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# Influence of ethanol and ethylene on the seed germination of three nymphaeid water plants

A.J.M. SMITS, G.H.W. SCHMITZ, G. VAN DER VELDE† AND L.A.C.J. VOESENEK\*  
Laboratory of Aquatic Ecology and \*Department of Experimental Botany, University of Nijmegen, Toernooiveld, 6525 ED Nijmegen, the Netherlands

†Author to whom correspondence should be addressed.

## SUMMARY

1. Under anaerobic conditions cold stratified seeds of *Nymphaea alba* and *Nuphar lutea* germinated readily and released ethanol (up to 6–7 mM ethanol g<sup>-1</sup> DW), whereas seeds of *Nymphoides peltata* did not germinate and hardly any ethanol was released (up to 1.5 mM ethanol g<sup>-1</sup> DW). Ethylene release by seeds of *Nymphaea*, *Nuphar* and *Nymphoides* incubated under hypoxic conditions could not be detected.
2. Under aerobic conditions all *Nymphaea* and *Nuphar* seeds germinated, but at a lower rate compared with anaerobic incubation. Ethanol release under aerobic conditions was low (1.5–2 mM ethanol g<sup>-1</sup> DW). Under aerobic conditions the seeds of *Nymphoides* germinated promptly and ethanol release was low (0–0.5 mM ethanol g<sup>-1</sup> DW).
3. Germination of *Nymphaea* and *Nuphar* seeds in an ethanol solution (350 mM) was generally stimulated compared with that in water, but no significant effect was recorded if seeds had not received a cold treatment or had been stratified for 8 weeks. Germination of *Nymphoides* seeds was inhibited in the ethanol solution unless they had received a cold treatment of 12 weeks.
4. Germination of *Nymphaea* and *Nuphar* was stimulated by ethylene (5 µg l<sup>-1</sup>) but germination in moist air was lower than under normal submersed conditions. A stimulating effect of ethylene on the germination of *Nymphoides* seeds was also evident.
5. It is suggested that ethanol and ethylene play an important role in determining niches for germination, contributing to the zonation of these nymphaeids in a water body.

## Introduction

*Nymphaea alba* L., *Nuphar lutea* (L.) Sm. and *Nymphoides peltata* (Gmel.) O. Kuntze share a nymphaeid growth form but often occur at different sites in water bodies (Brock, 1985; Van der Velde, Custers & De Lyon, 1986). *Nymphaea* is frequently found in sheltered waters with an organic sediment, whereas *Nuphar* roots in a similar substratum but is capable of maintaining itself at locations subject to water currents and wave action (Kirchner, Loew & Schröter, 1927; Brock, 1985). *Nymphoides* is frequently found in water bodies that have a fluctuating water level and are regularly subjected to water currents and erosion. *Nymphoides* is a 'ruderal' water plant, capable of swift colonization of eroded areas of the littoral zone. *Nymphoides* can also occur

on sediments with a relatively high organic matter content (Grote, 1980; Van der Velde *et al.*, 1986) but probably does not tolerate too great an accumulation of organic material (Van der Voo & Westhoff, 1961; Westhoff *et al.*, 1971). Due to water level fluctuations the physicochemical processes in the sediment in the littoral zone resemble that of soils which become periodically waterlogged.

The ethylene production of waterlogged soils has been described by Smith & Russell (1969) and Smith & Restall (1971). Ethylene is produced in the waterlogged soil during the depletion of oxygen as a result of microbial respiration during submersion. If oxygen concentrations drop below 2% by volume, ethylene

production occurs. Experiments with sterilized soils demonstrated that the ethylene production originates from an enzymatic and not a chemical conversion process. Accumulation exceeding  $20 \mu\text{g l}^{-1}$  ethylene was measured in gas spaces of several soil types after 10 days at a temperature of  $20^\circ\text{C}$ . The quantity of ethylene evolved after 10 days of incubation was approximately proportional to the organic matter content of the soil, up to about 10% by volume. After this period, the oxygen has been completely consumed and ethylene production ceases. Thus, no ethylene production by microbial activity will occur in a permanently submerged soil where the oxygen consumption rate is higher than the rate of oxygen supply by diffusion.

In aquatic sediments both anoxic and hypoxic conditions are present (in the deeper, organic parts and the littoral zone, respectively). The observation that *Nymphaea*, *Nuphar* and *Nymphoides* roots have a capacity for ethanol fermentation under anaerobic conditions (Smits *et al.*, 1990a), leads to the question whether the seeds also have this capacity for ethanol fermentation and if so, whether ethanol plays a regulating role in the germination process (Fig. 1). Furthermore, it will be of interest to know whether the seeds of *Nymphaea*, *Nuphar* and *Nymphoides* are able to produce the phytohormone ethylene under hypoxic conditions and if so, whether this affects germination. The present paper discusses the results of a number of seed germination experiments designed to provide an answer to these questions.

## Materials and methods

### Seed collection and storage

Ripe *Nymphaea* fruits were collected from Achterste Goorven (coordinates N  $51^\circ34'54''$ , E  $5^\circ12'18''$ ); *Nuphar* fruits from Oude Waal (coordinates N  $51^\circ51'14''$ , E  $5^\circ53'41''$ ) and Breukelen (coordinates N  $52^\circ10'50''$ , E  $5^\circ01'16''$ ); *Nymphoides* fruits from the Oude Waal and Millingerwaard (N  $51^\circ51'53''$ , E  $5^\circ59'57''$ ). All fruits were stored in open plastic containers filled with tap-water at room temperature (c.  $20^\circ\text{C}$ ). Pressurized air provided stirring and aeration of the seed stock. After the seeds were released from the fruits they were stored at  $1\text{--}4^\circ\text{C}$  (cold stratification). Tap-water was changed frequently during storage.

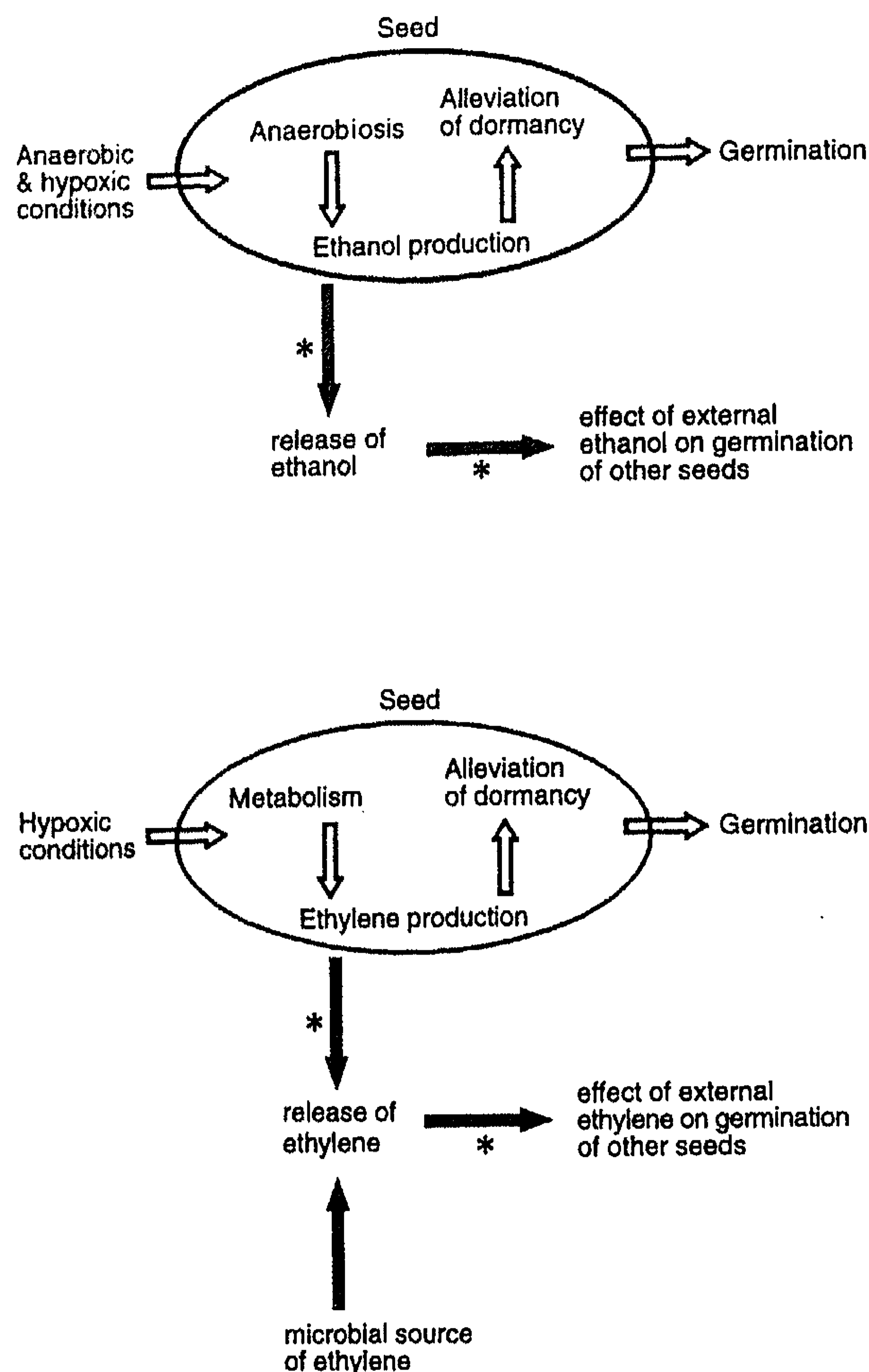


Fig. 1 Diagram of the hypothetical events that occur during the germination of *Nymphaea*, *Nuphar* and *Nymphoides* seeds. The events marked with an asterisk were investigated in the present study.

### Release of ethanol by seeds

To rule out the possibility of ethanol production by micro-organisms attached to the seed coat, the seeds were sterilized. Twenty-five, 9-week stratified seeds of each of *Nuphar*, *Nymphaea* and *Nymphoides* were sterilized by placing them in a 1% chlorine solution for 20 min. The seeds were subsequently placed in serum flasks (30 ml) filled with sterilized aerobic or anaerobic water. Anaerobic water was obtained by saturating water with nitrogen gas for 1 h. The flasks were made airtight with the help of a screw cap. The seeds were allowed to germinate in a controlled temperature room at  $20^\circ\text{C}$ . Light was provided during a 16 h light period by means of white fluorescent tubes. The light intensity was approximately  $100\text{--}130 \mu\text{Einstein m}^{-2} \text{s}^{-1}$ . A rubber sealing pad in the

screw cap allowed sampling with hypodermic syringes. Samples of 0.5 ml were taken for ethanol determinations at regular intervals during the incubation. The removed sample volume was replaced by an identical volume of aerobic or anaerobic water, as appropriate. Ethanol was assayed enzymatically according to Bernt & Gutmann (1974). The incubations were conducted in duplicate.

#### *Ethanol and germination*

After a period of cold stratification of 0, 4, 8 and 12 weeks, four batches of twenty-five non-sterilized seeds per species were placed in a Petri dish filled with a 350 mM ethanol solution. This ethanol concentration was chosen because it was the maximal ethanol concentration produced by *Nymphaea* and *Nuphar* seeds under anaerobic conditions in the present study. Four batches of twenty-five seeds placed in twice-distilled water served as controls. The seeds were allowed to germinate under conditions similar to those described above. Seeds with a protruding radicle (*Nymphoides*) or first subulate leaf (*Nymphaea* and *Nuphar*) were scored as germinated. The ethanol solution and the twice-distilled water were renewed weekly.

#### *Release of ethylene by seeds*

Because the sterilization of seeds may affect the vitality of the seeds it was decided to determine first whether ethylene is released by non-sterilized seeds. Cold (8–9 weeks) stratified seeds (fifty and 100 seeds per species), were placed in 6 ml capped serum flasks. Subsequently about 3 ml of nitrogen saturated water was added so that the seeds were fully submersed and hypoxic conditions were created. After 20 days of incubation the flasks were shaken and the air in the flasks was sampled and analysed for the presence of ethylene. A gas-tight syringe was used to collect 1 ml air samples and inject them into a Chrompack Packard gas chromatograph, model 438 A, with a packed Poropack Q column (length 100 cm), filled to a density of 0.34 g cm<sup>-3</sup>, at 60 °C.

#### *Ethylene and germination*

In order to determine the effect of external ethylene on germination, 25 (8–9 weeks) stratified, non-sterilized seeds were incubated in a glass serum flask (650 ml)

filled with an air–ethylene mixture. In order to mimic an environment in which ethylene is produced (namely above a soil producing ethylene), the seeds were placed on moist filter paper. Four serum flasks were used per species and per treatment. Preliminary experiments demonstrated that the germination response of *Nymphaea*, *Nuphar* and *Nymphoides* seeds exposed to 0, 1, 5 and 10 µg l<sup>-1</sup> ethylene mixtures was maximal at 5 µg l<sup>-1</sup> ethylene. Hence, four flasks were filled with a 5 µg l<sup>-1</sup> ethylene mixture (AGA, Amsterdam, the Netherlands) and four serum flasks with medicinal air (AGA).

The flasks were made airtight with the help of a screw cap. A rubber sealing pad in the screw cap allowed the gas mixtures to be renewed with two hypodermic syringes. The gas was led through the first syringe into the serum bottle, while the displaced gas could escape through a second syringe. The air in the serum flasks was replaced by flowing 2 l of ethylene–air mixture through the flasks. This was repeated during the second day of the experiment. Subsequently 1.4 l of gas were flown through the flasks every third day in order to maintain the composition of the gas mixture as it was at the beginning of the experiment. The ethylene concentration in the serum flasks was determined as described above. The temperature and light conditions and the criteria for seed germination used in the ethylene experiment were identical to those in the ethanol experiment.

#### **Statistical methods**

The regression coefficients of the ethanol release rates during anaerobic and aerobic incubation were compared by means of an *F*-test (SAS, 1986). The data obtained in the ethanol and ethylene experiments were analysed by a distribution-free test for curve analysis (Kozioł *et al.*, 1981).

#### **Results**

##### *Release of ethanol by seeds*

The seeds of *Nymphaea* and *Nuphar* germinated in both aerobic and anaerobic water. After 15 days of anaerobic incubation 60–70% of the seeds had germinated, as opposed to approximately 10–20% in the aerobic incubation (results not shown). The seeds of *Nymphoides* only germinated in aerobic water. *Nymphaea*

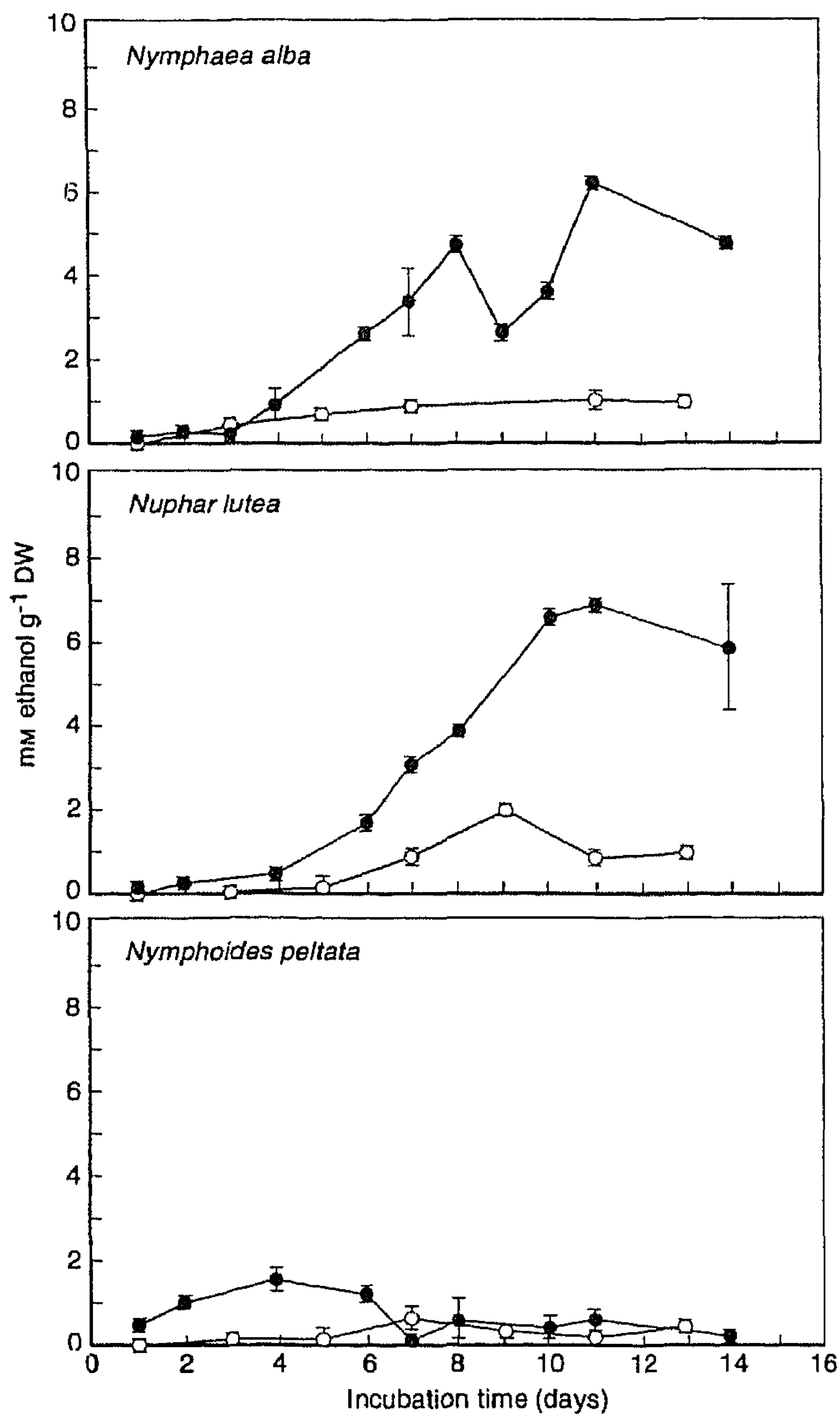


Fig. 2 Accumulation of ethanol released by sterilized *Nymphaea alba*, *Nuphar lutea* and *Nymphoides peltata* seeds during aerobic (open symbols) and anaerobic (closed symbols) incubation. Mean numbers are shown; each replicate contained 25 seeds and  $n = 2$ . Bars represent standard error of the mean.

and *Nuphar* seeds released more ethanol in the anaerobic incubation (up to 6–7 mm ethanol g<sup>-1</sup> DW) than in the aerobic incubation (1–1.5 mm ethanol g<sup>-1</sup> DW) (Fig. 2). The seeds of *Nymphoides* released a small amount of ethanol during the first phase of anaerobic incubation (up to 1.5 mm ethanol g<sup>-1</sup> DW) but this ceased after day 6. Under aerobic conditions the seeds of *Nymphoides* germinated promptly (70% within 15 days; results not shown) and ethanol release was low.

The ethanol release by *Nymphaea* and *Nuphar* seeds during anaerobic incubation was significantly different from that during aerobic incubation ( $P < 0.0001$ ).

There was no significant difference in ethanol release by *Nymphoides peltata* seeds under aerobic and anaerobic conditions.

#### Ethanol and germination

Without a cold treatment only one or two *Nymphaea*, *Nuphar* and *Nymphoides* seeds germinated in the ethanol solution or in twice distilled water over a period of 60 days. This erratic germination is not considered representative of the germination process of these nymphaeid water plants. The germination rate of all three plant species increased with increasing duration of the cold treatment (Fig. 3). After a cold treatment of 4 weeks ethanol stimulated the germination of *Nymphaea* seeds. Due to fluctuations in the numbers of germinated seeds which were stratified for 8 weeks, this effect was not significant. The stimulating effect of ethanol could once more be observed when seeds were used which had undergone a cold treatment for 12 weeks. The course of the germination process of *Nuphar* resembled that of *Nymphaea*. Once again, there was a stimulating effect of the ethanol on the germination of the seeds.

Unlike *Nymphaea* and *Nuphar*, the germination of *Nymphoides* seeds was inhibited by ethanol. However, increasing the stratification period reduced the magnitude of this effect and after a cold treatment of 12 weeks the germination rate was so high that the inhibitory effect of ethanol was no longer noticeable.

#### Ethylene and germination

No ethylene production by *Nymphaea*, *Nuphar* or *Nymphoides* seeds could be detected after 20 days of incubation.

Ethylene stimulated the germination of *Nymphaea*, *Nuphar* and *Nymphoides* seeds (Fig. 4). However, the total numbers of germinated seeds of *Nuphar* and in particular *Nymphaea* were low compared with those in the ethanol experiment. The stimulating effect of ethylene was most evident in the germination of *Nymphoides*.

## Discussion

### Ethanol

Under anaerobic conditions the seeds of *Nymphaea* and *Nuphar* produce a substantial amount of ethanol,

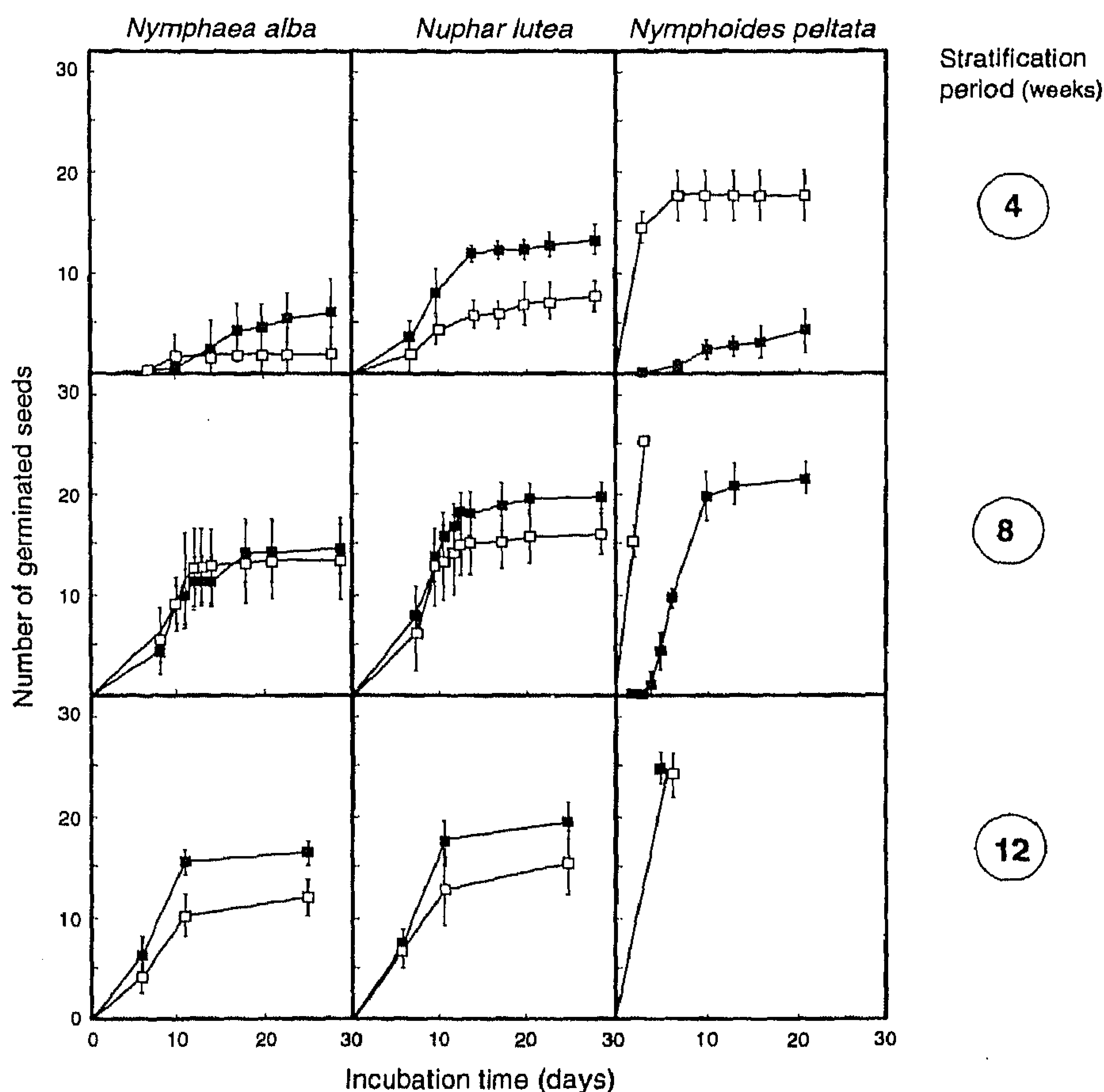


Fig. 3 Germination of *Nymphaea alba*, *Nuphar lutea* and *Nymphoides peltata* seeds in a 350 mM ethanol solution (closed symbols) and in a water control (open symbols). Before the seeds were allowed to germinate they were stored for various stratification periods at 1–4 °C (4, 8 and 12 weeks). Mean numbers ( $\pm$  SE) are shown; each replicate contained 25 seeds,  $n = 4$ . The data were analysed by a distribution-free test for curve analysis (Kozioł *et al.*, 1981). Abbreviations: NS= not significant; \* =  $0.05 > P > 0.01$ ; \*\* =  $P < 0.01$ . *Nymphaea alba*, 4 weeks, NS; 8 weeks, NS; 12 weeks, \*. *Nuphar lutea*, 4 weeks, \*; 8 weeks, NS; 12 weeks, NS; *Nymphoides peltata*, 4 weeks, \*; 8 weeks, \*\*; 12 weeks, NS.

which is released into the surrounding water. Under these conditions there is no germination of *Nymphoides* seeds and ethanol release by the seeds is low and temporary.

Regardless of ethanol release, ethanol by itself (i.e. externally applied ethanol under aerobic conditions) appears to stimulate the germination of *Nymphaea* and *Nuphar* seeds. The effect of externally applied ethanol (this study) does not match germination under anaerobic conditions (see also Smits *et al.*, 1990b) and this is probably because externally added ethanol can only partly simulate the effect of endogenously produced ethanol. Both concentrations and sites of action differ for external and endogenous ethanol.

A few other studies have demonstrated that externally added ethanol alleviates seed dormancy. Taylorson (1988) showed that not only ethanol but also other compounds with an anaesthetic effect stimulate germination. The explanation given was that compounds with an anaesthetic effect increase the permeability of the plasmalemma, thereby also increasing the transport of ions. This activates a number of metabolic events which precede germination.

The observations made in this study indicate that stimulation of germination in anaerobic mud is, at least partly, mediated by ethanol. It is suggested that ethanol production by *Nymphaea* and *Nuphar* seeds under anaerobic conditions and the germination-stimulating effect of ethanol together act as a self-accelerating germination process.

The fact that the germination of *Nymphoides* seeds, which takes place exclusively in the presence of oxygen, is inhibited by ethanol emphasizes the limited adaptation of this plant species to anaerobic conditions.

#### Ethylene

It was not possible to demonstrate release of ethylene by the seeds. Either no ethylene production occurred or the concentration of ethylene in the gas space of the incubation flasks was too low for detection. We cannot exclude the possibility that ethylene can be produced by *Nymphaea*, *Nuphar* and *Nymphoides* seeds.

The germination rates of *Nymphaea* and *Nuphar* seeds in the ethylene experiment were strikingly low

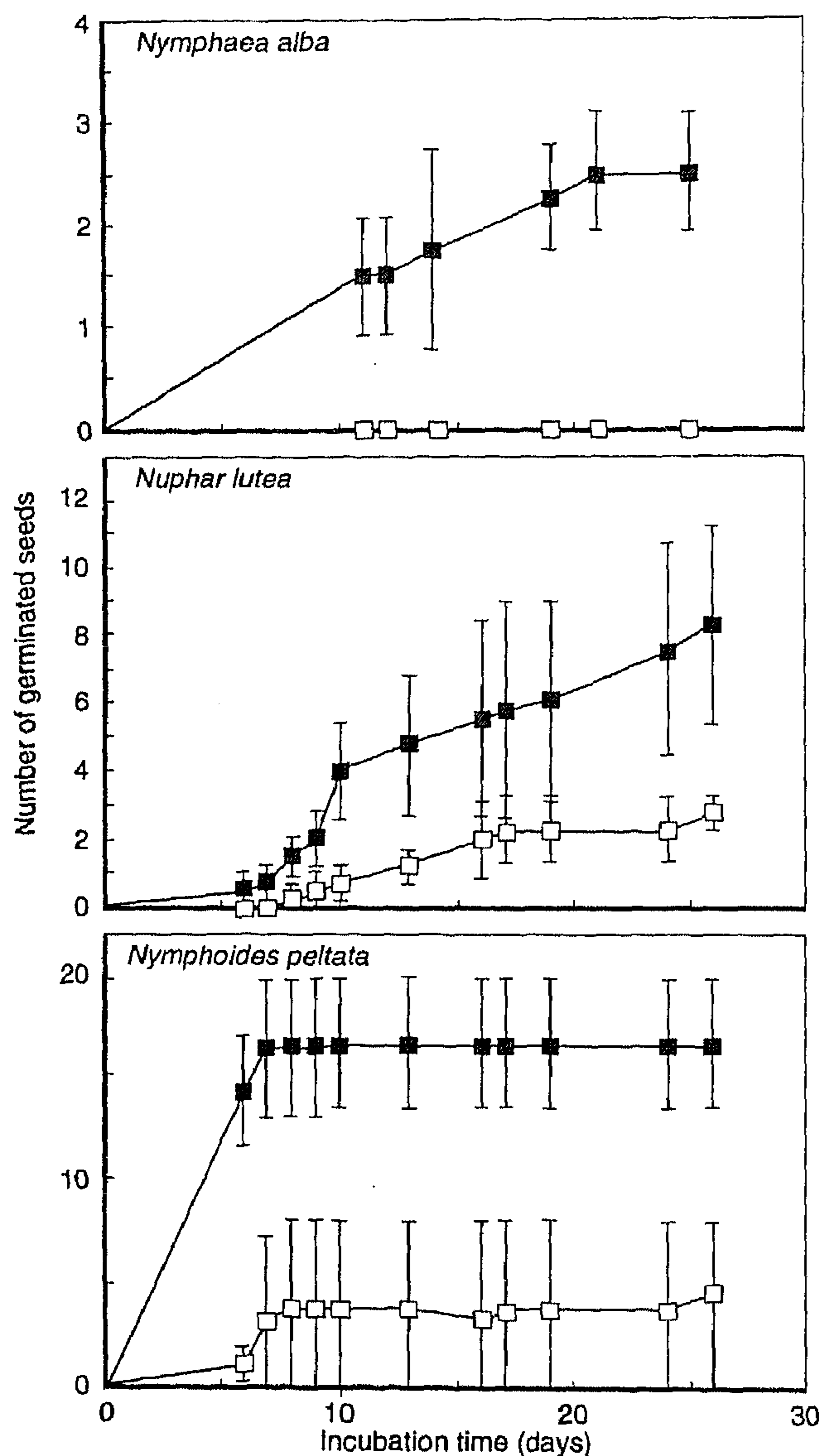


Fig. 4 Effect of  $5 \mu\text{g l}^{-1}$  ethylene (closed symbols) on the germination of *Nymphaea alba*, *Nuphar lutea* and *Nymphoides peltata* seeds. Open symbols = air (control). Mean numbers ( $\pm$  SE) are shown, each replicate contained 25 seeds;  $n = 4$ . Duration of the cold treatment of the seeds exposed to ethylene was 8 weeks. Germination in the moist ethylene-air mixture was higher for all three species than that in moist air ( $P < 0.05$  in all cases).

compared with those in the ethanol experiment. It appears that the observed germination of *Nymphaea* and *Nuphar* seeds in moist air is retarded.

As far as could be ascertained, only one study so far has dealt with the effect of ethylene on the germination of seeds of aquatic macrophytes. The germination of seeds of *Nymphaea odorata* Ait., an American white waterlily, was found to be stimulated

by 2-chloroethylphosphonic acid (ethyphon) and inhibited by aeration and  $\text{CO}_2$  (Else & Riemer, 1984). These authors also concluded that ethylene was a germination stimulating factor.

#### *Ethanol and ethylene characterize niches for germination*

In the competition process a swift colonisation of the substratum and a gain in biomass during the growth season are important. Therefore, seeds must be able to sense their environment and to 'recognize' a suitable site for germination and seedling establishment. In this process physical conditions and/or the presence of compounds that are characteristic of a certain niche, and that influence the germination of seeds, may play an important role. For example, Stockey & Hunt (1992) demonstrated that fluctuating water conditions characterize niches for germination in *Alisma plantago-aquatica* L. In this sense ethanol, as a characteristic compound of anaerobic metabolism that stimulates the germination of *Nymphaea* and *Nuphar* seeds, could be designated as a niche-specific compound for these species, indicating the presence of organic, anaerobic soils.

Analogous to this function of ethanol, ethylene could act as a niche-identifying compound for *Nymphoides*. The presence of ethylene can be expected in and just above sediments which are only periodically submersed and in which hypoxic conditions prevail, a prerequisite for ethylene production. This environment is most likely to be found in shallow water zones in combination with fluctuating water levels, the habitat where *Nymphoides* occurs frequently. The ethylene which is produced by micro-organisms in the sediment (or possibly released by the seeds themselves) stimulates the germination of *Nymphoides*. Therefore, it is interesting that Lammens & Van der Velde (1978) and Brock, Van der Velde & Van der Steeg (1987) reported massive germination of *Nymphoides* seeds on an exposed bed after a sudden drop in the water level. Of course the observed increase in germination rate may have been due to a number of factors, such as increased oxygenation, temperature and light intensity, but an elevated release of ethylene by the wet mud exposed to the air could also have contributed to this phenomenon. The germination of *Nymphaea* and *Nuphar* is also stimulated by ethylene, but exposed conditions appear to hamper germination

of these seeds, so that the overall effect is a low germination rate. In contrast to *Nymphoides* the seedlings of *Nymphaea* and *Nuphar* desiccate readily after emergence.

It was suggested above that ethanol and ethylene affect germination in such a way that they could act as niche-identifying substances. However, their action is just part of a chain of events which eventually lead to stimulation or postponement of germination. Fluctuating temperatures, carbon dioxide concentrations, sensitivity to toxic substances in the sediment, among other things, may also affect the germination process in one way or another. Our current understanding of the germination responses of *Nymphaea*, *Nuphar* and *Nymphoides* seeds (Smits, Van Avesaath & Van der Velde, 1990 and this study) can be summarized as follows. A clear distinction can be made between conditions or factors associated with organic sediments present in the deeper parts of a water body and less organic sediments present in the littoral zone of a water body. Anaerobic conditions, the presence of ethanol and darkness are associated with the deeper parts of a water body. The germination of *Nymphaea* and *Nuphar* seeds is stimulated by anaerobic conditions and ethanol. Germination of *Nymphoides* seeds does not occur under anaerobic conditions, whereas ethanol inhibits the germination of these seeds.

In the littoral zone, aerobic or hypoxic conditions prevail in the sediment, which contains less organic material and is subjected to fluctuating water levels. Under aerobic conditions, low germination rates of *Nymphaea* and *Nuphar* seeds have been recorded, while the germination rate of *Nymphoides* seeds is high. Moreover, in contrast to *Nymphoides* seeds the germination of *Nymphaea* and *Nuphar* seeds under conditions where ethylene can be produced is low.

The seeds of all three plant species are able to germinate under dark conditions. The germination of seeds of all species, in particular *Nymphoides*, is stimulated by light. From these data it can be deduced that the germination of *Nymphaea* and *Nuphar* is stimulated by factors associated with soft, organic sediments, in which dark and anaerobic conditions prevail. The germination of *Nymphoides* seeds is stimulated by factors associated with littoral sediments, in which light and oxygen are most likely to be available, and is inhibited by those which are associated with organic sediments in the deeper parts of a water body.

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