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ORIGINAL ARTICLE

How fast can angiosperms grow? Species and clonal diversity of growth rates in the genus *Wolffia* (Lemnaceae)

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Abstract Species of the genus *Wolffia* (duckweed) are harvested from natural water bodies in many countries for human consumption. Relative growth rates (RGR) of 25 clones (ecotypes) representing all 11 species of the genus *Wolffia* were investigated under standardized laboratory conditions in search for potential candidates for production of *Wolffia* biomass at a biotechnological scale. This is the first report of large-scale screening of physiological properties of *Wolffia* species. Large differences in RGR of different clones were detected, e.g., in *Wolffia globosa*. Interestingly, intraspecific differences, i.e., at the level of clones are much higher than differences between species. Rate of photosynthesis (oxygen production in light) and respiration (oxygen consumption in dark) in clones of *W. globosa*, measured under standardized conditions, are in positive correlation with their respective RGR. Higher rate of photosynthesis seems to be a determining factor for higher RGR. The RGR of the first available axenic clone of the re-discovered species, *Wolffia microscopica* (clone 2005), depends strongly on the nutrient medium used, in contrast to other investigated species. This clone of *W. microscopica* has a doubling time of 29.3 h and represents the fastest growing flowering plant known till date.

Keywords Duckweed · Growth rates · Lemnaceae · *Wolffia* · *Wolffia globosa*

Abbreviations

DT	Doubling time (h or day)
RGR	Relative growth rate (day^{-1})
RY	Relative yield ($g\ g^{-1}\ week^{-1}$)

Introduction

Lemnaceae is a family of monocotyledonous water plants classified into 37 species arranged in five genera (Appenroth et al. 2013; Borisjuk et al. 2015). This family represents the smallest flowering plants (Landolt 1986; Bog et al. 2013). More interesting, however, is the fact that they are gaining increasing importance in terms of their practical applications because of the following reasons: (1) they are the fastest growing angiosperms known till date (Ziegler et al. 2015) producing high amounts of biomass which can be used as feedstock, e.g., for biogas production (Fedler and Duan 2011). (2) Under optimal growth conditions, i.e., light, temperature and nutrient availability, the resulting biomass contains high amounts of valuable proteins that makes it suitable for animal and human nutrition (Bhantumnavin and McGarry 1971; Appenroth et al. 1982; Suppadit et al. 2012; Zetina-Cordoba et al. 2013). (3) When subjected to certain stress conditions, the protein content decreases but the starch content in the duckweed

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biomass increases to a considerable level. Biomass obtained from such conditions can be used for production of bioethanol via starch degradation and subsequent sugar fermentation (Sree and Appenroth 2014; Cui and Cheng 2015; Sree et al. 2015a, b; Zhao et al. 2015). (4) Duckweeds can grow efficiently on wastewaters. In this process, they take up nutrients from wastewater for their growth and in turn clean it up, thus, eliminating the requirement of external application of fertilizers that can create an additional threat to the environment (Fujita et al. 1999; Verma and Suthar 2014; Cui and Cheng 2015; Zhao et al. 2015).

The natural genetic variability of duckweeds has hardly been tapped for practical applications and there is an urgent need to screen physiological properties at the level of species and clones. This holds true for the rate of biomass production, capacity of protein and starch accumulation in the biomass and so on. Some of the recent physiological investigations demonstrate that such natural variance exists not only exists between different duckweed species but also is intraspecific, i.e., between different clones (ecotypes) of the same species collected from different geographic locations of the world (Kuehdorf et al. 2014; Ziegler et al. 2015).

Wolffia species have been used for many generations as human food in Asian countries like Laos, Cambodia and Thailand (Landolt and Kandeler 1987). The authors have witnessed the use of *Wolffia globosa* for nutrition of children in Bangladesh. The fact that *Wolffia* species, unlike duckweeds belonging to other genera, contain oxalate in the soluble form and not as calcium oxalate crystals (that might create problems in digestion), makes them well suited as human food (cf. Landolt and Kandeler 1987). Moreover, the rootless nature of *Wolffia* species might increase their palatability just by esthetic reasons. *Wolffia* multiplies mainly by vegetative propagation by the budding of daughter fronds from one single pouch of the mother frond (Sree et al. 2015c). Consequently, clonal offsprings with identical genetic properties are produced. In a previous paper, we investigated rates of vegetative growth of 13 species of duckweeds belonging to all five genera, i.e., *Spirodela*, *Landoltia*, *Lemna*, *Wolffiella*, and *Wolffia* (Ziegler et al. 2015). It was shown that the variation in vegetative growth rates exists at the level of clones rather than at the level of species or genera. As biomass production is a key point for any practical application and considering the potential of *Wolffia* as human food, in the present project we focused on large-scale screening of different species and clones of this genus.

The screening strategy in the present investigation is based on the exponential growth rate of the fronds under optimal growth conditions in order to examine their potential for biomass production. It was not intended to imitate any of the ecological conditions concerning temperature, light intensity, photoperiod or abiotic or biotic stress in order to predict the behavior of the clones under out-door conditions. For the first time after several decades, the species endemic to India, *Wolffia microscopica*, could be integrated into the current investigation after its recent re-discovery (Sree et al. 2015c). During our investigations, we found that the growth of *W. microscopica* depends largely on the nutrient medium being used. Thus, we compared the growth rates of six selected clones of different *Wolffia* species in three different growth media. Finally, in order to explain the varying growth rates of different clones of the same species in the genus *Wolffia*, we selected the clones of *W. globosa* and tested the hypothesis whether there is any correlation between growth rate and photosynthetic rate.

Materials and methods

Plant material, pre-cultivation and cultivation

All plant material was taken from the collection of the Department of Plant Physiology of the University of Jena. The species identities of the clones were originally defined by the late Elias Landolt, ETH Zurich and confirmed again before the experiments by one of the present authors (KSS). Moreover, 19 out of the 33 clones were characterized before by molecular barcoding (Bog et al. 2013; Borisjuk et al. 2015). The clones investigated are listed in Table 1. The duckweed clones were taken from stock cultures and pre-cultivated under axenic conditions at 25 ± 1 °C and 100 µmol m^{-2} s^{-1} photosynthetically active radiation from fluorescence tubes TLD 36W/86 (Philips, Eindhoven, The Netherlands) following the ISO 20079 protocol (Naumann et al. 2007). The plants were pre-cultivated for 4 weeks and nutrient medium was replenished every week. This step of pre-cultivation was necessary to adapt the plants to the different nutrient media, light and temperature conditions, otherwise irreproducible results would be obtained. A modified Schenk-Hildebrandt medium (Schenk and Hildebrandt 1972) was employed with the following composition: $CaCl_2 \cdot 2H_2O$ 0.68 mM, KNO_3 12.4 mM, $MgSO_4 \cdot 7H_2O$ 0.81 mM, $(NH_4)H_2PO_4$ 1.3 mM, $MnSO_4 \cdot H_2O$ 30 µM, H_3BO_3 40 µM, $ZnSO_4 \cdot 7H_2O$ 1.74 µM, KI 3.0 µM, $CuSO_4 \cdot 5H_2O$ 0.4 µM, $Na_2MoO_4 \cdot 2H_2O$ 0.21 µM, $CoCl_2 \cdot 6H_2O$ 0.21 µM, FeNaEDTA 27.0 µM, $Na_2EDTA \cdot 2H_2O$ 2.74 µM. The pH of the medium was adjusted to 5.50. For some experiments, modified Hoagland medium with 100 µM EDTA (Venkataraman et al. 1970) or N-medium (Appenroth et al. 1996) were used to test the influence of the nutrient media.

The main phase of cultivation used for determining the growth rates employed the same conditions as described

Table 1 *Wolffia* clones investigated in the present and previous project (indicated with *; Ziegler et al. 2015): their species identity, clone number and origin, relative growth rate (RGR) and doubling time (DT) together with their standard error of the means (SE)

Wolffia species	Clone	Origin	RGR ± SE (day^{-1})	DT ± SE (days)
W. angusta	7274	Australia, New South Wales	0.248 ± 0.023	2.79 ± 0.28
	8878	Malaysia, Kuala Lumpur	0.526 ± 0.010	1.32 ± 0.03
W. arrhiza	7246	South Africa, Cape	0.243 ± 0.012	2.89 ± 0.15
	9528*	Germany, Jena	0.255 ± 0.019	2.72 ± 0.29
	8853	Brazil, Rio de Janeiro	0.301 ± 0.011	2.32 ± 0.09
	7421	Croatia, Zagreb	0.307 ± 0.010	2.27 ± 0.07
	8953	Greece, Macedonia	0.318 ± 0.007	2.18 ± 0.05
	8871	Togo, Mango	0.325 ± 0.007	2.14 ± 0.04
	9567	Poland, Topilo	0.338 ± 0.010	2.06 ± 0.06
	7215	Ivory Coast, Azagui	0.339 ± 0.009	2.05 ± 0.06
	9006*	Japan, Kyoto, Yodo	0.344 ± 0.083	2.01 ± 0.19
	8649	Senegal, Dakar	0.357 ± 0.004	1.94 ± 0.02
	9412	Italy, Po Delta	0.363 ± 0.008	1.91 ± 0.04
	8618*	Kenya, Wadi, Dema	0.426 ± 0.020	1.65 ± 0.08
W. australiana	7540	New Zealand, North Vaterbury	0.501 ± 0.008	1.39 ± 0.02
W. borealis	9123	USA, California	0.426 ± 0.003	1.63 ± 0.01
W. brasiliensis	9396	Venezuela, Sucre	0.412 ± 0.007	1.69 ± 0.03
	9597	India, UP, Mathura	0.428 ± 0.014	1.66 ± 0.04
	7925	Argentina, Buenos Aires	0.502 ± 0.011	1.38 ± 0.07
W. columbiana	9379*	Venezuela, Guarico	0.249 ± 0.012	2.78 ± 0.15
	7972	USA, Alabama	0.294 ± 0.013	2.38 ± 0.12
W. cylindracea	9056	Zimbabwe, Urungwe Safari	0.313 ± 0.009	2.22 ± 0.06
W. elongata	9188	Colombia, Atlantico	0.441 ± 0.006	1.57 ± 0.02
W. globosa	9196	Colombia, Cordoba	0.155 ± 0.040	4.47 ± 2.20
	9582	Poland, Topilo	0.322 ± 0.007	2.16 ± 0.05
	9331*	China, Wuhan	0.329 ± 0.022	2.11 ± 0.14
	9667*	China, Sichuan	0.369 ± 0.047	1.88 ± 0.24
	9659*	China, Sichuan	0.386 ± 0.047	1.80 ± 0.17
	9299	India, West Bengal	0.413 ± 0.002	1.68 ± 0.01
	9527*	India, Delhi, Northern border to Haryana	0.457 ± 0.003	1.52 ± 0.01
	9498	India, UP, Mathura	0.559 ± 0.003	1.24 ± 0.01
W. microscopica	2005	India, Gujarat	0.305 ± 0.011	2.29 ± 0.08
W. neglecta	9149	Pakistan, Karachi	0.428 ± 0.005	1.62 ± 0.02

All plants were cultivated in Schenk-Hildebrandt medium. Each value represents the average of six samples

for the pre-cultivation, except that 400 mL glass beakers containing 300 mL autoclaved media were used for cultivation. Ten to 20 fronds were randomly selected from the pre-culture as inoculum for initiating the main (measurement) phase of growth, which lasted for 7 days. The fronds never completely covered the surface of the medium that would lead otherwise to growth limitation.

Growth parameters

The number of fronds (FN) was determined at the onset of the respective experiment ($t = 0$ day: "$t0$") and 7 days later ($t = 7$ days: "$t7$") present in each of the 6 parallel samples. In preliminary investigations counting the number of fronds at 0, 1, 2, 4 and 7 days it was confirmed that all clones in all three media tested followed the law of exponential growth. Therefore, the experiments were simplified using only two data points. Relative growth rates per day (RGR) were calculated using Eq. (1) (Naumann et al. 2007). For measurements involving only two time points, this equation simplifies to Eq. (2) with $t7$ at the end of the 7-day test period, for each replicate separately.

$$X_t = x_{t0} \times e^{\mathrm{RGR} \times t} \quad (1)$$

$$\mathrm{RGR} = (\ln x_{t7} - \ln x_{t0})/(t_7 - t_0) \quad (2)$$

"RGR" is the increase in parameter value per the unit of time of 1 day.

Doubling time DT (in days) was calculated as:

$$DT = \ln 2/RGR \quad (3)$$

The yield obtained from the inoculum of 1 frond (or 1 g duckweed biomass) after 7 days of cultivation was termed "relative yield" (RY: analogous to the RGR, but on the basis of the unit of time of 1 week). It was calculated by solving for $\ln x_{t7}$ in Eq. (2) in the following equation:

$$\ln x_{t7} = \ln x_{t0} + RGR \cdot (t7 - t0) \quad (4)$$

Since $t7$ is 7 day and x at $t0$ is 1 frond or 1 g, x_{t7} is then equal to $e^{\ln xt7}$. The RY on a frond number basis has the dimension of g g^{-1} 7 day^{-1} or g g^{-1} $week^{-1}$.

All quoted data give the mean values obtained from 6 replicate measurements, together with the standard error of the means.

Microelectrode measurements

The oxygen production and oxygen consumption of five different clones of *W. globosa* were measured using an oxygen microelectrode with a tip diameter of 8–12 µm connected to a microsensor-multimeter OX10 (Unisense, Aarhus, Denmark). The oxygen microelectrode was calibrated using water saturated with air (21 % O_2) and 100 % nitrogen (0 % O_2). This set up was used with two replicates for each clone of *W. globosa* weighing approximately 100 mg in fresh weight suspended in 1 ml of the nutrient medium in a sterile GC vial. Each replicate was initially allowed to stabilize over a period of 5 min (steady state) before being plunged into darkness for 150 s to record the rate of oxygen consumption. It followed measurement of the rate of oxygen production for 150 s in the presence of light after a new steady state was reached. The light intensity was set to 100 µmol m^{-2} s^{-1} at 25 °C. Results were given as change of relative oxygen concentration (arbitrary units) per g fresh weight of *W. globosa* per minute.

Statistics

The mean values of the RGR and DT of each clone were calculated from two sets of three parallel samples each from two independent experiments ($n = 6$). They are presented in Table 1 and Fig. 1 together with the standard errors of the means. In Fig. S1 each "error bar" for a particular column indicates the span of the lowest and the highest mean values for the individual clones associated with that column.

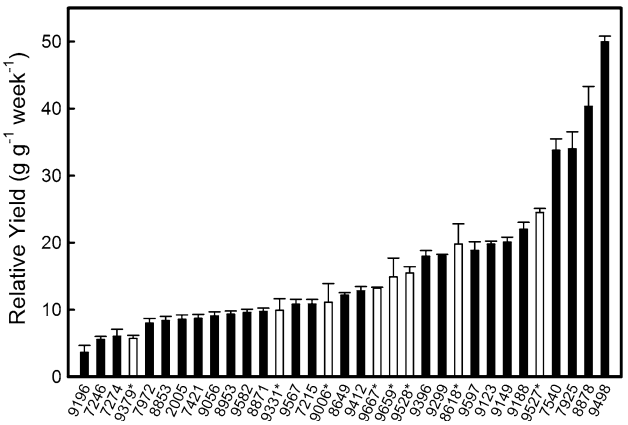

Fig. 1 Relative yield (RY) of all the 25 clones of *Wolffia* investigated in the present project (*black bars*) compiled together with 8 of those investigated previously (*white bars*; Ziegler et al. 2015). Schenk-Hildebrandt medium was used in all cultivations. More details about these 33 clones are available in Table 1

Results and discussion

In the present project, RGRs of 25 clones spanning all 11 species of the genus *Wolffia* were determined. The previously published RGRs of 8 additional clones belonging to three of the *Wolffia* species (Ziegler et al. 2015) were integrated in the present data in order to make the data set more comprehensive. Thus, in the present paper growth rates of 33 clones of different *Wolffia* species investigated under identical, standardized growth conditions were analyzed. The RGR and the DT of all clones were summarized in Table 1, and RY is depicted in Fig. 1.

In Schenk-Hildebrandt nutrient medium, the lowest growth rate was recorded in *W. globosa,* clone 9196, with an RGR of 0.155 day^{-1} and a DT of 4.47 days (Table 1; Fig. 1). In terms of RY, this clone produced 2.96 g biomass from a starting material of 1 g in 1 week. Interestingly, under the same cultivation conditions, the highest growth rate was also recorded in the species *W. globosa,* clone 9498, with an RGR of 0.559 day^{-1} and a DT of 29.8 h. In addition, in terms of RY, this clone produces 50 g biomass per week from a starting material of 1 g. Evidently, there is a huge variation in the growth rates of these two clones. These results were obtained under standardized laboratory conditions in Schenk-Hildebrandt nutrient medium. The performance of the clones will, of course, be different under natural conditions. However, cultivation under standardized, optimal conditions gives information about the growth capacity of the investigated clones and makes it possible to perform a comparative analysis of the clones under the same defined conditions. As discussed above, both the slowest and the fastest growing clones belong to

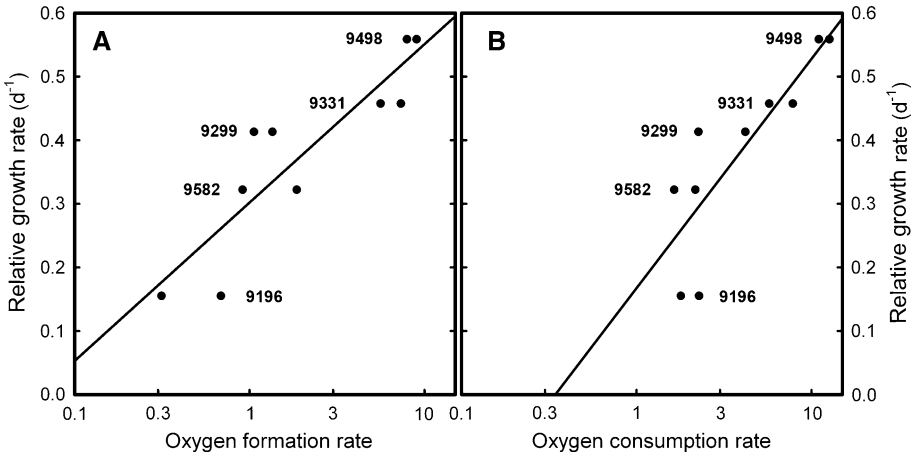

Fig. 2 Correlation of the relative growth rates (RGR) of five clones of *Wolffia globosa* with rates of oxygen formation (**a**) and consumption (**b**), respectively. Oxygen formation and consumption were measured in relative units (see "Materials and methods" section). The clone numbers are indicated parallel to the two data points that represent the replicate measurements of the oxygen formation or consumption correlating to the RGR of that particular clone

the species *W. globosa* demonstrating a very high intraspecific variation. Intraspecific variations of vegetative growth can also be seen in *W. angusta*, *W. arrhiza*, *W. brasiliensis* and *W. columbiana* (Fig. 1; Table 1). The intraspecific variations in the growth rates between some of the clones of a species are larger than the interspecific variation of the averages of growth rates of all the clones of a species (Fig. S1, supplementary material). It can be concluded that the rate of vegetative propagation of a clone is not completely defined by the species it belongs to. In general, clones of a duckweed species are isolated from locally adapted populations. Therefore, it can be assumed that the adaptation of plants to local ecological conditions must be a crucial factor in determining their physiological properties, e.g., rate of vegetative growth. This is in accordance with our previous results for representative clones belonging to different species and genera of duckweed (Ziegler et al. 2015), however, this assumption can now be firmly based on the present, more detailed analysis of the data comprising of 33 clones, all belonging to the genus *Wolffia*.

According to Venkataraman et al. (1970), the growth rate of one of the clones of *W. microscopica* was reported to be 0.78 day^{-1}, equivalent to a DT of 21 h and RY of 235 g g^{-1} week^{-1}. This growth rate is much higher than any of the ones in the present data set. Unfortunately, this clone was lost in all stock collections and no representative of the species, *W. microscopica,* was available (Bog et al. 2013; Lam et al. 2014) until its recent re-discovery (Sree et al. 2015c). We were now able to include the very first data about the re-discovered *W. microscopica,* clone 2005 (Table 1). However, the RGR of this clone measured in Schenk-Hildebrandt medium was not especially high in comparison with other *Wolffia* clones (Fig. 1).

In an effort to understand the large intraspecific differences in growth rates of duckweeds, we hypothesized that RGR correlates positively with photosynthetic rate of the given clone. Clones belonging to *W. globosa* were selected for testing this hypothesis because they displayed large intraspecific differences in growth rates. As shown in Fig. 2a, there is a positive correlation between the logarithms of the oxygen evolution (OE) and growth rates of the clones following the equation:

$$\mathrm{RGR} = (0.302 \pm 0.026) + (0.108 \pm 0.019) \times \lg(\mathrm{OE})$$

This relationship has a coefficient of determination of $R^2 = 0.796$ and the statistical test indicated high significance ($F = 31.159$, $P < 0.001$). It can be concluded that the clones with high apparent photosynthetic rates also possess high growth rates. The high rates of photosynthesis might make high growth rates possible. This inference does not exclude the influence of other factors on a complex physiological process like growth but demonstrates that it is a factor of significant impact.

We tested also the respiration in darkness in all five clones by measuring the consumption of oxygen (OC) in darkness (Fig. 2b). The following equation describes the relationship between the logarithm of the rate of oxygen consumption and the growth rates with an $R^2 = 0.771$; with the statistical test indicating high significance ($F = 19.776$, $P = 0.003$):

$$\mathrm{GR} = (0.167 \pm 0.055) - (0.157 \pm 0.035) \times \lg(\mathrm{OC})$$

From the results, it can be concluded that high growth rates are associated with high respiratory activity or, in a broader sense, may indicate high metabolic activity. It should be finally added that the logarithm of gross photosynthetic oxygen production (net photosynthesis) and the growth rate also correlated significantly with an $R^2 = 0.777$ (data not shown). Already many years ago, Zelitch (1982) stressed that measuring photosynthesis of single leaves of a plant cannot result in satisfactory results to comprehend the rate of plant growth and postulated the requirement of measuring photosynthesis and respiration of

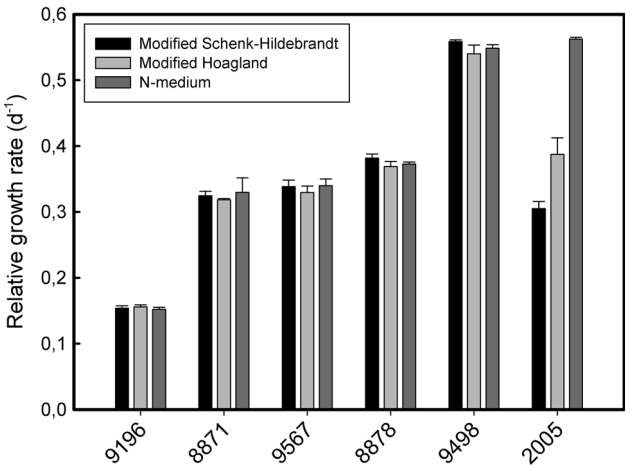

Fig. 3 Relative growth rates of six clones of *Wolffia* in three different nutrient media. For the composition of the nutrient media refer to the "Materials and methods" section. The investigated clones belong to the following species: *W. globosa* (9196, 9498), *W. arrhiza* (8871, 9567), *W. angusta* (8878), *W. microscopica* (2005). The values are represented by means together with the standard errors of the means

whole plants, which was easily possible using duckweed system in the present study. Poorter et al. (1990) demonstrated a significant relationship between photosynthesis and dry mass of the leaves in many wild species. This is similar to the present results with different clones of *W. globosa* showing a significant correlation between photosynthesis and growth rate.

In general, for the measurement of growth rates of duckweeds, modified Schenk-Hildebrandt medium is used as in Ziegler et al. (2015) and also in the present work because a preliminary experiment with a set of selected duckweed clones belonging to all five different genera including *Wolffia* showed a good performance in this medium in terms of their growth and appearance (data not shown). Apart from this, we wanted to replicate the method of Venkataraman et al. (1970) for the measurement of growth rate especially of *W. microscopica* in order to be able to compare their published results with those of the present ones. Therefore, we decided to measure the RGR of *W. microscopica* in three different nutrient media (modified Schenk-Hildebrandt medium, modified Hoagland medium, and N medium; cf. "Materials and methods" section). For comparison the clones of other *Wolffia species*, i.e. *W. globosa*, *W. arrhiza*, and *W. angusta* which showed fast and slow growth in modified Schenk-Hildebrandt medium (Fig. 1) were also tested in modified Hoagland medium and N medium (Fig. 3). Interestingly, there was no significant difference in the growth rates of *Wolffia* clones in different nutrient media except for that of *W. microscopica* clone 2005. This clone showed huge differences in its growth rates when cultivated in different nutrient media, the slowest being in modified Schenk-Hildebrandt medium and the highest in N medium (Fig. 2). The reason for this exceptional behavior of *W. microscopica* is not clear. Of course, there exist differences in the composition of the three nutrient media used but it is very intriguing why only *W. microscopica* reacts so sensitively to different growth media. In N medium, the RGR of *W. microscopica* clone 2005 was 0.568 ± 0.006 day^{-1} (DT = 29.3 h, RY = 51.2 g g^{-1} $week^{-1}$). This is the fastest growth rate out of all the investigated clones, significantly higher than that of *W. globosa* clone 9498 grown in the N medium or in modified Schenk-Hildebrandt medium. This means that this clone is presently the fastest growing Angiosperm.

Although the fastest growing in the present data set, *W. microscopica* clone 2005 showed much lower RGR than that of the lost clone which was investigated by Venkataraman et al. (1970). The huge difference between the growth rates of the two clones of *W. microscopica* is not very surprising; such differences were also detected in *W. globosa,* e.g., in the present study. These results provide an additional proof that the growth rate is essentially defined at the level of clones rather than at the species level.

Author contribution statement K. Sowjanya Sree and Klaus-J. Appenroth designed and carried out the experiments and evaluated the data. The measurements of the oxygen formation and consumption were carried out by Sailendharan Sudakaran. The manuscript was written in cooperation of all three authors.

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