Modeling laboratory culture of *Gracilaria verrucosa* (Hudson) Papenfuss according to nutrients concentrations and salinities levels

Fethi MENSI[1*], J. KSOURI[1], E.G. SEALE[2] and M. S. ROMDHANE[3]

[1] *Institut National des Sciences et Technologies de la Mer, Laboratoire de biodiversité et biotechnologies marine, 29 Rue Géneral Kheireddine 2015 Le Kram, Tunisie.*

[2]*Daithi O'Murchu Marine Research Station, Gearhies, Bantry, Co. Cork, Ireland*

[3] *Institut National Agronomique de Tunisie, Unité de recherche Ecosystèmes et Ressources Aquatiques, 43 av. Charles Nicolle 1082 Tunis. Tunisie*

[*]Corresponding author. Tel.: +216 71 276 121; Fax: +216 71 276 121.
mensi.fethi@instm.rnrt.tn

ملخّص

نمذجة زراعة طحلب الغراسيلاريا داخل المختبروفقا لتركيز الاملاح ومستويات الملوحة : تطوّر إستغلال الطحلب الأحمر *Gracilaria verrrrucosa* في العقود الأخيرتس ظرا لأهمّية مادّة الأغرة المستخرجة منه. وهذا النوع من الطحالب مرشّح للاستزراع ببحيرة بنزرت بالشّمال التوسي حيث لا ينمو إلا في فصل الرّبيع . إن الهدف من هذه الدّراسة هو جمع معلومات أساسية عن نمو هذا الطحلب وذلك باستخدام منهجية الاستجابة السطحيّة على نطاق المختبر. وللاكتشاف التفاعل بين المتغيّرات والمستويات المثلى من هذه المتغيّرات استعملنا خطة مركزية مركّبة (Plan Central Composé). كما حددت درجة الحرارة كامل فترة التجربة ب 20 درجة مأوية إن معامل التحديد للنموذج الرياضي الذي تحصلنا عليه من خلال البيانات التجريبية مرتفعة (0.8) كما تبين أن الطحلب يموت عندما تكون درجة الملوحة أقل من 25‰.

كما بينت النتائج أن وزن الطحلب قد إرتفع عندما إرتفعت نسبة تركيز الأمونيوم والنترات من 0.01 إلى 2,2,5 مغ /ل على التوالي . ولكن عندما تتجاوز هذه النسب فإن نمو الطحلب يتراجع بصفة ملحوظة فحين أن مادة الفسفاط ليس لها تأثير إيجابي على النمو . هذه النتائج الأوليّة تثبت محدودية إمكانية استزراع طحلب الغراسيلاريا ببحيرة بنزرت .

كلمات مفاتيح : بحيرة بنزرت , *Gracilaria verrrrucosa* , ملوحة ,الأمونيوم , تبترات , الاستجابة السطحية

RESUME

Modélisation de la culture de *Gracilaria verrucosa* (Hudson) Papenfuss au laboratoire en fonction des concentrations en nutriments et différents niveaux de salinité : L'exploitation des algues rouges appartenant au genre Gracilaria pour l'extraction de l'agar a considérablement augmenté au cours des dernières décennies. En Tunisie *Gracilaria verrucosa* (Hudson) Papenfuss 1950 (Rhodophyta), est candidate pour la culture dans la lagune de Bizerte (Nord de la Tunisie) où ne pousse que sur une courte période de l'année. Ainsi, le but de cette étude est de recueillir des informations de base sur sa croissance dans cette région. Les effets de la salinité des sources d'azote et du phosphore ainsi que leurs concentrations sur la croissance de cette algue ont été étudiés à l'échelle de laboratoire en utilisant la méthodologie de surface des réponses. Afin de déterminer les interactions entre les variables et les niveaux optimaux de ces derniers, un plan central composite (PCD) a été employé. La température a été maintenue à 20 ° C. Le coefficient de détermination (R^2) est de 0,80, ce qui montre que le modèle quadratique développé est satisfaisant. La mortalité des thalles de *G. verrucosa* s'est produite lors que la salinité est inferieur à 25 ‰. Les résultats du PCD utilisé montrent que le poids final des thalles a augmenté lorsque les concentrations d'ammonium et de nitrate passent de 0,01 mg / l à 2,5 mg / l et 2 mg / l respectivement. Lorsque les concentrations dépassent ces valeurs, le taux de croissance a chuté considérablement. Ces résultats préliminaires démontrent que la culture de *G. verrucosa* dans la lagune est limitée.

Mots clés : Lagune de Bizerte, Gracilaria verrrrucosa, Salinité, Ammonium, Nitrates, surface desréponses

ABSTRACT

The exploitation of macroalgae of the genus *Gracilaria* for agar extraction has increased significantly in recent decades. In Tunisia *Gracilaria verrucosa* (Hudson) Papenfuss 1950 (Rhodophyta), is a candidate species for mariculture in Bizerte lagoon (North Tunisia) where grows only in late spring and early winter. Thus, the aim of this study was to gather basic information about the growth rates of G. *verrucosa* in this region. The effects of salinity, nitrogen and phosphorus sources and concentrations on growth rates of *G. verrucosa* were investigated using response surface methodology at a laboratory scale. In order to discover the interactions between the variables and the optimum levels of these variables, a Central Composite Design (CCD) was employed. Temperature was maintained at 20°C. Analysis of variance exhibited a high coefficient of determination (R^2)

value of 0.80 and ensured that the quadratic model with the experimental data was a satisfactory choice. Mortality of the *G. verrucosa* thalli occurred when salinity dropped below 25 ‰. The CCD results showed increased growth rate when the ammonium and nitrate concentrations increased from 0.01mg/l to 2.5mg/l and 2mg/l respectively, but when concentrations exceeded these values, the growth rate dropped significantly while no change occurred with increasing phosphate concentration. These results demonstrate the limited potential of *G. verrucosa* for mariculture in this area.
Keywords: Bizerte lagoon; *Gracilaria verrucosa*; Growth; Salinity; Ammonium; Nitrate; Response surface

INTRODUCTION

Seaweeds belonging to the genus *Gracilaria* are very important as a food for humans and marine animals, and also as a source of industrial agars (Zemke-White and Ohno, 1999). *Gracilaria* spp are currently the most economically important cultured agarophyte, producing approximately 60% of the word's agar production (Tseng, 2001). Commercial cultivation is performed on a very large scale in several countries such as Chile, China and Taiwan (Dawes, 1995) but its potential as a commercial crop in Tunisia remains undeveloped (Mensi et al., 2009).

The distribution of *G. verrucosa* in Tunisia is restricted to lagoon areas where salinity undergoes seasonal fluctuations from 20‰ or less in winter to 40 ‰ or more in summer (Ksouri et al., 1998). This study addresses the question of whether extreme values of salinity are limiting to *G. verrucosa*. Nutrients supply fluctuates strongly with season. Ammonium (NH_4^+), nitrate (NO_3^-) and phosphate (PO_4^{3-}) reach an annual maximum of around 5 mg/l (ammonium and nitrate concentration) and 2mg/l (phosphate concentration) in later winter and early spring. Nitrate and ammonium, is quickly consumed by spring phytoplankton blooms and falls below 1mg/l by late spring, remaining at that low level through the summer (Sakka et al.,2008). Intermediate concentrations were observed in autumn (Mansouri, 1996). Most of the available dissolved inorganic nitrogen came primarily short-term pulsing of high concentrations of N and P that is routinely used locally to maintain mass cultures of *G. tikvahiae* (Ryther *et al.*, 1981; Lapointe, 1985). Thus it is important to study the possible impact of the exposure of these N and P pulses on *G. verrucosa* over a longer period of time.

Correlations of *Gracilaria* growth with various environmental factors have been suggested under laboratory cultures by various studies (Wong and Chang, 2000; Costanzo et al., 2000; Liu and Dong, 2001). It has not been possible to determine any clear individual cause and effect relationships under natural conditions, as one cannot distinguish between individual factors that may simultaneously fluctuate. However, statistical experimental designs provide an efficient approach that accounts for these interactions is possible, and could identify the most significant factors affecting *G. verrucosa* growth. A combination of factors generating a certain optimal response can be identified by using factorial design and Response Surface Methodology. Response Surface Methodology (RSM) is a statistical technique, based on the fundamental principles of statistics, randomization, replication and, duplication, which simplifies the optimization by studying the mutual interactions among the variables over a range of values in a statistically valid manner. It is an efficient statistical technique for optimization of multiple variables in order to predict the best performance conditions with a minimum number of experiments.

The main objective of our work was to study the chemical and physical conditions (salinity, ammonium, nitrate and phosphate concentrations) wherein *G. verrucosa* has an optimal growth. A Central Composite Design (CCD) was used to elucidate the influences of fluctuating salinity and nutrient availability on seasonal variations of biomass/abundance of *G. verrucosa*. The growth rate, salinity range, optima, and the interactions between nutrients and salinity on algal growth were determined by exposing algae to varying salinity ranging from 20 to 40 ‰ in seawater enriched with or without nutrients. The "minimum" level of ammonium, nitrate and phosphate in the experimental design was ambient seawater. The high, central and star points of CCD design were much greater than concentrations encountered in ambient seawater environment. However, we attained these values in Bizerte lagoon.

MATERIALS AND METHODS

1. Seaweed collection and pre-culture

Clean healthy *G. verrucosa* (Hudson) Papenfuss were selected and collected, in March, 2010 from Bizerte lagoon located on the north of Tunisia (37°8'-37°14'N, 9°48'- 9°56'E) and transported back to the laboratory in 25 litre plastic containers. The site is a sheltered estuary (1m water depth), with a salinity range of 30-35‰, a temperature range of 17-20°C and low visibility (Mensi et al., 2009).

Algae were brought to the laboratory, cleaned of epiphytes, and washed in filtered seawater. Stock cultures were maintained in 14 litres of medium in five aerated 20 litre glass aquarium in a "walk in" culture room under standard conditions of 20°C, 36‰, 12 h: 12 h light: dark (L:D) cycle provided by cool-white fluorescent light (1000 lux). This

experimental step was carried out to deplete nutrients stores within the algae and obtain homogenous thalli.

2. Experimental *G. verrucosa* culture

Algae sample: Only thalli of the same colour and at the same stage of the development cycle with similar ratio surfaces/volume were selected. This procedure was adopted to minimize the variation coefficient of our experimental units, thus thalli with similar lengths and morphology were selected based on the model suggested by Hanisak et al., (1990).

Natural seawater treatment and storage: Offshore seawater free from pollution was collected in plastic bottles, in April 2010. Bottles were filled and returned to the laboratory for filtration. The water was filtered to 0.45 mm with membrane filters (GF Whatman filter with 40µm of diameter). No special treatment was performed to remove dissolved inorganic matter. Seawater salinity was 36‰. Filtered seawater was stored in 20 litre plastic carboys and kept cool in refrigerated dark room for subsequent use. Sterilization was performed by the addition of sodium hypochloride bleach. Typically, 1 to 5 ml of this commercial bleach was added per liter of water, and after gentle mixing, the water was left to stand (without mixing or aeration) for several hours in dark room. Bleach treated water was neutralized with sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$). One ml of the sodium thiosulfate solution was added for each 4 ml of bleach used. The sodium thiosulfate solution consisted of 250 g of sodium thiosulfate dissolved in 1 litre of water.

Enriched natural seawater preparation: The enriched seawater medium used was a modified Von Stosch (Grund) medium according to Harrison and Berges (2005). This medium is suitable for growing many different red types of seaweed (Guiry and Cunningham, 1984). In an effort to reduce bacterial growth the Tris buffer used in Grund Medium was removed from the protocol. To prepare the medium, 940 ml of treated seawater (as indicated above) were pasteurized and 10 ml each of the stock solutions (table I) were added aseptically; the medium was subsequently autoclaved. All constituents and vitamins used to prepare stock solution are indicated in table II.

Stocks solutions of nutrients: ammonium, nitrate and phosphates were prepared according to Table III. $NaNO_3$, NH_4Cl and KH_2PO_4 were used to prepare ammonium, nitrate and phosphate stock solutions used in experiment.

Experimental culture setup: After three weeks without any nutrient addition, algae were removed from aquaria and placed into 2 litre glass flasks. About 2g (fresh weight) of *Gracilaria* was cultured continually in 0.6 litres of treated natural seawater which was replaced every three days. To maintain the required nutrient concentration, 0.6 ml of nutrient stock solution corresponding to the desired experimental run and 0.6 ml of enriched natural seawater were added. The frequency of the replacement of the culture medium was determined by preliminary experiments. Algae were gently aerated to facilitate nutrient uptake by preventing excessive diffusion. The treatment was performed in triplicate for duration of three weeks. On 21st day of the experiment, a final weight rate was recorded for the algae.

3. Experiment designs and statistical analysis

As the first step in analysis, the optimization of *G. verrucosa* growth, RSM and central composite design were used to analyze the main factors influencing growth. This design was composed of 2^4 factorial design (run1-16), 8 star points (run 16-24) and 2 replicates (run 25 – 26) thus 26 experiments were needed in total. Table III lists independent variable levels (-2,-1,0,1,2) and their values used for central composite rotatable design. The variables were coded according to Eq.(1):

$$x_i = (X_i - X_o)/\Delta X_i;\ i=1,2,\ldots\ldots,k \qquad (1)$$

Where xi and Xi are the dimensionless and the actual values of the independent variable i, X0 is the actual value of the independent variable at the centre point, and ΔXi is the step change of Xi corresponding to a unit variation of the dimensionless value. The experimental data allowed the development of empirical models describing the interrelationship between operational and experimental variable by equation including linear, interaction and quadratic terms. The quadratic model for predicting the optimal point was expressed as Eq. (2) :

$$Y = \beta_0 + \Sigma\beta_i x_i + \Sigma\beta_{ii} x_i^2 + \Sigma\Sigma\beta_{ij} x_i x_j;\ i = 1; 2; \ldots; k;\ j= 1; 2; \ldots; k;\ i \neq j \qquad (2)$$

Here Y represents the growth, β_0 is the value of fitted response at the centre point of design; β_i, β_{ii} and β_{ij} are the linear, quadratic and interaction terms respectively. In the study, *G. verrucosa* growth was processed via Eq. (2) including ANOVA to obtain the interaction between the variables and the responses. The determination coefficient R^2 value, coefficients of variation (CV) and model significance (*F*-value) were used to judge the adequacy of the model. Where R^2 (the coefficient of determination), is the proportion of variation in the response attributed to the model rather than to random error. The *P*-values are used as a tool to check the significance of each of the coefficients. The coefficient of variation (CV) is

Table I: von Stosch (Grund) Medium (from Harrison and Berges, 2005)

Component	Stock Solution ($gL^{-1}dH_2O$)	Volume (ml)	Concentration in Final Medium (M)
Thiamine HCl (vitamin B1)	-	10	5.93×10^{-6}
Biotin (vitamin H)	0.1	1	4.09×10^{-9}
Cyanocobalamin (vitamin B12)	0.2	1	1.48×10^{-9}

Table II: Vitamins Stock Solution (from Harrison and Berges, 2005)

Component	Stock Solution ($gL^{-1}dH_2O$)	Volume (ml)	Concentration in Final Medium (M)
$Na_2\beta$-glycerophophate	5.36	10	$2.48x10^{-4}$
$NaNO_3$	42.52	10	$5.00x10^{-3}$
FeSO4.7H2O	0.28	10	$1.00x10^{-5}$
MnCl2.4H2O	1.96	10	$1.00x10^{-4}$
Na2EDTA.2H2O	3.72	10	$1.00x10^{-4}$
Vitamins stock solution	(see Table 2)		

Table III. Process variables in coded and actual units

Variables	Salinity ‰	Ammonium (mg/l)	Nitrate (mg/l	Phosphate (mg/l)
Symbole	x_1	x_2	x_3	x_4
-2	20	0	0	0
-1	25	2	2	0.5
0	30	3	3	1
1	35	4	4	1.5
2	40	5	5	2

the ratio of the standard error of estimate to the mean value of the observed response and is expressed as a percentage. The effects of each variable were determined by statistical software, STATISTICA (StatSoft, Inc., 2008).

In the second step of analysis, the response functions was analyzed by canonical analysis, a method of rewriting a fitted second-degree equation in a form that describe the nature of the stationary point and the nature of the system around the stationary point (in which it can be more readily understood). This is accomplished by a rotation of axes that remove all cross-product terms. If desired, this may be accompanied by a change of origin to remove first-order terms. Using this new coordinate system, the second-order model equations are simplified and its geometrical nature becomes apparent. The equation developed by the transformation called the canonical form of the model was illustrated in Eq. (3):

$$\mathbf{Y = Y_s + \Sigma \lambda_j w_j^2} \quad \mathbf{(3)}$$

where w_j (j = 1, 2, 3,4) denotes the transformed independent variables or the canonical variables. λj is the eigenvalues which will describe the curvature of the response. The constant *Y*s is the calculated response value at the stationary point. The stationary point, if it exists, is the solution to Eq (4) and could represent a point of maximum response, a point of minimum response, or a saddle point.

$$\frac{\partial Y}{\partial x_1} = \frac{\partial Y}{\partial x_2} = ... = 0 \quad \mathbf{(4)}$$

The algebraic signs of the eigenvalues provide an idea about the nature of its stationary point. If the values are all negative, it is a maximum; if all positive, it is a minimum, and if the signs are mixed; it is a saddle point.

RESULTS AND DISCUSSION

Table IV shows the independent variables which were used to optimize *G. verrucosa* growth and their values at different coded and actual levels. As shown there was a considerable variation in the *Gracilaria verrucosa* growth depending on the four chosen variables. The maximum growth (>3.20) was

Table IV : Central composite design (CCD). Factors are x_1-salinity (‰); x_2-ammonium (mg/l); x_3-nitrate (mg/l); x_4-phosphorus (mg/l). The factors levels are 20 and 40 ‰ for x_1, 0 and 6 mg/l for x_2, 0 and 6 mg/l for x_3, 0 and 2 mg/l for x_4.

Run	x_1	x_2	x_3	x_4	Weight (g)
1	-1	-1	-1	-1	0
2	-1	-1	-1	1	0
3	-1	-1	+1	-1	0
4	-1	-1	+1	1	0
5	-1	+1	-1	-1	0
6	-1	+1	-1	1	0
7	-1	+1	+1	-1	0
8	-1	+1	+1	1	0
9	+1	-1	-1	-1	3.27
10	+1	-1	-1	1	2.95
11	+1	-1	+1	-1	3.06
12	+1	-1	+1	1	3.10
13	+1	+1	-1	-1	3.01
14	+1	+1	-1	1	2.62
15	+1	+1	+1	-1	2.36
16	+1	+1	+1	1	0
17	-2	0	0	0	0
18	2	0	0	0	2.96
19	0	-2	0	0	2.88
20	0	2	0	0	0
21	0	0	-2	0	2.90
22	0	0	2	0	0
23	0	0	0	-2	2.77
24	0	0	0	2	2.45
25	0	0	0	0	2.50
26	0	0	0	0	2.80

achieved in run number 15, whilst mortality was observed in many runs particularly in salinity treatments lower than 30 ‰.

An examination of the behavior of the system and a determination of optimum conditions were performed. Canonical analysis was conducted, using the normal form of the second-order model in :

$$\mathbf{Y = 3.54 - 0.33}w_1^2 \mathbf{- 0.34}w_2^2 \mathbf{- 0.33}w_3^2 \qquad \mathbf{(6)}$$

Eigenvalues from the canonical transformation of the response surface were used to interpret the nature of the stationary point. The eigenvalues of the transformed system were equal to - 0.33, - 0.34, - 0.33. Thus, the stationary point is a maximum. If the stationary point is outside the region of exploration for fitting the second-order model and one or more eigenvalues are near zero, another canonical should be used.

Three-dimensional response plots and their corresponding contour plots for the *G. verrucosa* growth by the above model are shown in figures 1. Figure 1a depicts the three-dimensional plot and its respective contour plot showing the effects of salinity (x_1) and ammonium concentration (x_2) on *G. verrucosa* growth, while x_3 and x_4 were fixed at it mid levels. The contour plots indicated that no interactions between salinity and ammonium concentration were found to contribute to the response at a significant level. *G. verrucosa* growth increased gradually with the increasing salinity and ammonium concentration (figure 1a). When salinity levels were low the effect of ammonium on the response was insignificant. *G. verrucosa* growth increased with increasing salinity and ammonium up to 38‰ and 2.5 mg/l but subsequently decreased slowly beyond these respective points of salinity and ammonium concentration. Figure 1b shows the effects of salinity (x_1) and nitrate concentration (x_3) on *G. verrucosa* growth, whilst the other variables are fixed at mid level. There were no evident interaction relationships existing between the two independent variables and the response variable. It was evident that at low nitrate concentration, the effect of salinity on *G. verrucosa* growth was negligible. Growth increased when the salinity and nitrate concentration increase up to 38‰ and 2 mg/l and decrease slowly when nutrients concentrations increased.

Reducing salinity to 30 ‰ or lower had a significant effect on the growth rate of *G. verrucosa*. In batch

cultivation, the growing apexes of the thalli turned white and the alga stopped growing in advance of the whole thalli turned white and dying. Deterioration of the thalli was observed after one week of culture, indicating low tolerance of this species to reduced salinity. The results in our study concur with previous studies (Bird, 1988 and Choi et al., 2006) who indicated that salinity of less then 25‰ negatively affected *Gracilaria* growth. Sfriso et al. (1987); Kamer and Fong, (2000) also recorded decreased weight and accumulation of NH_4^+ in the water column with low salinity. Salinities above 38‰ threshold reduced *G. verrucosa* growth significantly (Marinho-soriano et al., 2006).

Lower salinity occurs naturally in Bizerte lagoon for long periods throughout the year due to large inputs from precipitation and runoff from the surrounding watershed. Winter months are cool with high rainfall, and summers are warm and dry with little or no rainfall. Consequently salinity in winter drops below 30 ‰ and in summer rises to reach 40 ‰ (Harzallah, 2003). *G. verrucosa* was largely absent in the lagoon during these two periods of the year, but the lagoon experienced significantly increased biomass in spring and autumn when salinities were between 30 and 38 ‰ (Ksouri et al., 1998). Spring growth is superior to that in autumn. However *G. verrucosa* growth may be affected not only by salinity but by others factors.

Growth rate of *G. verrucosa* increased with increasing NH_4^+ and NO_3^- concentration simultaneously. This result is consistent with those of Bjornsater and Wheeler (1990) who found that both photosynthesis rate and growth rate of *G.asiatica* and *G.tikvahiac* increased under high nitrogen concentrations. Maximal growth of *G. verrucosa* was supported by equal concentrations of NH_4^+ and NO_3^-. Our results were consistent with those of Thomas *et al.*, (1987), but not with D'Elia and DeBoer, (1978) ; Ryther et al., (1981); Thomas et Harrison, (1985) and Rees,(2003), who found that *Gracilaria* grows more successfully on NH_4^+ than NO_3^-. However, it appears that the difference between these studies may be due to environment conditions, essentially temperature. A positive correlation between absorption rate of nitrate and temperature was reported (Nishihara and al., 2005). According to Smit (2002), *Gracilaria sp.* appears to have a higher affinity for NH_4^+ than for NO_3^- at low temperatures, but this difference is lost at 20 °C. Our results also showed that the growth rate of *G. verrucosa* fell when NH_4^+ and NO_3^- concentration went beyond critical points, which were 2.5 and 2mg/l respectively. As concentration of these two nutrients increases, the growth rate of G. *verrucosa* gradually fell, which is in agreement with the results reported by Hanisak (1990) and Yu and Yang (2008).

Response Surface Methodology plots was generated using the data shown in Table V. Inputs were the 26 experimental runs carried out under the conditions established by the Central Composite Design. (a) Final weight (g) as a function of ammonium and salinity. (b) Final weight (g) as a function of salinity and nitrate. (c) Final weight (g) as a function of ammonium and nitrate. (d) Final weight (g) as a function of ammonium and phosphate. (e) Final weight (g) as a function of nitrate and phosphate. (f) Final weight (g) as a function of salinity and phosphate. The value of the missing independent variable in each plot was kept at the center point

Figures 1d, 1e and 1f, shows the interaction occurring between phosphate concentration ($\boldsymbol{x}_{4)}$ and others factors. the surface presents a stationary ridge (not a single point maximum or minimum, but a line of maxima). The contour plots indicated that the interactions between phosphate and others factors were not significant. Furthermore the effect of phosphate on *G. verrucosa* growth was the same with low or high levels. In this study,comparison of the dissolved inorganic nitrogen to phosphate suggested that nitrogen, rather than phosphate limit productivity. In general, a 10:1 proportion of N/P is used to obtain the best growth responses in seaweeds (Friedlander and Levy, 1995). These results were contrary to our study; we did not find differential growth rates of G. *verrucosa* between N/P ratio of 10:0 and 10:2. Furthermore, the effect of phosphate on G. *verrucosa* growth was not observed to be negative in nature. According to Navarro-Angulo and Robledo, (1999), at ratios of 10:0; G. *cornea* presented lower growth rates than at 10:1 ratios, whereas a negative effect of P was detected when 10:10 ratios were tested. Differences in results may be attributed to species specific responses to P ratios.

CONCLUSIONS

Results from this study have demonstrated the feasibility of using response surface methodology in studying *G. verrucosa* growth under lagoon condition. In conclusion, *Gracilaria verrucosa* growth was mainly affected by salinity. By reducing salinity to 25 ‰ or low growth in apex thalli was inhibited, thalli subsequently turned white and died. An excess concentration of individual variables had no effect in the case of phosphate but did have a negative effect (in the case of salinity, ammonium and nitrate) on *G. verrucosa* growth. We conclude

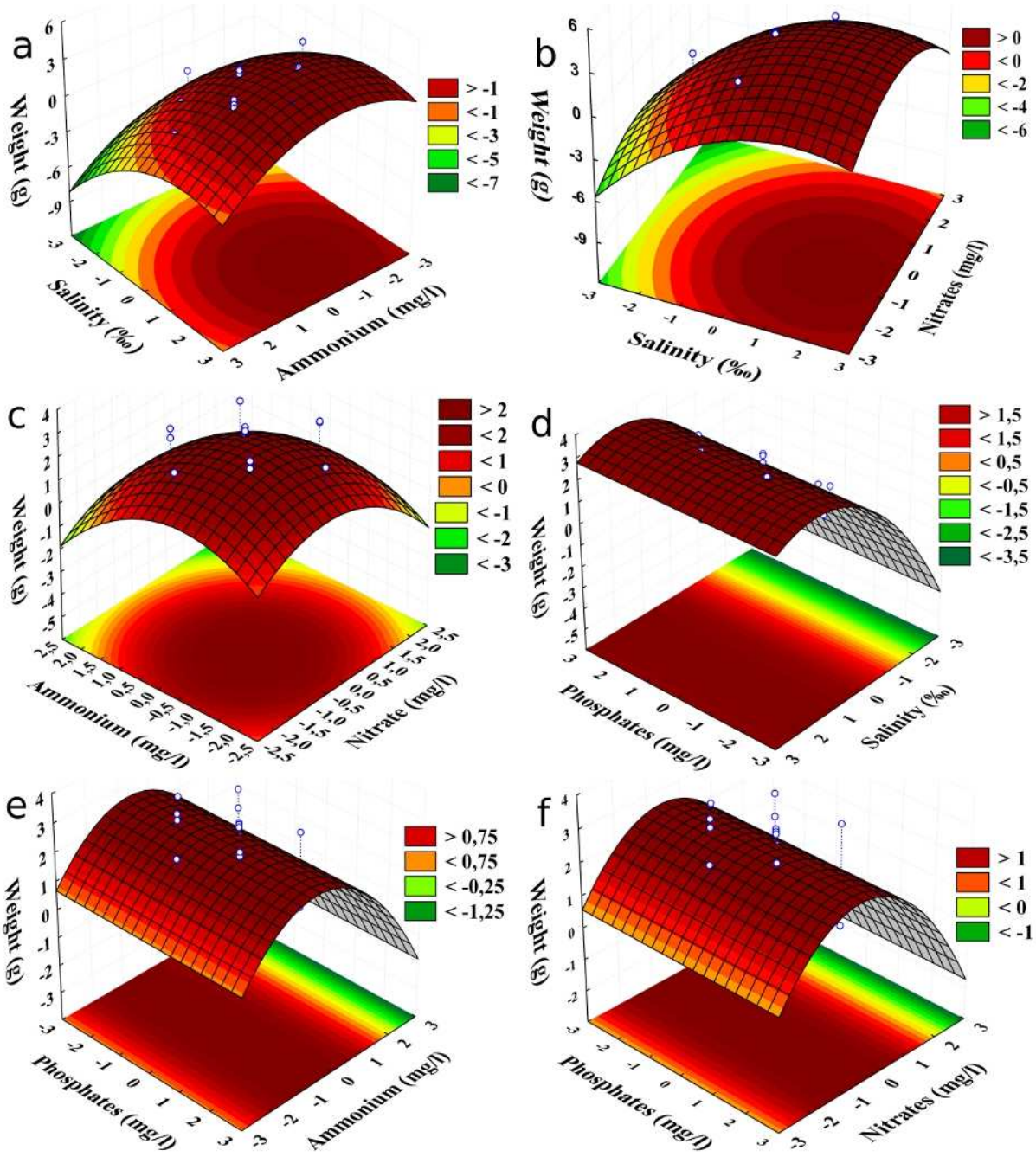

Fig.1. Three dimensional contour plots for the maximum final weight.

from this study that the optimal growth is obtained with the salinities ranging between 30 and 38 ‰, with equal concentrations of ammonium and nitrate and lower phosphate concentrations.

The results of this study show that the growth values obtained by *G. verrucosa* in this lagoon were not satisfactory to suggest implementing viable commercial scale cultivations in this particular lagoon area according to our model.

Table V : Analysis of variance (ANOVA) for the response surface quadratic model

	Degrees of freedom	Sum of Squares	Mean Squares	F test	P value
Linear					
Nitrates	3.47	1.00	3.47	5.73	0.03
Ammonium	4.29	1.00	4.29	7.08	0.02
Salinity	28.80	1.00	28.80	47.50	0.00
Square					
Nitrates	2.52	1.00	2.52	4.15	0.06
Ammonium	2.56	1.00	2.56	4.22	0.05
Salinity	2.41	1.00	2.41	3.97	0.06
Residual error	11.52	19.00	0.61		
Total	52.69	25.00			

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