Effects of depth and initial fragment weights of *Gracilaria gracilis* on the growth, agar yield, quality and biochemical composition

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Introduction

Globally, seaweeds are increasingly used as food, feed and fertilizers in agriculture thus representing a valuable source of income and a sustainable livelihood for coastal communities (Rebours et al.2014). In the industry, algae are used in various sectors such as pharmaceutical, nutraceutical and cosmetic. The production of aquatic plants, mostly seaweeds reached 27.3 million tons in 2014 (FAO 2016). Several interesting compounds are obtained from seaweeds. Among these, hydrocolloids such as alginates, agars, and carrageenan are considered the main natural substances which have a large use spectrum (Bixler and Porse 2011). The Gelidiales and the Gracilariales are among the red seaweeds the most used in agar industry. Nevertheless, the wild raw material is not always sufficient to supply a viable industry facing the increasing demand of seaweeds. Accordingly, seaweed cultivation is actually practiced in several countries such as China, Japan, Chile, Argentina, Philippines, Indonesia and Malaysia to supply hydrocolloid factories with substantial biomass of agarophyte and carragenophyte macroalgae (Ren-Zhi et al.1984; Xing-Hong and Juan Wang 1993; Pizarro and Santelices 1993; Bixler and Porse 2011; Zuldin et al. 2016).

Approximately 100 species of red algae, including *Gracilaria* spp are cultured worldwide for the agar industry. In fact, *Gracilaria* is well known for its high quality of both agar and agarose which are mainly used for food, pharmaceutical and biotechnology applications (Hurtado et al.2014). In addition, interest in other *Gracilaria* compounds such as carbohydrates, proteins and lipids is increasing worldwide, in order to investigate their potential nutritional and energy values. An increased interest in different pigments, namely the R-phycoerythrin and other secondary metabolites is also noted in several studies (Mensi et al.2012; Francavilla 2013). Indeed, three kinds of pigments are directly involved in the photosynthesis: chlorophylls, phycobiliproteins and carotenoids (Meeks 1974; Goodwin

1974; Ragan et al. 1986; Rowan 1989; Dring 1990). Among the phycobiliproteins, the R-phycoerythrin is considered to be a predominant pigment in red algae. This pigment is at present used in food industry as colorant. Nevertheless, because of the spectral properties, it is used mainly in clinical and immunological analysis (Mensi et al.2012), in fluorescence microscopy and flux cytometry (Glazer and Stryer 1984; Kronick 1986). It is also reported that R-phycoerythrin and phycocyanin act as a part of protective mechanism in seaweeds in general (Sinha et al.1995) and in *Gracilaria caudata* particularly (Araujo et al.2014). To our best knowledge, few studies (Molloy and Bolton 1996; Mensi et al. 2009) were published regarding the effect of both depth and initial fragment weights on growth and proximate chemical composition of *Gracilaria gracilis* (previously known as *G. verrucosa*).

Presently, there are neither large scale seaweed cultivation nor agar industry in Tunisia. Nevertheless, several attempts of *Gracilaria* farming were performed in the two last decades, using both spores (Ben Said and Aouini 2014) and cuttings/fragments (Ksouri et al.1999, 2000; Mensi et al.2009). Four farming methods were used: ropes, sand filled plastic tubes, spade and suspended system in hoop-nets. On the other hand, some experimental agar extractions have been carried out on *Gracilaria gracilis* (Ben Said and Ksouri 1999; Ben Said et al.2015).

This study aimed to determine the ability of *Gracilaria gracilis* to be cultivated at two different water depths in Bizerte lagoon, using net pockets. Additionally, relatively low initial fragment weights, compared with previously reported studies (Ksouri et al.1999, 2000; Mensi et al.2009) were also tested in order to have an appreciable biomass with a high daily growth rate (DGR) in relation to environmental factors. The biomass could be firstly used for agar extraction and secondly for other potentially interesting compounds. Hence, agar yield, gel strength, gelling and melting temperatures were investigated because of their properties to

correlate with the 3,6-anhydrogalactose and sulfate contents (Minghou et al.1985; Oyieke 1993; Rodriguez et al.2009). Furthermore, IR spectroscopy was performed to study agar extracts since this technique is considered as an appropriate tool tool to distinguish different phycocolloids, especially agars and carrageenans (Gomez-Ordenez and Rupérez 2011). Also, the proximate biochemical composition of farmed seaweeds was studied. The main objective of this work is to determine which starting weight and which depth could lead to better results, mainly agar yield, quality and biochemical characterization for various potential valorizations in the future.

Material and methods

Sampling site and culture system preparation

Gracilaria gracilis was collected in Bizerte lagoon, in the North of Tunisia (37°12'N; 9°55'E) nearby a local shellfish farm, at Menzel Jemil, in February 2015. Young; healthy thalli were thoroughly cleaned of sand, epiphytes such as invertebrates and other undesirable seaweeds. They were further washed with freshly collected seawater. Seaweeds were then cut to have cuttings/fragments with different weights. Thus, three initial fragment weights were tested: 5, 20 and 40 g, using a field scale. Fragments (5) were tied using a nylon string and then placed in netting pockets which were fixed to a nylon rope. The latter was fixed to a woody “bridge” used by the local shellfish farmers (Fig.1).The experiment was performed in triplicate at both the two depths: depth 1(0.5m) and depth 2 (2.5m).

Environmental parameters

Water samples were collected in Bizerte lagoon at approximately three week intervals in plastic bottles and kept in an ice cooler box. They were then brought to the laboratory for chemical analysis. The water temperature was recorded “*in situ*” at the two depths, using an

electronic thermometer SA880SSXH (Huger type). Salinity, pH, and dissolved oxygen (DO) were recorded with a multiparameter apparatus (HACH, HQ40d). Nutrients: dissolved inorganic nitrogen (DIN) composed of nitrates (NO_3^-), nitrites NO_2^- and ammonium (NH_4^+), total nitrogen (TN), orthophosphates (PO_4^{3-}) and total phosphates (TP) were determined with an auto analyzer 3 Bran Luebbe.

Growth rate

Gracilaria gracilis was cultivated during 112 days (since February 12, 2015 to June 3, 2015). The daily growth rate (DGR) was calculated every five (5) time intervals, using the following formula:

$$DGR(\%d^{-1}) = \ln((W_t/W_0)/t) * 100$$

where W_t is the final wet weight (g) at t day, W_0 is the initial wet weight (g) and t is the time of culture in days.

Agar extraction and quality determination

At the end of 112 days of culture, dry cleaned seaweed samples (15g each) were firstly washed with tap water to remove salts, then placed in 400 ml of H_2SO_4 (5 %) solution for 1 h at room temperature and further rinsed thoroughly with tap water. Agar extraction was performed in 500 ml of a 5% alkaline solution (NaOH) at 100°C for 80 min. The heated solution was then filtrated using a büchner filter and a vacuum pump. The filtrate obtained was then transferred to a flat steel recipient until it was cooled at room temperature for 15-20 min and then frozen overnight at -18°C. On the next day, the filtrate was thawed at room temperature until a thin agar film was formed. The latter was bleached using a 12 ° sodium hypochlorite solution for 2-3 min, rinsed with tap water and finally oven dried at 105°C

overnight. The dry agar obtained was weighed using a Bosh electronic scale. The agar yield was calculated as follows:

$$\text{Agar yield (\%)} = (W_a/W_s) * 100$$

Where W_a is the dry agar weight and W_s is the dry seaweed weight (g)

Agar quality was determined by measuring gel strength (g cm^{-2}), gelling and melting temperatures ($^{\circ}\text{C}$) as described by Lee et al. (2014) and Ben Said et al. (2011), with a little modification which consists of the use of a steely bead instead of glass one to determine the melting temperature.

Fourier transform infrared spectroscopy (ATR-FTIR)

The agars obtained from farmed seaweeds at the two depths (six samples) were analyzed, using PerkinElmer Spectrum Two ATR-FTIR, over the wave number range between 4000 and 500 cm^{-1} . Other FTIR spectra were also performed to improve resolution of overlapped bands in the original spectra (Matsuhiro and Rivas 1993; Gomez-Ordenez and Rupérez 2011). The farmed seaweed agar samples were compared to each other as well as to the commercial agar to detect any difference.

Proximate biochemical composition

All the following analyzes were performed using the seaweeds harvested at the end of 112 days of culture.

Dry matter and ash content

The dry matter content of cleaned wet seaweeds was determined using the oven method (Mollet et al.1998) at 50 $^{\circ}\text{C}$ until the constant dry weight was obtained. Dry matter (DM) was calculated as follows:

$$\text{Dry matter DM (\%)} = \frac{W_f}{W_i} * 100$$

140

141 Where W_f is the final weight and W_i is the initial weight. Ash was determined by heating
 142 seaweeds in a muffle furnace at 550°C for 4 h and then weighing the residue (AOAC
 143 2000). Ash contents (AC) were calculated as follows:

$$\text{Ash content AC(\%)} = \left(\frac{W_f}{W_i} \right) * 100$$

144

145 Where W_f is final weight and W_i is the initial dry weight

146 ***Carbohydrate, 3,6-anhydrogalactose, sulfate contents and monosaccharide composition***

147 The total carbohydrate content was determined according to the Phenol-Sulfuric acid method
 148 (Dubois et al.1956) as the percentage of dry weight. Briefly, 4 mL of distilled water was
 149 added to 0.5 g of dry powdered seaweed. Furthermore the seaweeds were homogenized using
 150 the vortex and ultra-turrax apparatus. After which, centrifugation was performed during 15
 151 min at 12000 x g, the supernatant (1mL) was placed in an eppendorf tube and frozen at -80°C
 152 until analysis was made. A known volume (20µL) of the extract was put in a test tube to
 153 which was added 80µL of distilled water, 2.5 mL of H₂SO₄ and 0.5 mL of a 5% phenol
 154 solution. After which, the test tubes were homogenized using the vortex apparatus and then
 155 incubated in a water bath at 30°C during 20 min. Finally, the absorbance was measured at 490
 156 nm using a UV-visible Jenway spectrophotometer (6704 type). The glucose was used to
 157 establish the standard curve using different concentrations (0; 20; 50; 75; 100; 150 and 200 µg
 158 mL⁻¹).

159 The resorcinol reagent method of Yaphe and Arsenault (1965) was used to determine the
 160 3,6-anhydrogalactose content. The sulfate content was determined by the turbidimetric

method, using sodium sulfate (Na₂SO₄) after acid hydrolysis (2 M HCl at 100°C for 2 h) of the polysaccharide (Salem et al.2017).

The monosaccharide composition was performed using a 1200 Agilent HPLC chromatography (High Performance Liquid Chromatography) equipped with a Refractive Index detector (RID). Agar extracts (300 mg) were dissolved in a 75% Acetonitrile solution. Polysaccharide solution (20 µL) was automatically injected into the column at a flow rate of 0.8 mL min⁻¹.

Crude protein and R-phycoerythrin

Crude protein content was determined as described by Bradford (1976). Fresh alga (1 g) was first milled with a mortar and placed in 20 mL of distilled water. After which, incubation in bath water was made during 10 h at 50°C. Samples were then filtered through a 250 µm nylon cloth filter. The filtrate was centrifuged at 4000 x g during 15 min. The supernatant of seaweed extract sample (1 mL) was then put in an hemolytic tube to which was added 2 mL of Coomassie Blue reagent and then homogenized with vortex homogenizer. Absorbance was read at 595 nm after 5 min, using a UV-visible spectrophotometer (6405 Jenway type). Curve calibration was performed using a Bovine Serum Albumin (BSA) solution with concentrations ranging from 0 to 2.0 mg mL⁻¹. Protein content Q (mg) in seaweed samples was calculated as follows:

$$Q = V * C$$

Where C is the protein concentration (mg g⁻¹ fresh algae), obtained using the calibration curve; V= initial sampling volume (mL). Results are presented as percentage of dry weight (% dw), given the average dry matter /fresh matter ratio is approximately 15%.

R-phycoerythrin content was determined as described by Mensi et al. (2012) and the absorbance was measured at 565 nm, which is the maximum absorbance of R-phycoerythrin. Beer-Lambert law established the absorbance at 565 nm as follows:

$$A = \varepsilon * L * C_1 = \varepsilon * L * \frac{C_2}{MW}$$

Where:

$$C_2 = A * \frac{260 * 10^3}{2 * 10^6} = 0.13 * A$$

A: absorbance at 565 nm

ε : R-phycoerythrin extinction coefficient ($2.10^6 \text{ M}^{-1} \text{ cm}^{-1}$)

L: optic length (= 1 cm)

C_1 : molar concentration of R-phycoerythrin (M)

C_2 : Concentration of R-phycoerythrin (mg mL^{-1})

MW: molecular weight of R-phycoerythrin (260000 da).

The absorbance was measured using an UV-visible spectrophotometer (6405 Jenway type).

Total lipid content

Total lipids of the farmed seaweeds samples were extracted according to the modified method of Floc'h et al. (1957). Dry powdered seaweed (1 g) was placed in a 50 mL conic tube to which was added 30 mL of Floc'h reagent (Dichloromethane-methanol, 2v/1v) and homogenized with the vortex, with the addition of 5 mL of a 0.73 % NaCl solution. Finally, the solution was filtrated using the cotton inserted in a medical syringe. The extract was then centrifuged during 10 min at 4°C. After which, the bottom part was transferred to test tubes.

205 The evaporation of the solution was performed using the nitrogen gas. The lipid content was
206 determined as follows:

$$\text{Lipid content (\%)} = \left(\frac{\frac{W_f}{W_i}}{W_s} \right) * 100$$

207

208 Where: W_f is the final weight of the test tube (g), W_i is the initial weight of test tube (g) and
209 W_s is the dry weight of seaweed sample (g).

210 Results were expressed as % of dry weight. The experiments were performed in triplicate to
211 determine the agar yield, quality and proximate biochemical composition.

212 **Statistical analysis**

213 The data were presented as the mean $\pm$ standard deviation (SD). The analysis of variance
214 ANOVA (two ways) was performed using the SPSS (version 20.0) software to study the
215 effect of the depth and the initial fragment weight on the DGR, agar yield, quality and
216 proximate biochemical composition. The homogeneity of variance was verified and the
217 Turkey's test was used for the multi comparison of means at the level confidence of 5 %.
218 Pearson correlations were also investigated to have some information on the relationship
219 between all the studied parameters.

220 **Results**

221 *Environmental parameters*

222 The highest seawater temperature was recorded in April at depth 1 (25.3 °C) and the lowest
223 was 13.2 °C in February at the two depths (Table 1). The seawater salinity ranged from 33.7
224 to 39.3 psu. The dissolved oxygen (DO) varied from 7.19 to 10.48 mgL⁻¹. The pH fluctuated
225 between 6.69 and 7.82. The minimum dissolved inorganic nitrogen (DIN) content was 2.81
226 μ M in April at depth 1 and the maximum was 13.64 μ M in June at depth 2. The total nitrogen

(TN) content ranged from 11.959 to 21.346 μM . The inorganic phosphate (orthophosphate PO_4^{3-}) content varied from 0.190 to 0.371 μM , while the total phosphate (TP) content varied from 4.125 to 5.397 μM . The peak was obtained in June 2015 at depth 1.

Growth Rate

Daily Growth Rate (DGR) fluctuation is illustrated in Fig.2 and Fig.3. As shown, the highest DGR was always recorded when using an initial seedling weight of 5 g, followed generally by 20 g and finally 40 g. The maximum value ($5.98 \pm 1.16 \% \text{ d}^{-1}$) was recorded within three weeks of culture ($T_1 = 20$ days) at depth 1 (Fig.2). At the end of the culture, the DGR ranged from 3.08 ± 0.74 to $3.5 \pm 0.49 \% \text{ d}^{-1}$. At depth 2 (Fig.3), the highest DGR was also obtained within three weeks of culture, using 5g as initial seedling weight ($5.14 \pm 1.93 \% \text{ d}^{-1}$), followed by 20g and 40g. At the end of the experiment and after 112 days of cultivation, the DGR varied from 0.75 ± 0.63 to $2.00 \pm 0.62 \% \text{ d}^{-1}$ (Fig.3). It was noted that at T_3 (69 days), seaweeds cultured from initial weight of 40g lost many of branches and consequently the weight drastically decreased. The results of ANOVA showed a highly significant effect of the depth and the initial weight on DGR ($p=0.000$ and $p=0.000$, respectively) and there was a highly significant interaction between both factors ($p=0.004$). Table 2 indicates the different percentages of weight increment during the cultivation period (112 days) As shown, a marked increase of average weight was noted in farmed algae at depth 1, compared to those farmed at depth 2, especially in algae which had 5 g as initial weight (37.6 fold).

Agar yield and quality

The agar yield varied from 13.13 ± 1.03 to $14.87 \pm 1.91 \% \text{ dw}$ at depth 1 and from 9.89 ± 0.34 % to $14.53 \pm 0.29 \% \text{ dw}$ at depth 2. The results of the ANOVA indicated that there was a highly significant effect of initial cutting weight ($p=0.012$). However, there was not a significant effect of the depth ($p=0.069$) and there was a highly significant interaction

between both factors ($p=0.018$). The maximum was recorded at depth 1 in seaweeds which had a starting weight of 5 g, while the minimum was recorded at depth 2 in farmed algae with initial weight of 40 g. The highest agar yields were obtained in algae cultured from initial weight of 5g at both depth 1 and depth 2 (Table 3). The gel strength varied significantly from $171.66 \pm 7.64 \text{ g.cm}^{-2}$ to $356.67 \pm 15.25 \text{ g.cm}^{-2}$ and there was a highly significant effect of starting weight, depth and the interaction of both variables ($p=0.000$). The highest value was obtained at depth 2 in farmed algae which had 20g as initial fragment weight, while the minimum was obtained at depth 1 in algae, which had 5 g as initial weight (Table 3). The gelling temperatures ranged from $34.33 \pm 0.57^\circ\text{C}$ to $35.50 \pm 0.57^\circ\text{C}$ (Table 3). The results of the ANOVA analysis showed no significant effect of depth and initial fragment weights ($p=0.773$; $p=0.067$, respectively). The melting temperature ranged from $77.00 \pm 1.00^\circ\text{C}$ to $85.00 \pm 0.50^\circ\text{C}$ and there was significant effect of initial weights, depth and the interaction between both variables ($p=0.001$, $p=0.000$, $p=0.000$, respectively). The maximum was recorded at depth 1, using an initial weight of 20 g, while the minimum was obtained depth 2 in algae having an initial fragment weight of 5g ($77.00 \pm 1.00^\circ\text{C}$).

Infrared spectroscopy

As presented in Fig.4a, all the spectra (1-6) were comparable to the spectrum (7) of commercial agar via distinctive absorption bands. In fact, a broad band from 3000 to 3600 cm^{-1} was assigned to C-OH elongation vibration. The band at around 2910 cm^{-1} was probably attributed to the 6-O-methyl D-galactose stretching vibration. These results are consistent with those found, according to Mouradi-Givernaud (1992) and Mouradi-Givernaud et al. (1992) from the agar of *Gelidium latifolium*. In addition, we have noticed that depth has an influence on the amount of the residue 6-O-meyhyl D galactose. This was noted by the spectrum 6 (depth 2, initial weight 40 g), compared with the commercial agar and other agar extract

spectra. Moreover, we have noticed that agars obtained from farmed seaweeds contained probably more sulfur and nitrogen than commercial agar via the presence of a little band of N-C-S at around 2160 cm^{-1} . (Spectrum 1; depth 1, initial weight 5g). The spectrum 7 doesn't show such a band. It means that this band is generally obtained from seaweeds belonging to the Gracilariales. In fact, the Gelidiales are well known as source of bacteriological agars, while agars from the Gracilariales are known being used especially in food industry and have lower quality. The additional bands comprised between 1630 and 1643 cm^{-1} were referred to (N-H) absorption stretching vibrations. The band at about 1420 cm^{-1} indicated the presence of ester sulfate, according to Cross (1964) and Mollet et al. (1998). Also, the pick assigned to 1240 cm^{-1} corresponded to the asymmetric stretching S=O (Mollet et al. 1998). This was comparable with works of Christiaen and Bodard (1983) which reported that sulfate ester link was recognized at the bands 1060 , 1180 cm^{-1} and 1370 cm^{-1} in agar film of *Gracilaria verrucosa*. The sulfate esters bands absorption was noted at 1250 cm^{-1} according to Deslandes (1988). In the current study, the bands which appeared at 1164 cm^{-1} were assigned to β -D galactose (Sekkal, 1990), the band at 1070 cm^{-1} was attributed to the skeleton mode of the galactan (Sekkal et al.1993) and the bands absorption at around 1046 cm^{-1} were attributed to 3, 6- anhydrogalactose. For the band absorption at 930 cm^{-1} , it may be attributable to the stretching vibration of C-C of 3, 6 L- galactose (Stanley, 1963). According to Christiaen and Bodard (1983), the bands at 1070 cm^{-1} were attributed to the vibration of the C-O-C bridge of 3, 6 anhydrogalactose. Also, the band comprised at 800 cm^{-1} was specific to the galactose skeleton (Mouradi-Givernaud, 1992). The two bands at 740 and 716 cm^{-1} in the extracted agars were assigned to the C-O-C bending mode in glycosidic linkages (Sekkal et al.1993). Finally, the bands at around 528 cm^{-1} in the spectrum 4 (depth 2, initial fragment weight of 5 g) was attributed to the presence of C-Br absorption stretching vibrations.

The Fig.4 b depicts agar spectra with more resolution in the region of 2000 to 600 cm^{-1} . In agreement with that reported by Gomez-Ordóñez and Rupérez (2011) who found two characteristic agar signals at 790.9 and 715.3 cm^{-1} , the different agar spectra (1-6) of farmed seaweeds and the commercial agar spectrum (7) showed two diagnostic bands in the region 800-700 cm^{-1} . Matsuhira (1996) observed two important bands at 790.8 and 717.0 cm^{-1} in FTIR and its second derivative spectra of the agar from *Gracilaria chilensis*. In the current study, the two similar bands were recorded at around 785-790 cm^{-1} and 715-720 cm^{-1} , respectively. These bands are usually used to distinguish between agar- and carrageenan- type galactans in red seaweeds within a few minutes (Matsuhira 1996). The signals at 893-882 are probably due to the equatorial CH deformation as found by Neely (1957). The band at 925-936 cm^{-1} indicates the presence of 3, 6-anhydrogalactose in agar extracts and in the commercial one. A series of bands in the region of 1166 and 1000 cm^{-1} , especially at 1043-1053 cm^{-1} assigned to the sulfure-oxygen stretching vibrations for the sulfate group (Conley, 1966, Nakamoto, 1986, Mouradi-Givernaud et al.1993; Matsuhira 1996) and the presence of 3,6 anhydrogalactose, as mentioned above are observed in all the spectra.

Proximate biochemical composition

The biochemical composition of farmed seaweeds is summarized in Table 3.

Dry matter and ash content

The dry matter varied from 14.88 ± 0.08 to $22.68 \pm 1.77\%$. The results of the ANOVA indicated that depth and initial weight had a highly significant effect on dry matter ($p=0.009$; $p=0.000$; respectively) and there was a highly significant interaction between both variables ($p=0.000$). The highest value was recorded in samples which had an initial weight of 20g at depth 2, while the minimum was recorded in cultured algae with an initial weight of 5g at depth 1. The ash content ranged significantly from 19.04 ± 0.16 to $35.25 \pm 0.02\%$. The results of the

ANOVA showed a significant effect of the initial fragment weights, depth and the interaction between both parameters ($p=0.000$). The maximum was recorded in algae with starting fragment weight of 5 g at depth 2, while the minimum was recorded in algae of starting weight of 40g at the same depth (Table 3).

Carbohydrate, 3,6-anhydrogalactose, sulfate contents and monosaccharide composition

The carbohydrate content varied significantly from 5.38 ± 0.35 to 9.52 ± 0.36 % dw. The results of the ANOVA indicated a highly significant effect of depth and initial weight ($p=0.000$, $p=0.005$, respectively) and there was a significant interaction between both variables ($p=0.000$). The maximum was obtained at depth 1 in farmed algae which had an initial cutting weight of 5 g, while the minimum was obtained at depth 2 for algae which had a starting weight of 40 g (Table 3). The 3, 6-anhydrogalactose content varied significantly from 20.12 ± 8.93 % to 47.17 ± 5.07 % (Table 3). The minimum and the maximum values were obtained at depth 1. The results of ANOVA showed a highly significant effect of initial weight ($p=0.034$), while there was not a significant effect of depth ($p=0.589$). The sulfate content ranged from 4.11 ± 0.19 % to 5.51 ± 0.41 %. The highest value was obtained with the agar samples at depth 1 and from initial weight of 5g. On the contrary, the lowest value was recorded with agar sample obtained from seaweeds cultivated at depth 2 with initial weight of 40g (Table 3).

The analysis of the monosaccharide composition, using a liquid chromatography system revealed the presence of the glucose in all the agar extracts and in the commercial one. The amount ranged from 1.62 ± 0.04 % to 14.06 ± 0.09 %. The xylose was solely detected in two extracts with small amount (Table 3). The highest content was obtained in the agar extract of farmed seaweeds at depth 1 with an initial weight of 40g, while the lowest was obtained from agar extract of farmed seaweeds at depth 2 with the same initial weight. The statistical

analysis showed significant effects of initial weight, depth and the interaction between the two factors ($p=0.000$).

Crude protein and R-phycoerythrin content

The crude protein content varied significantly from 2.96 ± 0.28 to 5.83 ± 0.70 % dw. The statistical analysis showed a highly significant effect of depth, initial fragment weight and the interaction between both variables ($p= 0.009$; $p=0.006$; $p= 0.000$, respectively). The highest value was recorded at depth 1 in seaweeds cultured with starting fragment weight of 5 g, while the lowest one was obtained at depth 2 in algae which had a starting fragment weight of 40g (Table 3). The R-phycoerythrin content ranged from 0.011 ± 0.006 mg g⁻¹ to 0.050 ± 0.007 mg g⁻¹) and there was a significant effect of depth, initial weight and the interaction between both variables ($p=0.000$). The maximum was recorded at depth 1 in farmed algae with initial weight of 5g, whereas the minimum was recorded at depth 2 in algae which had a starting weight of 40g.

Total lipids content

The total lipid content varied from 1.37 ± 0.11 to 3.58 ± 0.63 % dw. The highest lipid content was obtained in algae cultured at depth 2 from initial weights of 5 g, while the minimum was recorded at depth 1 in algae cultured from initial fragment weights of 5g (Table3). The results of the ANOVA showed a significant effect of initial weights ($p=0.004$). On the contrary, there was no significant effect of depth ($p=0.133$), whereas there was a highly interaction between the two factors ($p=0.000$).

Discussion

The present study has pointed the ability of *G. gracilis* to be cultivated at two different depths: depth 1(0.5 m) and depth 2(2.5 m). On the other hand, this alga can propagate using relatively low initial fragment weights ranging from 5 to 40g. Nevertheless, the highest DGR was

370 recorded at depth 1, using a starting fragment weight of 5g ($5.98 \pm 1.98 \% d^{-1}$, after 20 days of
371 cultivation and $3.50 \pm 0.49 \% d^{-1}$ after 112 days of the study). The results obtained were
372 higher than those reported by Ksouri et al. (1999) and Mensi et al. (2009) who found a DGR
373 ranging from 1.3 to 3.7% d^{-1} , respectively. In the latter study, the cultivation periods of
374 *Gracilaria verrucosa* (as *G. gracilis*) at Bizerte lagoon were 58 and 35 days, respectively, at
375 depths ranging from 1 m to 4 m and using initial fragment weights of 200 g. However, results
376 obtained in the current study were lower than those of Molloy and Bolton (1996) working
377 with *Gracilaria gracilis* at Lüderitz, Namibia. The DGR recorded in this area ranged from 5.7
378 to 12.1 % d^{-1} . The optimal depth range for growth was found to be 0.5-2.5 m, which is in
379 agreement with our results. Nevertheless, the results were in accordance with those of Molloy
380 and Bolton (1996) regarding the decrease of DGR with an initial fragment weights increasing
381 (10 to 70 g). Accordingly, the main differences between DGR at the two depths could be
382 explained by the light availability for the seaweeds and the self shading which influenced the
383 seaweed light harvesting that caused the decrease of the photosynthesis process. On the other
384 hand, the water current at depth 2 had conspicuously an important impact on the farmed
385 seaweeds which lost many branches during the study, compared to the farmed seaweeds at
386 depth 1. This was reflected by a negative growth in some cases. Despite of the occurrence at
387 relatively high depth, the genus *Gracilaria* is well known to prefer calm waters in estuaries,
388 bays and lagoons and develops generally in shallow waters. That is very likely the reason why
389 growth at depth 2 was lower than at depth 1, while, the physico-chemical parameters
390 measured in this study didn't vary greatly between the two depths

391 Regarding the agar yield which ranged from 9.89 ± 0.32 to $14.89 \pm 1.91 \% dw$, the results
392 obtained were generally comparable to those reported by Mollet et al. (1998) for *Gracilaria*
393 *gracilis* harvested in Brittany coast (France). The agar yield varied in this area from 11.1 % to

394 18.7 % dw. But in similar period of harvest (June), our results were higher than those found
395 by these authors (12.7 % dw). Marinho-Soriano and Bourret (2003) reported that the agar
396 yield of *Gracilaria gracilis* collected in the Thau Lagoon (France) varied from 19 to 30.5 %
397 dw and the gel strength ranged from 229.5 to 828 g cm⁻¹. These results are generally higher
398 than those found in this study, suggesting that geographical location and seasonal
399 environmental factors may influence the agar synthesis and rheological properties of extracted
400 hydrocolloids. Rebello et al. (1996) reported that *Gracilaria gracilis* had the highest agar
401 yield and gel strength in early summer and spring, which is in conformity with those of
402 Martin et al.(2013).These authors working with *Gracilaria gracilis* in the Patagonian coast of
403 Argentina have generally recorded lower values of agar yield and gel strengths than those
404 found in the current study. On the other hand, several studies (Bird, 1988; Molloy and Bolton,
405 1996; Arano et al.2000) showed that light, nutrient levels, especially nitrogen level have been
406 reported to be crucial factors affecting the growth, the agar yield and quality of *Gracilaria*.
407 Nutrient enrichment is known to result in a decrease in agar yield of many red types of
408 seaweed (Bird, 1988; Freile-Pelegrin, 1996). Arano et al. (2000) reported that higher gel
409 strength in different species belonging to the genus *Gracilaria* was observed in plants grown
410 in culture under low light treatment and the highest nitrogen enrichment that conflicts with
411 other studies (Bird and Ryther 1990; Penniman and Mathieson 1985). It was also reported that
412 the growth and chemical constituents of *Gracilaria tenuistipitata* were affected by
413 concentration of salts and nutrients (Israel et al. 1999). In addition, intrinsic factors such as
414 genetic characteristics can influence agar yield and quality. In fact, the gel strength, gelling
415 and melting temperatures are the result of the agar composition. Low gel strength is the result
416 of the lowly methylated and high sulfated galactan. This agarocolloid is mainly composed of
417 the galactosyl-6 sulfate residues considered as the putative precursor of the 3,6-

anhydrogalactose (Mollet et al. 1998). Whereas the methoxyl groups influence the gelling temperature (Guiseley, 1970), the sulfate groups influence the gel strength and melting temperature (Duckworth and Yaphe 1971). On the contrary, Lee et al. (2014) reported that both the 3, 6 anhydrogalactose and total sulfate ester content were found to have no relationship with the agar gel strength. Roleda et al. (1997) reported that the variation of gelling temperature is related to the increase in gel strength. Furthermore, Murano et al. (1992) found that a relationship exists between the increase of methoxyl groups and the gelling temperature as well as the molecular weight of the polymer (Kapraun, 1994).

Main correlations found between agar yield, quality and biochemical composition of farmed seaweeds and agar extracts are presented in Table 4. The gel strength was positively correlated to gelling temperature at depth1, while the melting temperature was inversely correlated to the carbohydrate content at depth 2. On the other hand, the lipid and protein contents were inversely correlated both at depth 1 and depth 2. The lipid content was inversely correlated to the carbohydrate content at depth 1, as mentioned by Jayasankar *et al.* (2005). However, there was no significant correlation at depth 2. Also, it was observed that the content of the main organic components of the farmed seaweeds (the proteins and the carbohydrates) varied generally in the same way and inversely to the lipid synthesis trend. The relationship between the R-phycoerythrin and these constituents wasn't very clear. This may be explained by the synergistically interaction of many factors or the inhibitory effect of some factors which orientated the algae metabolism process to produce different cellular components as a response to environmental factors. In this context, Falkowski and LaRoche (1991) reported that when the seaweeds are shifted from high to low irradiance, they suffer an "energy crisis", to which they may respond by diverting macromolecule biosynthesis from lipids and carbohydrates to proteins (for light-harvesting complexes) and then back to lipids

(for photosynthetic membranes). When algae are shifted to high light, their pigments decrease via dilution (through growth) and degradation. These changes are called photoacclimation (Falkowski and LaRoche, 1991). Floreto and Teshima (1998) reported that the exposure of three different seaweeds (*Ulva*, *Grateloupia* and *Sargassum*) to low irradiance resulted in the increase of poly-unsaturated fatty acids. This was explained as a result of the increased galactolipid synthesis, thylakoid membrane stacking and chloroplast volume (Sikko-Goad et al. 1988; Sukenik et al. 1989) as the plant maximizes the capture of light. At depth 2, light intensity is evidently lower than at depth 1. In these conditions, the response of farmed seaweeds may likely be explained by the concomitant effect of light availability, water turbidity and motion. Other factors could induce the lipid synthesis, in addition to the mineral accumulation in the alga cells of the farmed *Gracilaria gracilis*, namely in seaweeds which had a starting weight of 5 g.

On the other hand, the results reported in this study indicated that the maximum values of dry matter were recorded at depth 2, regardless of the starting cutting weight. Nevertheless, the highest value was obtained in seaweeds which had an initial weight of 20 g (22.68 ± 1.77 %). These results showed that in such samples, the organic fraction and mainly the mineral one were probably more presented than in the other seaweed samples. Our results are lower than reported by Turan et al. (2015) in other red algae harvested in Turkish coasts. The dry matter in this area ranged from 27.35 ± 0.51 % to 32.68 ± 0.69 %. The results with respect to the ash content showed that the highest value was recorded at depth 2, using seaweeds which had a starting weight of 5 g. Nevertheless, and regardless of the depth at which seaweeds have grown, the maximum ash contents were observed in seaweeds having an initial weight of 5 g and 20 g. Therefore, the accumulation of mineral fraction in these seaweeds was higher than in the others. The high proportion of ash content, namely at depth 2 was related to the ability

to absorb mineral and trace elements from the surrounding environment easily with remarkable amount. This finding may explain in part the high percentage of dry matter in these conditions as discussed above. In this study, the ash contents were in general higher than those found by Zuldin et al. (2016) in the red alga *Kappahycus striatum* and *K. alvarezii* and in three different red algae (*Laurencia obtuse*, *L. papillosa* and *Jania rubens*). This finding could be explained by the differences between seaweed species, in addition to the environmental factors in each sampling location.

Concerning the carbohydrate content, our results are in accordance with those found by Mensi et al.(2009) which indicated that the maximum values were recorded at lower depth (0-1 m) and the minimum were recorded at -3 m depth. Nevertheless, our results were generally higher than those reported by these authors. The carbohydrate content of *Gracilaria edulis* cultured from spores in the open sea of India didn't exceed 20.5 % dw (Jayasankar et al. 2005). But the depth of raft culture wasn't mentioned in this study. On the other hand, Turan et al. (2015) reported that the carbohydrate content varied from 199.69 ± 9.19 mg g⁻¹ to $374,02 \pm 7.29$ mg g⁻¹ dw in three red algae and from 506.69 ± 9.19 to 643.93 ± 4.68 mg g⁻¹ dw in two other green seaweeds. Thus, the fluctuation of the carbohydrate content seems to be species dependent and also probably related to several factors such as environmental ones (temperature, salinity, nutrients, etc.). The high carbohydrate content recorded at depth 1 with seaweeds which had the lowest initial weight (5g) was probably due the bulk photosynthesis carbon fixation in these conditions (Martin et al. 2013).

The 3, 6-anhydrogalactose contents found in the current study are generally in accordance with the findings of Mollet et al.(1998) for three different species that belong to the Gracilariaceae harvested in Brittany, France. For *Gracilaria gracilis*, the 3, 6-anhydrogalactose content varied from 36.6 % to 50.4 %, according to the intertidal level in

the Brittany coast. The highest value was recorded at the upper level, while the lowest one was at the lower one. The results reported by Minghou et al.(1985) showed that 3,6-anhydrogalactose of crude agars varied from 22.3% to 35.2% for different species of *Gracilaria*, while the sulfate content ranged from 3.2% to 5% , which is in accordance with our results. In the current study, a positive correlation was found between sulfate content and 3,6-anhydrogalactose content at depth 2, while an inverse correlation was found at depth 1 (Table 4). Oyieke (1993) found that the sulfate content varied markedly, with the highest amount recorded for *Gracilaria corticata* (3.05 %) and the lowest for *Gracilaria sp* (0.3%) native agars. Considerable degree of sulfation on different native agar extracts from *Gracilaria gracilis*, ranging from 4.6% to 16.8% were reported by Rodriguez et al. (2009). Lee et al. (2014) observed that gel strength is not solely dependent on the amount of 3,6-anhydrogalactose as mentioned above, which is in conformity with our findings. The sulfate esterification level at various positions, molecular weight of agar and methylation level may influence agar gel strength (Murano 1995).

On the other hand, the occurrence of different sugars such as the glucose and xylose in the agar extracts is considered as a contamination (Yenigul 1993). Therefore, it could affect rheological properties. Floridean starch is usually extracted with hot water (Rodriguez et al.2009). Results reported in this study showed the presence of glucose in agar extracts with different amounts according to the initial weight and depth, indicating the degradation of the floridean starch at different levels as a result of differences between depths and initial seaweed weights. The starch has been already reported to affect gel properties, mainly gel strength, melting and gelling temperatures (Mouradi,-Givernaud 1993). The results obtained in this study were generally in the range (1.6-5.5%) of those previously reported by Mollet et al.(1998) for *Gracilaria gracilis* and lower than observed by Rodriguez et al.(2009) for the

same species. The glucose content in the commercial agar was the lowest at all the agar extracts (1.49 ± 0.03 %). Indeed, the commercial agar for laboratory research is generally obtained from seaweeds that belong to the Gelidiales and is with high grade, while agars from Gracilariaceae are known to be of lower quality. Table 4 depicts significant correlations between glucose content and other studied parameters. Thus, glucose content was inversely correlated with melting temperature at both the two depths ($r = -0.88$, $r = -0.83$, respectively). Xylose was not detected except in two extracts. It was considered with the mannose as a trace (Mouradi-Givernaud 1993; Rodriguez et al.2009).

The crude protein and R-phycoerythrin contents varied closely similarly at depth 1. In fact, the maximum values were obtained in seaweed samples cultured from initial weight of 5 g, followed by those of 20 and 40 g. At the two depths, the variation of R-phycoerythrin content varied inversely to the increase of the initial weight. In addition, at depth 2, all the values were lower than those recorded at depth 1. This was explained by the availability of the light to the farmed seaweeds at this depth. In fact, despite of the ability to grow until -5 m (FAO 1987), *Gracilaria gracilis* can't reach high growth because of the decrease of the photosynthesis due to the decrease of light availability and the water transparency. Martin et al.(2013) reported that high nitrogen and phosphate content in water coincided with important thylakoidal development in algal cortical cells and higher protein content in the products extracted from autumn-winter (May–August) plants of *Gracilaria gracilis* in Argentina coasts. In the present investigation, the period of the harvest of the farmed seaweeds was performed in early summer (June). It coincided with the highest value of total nitrogen and total phosphate, namely at depth 1 (Table 1). In these conditions, we have recorded the highest values of carbohydrate, protein and R-phycoerythrin contents, especially in algae which had an initial weight of 5 g. At depth 2, the pigments, namely the R-phycoerythrin

538 didn't harvest sun light with high efficacy. As a result, farmed seaweeds at depth 2 showed
539 lower red pigmentation and were rather yellow colored and suffered from epiphytes like
540 shellfishes and hydrozoa. Whereas the results obtained in the present study were higher than
541 those reported by Mensi et al.(2009) with respect to the protein content in *Gracilaria*
542 *verrucosa* (as *G. gracilis*), they were in accordance regarding the R-phycoerythrin content
543 which ranged from 0.35 to 0.75 mg g⁻¹ dw for farmed *G. verrucosa* at similar depth(0-1 m).

544 The lipid content varied according to the taxonomic entity, season, and location and
545 growing conditions in both macroalgae and microalgae (Khotimchenko, 2006). In this
546 context, the lipid contents in seaweeds ranged from 1 to 6 % dw (Fleurence et al.1994). In the
547 present study, the level of lipid content varied from 1.37 to 3.58 % dw, which is higher than
548 the results found by Fleurence et al. (1994) for *Gracilaria verrucosa* of the Brittany coast
549 (1.3-1.5 % dw). On the other hand, Khotimchenko (2006) reported that the lipid content
550 varied from 2.1±0.3 to 3.1 ±0.5 % fw, according to the development stage of *Gracilaria*
551 *verrucosa* collected from natural habitats in the Great Peter Bay in the Sea of Japan. In the
552 current study, the highest lipid content was recorded at depth 2. It was closely similar to that
553 obtained for the red alga *Grateloupia turuturu* collected from the intertidal zone at Piriac-sur
554 Mer, in the Atlantic coast of France (Kendel et al.2013). Yong et al.(2015) working with the
555 carrageenophyte *Kappaphycus alvarezii* cultured in Malaysia, registered that the lipid content
556 varied from 2.06 ±0.15 to 3.00±0.29 % dw. The results reported in this study suggested that
557 the water depth in concomitance with turbidity may cause a stress to the seaweeds which
558 orientated their metabolism to produce lipids with relatively high level. It was also observed
559 that the maximum and the minimum lipid contents were inversely recorded at both depths in
560 farmed seaweeds which had 5 and 40 g as initial weights. This statement was recorded with

an inverse trend of the protein contents as described above. This may be also related to the photosynthesis process and accordingly all the metabolism process.

Conclusion

The present study evidenced the ability of *Gracilaria gracilis* to be cultivated at two different depths, using relatively low starting fragment weights (ranging from 5 to 40 g). Nevertheless, the DGR was the highest at depth 1(0.5m). It also revealed that the maximum values of agar yield and melting temperature were recorded at depth 1, while the gel strength and the gelation temperature were obtained at depth 2.

The infrared spectroscopy showed generally the same trend of the agar extract spectra when compared to each other and to the commercial one, with characteristic signals at around 790 cm^{-1} and 715 cm^{-1} . However, some different signals were recorded and related to the amount of some agar constituents such as 3,6-anhydrogalactose and sulfates.

Regarding the proximate biochemical composition of the farmed seaweeds, the results generally showed that the highest values of the carbohydrate, crude protein and R-phycoerythrin contents were obtained in algae cultivated at depth 1 from a starting weight of 5 g, whereas the maximum values of dry matter, ash and lipid contents were recorded at depth 2, regardless of the initial fragment weights. All the results suggested that the parameters tested (depth and initial weight) had clear effects on the growth and biochemical composition of *G.gracilis*.

These findings could firstly help the potential seaweed farmers to choose the depth at which seaweed cultivation could lead mainly to high biomass and in parallel high agar yield with best rheological properties. Based on the results obtained in this current study, farming seaweed valorization could be extended in a second step to other constituents such as minerals, carbohydrates, proteins, lipids, R-phycoerythrin,etc.). On the other hand, when

using a low starting weight, the seaweed farmers could preserve natural resources and in parallel have substantial farming area with numerous cuttings which give an appreciable biomass and consequently a sustainable seaweed farming activity. Further studies are needed to have more knowledge and information on the amino and the fatty acid composition and the different minerals of farmed seaweeds.

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Figure captions

Fig. 1 Farming technique of *Gracilaria gracilis* in Bizerte lagoon (North of Tunisia)

Fig. 2 Variation of Daily Growth Rate (DGR % d⁻¹) in farmed *G. gracilis* at depth 1 (February to June 2015); Values of bars marked with different letters indicate significant difference according to the Tukey's multiple comparison test ($P=0.05$)

Fig. 3 Variation of Daily Growth Rate (DGR % d⁻¹) in farmed *G. gracilis* at depth 2 (February to June 2015); Values of bars marked with different letters indicate significant difference according to the Tukey's multiple comparison test ($P=0.05$)

Fig. 4 a IR Spectra of different agars in the region 4000-400 cm⁻¹. 1: from farmed *Gracilaria gracilis* at depth 1 and with initial weight of 5 g; 2 from farmed *Gracilaria gracilis* at depth 1 and with initial weight of 20 g; 3 from farmed *Gracilaria gracilis* at depth 1 and with initial weight of 40 g ; 4 from farmed *Gracilaria gracilis* at depth 2 and with initial weight of 5 g; 5 from farmed *Gracilaria gracilis* at depth 2 and with initial weight of 20 g; 6 from farmed *Gracilaria gracilis* at depth 2 and with initial weight of 40 g; 7 from commercial agar

Fig. 4 b Spectra of different agars in the region 2000-600 cm⁻¹. 1: from farmed *Gracilaria gracilis* at depth 1 and with initial weight of 5 g; 2 from farmed *Gracilaria gracilis* at depth 1 and with initial weight of 20 g; 3 from farmed *Gracilaria gracilis* at depth 1 and with initial weight of 40 g ; 4 from farmed *Gracilaria gracilis* at depth 2 and with initial weight of 5 g; 5

816 from farmed s *Gracilaria gracilis* at depth 2 and with initial weight of 20 g; 6 from farmed
817 *Gracilaria gracilis* at depth 2 and with initial weight of 40 g; 7 from commercial agar

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822 **Table captions**

823 **Table 1** Physico-chemical parameters at the Bizerte lagoon at two different depths

824 **Table 2** Percentage of weight increment

825 **Table 3** Agar yield, quality and proximate biochemical composition of *Gracilaria gracilis* cultivated
826 in the Bizerte lagoon at two different depths and from different initial fragment weights. Values are
827 means $\pm$ SD; Means with the same superscripts are not significantly different according to the Tukey's
828 multiple comparison test (P=0.05)

829 **Table 4** Main correlations between different parameters studied

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