Influence of the Growth Regulators Kinetin and 2,4-D on the Growth of Two Chlorophyte Microalgae, *Haematococcus pluvialis* and *Dunaliella salina*

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Abstract: *Haematococcus pluvialis* Flotow and *Dunaliella salina* Teodoresco are commercially important because of their ability to accumulate very high carotenoid contents. However, their use is hindered by their slow growth rates. This paper reports a study on the effects of two growth regulators, 2,4-D (2,4-dichlorophenoxyacetic acid) and kin (kinetin), in concentrations of 0, 0.5, 1.0 and 2.0 mg l^{-1} each in a factorial design (2^4 combinations), as a possible means of enhancing the growth rates.

After 12-13 days of treatment with plant hormones, *D. salina* showed a significant increase in growth with all the hormone concentrations and combinations used and under 15% salinity (NaCl, w/v), (except for 0.5 mg l^{-1} 2,4-D and no kin), with up to 410% more cells than the control; under 10% salinity (NaCl, w/v), the increase in growth was significant with 0.5 mg l^{-1} 2,4-D and no kin (180% more cells than the control), and also with 1.0 mg l^{-1} 2,4-D and no kin (126% more cells than the control) and 2.0 mg l^{-1} 2,4-D and 0.5 mg l^{-1} kin (134% more cells than the control) in the culture medium. Cultures of *H. pluvialis* were significantly influenced under 1.0 mg l^{-1} 2,4-D (with 320% more cells than the control), but also showed a significant increase in the growth rate when the ratio auxin to cytokinin was 1 (equal concentrations of 1.0 mg l^{-1} of both growth regulators) with more than 290% cells than the control, and with 0.5 mg l^{-1} 2,4-D and 2.0 mg l^{-1} kin (200% more cells than the control) in the culture medium.

Keywords: *D. salina*, *H. pluvialis*, 2,4-D, kin, growth regulators.

INTRODUCTION

Haematococcus pluvialis and *Dunaliella salina* are two of the most cultivated microalgae. *H. pluvialis* is a freshwater microalga and *D. salina* can tolerate salinities up to 35% NaCl (w/v) [1]; both of these microalgae are chlorophytes and produce high amounts of carotenoids.

As a reaction to one or combination of stressors — high temperature, high light intensity, or even a nutrient depletion (nitrates or phosphates) —, *H. pluvialis* may accumulate up to 2% of its dry weight in astaxanthin, which is the ketocarotenoid β,β-carotene-3,3'-dihydroxy-4,4'-dione, mostly in the form of 3S,3'S isomer [2, 3], whereas *D. salina* can accumulate 5-15 % in β-carotene, β,β-carotene, in a dry weight basis [1, 2].

Due to the slow growth rate of *H. pluvialis* and *D. salina*, several methods have already been used to enhance their productivity in a first, pre-carotenogenic phase, such as the manipulation of culture conditions or medium composition. In the present study another approach was used in order to increase the biomass productivity.

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In most plants, auxins appear essentially in the form of IAA (indole-3-acetic acid), although substances as indolacetaldehyde, which act as IAA precursors, can also have auxinic activity [4]. In various algal taxa, both macro and microalgae, the occurrence of endogenous growth substances (auxines, cytokinins and gibberelins), which act like phytohormones has been reported for several decades [5-13]. In addition, the requirement for or the stimulating influence of cytokinins, auxins and other growth promoters has also been evidenced in marine and freshwater, both in multicellular and unicellular species [14-23]. Nevertheless, there are a few reports on the effects of 2,4-D (2,4-dichlorophenoxyacetic acid), on *Dunaliella salina* and other two marine microalgae [23], and none on *Haematococcus pluvialis.* Information on the combined action of kin (kinetin) and 2,4-D are even more scarce, if not null. The information about hormone metabolism in algae is also scarce [24], but different biosynthetic pathways and regulatory mechanisms for microalgae were already suggested [13]. According to some authors, the stimulation of cell enlargement and/or cell division, either by endogenous or externally applied phytohormones, can be associated to the acidification of the culture medium, carried out by the tissues/cells. Plasma membrane-bound proton pumps are probably activated by phytohormones activity that induces the acidification of free space in cell walls, therefore loosening cell wall and increasing plasticity.

ISSN: 1814-8085 / E-ISSN: 1927-5129/13

This causes an increase in the elongation rate of cell tissue [21, 25, 26]. This (acidic) theory also suggests that proton pumps could be targets for auxin receptors [25, 26]. The site of cytokinin activity has also been identified in *Euglena gracilis* var. *bacillaris*, as being three ribonucleosides from tRNA [10]. And Dibb-Fuller and Morris [27] had evidenced transmembrane transport for auxin *via* carrier systems in the multicellular alga *Chara vulgaris*. Because of their greater stability, potency and cost, synthetic auxins (like 2,4-D) and synthetic cytokinins (like kinetin) present great advantages mainly if we think in large scale applications.

Auxins usually act as promoting the elongation of cells and, with cytokinins, stimulate cellular division [28-30]; these last ones also promote chloroplast development. It is known that cytokinins exist also in brown and red algae and, apparently, also in Chlorophyta, and sometimes promote the algal growth [13, 31, 32]. In addition, one of the effects of the exogenous application of cytokinins is the induction of cell division in tissue cultures, in the presence of auxin [33]. However, most of the times it is the balance of hormones present in a plant cell that produces the effect, more than the presence or absence of one particular hormone [34, 35].

For all these reasons, this research work proposes to evaluate the effect of exogenously applied auxin 2,4-D and cytokinin kin on the growth of *Dunaliella salina*, at two salinities, 10 and 15% (NaCl w/v), and on the growth of *Haematococcus pluvialis*. The effect of different concentrations (from 0 to 2 mg/l) of two growth regulators on the microalgae growth was studied.

MATERIALS AND METHODS

Strains and Growth Conditions

Haematococcus pluvialis Flotow (strain 34/7 from CCAP, Windermere, UK) was cultivated in modified Bold's Basal Medium [36]: urea 1.5 mM (1.08487, Merck KGaA, 64271 Darmstadt, Germany) was used instead of sodium nitrate; pH 7.02-7.38 was adjusted before autoclaving. *Dunaliella salina* Teodoresco strain 10/30 (CCAP) was cultivated in modified Johnson's Medium [37]: 1.0 g potassium nitrate (1.05063, Merck KGaA, 64271 Darmstadt, Germany) per litre medium was used at 10% and 15% NaCl, w/v, (1.06404, Merck KGaA, 64271 Darmstadt, Germany), pH 8±0.1 was adjusted before autoclaving.

Cultures were maintained axenic all along the experiments. Experiments were carried out during 13 days for *Haematococcus* and 12 days for *Dunaliella*, in a *walk-in* chamber (Aralab, Parede, Portugal), in glass tubes with 40 ml sterile culture medium, supplemented with the hormones to be tested. Tubes with medium were inoculated with $2.8x10^5$ cells of *Haematococcus*/ml culture, or $3x10^4$ cells of *Dunaliella*/ml culture. Both strains were axenic. Growth was carried out under constant temperature (21 ºC) and light (white fluorescent tubes, 62 µmol photons m^{-2} s^{-1}). Aeration was provided by bubbling sterile compressed air (0.20µm filters Millex, Millipore, Bedford, USA) into the cultures. Illumination was measured with a LI-1000 DataLogger (Li-Cor, Magnetrom, Lisboa, Portugal). Experiments were carried out twice. Three independent vessels were used for each condition/treatment in all the experiments and, for each vessel, a 1ml-sample was taken, on days

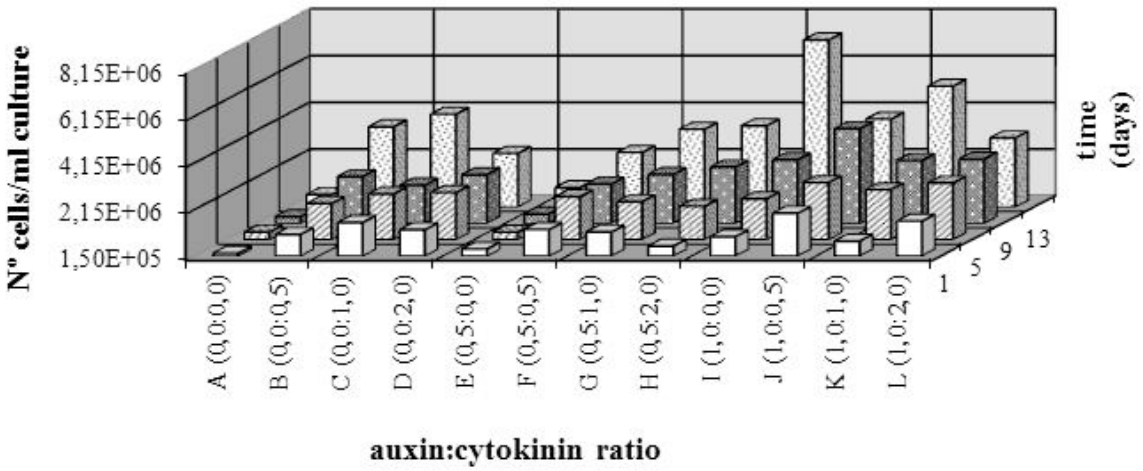

Figure 1: Relation between the concentrations of auxin and cytokinin used and growth of *H. pluvialis* cells (number of cells are related to concentration of cultures from the beginning until the end of the experiment).

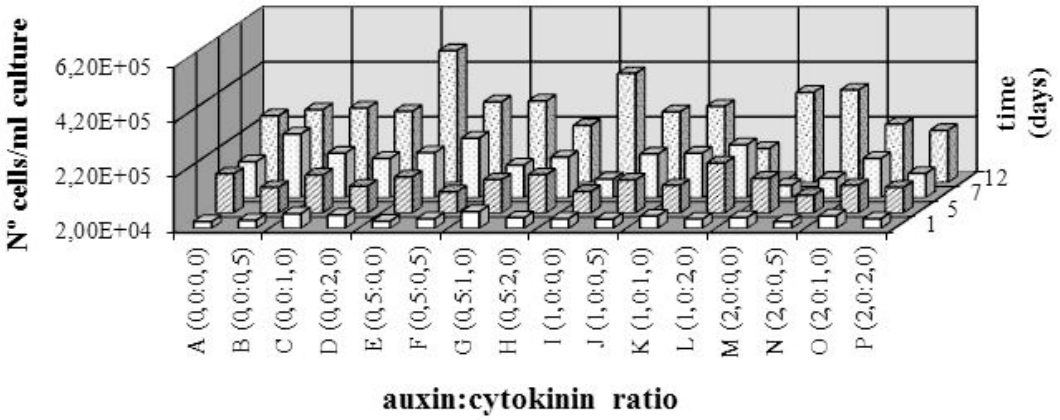

Figure 2: Relation between the concentrations of auxin and cytokinin used and growth of *D. salina* cells at 10% salinity (number of cells are related to concentration of cultures from the beginning until the end of the experiment).

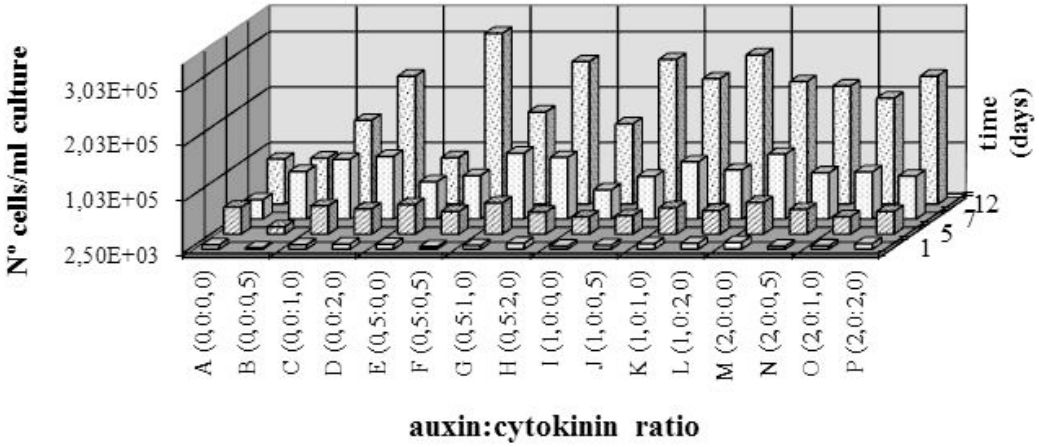

Figure 3: Relation between the concentrations of auxin and cytokinin used and growth of *D. salina* cells at 15% salinity (number of cells are related to concentration of cultures from the beginning until the end of the experiment).

reported in Figures **1-3**. Cells were counted twice, using a Neubauer improved chamber. Results in figures represent the average.

Growth Regulators

Growth regulators kinetin (K-0753 Sigma Chemicals Inc., St Louis, MO, USA) and 2,4-D (D-8407 Sigma Chemicals Inc., St Louis, MO, USA) were dissolved in 1ml NaOH 1M (Pronalab, J M Vaz Pereira, Distr., Lisboa, Portugal) and the final volume was complemented with deionised water in order to obtain 1 mg ml^{-1} solution. The final concentrations in the cultures were 0.5, 1.0 and 2.0 mg l^{-1}. Sixteen combinations (2^4) were tested (Table **1**), the control (A) did not have either kinetin or 2,4-D.

Statistical Analysis

Statistical analysis for the significant effect and time/hormone interactions was performed using Statistica (Anova/Manova) (StatSoft Inc., 1993). Significance between means was assessed by Tukey's Honest Significant Difference (HSD) test. Means were considered significantly different at $p<0.05$.

Despite controlling microalgae concentration of the inocula, by cell counting, the initial number of cells in culture was slightly different (differences <100). Hence, statistical analysis was also based on the ratio (Gi/G_0), meaning the growth of microalgae along time (Gi) in

Table 1: Experimental Design of the Experiments with Two Growth Regulators

		kinetin (mg/l)			
		0.0	**0.5**	**1.0**	**2.0**
2,4-D (mg/l)	0.0	A	B	C	D
	0.5	E	F	G	H
	1.0	I	J	K	L
	2.0	M	N	O	P

relation to the initial number of cells (G_0), for each combination of the growth regulators.

RESULTS

Both microalgae, *Haematococcus* and *Dunaliella*, showed positive responses to some of the auxin:cytokinin combinations (Figures **1-4**), especially *D. salina* grown at 15% salinity.

Haematococcus pluvialis presented a significant increase in the cell number under auxin stimulation, after 13 days of culture **(**1.0 mg 2,4-D l^{-1}**)**, with 355% more cells than the control, just followed by treatments 1:1 mg l^{-1} of 2,4-D:kin and 0.5:2.0 mg l^{-1} of 2,4-D:kin, with an increase of growth by 297% and 275% over the control, respectively. As a matter of fact, these two combinations had significantly shortened the lag phase, resulting in a cell number almost three times higher on the 7th day of culture, with 239% and 250% more cells, respectively, than the control, (Figure **1**). On day 11th, the number of cells in the culture under these two combinations (1:1 and 0.5:2 mg l^{-1} of 2,4-D:kin) increased up to 316% and 310% as compared to the control.

At 10% salinity (Figure **2**), the growth of *Dunaliella salina* only presented a significant increase for three combinations of auxin:cytokinin, after 12 days of culture: 0.5:0.0, 1.0:0.0 and 2.0:0.5 mg l^{-1} 2,4-D:kin. Either by the 7th or 12th day, the number of cells in culture under 0.5 mg l^{-1} 2,4-D showed an increase of 184% over the control. With 2.0:0.5 mg l^{-1} of 2,4-D;kin, cultures of *D. salina*, at 10% salinity, presented a very long lag phase, but by the 12th day cultures were still in the middle of the exponential phase, with 4.2 times more cells than on the 5th day (Figure **2**). However, it was at 15% salinity that *D. salina* presented the highest stimulation of the growth, when under the influence of the growth regulators 2,4-D and kinetin (Figure **3**). As a matter of fact, except for the combination E (0.5:0.0, 2,4-D:kin), where algal growth was similar to the control, all concentrations of these growth regulators tested resulted in a significant increase in the growth ratio G_i/G_0 of *Dunaliella salina*: combination 0.5:0.5 mg l^{-1} 2,4-D:kin dramatically increased the growth and the number of *D. salina* cells by 412% over the control, followed by the other two combinations with 0.5 mg kin / l^{-1} (1.0:0.5 and 2.0:0.5, 2,4-D:kin), with, respectively, 291% and 270% more cells than the control, on the 12th day of culture (Figure **4c**).

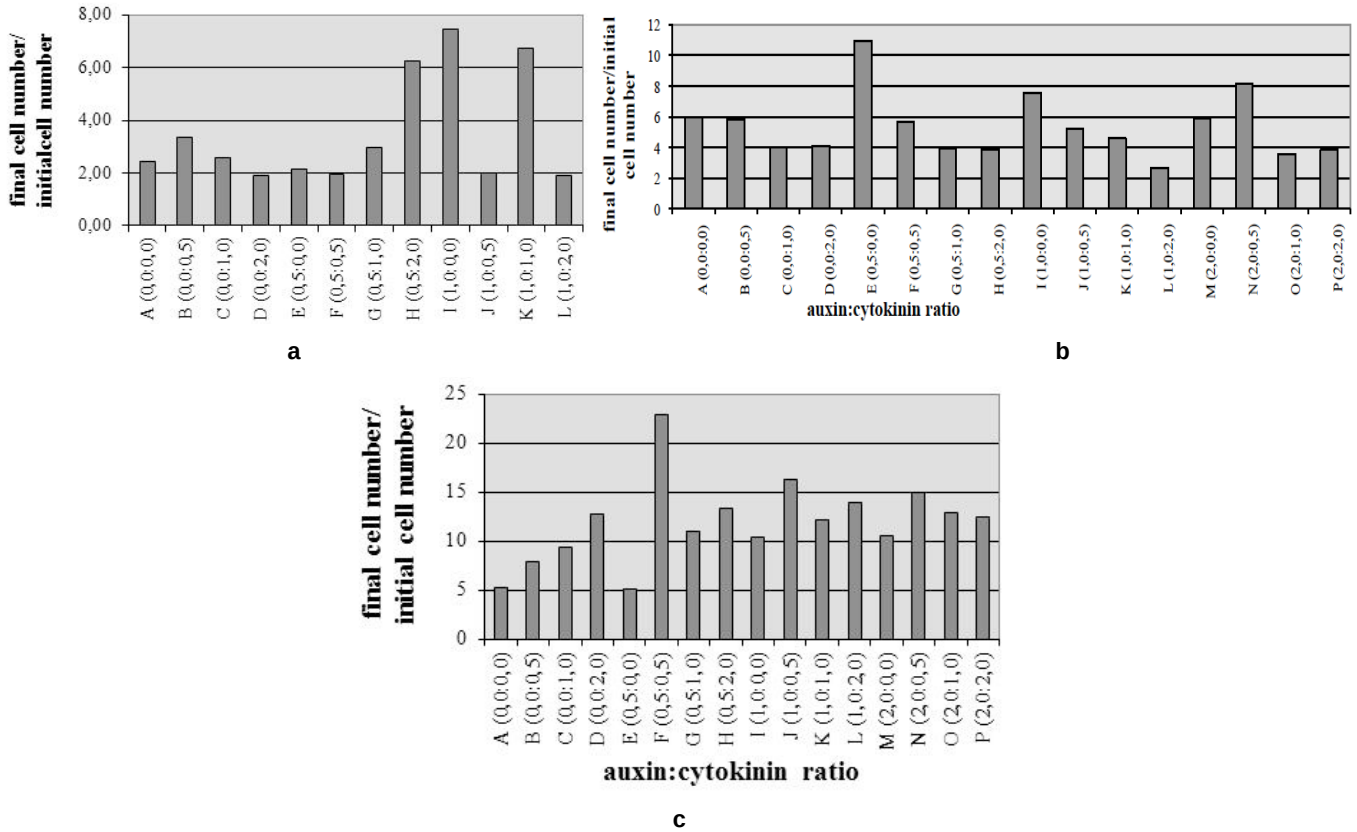

Figure 4: Relation between the concentrations of auxin and cytokinin used and growth of *H. pluvialis* **a**), and *D. salina* cells at 10% salinity **b**) and 15% salinity **c**).

DISCUSSION

No experiments concerning the influence of phytohormones kinetin and 2,4-dichlorophenoxyacetic acid on *H. pluvialis* and *D. salina* had been conducted until now, and neither with combinations 2,4-D:kin on both algae. The results presented in this paper proved that both 2,4-D and kin, exogenously applied, can significantly stimulate the growth of cells of *H. pluvialis* and *D. salina*. But results also show that the effects of these two regulators depend either on the algal species or on the culture conditions. Similar findings were reported by Burkiewicz [20] for the influence of gibberellins on three other species of microalgae.

In the case of *Dunaliella salina,* grown at 10% salinity, and *H. pluvialis* a gradual decrease was observed in the growth, when increasing the concentration of kin. These results are somewhat confirmed by Burkiewicz [20], who found that, under the influence of cytokynins, the biomass of *Chlorella pyrenoidosa* also suffered a gradual decrease when the dose of cytokinin was increased. In addition, for some of the combinations of 2,4-D:kin used in this work, as is the case of treatment D (1.0:2.0) in *Haematococcus*, a significant shift in cell number during the first seven days could be observed but, after that, a stagnation in the growth was noticed. Similar observations were reported by Bajguz and Czerpak [22] for *Chlorella vulgaris* when exposed to brassinosteroids, and also by Burkiewicz [20], who noticed an initial stimulation of cytokinins in cell division and dry weight of three species of microalgae followed by a decrease in the dry weight. Burkiewicz [20] even suggested that cell division could be the main effect of cytokinins, the increase in dry weight being a secondary influence. Very little influence of kin was also found by Bentley-Mowat [17] on young cultures of *Nannochloris*.

In contrast with these results, we observed a strong stimulation, dose-effect dependent, when kinetin was exogenously applied to the cultures of *Dunaliella* at 15% salinity**,** perhaps due the high salinity of the cultures, which could have somewhat modified the permeability of the cell membrane. These findings are in accordance with those obtained and reported by Sundaralingam and Govindaraj [38], who referred that the application of kin affected the multiplication and cell size of *Cosmarium subtriordinatum.* Furthermore, it is known that ABA (abscisic acid) can play a morphogenic effect on cells of some microalgae and that, in some other stress conditions, such as the increase in salinity, it may cause an increase in endogenous ABA [39-41]. Therefore, it can be stressed that a synergistic effect between endogenous ABA and exogenously applied 2,4-D and kin could have happened, giving such a stimulating answer in the growth of *Dunaliella salina* in 15% salinity.

Czerpak *et al.* [42, 43] reported the stimulating effects of natural and synthetic auxins on the growth of algae, their metabolic activity being significantly higher than in control cultures. These findings are in agreement with the present work since concentrations of 0.5 and 1.0 mg l^{-1} 2,4-D significantly increased the number of cells in *Dunaliella* at 10% salinity and *Haematococcus*. An increase in the number of cells was also reported for *Skeletonema, Chlorella, Scenedesmus*, and other algae under IAA [14, 17, 21, 44] and NAA (1-naphtalenic acetic acid) treatment [45].

With respect to the combined action of auxins and cytokinins, Bradley and Cheney [46] had already suggested that combined activity of different growth regulators enhanced the growth of cultured algae, stimulating the biomass productivity. Hunt *et al.* [45] also referred that the exogenous application of auxins in combination with cytokinins (such as NAA and zeatin), under certain concentrations, could increase the biomass productivity of *Chlorella sorokiniana*, but some other concentrations may also cause the opposite effect. Similar stimulatory/ inhibitory answers are reported in this work. In *Dunaliella salina* grown in 10% salinity, combinations with 1.0 and 2.0 mg l^{-1} kin were always inhibitory, after 12 days of treatment, whatever the concentration of 2,4-D, even when it was absent. Strongest inhibition (number of cells 40% lower than the control) was verified for the treatment O (2.0:1.0, 2,4-D:kin). Inhibition or no effect was also observed in *Haematococcus*, 13 days post-treatment, except for the combinations 0.5:1, 0.5:2.0 and 1.0:1.0 (2,4-D:kin). Perhaps photon flux density and/or temperature, and/or pH were not optima and, thus, affected the uptake of the regulators, in those concentrations, by *Dunaliella* at 10% salinity and *Haematoccus*. Or, under these culture conditions, such phytohormone combinations had just an antagonistic effect, or total hormone concentration might be too high.

The existence of compounds with auxin- and cytokinin-like activity had already been noticed and reported in Cyanophyta [32] and chlorophyte microalgae [12]. But pathways of auxin and cytokinin synthesis and locations have still to be investigated

[24]. Also, the presence of ABA and jasmonic acid was already found in some microalgae, such as the chlorophytes *Haematoccoccus*, *Dunaliella* and *Chlorella* [24, 39-41, 47].

Auxin carriers and auxin receptors have already been associated to cell-membrane in maize coleoptiles [48] and suspension-cultured tobacco cells [49], and evidence of a carrier system for the auxin transmembrane transport has also been discovered in a multicellular chlorophyte, *Chara vulgars* [27]. Nevertheless, evidence of an auxin carrier system in unicellular algae has not yet been found, despite the finding of a pH-sensitive diffusion of IAA in *Chlorella vulgaris* [12]. Hobbie *et al.* [26] referred that the mechanism of cytokinin action had still to be discovered. Nevertheless, it is thought that there could be a specific protein receptor interacting with cytokinins and, moreover, several cytokinin binding proteins had already been identified [50]. In addition, Swaminathan and Bock [10] indicated three ribonucleosides from t-RNA as being the site of cytokinin activity in *Euglena gracilis* var. *bacillaris*. But as Tarakvoskaya and co-workers observed recently [24], the dynamics of the endogenous cytokinin in algae needs still needs to be investigated.

Analysing all the results, it can be concluded that, except for *D. salina* growing in 15% salinity and which showed an enormous increase in cell number with almost all the concentrations and combinations of 2,4-D:kin studied, the highest ratios G_i/G_0 were obtained with 2,4-D alone, either for *H. pluvialis*, with 1.0 mg l^{-1} of culture or *D. salina*, grown in 10% salinity, with 0.5 mg l^{-1} of culture. Furthermore, the study on the production of carotenoids will be a future challenging step.

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Received on 30-01-2013 Accepted on 18-04-2013 Published on 25-04-2013

http://dx.doi.org/10.6000/1927-5129.2013.09.40