In vitro proliferation of Lebanese *Lemna minor* and *Lemna gibba* on different nutrient media

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1. Introduction

*Lemna* (duckweed) is a small-sized freshwater floating macrophyte from the family *Lemmaceae*. The individual plant consists of a leaf-like structure, a frond, connected to a fine rootlet [1]. The species inhabits stagnant to gently flowing surface waters and reproduces mainly vegetatively giving rise to genetically uniform plant populations [1]. *Lemna* sp. are commonly cultured for wastewater treatment in the Mediterranean climate [2–5].

Two *Lemna* species are reported to be naturally found in Lebanese water bodies and precisely in Litani River, Bekaa [6–8]. In spite of the number of reported studies on pollution concerning the Lebanese fresh waterbodies [9–11], few studies are concerned with the phytoremediation using macrophytes of the polluted waterbodies [12]. These efforts were mostly limited to the investigation of bio-filtration in the wetland in the Upper Litani River basin to evaluate the constructed wetland performance and determine the treatment efficiency and improvement of the water quality [13].

Duckweeds grow rapidly, and this is vital for their ability to colonize open water surfaces in nature [1,14] whereby accumulation of the biomass is often coupled with nutrient and heavy metals removal from wastewater [3,4,15]. When conditions, i.e. water temperature, pH, light and nutrient concentrations, are optimal, duckweed often demonstrates near exponential growth rates and double the biomass in between 2 and 3 days depending on the environmental conditions [16]. Vegetative growth in *Lemna* sp exhibits cycles of senescence and rejuvenation under constant nutrient availability and consistent climatic conditions [14].

Axenic cultures of duckweeds established under *in vitro* conditions are often used for biological research and stock-culture maintenance. *Lemna* plants can be grown in aseptic nutritive solutions that supply essential macro- and micro-nutrients. Several basic solutions were reported for the proliferation of *Lemna* species, e.g. Murashige and Skoog (MS) [17], Schenk and Hildebrandt (SH) [18] and Algal Assay Procedure (AAP) [19]. These nutrient solutions have been widely used in plant tissue culture [16] and in ecotoxicological studies according to the standardized methods and protocols [19,20]. Duckweeds require many nutrients and minerals to support its optimal growth. Duckweeds appear to be able to concentrate many macro and micro minerals from water [21]. Duckweeds have developed many mechanisms to concentrate trace minerals from slowly decaying plant materials for growth which is often more affected by the concentrations of nitrogen, phosphorous, potassium and sodium levels [21]. There is a large number of nutritive media in use for duckweed cultivation which implies that duckweed can adapt to a broad range of conditions, while the physiological responses are not always the same [22].

*In vitro* culture, if properly optimized, can satisfy all requirements for *Lemna* proliferation. It provides a...
reliable approach for a sustainable and high rate multiplication of *Lemna*, which in turn may enhance remediation ability by providing a tool to understand the uptake of heavy metals for phytoremediation applications [23].

This study aimed to investigate the growth potential under *in vitro* conditions of two Lebanese duckweed species *L. minor* L. and *L. gibba* L., collected from two different sites at the Litani River Ghouzel River and Haouche Harimeh tributaries, respectively, in Bekaa region. The proliferation ability is assessed under axenic and controlled conditions, in response to different nutritive solutions MS, SH and AAP. Such proliferation system is the first step towards enabling trials for future phytoremediation research.

2. Materials and methods

2.1. Pre-culture of plant materials and culture conditions

Plants of *L. minor* and *L. gibba* collected respectively from Ghouzel River and Haouche Harimeh tributaries (Bekaa) in October 2017. Plant individuals were introduced in the laboratory where they were subject to thorough washing with tap water for 10 times. Fronds were then surface-sterilized for one minute by immersion in 0.5% sodium hypochlorite solution, before being rinsed five times with sterile distilled water. Sterilized plants were periodically transferred to 500-ml Erlenmeyer flasks containing 250 ml of sterile culture nutritive solutions. Three nutritive solutions were tested: MS [17], SH [18] and Algal Assay Procedure (AAP) [19] differing by their mineral composition (Table 1). Daughter plants derived from single mother frond were kept and maintained as stock cultures while being exposed to continuous light conditions (24 h) at 100 μmol/m²/s light intensity from fluorescent light tubes (18W/54) at 25°C [20]. Only individual plants with three fronds were used for the experiments of proliferation.

2.2. In vitro growth proliferation

Fronds of both species were chosen randomly from a mixture of at least four flasks of pre-cultured plants. Cultures were grown aseptically under the same conditions described above, in three replicates of 10 plants of three fronds each per medium. Subcultures are renewed every seven days to prohibit nutrient limitation. *Lemna* proliferation was monitored on the different growth media based on the visible fronds number and fresh weight scored during the experiment and at t = 0 and 2, 5, 7, 9, 12, 14, 19, and 21 days of culture and using the following variables:

- **Doubling time (DT)** determined in terms of frond numbers at different days of culture according to the formula of Zeigler et al. [14]:

\[ DT = \ln 2 / RGR \]

where RGR is relative growth rate per day [24]:

\[ RGR = (\ln N_t - \ln N_0) / t \]

where \( N_0 \) = initial number of fronds, \( N_t \) = number of fronds scored at t days of culture, and \( t \) = days of culture.

As well the doubling time after 7 days of culture was recorded according to the OECD guidelines [19].

**Growth index (GI)** calculated according to Khellaf et al. [25]:

\[ GI = FWt / FW0 \]

where \( FW0 \) = initial fresh weight (biomass production) of fronds and \( FWt \) = fresh weight of fronds (biomass production) scored on different days of culture.

2.3. Data analysis

All quoted data give the mean values obtained from six replicate measurements, together with the Standard Error (SE). Data were analysed using ANOVA and Duncan test. The regression equations for the best-fitted prediction model for growth index variable were built in function with the time of cultivation. Statistical analysis was performed with the statistical software processor IBM SPSS 22. Differences between treatments were considered as statistically significant at \( P < .05 \).

3. Results

Different effects of the nutrient media SH, MS and AAP on the vegetative growth of *L. minor* and *L. gibba* during *in vitro* cultivation are demonstrated in this study.

| Mineral composition | SH [18] | MS [17] | AAP [19] |
|---------------------|---------|---------|---------|
| KNO₃                | 2.48 M  | 752 mM  | 0       |
| NH₄H₂PO₄            | 260 mM  | 0       | 0       |
| NaNO₃              | 0       | 0.305 M | 0       |
| NH₄NO₃             | 0       | 824 mM  | 0       |
| K₂HPO₄            | 0       | 50 mM   | 0       |
| K₃H₂PO₄.H₂O        | 0       | 0       | 6.13 mM |
| CaCl₂.2H₂O       | 136 mM  | 120 mM  | 29.9 mM |
| MgSO₄.7H₂O       | 162 mM  | 60 mM   | 60.85 mM|
| MgCl₂.6H₂O        | 0       | 60 mM   | 0       |
| H₂B₂O₃            | 8.1 mM  | 4 mM    | 3.07 mM |
| NaHCO₃            | 0       | 0       | 178.8 mM|
| Na₂MoO₄.2H₂O      | 41 μM   | 0.04 M  | 30.17 μM|
| FeCl₃.6H₂O       | 0       | 0       | 0.761 mM|
| MnSO₄.H₂O        | 5.92 mM | 4 mM    | 0       |
| MnCl₂.4H₂O       | 0       | 0       | 0.805 mM|
| ZnSO₄.7H₂O       | 0.35 mM | 1.2 mM  | 0       |
| ZnCl₂             | 0       | 0       | 0.024 mM|
| CuSO₄.5H₂O       | 80.1 μM | 0.004 M | 0       |
| CoCl₂.6H₂O       | 42 μM   | 0       | 5.884 μM|
| FeNaEDTA          | 0.6 mM  | 0.2 mM  | 0       |
| Na₂EDTA          | 5.39 mM | 0       | 0       |
| FeIIIEDTA        | 0.55 μM | 0       | 0.806 mM|

Table 1. Composition of nutrient solutions utilized for the cultivation of *L. minor* and *L. gibba*.
Many constituents are commonly present in SH, MS and AAP media at different concentrations, including KNO₃, MgSO₄, KH₂PO₄, H₃BO₃, NaMoO₄ and Na₂EDTA (Table 1). With the exception of ZnSO₄·7H₂O, the macro and micro nutrients concentrations in SH are higher than those in MS solutions. SH also contains the CoCl₂·6H₂O while this micro-nutrient is absent in MS [22]. The nutrient solution AAP recommended by the OECD guidelines [19] has higher concentrations of macro and micro nutrients. Moreover, NaHCO₃ is added to AAP to stabilize the medium and is actually a good bicarbonate source needed for growth.

3.1. Fronds number and doubling time

In terms of fronds number, *L. minor* and *L. gibba* recorded different proliferation responses along the cultures on the different nutritive media (Figure 1). *Lemna minor* displayed a higher proliferation rate on SH medium with 176 ± 4.9 and 389 ± 13.5 newly developed fronds by the 7th and 14th day of start respectively (Figure 4). Whereas, the proliferation rate recorded 129 ± 3.18 and 227 ± 3.21 on AAP and 100 ± 1.6 and 72 ± 3.84 fronds on MS medium during the same corresponding time intervals. For *L. gibba*, higher proliferation rate was found on AAP medium with 174 ± 4.06 and 342 ± 1.76 newly developed fronds after 7th and 14th day respectively vs. 115 ± 2.85 and 168 ± 9.13 new fronds obtained on SH medium and 119 ± 1.4 and 131 ± 0.67 fronds on MS medium during the same time interval. Remarkably, an exponential frond proliferation is recorded up to the 30th day of culture for *L. minor* on SH medium (1096 ± 17.9 fronds) and for *L. gibba* on AAP medium (650 ± 12.3 fronds).

The DT increased for both species along cultures on the three media, resulting from the deceleration of the frond proliferation, with significant effects recorded for both species and nutritive media (Table 2). As to *L. minor*, DT passed from 2.5 at day 5 to 4.4 and above on both SH and AAP while DT becomes nul after day 14 on MS medium. Similarly, for *L. gibba*, DT increased along cultures from 2.4 at day 5 to 5.4 reached at day 21 on AAP medium vs. 7.6 on SH medium, while DT was nul after day 14 on MS medium.

At seven days of culture, doubling time of frond number of both *L. minor* and *L. gibba* differed between the different test media (Table 2; Figure 2). Lowest growth was found on MS medium for *L. minor* and *L. gibba* where average DT values ranged from 3.3–4.0 days. Best

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**Table 2.** Mean values and standard errors of DT of proliferation of *L. minor* and *L. gibba* along 21 days of culture.

| Species | Media | Day 2 | Day 5 | Day 7 | Day 9 | Day 12 | Day 14 | Day 16 | Day 19 | Day 21 |
|---------|-------|-------|-------|-------|-------|--------|--------|--------|--------|--------|
| *L. minor* | SH    | 2.5 ± 0.39 | 2.6 ± 0.08<sup>a</sup> | 2.7 ± 0.07<sup>b</sup> | 3.2 ± 0.04<sup>b</sup> | 3.8 ± 0.07<sup>b</sup> | 3.7 ± 0.09<sup>b</sup> | 3.9 ± 0.10<sup>b</sup> | 4.2 ± 0.08<sup>b</sup> | 4.4 ± 0.05<sup>a</sup> |
|         | MS    | 2.5 ± 0.31 | 3 ± 0.14<sup>a</sup> | 4 ± 0.09<sup>b</sup> | 5.2 ± 0.11<sup>b</sup> | 7.8 ± 0.78<sup>b</sup> | 11.4 ± 1.2<sup>b</sup> | 0       | 0       | 0       |
|         | AAP   | 2.6 ± 0.23 | 3.1 ± 0.20<sup>a</sup> | 3.3 ± 0.10<sup>a</sup> | 4 ± 0.08<sup>a</sup> | 4.7 ± 0.07<sup>b</sup> | 4.8 ± 0.06<sup>b</sup> | 4.4 ± 0.10<sup>b</sup> | 4.4 ± 0.03<sup>b</sup> | 4.8 ± 0.03<sup>b</sup> |
| *L. gibba* | SH    | 2.5 ± 0.13 | 2.7 ± 0.23 | 3.6 ± 0.11<sup>a</sup> | 4.2 ± 0.10<sup>a</sup> | 5.1 ± 0.31<sup>a</sup> | 5.6 ± 0.32<sup>a</sup> | 6.2 ± 0.17<sup>a</sup> | 7.1 ± 0.13<sup>a</sup> | 7.6 ± 0.14<sup>a</sup> |
|         | MS    | 2.4 ± 0.16 | 2.9 ± 0.10 | 3.4 ± 0.08<sup>b</sup> | 4.2 ± 0.10<sup>a</sup> | 5.5 ± 0.05<sup>b</sup> | 6.6 ± 0.03<sup>b</sup> | 0       | 0       | 0       |
|         | AAP   | 2.5 ± 0.34 | 2.6 ± 0.08 | 2.7 ± 0.06<sup>b</sup> | 2.9 ± 0.03<sup>b</sup> | 3.6 ± 0.03<sup>c</sup> | 3.9 ± 0.01<sup>c</sup> | 4.4 ± 0.07<sup>b</sup> | 5.0 ± 0.14<sup>b</sup> | 5.4 ± 0.02<sup>b</sup> |

<sup>a</sup>Significantly different on SH and MS and AAP on day 2, 5, 7, 9, 12, 14, 16, 19, and 21, (<i>P</i> < .05). Superscripts a, b, c indicate homogenous value subsets that significantly differ by ANOVA.
doubling time was found on SH medium for \( L. \) minor and on AAP for \( L. \) gibba with 2.7 days.

### 3.2. Fresh weight and growth index

The ANOVA analysis shows that the fresh weight (biomass production) of \( L. \) minor and \( L. \) gibba, determined by GI, is relatively similar on SH, MS and AAP media after five days of culture, before becoming significantly higher \((P < .05)\) on SH medium later on (Figure 3). The growth index of both species decreases in MS medium where the proliferation ceased after 14 days of culture.

\( \text{Lemna} \) minor displayed a higher biomass production on SH medium of 196.43 ± 4.2 mg and 597.63 ± 35 mg of fresh weight by the 7th and 14th day of start respectively. While the fresh weight was 166.53 ± 6.7 mg and 282.73 ± 36.3 mg on AAP and 114.13 ± 8.2 mg and 124.63 ± 21.6 mg on MS medium by the 7th and 14th day of culture. For \( L. \) gibba, higher biomass production was found on AAP media with 459.7 ± 22.6 mg and 813.6 ± 15.4 mg fresh weight after 7th and 14th day respectively vs. 305.6 ± 5.5 mg and 643.3 ± 47.5 mg obtained on SH medium and 242.9 ± 10.3 mg and 171.7 ± 12.9 mg on MS medium during the same time interval (Figure 4).

After 21 days of cultivation, the growth index of \( L. \) minor on SH medium was 41.6 ± 3.08 vs. 15.4 ± 1.33 recorded on AAP medium (about 2.7 times higher than on AAP medium). For \( L. \) gibba, GI on AAP medium at 21 days was 15.51 ± 1.04 vs. 9.3 ± 0.6 recorded on SH medium (about 1.66 times higher than on SH medium).

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**Figure 3.** Growth index of \( L. \) minor and \( L. \) gibba on SH, MS and AAP media on day 2, 5, 7, 9, 12, 14, 16, 19 and 21.

**Figure 4.** Proliferation of \( L. \) minor and \( L. \) Gibba on different nutrient media (SH, MS and AAP) after 7 days of culture (bar = 5 mm).
The linear regression model built to describe growth index as a function of cultivation time and on different nutrient media resulted in the below equations (1-6); $R^2$ ranged from 0.40 - 0.95 for both species.

For *L. minor*:

\[
\hat{Y}_{GL} = 1.96 \times \text{Time} - 5.23, \quad R^2 = 0.89 \text{ on SH medium}
\]  
(1)

\[
\hat{Y}_{GL} = 0.23 \times \text{Time} + 2.4, \quad R^2 = 0.40 \text{ on MS medium}
\]  
(2)

\[
\hat{Y}_{GL} = 0.65 \times \text{Time} + 1.03, \quad R^2 = 0.88 \text{ on AAP medium}
\]  
(3)

For *L. gibba*:

\[
\hat{Y}_{GL} = 0.43 \times \text{Time} + 1.02, \quad R^2 = 0.91 \text{ on SH medium}
\]  
(4)

\[
\hat{Y}_{GL} = 0.026 \times \text{Time} + 2.4, \quad R^2 = 0.04 \text{ on MS medium}
\]  
(5)

\[
\hat{Y}_{GL} = 0.71 \times \text{Time} - 0.337, \quad R^2 = 0.95 \text{ on AAP medium}
\]  
(6)

The growth index of *L. minor* in the three different media showed that the coefficients for time (days) were 1.96, 0.23, 0.65 for SH, MS and AAP media respectively; whereas, the coefficients for time (days) for *L. gibba* were 0.43, 0.026 and 0.71 for SH, MS and AAP media respectively. This confirms that *L. minor* displayed maximum GI on SH, with an expected increase of 1.96 average for every additional day in time. As to *L. gibba*, it displayed its highest GI on AAP medium, for every additional day in time GI is expected to increase by an average of 0.71.

4. Discussion

The effects of SH, MS and AAP nutrient solutions on the in vitro vegetative growth of *L. minor* and *L. gibba* are investigated. Proliferation of axenically grown *L. minor* and *L. gibba* is determined based on the frond doubling time and growth index.

Our results indicate that the biomass production of *L. minor* and *L. gibba* in SH and AAP solutions, respectively, are significantly higher than those obtained in MS. These results are in line with the suggestions of OECD [19] which recommends the growth of *L. gibba* on AAP, while MS is rather suitable for maintaining stock cultures of *Lemma* species [16, 22]. Actually, the macro and micro nutrients concentrations are generally higher in SH than MS solutions [22]. It is also worthy noted that the high concentration of phosphate in SH and AAP normally accelerates *Lemma* proliferation and its frond doubling time while this process remains slower in MS medium. Horemans et al. [26] found that *L. minor* growth rates were similar in SIS-medium and K-medium and generally decreased with lowering phosphate concentrations.

Previous works showed that *L. minor* and other duckweeds grow on many diluted inorganic salt solution with essential macro-and micro nutrients such as SH, MS and Hoagland [16], with however distinct growth and physiological responses according to the composition of the nutritive solution [14, 27]. Yu et al. [28] reported an efficient frond proliferation for *L. minor* in SH solution.

Other examples are reported in the literature where floating plant species exhibited different average growth rates across the tested media. Lemon et al. [29] examined the growth of *L. minor*, *Spirodea polyrhiza*, and *Wolffia borealis*, at high nutrients levels (33% v/v Hutner’s medium) and found that *W. borealis* has the highest growth rate, while *S. polyrhiza* has the lowest in terms of frond proliferation. Lemon et al. [29] explained that differences in growth rates can be the result of faster development and release of daughter fronds and a longer life span. Ge et al. [30] recorded a growth cycle of 27 days for *L. minor* when measuring the biomass production and starch accumulation grown in SH medium under sterile conditions compared to wastewater. The biomass increased almost linearly between day 6 and 21 with an average growth rate of 14.1 g dry weight per day and a doubling time of 2.1 days [30]. Patel et al. [31] inspected the effects of MS, B5 and White media on the rates of frond proliferation of *L. gibba* based on the frond doubling time after 28 days of cultivation. Plant responses of *L. gibba* to White medium exhibited a higher biomass production and rapid frond proliferation in in vitro culture.

*Lemma minor* exhibited a better growth index on SH medium as resulted from the regression model (regression coefficient 1.96, $R^2 = 0.89$) while *L. gibba* grew better on AAP medium (regression coefficient 0.71, $R^2 = 0.95$). This could be a good predictive model for the growth of duckweed for further studies for phytoremediation purposes for nutrients and heavy metal uptake. The growth index of *L. minor* and *L. gibba* reached after 21 days 41.6 and 15.51, respectively, on SH on AAP media. Similar results were also recorded by Ziegler et al. [14] for five floating plant genera grown in SH medium. Conversely, Zhang et al. [32] indicated that the nutrient concentrations had no significant influence on the growth rate of *L. minor* if the nitrogen concentration was between 1 and 5 mg/L in the culture medium. They pointed that the kinetics of duckweed growth and nutrient uptake may provide insights into the uptake mechanisms and facilitate the predictive modelling of nutrient uptake in nature [32]. As stated by Goulet et al. [33] the composition of the plant nutrient medium, e.g. the higher amounts of phosphate necessary to sustain *L. minor* growth, could greatly influence heavy metal uptake. The nutrient medium described in the guidelines for a standard *L. minor* and *L. gibba* growth inhibition test [19] is indeed rich in phosphate and other ions.
Following 14 days of cultivation in MS medium, the biomass of our both species did not increase further, indicating that the growth potential for duckweed in MS culture was not more than 14 days. MS medium is recommended to provide a slower proliferation needed for stock culture cultivation, while SH medium is suggested by Appenroth [22] to be an optimized culture medium for *L. minor*, and AAP is recommended by OECD [19] to grow *L. gibba*. The duckweed plants grown in the SH and AAP media had a longer growth cycle due to proper nutrient ingredients. When grown in MS medium, the duckweed biomass was usually lower compared to that in the SH and AAP media, due to the low nutrient levels.

Frond number DT increased for both species along cultures on the three media, resulting from the decrease of the frond proliferation, with significant effects recorded for both species and nutritive media. Horemans et al. [26] stated that growing plants for longer periods in any of the different experimental media resulted in lower doubling time for the tested media. In other similar studies, Kittiwongwattana and Vutipongchakij [27] found that under experimental conditions, *L. minor* cultivated in Hoagland medium displays a faster proliferation rate than in MS medium with 2.3 days doubling time and a growth index of 24.03, around 2.2 times higher than in MS medium.

The rapid growth of duckweed together with the simplicity of *in vitro* cultivation, have contributed to the importance of the use of duckweed as an experimental plant model [2, 4, 14, 24, 34, 35]. *Lemna* species are commonly used for bioassays for heavy metals and nutrients uptake over a standardized 7-day growth inhibition test according to the standardized methods and protocols [19,20] with specified DT of *Lemna* species of 2.5 days as a validity criterion.

Based on our observation, at seven days culture, DT of both *L. minor* and *L. gibba* differed between the different test media. Lowest growth was found on MS medium which has lower phosphate concentration, for both *L. minor* and *L. gibba* (3.3 and 4.0 days, respectively). These values were all significantly higher than those obtained at 2.5 days, which is the validity criteria indicated in the OECD guidelines [19] for the growth inhibition test for *Lemna* spp. Best growth was found on SH medium for *L. minor* and on AAP for *L. gibba* with 2.7 days. Similarly, lower growth was previously recorded for *L. minor* with the decrease of phosphate concentrations contained in the nutritive solutions as evidenced by a doubling time significantly higher than 2.5 days [26].

Depending on the conditions of the growth medium, species of duckweed differ in growth rates and biomass production. These species-specific differences are important because nutrient uptake differs depending on the source of nutrient loading and other various factors [14, 36].

5. Conclusion

The findings of this study showed that SH and AAP media may be efficiently used for growing Lebanese duckweeds when high biomass production is needed. In contrast, the slower growth rate can be obtained by using MS medium which may therefore be used for sustaining stock cultures. Additionally, the effects of media on plant growth may also be a determining factor for the selection of medium that can be used in further investigations. Moreover, the variation in growth rates of different *Lemna* sp. provides a particularly good understanding of how different the response and productive potentials of the various duckweeds can be. Therefore, further research tackling the responses of *Lemna* sp. to nutrients will have practical recommendations for both management and phytoremediation applications.

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No potential conflict of interest was reported by the authors.

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