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Use of Azolla as a test organism in a growth chamber of simple design

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Summary The construction and application of a new type of growth chamber, in which different growth conditions *i.e.*: temperature, humidity, pH, light intensity, light colour, change in nutrient composition and gas exchange can easily be controlled, are presented.

The method has previously been applied to two *Azolla* species *viz. Azolla filiculoides*, which is cold tolerant and *Azolla pinnata* (distinguished in Vietnam as the form Xanh), which is heat tolerant.

In the growth chamber natural growth conditions of the Azolla — *Anabaena azollae* symbiotic association were imitated as much as possible.

For testing the system, methods discussed earlier[8,14] and some previously presented data, concerning photosynthetic activities, such as oxygen evolution and nitrogen fixation (acetylene reduction) of two *Azolla* species[39], were partially used. Biomass of *A. filiculoides* was measured and reactions to its environment at conditions when grown in the field and in the growth chamber, were studied.

Growth and photosynthesis measurements were performed under special light conditions and with whole plants grown under laboratory conditions.

Anthocyanin synthesis was studied in relation with humidity.

Anthocyanin spectra were analyzed by means of a spectrum-deconvolution method.

Introduction

For many years researchers around the world have tried to grow several strains of Azolla (Gr. azo, parched, killed by drought)[5,20] under laboratory conditions[13,34–39].

Azolla is a small, free-floating aquatic fern, which lives in symbiosis with a filamentous blue-green alga, *Anabaena azollae*. The alga is capable of fixing molecular nitrogen, and it meets the entire nitrogen requirement of the association. Photosynthesis is performed by the alga as well as by the fern[22–24].

It has been shown that the growth rate and the photosynthesis rate of various species of Azolla grown under the same environment conditions, differed from each other[26,33,39].

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Azolla is widely distributed throughout the world, but most of all in tropical- and subtropical areas with different temperature- and humidity ranges[28]. The tropical *A. pinnata* has been domesticated in Vietnam and China for several centuries, where it has been grown as a fertilizer crop on over 400 000 hectares of fallow rice fields, providing sufficient nitrogen to produce 5 metric ton/ha of paddy yield[36]. The Azolla — *Anabaena azollae* association can be used as a green manure for upland crops; *e.g.*: potatoes, vegetables, maize.

Azolla as a compost decomposes very rapidly and mixed with potash and other fertilizing agents, becomes an almost perfect fertilizer[20,36]. The association can also be used as a forage for almost all domestic animals and for many species of herbivorous fish. Other advantages of the association are, that it can be used as a purification plant to study and regulate the water quality[13], to study the effects of 'acid rain'[39] and also the environmental changes in relation with food production.

The presented growth chamber allows for small-scale investigations of the many varieties of Azolla grown under well controlled 'natural conditions', *e.g.*: determination *in vitro* of the effect of shading, of a soil growth medium, of variations in the microclimate (humidity, temperature), the development of effective physical and chemical methods for oversummering and overwintering of Azolla under simulated tropical- and temperate climates, and the development of artificial methods for the induction of the sexual cycle (to find effective ways to produce and collect large amounts of spores as planting material).

Materials and methods

Design of growth chamber and accessories

For the construction of the growth chamber, we used a special type of UV Plexiglass type *GS Clear 2058* which is transparent between 280–800 nm and has a thickness of 5 mm. The material was chosen because it gives the possibility of simulating natural light conditions. The dimensions of the growth chamber (Fig. 1) are 520 × 380 × 180 mm (l × w × h). The chamber consists of three parts, *i.e.*: a dismountable thermostated top compartment, which functions as a lid, a plant growth chamber and a bottom compartment, which is also thermostated and fixed to the growth chamber. For a more efficient temperature exchange the water controlling devices (CD) were constructed in the bottom compartment. By their shape and with the aid of a doubleacting pump thermostate (*Colora tye P3*) the water circulation is very effective. To improve the temperature exchange with the plant material in the growth chamber, distilled water was added, and in some experiments saturated salt solution, for humidity control, up to the level of the growth media inside the growth dishes.

For ventilation and gas-exchange experiments the growth chamber contains gas inlet and outlet connectors. The ventilation was performed by flushing pre-moisted air through the growth chamber, every hour, during 15 minutes and with 15 s pulse intervals. When humidity control and gas exchange are of minor importance, it is possible to ventilate the growth chamber by simply lifting and supporting the thermostated top compartment as pleased. Bottom- and top plates were made dismountable for cleaning purposes. For more details, see Fig. 1 and the accompanying legend.

For illumination we used four *Silvania Gro-lux F 36 W Gro ES* fluorescent tubes, placed at a distance of 11 cm from each other and at a variable height (between 12.5 and 40 cm) above the

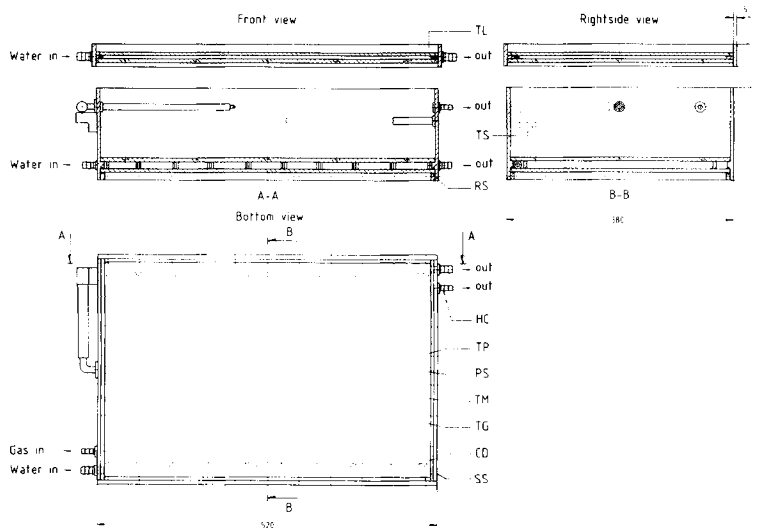

Fig. 1. Design of growth chamber. Front view, Right side view and bottom view with description of components (see text). TL — top lid, RS — rubber sealing, TS — thermometer support, HC — hose connector, TP — temperature probe, PS — probe support, TM — thermometer, TG — thermometer guide, CD — water-controlling devices and SS — stainless steel screws. The scale dimensions are in mm.

plants. By changing the height of the fluorescent tube fixtures, it was possible to control the light intensity (*cf.* Fig. 2). Cultures were subjected to a photoperiodic cycle of 15 h light/9 h dark, while the light intensity was maintained at $650\,\mu\,W/cm^2$, which is more or less the light intensity under the rice canopy in Vietnam at noon. When higher light energy and more far-red light was required, Gro-Lux WS fluorescent tubes more closely matched the light requirements of the photosynthesis of the association in study.

The day temperature of the growth medium was generally kept at 25°C, the night temperature at 18°C, while the respective day/night-air temperatures were controlled within 1°C higher, except otherwise stated. This was to prevent condensation at the bottom plate of the thermostated top compartment if the relative humidity in the growth chamber should reach 100%, which would affect light penetration and plant growth. A more efficient temperature control was obtained by the water-controlling devices (CD) and by equaling the water and growth-media levels in the growth chamber as mentioned above. The air temperature was controlled by means of a temperature controller of the *United Electric Control Co.*, type *UE 800* model ICS 15 A/250 VAC connected to a temperature probe inside the growth chamber and regulated by a magnetic shut-down valve, incorporated in the water-circulation system. When the temperature inside the growth chamber differed more than 1°C from the preset-probe temperature, the water in the top lid started to circulate until the pre-set temperature was reached again. This method was specially chosen to control the relative humidity inside the growth chamber within the range of 60–95%, which is more or less the natural humidity in summer time in Vietnam and also the range at which the Azolla plants grow best[20,28,37,39]. When the application of low humidity was needed, saturated salt solutions were used[41].

The relative humidity was measured by means of two well-matched thermometers and according to the condensed table of the U.S. Weather Bureau Bulletin No. 1071[41] at a wind speed of 2 m/s. One of the thermometer bulbs (wet) was covered with muslin and kept moisted by being in contact with the water reservoir inside the growth chamber, while the dry bulb was mounted in the thermometer guide (TG) at about 5 cm above the Azolla plants (cg. Fig. 1). The dry-wet bulb thermometer method is a standard application in most of the more sophisticated incubators, which

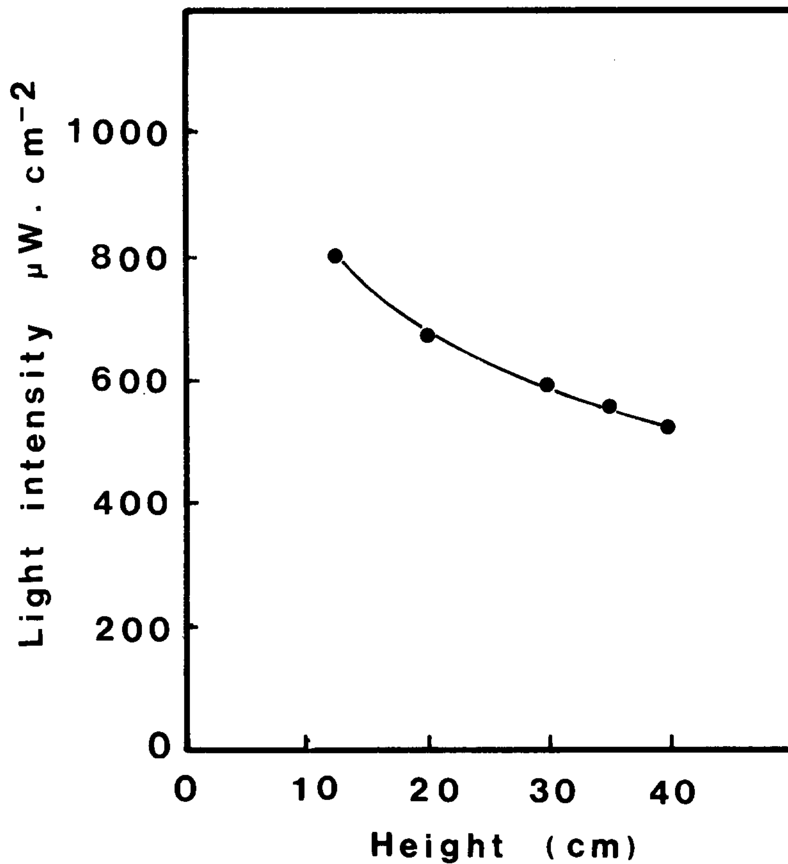

Fig. 2. Light intensity curve of four Sylvania Grolux fluorescent tubes, type Gro F36W/Gro-ES (at a distance of 11 cm from each other) as function of the height between 12.5 cm and 40 cm above the plants. The light intensity was measured after the light has passed through the top lid of the growth chamber and at the level of the plants.

are combined with CO_2- and humidity controlling systems. It is also the method for calibration of hair hygrometers in general and is still in use at several Metereological Institutes and Airports, *e.g.* *Schiphol Airport.*

The wind speed was measured by means of a *Wallace Thermo* Anemometer type GGA 235 connected to a NI-125 ANE 760983 probe, both from *Wallace Control Instruments* and calibrated according to their instruction manual. Incidentally, the relative humidity of the room and in the field was measured with a *Thiës- Göttingen* self-recording thermo hydrograph, type 620–9378, which particularly in tropical climates needs frequent recalibration.

Construction of the growth chamber

The construction of the growth chamber was performed by means of a *Deckel* Universal milling-drilling Machine, type FP1, fitted with a fixed-angular table with overall dimensions of 300 × 150 (manual, longitudinal × transversal) a maximal stretch plane of 600 × 260 mm and a maximal vertical adjustment of 340 mm.

The Plexiglass GS 2058 was manufactured according to the manufacturer's instructions for sawing, drilling, milling and glueing. For glueing, *Casol 50*, combined with the accelerator 24 was used.

For consolidation of the bottom part, as well as the construction of the thermometer guide and temperature-probe support, round perspex material was used.

On special request a set of detailed construction schemes will be obtainable.

Analytical methods

In order to study the effects of the growth chamber on the growth of *Azolla* species, we have used methods and results of experiments with two *Azolla* species, grown and studied in the same growth chamber[39]. The measurement of nitrogenase activity (acetylene reduction) and of the oxygen evolution (pH 7,2), and the composition of the growth media and the reaction medium for oxygen evolution, were in accordance with the methods described by Vu Van Vu *et al.*[39].

Spectral analysis was done by means of a computerized Aminco DW-2a UV-VIS spectrophotometer and a spectral deconvolution method[41], developed in the Department of Microbiology of the Vrije Universiteit.

The Anthocyanin pigments were extracted from 1 g fresh plant material with 0.1% methanolic-hydrochloric acid solution[10,11,15,25] (*cf.* Fig. 3).

The dry-weight experiments were performed in a *Hereaus-Hanau* vacuum oven model *RVT 360*, attached to a two-stage *Speedivac* High vacuum pump model 25C 20A, from *Edwards High Vacuum* Ltd.

Plant material (about 2 g) randomly collected from the field and growth chamber, respectively, was washed, blotted with paper to get rid of the water adhering to the Azolla surfaces, after which it was weighted in triplicate (fr. wt.). During 48 h the plant material was dried at a temperature of 80°C, while it was kept *in vacuo* at about 2.104 Pa. Upon terminating the pre-treatment, the plant material was cooled to room temperature, transferred into a dessicator, weighted and heated to 50°C. It was then cooled to room temperature and weighted again. The procedure was repeated at 1 h intervals till the weight was constant (dry weight).

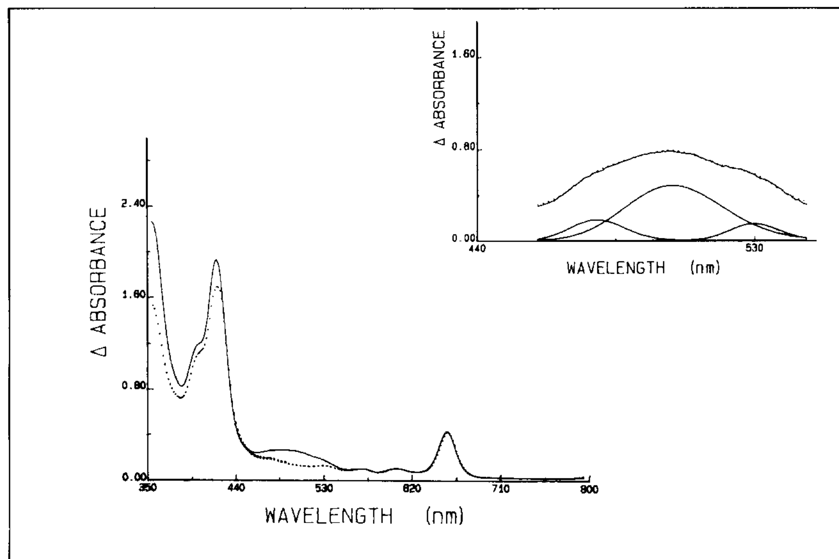

Fig. 3. Spectra of extracts of whole *Azolla filiculoides* plants in 0.1% methanolic-hydrochloric acid. *Dotted curve* is of extract of *A. filiculoides* before visual anthocyanin synthesis in the leaves occurred. *Solid curve* is of *A. filiculoides* after anthocyanin synthesis was clearly visual in the leaves. The *inset* represents the deconvolution of the *solid curve*, revealing three components with absorption at 479, 503 and 530 nm, respectively. The spectra have been normalized with 656.3 nm as reference wavelength.

The relative growth rate (rgr) and the biomass-doubling time (t_d) were calculated with the aid of the formulae:

$$\text{rgr} = \frac{\text{Ln}\,W_f - \text{Ln}\,W_i}{t} \quad \text{and} \quad t_d = \frac{\text{Ln}\,2}{\text{rgr}}$$

in which W_i and W_f are initial and final washed dry weights and t the time elapsed in days between the two weight measurements, respectively. Results of rgr (Fig. 4) and t_d (results) are expressed in $mg \cdot g^{-1} \cdot d^{-1}$ and in days respectively.

The pH measurements were done by means of a *Knick* type 641 digital pH meter.

The conductivity measurements were done with a *Philips* PW 9501 conductometer.

Results

To make the test procedure complete, we present in brief some data which deal with a comparison study between two *Azolla* species, *i.e.*: *A. filiculoides* and *A. pinnata*[39]. It has been shown that *A. filiculoides* has an optimum temperature for both nitrogen fixation and oxygen evolution at 25°C and *A. pinnata* at 30°C.

The Azolla plants showed phenotipical adaption to temperature and humidity, which seems to indicate a possible lipid-protein complex change in the membrane structure.

In the present study we found that *A. filliculoides* fronds which had been frozen for at least two weeks in the field (between −10°C and −15°C) after which they were transferred to the growth chamber at a day/night temperature of 25°C/18°C, a relative humidity of 95% and a light intensity of 650 $\mu W/cm^2$ started to grow again, but had no measurable nitrogen fixation activity at the start of the growth period.

Microscopy study of the cavity showed a relative poor quantity of *Anabaena azollae* algae. The easily identified heterocysts seemed to be in a developing stage.

From the nitrogen fixation data of Becking[4] we estimated a dry weight (dr.wt.)/fresh weight (fr.wt.) ratio of 0.024. However, we found this value to vary with the growth conditions. *A. filliculoides* plants which have been collected from the field with their leaves compact, gave a dr.wt./fr.wt. ratio of 0.084. After growing them in contact with their own ditch soil and ditch water in the growth chamber at 25°C and a humidity of 70%, the ratio was 0,094 but the leaves were still compact (unfolded). When grown in the growth chamber during five days at a humidity of 95% and a day/night temperature of 25°C/18°C the shape of the leaves changed from a compact leaf structure to a curly appearance and large upper lobes of the leaflets with broad membraneous margins. This was in good agreement with the description of Pieterse[26] for typical luxuriously growing Azolla plants. In that case we found a dr.wt./fr.wt. ratio of 0.064. *A. filiculoides* fronds, which have been grown at several initial pHs ranging from pH (5–9), seemed to be influenced in their growth rate

by the pHs. We found a broad pH range from pH (5–8), which compared with the growth rate at pH 7 has a small deviation of 2.8 and 5.0% at pH 6 and 8, while at pH 5 and 9 it was more drastically influenced, 17.3 and 27.3%, respectively. We found biomass doubling times (t_d) at pHs 5, 6, 7, 8 and 9 to be 5.4, 4.6, 4.4, 4.7 and 6.1 d, respectively.

In comparison with these date, the nitrogen fixation activity (Fig. 3) has not been influenced so much, probably because of the membrane barriers between the buffer outside and the heterocysts of the *Anabaena azollae* algae inside the leaflob cavity. According to Peters *et al.*[23] the heterocysts seems to be responsible for the nitrogenase activity. *A. filiculoides* plants collected from the greenhouse of the University of Leiden, grown for two weeks at 15°C/10°C (day/night temperature, a humidity of about 60% and a light intensity of 650 μW/cm^2, gradually changed colour from green to violet reddish (synthesis of anthocyanin cf. Fig. 3).

A. filiculoides fronds collected from the field in Amstelveen, The Netherlands, (field conditions: rel. humidity 70% at a ditch water temperature 10°C, conductivity, 1180 μS/cm and pH 7.24) changed their colour from deep violet reddish to green, within 5 d of growth in the growth chamber at a humidity of 95% and a growth temperature of 25°C/18°C. A check of the growth medium after the growth period, does not show any anthocyanin extrusion. We found that a combination of low humidity (about 60%), low temperature (10°C) and much blue and red colour in the applied light spectrum, (Gro-lux fluorescent light) resulted in the development of three anthocyanin pigments (cf. Fig. 3). For clearness sake it should be noted that, the original spectra of anthocyanins were multiplied by six. The deconvolution method was applied within the range of 460-458 nm (Fig. 3 inset).

According to previous anthocyanin studies[10–13,15,26] the three anthocyanin pigments could possibly be pelargonigin-3-glucoside with λ_{max} at 503 nm (505[10,12]), cyanin-3-glucoside λ_{max} 479 nm (478[26]). The data between brackets are values found by previous work as indicated. The distribution of the peak areas were 15.6% at λ_{max}, 73,9% at λ_{max} 503 nm and 11.1% at λ_{max} 530 nm respectively.

Discussion

Previous efforts to keep *Azolla* species in laboratory conditions in Vietnam alive, sometimes failed. These failures were caused by the extreme temperatures (above 35°C) and the bright sunlight radiation in summertime, outside as well as inside the laboratory (window-sill).

Growing *Azolla* species under these environmental conditions in conical flasks or Petridishes, has some disadvantages, *i.e.*:

— Depending on the brightness of the light radiation, the temperature inside the growth vessels can easily increase 3–5°C higher, than outside. This necessitates a proper temperature control, which is difficult to apply in that system. At higher temperatures (above 35°C), this temperature shift sometimes caused the death of the Azolla plant material (over-summering problems).

— The O_2-CO_2 gas exchange, as well as the ventilation and humidity, are also difficult to control.

More success was gained when *Azolla* species were grown in contact with wetted soil (unpublished).

Pieterse *et al.*[26] reported poor conditions of Azolla when cultivated in the greenhouse during the winter-season. Schapendonk *et al.*[32,32] emphasize the fact, that any interaction between factors determining the climate in the greenhouse will increase the complexity of the climate control, because for many plants grown in the greenhouses, long days and intense sunlight in summer-time means too much light, and without natural shading and good ventilation, the greenhouses may get too hot, while in winter the plants need higher light intensity on cloudy days and a more effectivetemperature-controlling system.

These disadvantages, have made us to design and construct the presented growth chamber. It is of great importance to have the possibility of controlling climatic factors, *e.g.*: temperature, humidity, CO_2 *etc.*, independently of each other. With the presented growth chamber the control of climatic factors can easily be realized on a more economical small scale. The growth chamber can easily be adapted to other applications too.

In most of the literature on the growth of *Azolla* species, the application of humidity control during the growth period in the laboratory is not mentioned[1,2,9,15,20–24,26,27,33].

In harmony with earlier membrane-lipid studies[29,30,40,43] we explain the phenomenon of the variation in the dr.wt. over fr.wt. ratio of *A. fillicu-loides* grown at different humidities, at present as water-binding capacity of the dominating membrane lipids, *i.e.*: monoglactosyldiacylglycerol, diagalactosyldiacyl-glycerol. whereas the phospholipids and addition of magnesium and calcium ions increase the hydration capacity. In winter-time the air humidity is normally dry, while because of the cold, the water transport via the roots and the solubility of magnesium and calcium salts will decrease. In that case there will be less water bound to the lipids and the dr.wt./fr.wt. ratio will increase, which seems to be in agreement with our findings.

According to Holst[15] strong sunlight is the activator of anthocyanin

synthesis and in *A. mexicana* he identified the anthocyanin as Luteolinidin-5-glucoside. Pieterse[26] reported that anthocyanin production was influenced by temperature and water chemical composition and not by photoperiod, while Dubois[9] stated that Azolla plants, which have been grown under a glass cover, as well as in the shadow, did not show any anthocyanin synthesis. Kuilman[19] stated that anthocyanin in Buckwheat germ plants and other green plants are essentially formed by alternating exposure to light and high temperature followed by a period of darkness and low temperature. He also stated that it is out of question that an increase of sugar concentration in the tissue could probably be the cause of anthocyanin synthesis in green plants. The reason why Dubois[9] and others did not see any anthocyanin synthesis when Azolla plants were grown under a glass cover or in the shadow, can be explained by the fact that in a closed vessel and in the shadow the evapo-transpiration differs from that in an open vessel, and open water surfaces. In the first situation, the relative humidity reaches saturation very fast (no wind action in the closed vessel or breaking of the wind in case of shadowing plants) and in the closed vessel, the temperature rises rapidly by the solar radiation.

In the second situation the relative humidity will not reach saturation so fast, because the wind removes the evaporated water and also causes a cooling effect on the Azolla plants and the water surface. The evapotranspiration will be less pronounced and the relative humidity will decrease markedly. In our system we have only found anthocyanin synthesis when the temperature was low (about 15°C) and the humidity about 60%. When the humidity was high, the plants changed colour to green again.

The light conditions were the same in both situations of growth in the growth chamber. So, from our results we conclude that low temperature combined with low humidity are the activators of anthocyanin synthesis.

An other far more important effect of humidity on Azolla is reported by Reynoud[28] who found that the growth stopped when the dry season was well established, the night temperature had decreased and the relative humidity was in the range of 90% at 6 AM to 40% at 2 PM. At the same time, micro and megasporangia appeared in the ventral lobe of the fern. This observation suggests that humidity control may be a key factor in the experimental induction of sexual reproduction.

Together with the screening of the 30 varieties of *A. pinnata* native in Vietnam[20,37] with respect to their usefulness for agricultural purposes, the study of the sexual reproduction will be the subject of our forthcoming investigations.

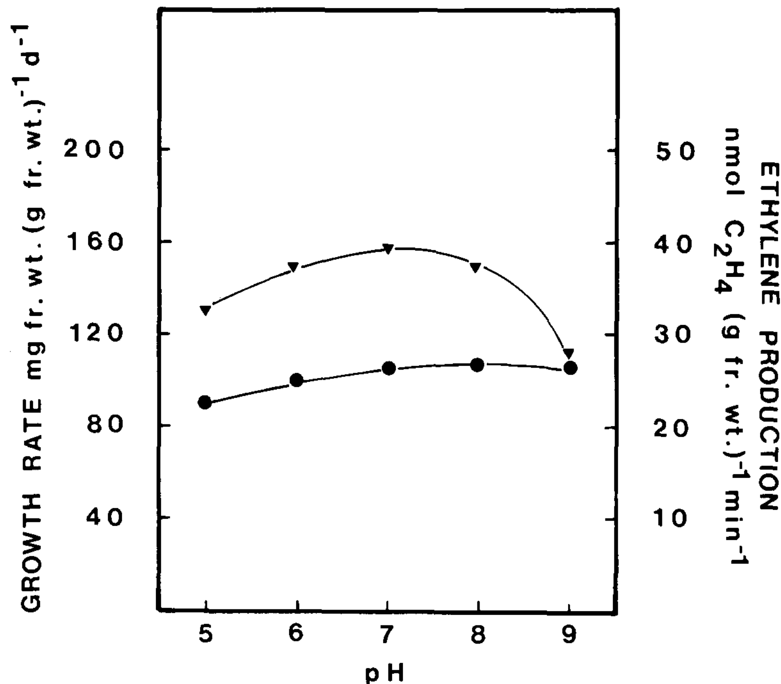

Fig. 4. Growth rate ▼ — ▼ and ethylene production ● — ● of *A. filiculoides* Lam. grown during five days (day/night temperature, 25°C/18°C) at several initial pHs. During the experiments the growth media (R-N) have been refreshed once after three days of growth. The growth values are mean values from five separate experiments of which the errors never exceeded 3% of the main values. The ethylene production was performed according to the method described in analytical methods. Upon terminating the growth rate experiments the Azolla plants have been incubated at their initial pHs and in 10% acetylene-air atmosphere, while illuminated during three hours with Grolux fluorescent light. At one hour interval samples were taken from measurement of ethylene production.

Conclusions

With the presented rather simple growth chamber we have made a first step toward controlled growth of *Azolla*species under well-defined conditions and in an economical way. For low-budget laboratories it is very easy to build, portable and easily adaptable to particular demands.

When growing *Azolla* species under laboratory conditions the humidity conditions are of crucial importance. From this study it was shown that humidity affects several processes in the Azolla plants.

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