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Nitrogenase activity by *Phaeoceros-Nostoc* symbiosis as affected by the incubation time and temperature.

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Riassunto

È stata misurata l'attività di riduzione dell'acetilene (ARA) in gametofiti dell'associazione simbiontica *Phaeoceros laevis* (L.) Prosk. e *Nostoc* sp. L'ARA è stata esaminata in funzione del tempo di incubazione e della temperatura.

I gametofiti incubati per 56 ore a 24°C ed a 60 μE m^{-2} sec^{-1} hanno mostrato una produzione di etilene lineare per circa 30-35 ore; successivamente l'attività nitrogenasica è declinata lentamente, cessando a circa 48 ore. Il valore finale di etilene prodotto è stato di 178,51 nmoli mg^{-1} di peso fresco.

I gametofiti, incubati in primavera ed in inverno a temperature comprese nell'intervallo di 1° — 45°C ed alla intensità luminosa di 60 μE m^{-2} sec^{-1}, hanno mostrato la massima attività nitrogenasica a 24°C; valori minimi di attività nitrogenasica sono risultati alle temperature estreme di 1° e 45°C. Il valore massimo di etilene prodotto è stato di 10 nmoli h^{-1} mg^{-1} di peso fresco in primavera e di 9,65 nmoli h^{-1} mg^{-1} di peso fresco in inverno.

I gametofiti sono stati incubati inoltre ad 1° ed a 45°C e quindi reincubati alla temperatura ottimale (24°C) alla quale risultava la massima attività nitrogenasica. I gametofiti incubati ad 1°C hanno recuperato completamente l'attività nitrogenasica; quelli incubati a 45°C hanno mostrato bassissimi valori di attività nitrogenasica.

INTRODUCTION

Nitrogen fixing symbioses are very useful systems in the study of a wide variety of biological features. They are used in research on the interspecific relationships, on the structure, bio-

Key words: Symbiosis, *Phaeoceros laevis*, *Nostoc*, nitrogen fixation.

chemistry and physiology of plants, as well as on nitrogen fixation, a metabolic process that sometimes reaches highest rates of efficiency in symbiotic associations. It is for there reasons that outstanding research on several nitrogen fixing symbioses is currently being carried at the Department of Plant Biology of Naples, Italy. The symbioses under examination include representatives of several taxa in which both nitrogen fixing bacteria and cyanobacteria are represented (Bozzini *et al.*, 1982 and 1983; Moretti *et al.*, 1982; Barone Lumaga *et al.*, 1985; Moretti & Siniscalco Gigliano, 1988).

In order to examine new symbiotic plant taxa that can provide additional data on the nitrogen fixation process, the number of symbioses has been enlarged lately by including associations between Anthocerotales and cyanobacteria. The first report on symbionts of the Anthocerotales tallus was by Hofmeister (1862). Later, Leitgeb (1878) referred these cyanobacteria to *Nostoc* and reported their localization in mucilagineous cavities of gametophytes. The endophytism is created by the movements of cyanobacteria hormogonia from the soil to the tallus through stomata; hormogonia reach mucilagineous cavities where *Nostoc* globular colonies are formed (Schuster, 1966). The symbiosis is facultative and not specific. Pierce (1906) grew the gametophyte on a *Nostoc*-free culture, and showed that the symbyosis is not indispensable for gametophyte growth. Rodgers & Stewart (1974) isolated the symbiont from *Blasia* and *Anthoceros* and showed that the symbiont from *Blasia* was able to infect *Anthoceros* gametophytes and the symbiont from *Anthoceros* to infect *Blasia* gametophytes. Nitrogen fixing ability by symbioses in Anthocerotales was first shown by Bond & Scott (1955). Watanabe & Kiyohare (1963) were able to confirm it through the use of isotopic nitrogen.

In the present paper preliminary data from the study on nitrogenase activity by the symbiosis between *Phaeoceros laevis* (L.) Prosk. (*Anthoceros laevis* L.) and *Nostoc* sp. are shown. Acetylene reduction activity (ARA) as affected by the incubation time and temperature in two different seasons of the year was measured.

Materials and methods

Phaeoceros laevis gametophytes were collected at the Botanical Garden of Naples, Italy, where they grow abundantly on tuff layers. Their nitrogenase activity was determined by the acetylene reduction method (Hardy *et al.*, 1968). For this purpose the gametophytes were washed, sterilized in sodium hypoclorite 1.5-2%, and sealed in 10 ml leak-proof tubes. Wet paper discs were placed in the tubes to prevent dehydration. The tubes were incubated at 60 μE m^{-2} sec^{-1} and at different temperatures as required in each experiment. After each incubation, 0.5 ml gas samples were withdrawn by a gas-tight syringe and injected directly into the gas chromatograph. Gas chromatography was performed in a 1.5 m X 4 mm glass column packed with Silica gel 100-120 mesh. Isothermal 75°C, carrier gas was N_2 at 30 ml min^{-1}. Injector temperature and FID detector temperature were 100°C. Sample peak heights of ethylene were compared with prepared and analyzed standards. Calibration curves were drawn and response factors determined. Peak areas were measured by a triangulation method.

Results and discussion

In order to determine the appropriate incubation temperature and time values to adopt for ARA measurements, preliminary tests were performed on the association. Gametophytes were incubated at 5°, 20°, 25°, 35°, and 45°C, and, for each temperature value, for 30, 60, 120, and 180 min. Maximum ARA value occurred at 25°C, and within each temperature value, the ethylene production increased linearly over the whole incubation time (Fig. 1). These data suggested that 25°C and 1 h were the most suitable temperature and time values to adopt in the incubations in following experiments.

In Fig. 2, the time effect on the ARA by the association incubated for 56 h is showed. Ethylene production was almost linear in the first 30-35 h, then it slowly declined, ceasing at 48 h. The final quantity of ethylene produced was 178.5 nmoles mg^{-1} fresh weight.

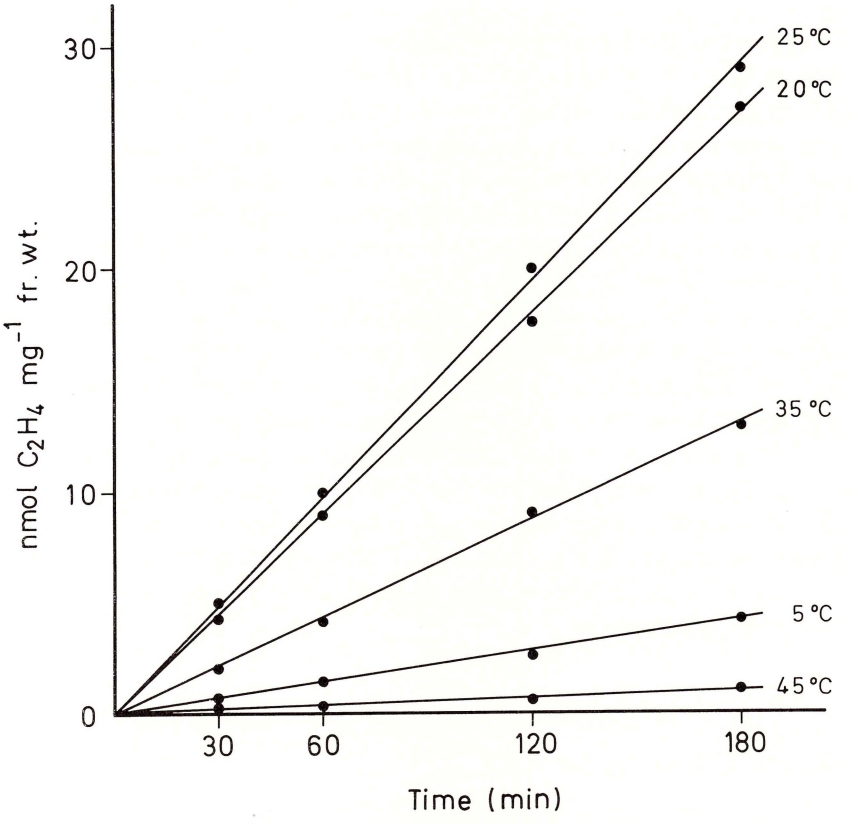

Fig. 1 - Effect of incubation time and temperature upon acetylene reduction of *Phaeoceros laevis* at 60 μE m^{-2} sec^{-1}. Each point represents the mean of triplicate determinations.

In Fig. 3, the temperature effect on the acetylene reduction measured in the Spring and in the Winter (May 1988 and January 1989, respectively), and in the 1° — 45°C range, is showed. No significant differences were seen between the two seasons, except that the maximum ethylene production was sligthly higher in the Spring than in the Winter. In both seasons, optimum temperature for ARA was about 24°C. At this temperature, the ethylene amount produced was 10 nmol h^{-1} mg^{-1} fresh weigth. There was almost no activity at 1° and 45°C.

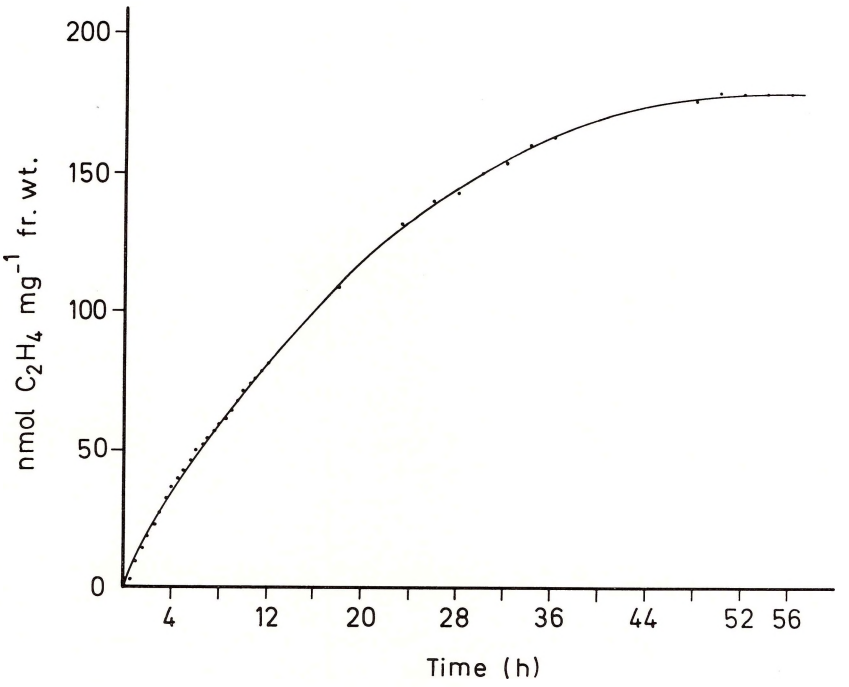

Fig. 2 - Effect of incubation time on acetylene reduction of *Phaeoceros laevis* at 25°C and 60 μE m^{-2} sec^{-1}. Each point represents the mean of triplicate determinations.

Curves from Fig. 3 shows that *P. laevis* gametophytes have a low ARA in the 1°-5°C range and an exponential response from 5° to 24°C. Above the optimal temperature, ARA quickly decreases, and ceases almost completely at 45°C.

Values for both the optimum temperature and ethylene product are in agreement with data achieved by RODGERS & STEWART (1977) for *P. laevis*. They incubated *P. laevis* under conditions similar to those here adopted, and obtained, as optimal temperature and maximum ARA value, 24°C and 13 nmol h^{-1} mg^{-1} fresh weigth, respectively. The correspondence between data would suggest that both *P. laevis* examined in the present paper and *P. laevis* examined by RODGERS & STEWART (1977) are associated with the same *Nostoc* species, although the two populations of *P. laevis* live in very separate localities (Naples, Italy and Arbroath, Scotland).

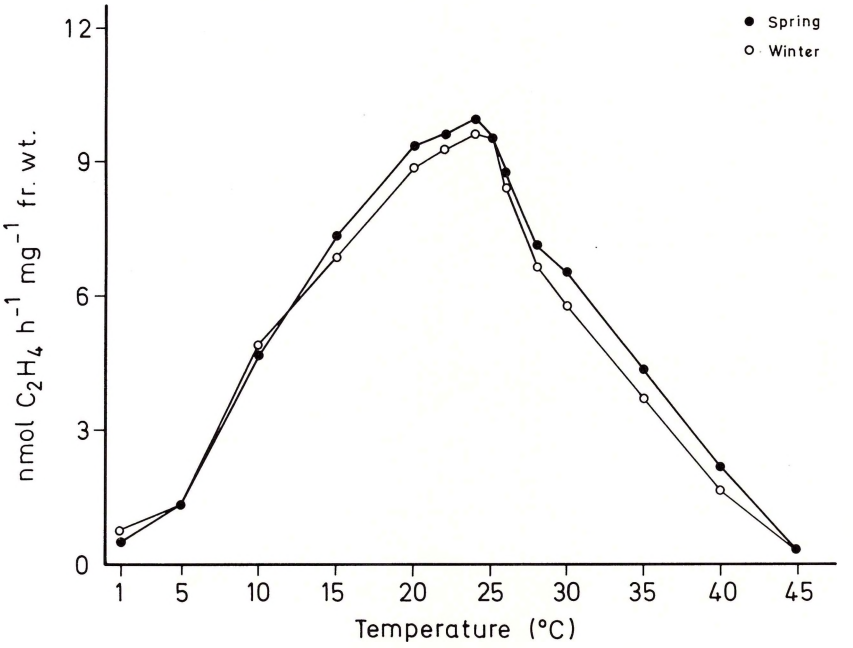

Fig. 3 - Effect of temperature on acetylene reduction of *Phaeoceros laevis* at 60 μE m^{-2} sec^{-1}. Each point represents the mean of triplicate determinations.

In order to know if the very poor ARA obtained at the extreme temperature (1° and 45°C) was due to some kind of damage that involves the nitrogenase apparatus, a few gametophytes were incubated at 1° and 45°C and then reincubated at their optimum ARA temperature (24°C). Results from this additional test are shown in Fig. 4. The gametophytes were able to recover their normal ARA at the optimal temperature after exposure at 1°C. By contrast, they were not able to do so when they were first incubated at 45°C. The reason for these two different responses by *P. laevis* is not clear, and needs further investigation. However, the results allow a conclusion of ecological interest. The *P. laevis* symbiosis prefers to live in temperate areas rather that in areas where high temperature are usually reached.

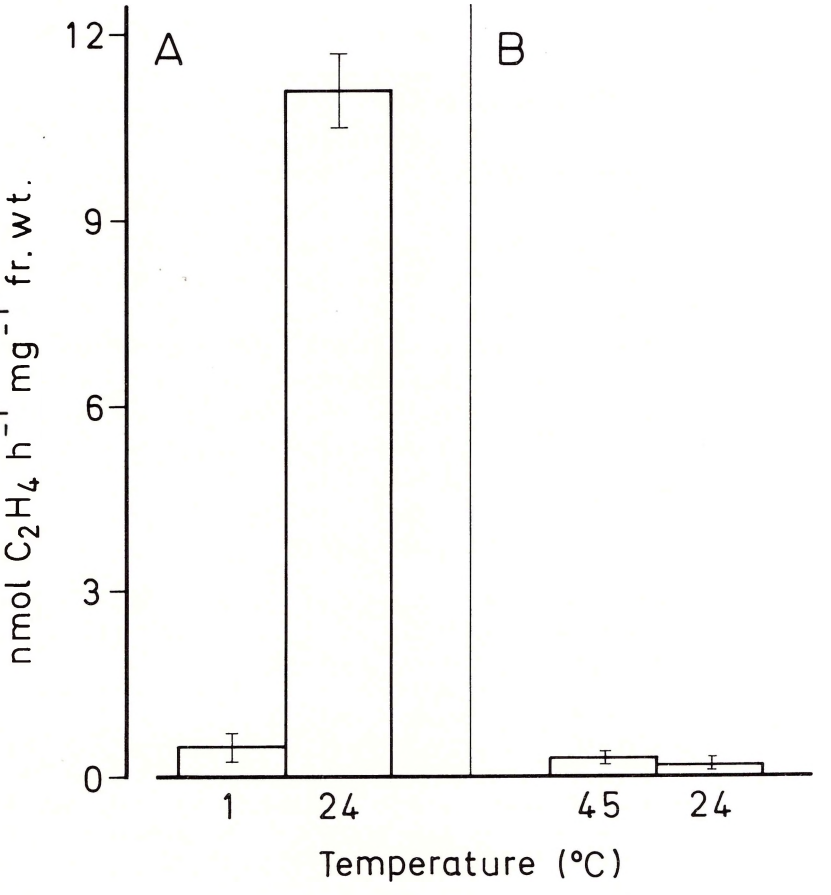

Fig. 4 - Histograms representing the remaining nitrogenase activity by *Phaeoceros laevis* incubated at its optimal temperature for ARA and at 60 μE m^{-2} sec^{-1} after low and high temperature treatments.
A: ARA values of *Phaeoceros laevis* incubated at 1°C for 1 h, and reincubated at 24°C. B: ARA values of *Phaeoceros laevis* incubated at 45°C and reincubated at 24°C.
Each point represents the mean of triplicate determinations.

ABSTRACT

The effect of incubation time and temperature on acetylene reduction activity (ARA) has been studied in gametophytes of the nitrogen fixing association *Phaeoceros laevis* (L.) Prosk. and *Nostoc* sp.

ARA by gametophytes incubated for 56 h at 24°C and 60 μE m^{-2} sec^{-1} was linear in first 30-35 h and then it slowly declined, ceasing at 48 h. The final amount of ethylene produced was 178.51 nmol mg^{-1} fresh weight. Maximum values of ARA by gametophytes incubated in the 1° — 45°C range, both in the Spring and in the Winter occurred at 24°C; minimum ARA values occurred at 1° and 45°C. At 24°C, the ethylene production was 10 nmol h^{-1} mg^{-1} fresh weight in the Spring and 9.65 nmol h^{-1} mg^{-1} fresh weigth in the Winter.

Gametophytes incubated for 1 h at 1°C and then reincubated at the optimum temperature for ARA (24°C) showed a total ARA resumption. By contrast, a very low ARA resumption occurred in gametophytes incubated at 45°C and then reincubated at 24°C.

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