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In situ acetylene reduction activity of *Scytonema julianum* in Vapor cave (Spain)

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Abstract:

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Nitrogen fixation was measured *in situ* for the first time by acetylene reduction for a greyish mat composed of *Scytonema julianum* in cave-like environments. Mat-specific rates (129.9-215.7 nmol C_2H_4 m^{-2} s^{-1} for daytime fixation and 65.1-120.6 nmol C_2H_4 m^{-2} s^{-1} for nighttime fixation) recorded in the Vapor cave differed considerably due to the energy reserves stored during photosynthesis being exhausted and used in the dark phase. The most influential environmental parameter for nitrogen fixation in the Vapor cave is temperature in the daytime and nighttime fixations. Nitrogen fixation by cyanobacteria may contribute considerably to the overall nitrogen cycle in harsh environments such as caves. Nitrogenase activity in *Scytonema julianum* was roughly 30 times higher than that of *Scytonema mirabile*, which also grew in cave environments, which is due to the characteristics of each site. The entrance of Vapour cave (Spain) faces SE, measures 0.75 x 0.6 m and opens to shafts of a total depth of 80 m. Its dimensions and environmental conditions (relative humidity up to 100%; maximum temperature, 43ºC) imply that it is isolated from external influences, and that the microclimate differs substantially from that experienced externally. Nitrogen fixation, photon flux density, relative humidity and temperature in the Vapor cave were taken hourly over a 24-hour period in winter.

Keywords: caves, cyanobacteria, nitrogen fixation, *Scytonema julianum*, SE Spain

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INTRODUCTION

Nitrogen is a nutrient that limits the growth of microorganisms in all kinds of environments, especially N-poor ones, like caves where nutrients are provided fundamentally thanks to the visits of certain animals that use caves as shelters (Hill, 1987).

Cyanobacteria are the most abundant phototrophic microorganisms to grow in caves. Most of them have structures called heterocysts in which the enzyme responsible for nitrogen fixation, nitrogenase, is found to protect the enzyme from oxygen since this gas irreversibly inhibits this enzyme (Bothe, 1982). The capacity to fix atmospheric nitrogen allows cyanobacteria to play an important role in extreme environments (Houssman et al., 2006; Zielke et al., 2005).

Scytonema julianum (Meneghini ex Franck) Richter is one of the most abundant cyanobacteria in caves (Hoffmann, 1989). It is an aerophytic, filamentous, branched, heterocysted species characterized by the presence of calcium carbonate crystals on sheaths. Most authors consider this species as typical of lit walls close to cave openings (Hoffmann, 1989), whereas others have detected it in poorly lit conditions (Aboal et al., 1994).

Scytonema julianum has been studied from morphological (Aboal et al., 1994; Couté & Bury, 1988; Friedman, 1973; Hoffmann, 1992) and biochemical (Bellezza et al., 2006; Antonopoulou et al., 2002, 2005) viewpoints, but nothing is known about its atmospheric nitrogen fixation capacity.

There are very few previous studies on *in situ* nitrogen fixation by cyanobacteria in caves (Griffiths et al., 1987; Asencio & Aboal, 2010), despite its importance owing to the fact that certain environmental factors, which cannot be reproduced in the laboratory, intervene in this process. This research work focuses on investigating the *in situ* nitrogen fixation capacity of a typical cave species, *Scytonema julianum*, in special environmental conditions (maximum temperature, 43ºC and relative humidity up to 100%) where it grows.

STUDY AREA

The Vapor cave (Fig. 1) is located in Alhama (Murcia Region, SE Spain), a municipal area crossed by the Alhama Fault. This Fault is considered one of the most active in the Iberian Peninsula, and is responsible for heating ground waters in this region.

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Fig. 1. The Vapor cave entrance in Murcia (SE Spain).

This enclave is located on the Castillo Hill at an altitude of 295 m (UTM 30SXG389912) and calcite is the dominant mineral (95%). At the bottom of this hill we find a warm water spring, used by different civilizations for centuries. The phreatic level of the region has dropped by more than 100 m over time, and currently the Alhama warm-water spring does not actually flow; but when approaching the Vapor cave a constant steam flow can be observed (Fig. 2).

Fig. 2. Panoramic view of Vapor cave with the steam flowing (arrow) from the entrance.

The entrance to this cave, facing SE, is oval-shaped and the axes measure 0.6 x 0.75 m. It opens to form a diaclase with the strata of conglomerated Triassic limestone edges. Then a small 3.5 m deep vertical shaft is found in which only plant organisms have been registered. The initial 0.5 m are covered by bryophytes giving it a greenish tone whereas the following 3 m are made up of cyanobacteria that create a greyish hue (Fig. 3).

The cave continues over approximately 50 m along a sharp downward sloping passage, which runs unlevel at -30 m in a SE-NW direction until it reaches the top of the Agobio shaft (Fig. 4). This shaft is split in two sections: one measuring 18.30 m that reaches the Chopard shelf and the other is the bottom measuring 13.70 m. The total shaft length comes to 32 m where the uneven level lies at -61.41 m. From this point,

Fig. 3. Wall of Vapor cave showing the *Scytonema julianum* mat.

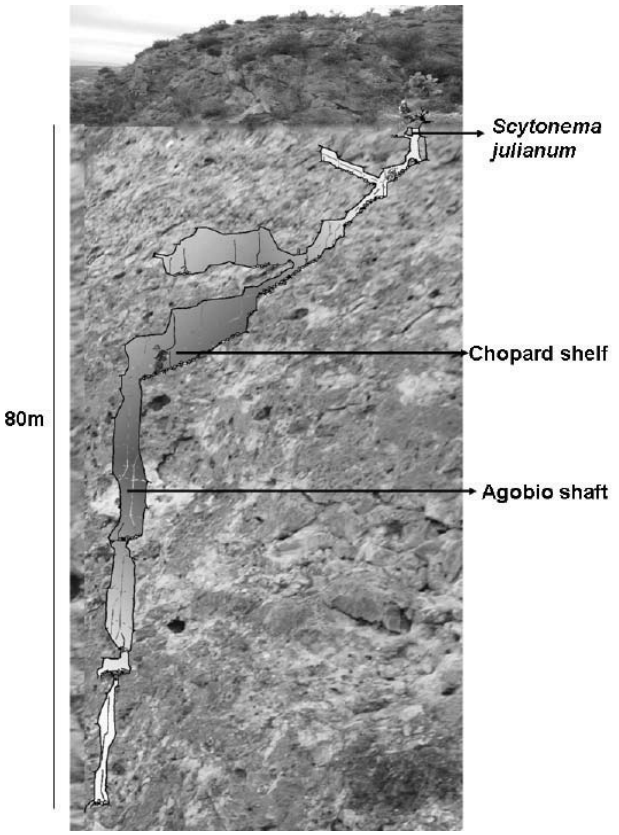

Fig. 4. Drawing showing the different passages and shafts in the Vapor cave.

two descents commence with an uneven level at approximately -20 m, which places the cavity on a final uneven level of -80 m.

The relative humidity inside the cavity is 100%. Temperatures vary and increase with depth, ranging between 27ºC and 43ºC. The carbon dioxide and oxygen concentrations inside the cave are 1.8% for CO_2 and 18.5% for O_2 as opposed to 0.03% and 20.93%, respectively, recorded in the atmosphere. Given these environmental conditions, the visitors only can access the cave for short periods of time.

MATERIALS AND METHODS

Material was collected aseptically from the wall near the Vapor cave opening where *Scytonema julianum* grows. We were careful not to remain inside too long to avoid any ill effects to our organisms because of the high temperature and high relative humidity of this cave (Ruíz de Almirón, 1998).

Non-hydrated samples were incubated *in situ* from sunrise to sunset in triplicate inside transparent and opaque sterile vials to reproduce the light and dark conditions, respectively. Then 10% of the gaseous phase of these vials was replaced with acetylene by placing syringes through the rubber vial stoppers. Gaseous samples were collected on an hourly basis over a 24-hour period in winter between sunrise and sunset. The following values were recorded hourly throughout the incubation period: photon flux density (PAR - photosynthetically active radiation), air temperature and relative humidity. A LI-1400 datalogger model (LICOR) with a LI-192 sensor and a Delta Ohm HD 8501 H thermohygrometre were used. Electrodes were placed on the rock surface. The concentrations of CO_2 and O_2 in the cave air were measured with a hand pump (Gastec Corporation, Japan) and detector tubes (measurement range: CO_2, 300-5,000 ppm; O_2, 3-24%). Nitrogen fixation was quantified by acetylene-ethylene reduction and was subsequently analyzed in a Shimadzu GC 14 A gas chromatograph.

RESULTS

Nitrogen fixation by *Scytonema julianum* (Fig. 5), a species growing in the Vapor cave forming a greyish covering, was measured for the first time.

During the daytime, nitrogenase activity ranged between 129.9 and 215.7 nmol of $C_2 H_4$ m^{-2} s^{-1} (Fig. 6). It reached its peak value after a series of high values followed by low values. Thus, the graphic representation of nitrogenase activity is a zigzag pattern. The highest values were recorded at 11:00h, 13:00h and 15:00h at 201.2, 215.7 and 213.0 nmol of $C_2 H_4$ m^{-2} s^{-1}, respectively, while the lowest values were observed at 08:00h, 10:00h, 12:00h, 14:00h and 17:00h at 125.1, 129.9, 157.8 and 130.4 nmol of $C_2 H_4$ m^{-2} s^{-1}, respectively.

The PAR values in the Vapor cave ranged between 5.2 and 57.7 $\mu E.m^{-2}.s^{-1}$ (Fig. 7). From 08:00h to 10:00h, these values increased from 7.5 to 18.9 $\mu E.m^{-2}.s^{-1}$. Then at this time, a substantial increase took place at 11:00h reaching 48.6 $\mu E.m^{-2}.s^{-1}$. Finally, these values continued rising until a peak value of 57.7 $\mu E.m^{-2}.s^{-1}$ was noted at 13:00h, to drop later.

The minimum value of relative humidity (Fig. 7) was 72% recorded at 08:00h. Relative humidity progressively increased to a maximum value of 100% at 11:00h, and remained so until 17:00h when it dropped slightly to 99.2% at 18:00h.

Temperatures in the Vapor cave ranged between 27.2ºC, recorded at 08:00h, to 31.0ºC noted at 13:00h (Fig. 7). The highest temperatures were 28.9, 31.0 and 29.5ºC recorded at 11:00h, 13:00h and 15:00h, respectively, while the lowest temperatures were 27.2, 27.5, 28.1, 29.1 and 27.4ºC noted at 08:00h, 10:00h, 12:00h, 14:00h and 18:00h, respectively.

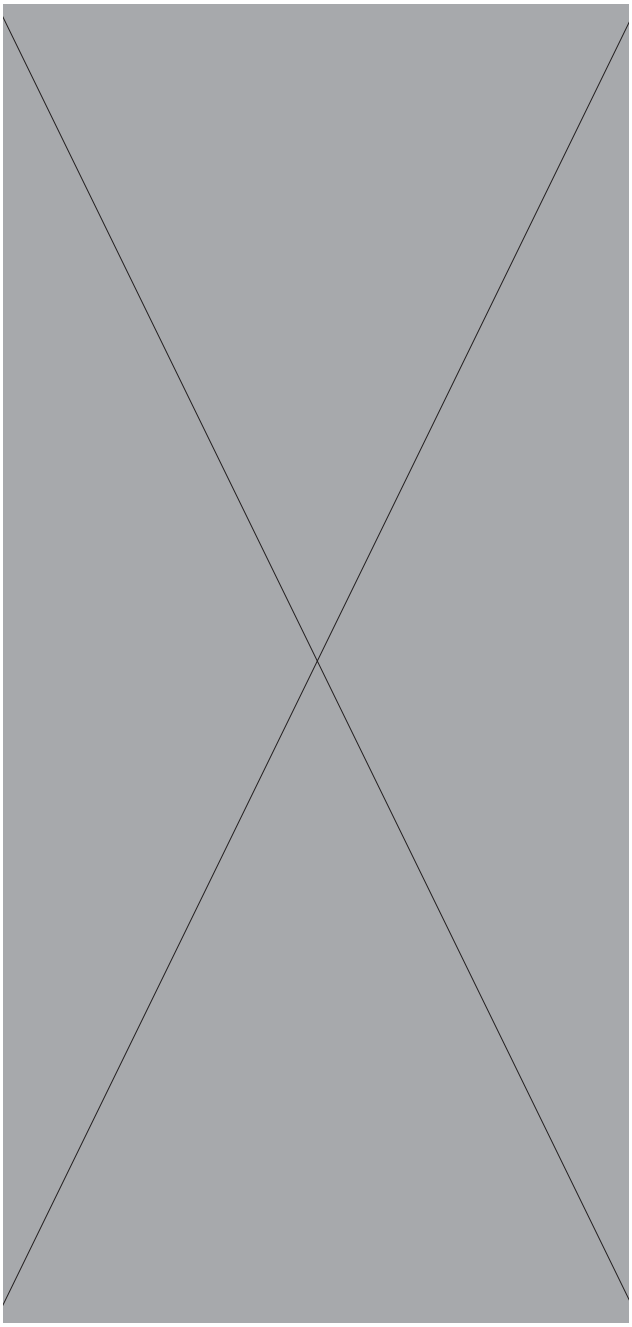

Fig. 5. Light micrographs [scale bar: 10 µm] of *Scytonema julianum*.

Nighttime nitrogen fixation varied from 65.1 to 120.6 nmol of $C_2 H_4$ m^{-2} s^{-1} (Fig. 6) and nitrogenase activity was graphically depicted as a zigzagging line. At 08:00h, a value of 79.2 nmol of $C_2 H_4$ m^{-2} s^{-1} was noted, which rose to 120.6 nmol of $C_2 H_4$ m^{-2} s^{-1} at 10:00h. After this time, it dropped to 78.3 nmol of $C_2 H_4$ m^{-2} s^{-1} at 11:00h. Nitrogenase activity started rising again to reach 94.4 nmol of $C_2 H_4$ m^{-2} s^{-1} at 12:00h and then dropped to 75.8 $C_2 H_4$ m^{-2} s^{-1} at 13:00h. Finally, at 14:00h it rose to 97.3 $C_2 H_4$ m^{-2} s^{-1} at 15:00h, from which time nitrogenase activity descended to reach a minimum value of 65.1 to 120.6 nmol of $C_2 H_4$ m^{-2} s^{-1} at 17:00h.

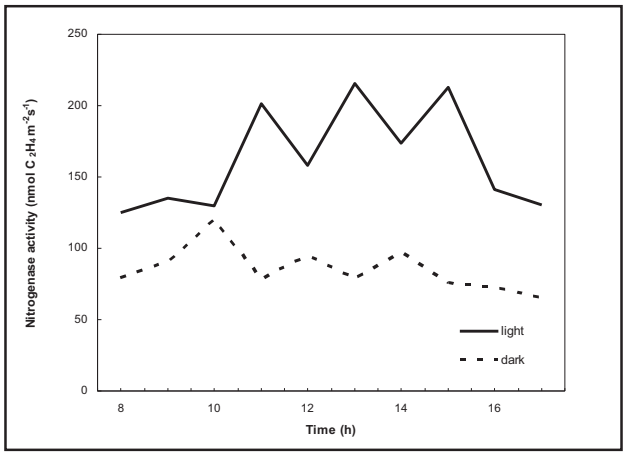

Fig. 6. Nitrogenase activity by *Scytonema julianum* (light and dark) in Vapor cave.

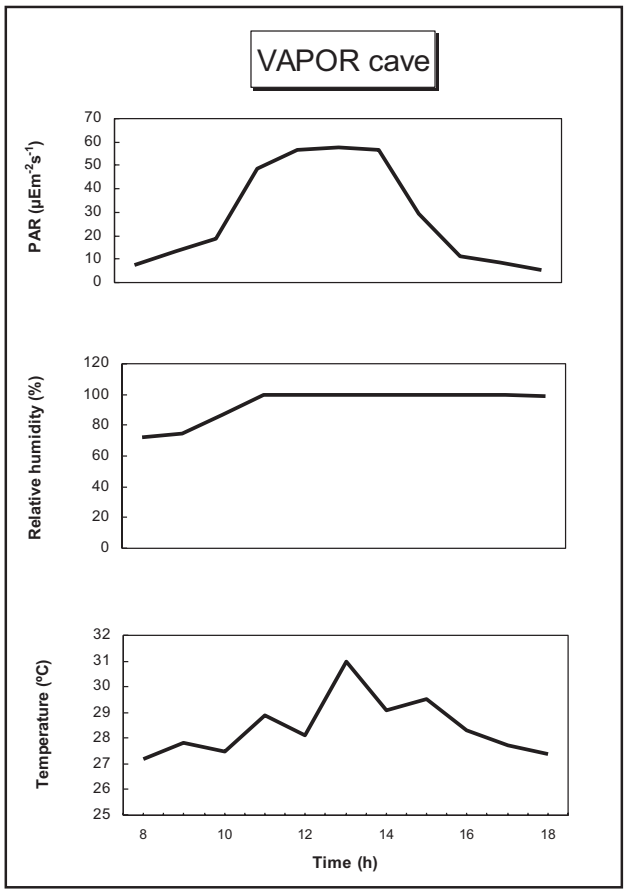

Fig. 7. PAR (Photosynthetically active radiation), temperature and relative humidity from sunrise to sunset in Vapor cave from a cyanobacterial mat of *Scytonema julianum*.

DISCUSSION

Nitrogenase activity by *Scytonema julianum* recorded in the Vapor cave over a 24-h period ranged from 129.9 to 215.7 nmol of $C_2 H_4$ m^{-2} s^{-1}. These values are very close to those obtained for the *Calothrix* genus: 284 nmol of $C_2 H_4$ m^{-2} s^{-1} in tropical environments (Jones, 1992), where similar relative humidity and temperature conditions to those in the Vapor cave were registered.

If we compare the aforementioned values with the mean 28.2 nmol value of $C_2 H_4$ m^{-2} s^{-1} recorded in *Scytonema* sp. crusts from semi-arid areas of the U.S.A. (Jeffries et al., 1992), we notice a vast difference, despite previously rehydrating samples, which confirms the importance of a high, constant relative humidity in the nitrogen fixation process, which also occurs in the Vapor cave.

We also noted that the greater the light intensity at the Vapor cave study site, the higher the nitrogenase activity. This coincides with the results of Dodds (1989), even to the point that the times the highest nitrogenase activities were recorded coincide with the maximum PAR, temperature and relative humidity values.

Ethylene nighttime production by *Scytonema julianum* was between 65.1 and 120.6 nmol of $C_2 H_4$ m^{-2} s^{-1}. These values were lower than those obtained in the daytime, which occurred with all the heterocysted cyanobacteria. Although these prokaryotes are considered totally photoautotroph organisms, some may grow in the darkness at a slower rate (Stewart, 1973; Jones, 1992).

Temperature inside the Vapor cave changed in accordance with the daytime or nighttime nitrogenase activity. When temperatures were lower, nitrogenase activity dropped, which is clearly demonstrated in the daytime when ethylene production dropped sharply as temperatures lowered, and again increased at higher temperatures. The exact opposite took place with nighttime nitrogen fixation as nitrogenase activity dropped at higher temperatures, and increased with lower temperatures.

Nitrogenase activity by *Scytonema julianum* was roughly 30 times higher than that of *Scytonema mirabile*, which also grew in a similar environment (Asencio & Aboal, 2010), due to the characteristics of each site. The Andragulla cave was 2.0 m deep, 2.0 m high and 6.0 m wide. Its lack of depth meant that the microclimate was very similar to that experienced externally, which explained its extreme PAR (0.5-582.7 $\mu E.m^{-2}.s^{-1}$), temperature (1.5-20.3 ºC) and relative humidity (24.0-79.9 %) winter values. The Vapor cave, however, was very deep so it was isolated from external influences. Therefore, its PAR values remained constant and it had very high temperature and relative humidity values.

The first ever daytime and nighttime nitrogen fixation values by *Scytonema julianum* recorded in the Vapor cave differed considerably, but coincided with *Calothrix* in tropical environments (Jones, 1992). This was likely due to the energy reserves stored during photosynthesis being exhausted and used in the dark phase, which occurred with *Nostoc* in Californian streams (Horne & Carmiggelt, 1974). This fact suggests that *Scytonema julianum*, like other tropical terrestrial cyanobacteria (Jones, 1981, Saino & Hattori, 1978), preferred high-levels of sunlight which could substantially contribute to the overall nitrogen cycle in N-poor environments, such as cave entrances and other lighted areas.

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