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RESEARCH ARTICLE

Starch accumulation in duckweeds (Lemnaceae) induced by nutrient deficiency

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ABSTRACT

The aim of the present project is to demonstrate the potential of duckweeds in fast production of starch-rich biomass that could render these plants as promising next generation crop plants. Starch-rich biomass can be produced by several methods of starch induction. In the present project the effects of nutrient deficiency (phosphate, nitrate and sulphate) on starch accumulation were investigated in 21 clones (strains) of duckweeds covering 11 species and all 5 genera of the duckweed family encompassing a wide geographic area. The magnitude of change in the starch content after treatment with nutrient deficient medium varied widely within the genera, the different species and different clones of the same species. Highest values, between 40 and 50 %, of starch on dry weight basis of the whole plant biomass were obtained after two weeks of application of phosphate- or nitrogen-lacking nutrient media whereas sulphate deficiency showed minor effects. It is concluded that various clones of the species *Landoltia punctata*, *Lemna minor*, and *Spirodela intermedia* proved to be promising candidates for starch rich biomass production.

Keywords: Duckweed; Nitrogen limitation; Phosphate limitation; Starch accumulation; Sulphate limitation

INTRODUCTION

Duckweeds represent the fastest growing Angiosperms (Sree et al., 2015a; Ziegler et al., 2015) having the potential as novel sustainable crop with high biomass production. Therefore, they attract increasingly the interest of researchers in the field of basic and applied research (Acosta et al., 2021; Ishizawa et al., 2021). Under certain stress conditions duckweeds accumulate high amounts of starch (Sree and Appenroth, 2014; Tian et al., 2021) in the whole vegetatively growing plant body under laboratory as well as under field conditions (Cui and Cheng, 2015; Zhao et al., 2015a, b). Subsequent production of bio-alcohols by hydrolysis of starch and fermentation of resulting carbohydrates has a high biotechnological potential (Cui and Cheng, 2015; Rana et al., 2021; Su et al., 2015).

Stress from limitation or lack of nutrients has been known to be an inducer of starch accumulation in duckweeds (Reid and Bieleski, 1970). The mechanism of starch accumulation has been investigated in several duckweed-related projects (Guo et al., 2020; Huang et al., 2014, 2015; Liu et al., 2015; Tao et al., 2013; Zhao et al., 2015b). Under lack of phosphate or nitrate, the decreased export of photosynthetic products out of plastids results in accumulation of carbohydrates and formation of starch (Thorssteinssen and Tillberg, 1987; Thorsteinssen et al., 1987). Most of the crucial components of the complicated molecular mechanism of starch accumulation in duckweed have been uncovered in the recent years by genomic and proteomic analyses, especially of the species *Landoltia punctata* (for a review see Appenroth et al., 2021). The expression of genes of the key enzyme of starch synthesis, ADP-glucose pyrophosphorylase, was enhanced under stress condition while that of the genes encoding starch degrading enzymes were down-regulated (Tao et al., 2013). Further, the genes encoding ADP-glucose pyrophophorylase and soluble starch synthesis related ones were also investigated in stress exposed *L. punctata* (Huang et al., 2014; Li et al., 2021; Zhao et al.,2015b).

The quantitative metrics of different physiological properties of duckweeds vary from clone to clone and cannot be characterized with a particular species or genus of Lemnaceae (Kuehdorf et al., 2014; Sree et al., 2015b; Ziegler et al., 2015).

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However, until now only a few species or clones have been tested for content and kinetics of starch accumulation under controlled environmental conditions, i.e. few clones of *La. punctata* and *Lemna aequinoctialis*. Investigating the response of a larger number of duckweed clones to nutrient limitation, i.e. phosphate, nitrogen and sulphate limiting conditions, was one of the goals of the present project. The inclusion of the condition of lack of sulphate was especially done keeping in view that this condition also induces the formation of turions, whose development is assumed to be closely connected with the accumulation of starch, as tested in *S. polyrhiza* (Appenroth et al., 1989, and references therein; Wang and Messing, 2012). Therefore, we investigated duckweed species belonging to all five genera (*Spirodela, Landoltia, Lemna, Wolffiella* and *Wolffia*) in terms of their potential for production of starch-rich biomass induced by nutrient limitation. The analyses of the data thus obtained is aimed at revealing the clones with high potential for production of starch-rich biomass that can be used for conversion into bio-energy. Moreover, the effectiveness of starch induction by nutrient limitation compared to other starch-inducing factors is discussed.

MATERIALS AND METHODS

Plant material

The clones used in the present investigation were taken from the duckweed stock collection at the Department of Plant Physiology, Friedrich Schiller University of Jena, Germany (Appenroth et al., 1996). Twenty-one clones encompassing all five duckweed genera and 11 species (Table 1) were selected for testing the response of lack of specific nutrients. The species identity of the clones was confirmed on the basis of morphology by E. Landolt, ETH Zurich († 2013) and by one of the present authors (KSS) and, in most cases, also by methods of molecular taxonomy (Bog et al., 2010, 2013, 2015, 2018). It should be mentioned that out of the investigated clones, for all *S. polyrhiza* clones the complete genome sequence is already known (Xu et al., 2019), *Le. minor* 9441 is the clone recommended for phytotoxicity measurements following the standardized ISO20079 protocol (Naumann et al., 2007) and *Lemna gibba* 7796, the well-known clone G3, is used e.g. for photoperiodic research (Kandeler, 1955) as well as for phytotoxicity measurements (Mkandawire et al., 2007). In contrast to the general taxonomic rule, abbreviations of the genera in the text were given by two letters to distinguish *Landoltia* (La) and *Lemna* (Le), and *Wolffiella* (Wa) and *Wolffia* (Wo) (cf. Sree et al., 2015b).

Pre-cultivation and cultivation

Duckweed clones were pre-cultivated under axenic conditions at 25 ± 1°C and 100 µmol m^{-2} s^{-1} (photosynthetically active radiation) continuous white light following the ISO 20079 protocol (Naumann et al., 2007) for four weeks in order to ensure reproducible results. A modified Schenk-Hildebrand medium (SH medium; Sree et al., 2015a, b) was used, replaced weekly by fresh medium.

The experimental phase of cultivation employed the same conditions as described for the pre-cultivation, except that 400 mL- glass beakers containing 300 mL autoclaved medium (complete medium or media lacking one of the nutrients) and covered with glass plates were used. Depending on the frond size, 50 to 200 fronds were randomly selected from the pre-culture as inoculum for initiating the experimental phase up to 14 days (Fig. 1). Six plant samples were examined as described below in each of the two independent experiments with each of the clones investigated and in each of the nutrient media (n=6).

Nutrient media

For control experiments (complete medium) SH medium was used (Schenk and Hildebrand, 1972). Phosphate-free (SH-P) medium was prepared by replacing $(NH_4)H_2PO_4$ by equimolar concentrations of NH_4Cl. For Nitrogen-free medium (SH-N), KNO_3 was replaced by equimolar concentrations of KCl, and $(NH_4)H_2PO_4$ by NaH_2PO_4. For sulphate-free nutrient medium (SH-S), sulphate was replaced by chloride in $MgSO_4$, $MnSO_4$, $ZnSO_4$, and $CuSO_4$.

Determination of starch content

In preliminary experiments, the starch content of clones 9525 and 9441 (Table 1, Fig. 2) was measured at the beginning (t=0), and after 1, 2, 4, 7, and 14 days of treatment. Thereafter, the starch contents were measured for all clones after t=0, 7 d and 14 d. As the initial starch content was always between 4 and 10 % per DW, only the starch content after 14 d of treatment were given in most of the figures. Two samples (100 mg fresh weight (FW) each) were harvested at each time point from each culture and either used for starch determination or for the determination of the ratio of FW to DW. For starch content determination (Appenroth et al., 2003), starch was extracted by 18 % (w/v) HCl and measured spectrophotometrically at 605 nm and 530 nm after addition of Lugol´s solution (Appenroth et al., 2010). Dry weight was determined by drying the samples for 12 hours at 105°C in an electrical oven.

Statistics

All presented data were based on the average of the starch content per DW of six samples. Data were given as means ± standard error of the means. Means were compared by one-way ANOVA using the Student-Newman-Keuls test ($p \leq 0.05$). The starch contents of control samples,

and samples from SH–P, SH-N, and SH–S media were compared.

RESULTS

In the present project the effect of deficiency of phosphate, nitrogen or sulphate on starch accumulation capacity of 21 clones of duckweeds was investigated (Table 1). Lack of any of the other 14 ions of the Schenk-Hildebrand medium did not show the effects described below, as tested with *S. polyrhiza* 9500 and *Le. minor* 9441 (data not shown).

Kinetics of starch accumulation

In the fronds of *Le. minor* 9441 and *Wa. hyalina* 9525 (Table 1), in complete medium, there was only a small, transient increase of the starch content within the first days after the start of the experiment. In SH-P and SH-N media, however, there was a strong increase of the starch content in *Le. minor* 9441, reaching approximately 46 ± 2% and 36 ± 1% on a DW basis, respectively (Fig. 2A). In SH-S medium, no transient increase of starch content in the first week of treatment was observed and after 14 days of treatment, the starch content was slightly but significantly higher than in the control (7.7 ± 0.5% per DW). Clone *Wa. hyalina* 9525 accumulated maximum starch content after being exposed to SH-N and SH-S for 14 days which was not the case with SH-P medium (Fig. 2B). Considering these results, we decided to present in the following only the starch content of the different investigated clones after 14 days of treatment.

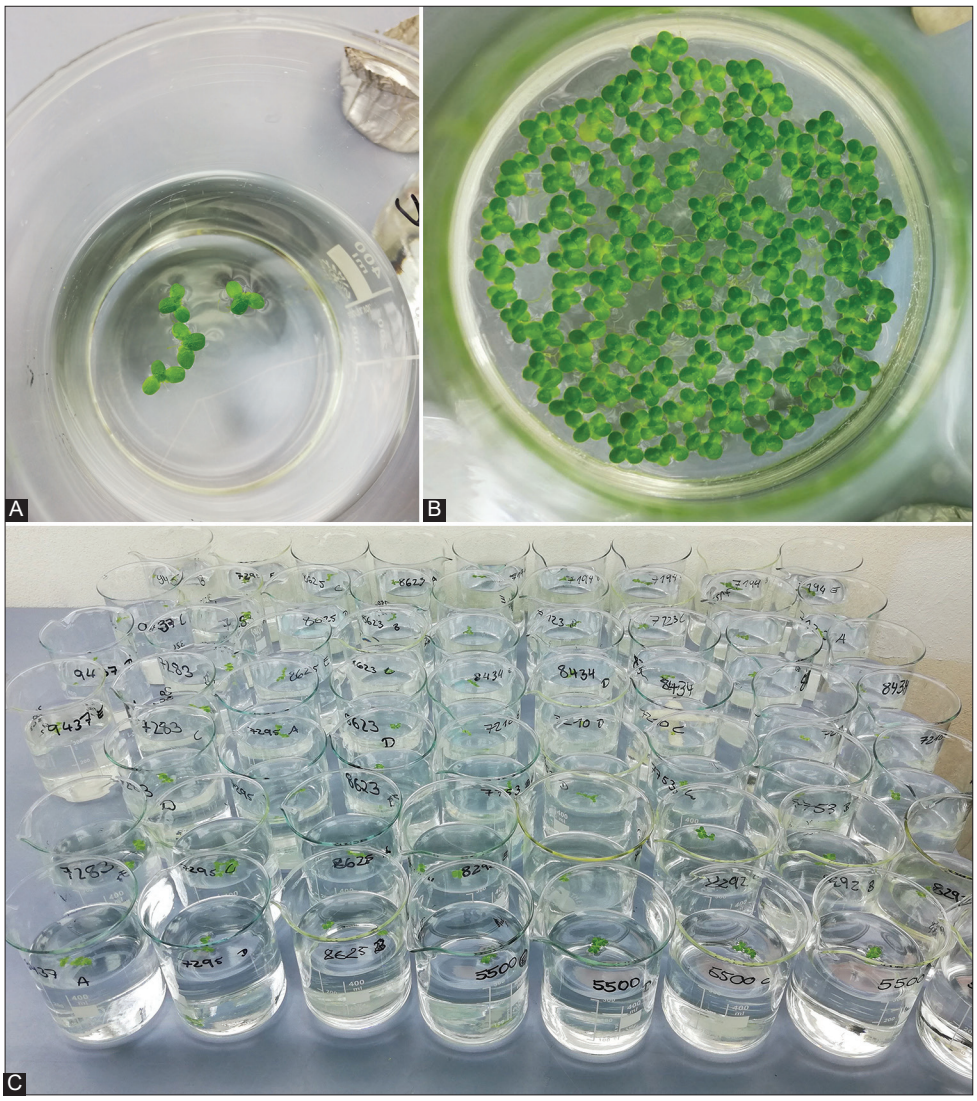

Fig 1. Experimental set up of duckweed cultivation in different nutrient media (cf. Fig. 2) A: Start of the experiment with fronds of Lemna minor. B: After 7 days of cultivation in control medium. C: Cultivation under continuous white light in 400 ml-glass beakers. Photo credit: Maxi Heller, Angelina Nolle, Emma Schwarz; Erfurt.

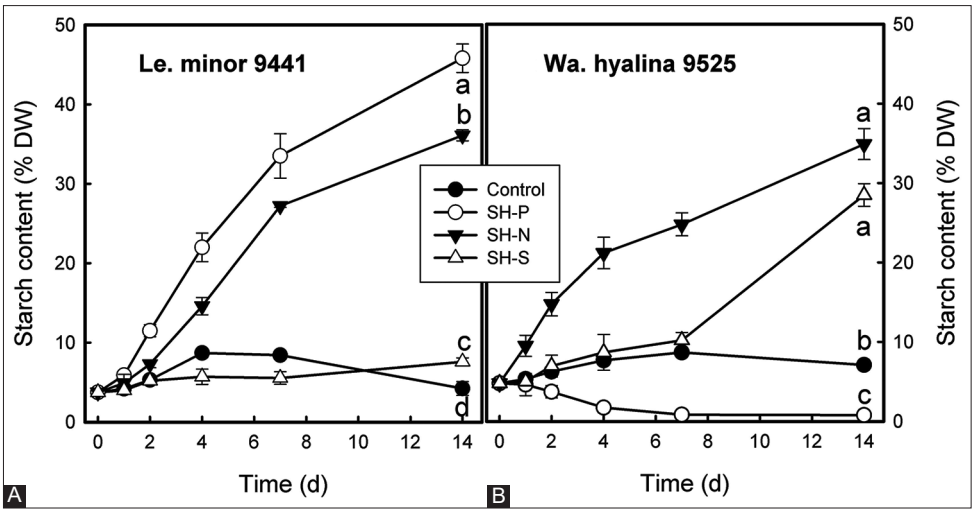

Fig 2. Kinetics of starch accumulation during cultivation in complete Schenk-Hildebrand (SH) medium, or media lacking phosphate (SH-P), nitrogen (SH-N) or sulphate (SH-S). Part A: Lemna minor 9441, part B: Wolffiella hyalina 9525. The lower case letters indicate the results of one-way ANOVA test; different letters indicate significant differences between the starch contents after 14 days of treatment. Data are given as means ± standard errors of six parallel samples. DW = dry weight.

Table 1: Species, clone numbers and place of origin of clones of duckweeds investigated in the present study

Genus	Species	Clone	Origin
Spirodela	*polyrhiza* (L.) Schleid.	7498	USA, NC, Durham
		9500	Germany, Jena
		9505	Cuba, Havana
	intermedia W. Koch	7125	Uruguay, Canelones
		7797	Peru, Lima
		9394	Venezuela, Sucre
Landoltia	*punctata* (G. Meyer) Les & Crawford	0049	China, Sichuan, Xinjin
		9234	Ecuador, Esmerelda, Viche
		9589	India, Delhi
		9637	Australia, NSW, Armidale
Lemna	*aequinoctialis* Welw.	6746	USA, CA, Plainsburg
	minor L.	6580	USA, NJ, Harrington
		9441	Germany, Marburg
	gibba L.	7742	Italy, Sicilia
		7796 (G3)	Italy, Sicilia
	minuta Kunth	9260	Italy, Trentino-Alto Adige
Wolffiella	*hyalina* (Delile) Monod	9525	India, Telangana, Hyderabad
Wolffia	*microscopica* (Griff.) Kurz	2007	Bangladesh, Jessore
	arrhiza (L.) Horkel ex Wimm.	9528	Germany, Jena
		9682	Poland, Podlasie
	globosa (Roxb.) Hartog	9498	India, UP, Mathura

Starch accumulation in different duckweed species and clones

Three clones of *S. polyrhiza* were investigated (Fig. 3A) for a possible increasing of the starch content by lack of one of three nutrients in the growth medium. In all three clones of this species, the level of accumulated starch was highest in SH-N, reaching between 20 and 35 % starch on DW basis after 14 days of treatment. The effect of SH-P was significantly lower and in all three clones it was around 15 %. Lack of sulphate in SH-S resulted in starch content values as low as the control samples (clone 7498), or slightly but significantly higher than in the control medium (clone 9505), or very similar to those in SH-P (clone 9500). From the same genus (*Spirodela*), three clones of *S. intermedia* were investigated (Fig. 3B). Whereas in clone 7797 the stimulating effect of SH-P and SH-N was similar (approximately 40 % starch per DW), and exposure to SH-S increased the starch level significantly higher than in the control, the situation was quite different in clone 9394. In this clone, the highest starch level in case of SH-N was lower than 25 %, whereas in SH-P the level was significantly lower and SH-S was comparable with the control, i.e. approximately 5 %. Although clone 7125 followed a similar trend as clone 9394, the starch accumulation capacity was higher, reaching up to 30 % in SH-N medium.

In *La. punctata* (Fig. 3C), the highest starch levels were between 30 and 40 %, that were induced either in SH-N medium (clones 0049 and 9234), in SH-P medium (clone 9589) or in both SH-N and SH-P (clone 9637). Except for clone 0049, the starch content in SH-S was significantly higher than in the control although it was in all clones lesser than 15%.

From the genera *Lemna*, 4 different species, *Le. aequinoctialis*, *Le. minor* (2 clones), *Le. gibba* (2 clones) and *Lemna minuta* were investigated (Fig. 4A). The highest starch content was

measured in the two *Le. minor* clones cultivated in SH-P (40 and 46 %). *Le. gibba* responded strongest in SH-N (30%) whereas *Le. aequinoctialis* and *Le. minuta* showed no difference in the starch content between treatment in SH-P and SH-N (ca. 33%). The effect of SH-S was always lower than the response in the two other nutrient-limited media but the starch levels reached between 15 and 20 % in five of the clones.

Three species represented by four clones of Wolffia were investigated (Fig. 4B). Only *Wolffia microscopica* 2007 accumulated a starch content of above 20%. Under all other conditions and in all other clones, the starch content was lower than 10%. Some of the *Wolffia* clones formed submerged forms. Therefore, starch content of both floating and submerged forms was investigated in *Wolffia arrhiza* clone 9528 in an independent experiment (Table 2). After cultivation in SH-P and SH-N, the submerged forms that were formed had several-fold higher starch content than the floating fronds. No submerged forms were formed in control and in SH-S media.

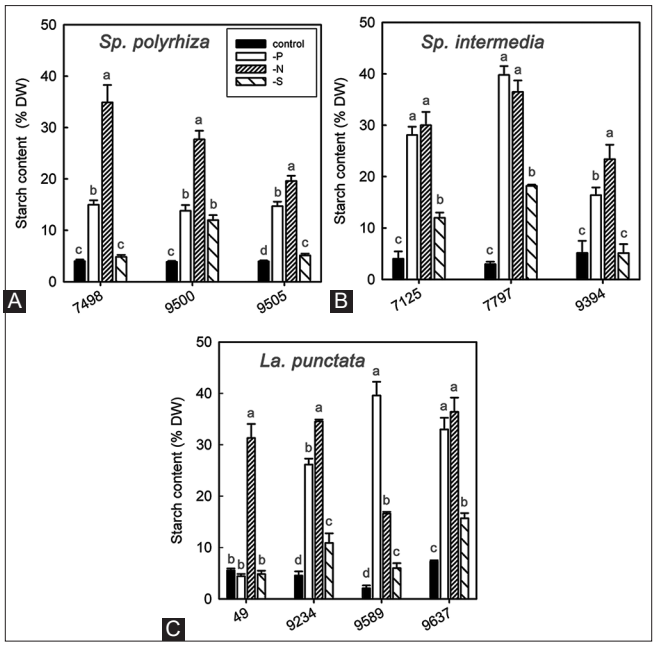

Fig 3. Starch content after cultivation of different clones for 14 days in complete SH medium or in media lacking nutrients. Part A: clones of the species Spirodela polyrhiza, part B: clones of the species Spirodela intermedia, part c: clones of the species Landoltia punctata. The origin of the clones is given in Table 1. Starch content was investigated by one-way ANOVA tests for the same clone grown in different nutrient media. For further explanations, cf. Fig. 2

Table 2: Starch content of floating and submerged forms of *Wolffia arrhiza* 9528 after 14 days of treatment in Schenk-Hildebrand medium (SH, control), lacking phosphate (SH-P), nitrogen (SH-N), or sulphate (SH-S). Six samples were investigated for each condition and the results were given as mean ± standard error of starch percentage per dry weight

Nutrient medium	Floating forms	Submerged forms
SH	6.60±0.97	no submerged forms
SH-P	1.09±0.11	25.3±1.1
SH-N	0.99±0.23	6.62±0.22
SH-S	1.07±0.09	no submerged forms

Fig. 4B shows the kinetics of starch accumulation of the only investigated species of the genus *Wolffiella*, *Wa. hyalina* 9525. Both under SH–N and SH–S conditions, starch content of approximately 30% was accumulated. This is the only investigated clone where higher amounts of starch were accumulated in SH–S than in SH–P or SH-N.

DISCUSSION

Variance in starch accumulation in different genera, species and clones of duckweeds

Several physiological properties of duckweeds show large natural variance between not only species but also between clones or strains of the same species, as already demonstrated for growth (Sree et al., 2015a; Ziegler et al., 2015), influence of salinity stress (Sree et al., 2015b) or turion formation (Kuehdorf et al., 2014). Hence, in order to tap the highest potential of these plants, it is necessary to undertake screening of large numbers of duckweed species and clones. Ma et al. (2018) investigated two species with 8 strains of *Le. aequinoctialis* and 12 strains of *S. polyrhiza*. The present screening for starch accumulation in duckweeds under deficiency of nutrients is the first study that compared a larger set of species in this family of aquatic plants. The 21 clones investigated in the present study belonged to 11 out of 36 species from all five genera (Bog et al., 2020; Sree et al., 2016). The clones 7797 (*S. intermedia*), 9589 (*La. punctata*) and 9441 (*Le. minor*) accumulated more than 40 % starch per DW under SH-P medium. For a large scale set-up, the use of a two-step procedure is recommended which involves the production of biomass under optimal conditions as a preliminary step followed by the transfer of this well grown biomass to the nutrient limiting conditions for starch accumulation (Appenroth et al., 2021; Xu et al., 2011).

In the genus *Wolffia*, however, it is known that many species form turions very quickly that sink to the bottom. This response is known in *Wo. australiana* (Bernard and Bernard, 1990), *Wo. arrhiza* (Fujita et al., 1999; Soda et al., 2013), and in *Wo. globosa* (Fujita et al., 2016; Soda et al. 2015). Turions are induced under very similar conditions as for the accumulation of starch and turions are very rich of starch (Dolger et al., 1997; Soda et al., 2015). In *Wo. arrhiza* 9528, starch formation has been induced in SH-P and SH-N but directed toward formation of submerged forms, i.e., turions (Les et al., 1997), whereas the floating fronds had low content of starch. Similar

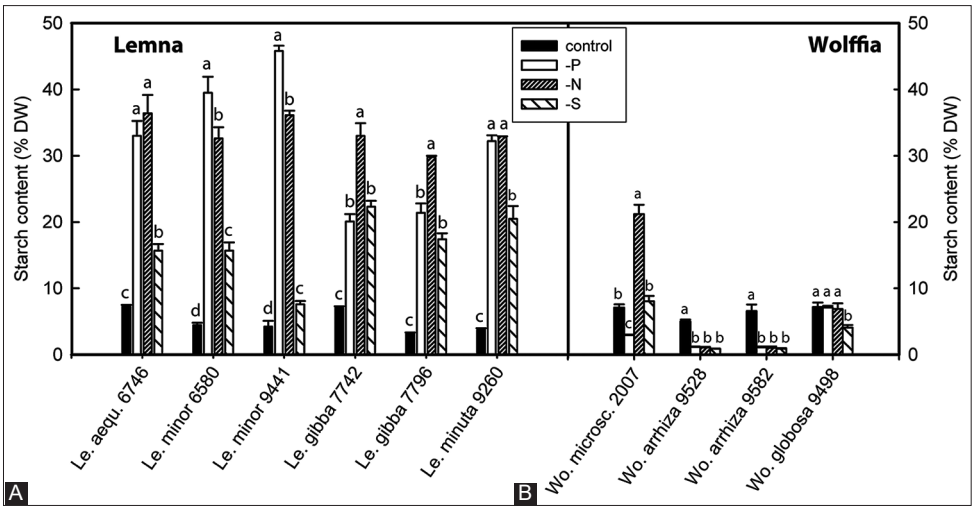

Fig 4. Starch content in clones of the genus Lemna (part A) and Wolffia (part B) after cultivation for 14 days in complete SH medium or media lacking nutrients. For further explanations, cf. Fig. 2 and Table 1.

results were reported by Soda et al. (2015) for *Wo. globosa* using highly diluted nutrient medium for induction of starch.

Comparison of nutrient deficiency with other external signals for starch accumulation

Nutrient deficiency is not the only inducer of starch accumulation in duckweeds. It can be achieved by several stress conditions, like low temperature, high light intensity, salt stress, or heavy metal stress (Acosta et al., 2021; Appenroth et al., 2021). The influence of lower temperature (5°C and 15°C vs. 25°C) at different light intensities was investigated by Cui et al. (2011) in *S. polyrhiza*. There was clearly an effect of lower temperature on starch accumulation, increasing the content from ca. 7% at 25°C to 15% at 5°C. However, this effect is not very high and may hardly justify the energy supply for decreasing the water temperature for several days. Cui et al. (2011) reported also a positive effect of increasing the light intensity from 28 μmol m^{-2} s^{-1} to 54 μmol m^{-2} s^{-1}. As both light intensities are suboptimal for growth of *S. polyrhiza*, the slightly increased starch content at the higher intensity might be caused by higher photosynthetic rates. The presently available data do not permit the conclusion that higher light intensity represents a signal that induces starch accumulation. Zhong et al. (2022) discovered that red light stimulated starch accumulation in *S. polyrhiza* more than blue light but concluded that the molecular mechanisms underlying this response of duckweed remain unclear.

Accumulation of starch under salt stress in duckweeds has not been investigated frequently. In most previous studies, only single clones were investigated (Chang et al., 2012; Radic and Pevalek-Kozlina, 2010; Sikorski et al., 2013). Xiao et al. (2013) investigated 4 clones from three different species in a field trial. However, the different results cannot be related to a certain species and compared with each other since the growth conditions varied from experiment to experiment under field conditions. Xu et al. (2011) tested the effect of low concentrations of NaCl (10, 20 and 30 mM) and observed only a small increase in one single clone of *S. polyrhiza*. In contrast to the observations with *S. polyrhiza*, much higher values in starch content of between 40 and 50% DW can be induced in several other duckweed clones (Appenroth et al., 2021; Sree et al., 2015b) upon applying 150 mM NaCl for 4 or 7 days. For the mechanism of starch accumulation in duckweeds our hypothesis raised previously (Sree and Appenroth, 2014) is that some stress factors, suppress vegetative growth more effectively than photosynthesis. The photosynthetic low-molecular carbohydrates, no longer used by growth-related metabolism, are thus converted into storage starch. In contrast to the two step-method used by Xu et al. (2011), salt application permits an alternative approach (Sree et al., 2015b): in many clones, the EC_{10} (DW) values for inhibiting growth are rather high, i.e. between 50 and 100 mM NaCl. As a consequence, the NaCl concentration that induced effectively starch accumulation does not inhibit too much growth, e.g. around 10%. In contrast to the situation under nutrient-limitation, duckweed can be well cultivated with high growth rates (in order to obtain large amounts of biomass) and induce accumulation of starch in the same medium adding e.g. between 50 and 100 mM NaCl. This one-step procedure could potentially result in much lower labour costs.

Starch accumulates in duckweeds also in the presence of several heavy metals. As an example, *Le. minor* 9441 accumulated approximately 45 % starch under cadmium stress (Sree and Appenroth, 2014). Similar results were obtained with chromate (Appenroth et al., 2003), nickel (Appenroth et al., 2010) and cobalt (Sree et al., 2015c). In

general, it has been demonstrated that under the influence of heavy metals, the starch synthesizing pathways were activated whereas starch degrading pathways were much less influenced (Appenroth et al., 2021). The speed of this response, within a few days, makes this method very attractive in the laboratory. In large-scale production of starch-rich biomass, however, the threat of the heavy metals to the environment, makes it impractical and unacceptable.

The application of the plant growth regulator uniconazole also results in intensive accumulation of starch and in parallel enhances the level of abscisic acid and cytokinins (Huang et al., 2015; Liu et al., 2015). However, it is not yet clear whether these effects of plant growth regulators help to understand the mechanism of starch accumulation under nutrient stress. Moreover, the signal transduction pathway from sensing lack of phosphate, nitrogen or sulphate in the medium, to the gene expression of starch synthesizing enzymes has not yet been comprehended in detail.

CONCLUSIONS

The presently tested method of treatment with nutrient media lacking phosphate, nitrogen or sulphate, or alternatively using clean water pure of such nutrients (Cheng and Stomp, 2009), results in some clones to accumulate 40 to 50 % starch on DW basis within 14 days. In some clones, only slightly lower values were obtained already after 7 days of treatment. Under conditions of large-scale production, it is required to change the liquid medium from nutrient-rich, e.g. eutrophic wastewater in order to produce biomass under good growth conditions, to clean source water to induce starch accumulation as demonstrated by Xu et al. (2011) for *S. polyrhiza*. Also, use of polyculture consisting of clones belonging to different species (*S. polyrhiza, Le. minor, La. punctata*) showed higher starch accumulation capacity (Chen et al., 2018). The use of already available by-products in a certain process like diluted anaerobically digested dairy manure can act as a good medium for duckweed cultivation for starch enrichment e.g. in *La. punctata* (Kruger et al., 2020). Such use can add value to the small scale dairy farmers encouraging them to take up cultivation of starch enriched duckweed bio mass production with no or little extra cost involvement. Moreover, these aquatic plants do not compete with land plants or crops for fertile soils and hence can be a potential alternative resource for starch-rich biomass production.

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Authors' contributions

Both authors developed the concept, planned and carried out the analyses together and wrote and proofread the manuscript.

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