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Wolffia columbiana Can Switch Between Two Anatomically and Physiologically Separate States: Buoyant for Invasion and Starch Rich for Colonization

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Abstract: Turion formation is poorly studied in all of the *Wolffi*a species and only narrow studies have been done. In this study details of *Wolffia columbiana* ecology and physiology are related to anatomy and histochemistry. We used a combination of histochemistry and a new method of pressing tissue into one focal plane to reveal anatomical features that are not visible using conventional methods. Previously unknown organs, wax coated substomatal cavities, are described which play a crucial role in *Wolffia* ecology. Tank experiments were used to determine the behavior of *Wolffia columbiana* plants in response to light and dark conditions. A physiological mechanism for transition between an invasive floating population and dormant benthic population using oxygen floatation is described, which involves *Wolffia columbiana* behaving like a small bathyscaphe. This mechanism is combined with accumulation of large stores of starch and is an adaptation to colonization and overwintering.

Key words: Invasive, colonization, cuticle, sub stomatal cavity, amyloplast

INTRODUCTION

Eutrophication is a natural process which accelerates in lakes transformed by residential development because of increased fertilization, erosion of tributaries and formation of engineering works that prevent efficient flushing (Nixon, 1995). The Lemnaceae (duckweeds) are plants long recognized as an important members of aquatic plant communities (Hicks, 1937), especially in eutrophic systems (Santamaría, 2002; Frédéric *et al*., 2006). Godfrey lake is an example of eutrophication that has been strongly affected by human intervention. The lake is formed from a freshwater stream and salt marsh system that has been blocked by two causeways, one for pedestrian traffic and one for the Herberstville Road highway (Fig. 1). The causeways are pierced by drains allowing slow flow of water out of the lake. Godfrey lake is surrounded by high density residential development which includes a ring of minor roads and one main highway. Highly maintained gardens are a feature, though this lake is still characterized by native vegetation such as Pitch Pine (*Pinus rigida* Mill.) and Black-Jack Oak (*Quercus marilandica* Muenchh.), typical of pine barrens ecosystems (Gill, 1975). A conspicuous feature of Godfrey lake is the yearly bloom of water weeds that cover the lake in a continuous bright green surface layer. Other plants below the surface are starved of light, decay processes predominate (Balls *et al*., 2006) and a faint smell of sulfur compounds can be perceived periodically at this site. The

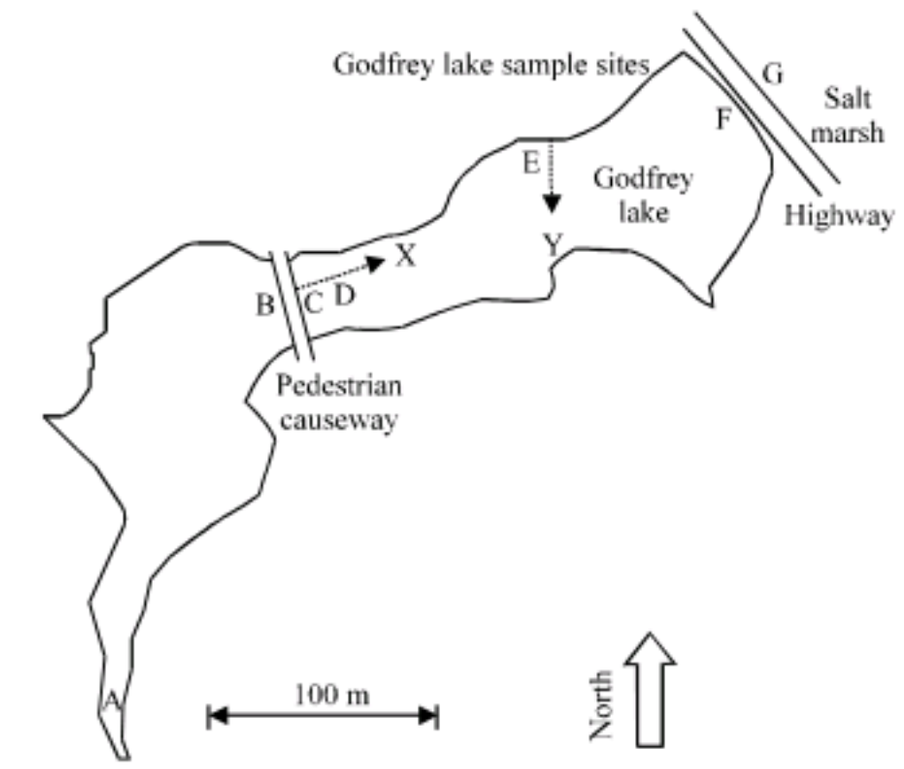

Fig. 1: Godfrey Lake sample sites. Sample sites A-G were used to estimate organic pollution, in particular coliform bacteria numbers which earlier study has shown originate predominantly from human and avifauna sources in Ocean County, New Jersey. Sites A-G are parallel to flow of water through the system and site E is near a roosting site for swans, as judged by accumulated feathers and droppings. Physical parameters for mid lake were estimated along transect X and parameters associated with the avifauna roosting site were estimated along transect Y. Plant material for growth in tanks was harvested from site C

most abundant member of these blooms are watermeal i.e., *Wolffia* species (Alexander *et al*., 2008; Kiage and Walker, 2009).

Watermeal reproduction is almost exclusively vegetative (Maheshwari and Chauhan, 1963) and they have average *in vitro* doubling times of approximately 17 days (Bernard *et al*., 1990) which allows for rapid and invasive growth during summer. Growth declines during autumn and watermeal are rarely seen in winter. *Wolffia* sp., are very small angiosperms which contain few gross anatomical features and only rare development of true seed. They have specialized epidermal cells including stomata for gas exchange and parenchyma with small intercellular gaps but almost no other features easily determined except by electron microscopy (White and Wise, 1998). Their survival is assumed to depend on rapid growth after invasion of new habitats and their mechanisms of overwintering in cold or temperate climates are unknown.

Some duckweeds produce anatomically distinct resistant structures called turions which overwinter at the bottom of lakes. These have been described in detail for natural populations of only a few species such as *Spirodela polyrhiza*, *Lemna gibba* (Jacobs, 1947), *Lemna minor* and *Lemna turionifera* (Dudley, 1987), but are poorly described for *Wolffia* sp. (Bernard *et al*., 1990). Some physiological studies of *Wolffia* have been carried out, but only in artificial laboratory conditions intended for biomass production (Fujita *et al*., 1999) which are very different to the natural environment of *Wolffia* sp. Despite having such a numerous and dynamically changing population this important member of the Urban Pine Barrens ecosystem is poorly studied. Few papers describing anatomy and physiology are published and none present experimental evidence which relates plant biology and the environment of aquatic plants with their mechanisms for survival and invasive ability. This study confirms the existence of a resistant stage for *Wolffia columbiana*, describes adaptive anatomical and histochemical features and proposes a mechanism for movement between separate benthic and floating populations. The research presented is for the year 2007 and observations of surface plants and turions in sediment samples confirm that the phenomena presented continue regularly from year to year.

MATERIALS AND METHODS

Technical grade ethanol (Fisher Scientific), commercial bleach (Chlorox Regular Bleach™, 6% sodium hypochlorite, 5.7% available chlorine), Acetocarmine Stain (0.5% w/v in 45% acetic acid), Iodine Solution (Fisher Scientific, 0.33%w/v iodine, 0.66% w/v KI), Sudan IV Stain (0.5% w/v in 70% ethanol) and Sudan Black Stain (0.5% w/v in 70% ethanol) were used. All other chemicals were reagent grade material purchased from the Sigma Chemical Company, St Louis.

Physical features of Godfrey Lake surface water, significant to ecology, were determined using a YSI 650 MDS Data Sonde (YSI, Yellow Springs, Ohio) in Summer 2007. The transects followed are shown in Fig. 1. Light intensity for tank studies was measured using a LiCor LI-250A light meter (Lincoln, Nebraska). Organic pollution (coliforms) was estimated using an abbreviated procedure based on EPA method 1603, which replaces membrane filtration with embedding 5 mL samples in 10 mL mTEC agar for rapid assessment of water quality.

Duckweed from sample site C (Fig. 1) was harvested and used to populate open tanks at the Monmouth University laboratory. Godfrey lake water was used and supplemented with distilled water to compensate for evaporation. Living plant material was examined and classified using a Nikon YS2-H bright field microscope and by consulting the Jepson Manual of Higher Plant Taxonomy (Hickman, 1993; Les *et al*., 2002). Floating and submerged individuals were separated and moved to 100 mL beakers containing 30 mL of Godfrey lake water using a disposable pasteur pipette and then incubated in low light which approximated environmental conditions (19 μmol/m^2/sec), medium light (42-54 μE/m^2/sec) or darkness. Floaters and Sinkers were periodically counted and rates of floatation calculated using linear regression. The various samples for anatomical and histochemical examination were prepared as follows. Material for examination of gross anatomy was prepared by simultaneously drying and pressing Floaters or Sinkers between a microscope slide and cover slip using water tension to apply a continuous moderate force over approximately 8 h. Starch staining was performed by a process of clearing in 1.5 mL polypropylene Eppendorf microcentrifuge tubes. This consisted of the following incubations: 2 min in 1 mL of 70% v/v ethanol at 100°C, 5 min in 0.5 mL of bleach at room temperature, 16 h in 100 μL of acetocarmine stain, 1 min in a drop of distilled water and 1 min in a drop of iodine solution on a microscope slide. A coverslip was lowered onto the microscope slide then each plant observed in bright field microscopy as before. Material for observing cuticle wax was prepared by transferring four Floaters and four Sinkers to separate 1.5 mL polypropylene Eppendorf microcentrifuge tubes. Most water was removed using a polypropylene pasteur pipette then 1 mL Sudan IV solution was added to both Eppendorf tubes. Sudan staining was performed overnight at room temperature. A

new Pasteur pipette was used to transfer individual plants to a microscope slide and a drop of distilled water used to wash away excess stain. Samples were observed using the Nikon YS2-H microscope as above.

RESULTS

Sample sites at Godfrey lake were selected to show parameters at varying points during flow through the lake (transect X) and to represent pollution at known roosting sites for avifauna, which include swan (*Cygnus olor*) and mallard (*Anas platyrhyn*) (transect Y). Godfrey Lake biological parameters are typical for a Pine Barrens freshwater lake with a low salinity and low pH (Table 1 and 2). The level of fecal pollution is moderate and distribution is associated with storm water flow through the lake rather than known avifauna roosting sites, indicating human rather than wildlife pollution (Table 3).

Samples of watermeal were identified as *Wolffia columbiana* primarily because of their rounded cross section, transparent upper surface and rounded plan profile. As expected, only vegetative structures were seen (Khurana and Maheshwari, 1983). Material from sample site C was used to establish colonies of floating *W. columbiana* in tanks grown from August 2007. These colonies had formed two populations by October 2007 which appeared to be able to exchange members: a floating population and a sunken population which were indistinguishable by the unaided eye. These are referred to as Floaters and Sinkers in this study.

Experiments with separated Sinkers that had already made the transition from Floater to Sinker showed movement from bottom to surface. Under low light conditions this reached an equilibrium that included both Floaters and Sinkers (Fig. 2) where the maximum rate of floatation was 6.4 plants day^{-1} (R^2 = 0.96). Floatation under low light did not proceed to completion. Further experiments showed that environmental factors influence the rate of floater formation, in particular light intensity. When Sinkers were incubated under medium light the rate of flotation increased dramatically to 17.5 plants day^{-1} (R^2 = 0.99) and floatation of this population carried on to

Table 1: Physical parameters of transect X, from site C North East

	Distance (m)					
Parameters	0.5	3.0	6.0	9.0	12.0	15.0
Temp. (°C)	10.29	11.39	11.66	11.69	11.68	11.64
Salinity	0.00	0.00	0.00	0.00	0.00	0.00
DO (mg L^{-1})	4.66	5.37	5.44	5.39	5.35	5.46
pH	6.16	5.99	5.94	5.91	5.88	5.87

Ecological parameters were sampled along the transect which starts from site C and continues West, towards Herbertsville Road. Data was collected on 2 November 2007

Table 2: Physical parameters of transect Y, from site E South

	Distance (m)			
Parameters	1	3	5	7
Temp (°C)	12.69	12.63	12.56	12.48
Salinity	0.00	0.00	0.00	0.00
DO (mg L^{-1})	5.59	6.41	5.66	5.57
pH	6.19	6.28	6.73	6.54

Ecological parameters were sampled along the transect which starts from site E and continues south. Data was collected on 2 November 2007

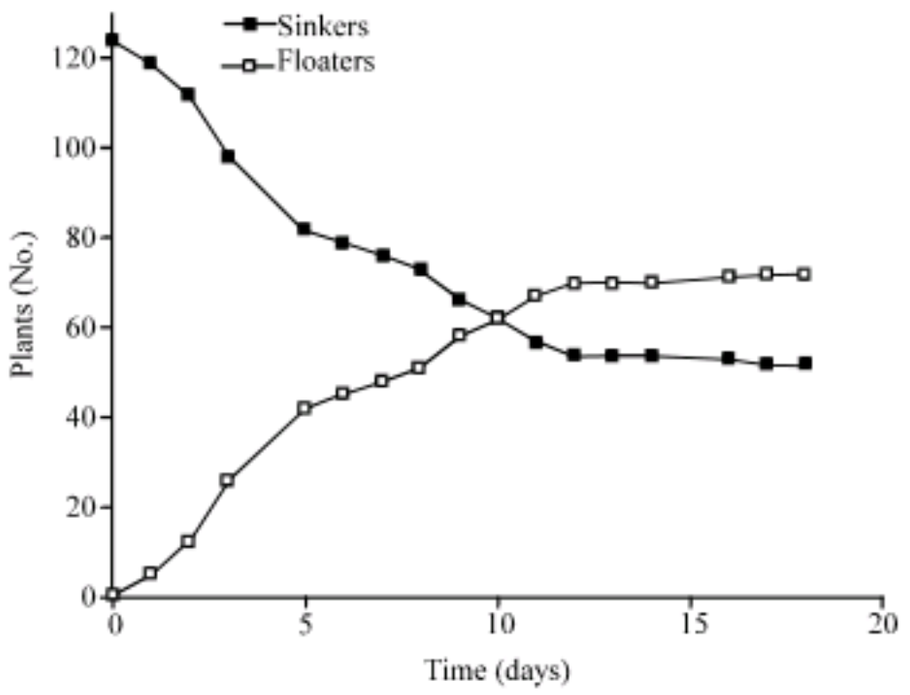

Fig. 2: Sinker and Floater Equilibrium in low light. Sinker plants, which had been introduced to laboratory tanks as floaters, were transferred to a 100 mL beaker containing 30 mL of Godfrey Lake water and incubated under low light intensity (19 µE/m^2/sec). The number of Floaters and Sinkers was counted periodically. The maximum rate of Floater formation (6.4 plants day^{-1}, R^2 = 0.96) was made measured using data from 0-12 days and linear regression, constraining the regression line to (0,0)

Table 3: Organic pollution

	Sample site						
Coliform No.	A	B	C	D	E	F	G
Description	Upper inlet	Upper drain	Lower inlet	Lower lake	Avifauna roosting	Lower drain	Salt marsh outlet
Coliforms (CFU/100 mL)	90	60	30	40	0	10	30

Samples for evaluation of organic pollution were collected from Godfrey lake sample sites A-G as 50 mL water samples in sterile Falcon tubes. These were transported to the Monmouth University Lab., where 5 mL was set in 10 mL mTEC agar and incubated overnight at 37°C. Detergent resistant and lactose fermenting colonies were counted

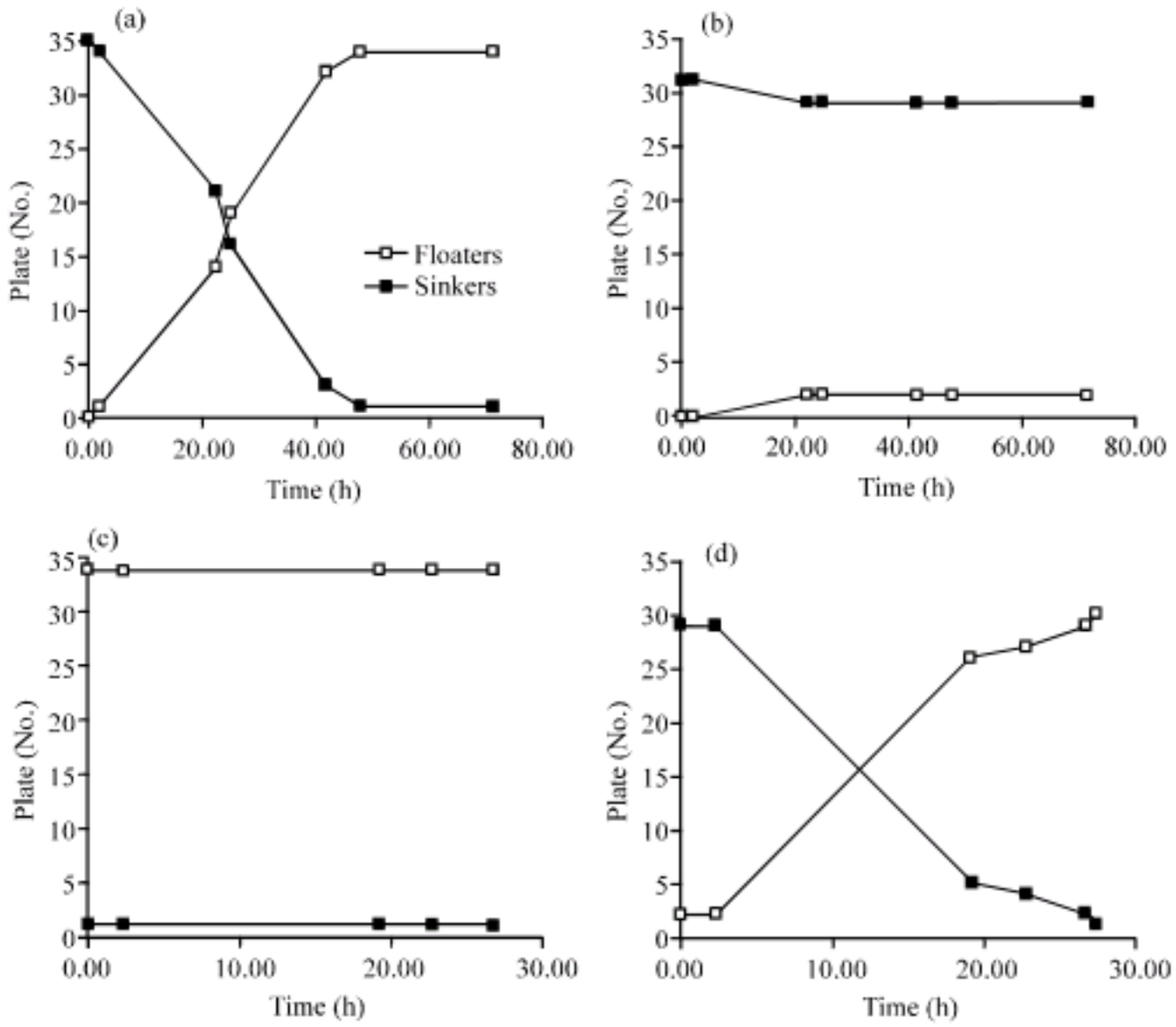

Fig. 3: Response of *Wolffia* to medium intensity light. Sinker plants were transferred to 100 mL beakers containing 30 mL beaker of Godfrey lake water as before and incubated under medium light intensity (42-54 μE/m^2/sec) or darkness (wrapped in aluminum foil). (a) Plants were treated identically to Fig. 2 except for the use of medium light intensity, (b) plants were treated identically to Fig. 2 except that they were incubated under dark conditions, (c) the beaker from treatment A was wrapped in foil, transferring the newly generated floaters to darkness and (d) the beaker from treatment B was unwrapped, transferring the hidden sinkers to medium light. In each treatment the number of Floaters and Sinkers was counted periodically. The maximum rate of floater formation (17.5 plants day^{-1} (R^2 = 0.99) was made measured using data from treatment A 0-2 days and linear regression, constraining the regression line to (0,0)

completion (Fig. 3a). Incubation of Sinkers in darkness prevented floatation which shows that the rate increase seen for Fig. 3a of approximately 300% depends on light (Fig. 3b), even though these dark grown plants are competent for induction of the Floater state as show by rapid floatation when they were brought out of darkness (Fig. 3d). However, when plants induced to the Floater state (Fig. 3a) were transferred to darkness (Fig. 3c), no Sinker formation was seen (Fig. 2c).

The rounded nature of *W. columbiana* makes observation of live material difficult because many cells in several planes of focus superimpose to form confusing images. Dry pressed material, however, slightly flattens whole plants and allows observation of many anatomically important features simultaneously (Witty, 2008). This method also reveals features not easily observed otherwise, including the distribution of small parenchyma air pockets and large substomatal air pockets (Fig. 4a). Floaters showed multiple large substomatal air pockets (Fig. 4b) while none were observed in Sinkers (Fig. 4c). These large substomatal pockets were not easily observable by conventional embedding and sectioning methods (data not shown).

Staining of cleared whole plants for Iodine shows that Floaters contain numerous small starch granules in cells previously observed to be green, including mesophyll parenchyma and stomata (Fig. 5a). In contrast, Sinkers stained intensely black and were distinguishable from Floaters by microscopy (Fig. 5b) and by unaided eye. Prolapse of meristem material from the budding cavity was a common post mortem change seen in Floaters and Sinkers upon use of cover slips or after slide storage (Fig. 5b).

During preparation for microscopy Sinkers displayed several physical and chemical features that were different to Floaters. Floaters interacted with the polypropylene swall of Eppendorf tubes, adhering strongly through hydrophobic interactions i.e., adherence was disrupted by

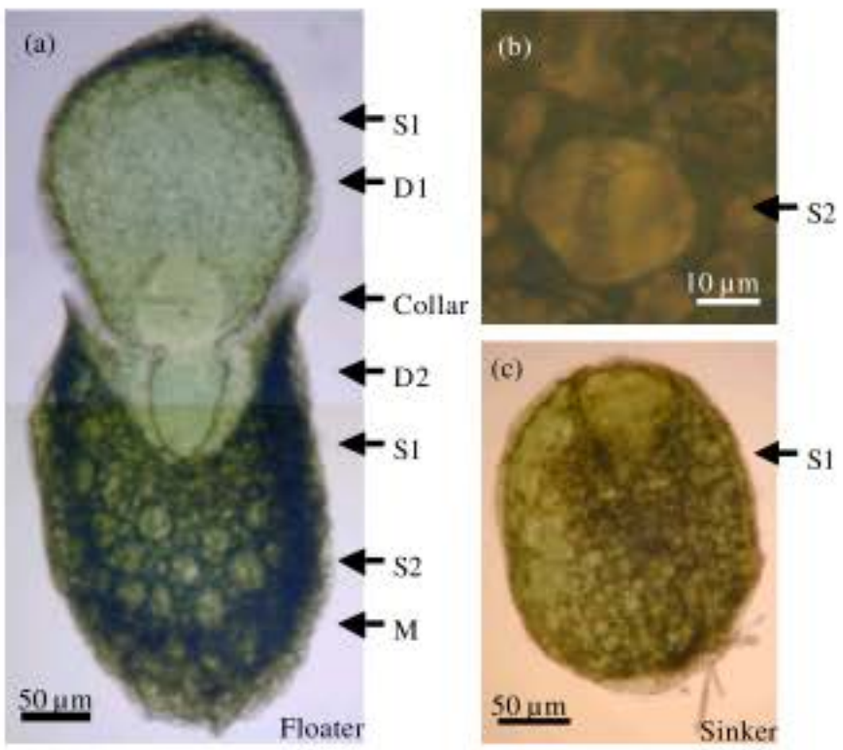

Fig. 4: *Wolffia columbiana* gross anatomy. (a) Dry pressed Floater showing a mother and daughter plant. Features seen were the mother plant (M) and two generations of daughter plant (D1 and D2), the collar around the reproductive pouch (Collar), conventional stomata (S1) and stomata with large substomatal cavities (S2). Magnification ×100. (b) Stoma with large substomatal cavity near a conventional stoma. Magnification ×400. (c) Dry pressed Sinker showing conventional stomata. Magnification ×100. Composites of several images were used

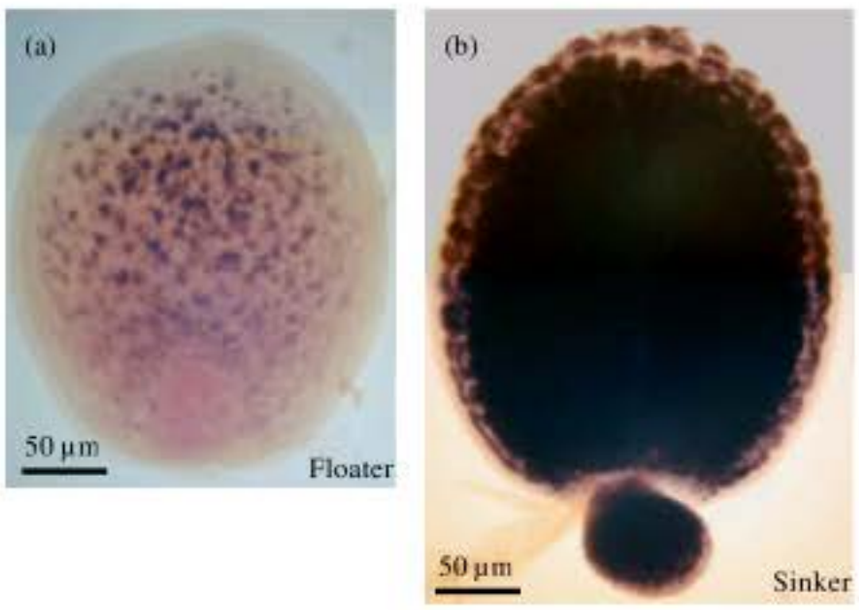

Fig. 5: Starch stain. Starch stained *Wolffia columbiana* after clearing which show the difference of intensity between (a) Floaters and (b) Sinkers. Magnification ×100. Composites of several images were used

adding the hydrophobic solvent 70% ethanol. Sinkers did not interact with polypropylene. Floaters rapidly bleached

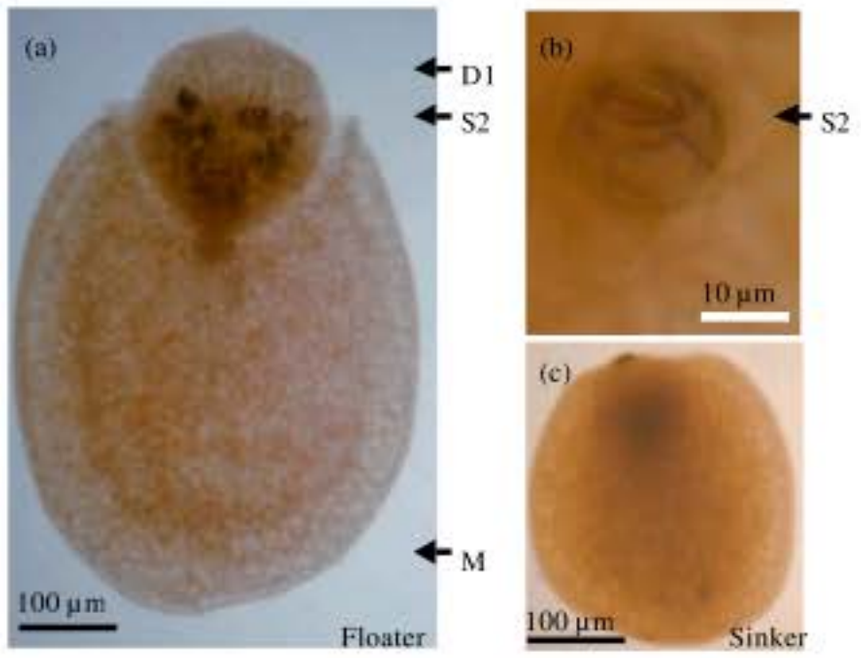

Fig. 6: Sudan stain. Sudan IV stained *Wolffia columbiana*. (a) Whole plant showing a ring of thickened cuticle for the mother plant and intensely stained stomata and substomatal cavities for the daughter. Magnification ×100. (b) Stoma and substomatal cavity. Magnification ×400. (c) Sinker. Magnification ×100. Composites of several images were used

in 70% ethanol at 100°C while Sinkers did not. This indicates strong differences in the hydrophobic nature of cuticle material between Floaters and Sinkers. Therefore, *W. columbiana* were stained with the hydrophobic dye Sudan IV. Mature Floaters showed a ring of thicker cuticle on their adaxial surface while immature Floaters showed some stomata and subsidiary cells in substomatal cavities with intensely staining cuticle (Fig. 6a, b). Few features of cuticle staining were seen for Sinkers, using Sudan IV. Sudan Black staining gave similar results (data not shown).

DISCUSSION

Earlier studies by White and Wise (1998) have suggested flotation by surface tension which allows two states: floating or sunk. We observed intermediate states of buoyancy including slow risers which shows a continuous spectrum of buoyancy during the research for this study and in subsequent samples of *W. columbiana* taken from Godfrey Lake. This study proposes a mechanism to explain the three states of floatation observed: there is movement from the sunken state as a response to light and the production of oxygen in photosynthesis. However, incubation in darkness did not stimulate sinking so photosynthesis is not the only factor involved in the buoyant state of *W. columbiana*. High levels of starch accumulation are seen in Sinkers

suggesting that achieving the submerged state requires continued photosynthesis but also response to an additional signal. This is presently unknown but may be light intensity.

A critical anatomical organ for buoyancy and control of buoyancy is the substomatal cavity which is seen in adapted Floaters but not Sinkers. Transformation from Sinker to Floater takes place in hours (Fig. 3d) showing that floatation is initially achieved using parenchyma air pockets rather than the large substomatal cavities of anatomically adapted Floaters.

During embedding Floaters and Sinkers behaved differently, in particular in their hydrophobic interaction with polypropylene and resistance to bleaching in 70% ethanol. This shows that these populations have differences in addition to the anatomical features described in this study and that Sinkers are chemically resistant. Sudan staining confirmed this biochemical difference. Formation of cuticle is important for floaters which must keep water away from stomata and especially important for immature Floaters which may be weighed down by their Mother before abscission. The concentric circle of thick cuticle on the adaxial surface of Floaters also orientates the plant at the water's surface.

Sinking and rising is an adaptation to an invasive lifestyle i.e., *W. columbiana* invade while floating and persist in one location by sinking. This tactic may be adapted to seasons, for example to resist winter floods by sinking as a response to reduced light flux. Floaters are adapted to be invasive by floating downstream. However, if floating in New Jersey watersheds is uninterrupted then all plant material will tend to be washed to the sea and killed. In addition to invasion and colonization, the Sinker phase is an adaptation to overwintering i.e., to avoid ice damage. Increased understanding of *Wolffia* physiology has suggested some methods of control which will be the subject of further research.

Duckweed including *W. columbiana* occupy two separate levels of Godfrey Lake. The floating and submerged populations have different gross anatomical and chemical properties, in particular, water resistant substomatal air pockets controllable using specialized stomata for opening or closing. Floaters and Sinkers are in equilibrium and that equilibrium can be disturbed by environmental factors such as small changes in light intensity. Floatation is achieved as a response to medium light intensity while the mechanism for Sinker formation is unknown. However, sinker formation is associated with starch accumulation and differences in cuticle hydrophobicity. It is assumed that attachment to the water surface is lost and dense starch granules cause sinking independently of photosynthetic activity.

CONCLUSION

These results confirm previously reported preliminary results regarding the formation of resistant vegetative structures (Bernard *et al*., 1990; Fujita *et al*., 1999). However, earlier study does not define biophysical mechanisms for turion function. This study describes formation of dense structures with largest stores of starch enabling long term survival in benthic detritus. Floatation by controlled accumulation of gas in substomatal air pockets is also described as a mechanism which enables emergence from benthic dormancy. This mechanism is similar to accumulation of gas in the lacunae of *Myriophyllum verticillatum* L. (Haloragaceae) winter buds, but has not been seen previously in any member of the lemnaceae (Weber and Noodén, 2005).

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