medium bubbling and maintain a good agitation of the suspension on photosynthesis cells. The experiments were conducted in the laboratory conditions, at 22 ± 2°C during the day and 17± 2° C during the night, with 12 hours photoperiod. The lighting was done with halogen lamps, 250 $\mu Em^{-2}s^{-1}$. Samples were taken axenically each 24 hours, and determinations were made for the exponential growth rate and doubling time (Wood *et al*., 2005) and lipid content (Cooksey *et al*., 1987).

Plant biostimulant production. A cascade process intended to assure a high yield of recovery of the main components of plant biostimulant ingredients (phytohormones, betaines, micro-algal protein hydrolysates), without reducing the yield of micro-algae lipids extraction, was developed. This process is scheduled in fig. 1 and will be briefly described here. After 4 day of cultivation, when micro-algae biomass reached more than 4 g/l, the photobioreactor was unloaded and biomass was harvested from culturing media by continuous centrifugation, on laboratory centrifuge Westfalia Laboratory Separator, model SA 1-02-175 (GEA Westfalia Separator Group, Germany, Oelde), which was operated at a speed of the centrifugal disks of 10.000 rpm, equivalent of 8.500 *x* g, and at a feeding rate of 0.3 liters/min, with continuous separation of clarified culturing media and a discontinuous one for algae concentrated, when this reach the density of 1100 kg/m^3. Algal biomass concentrate was homogenized on a high pressure piston homogenizer APV 2000 (APV, Albertslund, Denmark), with a knife edge valve, three cycle at 150 MPa, 0.3 liters / min. High pressure homogenization causes disruption of the algal cells by lysis induced by pressure variations and forced passing through the knife edge valve, with the expression of cell content and cell wall exposure.

The micro-algae homogenate was normalized to 10%, after refractometric estimation of dry matter content (with a RX- 5000 Digital Refractometer, Atago, Tokyo, Japan). A volume of re-suspended algal homogenate of 1 liter, corresponding to 100 g of dry microalgal matter, was added in a flat-bottom flask of 2500 ml, equipped with reflux condenser, adding funnel and thermoresistance for temperature monitoring. pH was corrected to 4.5 with 6 M HCl solution. 1 g of Glucanex 200 G (Novozyme a/s, Denmark, Bagvaerd), was added. Glucanex is a mixture of lytic enzymes of *Trichoderma harzianum*, which includes β-glucanase, cellulases and proteases, with an activity of 200 β-glucan Botrytis units per g. The resulted suspension was mixed vigorously, with a magnetic stirrer, and heated to 45°C. The adding funnel was removed and an ultrasonic probe was inserted. The suspension of micro-algae with enzyme was sonicated for 45 min, with 400 W at 45°C. After the completion of the sonication cycle, the micro-algae cell lysate was quantitatively passed in centrifuge tubes and the cell lysate was separated from cell debris by centrifugation at 7000 *x* g. The supernatant was recovered and was tangentially ultra-filtered on a Prostak system (Merck Millipore, Billerica, MA, USA), equipped with a membrane Ultracel PLAC (Merck Millipore) from regenerated cellulose, with exclusion limit of 1 KDa. Osmoprotectants and phytohormones concentration in permeate was continued, till reaching a concentration of 0.1% (mass/volume) of total betaine in permeate, controlled by determination of betaines by high pressure chromatography. The permeate was sampled periodically and analyzed for betaine content using the method described by MacKinnon *et al*., 2010, on Agilent 6224 Accurate Mass TOF LC/MS-MS (Agilent, Santa Clara, CA, USA). Ultrafiltration was stopped upon reaching the levels of concentration of 0.1% total betaines in permeate.

From the retentate the lipids were extracted with a mixture of chloroform: methanol 2: 1, in a ratio of 5 parts solvent mixture to 1 part dried matter of cell lysate (refractometric evaluated). Solvents with lipids were separated from aqueous phase, solvent was recovered under vacuum (Rotavapor® R-210, Büchi Labortechnik, Flawil, Switzerland) and algal lipids were separated.

Defatted retentate was passed on a 2500 ml flat-bottom flask, equipped with reflux condensers, adding funnel and thermoresistance for temperature monitoring. pH was corrected to 7 with 6 M NaOH solution. To 700 ml retentate, normalized to 10% dry matter, were added 0.7 g Alcalase AF 2.4 L (Novozyme), having a specific activity of 2.4 Anson units (AU) per gram, and 0.1 g of Flavourzyme 500 MG (Novozyme), with an activity of 500 LAPU/g. The mixture of suspension algal proteins – enzymes was maintained for 16 hours at 60°C.

To 1 ml hydrolisate were added 3 ml of solution 5% trichloroacetic acid (TCA). It did not result a precipitate, and this demonstrated that on hydrolisate are only free amino acids and di/tri-peptides. The hydrolysate was concentrated by evaporation under vacuum till 20% dry matter (determined by refractometry). This concentrated hydrolysate was added over osmoprotectants and phytohormones

concentrate, in a ratio of 8 parts of hydrolyzed protein concentrate to 2 parts osmoprotectants and phytohormones concentrate. The resulted solution represents the plant biostimulant, which was further characterized and tested on water stressed tomato.

Plant biostimulant characterization. In the resulted plant biostimulant were determined: total amino acids, with ninhydrin (Lee and Takahashi, 1966), proline (Abraham *et al*., 2010), total carbohydrates, with anthrone (Hedge and Hofreiter, 1962), betaines (MacKinnon *et al*., 2010) and cytokinins activity, by using the biotest of *Amaranthu*s caudatus cotyledons, proposed by Kubota *et al*., 1999, using kinetin (Sigma-Aldrich, St. Louis, MO, USA) as standard,.

Testing of plant biostimulant on water stressed tomato. Biostimulant composition was tested in the greenhouse condition, on tomatoes plant. Healthy plants of tomatoes (*Lycopersicum esculentum* cv. Cristal F1), 60 days transplants of the similar height, were re-potted into pots of 25 cm diameter and 50 cm height, containing 5 liters of growth substrate, enriched with plant nutrients for the first weeks of growth (Canna Terra Professional Plus, Canna International BV, Oosterhout, Netherlands). Pots were kept in a greenhouse, at 22 ± 2°C during the day and 17± 2°C during the night, with 12 hours photoperiod, supplemented with light intensity of 160 $\mu Em^{-2}s^{-1}$, derived from the halogen lamps, when light intensity decreased below 500 $\mu Em^{-2}s^{-1}$ threshold. Experiment was run for 56 days. The substrate contained reserves of nutrients, so the plants were fertilized only once, after 28 days from re-potting, by applying 100 ml of nutrient solution, 1 g/l of fertilizer 20-8-20 ($N\text{-}P_2O_5\text{-}K_2O$ TimacAgro Eurofertil, Romania). The experiment was organized in a randomized blocks, with 4 repetitions for each treatment, every repetition including five plants. Tested experimental treatments included control, treated with water, hydric stressed and not-stressed, and one standard product, Maxicrop Original (MaxiCrop, Corby, United Kingdom), containing 8% dry matter extracted from *Ascophyllum nodosum*. These experimental treatments were:

V_1 - control, hydric not-stressed, treated with water; 2 treatments *x* 2 ml per 100 l equivalent plant/ha;

V_2 - control, hydric stressed, treated with water, 2 treatments *x* 2 ml per 100 l equivalent plant/ha;

V_3 - hydric not-stressed, treated with the Maxicrop Original 2 treatments *x* 2 ml solution of 0.48% per plant, equiv. 6 liters with 8% dry matter in 100 l/ha;

V_4 - hydric stressed, treated with the Original Maxicrop 2 treatments *x* 2 ml solution of 0.48% per plant, equiv. 6 liters with 8% dry matter in 100 l/ha;

V_5 - hydric not-stressed, treated with microalgal plant biostimulant, 2 treatments *x* 2 ml of 0.5% solution per plant, equivalent to 0.5 kg in 100 l/ha

V_6 - hydric stressed, treated with the microalgal plant biostimulant, 2 treatments *x* 2 ml of 0.5% solution per plant, equivalent to 0.5 kg in 100 l/ha;

The treatments were applied in 2nd and 29th days after re-potting, by spraying each plant with a glass atomizer with metal top and plastisol bulb (model 15-RD, DeVilbiss Healthcare, Somerset, PA, USA). Control hydric not-stressed was watered once every five days at 100% field capacity, and hydric stressed treatments were watered every two weeks at 100% field capacity. At the end of the 8 weeks since transplants re-potting, the experiment was terminated, and the morphological parameters of plants, plant height, root length, number of leaves and the leaf surface, and ripe fruits per plant, were determined. The data were processed by analysis of variance, using ARM 8 software (Gylling Data Management, SD, USA).

RESULTS AND DISCUSSIONS

The used micro-algae strain, *Nannochloris* sp. 424-1, (CCAP 251/10) presented a better growth on mixotrophic media, with an exponential growth rate (R_{exp}, day $^{-1}$) of 0.723, comparing to 0.655 on autotrophic culture (tab.2). The doubling time, TD, was 0.72 day, comparing to 1.06 day on autotrophic

conditions. Mixotrophic grown micro-algae accumulated almost twice more lipid in the dry biomass (24.6%), comparing to 12.9% in dry microalgal biomass obtained in autotrophic conditions. This strain is suitable for growth in mixotrophic conditions for production of microalgal lipids, which could be further converted in biofuels (biodiesel, by transesterification, or bio-based jet fuel, by hydroprocessing).

The mixotrophic cultivation was done with 5 $g.l^{-1}$ technical glycerol (resulted from micro-algae lipid transesterification), added to the autotrophic Zarrouk medium. This concentration was proves also by other researchers to be optimal for mixotrophic cultivation of micro-algae, with higher biomass yield and accumulation of lipids, comparing to autotrophic media. Andruleviciute *et al.* (2014) reported that *Chlorella* sp., *Scenedesmus* sp., *Nannochloris* sp. and *Haematococcus* sp., grown in media with 5 g. l^{-1} technical glycerol, have higher lipid content than same strains cultivated on autotrophic concentration. Kong *et al*. (2013) shown that, on *C. vulgaris* cultivated on 5 $g.l^{-1}$ technical glycerol, the concentration of microalgae biomass increased to 2.13 $g.l^{-1}$. Liang *et al*. (2009) concluded that only the highest amount (10%) of technical glycerol had an inhibitory effect on algae growth, which is probably due to the impurities present in technical glycerol, like free fatty acids, residue of catalyst and soap. (Liang *et al*. 2010).

A cascade process was developed for the production of plant biostimulant from micro-algae biomass. This process is intended to assure a high recovery of main components of plant biostimulant ingredients (phytohormones, betaines, micro-algal protein hydrolysates), without reducing the yield of micro-algae lipids extraction. The main steps of the process are: micro-algae cell walls lysis by pressure homogenization and hydrolysis with mixture a lytic enzymes from *Trichoderma harzianum*, which includes β-glucanase, cellulases and proteases; separation of phytohormones, osmoprotectants, free amino acids and solubles carbohydrates by tangential ultrafiltration; extraction of lipids from ultrafiltration retentate; enzymatic hydrolysis of protein from defatted retentate; mixing protein hydrolisate with ultrafiltrate retentate, resulting the micro-algae based plant biostimulant. Prior separation of low molecular weight phytohormones, osmoprotectants and soluble carbohydrates aim to avoid the loss of these components (and especially of more amphiphilic / lipophilic phytohormones and osmoprotectants), during the lipid extraction with mixture of chloroform - methanol. Extensive cell wall lysis promotes lipid extraction and generates also cell wall fragments, which could have an elicitor effects on plant systemic defense activation (due to similarity with the damage-associated molecular patterns, DAMP, involved into network of plant defense signals – Pieterse *et al*., 2009). On commercial seaweed plant biostimulants such oligosaccharides based elicitors (derived from macro-algae polysaccharides cell walls) appears to be also responsible for the biological activity of seaweed extracts, related to conditioning the treated plant to tolerate biotic and abiotic stresses. (Shekhar Sharma *et al*., 2014).

The composition of the dry matter from the plant biostimulant, resulted from the developed process, is: 88.26% hydrolyzed algal protein, from which 5.63% is proline, 9.41% carbohydrates, 2.23 others compounds, including 0.023% total betaines and 0.012% cytokinins, kinetin activity equivalents. Cytokinins content is similar with those reported to be determined on commercial seaweed extracts (Craigie, 2011). Betaines are on the same level like in *A. nodosum* derived commercial extracts (MacKinnon *et al*., 2010). Presence of microalgal amino acids (micro-algae protein hydrolysates) should enhance the biological activity of obtained microalgal biostimulant. Protein hydrolysates are also included among the active ingredients of plant biostimulants (Calvo *et al*., 2014), promoting nitrogen assimilation in plants via coordinated regulation of C and N metabolism. Proline resulted from micro-algae hydrolysis is another active ingredient, regulating plant redox homeostasis and increasing plant tolerance to a variety of abiotic stresses, including salinity, drought, temperature and oxidative conditions (Ben Rejeb *et al*., 2014, Ertani *et al*., 2013, Apone *et al*., 2010, Ashraf and Foolad, 2007). Other amino acids, formed by hydrolysis of microalgae proteins, and having an effect on plant tolerance to abiotic stresses, could be included also among the active ingredients of a micro-algae based biostimulant. Exogenous application of glutamate can also enhance tolerance to salt stress (da Rocha *et al*., 2012). Arginine, with an important

role in nitrogen storage and transport in plants, accumulate in plants under abiotic and biotic stress (Lea *et al*. 2006) and is a metabolic precursor of putrescine, diamine from which start polyamine metabolism, as ubiquitous signal factors, required for different processes in plant development, and which participate in plant abiotic and biotic stress responses (Kusano *et al.* 2008).

The plant biostimulant composition obtained from micro-algae includes thus several components which stimulate physiological processes from cultivated plants, especially those related to plant tolerance to abiotic stress, including water stress. The obtained composition was tested on water stressed tomato. The results of the experiments are presented in the below table 3. The results demonstrate that the microalgal plant biostimulant, obtained from defatted biomass of *Nannochloris* sp. strain 424-1 by tangential ultrafiltration extraction of phytohormones and osmoprotectants (betaines) and further enzymatic hydrolysis of defatted microalgal biomass, stimulate the growth of the plants and improve the tomatoes plants resistance to hydric stress.

Morphological parameters values increased significantly after exogenous application of plant biostimulants produced from both micro- and macro- algae. Aplication of algae based plant biostimulants determine an increase of plant height by more than 10%. Plant biostimulant obtained from micro-algae determine a better development of root length (108.08% control), leaf number (120.31% control) and leaf area (105.16% control), comparing to seaweed extract – 105.98% control, 106.25% control, and, respectively, 104.45%. On leave number and leave surface the differences are statistically significant, micro-algae based bio-stimulant being more active.

These stimulatory effects partially compensate the negative influence of water stress. Plant height and root length decrease by almost 20% (from an average of 58.45 cm on control, well watered and not-treated with biostimulant control, to 48.87 cm on water stressed tomato plant) and, respectively, by more than 25% (from 58.82 cm average on control plants, to 39.85 cm on water stressed tomatoes). Application of algae based biostimulant reduced by almost 50% these negative effects.

Micro-algae based biostimulant, obtained according to the process presented in this work, have a more significant influence on development of the aerial part of tomato plant, comparing to commercial macro-algae extract. This is probably resulted from the combined effect of phytohormones, osmoprotectants and protein hydrolisate. But this better development of leaves it is not reflected in a much higher tomato fruits yield in the given experimental conditions. Average production of treated tomato plants (expressed as production per 30 days cycle of flowering and fructification) is similar for both algae based plant biostimulant. This is related also to the short period of experimentation, but further optimization experiments of the composition of the micro-algae based bio-stimulant, could exploit the effects on tomato leaves higher area (and resulted increased photosynthetic capacity) for a higher fruit yield.

Optimization of the micro-algae based plant biostimulant composition should consider the complementary and even synergic action of different components on major signals regulating metabolic pathways and plant response to stress. Macro-algae / seaweeds based plant biostimulant application increase plant tolerance to biotic and abiotic stress, and this is considered their main mechanism of action (Shekhar Sharma *et al*., 2014). All the main components of the macro-algae plant stimulants are acting on plant defense pathways. Cytokinins are priming plant immunity, stimulating the production of nitric oxide (Choi *et al*., 2011). Algae carbohydrates are generating a molecular pattern similar to DAMPs, eliciting plant defense and producing reactive oxygen species (ROS). (Meng and Zhang, 2013). Betaines sustain cultivated plant response to abiotic stress (Chen and Murata, 2008).

Micro-algae based plant biostimulant produced from the process described here contains all these components involved into plant immunity elicitation. The proposed micro-algae based plant biostimulant contains also amino acids / protein hydrolysate. These amino acids contribute also to an enhanced plant immunity. Arginine supports the formation of diamine putrescine, which is a substrate for nitric oxide generation by copper diamine oxidase (Wimalasekera *et al*., 2011). Proline regulates ROS homeostasis

(Ben Rejeb *et al*., 2014). Glutamic acids orchestrates crucial metabolic functions in plant defense, including cellular redox regulation (Seifi *et al*., 2013). Methionine participate in a number of essential metabolic pathways in plants, including those related to stress response (Margaret *et al*., 2013). Cysteine and related reduce glutathione are related to signaling crosstalk in light stress and immune reactions in plants. (Trotta *et al*., 2014).

Concomitant presence of nitric oxide (NO) and reactive oxygen species (ROS) determine the formation of reactive nitrogen species, (RNS, e.g., peroxynitrite, NOO-), with both regulatory and pathological implication (Gross *et al*., 2014). A proper balance between the components activating NO production and those leading to ROS generation should assure the precise level of RNS, which enhance plant response to stress, without pathological effects.

Supporting the metabolic pool of S-adenosylmethionine, linking the metabolic pathways of polyamine and of reduced thiols metabolites (Margaret *et al*., 2013), should support a proper balance of NO, ROS and RNS formation – fig. 2. An increase supply of betaines, osmoprotectant acting as donor of methyl groups (for recycling methionine from homocysteine) should support both the production of nitric oxide, via polyamines, which activates Methionine-adenosyl-transferase (MAT), and the metabolic process linked to high level of thiols scavengers, acting on excessive ROS / RNS. Balancing ROS / RNS pool should reduce the crosstalk between light stress and immune reactions in plants, and should improve the transfer and accumulation of plant photosynthesis compounds to edible fruits, which represent the useful yield. Further development of micro-algae based plant biostimulant will be done based on this proposed integrated mechanism of algae plant biostimulant action.

CONCLUSIONS

Nannochloris sp. 424-1, (CCAP 251/10) strain is suitable for growth in mixotrophic conditions. This strain presented a better growth on mixotrophic media, with an exponential growth rate (R_{exp}, day $^{-1}$) of 0.723, comparing to 0.655 on autotrophic culture. The doubling time, TD, was 0.72 days, comparing to 1.06 days on autotrophic conditions. Mixotrophic grown micro-algae accumulated almost twice more lipid in the dry biomass (24.6%), comparing to 12.9% in dry microalgal biomass obtained in autotrophic conditions.

A cascade process was developed for the production of plant biostimulant from micro-algae biomass. This process is intended to assure a higher recovery of the main components of plant biostimulant ingredients (phytohormones, osmoprotectants, oligosaccharides elicitors, micro-algal protein hydrolysates) and of micro-algae lipids. The main steps of the process are: micro-algae cell walls lysis by pressure homogenization and hydrolysis with mixture a lytic enzymes; separation of phytohormones, osmoprotectants, free amino acids and solubles carbohydrates by tangential ultrafiltration; extraction of lipids from ultrafiltration retentate; enzymatic hydrolysis of proteins from defatted retentate; mixing protein hydrolisate with ultrafiltrate retentate, resulting the micro-algae based plant biostimulant.

The composition of the dry matter of the resulted plant biostimulant is: 88.26% hydrolyzed algal protein, from which 5.63% is proline, 9.41% carbohydrates, 2.23 others compounds, including 0.023% total betaines and 0.012% cytokinins, kinetin activity equivalents. Cytokinins and betaines composition of this plant biostimulant is similar to that of seaweed commercial extracts, which are already largely used for cultivated plant treatments. The resulted plant biostimulant contains also a micro-algal protein hydrolisate, with amino acids which promote nitrogen assimilation in plants and increase plant tolerance to a variety of abiotic stresses. Oligosaccharides elicitors are also present in the resulted micro-algae plant biostimulant. These oligosaccharides have an elicitor effects on plant systemic defense activation, due to their similarity to the damage-associated molecular patterns, DAMPs, involved in the network of plant immunity signals.

Morphological parameters values of tomato plants increased significantly after exogenous application of plant biostimulants produced from both micro- and macro- algae. Application of algae

based plant biostimulants determines an increase of plant height by more than 10%. Plant biostimulant obtained from micro-algae determine a better development of root length (108.08% control) and determine an increase of leave number (120.31% control) and of leaves area (105.16% control), comparing to seaweed extract – 105.98% control, 106.25% control, and, respectively, 104.45%. On leave number and leave surface the differences are statistically significant, micro-algae based bio-stimulant being more active.

These stimulatory effects partially compensate the negative influence of water stress. Plant height and root length decrease by almost 20% (from an average of 58.45 cm on control, well watered and not-treated with biostimulant control, to 48.87 cm on water stressed tomato plant) and, respectively, by more than 25% (from 58.82 cm average on control plants, to 39.85 cm on water stressed tomatoes). Application of algae based biostimulant alleviated the effects of water stress on plants root development and reduced by almost 50% the negative influences on plants height.

Average production of treated tomato plants (expressed as production per 30 days cycle of flowering and fructification) is similar for both algae based plant biostimulant. Further optimization of the composition of the micro-algae based bio-stimulant, could exploit the effects on tomato leaves higher area (and resulted increased photosynthetic capacity) for a higher fruit yield.

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Tab. 1. The composition of the nutrient media Zarrouk and Zarrouk supplemented with glycerol and hydrolyzed algal protein (Zarrouk mixotrophic).

Media components	Zarrouk	Zarrouk mixotrophic
$NaHCO_3$	16.80 g/l	16.80 g/l
K_2HPO_4	0.50 g/l	0.50 g/l
$NaNO_3$	1.875 g/l	1.875 g/l
Protein hydrolyzed with 5% nitrogen content	-	12.5
Technical glycerol (from micro-algae lipid transesterification)	-	5
K_2SO_4	1.00 g/l	1.00 g/l
NaCl	1.00 g/l	1.00 g/l
$MgSO_4 \cdot 7H_2O$	0.20 g/l	0.20 g/l
$CaCl_2 \cdot 2H_2O$	0.04 g/l	0.04 g/l
Micronutrient stock solution *	1 ml	1 ml
Chelated Fe solution **	5 ml	5 ml

*Micronutrient stock solution (g/l): H_3BO_3, 2.860; $MnSO_4 \cdot 4 H_2O$, 2.030; $ZnSO_4 \cdot 7H_2O$ 0,222; MoO_3 (85%) 0,018; $CuSO_4 \cdot 5 H_2O$ 0.079; $Co(NO_3)_2 \cdot 6 H_2O$ 0.494.

**For preparation of chelated Fe stock solution were dissolved in 80 ml of distilled water 0.69g $FeSO_4 \cdot 7H_2O$ and 0.93g Na_2EDTA. After boiling for a short time and cooling to room temperature, was bring-out to the volume of 100 ml of the final solution.

Tab 2. Autotrophic and mixotrophic growth of *Nannochloris* sp. 424-1 on the integrated photosynthetic system, at an illumination of 250 $\mu Em^{-2}s^{-1}$, with 12 hours photoperiod.

Growth parameters	Autotrophic culture	Mixotrophic culture
Exponential growth rate, R_{exp} , day^{-1}	0.655	0.723
Doubling time, TD, day	1.06	0.72
Lipid content in dry biomass,% (g/100 g)	12.9	24.6

Tab. 3. Influence of treatments with compositions made in accordance with the invention of the tomatoes plants, hydric stressed and hydric not-stressed*.

Experimental version	Plant height (cm)	Root length (cm)	Leaf number	Leaf area (mm^2)	Average production * (g/plant ripe fruit)
V_1 control, hydric not-stressed, treated with water	58.45±5.04b	54.82±0.82b	32.00±5.2b	658.27±6.87b	315±63.5b
V_2 control hydric stress, treated with water	46.87±4.84c	39.85±4.02c	24.50±2.2c	532.80±3.27c	190±35.7c
V_3 hydric not-stressed, Maxicrop Original 2 *x* 2 ml 0.48%, equiv. 6 liter 8% d.w. in 100 l/ha	64.30±3.42a	57.20±3.29ab	34.00±2.1b	687.58±2.92ab	383±28.2a
V_4 hydric stressed, treated with the Original Maxicrop 2 *x* 2 ml 0.48%, equiv. 6 liters. 8% d.w. in 100 l/ha	51.70±4.12bc	54.20±1.93b	33.20±3.8b	673.16±5.84ab	310±42.6b
V_5 hydric not-stressed, micro-algae based biostimulant, 2 *x* 2 ml of 0.5%, equiv. 0.5 kg in 100 l/ha	65.15±2.49a	59.25±1.22a	38.50±2.4a	692.21±4.59a	374±22.4a
V_6 hydric stressed, micro-algae based biostimulant, 2 *x* 2 ml of 0.5%, equiv. to 0.5 kg in 100 l/ha	50.30±4.84bc	56.20±1.82b	34.01±2.6b	672.16±8.24b	309±35.6b

* The values are followed by the same letter does not differ significantly P > 0.05; ** production per 30 days cycle of flowering / fructification

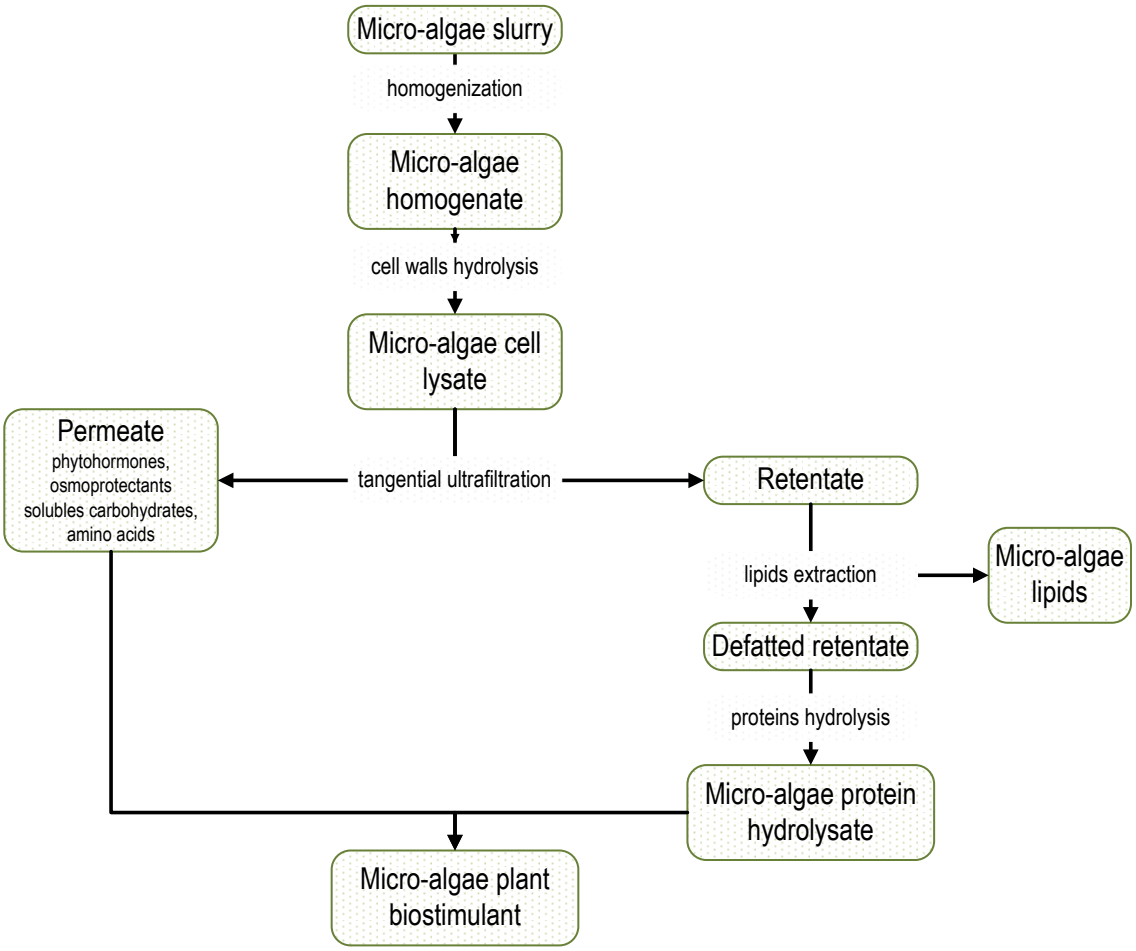

Fig. 1. Cascade processing of micro-algae biomass intended to assure a high recovery of main components of plant biostimulant ingredients (phytohormones from micro-algae, osmoprotectants, micro-algal protein hydrolysates), without interfering with the yield of micro-algae lipids extraction.

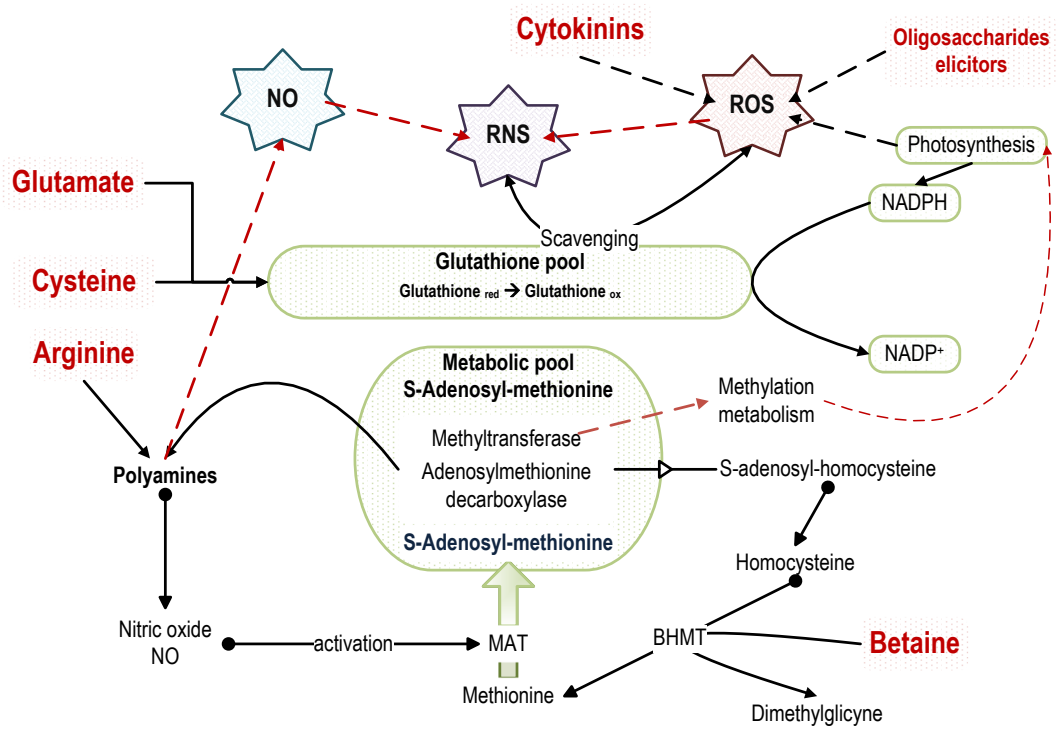

Fig. 2. Mechanism of action of the main ingredients of micro-algae based biostimulants, considered for composition optimization. Fine tuning of oxygen and nitrogen reactive species (ROS, respectively RNS) should be evaluated for an increased efficacy of micro-algae based biostimulant.