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Full Paper

Effects of nutrient media on vegetative growth of *Lemna minor* and *Landoltia punctata* during in vitro and ex vitro cultivation

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Abstract: Lemnaceous plants, namely *Lemna minor* and *Landoltia punctata*, have been used in various types of biological research. The effects of Murashige and Skoog (MS) and Hoagland media on vegetative growth rate of both species during in vitro and ex vitro cultivation were investigated. Under axenic conditions, frond proliferation of *L. minor* and *Lan. punctata* in Hoagland medium are 8 and 11.5% respectively faster than that in MS medium. Biomass production in Hoagland medium also increases 2.2-fold (*L. minor*) and 1.4-fold (*Lan. punctata*) compared to MS medium. The roots of both species in MS medium are distinctly shorter than those in Hoagland medium. In contrast, ex vitro regeneration of frond colonies in MS medium is 22.2% (for *L. minor*) and 17.1% (for *Lan. punctata*) faster than in Hoagland medium. Similarly, ex vitro biomass production of both species in MS increases 1.8-fold (for *L. minor*) and 1.3-fold (for *Lan. punctata*) compared to that in Hoagland medium. Root elongation of the frond colonies in MS and Hoagland media is comparable. The distinct effects of MS and Hoagland media on vegetative growth of both species and the pre-determination of ex vitro growth rates in each medium are demonstrated.

Keywords: *Lemna minor*, *Landoltia punctata*, effects of nutrient media, in vitro cultivation, ex vitro cultivation, frond proliferation, biomass production, root elongation

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INTRODUCTION

The Family Lemnaceae, generally recognised as duckweeds, comprises 38 different species in 5 genera, i.e. *Lemna, Landoltia, Spirodela, Wolffia* and *Wolffiella.* Duckweeds are small, free-floating, aquatic flowering plants that are widely distributed around the globe ranging from temperate to tropical regions [1, 2]. Duckweed plants lack true stems and leaves. The plant body generally consists of expanded, flat, leaf-like structures called fronds [2]. Each plant contains leaf-like fronds that float on the water surface or are slightly submerged. Fronds are primarily involved in photosynthesis and reproduction. The roots attach themselves on the lower surface of the fronds. The maximum root number is species specific [1, 3]. Generally, duckweeds reproduce vegetatively by generating daughter fronds from the meristematic tissue on the pocket cleft towards the base of the mother frond. Daughter fronds stay attached to the mother frond to form a colony and dissociate upon maturation. Alternatively, at low-temperature many duckweed species produce specialised fronds called turions that are starch-enriched and serve as an overwintering form. Duckweeds may also undergo sexual reproduction by forming flowers and setting fertile seeds [4]. Because of their relatively simple life cycle and rapid growth, lesser duckweed (*Lemna minor*), fat duckweed (*L. gibba*), dotted duckweed (*Landoltia punctata*), and greater duckweed (*Spirodela polyrhiza*) have been extensively used in biochemical and physiological research [3]. Previous studies have demonstrated that several species of duckweeds can be used to remove toxic heavy metals and organic compounds from waste water [5-9]. *L. minor* is currently used as a monitor for water quality according to the ISO 20079 protocol because of its high sensitivity to water pollutants [10]. The guideline for the substance toxicity test provided by the Organisation for Economic Cooperation and Development (OECD) also employs *L. minor* as well as other species in the same genus as test species [11]. *L. minor* chloroplast genome has been fully sequenced for public use and applied for molecular identification of lemnaceous species [2].

Axenic cultures of duckweeds are often used for biological research and stock-culture maintenance. Currently, axenic cultures of *L. minor* are used as the manufacturing platform for bioproduction of some pharmaceutical proteins [12]. Duckweeds can be grown on various types of media consisting of basic inorganic salts. Addition of 1% sucrose as the carbon source also supports frond growth [3]. Murashige and Skoog (MS) [13] and Hoagland [14] media are examples of nutrient solutions that have been widely used in plant tissue culture including duckweeds [15-18]. The aim of this study is to compare the effects of MS and Hoagland media on the vegetative growth of *L. minor* and *Lan. punctata* during in vitro and ex vitro cultivation.

MATERIALS AND METHODS

Preparation of Plant Materials and Culture Conditions

L. minor and *Lan. punctata* were collected from natural ponds in the northern and western regions of Thailand. Fronds were surface-sterilised using 10% chlorox solution supplied with a few drops of Tween-20. They were then thoroughly washed with sterile water three times to remove excess chlorox solution. Each frond was then placed separately on solid MS medium [13] containing 0.7% agar, pH 5.7, to allow frond regeneration. Daughter fronds of both species derived from a single mother frond were kept and maintained as stock cultures, which were grown under 16hr-light/8hr-dark photoperiod with 50 μmol $m^{-2}s^{-1}$ light intensity from fluorescent light tubes (36W/840) at 24$\pm$2^{o}C.

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Effects of Media on In Vitro Growth and Subsequent Ex Vitro Growth

Two-frond colonies of *L. minor* and *Lan. punctata* from the stock cultures were transferred onto fresh solid MS medium and grown for 14 days. Newly generated colonies of each species consisting of two fronds were pretreated with 0.7% solid agar without nutrients for 24 hours before being transferred to liquid MS and Hoagland media [14] with 1% sucrose, pH 5.7. The composition of MS and Hoagland media used in this study is shown in Table 1.

Table 1. Composition of MS and Hoagland media used in this study [13, 14]

Nutrient	Concentration (mg/L)	
	MS	Hoagland
KNO_3	1900	505.5
$MgSO_4.7H_2O$	370	492.74
KH_2PO_4	170	136.09
NH_4NO_3	1650	-
$CaCl_2.2H_2O$	440	-
$Ca(NO_3)_2$	-	820.45
H_3BO_3	6.2	2.86
$Na_2MoO_4.2H_2O$	0.25	0.025
$MnSO_4.H_2O$	16.9	-
$ZnSO_4.7H_2O$	8.6	-
$CuSO_4.5H_2O$	0.025	-
$CoCl_2.6H_2O$	0.025	-
$FeSO_4.7H_2O$	27.8	-
$CuCl_2.2H_2O$	-	0.05
$MnCl_2.4H_2O$	-	1.81
$ZnCl_2$	-	0.11
$FeCl_3.6H_2O$	-	27
KI	0.83	-
Na_2EDTA	37.2	44.8
Myo-inositol	100	-
Nicotinic acid	0.5	-
Pyridoxine.HCl	0.5	-
Glysine	2	-
Thiamine	0.1	-

The experiment was performed in six replicates for each medium. Each replicate contained 15 randomly chosen, pretreated fronds which were aseptically grown in closed cylindrical crystal-clear bottles (8 cm high, 4 cm in diameter) containing 40 mL of liquid medium. Growth conditions were similar to those of the stock cultures described above. To observe frond proliferation and biomass production, pictures of each culture were taken from atop with a digital camera on day 0, 1, 4, 7, 10 and 13 along with a standard rectangle of 25 mm^2. Frond proliferation was determined based on doubling time of the frond number (T_d) on day 4. T_d was calculated by the following equation: $T_d = \ln 2/\mu$. The parameter μ (day^{-1}) is the average growth rate that is obtained as follows:

$$\mu_{ij} = (\ln N_j - \ln N_i) / (t_j - t_i)$$

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where $i = 0$, $j = 4$, N = total frond number, and t = time (day) of cultivation [11].

The rate of biomass production is indicated by the growth index calculated by using total frond area as follows:

$$\text{Growth index} = T_{(i)} / T_{(0)}$$

where $T_{(i)}$ is the total frond area measured on different days of the cultivation (day 1, 4, 7, 10 and 13) and T_0 is the total frond area measured at the beginning of the test (day 0) [7]. The total frond area was measured by using the Adobe Photoshop CS3 program (Adobe Systems Inc.). Briefly, the frond area is selected by using the 'colour range' command that distinguishes the green colour of fronds from the background. The selected area is then determined as the number of pixel. This number is used to obtain the total frond area in mm^2 by comparing to the number of pixel representing the area of the standard rectangle (25 mm^2). Student's *t*-test was used to determine statistically significant differences ($P < 0.05$) between growth indices of samples in MS and Hoagland media on the same day of cultivation. After 13 days of cultivation, pictures of roots produced in both media were taken at the side of the bottles.

The in vitro pre-cultured frond colonies from each medium formula were then used to observe the vegetative growth rate after seven days of ex vitro cultivation in diluted pond water. The test was done in triplicate in the crystal-clear bottles similar to those for axenic cultures. Each replicate contained 10 randomly chosen three- or four-frond colonies grown in 40 mL of one-third-diluted pond water (distilled water: pond water = 1:3). The bottles were covered with glass plates to prevent evaporation. The growth conditions were 16 hr-light/ 8 hr-dark photoperiod with 50 µmol $m^{-2}s^{-1}$ light intensity from fluorescent light tubes (36W/840) and $28\pm2^{o}C$. Pictures of all fronds in each bottle were taken on day 0, 4 and 7 with a standard rectangle of 25 mm^2. T_d, total frond area, growth index and root elongation of the frond colonies were determined as described above.

RESULTS AND DISCUSSION

In Vitro Cultivation

Different effects of MS and Hoagland nutrient solutions on the vegetative growth of *L. minor* and *Lan. punctata* during in vitro and subsequent ex vitro cultivation are demonstrated. Because of their simple preparation, MS and Hoagland media have been used for growing axenic cultures of various duckweed species for stock-culture maintenance and callus production [15-18]. However, the effects of these two media on frond proliferation, biomass production and root elongation of the two duckweed species have never been described. Selection of a medium suitable for research is very important. For example, robust bioproduction of recombinant protein molecules using the duckweed platform requires optimal biomass production that may be optimised through environmental conditions and growing medium [3]. Many constituents are commonly present in MS and Hoagland media at different concentrations, including KNO_3, $MgSO_4$, KH_2PO_4, H_3BO_3, $NaMoO_4$ and Na_2EDTA. Micronutrients are sulphate and chloride salts in MS and Hoagland media respectively. Nine additional constituents are in MS medium but not in Hoagland medium. They are NH_4NO_3, $CaCl_2.2H_2O$, $CoCl_2.2H_2O$, KI, myo-inositol, nicotinic acid, pyridoxine-HCl, glycine and thymine. Various carbon sources are available for axenic culture of duckweeds depending on the purpose of a particular study. Glucose, fructose and mannitol support regeneration of *L. minor* intact plants while galactose and sorbitol are more efficiently utilised by *L. minor* calli [16, 19]. Sucrose is generally used as carbon source in plant tissue culture because of its nature as a native product from

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the carbon assimilation process. Sucrose at 1% is suggested as the optimal concentration for duckweed tissue culture [3] and thus is used in this study.

After four days of cultivation, the effects of MS and Hoagland media on the rates of frond proliferation of axenically grown *L. minor* and *Lan. punctata* are determined based on the frond doubling time (T_d). Under experimental conditions, *L. minor* cultivated in MS medium displays T_d of 2.5 days while in Hoagland medium it exhibits T_d of 2.3 days. Similarly, frond proliferation of *Lan. punctata* is relatively slow in MS medium (T_d = 2.6 days) compared to Hoagland medium (T_d = 2.3 days). This indicates that Hoagland medium produces a faster proliferation rate than MS medium by 8% and 11.5% for *L. minor* and *Lan. punctata* respectively.

The rate of biomass production of *L. minor* and *Lan. punctata* is determined by using the growth index, which is relatively similar for *L. minor* grown on MS and Hoagland media up to four days of cultivation (Figure 1a). However, the growth index of *L. minor* in Hoagland medium is significantly higher than in MS medium after that. From day 10 to 13, a 1.4-fold and 2-fold increase in growth index are observed in MS and Hoagland media respectively. At the end of the test, the growth index of *L. minor* cultured in Hoagland medium (growth index = 24.03) is 2.2 times higher than in MS medium (growth index = 10.85). In contrast, the growth index of *Lan. punctata* in both media remains relatively similar up to 10 days of cultivation (Figure 1b). From day 10 to 13, a 1.5-fold and a 2-fold increase in the growth index are observed in MS and Hoagland media respectively. This results in a 1.4-fold difference between the growth indices of *Lan. punctata* in MS (growth index = 13.78) and Hoagland media (growth index = 19.66) after 13 days of cultivation. Similar to frond proliferation, the rate of biomass production of the two duckweed species is elevated in Hoagland medium compared to that in MS medium.

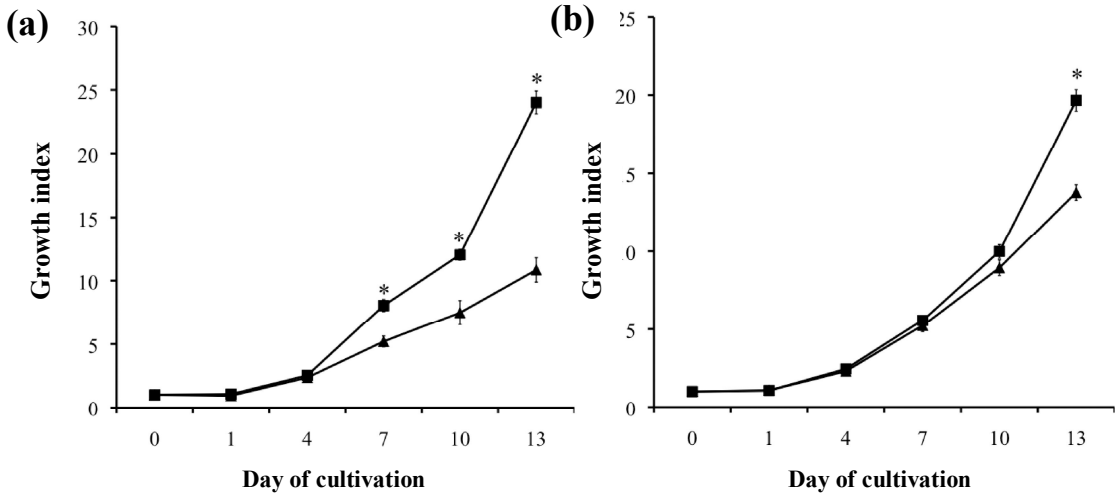

Figure 1. Biomass production of *L. minor* (a) and *Lan. punctata* (b) in MS (▲) and Hoagland (■) media on day 0, 1, 4, 7, 10 and 13 as determined by the growth index. *Vertical bars* represent standard error of the mean (n = 6). Asterisks indicate the statistically significant difference of the growth index of the plants in Hoagland and MS medium on the same day of cultivation ($P<0.05$)

The root growth from the lower surface of fronds is also examined. After 13 days of cultivation, roots of *L. minor* grown in MS medium are obviously shorter than those in Hoagland medium (Figure 2a-b). A similar effect is observed in *Lan. Punctata*, which displays a substantial

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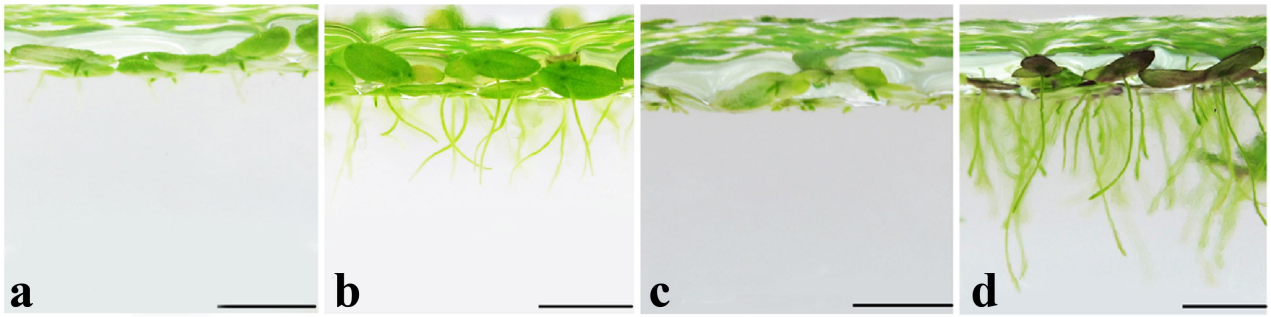

Figure 2. Effects of MS (a, c) and Hoagland (b, d) media on root growth of *L. minor* (a, b) and *Lan. punctata* (c, d) in axenic cultures (bar = 0.5 cm)

reduction in root elongation in MS medium compared to that in Hoagland medium (Figure 2c-d). This indicates an inhibitory effect of MS medium on root elongation in both species. The apparent effects of MS and Hoagland media on root elongation may be related to endogenous phytohormones. Gibberellin has been implicated in contributing to root growth in *L. minor* [20]. Exogenous application of uniconazole P, a gibberellin biosynthesis inhibitor, causes a significant reduction in both the root length and diameter. This inhibition is reversible upon addition of giberellic acid (GA_3) [20]. Another study has reported the effect of abscisic acid (ABA) on root growth. The application of exogenous ABA inhibits root growth of *L. minor* and induces starch accumulation in root cortical cells [21]. Auxin and ethylene are also plant hormones that regulate root elongation. Despite the lack of reports on root inhibition in lemnaceous plants, previous studies showed that auxin and ethylene affect root growth by synergistically reducing cell expansion in the central domain of the region of elongation of Arabidopsis [22, 23]. Further analyses are needed to better understand the effect of medium on root elongation of *L. minor* and *Lan. punctata*,. A comparative study on gibberellins, ABA and auxin levels in both plant species grown in MS and Hoagland media may confirm whether the reduction of root elongation in MS medium is related to these phytohormones.

Several previous studies showed distinct effects of the media on growth and physiological responses of other duckweed species. The rate of frond multiplication of *Lemna paucicostata* grown in a medium described by Boss et al. [24] is slower than in Hutner's medium [25]. The presence of EDTA, a chelating agent, in Hoagland medium prevents flowering of *Lemna perpusilla* but stimulates flowering of *L. gibba* under long-day conditions [26]. Frond proliferation and callus induction in *L. gibba* require different medium formula. While Nitsch-Nitsch, Schenk-Hildebrant and Gamborg media are efficient in frond proliferation, MS medium is more suitable for callus induction [27]. It was shown that Lemnaceae medium [28] is efficient for induction of turion formation as opposed to Hoagland medium, which promotes regeneration of daughter fronds [29]. According to our results, the rates of frond proliferation, biomass production and root elongation of *L. minor* and *Lan. punctata* in Hoagland medium are higher than those in MS medium. This indicates that Hoagland medium may be efficiently used for growing duckweeds when high biomass production and root elongation are needed. In contrast, slower growth rate can be obtained by using MS medium. Additionally, the effects of media on plant growth may also be a determining factor for the selection of medium that can be used in certain studies. In the Organisation for Economic Cooperation and Development (OECD) guideline, it requires T_d of the *Lemna* species to

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be less than 2.5 days for the substance toxicity test [11]. Based on our observation, Hoagland medium may be more preferable than MS medium for this particular toxicity test with *L. minor*.

Ex Vitro Cultivation

To examine the growth rates of pre-in-vitro cultured duckweeds during ex vitro cultivation, independent *L. minor* and *Lan. punctata* frond colonies in MS and Hoagland media are randomly selected and transferred to diluted pond water. Frond proliferation and biomass production are determined on day 4 and day 7 respectively after the transfer. It is found that the rates of vegetative growth of frond colonies in MS medium are generally higher than those obtained from Hoagland medium. While *L. minor* transferred from MS medium displays an observed T_d of 2.8 days, those from Hoagland medium exhibits a T_d of 3.6 days. *Lan. punctata* frond colonies from MS medium also show a faster frond proliferation (T_d = 3.4 days) compared to that from Hoagland medium (T_d = 4.1 days). For biomass production, the growth index of *L. minor* frond colonies from MS medium (growth index = 5.5) is 1.8 times higher than that from Hoagland medium (growth index = 3.07) (Figure 3). Similarly, *Lan. punctata* from MS medium exhibits a growth index (3.31) that is 1.3 times higher than that from Hoagland medium (growth index = 2.61) (Figure 3). Additionally, the root growth of *L. minor* and *Lan. punctata* frond colonies transferred from MS medium resumes and becomes comparable to what is observed in those from Hoagland medium after seven days of cultivation (Figure 4a-d).

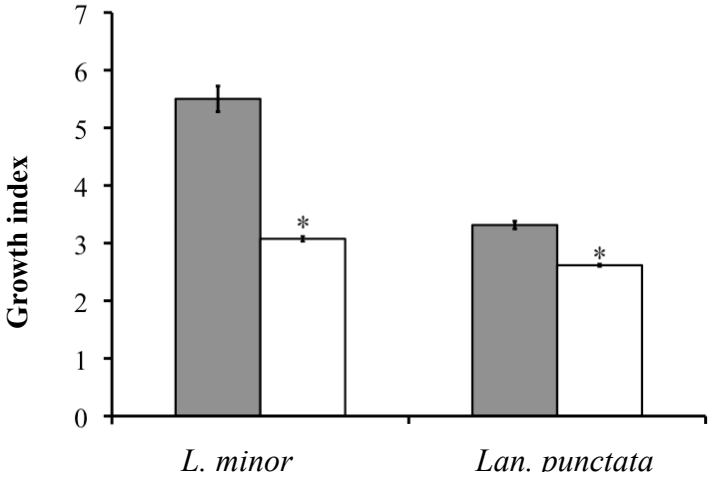

Figure 3. Biomass production of *L. minor* and *Lan. punctata* frond colonies from MS (gray bars) and Hoagland (white bars) media grown ex vitro in diluted pond water for seven days as determined by the growth index. Vertical bars represent standard error of the mean (n = 3). Asterisks indicate statistically significant difference of the growth index of the plants from Hoagland medium compared to MS medium ($P<0.05$).

In contrast to the observation during in vitro cultivation, the vegetative growth during ex vitro cultivation indicates the positive effects of MS medium on *L. minor* and *Lan. punctata* frond colonies in diluted pond water, which is strikingly different from the elevation of growth rates of axenic cultures by Hoagland medium compared to MS medium. This result shows that the growth rate of ex vitro cultures can be pre-determined by the previous medium formula. It also suggests that one may need to consider or investigate the effect of medium formula on plant growth before

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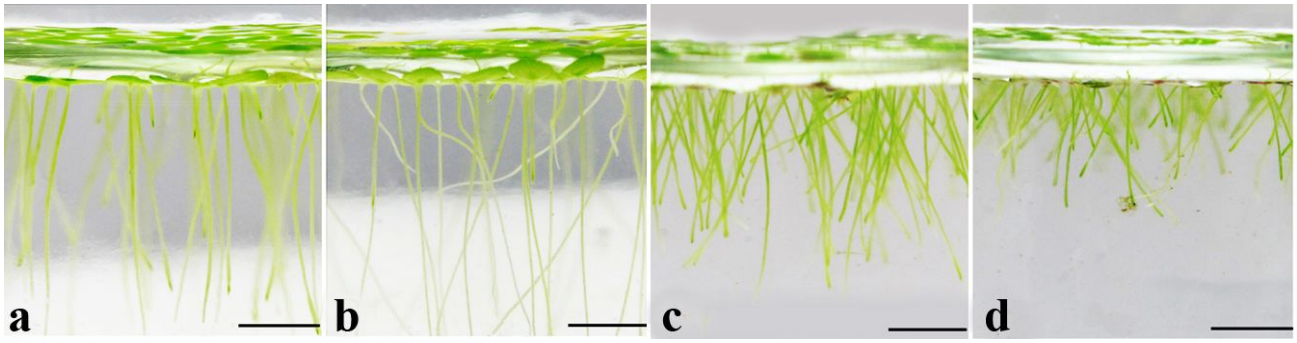

Figure 4. Root growth of *L. minor* (a, b) and *Lan. punctata* (c, d) after ex vitro cultivation for seven days in diluted pond water: frond colonies derived from MS (a, c) and from Hoagland (b, d) medium (bar = 0.5 cm)

planning a subsequent ex vitro toxicity test using axenic cultures of Lemnaceous plants. It is speculated that the MS medium may induce food storage which causes slower vegetative growth during in vitro cultivation. This reserved food then becomes readily available to support the growth during ex vitro cultivation in diluted pond water where nutrients are more limited. In contrast, Hoagland medium may stimulate a higher level of food consumption to sustain rapid frond and root growth in axenic cultures. This may result in a low level of food stored in the fronds and in turn cause a slower growth rate of the frond colonies in diluted pond water. Our speculation is partly supported by a previous study on the induction of turion formation in *S. polyrhiza*. Turions are the over-wintering form of several lemnaceous species that accumulate starch and sink to the bottom of the pond [26]. The use of Lemnaceae medium is more efficient to induce *S. polyrhiza* fronds to accumulate starch and transform into turions as opposed to Hoagland medium that stimulates daughter-frond regeneration [26]. Taken together, our results and the previous report implicate the medium relevance in starch or food-reserve accumulation. Further analysis of starch levels in the frond colonies of *L. minor* and *Lan. punctata* axenic cultures in MS and Hoagland media may confirm this speculation.

CONCLUSIONS

It has been shown that the rates of frond proliferation, biomass production and root elongation of *L. minor* and *Lan. punctata* are similarly dependent on the medium formula. This indicates the influence of the medium on the growth of both species. Plant responses to Hoagland medium are rapid frond proliferation and biomass production. The medium also supports root elongation and consequently is appropriate for further studies regarding root morphology and physiology of both duckweed species. In contrast, the growth rates are relatively low in MS medium. Thus, MS medium can be used to maintain a large collection of stock cultures where slower growth is preferred to reduce the amount of labour required for subculturing. It has also been found that *L. minor* and *Lan. punctata* frond colonies obtained from Hoagland medium grow slower than those from MS medium during ex vitro cultivation. This indicates the impact of medium formula on the use of axenic cultures under ex vitro conditions for standardised toxicity tests, etc., with lemnaceous species.

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