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Arsenic toxicity in the water weed *Wolffia arrhiza* measured using Pulse Amplitude Modulation Fluorometry (PAM) measurements of photosynthesis

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ABSTRACT

Accumulation of arsenic in plants is a serious South-east Asian environmental problem. Photosynthesis in the small aquatic angiosperm *Wolffia arrhiza* is very sensitive to arsenic toxicity, particularly in water below pH 7 where arsenite (As $(OH)_3$) (AsIII) is the dominant form; at pH >7 AsO_4^{2-} (As (V) predominates). A blue-diode PAM (Pulse Amplitude Fluorometer) machine was used to monitor photosynthesis in *Wolffia*. Maximum gross photosynthesis (Pg_{max}) and not maximum yield (Y_{max}) is the most reliable indicator of arsenic toxicity. The toxicity of arsenite As(III) and arsenate ($H_2AsO_4^{2-}$) As (V) vary with pH. As(V) was less toxic than As(III) at both pH 5 and pH 8 but both forms of arsenic were toxic ($>90\%$ inhibition) at below 0.1 mol m^{-3} when incubated in arsenic for 24 h. Arsenite toxicity was apparent after 1 h based on Pg_{max} and gradually increased over 7 h but there was no apparent effect on Y_{max} or photosynthetic efficiency (α_0).

1. Introduction

Accumulation of arsenic in plants, particularly rice, is a major public health concern in South-east Asia (Williams et al., 2009; Zhao et al., 2009). Arsenic toxicity is correlated with acid sulphate soils (Kennedy, 1994; Berg et al., 2007), arsenic contaminated well-water in much of South-east Asia (Brammer and Ravenscroft, 2009) and old tin mine ponds in much of southern Thailand and Malaysia (Yusof et al., 2001). Anaerobic conditions tend to mobilise arsenic (Greenwood and Earnshaw, 1984; Kennedy, 1994; Marschner, 1997; Younger, 1997; Pirrie et al., 2003; Dsa et al., 2008). Arsenic accumulation and toxicity is difficult to study in normal-sized higher plants with their complex anatomy of roots stems and leaves but using algae as simple models is not satisfactory because green algae are not closely related to flowering plants. *Wolffia arrhiza* (Lemnaceae) is a true angiosperm despite its very small size ($\approx$ 1–2 mm) and lack of roots or leaves. Results of studies using *Wolffia* are likely to be relevant to other flowering plants such as rice and other cereals and other crop plants than using algal models.

Photosynthesis is very sensitive to arsenic toxicity, particularly in water below pH 7 where arsenite ($As(OH)_3$) or As(III) is the dominant form. Growth kinetics can be used to measure toxicity effects very successfully using *Wolffia* (heavy metals: Piotrowska et al., 2009, 2010; arsenic: Duester et al., 2011) and *Lemna* (arsenic: Mkandawire and Dudel, 2005) but the method requires experiments lasting over a period of days. PAM (Pulse-Amplitude-Modulation) fluorescence techniques are very reliable measures of photosynthesis on vascular plants (Ritchie and Bunthawin, 2010a, b; Ritchie, 2012). Fluorometry methods have not been routinely used on *Wolffia* to monitor photosynthesis despite its favourable size and geometry. Wang G.-H. et al. (2010) used a Hansatech Plant Efficiency Apparatus (a type of PAM) to monitor the effects of UV stress on *Wolffia* but fluorescence technology has not been used to full advantage in *Wolffia*. PAM techniques give realistic estimates of photosynthesis in *Wolffia australiensis* (Rechmann et al., 2007) and *Wolffia arrhiza* (Ritchie and Mekjinda, 2014) and are more sophisticated than simple bioassay growth studies on arsenic toxicity in *Wolffia* (Duester et al., 2011). PAM machines are capable of detecting very short term (within minutes) responses to toxins and large amounts of data can be collected very quickly.

Arsenic is not an essential element for plants or animals (Marschner, 1997). Organoarsenate compounds do occur in organisms but these are associated with detoxification (Meharg and Hartley-Whitaker, 2002; Ye et al., 2012; Quaghebeur and Rengel,

Abbreviations: As(III), arsenite; As(V), arsenate, ATP; ETR, Electron Transport Rate; PAM, Pulse Amplitude Modulation; RAT, Reflectance Absorptance Transmission; Pg, gross photosynthesis

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2004; Zhao et al., 2009; Zhang et al., 2009; Duester et al., 2011). Inorganic arsenic exists in two forms under physiological conditions, as the reduced form, As(III) (Arsenite) and the oxidised form, As(V) (Arsenate) (Greenwood and Earnshaw, 1984; Ramirez-Solis et al., 2004; Zhao et al., 2009). Under the usual aerobic conditions arsenate is the predominant form as $HAsO_4^{2-}$ ($pKa_2=6.96$). Arsenous acid H_3AsO_3 (more accurately, $As(OH)_3$) has a pKa_1 of 9.29 (Greenwood and Earnshaw, 1984) and although present under aerobic conditions it becomes the dominant form under anaerobic conditions (Meharg and Hartley-Whitaker, 2002; Wang X. et al., 2010). Supplementary Table 1 is a summary of the acid-base and redox properties of arsenite (As(III)) and arsenate (As(V)) from Greenwood and Earnshaw (1984), Marschner (1997) and Duester et al. (2011). The speciation of arsenic in solution, at equilibrium, for various pH values and redox potential and in various types of culture media can be calculated using a geochemistry program such as the open software, Geochem-EZ (Shaff et al., 2010). Commercial software such as MINEQL+ are also available.

Arsenic accumulation has received great attention in rice (Berg et al., 2007; Brammer and Ravenscroft, 2009; Williams et al., 2009) but rice is a cereal plant with complex anatomy whereas *Wolffia* is a very simplified angiosperm. *Wolffia* is used as a vegetable in South-east Asia and is known as watermeal or khai-nam (Bhanthumnavin and McGarry, 1971). *Wolffia* is also routinely fed to herbivorous food fish such as *Tilapia* (Rahman and Hasegawa, 2011). Phytoaccumulation of arsenic by Lemnaceae (Duester et al., 2011; Rahman and Hasegawa, 2011) is therefore a public health tissue in South-east Asia because *Wolffia* is used as food and is also fed to food fish. This study will show that PAM technology can be used to detect arsenic toxicity effects in *Wolffia* and investigates the most useful PAM parameters to use as toxicity criteria in a vascular plant.

2. Materials and methods

2.1. Growing Wolffia

Wolffia arrhiza was a kind gift from Kasetsart University, Bangkok (Mr Suthin Somboon and Mr Sinchat Maneekat, Faculty of Fisheries). *Wolffia* was grown in exterior tubs in commercial NPK medium with the pH adjusted to pH 5. Experiments were run in the laboratory in modified NPK medium with zero phosphate and known concentrations of phosphate and arsenic compounds added. The control medium had the following major ion composition: KH_2PO_4, 527 mg/l; KCl, 145 mg/l; $(NH_4)_2SO_4$, 614 mg/l; $MgSO_4 \cdot 7H_2O$, 24.5 mg/l; $CaCl_2 \cdot 2H_2O$, 14.7 mg/l; pH 5 with 1 ml of trace element mix (final concentrations, Mn, Cu, Zn and B - 0.1 mg/l, Mo 0.0093 mg/l), 1 ml/l of 100 mmol m^{-3} Fe citrate. The phosphate-free medium was a modified version to replace the phosphate with as few other changes as possible: KCl, 428 mg/l; $(NH_4)_2SO_4$, 614 mg/l; $MgSO_4 \cdot 7H_2O$, 24.5 mg/l; $CaCl_2 \cdot 2H_2O$, 14.7 mg/l; pH 5 with a 1 ml of trace element mix, 1 ml/l of 100 mmol m^{-3} Fe citrate. Sodium (meta)arsenite (III) ($NaAsO_2$: MW 129.9) and Disodium hydrogen arsenate (V)($Na_2HAsO_4 \cdot 7H_2O$: MW 312.0) were purchased from Sigma-Aldrich, St Louis, USA.

2.2. Preparation of Wolffia for experiments

Wolffia plants are only about 1 mm in diameter and are close to ovoid in shape. *Wolffia* was prepared for as a pressed disk of packed plants on Glass Fibre disks (Whatman GF/C glass fibre disks, Whatman International, Maidstone, England, UK) using a Millipore apparatus designed for 25 mm filters. The absorptances of the pressed disks of *Wolffia* were then measured and then the disks were dark treated in a Petri dish with disks of filter paper impregnated with NPK medium for at least 10 min. Only one light saturation experiment was run on each filter to avoid confounding effects of multiple experimental treatments such as invalid estimates of baseline fluorescence (F_o). The inside diameter of the Millipore filtration apparatus was 16.2 mm and so the disks of *Wolffia* plants adhering to the glass-fibre filter had a surface area of 206×10^{-6} m^2.

Plants were washed in phosphate-free NPK medium before being placed in incubation treatments with phosphate-free NPK with known concentrations of added As(III) or As(V). Chlorophyll (Chl) was extracted from *Wolffia* using 3 ml of 100% ethanol and absorbances measured by spectrophotometer (664 nm and 649 nm) and Chl *a* calculated as described by Ritchie (2006).

In the present study, most measurements have been calculated on a mg Chl *a* basis but many routine studies in plant physiology are often expressed on a dry weight (DW) or freshweight (FW) basis. A Table of useful conversion factors are provided in Supplementary Table 2. 100–300 mg freshweight samples of *Wolffia* were weighed out and dried in a 100 °C drying oven to constant weight to determine the FW/DW ratio of *Wolffia* based upon 99 independent samples (Supplementary Table 2). The PAM machine calculates photosynthetic electron transport rates (ETR) on a surface area basis (mol (e^-) m^{-2} s^{-1}). The Chlorophyll *a* content of a pressed surface of *Wolffia* plants allowed the calculation of an estimate of Chl *a* m^{-2} of pressed *Wolffia* plants. This enabled ETR rates as mol (e^-) m^{-2} s^{-1} to be converted into mol (O_2) mg Chl a^{-1} s^{-1} on the basis of $4e^-$ is equivalent to one O_2.

2.3. Measurement of photosynthesis using Pulse Amplitude Modulation (PAM) Fluorometry

PAM measurements were made a Junior PAM portable Chlorophyll fluorometer (Gademann Instruments GmbH, Würzburg, Germany) fitted with a blue diode (465 ± 40 nm) light source. PAM parameters (Y, rETR, qN, NPQ) were automatically calculated using the WINCONTROL software (v2.08 & v2.13; Heinz Walz Gmbh, Effeltrich, Germany) as defined by Genty et al. (1989) van Kooten and Snel (1990) and Krause and Weis (1991), using the standard default settings for rapid light curves (default absorptance factor, $Abt_F=0.84$, PSI/PSII allocation factor=0.5) to calculate the relative Electron Transport Rate (rETR) (Schreiber et al., 1995; Rascher et al., 2000).

2.4. Monitoring effects of arsenic on photosynthesis

Photosynthesis is generally very sensitive to physiological stress. The basic experiment using a PAM machine is a rapid light curve to determine the response of a plant to a stepwise range of irradiances (White and Critchley, 1999; Ritchie and Bunthawin, 2010a, b; Ritchie, 2012). The key parameter measured with a PAM is apparent photochemical yield (Y) or more rarely as ΦPSII (Genty et al., 1989; van Kooten and Snel, 1990; Schreiber et al., 1995). Yield is a measure of the proportion of incident photons that are actually used for electron transport by PSII of the chloroplasts of a plant. The Electron Transport Rate (ETR) is an estimate of Pg $4e^- = 1$ O_2). The Walz software calculates relative ETR (rETR) assuming the default Abt_F of 0.84.

2.5. Absorptance measurements – the RAT

Absorptances of vascular plants are often considerably different to the standard value (McCree, 1972; Bjorkman and Demmig, 1987) and so it is better to measure it experimentally (Ritchie and Runcie, 2014; *Wolffia arrhiza*, Ritchie and Mekjinda, 2014). Absorptance measurements determined experimentally could then