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Isolation and polyphasic characterization of a salt tolerant, plant growth promoting Nostoc sp. from rice paddies in the Ebro Delta

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Doctoral Program: Plant Biology and Biotechnology

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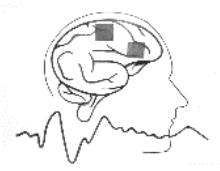
BABVE
Departament de Biologia Animal, de Biologia Vegetal i d'Ecologia

I am deeply indebted to him because he gave me this opportunity to finish my thesis.

For humanity,
He donated,
No price.



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Many thanks to my family and friends who always gave me motivation from thousands of kilometres away. Last but not least, I thank you that are reading my thesis. You have to be included in this list because I wrote this long manuscript for you. I hope you enjoy it and find answers and doubts along with it.

And remember "the works of the imagination always have a point of contact with the reality".

Nadia Bahavar

Abstract

Salinity and alkalinity stress are the prevalent environmental fluctuations in the Mediterranean paddy fields in the Ebro Delta; however, they have not been studied as yet in relation to terrestrial cyanobacteria. Among the diverse cyanobacteria, several species have been explored as plant growth-promoting biofertilizers to improve nutrient supply and tolerance to biotic and abiotic stress. In this thesis, we isolated different cyanobacteria from paddy fields to characterize their tolerance to saline-alkaline conditions and to make an initial evaluation of a selected strain as a biofertilizer for rice grown under saline conditions.

In chapter I, we described characterisation of cyanobacteria isolated from paddy fields at the Institute of Agrifood Research and Technology and Marquesa (Delta Ebre, Spain). Thirty axenic genera of homocytous and hetrocytous cyanobacteria were isolated. To our best knowledge, *Nostoc paludosum*, *Nostoc linckia*, *Nostoc muscorum*, and *Cylindrospermum tropicum* were recorded for the first time in this region.

Chapter II addresses polyphasic characterization of the diversity of the traits of the genus *Nostoc* under the influence of different environmental factors. We applied a combination of modern and classical taxonomy along with environmental fluctuation such as salinity and alkalinity and used advanced tools which may shed light into the complex diversity of this genus. We provide a polyphasic description of a *Nostoc sp*. (strain UAB 206) using morphological analysis (bright field, fluorescence, phase contrast, confocal and ultrastructural TEM microscopy), phylogenetic analysis (16s rRNA and the phycocyanin genome region) and ecophysiological studies (growth, oxygen evolution analysis, efficiency of photosystems I and II and phycobilisomes). As far as we know this is the first polyphasic description of cyanobacterium in the paddy fields of the Ebro Delta region and the first application of this methodology for description of cyanobacteria using different kind of characterization as diagnostic features.

We evaluate the combined effect of salinity (17-320 mM NaCl), alkalinity (pHs7-11) and age of the cultures (from the first to fourth day after

inoculation, daily) on the physiological acclimation of an unexplored terrestrial cyanobacterium, *Nostoc* sp. UAB 206 in chapter III. We look at growth, long and short-term photosynthesis, photosystem II and I activities, phycobilisome operations and exopolysaccharide production. The maximum growth rate and photosynthesis activity observed under combination of 80 mM salinity and pH 11, compared to pH 9 and 7. Increasing salinity did not have any considerable influence on growth rate until 160 mM at pH 9. The analysis of photosynthesis-irradiance curves showed that the maximal light-saturated photosynthesis activity (Pmax) was attained at 17 mM salinity and pH 11. The spectroscopy analysis (including Confocal) showed that increasing salinity (80mM) caused higher efficiency of both photosystem II and I under alkaline conditions. The ultrastructural analysis (Transmission Electron Micrograph) showed that the exopolysaccharide dimensions increased at the combination 80 mM salinity and pH 11.

In Chapter IV we first focused on the selection of suitable rice varieties for salinity stress and tolerance studies based on agronomic characteristics. With the selected sensitive cultivar, we then characterized the rice-cyanobacterium interaction under different Nitrogen (50& 100 %) and different saline conditions (17, 50 or 100 mM NaCl) in a salt sensitive variet. In this interaction, we observed a positive and beneficial influence of the cyanobacterium on the photosynthesis of the salt-sensitive rice cultivar Copsemar. This positive effect was more pronounced under low N supply.

Resumen

El estrés por salinidad y alcalinidad se debe a las fluctuaciones ambientales prevalentes en los arrozales mediterráneos en el Delta del Ebro; sin embargo, aún no se han estudiado en relación con las cianobacterias terrestres. Entre las diversas cianobacterias se han explorado varias especies como biofertilizantes promotores del crecimiento de las plantas para mejorar el suministro de nutrientes y la tolerancia al estrés biótico y abiótico. En esta tesis se aislaron diferentes cianobacterias de arrozales para caracterizar su tolerancia a condiciones salino-alcalinas y realizar una evaluación inicial de una cepa seleccionada como biofertilizante para arroz cultivado en condiciones salinas.

En el capítulo I, describimos la caracterización de cianobacterias aisladas de arrozales en el Instituto de Investigación y Tecnología Agroalimentaria y Marquesa (Delta del Ebro, España). Se aislaron treinta géneros axénicos de cianobacterias homocísticas y hetrocíticas. Hasta donde sabemos, *Nostoc paludosum*, Nostoc *linckia*, *Nostoc muscorum* y Cylindrospermum *tropicum* se registraron por primera vez en esta región.

El capítulo II aborda la caracterización polifásica de la diversidad de los rasgos del género *Nostoc* bajo la influencia de diferentes factores ambientales. Aplicamos una combinación de taxonomía moderna y clásica junto con la fluctuación ambiental como la salinidad y la alcalinidad y utilizamos herramientas avanzadas que pueden arrojar luz sobre la compleja diversidad de este género. Proporcionamos una descripción polifásica de un Nostoc sp. (cepa UAB 206) utilizando análisis morfológico (campo claro, fluorescencia, contraste de fases, confocal y microscopía TEM ultraestructural), análisis filogenético (ARNr 16s y la región del genoma de la ficocianina) y estudios ecofisiológicos (crecimiento, análisis de evolución del oxígeno, eficiencia de los fotosistemas I y II y ficobilisomas). Hasta donde sabemos, esta es la primera descripción polifásica de cianobacterias en los arrozales de la región del Delta del Ebro y la primera aplicación de esta metodología para la descripción de

cianobacterias utilizando diferentes tipos de caracterización como características diagnósticas.

En el capítulo III se evalúa el efecto combinado de la salinidad (17 a 320 mM NaCl), la alcalinidad (pHs 7 a 11) y la edad de los cultivos sobre la aclimatación fisiológica de una cianobacteria terrestre inexplorada, *Nostoc* sp. UAB 206. Analizamos el crecimiento, la fotosíntesis a largo y corto plazo, las actividades del fotosistema II e I, las operaciones de ficobilisoma y la producción de exopolisacáridos. La tasa máxima de crecimiento y la actividad fotosintética fue observada bajo la combinación de 80 mM de salinidad y pH 11, en comparación con pH 9 y 7. El aumento de la salinidad no tuvo ninguna influencia considerable en la tasa de crecimiento hasta 160 mM a pH 9. El análisis de las curvas de fotosíntesis-irradiancia mostró que la actividad máxima de fotosíntesis saturada de luz (Pmax) se alcanzó a 17 mM de salinidad y pH 11. El análisis espectroscópico (incluyendo microscopía confocal) mostró que el aumento de la salinidad (80mM) causó una mayor eficiencia tanto del fotosistema II como del I en condiciones alcalinas. El análisis ultraestructural (TEM) mostró que las dimensiones del exopolisacárido aumentaron en la combinación de 80 mM de salinidad y pH 11.

En el capítulo IV nos centramos en primer lugar en la selección de variedades de arroz adecuadas para estudios de estrés por salinidad y tolerancia basados en características agronómicas. Caracterizamos la interacción arroz-cianobacteria bajo diferentes suministros de nitrógeno (50 y 100 %) y diferentes condiciones salinas (17, 50 o 100 mM NaCl) en una variedad sensible a la sal (Copsemar). En esta interacción, se observó una influencia positiva y beneficiosa de la cianobacteria sobre la fotosíntesis. Este efecto positivo fue más pronunciado a un bajo suministro de N.

Resum

L'estrès de salinitat i alcalinitat es deu a prevalença de les fluctuacions ambientals en els arrossars mediterranis al Delta de l'Ebre; Tanmateix, encara no s'han estudiat en relació amb els cianobacteris terrestres. Entre els diversos cianobacteris, diverses espècies han estat explorades com biofertilitzadors que promouen el creixement de les plantes per millorar el subministrament de nutrients i la tolerància a l'estrès biòtic i abiòtic. En aquesta tesi, diferents cianobacteris van ser aïllats dels arrossars per caracteritzar la seva tolerància a les condicions salines alcalines i per dur a terme una avaluació inicial d'una soca seleccionada com a biofertilitzador per a l'arròs cultivat en condicions salines.

En el capítol I, descrivim la caracterització de cianobacteris aïllats de arrossars a l'Institut de Recerca i Tecnologia Agroalimentàries i Marquesa (Delta de l'Ebre, Espanya). Trenta gèneres de destral estaven aïllats de cianobacteris homocistics i hetrocístics. Segons el nostre coneixement, *Nostoc paludosum*, *Nostoc linckia*, *Nostoc muscorum* i *Cylindrospermum tropicum* es van registrar per primera vegada en aquesta regió.

El capítol II tracta sobre la caracterització polifàsica de la diversitat dels trets del gènere *Nostoc* sota la influència de diferents factors ambientals. Apliquem una combinació de taxonomia moderna i clàssica juntament amb fluctuacions ambientals com la salinitat i l'alcalinitat i utilitzem eines avançades que poden donar llum sobre la complexa diversitat d'aquest gènere. Proporcionem una descripció polifàsica d'un *Nostoc* sp. (Soca UAB 206) mitjançant anàlisi morfològica (camp clar, fluorescència, contrast de fase, microscòpia TEM confocal i ultraestructural), anàlisi filogenètica (16s rRNA i regió del genoma de la ficocianina) i estudis ecofisiològics (creixement, anàlisi de l'evolució de l'oxigen, eficiència dels fotosistemes I i II i ficobilisomes). Al nostre entendre, aquesta és la primera descripció polifàsica dels cianobacteris en els arrossars del Delta de l'Ebre i la primera aplicació d'aquesta metodologia per a la descripció dels cianobacteris utilitzant diferents tipus de caracterització com a característiques diagnòstiques.

El capítol III avalua l'efecte combinat de la salinitat (17 a 320 mM NaCl), l'alcalinitat (pHs 7 a 11) i l'edat dels cultius sobre l'aclimatació fisiològica d'un cianobacteri terrestre inexplorat, *Nostoc* sp. UAB 206. Analitzem el creixement, la fotosíntesi a llarg i curt termini, les activitats del fotosistema II i I, les operacions de ficobilisomes i la producció d'exopolisacàrids. La taxa màxima de creixement i l'activitat fotosintètica fou observada sota la combinació de salinitat de 80 mM i pH 11, en comparació amb el pH 9 i 7. L'augment de la salinitat no va tenir una influència considerable en la taxa de creixement fins a 160 mM a pH 9. L'anàlisi de les corbes de fotosíntesi-irradiància va mostrar que l'activitat màxima de fotosíntesi saturada de llum (Pmax) es va assolir a 17 mM salinitat i pH 11. L'anàlisi espectroscòpica (inclosa la microscòpia confocal) va mostrar que l'augment de la salinitat (80mM) va causar una major eficiència tant del fotosistema II com de l'I en condicions alcalines. L'anàlisi ultraestructural (TEM) va mostrar que les dimensions dels exopolisacàrids van augmentar en la combinació de salinitat de 80 mM i pH 11.

En el capítol IV ens centrem en primer lloc en la selecció de varietats d'arròs adequades per a estudis d'estrès i tolerància a la salinitat basats en característiques agronòmiques. Caracteritzem la interacció arròs-cianobacteri sota diferents subministraments de nitrogen (50 i 100%) i diferents condicions salines (17, 50 o 100 mM NaCl) en una varietat sensible a la sal (Copsemar). En aquesta interacció, es va observar una influència positiva i beneficiosa dels cianobacteris en la fotosíntesi. Aquest efecte positiu va ser més pronunciat en una baixa oferta de N.

List of abbreviations

A	Akinete
α	Photosynthetic efficiency
ANOVA	Analysis of variance
BLAST	Basic Local Alignment Search Tool
BTP	Bis-Tris Propane
CEPS	Cyano-exopolysaccharides
Chla	Chlorophyll a
CLSM	Confocal Laser Scanning Microscopy
Cyano	Cyanobacterium
DNA	Deoxyribonucleic acid
3D	Three-Dimensional space
EPS	Exopolysaccharides
EM	Emission
EX	Excitation
Fv/Fm	Quantum yield of photosystem II
G	Doubling Times
H	Heterocyte
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Ik	Light saturation point
ML	Maximum-Likelihood
MP	Maximum Parsimony
NaCl	Sodium chloride
NaOCL	Sodium hypochlorite
NJ	Neighbour-Joining
NCBI	National Center for Biotechnology Information
OD	Optical Density
PC	Phycocyanin
PCR	Polymerase chain reaction
PBS	Phycobilisome
P-I	Photosynthesis-irradiance
Pmax	The maximum photosynthetic rate
PSI, PSII	Photosystems I and II

PCA	Principal component analysis
SPR	Subtree-Pruning-Regrafting
TEM	Transmission Electron Microscopy
TSA plates	Trypticase Soy Agar or Tryptone Soya Agar
VG	Vegetative Cell
λscan	Lambda scan

Aims of the thesis

Cyanobacteria are a self-sufficient system that are capable of fixing atmospheric nitrogen and providing nutrients to plants. Moreover, diazotrophs contribute to the regulation of the dynamics of soil organic matter enhancing soil biological activities. As cyanobacteria can adapt to different adverse environmental condition, we hypothesize that in the saline-alkaline environment of paddy fields in the Ebro Delta cyanobacteria with tolerance to such stress conditions can be found. The ultimate aim of our study is to select the native cyanobacteria with high biomass production, that can survive and grow under saline-alkaline conditions and has plant growth promoting characteristics in order to be used as a biofertilizer in the paddy field. Based on this aim the specific objectives of this study were:

- Collection of the soil samples to extract, isolation and purification of the native cyanobacteria (heterocystous cyanobacteria).
- Polyphasic approach (morphological, ultrastructural, physiological, molecular, and ecological traits) to characterize and identify cyanobacterium isolated from the paddy fields of Ebro Delta, Spain.
- Selection of suitable and prevailing strain to emphasize on strong mechanism to biomass production, survive and grow under combination effect of salinity and alkalinity
- Selection of a rice cultivar based on both the economic importance of certain cultivars already used in the zone and the potential intrinsic tolerance to salinity.
- Use of a salt sensitive cultivar to explore the influence of a selected salt and alkaline tolerant cyanobacteria isolated from paddy field in the Ebro Delta on salt tolerance/sensitivity of a salt sensitive rice variety grown in this area
- Assessment of the possible influence of the level of inorganic Nitrogen supply on the rice-bacterial interaction

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Chapter 1: Isolation of cyanobacteria from paddy fields' soil and water

1.1 Introduction

Cyanobacteria (Cyanophytes / Cyanoprokaryotes / blue-green algae) are a gram-negative, diverse, fascinating, versatile, and ancient group of prokaryotic microorganisms. They are present in a wide range of habitats: marine, freshwater, soil, etc. Some of them have specialized cells; heterocyte can fix atmospheric nitrogen and thus enrich soils, akinetes enhance survival in stressed conditions and hormogonia help to dispersion. Nonetheless, nitrogen-fixing ability in cyanobacteria is not confined to the heterocystous cyanobacteria but also many other prokaryotes, have nitrogenase and can fix nitrogen as well (Bergman et al., 1997).

The association and the importance of cyanobacteria with paddy fields has been known since 1939 (Singh et al., 2014). In 1939, De established the agronomic potential of cyanobacteria, the diversity, distribution, and significant contribution of cyanobacteria with soil fertility (Mishra et al., 2005; Kishore Choudhary & Bimal, 2010). Cyanobacteria can be considered as "miniature factories" of the biological world and present an alternate source of a variety of bioactive compounds, including lipids / fatty acids, proteins, enzymes, hormones, pigments, and compounds of pharmaceutical, agricultural, and nutraceutical value (Ördög et al., 2004). Additionally, this diverse group of bacteria not only plays a crucial role in primary production in terrestrial and aquatic ecosystems but also participates in the utilization of global atmospheric carbon dioxide.

The agroclimatic conditions of the paddy field provide a favourable environment for the colonization of many cyanobacteria, and cyanobacteria have been considered as a major component of the fertility of rice field agroecosystems (Roger & Kulasooriya, 1980). Cyanobacteria play a fundamental role in the soil biological cycle by providing a large amount of nitrogen and phosphorus which are the essential nutrients for rice cultivation (Munagamage et al., 2020; Qu et al., 2015). Furthermore, cyanobacteria excrete several organic acids and release oxygen which

increase and maintain soil fertility, nutrient availability, and water holding capacity. Due to these beneficial properties, cyanobacteria can act as bio-fertilizers (Chittora et al., 2020; Saadatnia et al., 2009; Choudhary et al., 2007).

Compared to aquatic cyanobacteria, extracts of terrestrial cyanobacteria have higher biological activity based on their special growing conditions, survival, and adaptation mechanisms (Drobac-Cik, 2007). As terrestrial cyanobacteria are largely unexplored, they represent a rich opportunity of study. Hence, the present investigation deal with the isolation and identification of various heterocystous cyanobacteria from rice field soils.

1.2 Sampling area

Delta de l'Ebre (330 km^2, Fig. 1), one of the largest coastal wetlands in the Mediterranean Sea, is located in Tarragona province in southern Catalonia. Almost all the flat swampy area is used as agricultural land, especially for rice cultivation occupying about 20,500 hectares (Casanova et al., 2002; Genua-Olmedo et al., 2016; Prado et al., 2017). Despite the proximity to the river, human activities, sea-level fluctuations, flooding irrigation and environmental changes lead to soil salinity reaching levels ranging from around 5.5 dS m^{-1} to 23 dS m^{-1} (Benito et al., 2015; Genua-Olmedo et al., 2016), while alkalinity is reaching values up to pH 10 (Fores et al., 1986). Currently, rice cultivation represents an important economic activity and rice grain is an essential part of the Mediterranean diet. Spain after Italy is the second-largest producer of rice in Europe with an average annual yield of 154,000. Nevertheless, rice yield can be decreased when soil salinity and alkalinity increase too much. Furthermore, the excessive use of chemical fertilizers has generated several environmental problems including the greenhouse effect, ozone layer depletion, and acidification of water (Meena et al., 2017). These problems could be tackled using biofertilizers in paddy fields (Roger and Kulasooriya, 1980). Cyanobacteria play an important role in maintenance of nutrient availability and build-up of soil fertility, porosity, soil pH, water holding capacity and decrease

of salinity in soil, consequently increasing rice growth and yield can be achieved using appropriate cyanobacteria as a natural biofertilizer (Chittora et al., 2020). Most paddy field soils have a natural population of cyanobacteria which provides a potential source of nitrogen fixation at no cost (Saadatnia & Riahi, 2009). To this aim, using native cyanobacteria as biofertilizers and evaluating the viability of heterocystous cyanobacteria especially Nostocales in response to environmental fluctuations is essential (Boussiba 1987).

So far little attempt has been made to investigate the presence of terrestrial cyanobacteria in paddy fields of the Ebro delta. Most researchers have focused on cyanobacteria in microbial mats and their extension (Epping and Kühl, 2000; Esteve et al., 1992; Martinez-Alonso et al., 2004 a,b; Mir et al., 1991; Pérez and Carrillo, 2005; Solé et al., 2003; Urmeneta et al., 2003; Wierzchos et al., 2006). We collected soil samples from paddy fields of the Institute of Agrifood Research and Technology (IRTA, 40º 42' 29.8" N 000º37' 58.9" E) and Marquesa (40º 45'39.3" N 000º 47' 41.5" E) (Fig. 1).

1.3 Sampling technique

Cyanobacteria were collected with sterile instruments and placed into sterile containers. Materials used for samplings were GPS device, camera, scalpel, magnifying glass, a long narrow-bladed shovel or trowel (to collect soil samples), containers (small bin for mixing soil samples), sieve (remove big objects -rock, roots etc.), plastic bags, cup, gloves, label, formalin 4% (for aquatic samples), icebox, dropper, pipette, photometer, pH meter, and thermometer. The physical parameters such as temperature, salinity, and pH were recorded for each sampling site using conductivity (JENWAY 4071). Plastic bags and wide-mouthed sterile tubes (100-200 ml capacity) were used for dry and muggy soil samples, respectively; recipients were labelled with name, date, lab number, and soil extract. The chosen strategy for collecting and exploring the region was random. We divided the farm into lots and collected randomized 25 surface soil samples after removing the upper 5-7 cm of the soil crust in each region and poured

them into separate containers. We repeated this procedure at 10 different selected spots. After collecting soil samples, we mixed them in a container and removed foreign materials like roots, stones, pebbles, and gravels. Then, we poured the soil sample into a plastic bag and filled up the soil sampling information sheet and attached it to the plastic bag. To isolate cyanobacteria from muggy land we took larger samples: 100-200 ml sterile wide-mouthed sterile tubes, filled almost completely. Samples collected were kept in darkness at 4 °C temperature until transferred to the laboratory.

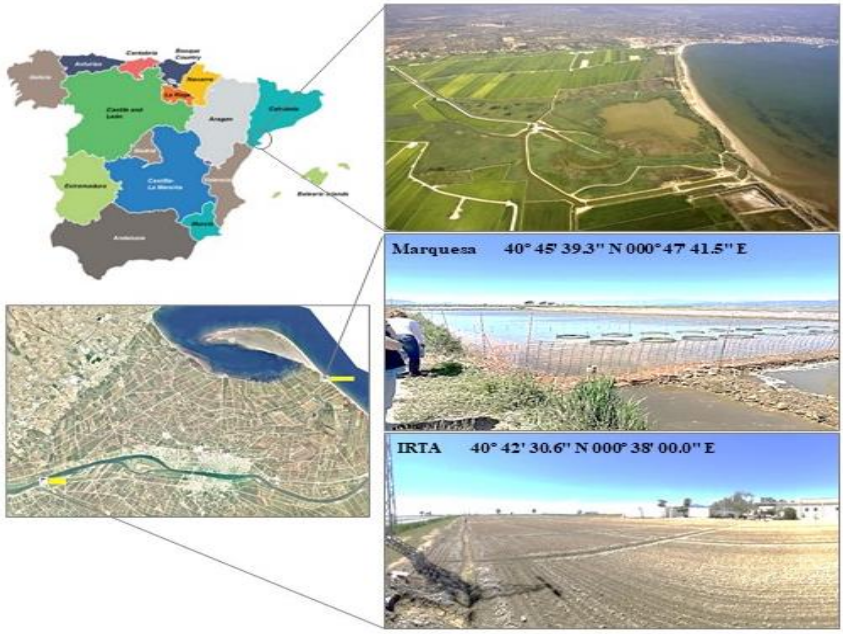

Fig. 1. Map showing the study locations of IRTA and Marquesa.

1.4 Storage of samples

The isolation and purification of cyanobacteria should be done as soon as possible in the laboratory. The transfer and long cultivation of cyanobacteria in laboratories can lead to changes, often resulting in modified morphology, physiological properties, and even changed genotypes and subsequent loss of features that were a result of acclimatization to biotope conditions (Komárek and Kaštovský, 2003).

To avoid alterations, the samples were processed immediately after return to the lab. After removal of any obvious strange materials, wet soils were dried in a drying oven at low temperature (40°C). Then the ground soil was passed through a sieve (2mm) to remove any large particles especify the mesh. For isolation of cyanobacteria, soil samples (1g) were inoculated into sterilized TSA plates (Trypticase Soy Agar or Tryptone Soya Agar) and mixed with liquid and solid BG-11 medium with or without combined nitrogen and the pH raised to 7.8 by the addition of KOH (Table. 1). TSA plates were placed in an incubator at 28±2°C with constant illumination of 100 µmol photon m^{-2} s^{-1} for two weeks (Stanier et al., 1971) (Fig. 2). Colonization of cyanobacteria appeared after 10-15 days. All strains were cultivated on 1% agar plates as well. We repeated streaking on agar plates till single colonies formed. Axenic populations were obtained by serial dilution (we isolated them by picking single cells and single filament from the original sample). At least 30 replicates were conducted for each strain (Stanier et al., 1971). Finally, we investigated both axenic cultures without supplementary aeration or stirring (standing condition) and aerated cultures (bubbled with air) (Soltani et al., 2010). Axenic cultures were examined microscopically, and the absence of bacteria was confirmed.

1.5 Identification and morphological characterization

The cyanobacterial colonies were identified by light transmission, phase contrast, and fluorescence microscopy. Morphological characterization was carried out based on a combination of their morphological features (aggregations, crustose form communities, shape and colour of the cells, the shape of the end cell, the presence/absence of calyptra, hormogonia, the form of the mucilage, and in which part of plate the colony will appear (margin, bottom, etc) and analysing cell dimensions (length and width of the vegetative cells, heterocyte, and akinetes) of each strain (Table 2). Regular visual examination was carried out in both mediums of BG 011 and BG 11 (liquid and solid) for heterocystous and non- heterocystous cyanobacteria respectively. Morphological determinations were carried out according to Desikachary, 1959, Prescott, 1962, Komárek and

Anagnostidis, 1989 and John et al., 2002. Semi-permanent slides were mounted with glycerine and used for identification.

Table 1. Composition of BG-11 and BG0-11 medium

BG0-11 medium				**BG-11 medium**	
$NaSo_4$	1.5 g			$NaNO_3$	1.5 g
K_2HPO_4	0.04 g			K_2HPO_4	0.04 g
$MgSO_4{\cdot}7H_2O$	0.075 g	**Trace metal mix A5**		$MgSO_4{\cdot}7H_2O$	0.075 g
$CaCl_2{\cdot}2H_2O$	0.036 g	H_3BO_3	2.86 g	$CaCl_2{\cdot}2H_2O$	0.036 g
Citric acid	0.006 g	$MnCl_2.4H_2O$	1.81 g	Citric acid	0.006 g
				Ferric ammonium	
$FeCl_3$	0.006 g	$ZnSO_4{\cdot}7H_2O$	0.222 g	citrate	0.006 g
EDTA (disodium				EDTA (disodium	
Salt)	0.001 g	$NaMoO_4{\cdot}2H_2O$	0.39 g	Salt)	0.001 g
$NaCO_3$	0.02 g	$CuSO_4.5H_2O$	0.079 g	$NaCO_3$	0.02 g
Trace metal				Trace metal	
mix A5	1.0 mL	$CoCl_2{\cdot}6H_2O$	49.4 mg	mix A5	1.0 mL
Agar (if				Agar (if	
needed)	10.0 g	Distilled water	1.0 L	needed)	10.0 g
Distilled				Distilled	
water	1.0 L			water	1.0 L

Table 2. Colony characteristics and morphology of cyanobacteria based on microscopic and macroscopic observations

Aggregation Color (Thallus)	**Texture**	**Colony**	**Filament**	**Trichome**	**Cells**	**Edge**
Green	Gelatinous	Clumpy	Long	Broad	Cylindrical	-Tend to
Greenish	Mucilaginous	Globular	Straight	With/Without	Ovoid	growth on
Brown	Expand	A Mat	Solitary	Sheath	Short	the
Yellow	Lumpy	Round	Compact	Flexible	Barrel	margin
Grey	Formless	Sticky	Curved	Smooth	Spherical	-Attached
Pale	Regular	Lumpy	Coiled	Confluently	Granulated	to bottom
Blue	Lamellate	Clathrate	Twisted		Oblong	- Air
Dark	Slimy	Entangled			Ellipsoid	bubbles
Light						

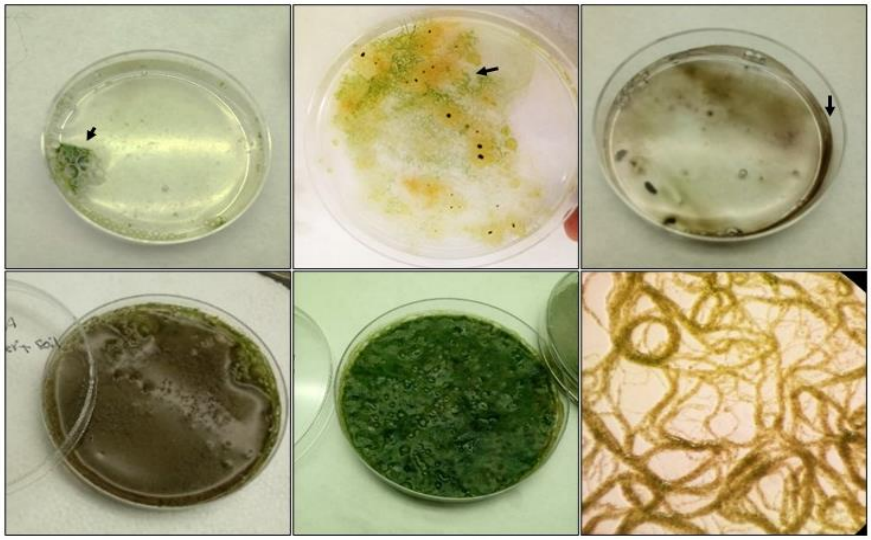

Fig. 2. Colony characteristics and morphology of unexploded cyanobacteria based on macroscopic observations

1.6 Microscopy

Isolated axenic cultures were observed with different microscopic techniques for preliminary identification of the species.

1.6.1 Bright field microscopy

Since the early 1950s, the diversity of cyanobacteria has traditionally been studied by Bright field microscopy which usually allows identification at the species level. Bright-field microscopy is one of the simplest which illumination lights is transmitted through the sample and the contrast is generated by the absorption of light in dense areas of the specimen (Wang and Fang, 2012). Bright field microscopy has different limitations such as low contrast and low resolution due to the blurry appearance of out-of-focus material (Wang and Fang, 2012),time-consuming, human-based and the fact that many taxa have overlapping morphological features and variations. Moreover, structure of cyanobacteria frequently is not recognizable with certainty. For these reasons, the classifications of cyanobacteria have been revised numerous times. Based on the plasticity of cyanobacteria, phenotypic characteristics may vary with environmental

fluctuation. Therefore, pictures should be taken using different microscopy techniques at the same time to accurately identify cyanobacteria (Grigoryeva and Chistyakova, 2018).

1.6.2 Fluorescence microscopy

Fluorescence microscopy allows the visualization of the cells by autofluorescence and is one of the essential tools to analyse the morphology of cyanobacteria and identify cells and cellular components. The disadvantage of this technique is the low depth of field. This limitation can be avoided with the Confocal Laser Scanning Microscope (Grigoryeva and Chistyakova, 2018). However, neither technique can reveal those structures without autofluorescence. Phase-contrast microscopy is an extremely useful technique for observing specimens that are in their natural state. One major advantage is that structures that cannot be seen under normal bright field microscopy, are observed with good contrast.

1.7 Results and discussion

Thirty axenic genera of homocytous and hetrocytous cyanobacteria were isolated from soil and preserved in the laboratory. In this work, we have focused on heterocyteous cyanobacteria. Most hetrocystous genera obtained were limited to *Nostoc* sp. Eight taxa (heterocyteous) were recorded as morphospecies. To our best knowledge, *Nostoc paludosum, Nostoc Linckia, Nostoc muscorum, Cylindrospermum tropicum, Anabaena sp., Nostoc sp*., have been recorded for the first time in this region. Morphological characteristics of the axenic strains of cyanobacteria are as follows:

1.7.1 *Nostoc commune*

Green-brown aggregations, dark green, rarely yellow-green, gelatinous, completely expanded; colonies round, globular, regular in shape, lumpy and clathrate; filaments long, not straight, coiled and irregular form, with a firm layer; sheath expanded, conspicuous using phase-contrast microscopy, hyaline, hardly visible or difficult to distinguish using bright

field microscopy; vegetative cells cylindrical to sub-cylindrical, ovoid or ovoid-spherical, 3.7 µ in diameter; spores nearly similar to vegetative cells, sometimes broader and spherical to ovoid-spherical granulated, intercalary; usually larger than the vegetative cells; heterocyte metameric (more or less regular spaced along trichrome), not so obvious without fluorescence and phase-contrast microscopy, larger than spores, yellow to yellow brown, disperse, cylindrical to sub-cylindrical, hyaline sheath able to distinguish by phase-contrast but no light microscopy.

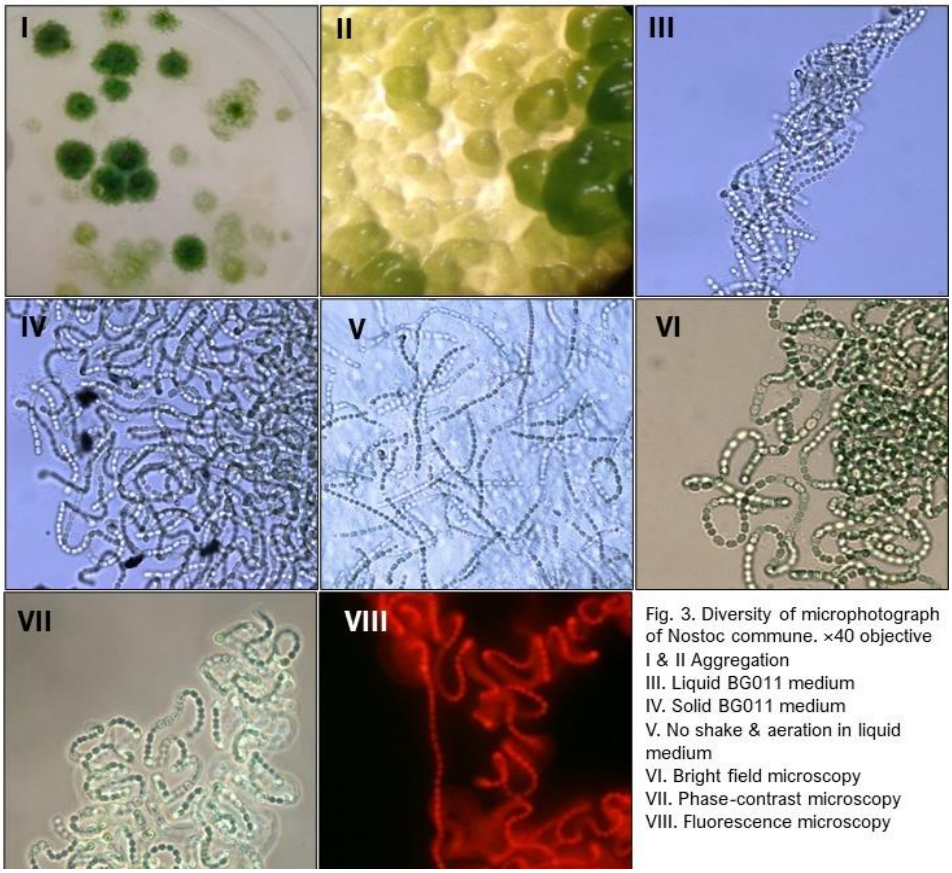

Fig. 3. Diversity of microphotograph of Nostoc commune. ×40 objective
I & II Aggregation
III. Liquid BG011 medium
IV. Solid BG011 medium
V. No shake & aeration in liquid medium
VI. Bright field microscopy
VII. Phase-contrast microscopy
VIII. Fluorescence microscopy

1.7.2 *Nostoc muscorum* Kutzing ex Borne. et Flah.

Green, dark green and brown aggregations, rarely yellow-green, lumpy, not so regular, slimy colonies, expanded, gelatinous, attached to margin;

filaments not straight, coiled and irregular form, unified into firm; sheath expanded, conspicuous using phase-contrast microscopy, hyaline hardly to seen or difficult to distinguish with bright field microscopy; cells variable in shape, short, subcylindrical, barrel-shaped 3.8 µm broad; constricted at the cross walls; vegetative cells spherical, ovoid or ovoid-spherical with 8.8 µm in diameter; spores oblong, oval-spherical, spherical and sometimes ovoid, many in a series, granulated, intercalary; mostly larger than the vegetative cells, 13.8 µm long and 9.4 µm broad, sometimes brownish, completely ovoid and showing grooves; heterocyte shorter than the spores, 6.15 µm broad, yellow to yellow-brown, sometimes between two spores, sometimes disperse, spherical to ovoid-spherical, hyaline sheath be able to recognizable by phase-contrast microscopy.

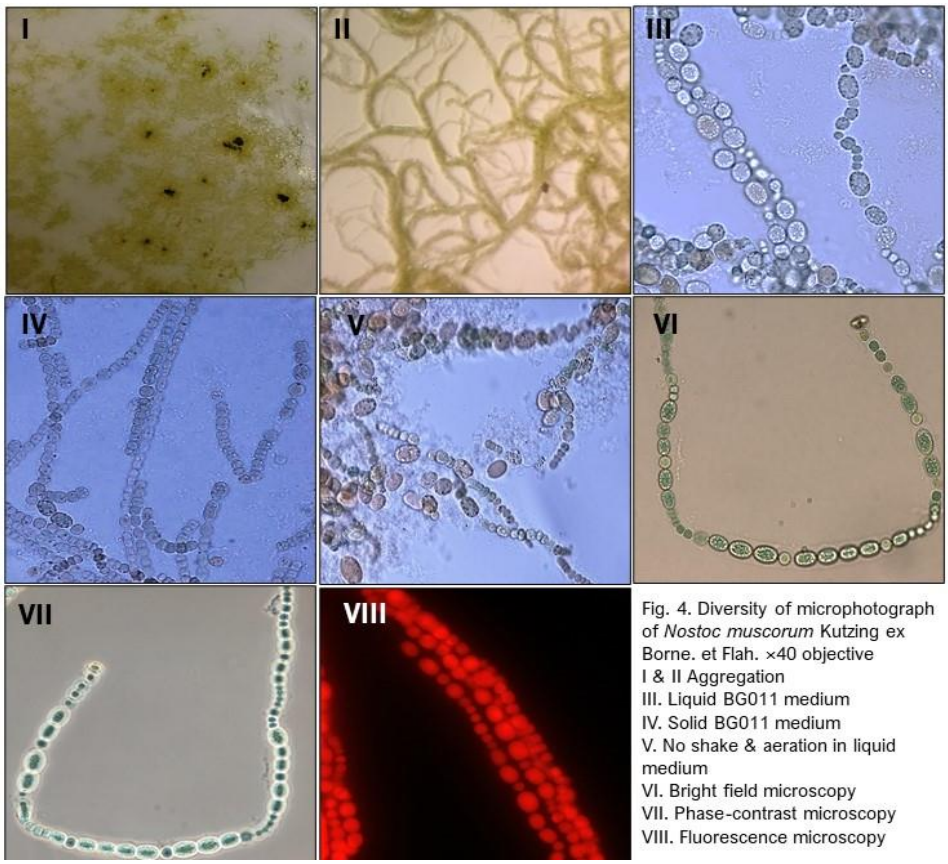

Fig. 4. Diversity of microphotograph of *Nostoc muscorum* Kutzing ex Borne. et Flah. ×40 objective
I & II Aggregation
III. Liquid BG011 medium
IV. Solid BG011 medium
V. No shake & aeration in liquid medium
VI. Bright field microscopy
VII. Phase-contrast microscopy
VIII. Fluorescence microscopy

1.7.3 *Anabaena* sp.

Aggregations brown, light brown, rarely green-brown, gelatinous, completely expanded, flexous; colonies lumpy, irregular in shape, entangled; filaments long, straight, curved and irregular form, constricted at the cross wall, isopolar; sheath expand, hyaline, colorless; vegetative cells spherical to short cylindrical, bent, pale blue-green to yellow-brown, 3.1 µm in diameter; spores cylindrical to ovate, remote from heterocyte, intercalary; heterocyte spherical to round, metameric (at regular interval along filament), larger than the vegetative cells, scattered, intercalary, 4.1 µm in diameter.

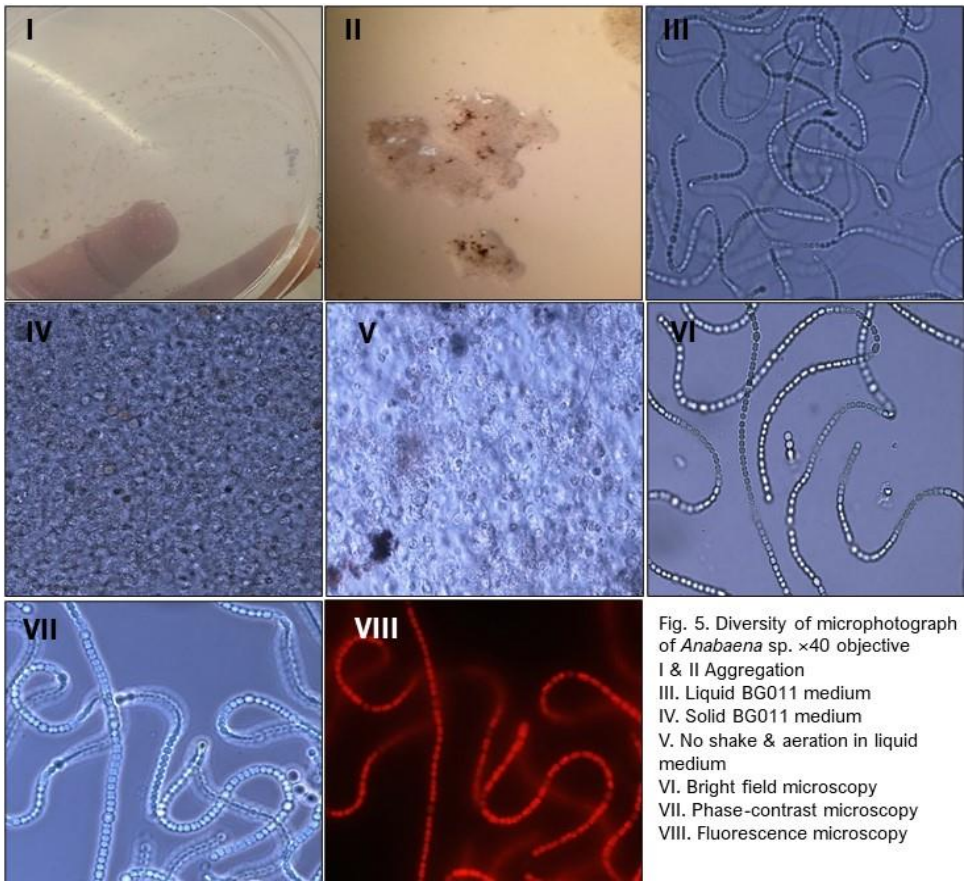

Fig. 5. Diversity of microphotograph of *Anabaena* sp. ×40 objective
I & II Aggregation
III. Liquid BG011 medium
IV. Solid BG011 medium
V. No shake & aeration in liquid medium
VI. Bright field microscopy
VII. Phase-contrast microscopy
VIII. Fluorescence microscopy

1.7.4 *Cylindrospermum tropicum*

Pale blue-green rarely green aggregations, tendency to grow on the margins of the Erlenmeyer; mucilaginous; compacted filaments, expanded, not straight, slightly curved; trichomes fine, colourless, homogeneous, symmetrical, slightly constricted at the cross wall; without sheath; cells quadrate to cylindrical 5.2µm long and 2.6µm broad, more or less isodiametric, no granulated; heterocytes terminal, single pored, intercalary, broadly ellipsoidal or conical, longer than cells, 3.2µm broad and 8.9µm long; spores brown, single 10µm broad and 23µm long ellipsoidal.

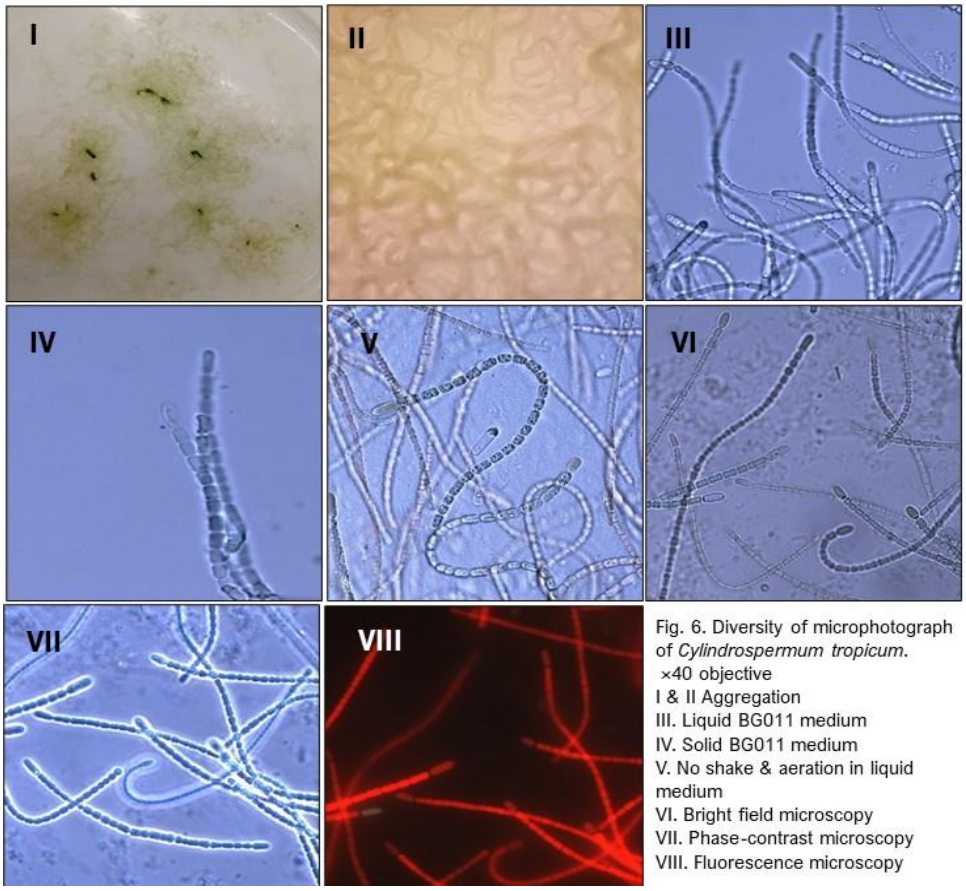

Fig. 6. Diversity of microphotograph of *Cylindrospermum tropicum*.
×40 objective
I & II Aggregation
III. Liquid BG011 medium
IV. Solid BG011 medium
V. No shake & aeration in liquid medium
VI. Bright field microscopy
VII. Phase-contrast microscopy
VIII. Fluorescence microscopy

1.7.5 *Nostoc linckia* (Roth) Bornet ex Born. et Flah

Aggregations yellowish brown to greenish-blue, gelatinous, expand, clathrate; colonies firm, clumpy, globular, irregular in shape; filaments entangled, twisted, often covered by lamellae; sheath colourless, difficult to distinguish, vegetative cells spherical or globular, 3.5 µm in diameter; Spores are more or less spherical; heterocyte spherical to ovate, 4.3 µm in diameter.

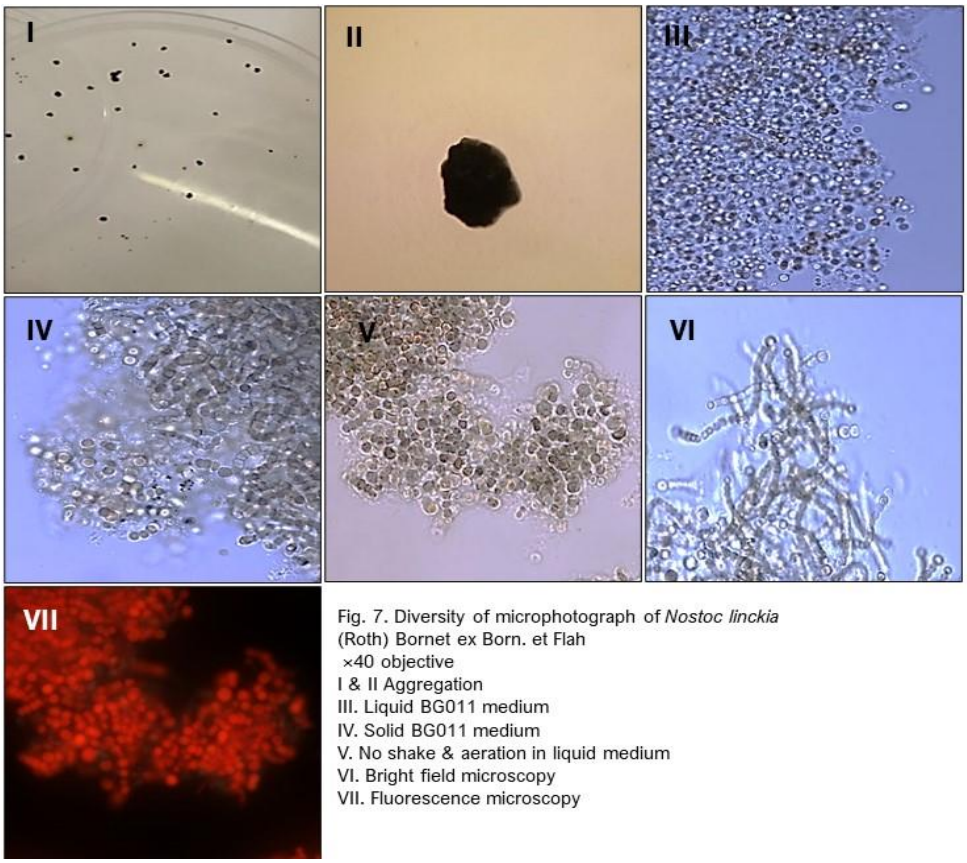

Fig. 7. Diversity of microphotograph of *Nostoc linckia* (Roth) Bornet ex Born. et Flah
×40 objective
I & II Aggregation
III. Liquid BG011 medium
IV. Solid BG011 medium
V. No shake & aeration in liquid medium
VI. Bright field microscopy
VII. Fluorescence microscopy

1.7.6 *Nostoc paludosum*

Aggregations green and brown, thallus microscopically not visible, more or less soft and formless, not so regular, expanded; filaments not straight more or less densely coiled and loosely arranged; gelatinous; sheath expand, colourless, conspicuous using phase-contrast microscopy,

hyaline, difficult to observe with bright field microscopy; cells as long as broad, barrel-shaped, light blue-green with 3.7*2.1µm in diameter; spores brownish, oval, larger than vegetative cells and difficult to distinction with heterocyte by bright field microscopy, only by Fluorescence microscopy; heterocyte intercalary, ovate, brown to yellow-brown, 5.1 µm in diameter.

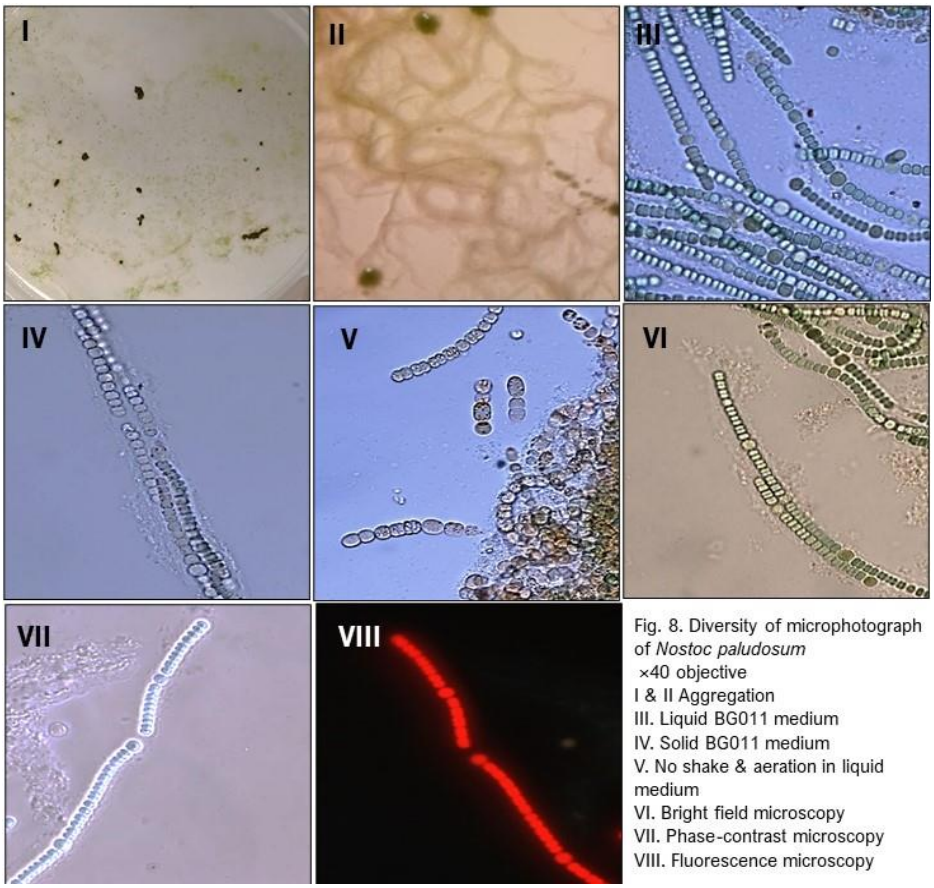

Fig. 8. Diversity of microphotograph of *Nostoc paludosum*
×40 objective
I & II Aggregation
III. Liquid BG011 medium
IV. Solid BG011 medium
V. No shake & aeration in liquid medium
VI. Bright field microscopy
VII. Phase-contrast microscopy
VIII. Fluorescence microscopy

1.7.7 *Nostoc* sp.

Aggregations green, gelatinous, completely expanded; colonies lumpy, almost round, globular, irregular in shape, attached to margin; filaments densely entangled, flexuous, not straight, coiled long, spirally twisted throughout their length; sheath distinct only at the periphery of the

thallus, colorless; vegetative cells short barrel-shaped or ellipsoidal, broad, pale blue-green to yellow-brown; heterocyte globular to round, larger than the vegetative cells, scattered; spores are difficult to distinguish. This species is quite similar to *Nostoc muscorum.*

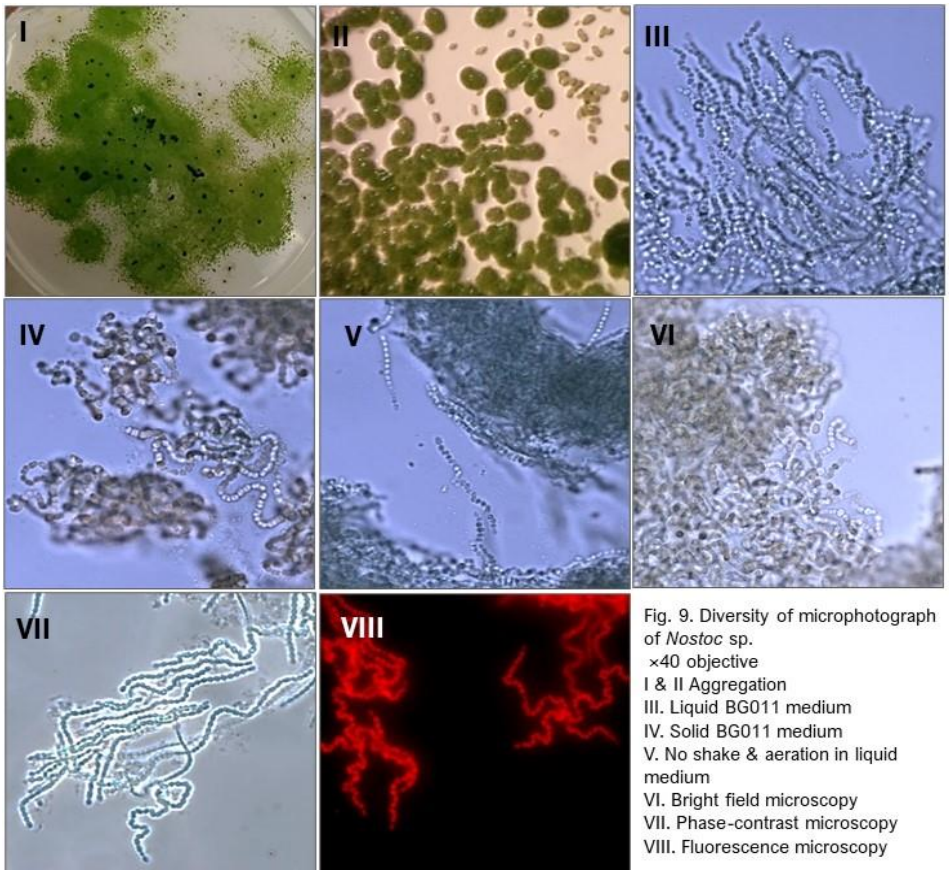

Fig. 9. Diversity of microphotograph of *Nostoc* sp.
×40 objective
I & II Aggregation
III. Liquid BG011 medium
IV. Solid BG011 medium
V. No shake & aeration in liquid medium
VI. Bright field microscopy
VII. Phase-contrast microscopy
VIII. Fluorescence microscopy

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Chapter 2: Taxonomy (Polyphasic approach)

2.1 Introduction

The aim of this chapter is to characterize the complex diversity of this *Nostoc sp*. by a combination of modern and classical taxonomy along with environmental fluctuation such as salinity and alkalinity using advanced tools.

Cyanobacteria as one of the most important organisms and an oxygenic photosynthesis appeared in the Precambrian Era (Komárek, 2016; Mloszewska et al., 2018), a period that can be considered chaotic from the environmental point of view. Since in chaotic systems it is difficult to find a stable point, high versatility is the natural consequence of survival under such conditions and high adaptability appears in different aspects of the organism including morphology, physiology, and molecular characteristics and may be one of the reasons why the classification of cyanobacteria is still so ambiguous. In consequence, previously described species may need to be continuously revised according to new findings and methodologies.

The traditional approaches of classification of cyanobacteria have primarily been based on morphological characters including level of morphological pigmentation, cell dimensions, cell shape, trichome width, presence or absence of a sheath (Komárek & Anagnostidis, 1989), cell arrangement (e.g., radially, in strict planes, or irregularly) and filaments (e.g., branched, coiled or straight) (Whitton and Potts, 2000). Nevertheless, phenotypes can change depending on environmental factors and growth conditions leading to the failure of this classification. Besides, morphological, physiological, and ecophysiological properties of cyanobacteria are influenced by environmental factors (Fernandez Valiente & Leganes, 1990; Islam & Whitton 1992; Grossman et al., 1993) and cyanobacteria with similar or identical morphology may have significantly different physiology.

Polyphasic approaches based on comparison of morphology, ultrastructure, molecular biology, and biochemistry, have been used in recent taxonomic revisions of various groups of cyanobacteria (Komárek, 2010b, 2010a). Nevertheless, this approach is still problematic and

doubtful since, often, molecular data are not consistent with morphological studies (Wright et al., 2001). As both are important characteristics for the final evaluation, this discrepancy generates confusion in the classification systems (Komárek, 2016; Řeháková et al., 2014).

Ecophysiological approaches to the environmental effects -light, temperature, salinity, pH, alkalinity, etc., on traits like growth, or photosynthetic efficiency important aspects of taxonomic studies. These environmental factors directly influence the occurrence, abundance, distribution, morphology, physiology and molecular biology of organisms. Physiological methods have been suggested as useful tools in the modern classification of cyanobacteria (Poryvkina et al., 2000; Ramírez et al., 2011; Yema et al., 2018). Nonetheless, they have been scarcely used compared to molecular biology, morphology or even phytochemistry.

Among environmental variables that have a strong influence on physiology temperature, irradiance, salinity and alkalinity have been recognized as important factors influencing phenotypic traits correlated with phylogeny (Gugger & Hoffmann, 2004). However, the influence of environmental fluctuations on the cyanobacteria are still poorly explored and cyanobacterial traits have mostly been evaluated under standard lab conditions, regardless of the influence of environmental factors (Epping & Kühl, 2000; Martínez-Alonso et al., 2004; Del Carmen Pérez et al., 2009).

Nostoc species are conspicuous components of terrestrial microbial populations worldwide (Dodds et al., 1995). However, *Nostoc* is a complex genus to differentiate. The morphological plasticity and its cryptic diversity and almost no distinguishable morphological characteristics make this genus incredibly difficult to evaluate on taxonomic scales (Bagchi et al., 2017). In this chapter, we make a polyphasic approach to characterize *Nostoc sp.* strain (UAB 206) isolated from Mediterranean paddy fields at the Ebro Delta in Spain.

Little attempt has been made to discern ambiguities and contradictions concerning cyanobacteria in the Ebro Delta (Pérez & Carrillo, 2005; Urmeneta et al., 2003). So, terrestrial cyanobacteria are still largely

unexplored and represent a rich opportunity for discovery. Most researchers have focused on microbial mats and their extensions, but not on individual strains (Martínez-Alonso et al., 2004; Wierzchos et al., 2006). Here, we employ different techniques to address some of the most prominent and stable physiological traits and bring them together with morphology and genetic traits to achieve a comprehensive characterization of an unexplored strain, *Nostoc* sp. UAB 206, under combination of salinity and alkalinity.

2.2 Materials and methods

2.2.1 Growth conditions and preliminary approach.

The *Nostoc* sp. strain UAB 206 isolated as described in the previous chapter was used for studying its physiological properties under different combinations of high pH and salinity.

Stock cultures were grown in nitrogen-free medium (BG-011). The temperature was maintained at 28±2 °C and cultures were incubated under a constant light intensity of 100 µmol photon m^{-2} s^{-1}. Cells from stock culture inoculated in 300 mL of BG-011 medium in 500 mL Erlenmeyer flasks stopped with cotton plugs during 3 weeks under aeration and constant shaking (25 rpm) and stirring according to (Poza-Carrión et al., 2001) (Fig. 10). Then, cells in exponential phase of growth were selected. Cyanobacteria were cultures under a combination of different pH values (7, 9 and 11) and salinity concentrations (17, 80 , 160 , 240 and 320 mM Nacl) in sterile petri dishes containing liquid or solid BG-011 medium (Soltani et al., 2010). Petri dishes were placed in a culture chamber under the same condition as those used for the stock culture (Fig. 10). Observations of cyanobacteria growth in Petri dishes were carried out daily. Culture media were adjusted to the desired pH with KOH.

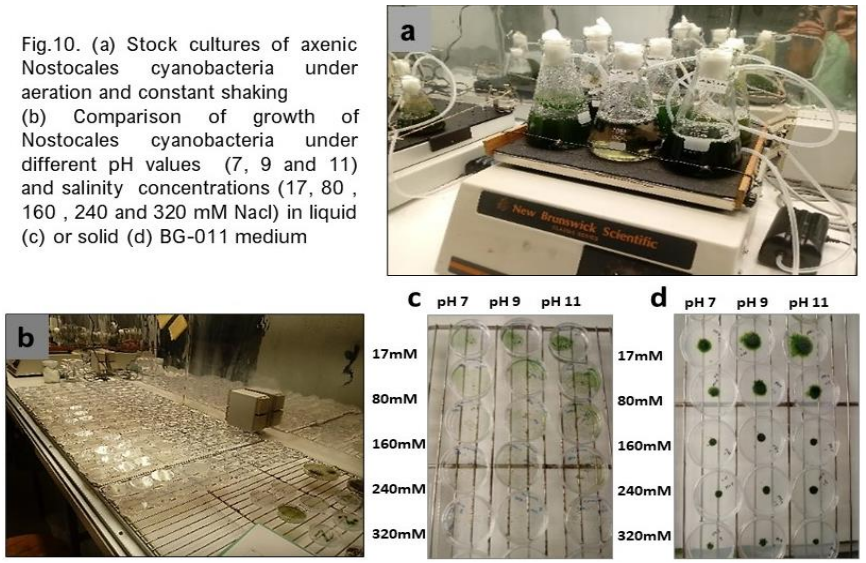


Fig.10. (a) Stock cultures of axenic Nostocales cyanobacteria under aeration and constant shaking (b) Comparison of growth of Nostocales cyanobacteria under different pH values (7, 9 and 11) and salinity concentrations (17, 80 , 160 , 240 and 320 mM Nacl) in liquid (c) or solid (d) BG-011 medium

2.2.2 Morphological characterization

To understand taxonomical classification and to accurately identify cyanobacteria morphology, we extended our imaging capabilities using different microscopes and advanced tools under different culture conditions. The morphological examination of the selected strain was carried out in liquid as well as on solid media. The form and colour of the aggregations was observed daily. Morphological and biometrical variations of the heterocyte, hormogonia and akinetes were studied daily under a bright field (Kaushik 1987), fluorescence, phase contrast (Shokravi and Soltani, 2011) and confocal laser microscopy (CSLM) (Ramírez et al. 2011, Roldán et al., 2013). Hormogonia formation was examined by bright field, fluorescence and confocal microscopy. CLSM allowed new perspectives to investigate detailed mechanisms and imaging under stress condition of single cells of our *Nostoc* strain. CLSM spectroscopy provided a unique opportunity to obtain high-resolution images and intrinsic fluorescence emission spectra from single cyanobacterial cells (Grigoryeva & Chistyakova, 2020).

2.2.3 Growth measurement and spectroscopy analysis

Growth under the different treatment conditions was estimated

spectrophotometrically by measuring optical density (OD) and biomass determinations (OD at 750 and 660 nm) for a week after inoculation (Śliwińska-Wilczewska et al., 2019). For chlorophyll determinations, samples of washed cells were extracted with acetone for 24 h at 4 °C. The chlorophyll concentration was estimated according to Marker, 1972. During the growth analysis, fluorescence excitation spectra of cultures were measured with a TECAN- multimode microplate reader SPARK, under an excitation 380 and 580 nm and emission ranges 600-760 according to Tiwari & Mohanty, 1996 and Fraser et al., 2013.

2.2.3.1 Specific growth rate

Specific growth rate (μ) was calculated using the equation

$$\mu = \frac{\mathrm{Ln}(N2/N1)}{(t2 - t1)}$$

being N1 and N2 optical density (750 or 660 nm) at the beginning (t1) and at the end (t2) of the exponential growth phase.

2.2.4 Phycobilisomes activity and photosystem operation

The phycobilisomes activity and photosystem operation (PBS and PSII and PSI activity) were identified using the lambda scan measurement *in vivo* to determine the emission spectra by CLSM. CLSM enables us to study different physiological processes including intensity of fluorescence emitted (as spectral unmixing) from single cells (Grigoryeva & Chistyakova, 2020). Variations in the position of the emission peak of autofluorescence from different photosynthetic pigments and phycobilisome were detected by recording fluorescence spectra with an excitation wavelength of 405 nm, and emission ranges from 423 to 544 and 622 to 763 nm-using 40X oil immersion objective (Ramírez et al., 2011).

2.2.5 Measurement of oxygen evolution

Measurement of changes in oxygen concentration was recorded daily with a Clark-type O_2 electrode (Thermo Scientific ORION 9708) (Soltani et al., 2006). Before starting the measurements, the oxygen initially present in an aliquot (10 ml) of cell suspensions was removed by bubbling helium gas. Measurements were taken at 28 ºC under constant illumination at a quantum flux density of 100 µmol photon m^{-2} s^{-1}. The amount of liberated oxygen was normalized based on the chlorophyll concentration in the sample (Poza-Carrión et al., 2001).

2.2.6 Transmission Electron Microscopy

Transmission electron microscopy (TEM) can be used to identify specific types of organisms and their physiological state through the identification of different cell structures like thylakoids arrangement, cytoplasmic granules, etc (Franks, 2007).
Cultured material was fixed with glutaraldehyde (2.5%) in 0.1M phosphate buffer followed by a post-fixing in 1% OsO_4 in the same buffer. Afterwards it was dehydrated with acetone and embedded in Spurr's resin. Ultrathin sections were cut and stained with 2% uranyl acetate and lead citrate. The sections were observed using a JEOL 1400 TEM at 100 kV accelerating voltage (Ramírez et al., 2011).

2.2.7 DNA extraction and PCR amplification

Total genomic DNA was extracted from 50–100 mg wet cyanobacteria samples by using the FastDNA™ SPIN kit (MP Biomedicals, Irvine, CA, USA) in accordance with manufacturer instructions. Amount and purity of extracted DNA was quality checked by using Nanodrop 2000 Thermo scientific spectrophotometry at 260–280 nm wavelength absorbance. Primers 16S rRNA (CYA359-Forward and CYA781-Reverse) (Nübel et al., 1997) and phycocyanin (PCβ-Forward and PCα-Reverse) (Neilan, 2002) were chosen to obtain a segment containing 16S ribosomal RNA gene and phycocyanin gene region. The PCR reaction had a final volume of 20 µl with 1 µl of template DNA (100-200 ng μl^{-1}), 10 µL IQ™ super mix (Bio-Rad

Laboratories, California, USA) and 1µL of each primer (10 µM). PCR amplification was performed using the iCycler (Bio-Rad Laboratories, California, USA) with a PCR profile of an initial denaturation step at 95 ºC for 4 min; 35 cycles of 95 ºC for 1 min (denaturation); 59ºC for 1 min (annealing); 72 ºC for 2 min (elongation); then the elongation hold step at 72 ºC for 8 min, and a final cooling to 4 °C. The PCR products was separated by 2% agarose gel electrophoresis and visualised using SYBR Safe DNA gel stain (Invitrogen, USA). The PCR products were sequenced, and results were aligned and edited using BioEdit program (Hall 1999); then the sequences with similarity found by the NCBI (www.ncbi.nlm.nih.gov/blast) taxonomy application were retrieved and checked by BLAST software (Altschul et al., 1997) to confirm their placement.

2.2.8 Bioinformatic and phylogenetic analysis

The obtained 16S rRNA and PC sequences were aligned using MEGA-X software (Kumar et al., 2018). The final phylogenetic trees were constructed using Neighbour-Joining (Saitou & Nei, 1987) Maximum-Likelihood and Maximum Parsimony (Felsenstein, 1985) with 1000 bootstrap replicates. Distances for the NJ and ML tree were estimated by the algorithm of Tamura-Nei algorithm (Tamura & Nei, 1993) using the complete deletion option. Maximum Parsimony was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm. Similar clustering was obtained with the NJ, ML and MP algorithms and combining them by TreeGraph 2 software (Stöver & Müller, 2010); hence, we have chosen to represent the sequence relationships with the ML tree.

2.2.9 Statistical analysis

Data were analysed using one-way Analysis of Variance (ANOVA) by JMP software. ANOVA test shows a significant difference between treatments with $p < 0.05$. Data and morphometric analysis are the mean and standard deviation of four replicates. Three-Dimensional reconstruction and image processing were carried out with IMARIS 7.4.2 (Leica GmBH, Germany)

and Image-Pro Plus 6.0 (Media Cybernetics Inc., USA). We applied dimension reduction via Principal Component Analysis (PCA) to visualize the distribution of data. Furthermore, we studied the variance along the principal component using MATLAB software. Since the salinity appeared as principal component, we performed the scatter plot to study the relationship between width, length and salinity of cells that are represented as points in three-dimension plot with the corresponding pH and time.

2.3 Results

2.3.1 Aggregations and colony morphology

The form and colour of the aggregates of *Nostoc* sp. UAB 206 varied daily either in liquid or solid media. In the liquid cultures under control (17 mM NaCl) conditions, the aggregates were gelatinous and almost completely expanded, while increasing salinity caused scattering and expansion. Light and phase contrast observations showed crustose to globular or irregular colonial aggregations, centripetal forms during the first days, but tending to centrifugal migration on the seventh day after inoculation (Fig. 11). We observed this trend under all conditions, regardless the alkalinity and salinity levels. Furthermore, the strain illustrated a clumpy shape under elevated pH values (9 and 11) combined with low salinity (17 mM NaCl), while the colour of the aggregation was between green and dark green.

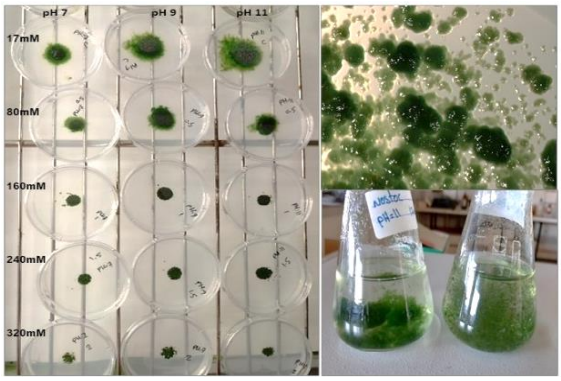


Fig. 11. Aggregation of *Nostoc* sp. at different pH (7,9,11, columns) and salinity concentration (17, 80, 160 , 240, 320 mM Nacl, rows) at 10th day after inoculation (Solid medium).

2.3.2 Trichomes and filaments

High alkalinity and salinity had a remarkable effect on the topology of the filaments. Trichomes were nearly straight, long, interwoven, irregular form with constriction at the cross walls and not narrowing towards the apical cell (Fig. 12 a & b). In general, under high alkalinity the most variable and flexible shapes were observed using CLSM (Fig. 12c).

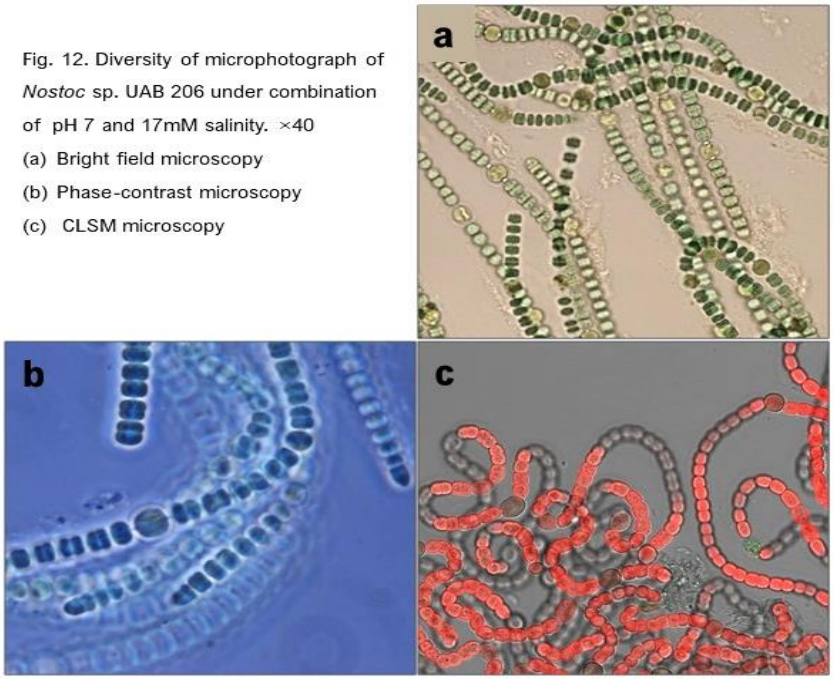


Fig. 12. Diversity of microphotograph of *Nostoc* sp. UAB 206 under combination of pH 7 and 17mM salinity. ×40
(a) Bright field microscopy
(b) Phase-contrast microscopy
(c) CLSM microscopy

Three-dimensional reconstruction from CLSM Z-stacks showed apparently true branches under the evaluated alkalinity and salinity (Fig. 13 a & b) which were not so different from main axes. Moreover, the TEM micrograph confirmed this true branching that the number of branches increased at pH 11 and all salinity compared to pH values of 7 and 9 (Fig. 13b).

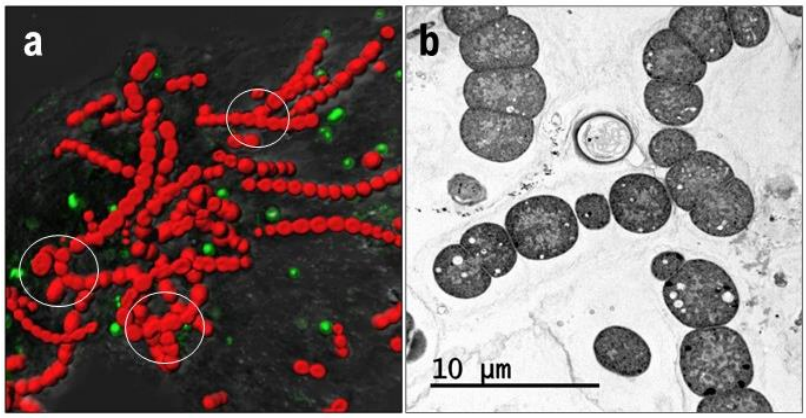


Fig. 13. Three-dimensional and TEM microphotograph of *Nostoc* sp. UAB 206 under combination of pH 11 and 80 mM NaCl (first day after inoculation).

The sheath of filaments is difficult to observe using bright field microscopy. However, phase-contrast and three-dimensional images revealed the existence of distinguishable thick and hyaline sheath which had a white (rarely white grey) colour and with a transverse wall around the trichomes that vary depending on pH and salinity (Fig. 14). TEM micrograph confirmed the thick sheaths. A transverse wall around the trichomes was observed (Fig. 14) that varied depending on pH and salinity.

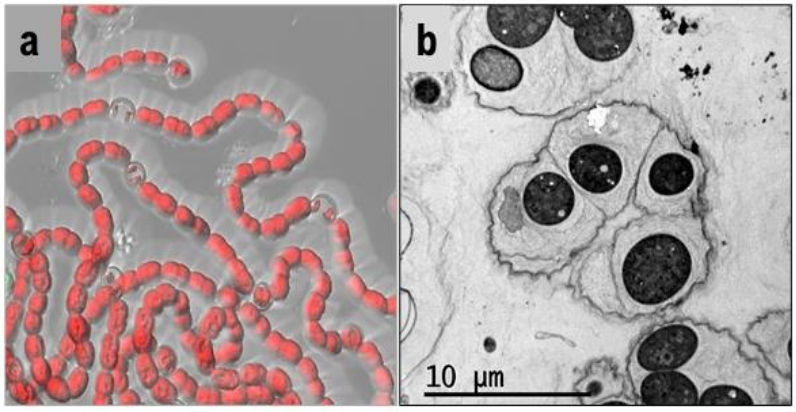


Fig. 14. Three-dimensional and TEM microphotograph of *Nostoc* sp. UAB 206 under combination of pH 9 and 80 mM NaCl (fourth day after inoculation).

The age of cultures had a strong influence on the sheath dimensions. In

general, increasing pH led to decreased sheath dimensions and vice versa ($P<0.05$) (Table 3). Sheath dimensions decreased under pH 9 from day 1 to day 4 after inoculation, regardless the salinity level. It was noticeable that the change of dimension was reduced by 17 mM NaCl under pH 11 whereas significantly increased when exposed to 80 mM NaCl ($P<0.05$).

Table 3. Comparison of sheath dimensions (µm) of *Nostoc* sp. UAB 206 at different pHs (9 and 11) and salinity concentration (17, 80, 160 mM); n = 10

	17 mM		**80 mM**		**160 mM**	
pH	**1st day**	**4th day**	**1st day**	**4th day**	**1st day**	**4th day**
9	10.18 ± 1.07^{a}	7.91 ± 0.28^{b}	8.86 ± 0.98^{ab}	5.95 ± 0.95^{bb}	7.15 ± 0.87^{ac}	6.50 ± 0.65^{bc}
11	5.14 ± 0.47^{a}	5.42 ± 0.73^{a}	4.30 ± 0.84^{ab}	5.38 ± 0.80^{aa}	3.29 ± 0.37^{ac}	5.74 ± 0.52^{bc}

2.3.3 Biometrical analysis

We explored correlations among trichome cell dimensions (length and width), environmental (salinity and alkalinity) fluctuations, and the age of the cultures (first and fourth day after inoculation) using Principal Component Analysis (PCA). We employed a PCA method to reduce the dimension of the underlying data from five to three for visualization purposes. The PCA analysis showed that the salinity is the principal component comparing pH and the age of the cultures (Fig. 15a). The maximum variance (morphological changes) was along the salinity axis. Time (days after inoculation) was the second component showing the importance of experiment duration on the distribution of data. Spread of data along the third component (pH) was less important compared to the first and second components**.** To study the relationship between different applied effects on cell dimensions we employed scatter plot and correlation analysis. The scatter plot visualization of the cell dimensions (Fig. 15b) showed almost linear correlation between width and length at low salinity. The correlation coefficient decreased with increasing salinity from 17 to 160 mM, while the variance of the cell dimensions was inversely proportional to salinity. The effect of time (first and fourth day after inoculation) was less noticeable than that of salinity, being the

highest correlation observed on the fourth day at 17 mM NaCl. The results of the biometrical analysis of the vegetative cells, heterocyte, and akinetes (Table 4 and 5) revealed the difficulty to find a unique pattern in the dimensional variation of the individual cells (especially vegetative cells and heterocyte) (P<0.05).

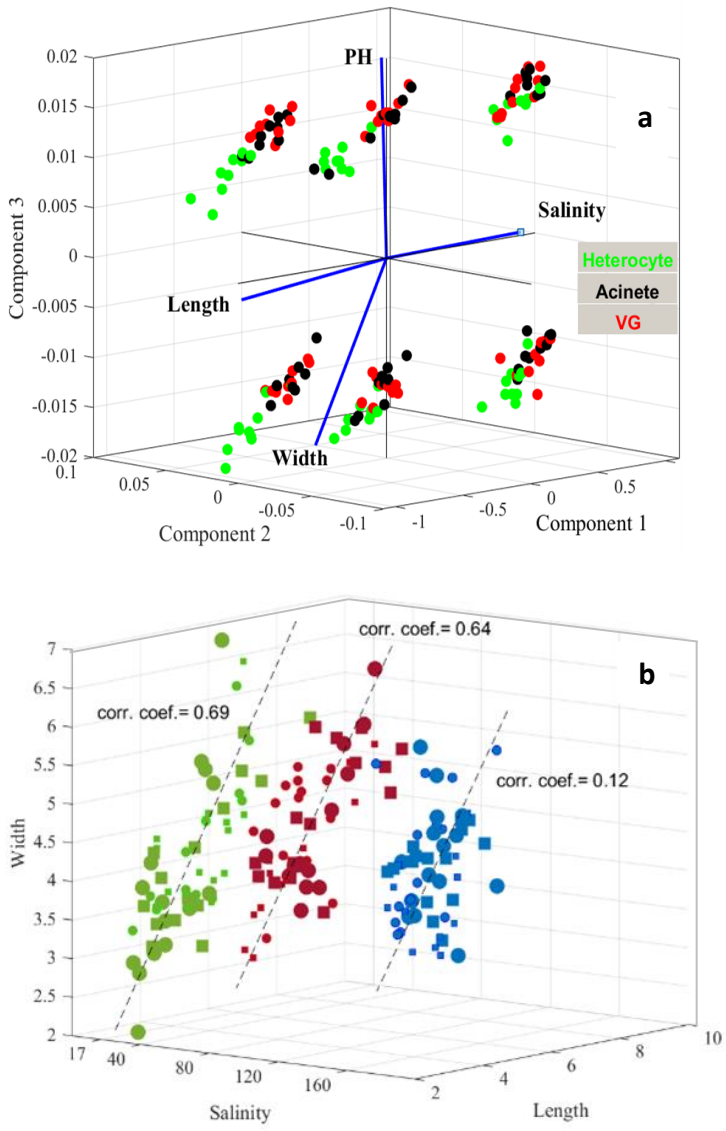


Fig. 15 **(a).** Statistical analysis of *Nostoc* sp. UAB 206. a) Principal Components Analysis (PCA) represent the combination effect of salinity, pH and time on heterocyte (green dots), akinete (black dots) and vegetative cell (red dots). Each dot points in the plot represents cells. **(b)**. The Scatter plot shows the relationship between salinity, pH and time, width and length. Each point on the plot represents heterocyte, akinete and vegetative cells. the cells at pH 11 and pH 9 are depicted in square and circle symbols respectively. The markers in small size represent day one and the big ones belong to day four.

Table 4. Comparison of vegetative cell (VG), heterocyts (H) and akinete (A) dimensions (µm) of *Nostoc* sp. UAB 206 at pH 9 and different salinity concentration (17, 80, 160 mM) after 1st and 4th day of inoculations. Values are means biological replicates ± standard deviation (n = 10).

pH9	17 mM		80 mM		160 mM	
	1st day	4th day	1st day	4th day	1st day	4th day
Length -H	5.91±0.58^{a}	5.20±0.21^{b}	4.60±0.20^{ab}	6.20±0.45^{ab}	5.07±0.72^{ac}	4.80±0.45^{ac}
Width -H	5.17±0.62^{a}	5.21±1.03^{a}	5.09±0.44^{aa}	5.63±0.58^{bb}	5.12±0.75^{bc}	5.24±0.48^{bc}
Length -VG	4.31±0.62^{a}	3.44±0.37^{b}	4.65±0.87^{aa}	4.37±0.61^{ab}	3.86±0.66^{ac}	4.36±1.04^{ac}
Width -VG	3.58±0.07^{a}	3.30±0.52^{a}	4.51±0.50^{ab}	4.27±0.26^{ab}	4.74±0.71^{ac}	4.05±0.27^{ac}
Length -A	3.91±0.76^{a}	3.66±0.54^{a}	4.48±0.85^{ab}	4.64±0.22^{ab}	3.61±0.14^{aa}	4.79±0.28^{ac}
Width -A	3.73±0.32^{a}	3.14±0.63^{a}	4.59±0.80^{ab}	3.98±0.21^{ab}	3.82±0.17^{aa}	4.29±0.62^{ac}

Table 5. Comparison of vegetative cell (VG), heterocyte (H) and akinete (A) dimensions (µm) of *Nostoc* sp. UAB 206 at pH 11 and different salinity concentration (17, 80, 160 mM) after 1st and 4th day of inoculations. Values are means biological replicates ± standard deviation (n = 10).

pH 11	17 mM		80 mM		160 mM	
	1st day	4th day	1st day	4th day	1st day	4th day
Length -H	5.79±0.45^{a}	6.54±0.87^{b}	5.94±0.69^{aa}	6.33±0.84^{bb}	4.72±0.80^{bc}	4.48±0.80^{ac}
Width -H	5.01±0.88^{a}	5.19±0.32^{a}	5.10±0.41^{aa}	5.45±0.37^{bb}	4.83±0.64^{ac}	4.60±0.07^{ac}
Length -VG	4.68±0.87^{a}	4.06±0.70^{b}	3.73±0.31^{ab}	4.61±0.45^{bb}	4.23±0.63^{bc}	5.28±0.23^{ac}
Width -VG	3.98±0.36^{a}	3.41±0.36^{b}	3.85±0.31^{aa}	4.12±0.20^{bb}	3.74±0.46^{ac}	4.63±0.39^{bc}
Length -A	4.92±0.62^{a}	4.12±0.48^{b}	3.58±0.41^{ab}	5.10±1.70^{ab}	4.19±0.85^{ac}	4.06±0.64^{bc}
Width -A	4.20±0.38^{a}	3.77±0.42^{b}	3.65±0.44^{ab}	4.92±0.63^{ab}	3.91±0.43^{bc}	4.01±0.42^{bc}

2.3.4 Hormogonia

Morphological changes associated with the induction of hormogonia formation are best revealed using different culture conditions (Mateo et al., 2011). In our study, at pH 11 and 240 mM salinity, hormogonia differed from vegetative cells by its small size, square shape, straight and very thin sheath. Daily observations revealed that the size and number of hormogonia decreased under pH 9 and 160 mM NaCl ($P<0.05$). The rest of the conditions caused increase of the hormogonia induction along with altered shape from straight to relatively bent (data not shown). The forms of the vegetative cells were variably influenced by environmental conditions. Square or rectangular forms prevailed under 17 mM NaCl and pH 7, while oval to oval-cylindrical were common under all other conditions. Most of the vegetative cells showed granulate cytoplasm; the granules tended to accumulate at the periphery of the cells (Fig. 16).

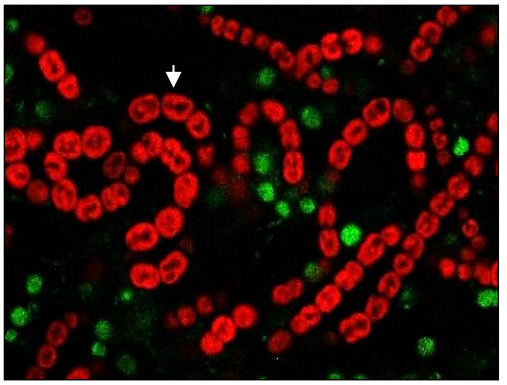


Fig. 16. Confocal laser scanning photomicrographs of *Nostoc* sp. UAB 206 of an overlap of inactive (green colour) and active, (red colour) autofluorescence under combination of pH 11 and 17 mM NaCl concentration (first day after inoculation
)

2.3.5 Akinetes and Heterocyte

CLSM analysis revealed that both the dimensions and the number of the akinetes, as well as their distance to the heterocyte were affected by the combination of pH and salinity treatments (Fig.17). Akinetes were spherical, spherical-elliptical and square or rectangular in the pH 7- 17 mM NaCl treatment.

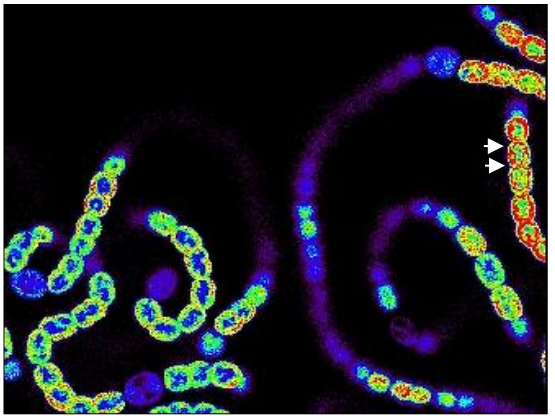


Fig. 17. CLSM microphotograph of *Nostoc* sp. UAB 206 under combination of pH 9 and 80 Mm NaCl (fourth day after inoculation).

Heterocyte were larger than vegetative cells, with a spherical or spherical-ellipsoid shape and visible polar nodules. CLSM analysis revealed different layers with a thick inner layer and a thin white outer layer. This layer could not be distinguished using bright field or phase contrast microscopy (Fig. 18). TEM microscopy confirmed these layers (Fig. 19 d and e).

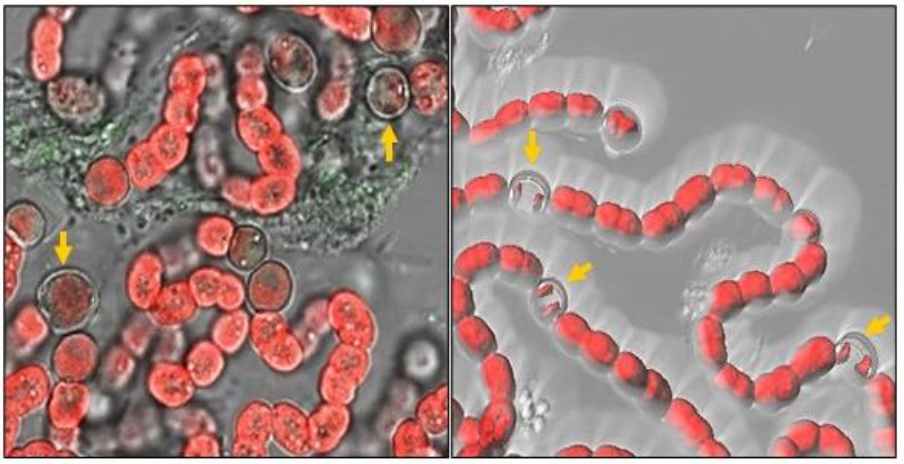


Fig. 18. Three-dimensional and CLSM microphotograph of *Nostoc* sp. UAB 206 under combination pH 9 and 160 mM NaCl concentration (fourth day after inoculation) revealed two layers around heterocyte.

2.3.6 Ultrastructural analysis

It was difficult to find a unique pattern in the internal thylakoid arrangement of the vegetative cells by increasing alkalinity and salinity

(Fig. 19a). The thylakoid organization in vegetative cells tended to scatter on the periphery of the cell's surface under 17 mM NaCl and pH 7 (Fig. 19a). Distribution of the thylakoids tended to a compact form and dense striate under pH 9 and 80 mM NaCl (Fig. 19b). Septa decreased by increasing pH and salinity and the filament changed from short rounded to barrel-shaped cells (Fig. 19c). The number of carboxysomes in the vegetative cells increased under 80 mM NaCl and pH 9 (Fig. 19b). Carboxysomes tended to migrate to the centre of the cells and decreased by increasing pH and salinity (pH 11 and 80 mM NaCl). Granules were observed to aggregate on the sides (adjacent to septa and margin) (Fig. 19c); while the density decreased at pH 9 and 80 mM NaCl. The granules were distributed centrally at pH 11 and 160 mM NaCl. We observed a regular expansion of granules in most cells under pH7 and different salinities. Vegetative cells were slightly thickened, striped and layered with a loose fibrillar outer envelope embedded in a mucilaginous matrix recognizable under high alkalinity and salinity (Figs. 19 a, b and c). Heterocyte were recognizable by layered and irregular thylakoid distribution. Moreover, they were less dense compared to vegetative cells (Figs. 19 e and d). The morphology of the heterocyte consisting of a thick cover that on the outside was dark and dense and on the inside white. This feature did not vary under any of the treatments. Also, the outer layer was a constant feature that was unaffected by different salinity and alkalinity levels.

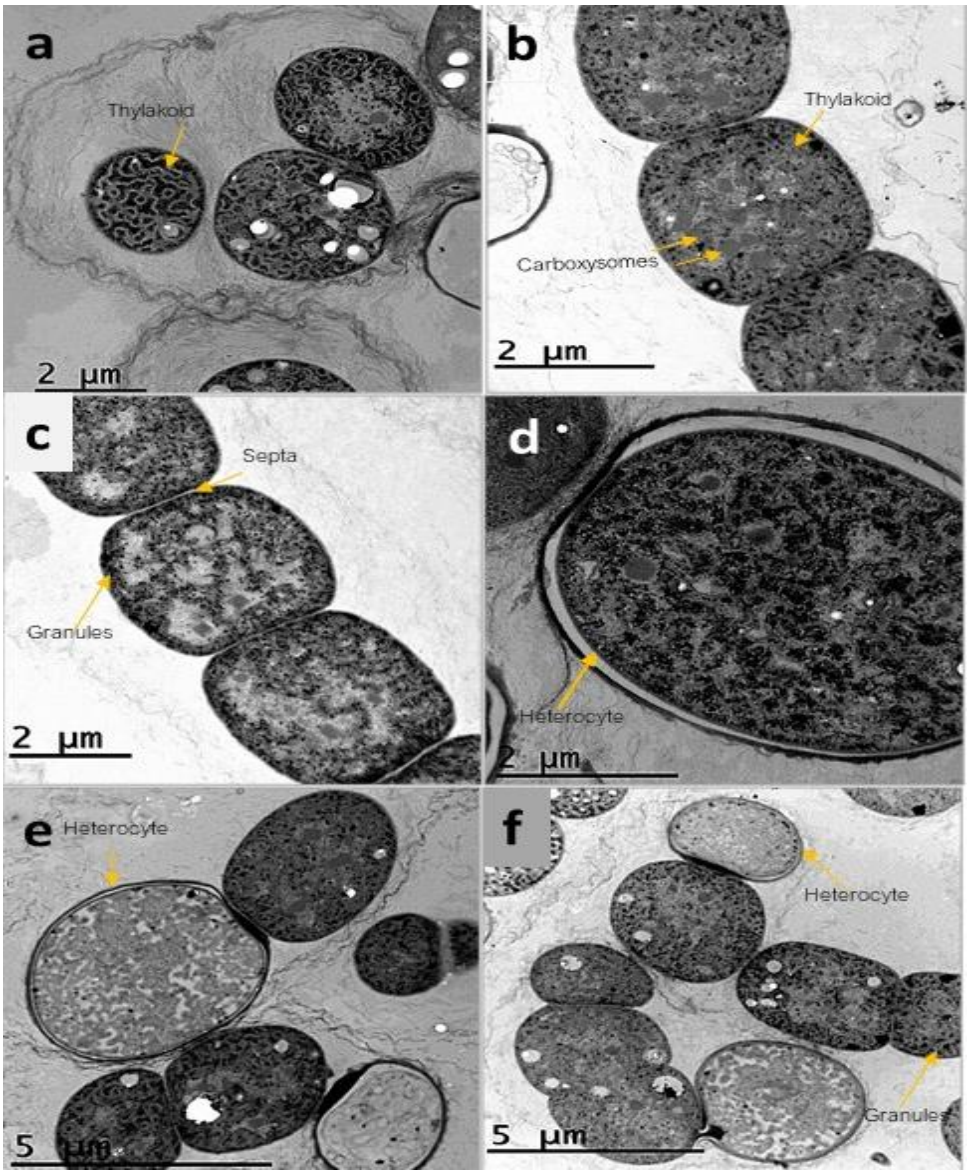


Fig.19. Transmission electron micrographs of *Nostoc* sp. UAB 206. (a) Thylakoid arrangement and expanded corrugated sheath of vegetative cells at pH 7 and 17 mM NaCl concentration (first day after inoculation). (b) the vegetative cell septa, carboxysomes arrangement almost central, glycogen granular tend to parietal clearly visible at pH 9 and 80 mM NaCl concentration (forth days after inoculation). (c) the vegetative cell septa, the thylakoid arrangement is not clearly visible at pH 11 and 160 mM NaCl concentration (forth days after inoculation). (d) Double-layered of heterocyte at pH 7 and 17 mM NaCl concentration (first day after inoculation), (f) the heterocyte thylakoid arrangement and glycogen granular at pH 9 and 80 mM NaCl concentration (forth days after inoculation).

2.3.7 Growth

To assess the effects of salinity and alkalinity on the growth of *Nostoc* sp. UAB 206, the optical density and Chl a concentration was monitored for 7 days (Table 6). The highest growth was observed under extreme alkaline conditions (pH 11) with 80 mM salinity (Table 6).

Table 6. Comparison of growth -Optical density OD 750 nm. Chl a concentration (µg mL^{-1}) of *Nostoc* sp. UAB 206 at different pHs (7, 9 and 11) and salinity (17, 80, 160 mM). Values are means ± standard deviation (n = 3).

	pH7			pH 9			pH 11		
	17mM	80mM	160mM	17mM	80mM	160mM	17mM	80mM	160mM
3rd day	0.076± 0.010^{a}	0.078± 0.005^{a}	0.074± 0.016^{a}	0.083± 0.004^{ab}	0.086± 0.031^{ab}	0.047± 0.008^{ab}	0.132± 0.017^{ac}	0.154± 0.037^{ac}	0.036± 0.002^{ac}
4th day	0.143± 0.021^{a}	0.139± 0.019^{a}	0.086± 0.011^{b}	0.202± 0.026^{ab}	0.158± 0.014^{ab}	0.105± 0.029^{ab}	0.274± 0.011^{ac}	0.182± 0.037^{ac}	0.046± 0.008^{ac}
5th day	0.202± 0.035^{a}	0.320± 0.031^{b}	0.179± 0.033^{c}	0.377± 0.017^{ab}	0.425± 0.038^{bc}	0.220± 0.032^{bc}	0.543± 0.089^{ac}	0.552± 0.102^{bc}	0.213± 0.052^{ac}

2.3.8 Photosynthetic activity

The long-term photosynthetic activity was measured daily as oxygen liberation, depending on the combination of pH, salinity and time (age of the cultures). The oxygen release increased from day 1 to day 3 at pHs 9 and 11 under all salinities ($p < 0.05$) (Table 7). However, under neutral pH, oxygen liberation increased after day three for all salinities. The oxygen liberation ranged from 39.16 ± 6.58 to 817.79 ± 80.98 µmol O_2 mg Chl^{-1} h^{-1} and was seriously affected by the age of cultures. Short-term photosynthesis measurements revealed that *Nostoc* sp. UAB 206 is not susceptible to photoinhibition even at high light intensities (1400 µE m^{-2} s^{-1}) and pH 11 (data not shown).

Table 7. Comparison of oxygen release (µmol O_2 mg Chl^{-1} h^{-1}) of *Nostoc* sp. UAB 206 at different pHs (7, 9 and 11) and salinity concentration (17, 80, 160 mM). The amount of liberated oxygen was normalized based on the chlorophyll concentration in each sample. Values are means ± standard deviation (n = 3).

O_2 liberation	pH 7			pH 9			pH 11		
	17mM	80mM	160mM	17mM	80mM	160mM	17mM	80mM	160mM
1st day	185.55 ±17.95 a	156.92 ±10.55 b	111.22 ±8.65c	179.68 ±15.23a b	39.16± 6.58ab	236.87 ±16.32a c	201.10 ±19.09a c	212.74 ±17.65a c	183.88 ±15.27a c
2sd day	182.54 ±13.75 a	187.21 ±15.22 a	205.75 ±29.25 b	208.83 ±19.68a b	144.55 ±11.78a b	228.53 ±19.68a b	261.25 ±16.95a c	232.87 ±15.75a c	192.17 ±14.95a b
3rd day	579.00 ±50.19 a	585.01 ±48.63 a	567.33 ±45.08 a	644.93 ±57.25a b	376.90 ±27.32a b	331.70 ±22.45a b	817.79 ±80.98a c	610.21 ±65.77a b	293.96 ±30.85a c

2.3.9. Photosystem (PS) and phycobilisomes (PBS).

Fluorometric analysis showed that the PBS activity was higher under pH 11 compared to pHs 7 and 9 (Fig. 20). The PBS and PSII activities at pH 11 and 80 mM NaCl were about 1.5 times greater than at pH 7. The increase of alkalinity from 7 to 9 also increased the activity of both PSII and PBS (Fig. 20 &21). The PSII activity was higher at pH 11 and 80 mM NaCl compared to pHs 7 and 9 under all salinity conditions (Fig. 21). CLSM revealed the PSI activity was higher at 80 mM NaCl and pH 9 compared to other salinities at day one and this trend was preserved over time (Fig. 22). The PSI activity showed the same trend at pH 11 (Fig. 22).

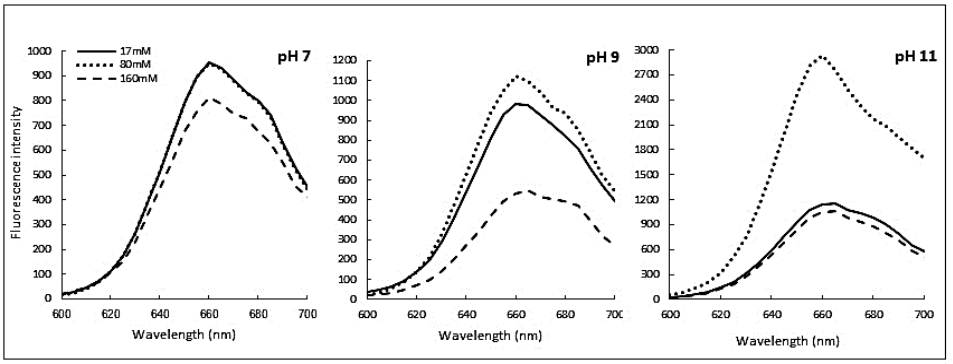


Fig. 20. Comparison of the fluorescence emission spectra of *Nostoc* sp. UAB 206 at different salinity and pHs after third day of inoculation. excitation 580 nm.

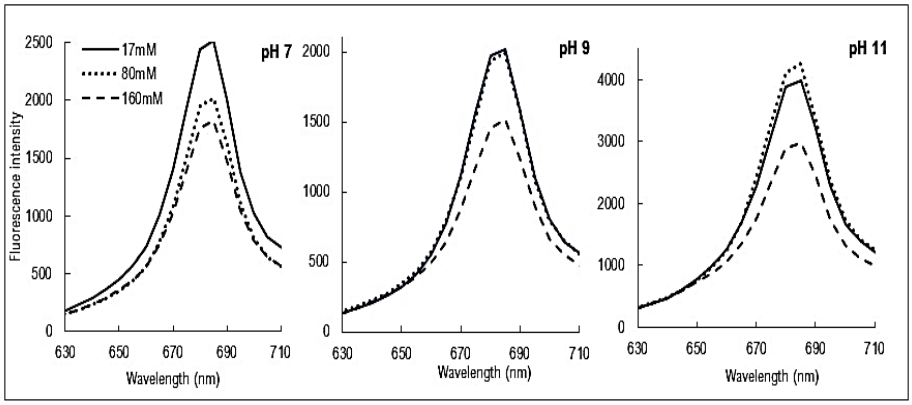
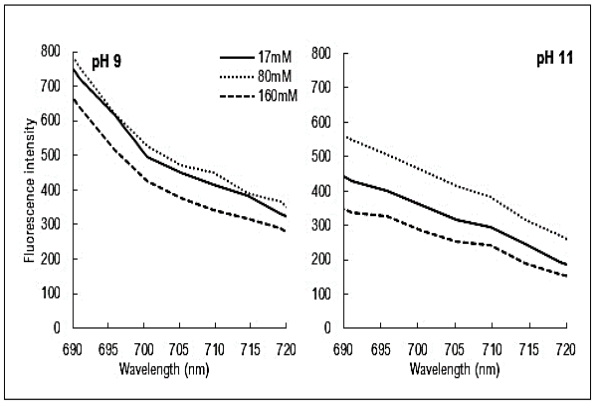


Fig. 21. Comparison of the fluorescence emission spectra of *Nostoc* sp. UAB 206 at different salinity and pHs after first day of inoculation. excitation 380 nm.

Fig. 22. Comparison of single cell fluorescence emission spectra (Lambda scan) of *Nostoc* sp. UAB 206 at different salinity and pHs after first day of inoculation. λexc= 405 nm.



2.3.10. Molecular analysis

The sequences of the 16s rRNA gene and the phycocyanine genes were aligned with those from other cyanobacteria from the databases (Fig. 23). Molecular analysis using 16s rRNAs and phycocyanin genes proved that the species belonged to the genus *Nostoc* (Fig 23 a and b).

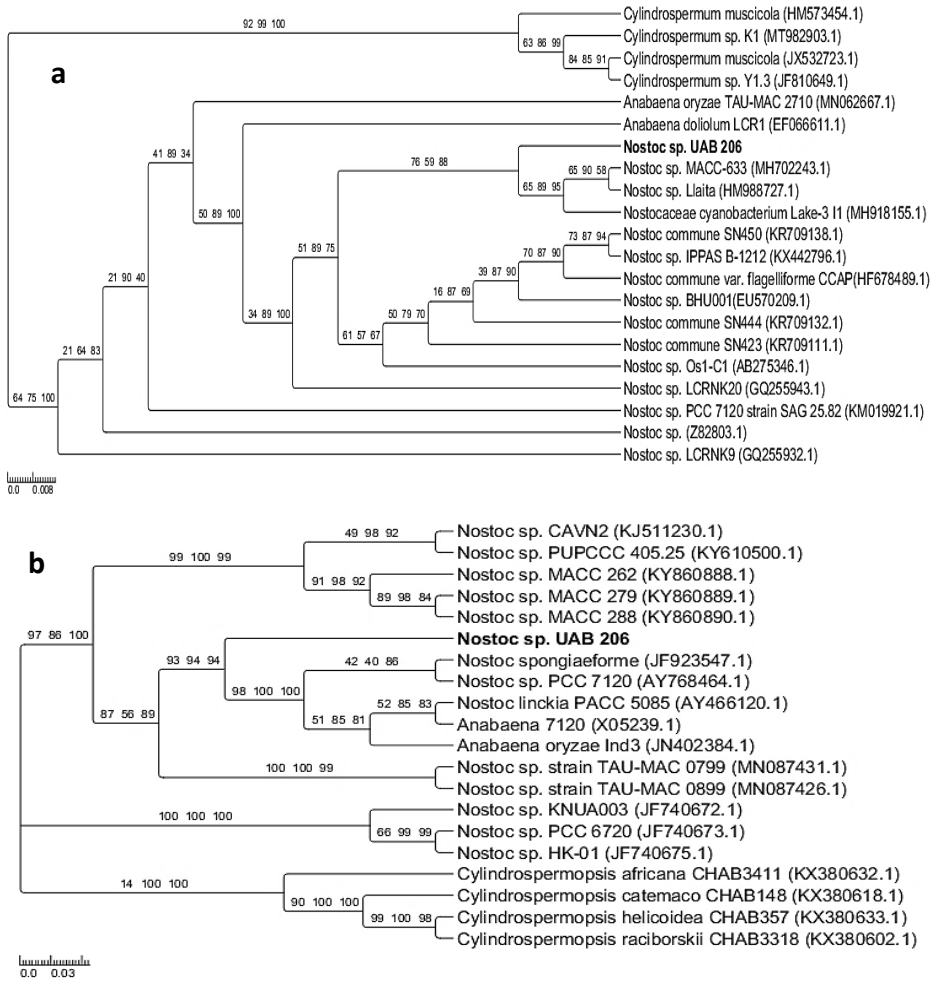


Fig. 23. Phytogenic tree of *Nostoc* sp. UAB 206 which presented Maximum likelihood tree. Numbers near nodes indicate percentage similarly of ML, NJ and MP methods respectively. Total 1000 bootstraps were performed. a) Phylogenetic relationship according to 16S rRNA sequences. b) Phylogenetic relationship according to Phycocyanin region sequences.

The phylogenetic analyses showed that *Nostoc sp.* UAB206 was clearly differentiated from Anabaena. According to 16s rRNA, our *Nostoc* sp. UAB 206 shares a common ancestor with *Nostocaceae* cyanobacterium Lake-3, *Nostoc* sp. MACC-633 and *Nostoc* sp. Llayta (Fig 23 a). According to the phycocyanine gene sequence *Nostoc* sp. UAB 206 evolved from a common ancestor from which also originated not only *Nostoc spongiaeforme*,

Nostoc sp. PCC7120, and *Nostoc linckia*, but also, on a different branch, *Anabaena* 7120 and *Anabaena oryzae* (Fig 23b).

2.4 Discussion

It is well known that morphological features alone do not reflect their evolutionary and phylogenetic relationships in cyanobacteria. After introducing molecular data, many cyanobacteria from different categories are proven as polyphyletic (Cai et al., n.d.; Jiří Komárek et al., 2014). Furthermore, cultivated strains of the same morphospecies, during transfer and long cultivation often significantly change in morphology, physiological properties and even genotypes under laboratory conditions (Jiří Komárek & Kaštovský, 2003; Mateo et al., 2011). This variation is an ability of cyanobacteria to adapt to environmental conditions and relies on the high genetic plasticity (Boussiba et al., 2000). But molecular biology is still not able to evaluate the environmental fluctuations and diversity of the cyanobacteria (Komárek, 2016; Mateo et al., 2011). Therefore, identification of cyanobacteria requires using a combined effect of morphology, ultrastructure and molecular indicators along with ecophysiological characteristic to evaluate stable traits.

Up to now, only few studies have focused on the effect of environmental fluctuation on the taxonomy and morphology of cyanobacteria. Our results from a polyphasic approach confirmed an unexplored strain, *Nostoc* sp. UAB 206 isolated from Mediterranean paddy field in Ebro Delta, related to: *Nostoc* sp. Vaucher, 1888, ex Bornet and Flahaul, strain UAB 206.

Our morphology description follows: edaphic; aggregations expanded, irregular, gelatinous; filaments mostly straight, long, interwoven, constricted at the cross walls; sheath thick with a transverse wall around the trichomes; statistical analysis reveals the strong effect of salinity on cell dimensions, the divergence of the dimension decreases by increasing salinity (isomorphism), decreasing salinity causes more divergence (paramorphism); apical cells conical, sometimes ellipsoid depend on the combination of salinity and alkalinity; vegetative cells square or

rectangular shapes (at pH7 and 17 mM salinity) oval and oval-cylindrical, granulate; akinetes spherical, oval and spherical, larger than vegetative cells, square or rectangular at 17 mM salinity and pH 7; heterocyte spherical, spherical-ellipsoid, larger than vegetative cells, with the visible polar nodules; hormogonia small, square, straight with tapered end cells. At ultrastructural level we observed regular arrangements of thylakoids in vegetative cells at pH 7 and 17 mM salinity (Fig. 19a); the number and distribution of the carboxysomes depends on salinity and pH; glycogen granules, distribution centripetal with increasing pH and salinity; thylakoids of the heterocyte less dense than in vegetative cells, two layers around heterocyte is a constant trait and unaffected by alkalinity and salinity fluctuations.

From the former observations, we observed that morphology characteristics of *Nostoc* sp. vary depending on the salinity and alkalinity and the age of culture. Mateo et al., 2011 observed varied morphological characteristics of trichome shape, cellular dimensions and branching in *Nostoc* sp which were dependent on the culture medium. Moreover, Berrendero et al., 2008 reported that *Rivularia* morphology varied in relation to nutrients' composition of the culture media. Our results of three-dimensional of confocal fluorescence microscopy and ultrastructure study (results not shown) revealed the appearance of true branching which increased number of branches at high pH and salinity (pH 11 and 240 mM NaCl). The occurrence of infrequent true branching was reported by (Dodds et al., 1995; Mateo et al., 2011; Mollenhauer, 1970) in this family.

To differentiate related taxa within the genus *Nostoc* is complex (Cai et al., 2019; Singh et al., 2016) and many genera using the polyphasic approach led to splitting from *Nostoc*. However, there are numerous limitations with molecular characterization and identification of *Nostoc* (Mateo et al., 2011). Using 16s rRNA sequence has a limited resolution to distinguish between cyanobacterial species. In the present study, diagnostic molecular biology described by using phycocyanin gene sequence along with 16s rRNA has revealed that this genus and species is related to

Nostoc. Especially complex is the separation between *Anabaena* and *Nostoc* both based on morphological and genetic data. An extensive analysis of 16S rRNA and rbcLX gene sequences on *Nostoc* and *Anabaena* strains of the Mosonmagyaróvár Algal Culture Collection (MACC) demonstrated that these genera are paraphyletic. Moreover, clearly revealed discrepancies between morphological and molecular results were found (Makra et al., 2019). Our results using the phycocyanin gene also confirm the paraphyletic character of *Nostoc* and *Anabaena*. Nonetheless, both the molecular approach and the morphology data definitely identify our strain UAB 206 as a *Nostoc* sp. UAB 206, clearly different from *Anabaena*. In the phylogenetic trees we found *Nostoc spongiaeforme* and *Nostoc* sp*.* Llayta close to our *Nostoc* sp UAB 206. *Nostoc spongiaeforme* has been described as a problem in paddy fields in California because big floating mats may damage the seedlings (Spencer et al., 2011). *Nostoc* sp*.* Llayta is a species occurring in the Andean wetlands (Galetovic et al., 2017). Contrastingly, *Nostoc sp*. UAB 206 is clearly separated from *Nostoc commune* including the variety flagelliforme which has been found in arid soils of SE Spain (Aboal et al., 2010). Our phylogenetic results in combination with the rather scarce information on the environmental conditions of the native habitats of the *Nostoc* sp. suggests that several wetlands adapted *Nostoc* sp. are relatively close from the evolutionary point of view.

Although previous studies (Fernandez - Valiente et al. 1998 and Poza-Carrion et al. 2001) reported that the optimum pH for growth, photosynthesis, and nitrogen fixation of terrestrial cyanobacteria from paddy fields in Spain was around 8 to 9, our comparison of growth curves showed that *Nostoc* sp. UAB 206 can significantly acclimatize to survive and grow at extreme alkalinity under laboratory conditions(pH 11) as well as under extreme conditions of salinity (320 mM NaCl significantly reduces but cannot stop the growth).

Our results show that increasing salinity (equal or above 80 mM NaCl) led to the activation of some cellular functions, such as growth and photosynthesis. Photosynthesis activity depends on the combination of

pH, salinity and time. We have seen that oxygen liberation increases after acclimatization to all salinities at pH 7 and 9, ranging between 39.16 to 817.79 µmol O_2 . $mgChl^{-1} \cdot h^{-1}$ depending on the age of the culture media. Moreover, this strain showed a resistance to photoinhibition at pH 11. Regarding photosystem activities, PSII activity varied with alkalinity conditions, being higher at pH 11 compared to pHs 7 and 9 at different salinities. PSI showed the highest activity at 80 mM salinity and pH 9. Phycobilisomes' activity depends on both salinity and alkalinity, being the highest activity at 80 mM NaCl at pH 9 and 17 mM NaCl at pH 11.

To the best of our knowledge, this is the first native paddy-fields' strain that can be considered as an alkaliphilic cyanobacteria and as a conclusion, a physiological description is suggested for this strain: alkaliphilic and extreme salinity resistant.

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Chapter 3: Ecophysiology (Combined effects of salinity and alkalinity)

3.1 Introduction

Cyanobacteria are a diverse and biologically versatile group of microorganisms that are widely distributed across terrestrial and aquatic environment (Diez and Ininbergs, 2014). Paddy fields provide a favourable environment for the colonization by cyanobacteria, which play a fundamental role in the biological cycle of the soil. They produce nitrogen and phosphorus, which are the most important nutrients for rice cultivation (Chittora et al., 2020; Munagamage et al., 2020). Under natural conditions in rice fields, cyanobacteria are exposed to the combined influence of several factors, including pH, irradiance, salinity, and temperature, which vary both during the day and over the crop cycle (Quesada and Fernández-Valiente, 1996; Bouazzara et al., 2020). Therefore, the survival of cyanobacteria in natural environments depends on their ability to acclimate to the fluctuating environmental conditions (Shokravi et al., 2014).

Soil salinity is an important factor that induces diverse alterations in the growth, photosynthesis, biochemical and physiological characteristics of cyanobacteria (Lee et al., 2021). Substrate pH is another factor that influences the abundance of cyanobacteria (Poza-Carrión et al., 2001). Most cyanobacteria grow in environments that are neutral to alkaline and, in laboratory cultures, the optimal pH ranges from 7.5 to 10 (Kaushik, 1994; Shokravi and Soltani, 2011). There is little information regarding the mechanisms of cellular survival, cell stability or growth of cyanobacterial cells under high alkaline conditions (Dwivedi et al., 1994; Jangir et al., 2021). Alkalinity and salinity can affect the structure and function of photosynthetic apparatus (electron transport system, PSI, cytochrome b6f and PSII complexes) and PBS activities. The effects of alkaline-salinity depend on the strain and other environmental conditions (Poza-Carrión et al., 2001; Inoue-Kashino et al., 2005; Abbasi et al., 2019).

Time is a further important factor influencing the efficiency of growth and photosynthesis of cyanobacteria. Few studies address the combined effects of environmental factors over time (Bouazzara et al., 2020; Jangir

et al., 2021). Nonetheless, there is evidence that under natural conditions in paddy fields stress effects on morphology and physiological activity can quickly manifest within less than 24 hours after stress onset (Abbasi et al., 2019). Moreover, there is increasing evidence that the effects of one environmental factor can be modulated by variations in other factors (Prosperi et al., 1992; Müller et al., 1993).

So far, poor information is available on the ecophysiology of native terrestrial cyanobacteria (croplands and paddy-fields) under different salinity and alkalinity conditions in the Ebro Delta (Spain). These largely unexplored bacterial community exposed to fluctuating alkaline saline conditions represent a rich opportunity for exploring adaptative responses at both the morphological and functional level.

Nostoc sp. UAB 206 is an unexplored cyanobacterium isolated from Mediterranean paddy fields in the Ebro Delta by the authors in 2019. This strain was selected among many other bacterial isolates form this area because of the prevailing characteristics of *Nostoc* species especially regarding nitrogen fixing activity (Dodds et al., 1995), which could be used to increase nitrogen input to rice paddies (Fernández Valiente et al., 2000) and their environmental stability (Ramírez et al., 2011).

This research aims to explore the morphological and physiological changes underlying acclimation and adaptation behaviour of *Nostoc* sp. (strain UAB 206) to different pH and salinity conditions in relation to the age of culture (short- and long-time conditions). We hypothesized that this *Nostoc* strain that in its natural habitat is exposed to fluctuating saline and alkaline conditions can respond to saline alkaline stress with adaptive changes in both photosynthesis and the growth patterns. This information will be useful not only for understanding fundamental biological responses to alkaline-saline stress in *Nostoc*, but also will provide valuable information for optimizing growth conditions for improving the photosynthesis and biomass production of the strain for possible future large-scale cultivation and economic programs.

3.2 Materials and methods

3.2.1 Collection and preservation of the strain

The strain was purified, identified, and described using multidisciplinary approaches (morphology, ultrastructure, physiology and molecular) based on the methods described in chapter 2.

3.2.2 Growth conditions

Stock cultures were grown in BG-011 liquid medium. The temperature was maintained at 28 ± 2 °C and culture was bubbled with supplementary aeration (Poza-Carrion et al. 2001). Light intensity was kept constant at an intensity of 100 µmol photon m^{-2} s^{-1}. The cells were harvested in an exponential growth phase and then transferred to culture media with different salinity and pH levels. The samples were treated with final NaCl concentrations of 17, 80, 160, 240 and 320 mM at different pH values (7, 9, 11). Culture media were buffered with 2.5 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) for pH 7 and 10 mM BTP (Bis-Tris Propane) for pH 9 and 11 adjusted to the desired pH with KOH (Shokravi & Soltani, 2011). Measurements were performed every day from the first to seventh day after inoculation.

3.2.3 Growth curves and analysis

Growth curves of the bacteria in different treatment media were analysed based on optical density (750 nm) according to Leganés et al., (1987), and Śliwińska-Wilczewska et al., (2019). Growth rates (µ) and doubling times (G) were calculated according to Li et al., (2014) and Khazi et al., (2018). Chlorophyll content was determined spectrophotometrically at 665 nm according to Marker, (1972).

3.2.4 Photosynthesis

Oxygen evolution was measured daily using Clark-type O_2 electrode at 28 ± 2 °C and under constant illumination at a quantum flux density of 100 µmol photon m^{-2} s^{-1} (Dhiab et al., 2007). Photosynthesis-Irradiance (P-I)

curves and parameters were calculated by measuring oxygen liberation rates during successive 2 minutes illumination periods with a stepwise increase from 0 to 1000 µmol photons m^{-2} s^{-1} (Inoue-Kashino et al., 2005). Parameters (P–I curves) were analysed according to (Henley, 1993), who gives:

$$P = P_m \cdot \mathrm{Tanh}(\alpha \cdot I/P_m) + R_d$$
$$I_k = P_m/\alpha \quad \text{and} \quad I_c = -R_d/\alpha$$

where I represent irradiance; P, photosynthetic activity at certain irradiance; P_m, light-saturated photosynthetic rate; R_d, dark respiration rate; I_k, saturating irradiance for photosynthesis; and I_c, light compensation point. The ascending slope at limiting irradiances, α, was calculated to assess the photosynthetic efficiency.
The amount of liberated oxygen was normalized by the amount of chlorophyll according to Poza-Carion et al. 2001.

3.2.5 Spectroscopy

The *in vivo* absorbance spectra were measured from 380 nm to 800 nm using a UV-2450 double beam spectrophotometer (SHIMADZU UV-2450). The absorbance spectra were normalized to biomass (OD 750) according to Tang & Vincent, (1999). Fluorescence emission and excitation spectra of the *in-vivo* cell were measured using a spectrofluorometer (TECAN-multimode microplate reader SPARK) at room temperature following the procedures in (Fraser et al., 2013; Tiwari & Mohanty, 1996). The fluorescence emission spectra were recorded from 630 nm to 760 nm. Excitation wavelengths were fixed at 440 nm and 550 nm. The 550 nm and 440 nm spectra were normalized on light intensity measured at 800 nm (Dhiab et al., 2007). The fluorescence intensity of a single cell was measured using the λscan of the confocal laser microscope system (Leica TCS-SP5 CLSM -Leica Microsystems Heidelberg GmbH, Mannheim, Germany). CLSM makes it possible to study different physiological processes, including the intensity of fluorescence emitted *in vivo* (as spectral unmixing) from single cyanobacteria cells (Grigoryeva & Chistyakova, 2020). Photosynthetic pigments excitation was carried out

with an argon laser at 405 nm. The fluorescence emission spectrum was collected by detection channels of wavelengths between 415 and 760 Software (Ramírez et al., 2011; Sugiura & Itoh, 2012). Analysis of the lambda scan data was carried out using the Leica LAS X v 3.7.0.20979 Confocal Software. The spectra were normalized to their maximum, and mean values and standard deviations were calculated.

3.2.6 Transmission Electron Microscopy (TEM)

TEM images were acquired using the same technique described in chapter 2. Images were taken daily in different combinations of salinity and alkalinity.

3.2.7 Statistical analysis

Data were analysed using one-way Analysis of Variance (ANOVA) with the SPSS-24 155 software. The ANOVA showed a significant difference between treatments with $p < 0.05$. All the experiments were carried out in four replicates and data are presented as mean values of four independent replicates. The contour plot of the MATLAB software was used to study the data relationships.

3.3 Results

3.3.1 Growth curves and analysis

Comparison of the growth curves of *Nostoc* sp. UAB 206 under the different culture conditions revealed that extremely alkaline conditions (pH 11) were most favourable significantly stimulating biomass production in comparison to neutral pH conditions (Fig. 24). Optimal growth was achieved under a combination of pH 11 in presence of 17 or 80 mM NaCl and under pH 9 combined with 80 mM NaCl (Fig. 24). Contrastingly, high salinity (≥160 mM) reduced the bacterial growth. Detailed growth rate analysis demonstrates that the combination of high alkalinity and relatively low salinity (pH 11 and 80 mM NaCl) increased growth rates and decreased the cell duplication time (Table 8). The

observed growth rate variation was mainly pH dependent and only extreme salinity (320 mM NaCl) had inhibitory effects.

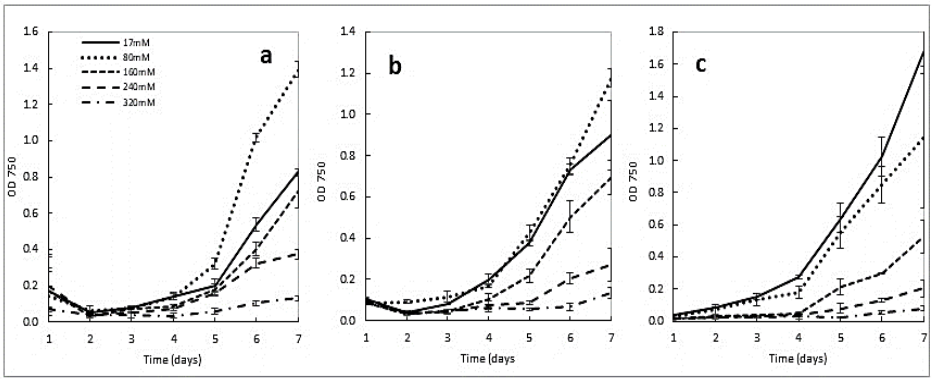

Fig. 24. Variation of optical density (OD 750 nm) of *Nostoc* sp. UAB 206 under different salinity (17, 80, 160, 240 and 320 mM NaCl) and alkalinity (pH 7, 9, 11) over 7 days. Line with error bars show significant difference at $P < 0.05$. (a) pH 7, (b) pH 9 and (c) pH 11.

Analysis of the growth rates showed that combined extreme alkaline and the relatively low salinity (pH 11 and 80 mM) increased growth rates and decreased the cell duplication time (Table 8). The growth rate variation was pH dependent and was not greatly affected by salinity. Comparison of growth curves, specific growth rates (μ) and doubling times (G) demonstrated that this strain is both an alkaliphile and relatively saline tolerant.

Table 8. Comparison of specific growth rate (μ) and doubling times (G) of *Nostoc* sp. UAB 206 at different salinity (17, 80, 160, 240, 320 mM NaCl) and pHs (7, 9, 11). Values are means biological three replicates ± standard deviation.

	Specific growth rate (μ)			Doubling times (G) hours		
Nacl	**pH 7**	**pH 9**	**pH 11**	**pH 7**	**pH 9**	**pH 11**
17 mM	0.665±0.040 a	0.644±0.009 a	0.771±0.018 b	25.107±0.008 a	25.832±0.089 b	21.571±0.054 c
80 mM	0.926±0.031 a	0.799±0.034 b	0.940±0.033 a	18.710±0.006 a	20.367±0.025 b	17.696±0.054 c
160mM	0.772±0.016 a	0.785±0.034 a	0.659±0.031 b	21.548±0.018 a	21.193±0.054 b	22.248±0.027 c
240mM	0.751±0.009 a	0.569±0.023 b	0.580±0.041 b	22.151±0.018 a	32.071±0.048 b	28.681±0.012 c
320mM	0.577±0.016 a	0.438±0.023 b	0.332±0.006 c	33.838±0.043 a	41.062±0.087 b	50.134±0.012 c

3.3.2 Photosynthetic parameters

The results of the photosynthetic activity of *Nostoc* sp. UAB 206 under the combination of pH, salinity and age of cultures revealed that oxygen evolution increased significantly from the third to fourth day at pH 11 exposed to 17- and 80-mM NaCl (Fig. 25). Growth and photosynthetic activity increased from day 3 to 4 at pH 11 in combination with 80 mM NaCl. The results of long-term photosynthesis measurements confirmed these results regarding the growth curve and growth analysis (Fig. 24 and Table 8). The contour plot visualization shows the relationship between growth, photosynthesis, and age of cultures at different pH and salinity levels (Fig. 26). A significant correlation was observed between growth and photosynthesis at pH 11 independently of salinity. Growth and photosynthesis activity increased from day 3 to 4 at pH 11 in combination with 80 mM salinity. The results changed over time. Noticeably, the operation of photosynthesis and growth decreased in all treatments on the second day after inoculation.

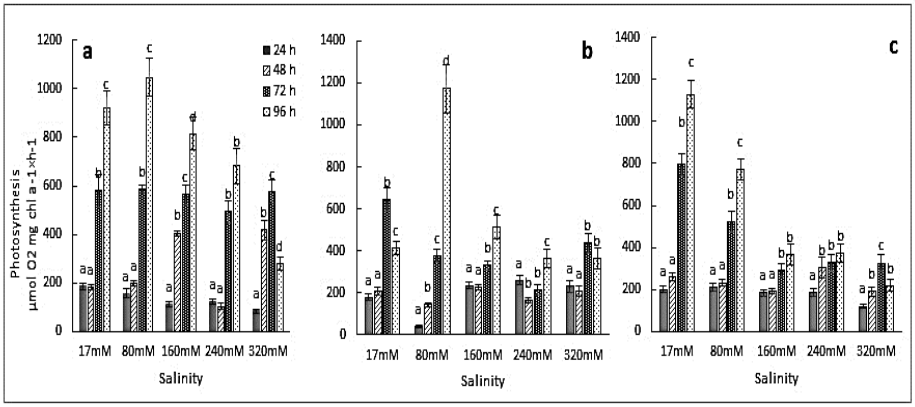

Fig. 25 Comparison of oxygen release (daily) (µmol O_2 mg $chl^{-1} \cdot h^{-1}$) of *Nostoc* sp. UAB 206 at different salinity, pHs and time. Bars with error bars show significant difference at $P < 0.05$. (a) pH 7, (b) pH 9 and (c) pH 11.

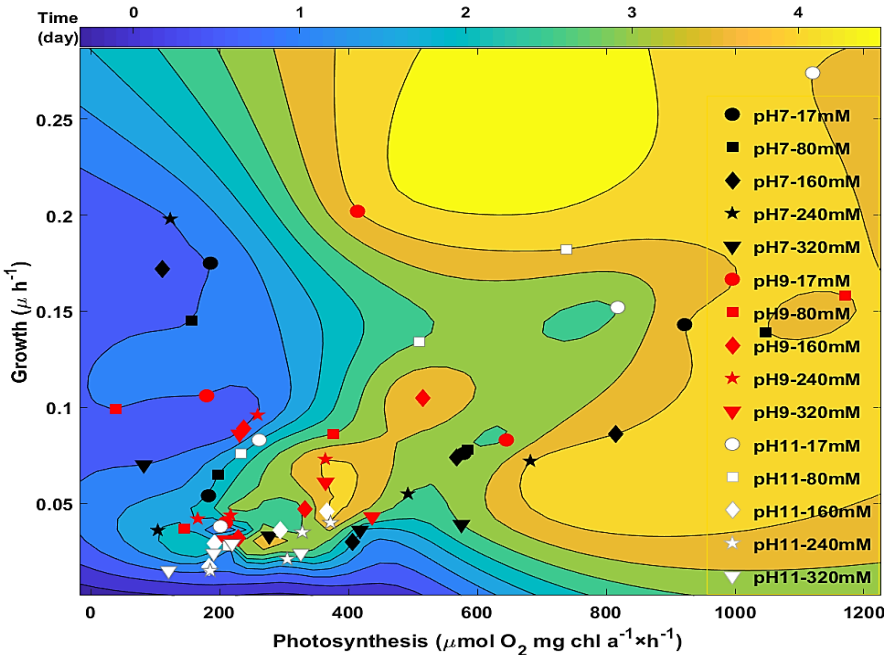

Fig. 26. Contour plot of *Nostoc* sp. UAB 206 presented the relation between growth, photosynthesis and time under different levels of salinity and alkalinity in the culture medium. Each data point in the plot represents specific salinity and pH.

3.3.3 Photosynthesis-irradiance (P-I) curves

The results of Photosynthesis-Irradiance (P-I) curves at pH 11 (the optimum pH) and different salinities is summarized in Table 9. The combined effects of salinity and alkalinity illustrated that *Nostoc* sp. UAB 206 was not susceptible to photoinhibition- even at high light intensities (up to 1000 µE m^{-2} s^{-1}) at pH 11 and 17 mM salinity (Fig. 27). Maximum light-saturated photosynthesis activity (P_{max}) was higher at 17 mM salinity, while increasing salinity resulted in a reduction in P_{max} (Table 9). Increasing salinity caused an increase in the quantum efficiency of photosynthesis (α) ($p < 0.05$), which led to acclimation to limited light intensity under relatively low salinity (≥80 mM NaCl). The pattern of saturating irradiance (I_k) was similar to that of photoinhibition, which was susceptible to salinity. Collectively, the resistance to photoinhibition decreased at high salinity (≥160 mM NaCl) at pH 11 and led to a significant reduction in O_2

liberation. Furthermore, the resistance to photoinhibition seems to contribute to the ability to survive under low-light intensity.

Table 9. Comparison of photosynthetic parameters of *Nostoc* sp. UAB 206 under different salinity (17, 80, 160, 240 mM NaCl) at pH 11 after 72 hours. Values are means biological three replicates ± standard deviation.

NaCl (mM)	**The maximum photosynthetic rate (P_{max})** (µmol O2 mg chl−1 h−1)	**photosynthetic efficiency (α)** µmol O2 mg chl-1 h-1)/ (µmol photon m^{-2} s^{-1}	**light saturation point (I_k)** (µmquanta.$m^{-2}s^{-1}$)
17	57.41±2.95 a	0.727±0.05 a	600
80	49.34±1.98 b	0.869±0.07 b	600
160	45.45±2.98 c	1.054±0.95 c	600
240	36.31±1.35 d	1.072±0.09 c	600

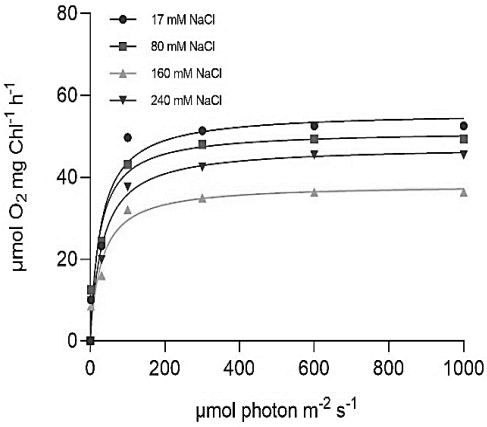

Fig. 27. Influence of different salinity (17, 80, 160, 240 mM NaCl) on Photosynthesis-Irradiance (P-I) curves of *Nostoc* sp. UAB 206 at pH 11 after four days.

3.3.4 Absorption spectra and fluorimetry

Comparison of the *in vivo* absorption spectra revealed that the main peaks correspond to chlorophyll blue (~ 440 nm), chlorophyll red (~ 680 nm) and phycocyanin (~ 620 nm) in all the treatments (Fig. 28). In addition, a shoulder appeared at 495 nm that was related to carotenoids. Our results revealed that the dynamism of photosystems (PSII) and light-harvesting

complexes (PBS) depended on the salinity at all pH levels and were in concordance with the growth curves. Results are shown in Fig. 28 for pH 11 only. Again, relatively low salinity (80 mM NaCl) led to a significant increase of the whole spectrum. Although the hights of the absorbance varied depending on the salinity, it is remarkable that salinity did not cause any peak shifts in the absorbance spectra (Fig. 28).

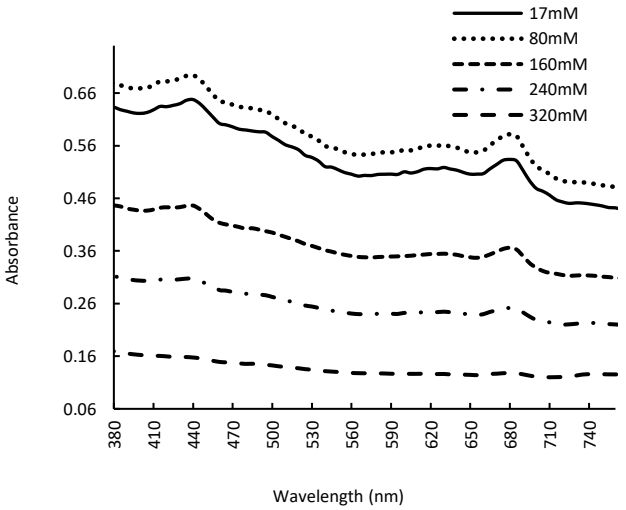

Fig. 28. The effect of different salinity concentrations and pHs on the *in vivo* absorbance of *Nostoc* sp. UAB 206 normalized to optical density (OD 750) after four days on inoculation.

The fluorescence emission analysis showed that the PBS activity was higher at pH 11 independent of salinity (compared to pH 7 and pH 9) (Fig. 29). PBS activity increased at 80 mM salinity at both pH 9 and 11. While, higher salinity concentration (≥160 mM) led to decreased PBS activity after four days of inoculation. These results (Fig. 29 a & c) confirmed that all cell activities drastically decreased at the second day after inoculation (Fig. 26). Time passing (3 day) led to an increase in PSII activity. Collectively, relatively lower salinity (80 mM) led to a significant increase in PBS and PSII activity at pH 11, which caused an increase in biomass production, growth, and photosynthesis.

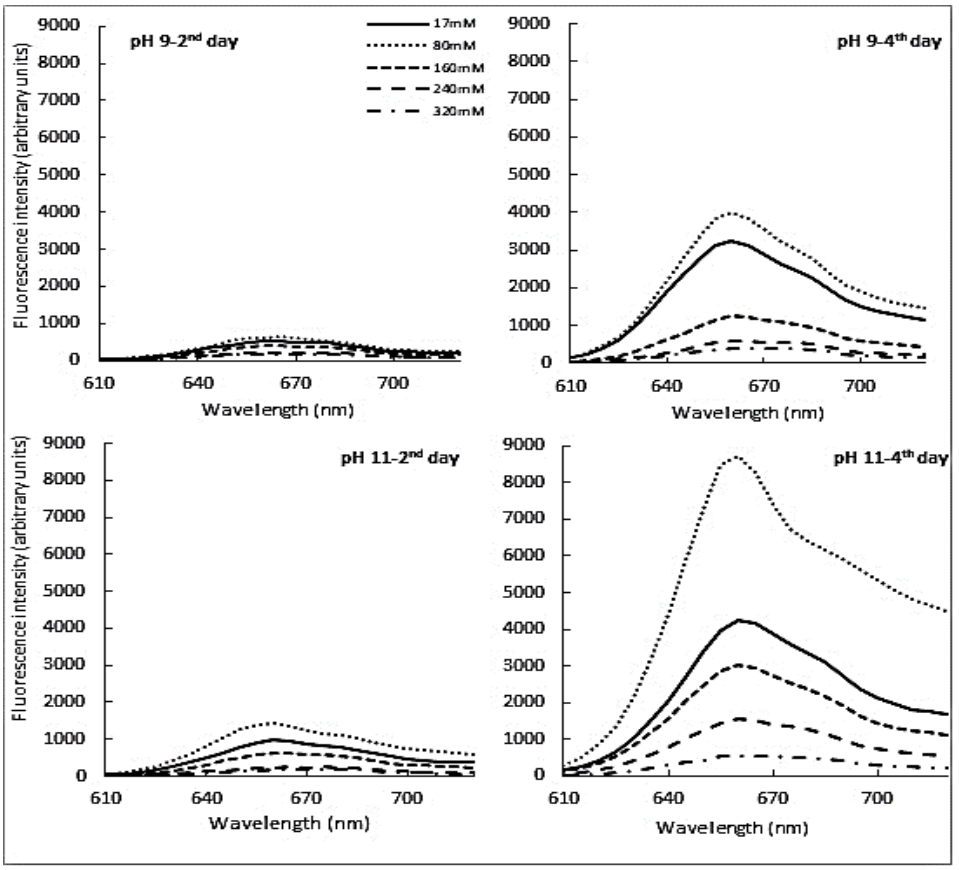

Fig. 29. Comparison of the fluorescence emission spectra of *Nostoc* sp. UAB 206 at different salinity and pHs after second and fourth day of inoculation. Excitation 550 nm.

Using CLSM enables us to study accurately the intensity of fluorescence emitted from a single cell as spectral unmixing and the distribution of excitation energy between the two photosystems (PSI and PSII) and PC (Fig. 30). In the single cell study (lambda scan), no significant difference was observed between the effects of 17 and 80 mM NaCl on PSI and PSII at pH 9 compared to pH 11(Fig. 30 a). Moreover, high salinity (160 µM NaCl) did not cause a severe reduction in photosystem activities at pH 9. PBS activity decreased with increasing salinity (≥ 80 mM) at pH 9 after four days. PBS activity was higher at pH 11 than at pH 9, regardless the salinity level (Fig. 30 b). At pH 11, we observed the maximum and minimum activities of both photosystems at 80 and 240 mM NaCl, respectively. High

salinity (≥160 µM NaCl) caused a decrease in the efficiency of both PSII and PSI after four days of exposure.

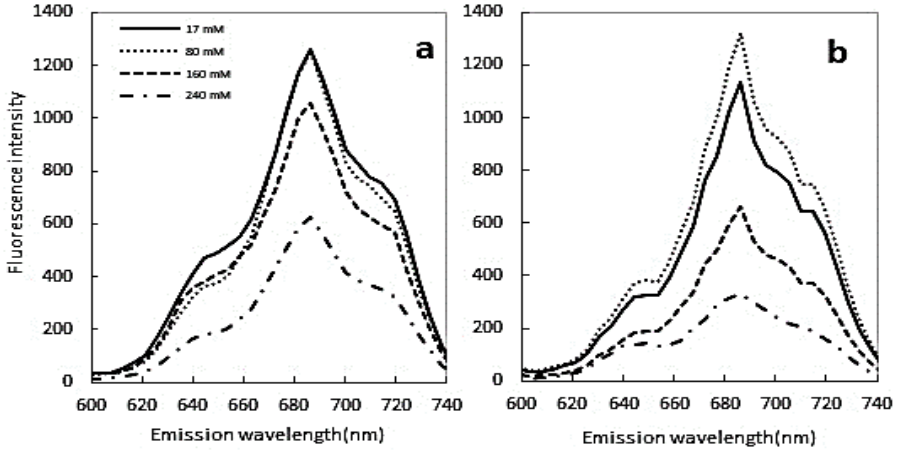

Fig. 30. Fluorescence spectra of individual cell (Lambda scan) of *Nostoc* sp. UAB 206 at different salinity and pHs after four day of inoculation. λexc= 405 nm. (a) pH 9 and (b) pH 11.

3.3.5 Exopolysaccharide production and dimension

Exopolysaccharides (EPS), a metabolic strategy for survival protecting cells under stress conditions, are among the most important compounds synthesized by cyanobacteria (Cruz et al., 2020). Although there is little evidence of the particular function of cyano-EPS in nature, cyano-EPS may play numerous physiological and ecological roles (Cui et al., 2017; Kamennaya et al., 2012). We observed that the dimension and viscosity of EPS (varied in the vicinity of the cell), were influenced by the combination of salinity, alkalinity, and the age of cultures (Fig. 31). The dimension of EPS was significantly higher at a salinity of 80 mM and pH 11 after four days of inoculation (Fig. 31c). EPS production decreased at pH 7 and pH 9 by adding > 80 mM salinity (Fig. 31 a & b). Our results revealed a direct relationship among growth, photosynthesis activity, and production of EPS in this strain. It seems that a higher growth rate and photosynthesis (80 mM salinity-pH 11) caused higher EPS production, which may be related to survival and cellular activities under these conditions.

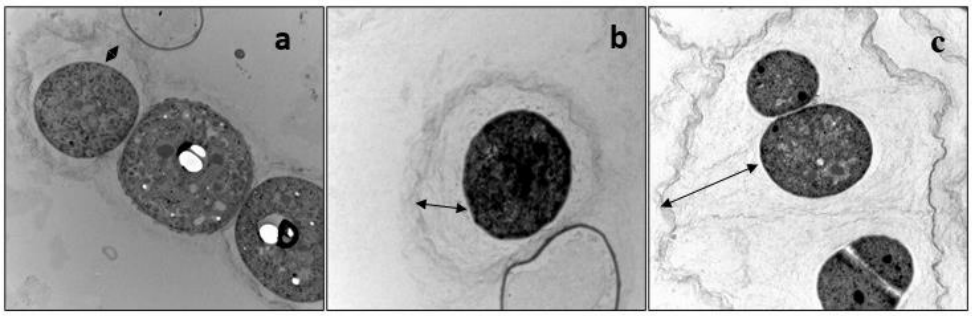

Fig. 31. Transmission electron microscopy (TEM) photomicrographs of exopolysaccharide production of *Nostoc* sp. UAB 206 under different salinity and alkalinity condition. black arrows show continuous outer layer and fibrillar sheath. Scale bar 5 µm. (a) Exopolysaccharide production at combine pH 7-17 mM after 1st day of inoculation, (b) pH 9 -80 mM-4th day, (c) pH 11 -80 mM- 4th day.

3.4 Discussion

To date, there are only few studies on the ecophysiology of free-living cyanobacteria related to the effect of environmental factors such as salinity and alkalinity in Mediterranean paddy fields in Spain. Most studies have been confined to the ecology and ecophysiology of microbial mats (Benito et al., 2015; Prado et al., 2017; Seder-Colomina et al., 2013) and previous works (Fernandez Valiente & Leganes, 1990; Poza-Carrión et al., 2001) reported that pH about 8-9 is optimum for growth, photosynthesis, and nitrogen fixation of cyanobacteria in paddy-fields.

Most of the studies on native cyanobacteria in Spanish paddy fields have been performed at fixed time (Mateo et al., 2011; Poza-Carrión et al., 2001). However, time-course studies of growth can better reveal the balance between photosynthesis and respiration (Nygård & Dring, 2008).

The behaviour of cyanobacteria under different salinity and pH conditions is highly variable. Amirlatifi et al., (2018) and Abbasi et al. (2019) showed that different combinations of salinity and alkalinity affected the growth and biochemical characteristics of *Calothrix* sp. and *Fischerella* sp. They observed that pH 11 decreased the growth rates of these cyanobacteria at salinities higher than 80 mM NaCl. In cyanobacteria, increasing pH

generally activates some cellular functions, such as growth rate and cell yield (de Souza Santos et al., 2011), enzyme activity (P. Li et al., 2013), biosorption (El.Din, 2017), resistance to oxidative stress (Summerfield et al., 2013) and protection (Pathak et al., 2018). Only few specialized groups of microorganisms may consider such an environment as optimal for growth and survival. Krausfeldt et al., (2019) revealed a strong and positively correlation between cyanobacterial abundance and pH. Singh et al., (2015) reported that the optimum growth and carbohydrate content of *Nostoc calcicola* was at 500 mM NaCl at pH 10.5 after 20 days, while above 500 mM NaCl, cell growth and carbohydrate content decreased. Contrastingly, Shamim et al., (2017) found that the growth of *Nostoc muscorum* stood in the lag phase for six days under 10 mM NaCl, while at higher doses (15 and 20 mM) of NaCl it did not show any growth even after 10 days.

These differences among *Nostoc* species may reflect the salinity level of the native habitats where they have evolved. Our *Nostoc* sp UAB 206 coming from paddy fields periodically invaded by sea water prefers a moderate salinity concentration (80 mM NaCl) as seen by significant increase in growth, biomass production and operation of different parts of the photosynthetic machinery when compared to the 17 mM NaCl treatment. In contrast, the highest salinity levels (240 and 320 mM) exceeded the tolerance limit of the strain causing severe growth inhibition (Fig. 24).

Our results are in line with Dhiab et al. (2007), who found that the increase in salt concentration enhances the growth, photosynthetic efficiency, and light-saturated maximum photosynthetic activity of *Arthrospira platensis*. Regarding the higher growth and photosynthesis at pH 11, *Nostoc* sp. UAB 206 displays a strong flexibility of its metabolic mechanisms with no reduction of activity at salt concentrations higher than 80 mM NaCl. This confirms the ability of *Nostoc* to acclimatize to extreme environmental conditions relying on the high genetic plasticity of this species (Boussiba et al., 2000).

Comparison of growth curves, specific growth rates (μ) and doubling times (G) demonstrated that *Nostoc* sp. UAB 206 is both alkaliphile and relatively saline tolerant (Fig. 24 and Table 8).

In alkaliphilic cyanobacteria, under deficiency of carbon dioxide, bicarbonate ions can be a major source of inorganic carbon for photosynthesis in cyanobacteria (Boyd, 2015). The carbon concentration mechanism (CCM) is the key process that enables them to adapt to alkaline conditions (Klanchui et al., 2017). Five different modes of inorganic carbon transport in cyanobacteria have been reported (Poschenrieder et al., 2018). The intracellular accumulation of inorganic carbon occurs against a concentration gradient and is activated and energized by light through photosynthesis (Young & Beardall, 2005), which requires a high activity of PSI, PSII and phycobilisomes (Mangan & Brenner, 2014). Bicarbonate uptake and fixation of this inorganic carbon by photosynthesis can be an effective way to maintain intracellular pH despite high external pH values.

In our experiment, the fluorescence emission analysis showed that the PBS activity was higher at pH 11 independently of salinity (Fig. 29). The strong relationships between growth, photosynthesis, and the age of the cultures (Fig. 26) indicate that in our case growth and photosynthesis were increased at pH 11 when compared to pH 9. Although, pH 9 is considered optimal in some respects (Fernandez Valiente & Leganes, 1990; Poza-Carrión et al., 2001).

In the present study, increasing salinity at pH 11 at 72 h of treatment caused decrease in P_{max} of *Nostoc* sp. UAB 206, besides the higher quantum yield (α). Inoue-Kashino et al., (2005) reported in *Synechococcus* 6803 that the decline of α value generally, depends on three processes: a decrease in the relative content of PS II, a decrease in the antenna size of PS II, and a decrease in the water oxidation rate in individual PS II. Our observation of the decrease of P_{max} with increasing salinity can be attributed to this low efficiency of water oxidation in PS II under evaluated salinity. Ye & Gao, (2004) observed the highest values of P_{max} and dark

respiration of *Nostoc flagelliforme* at 20 mM NaCl after 48 hours of incubation, while photosynthetic efficiency significantly decreased at salinity levels higher than 20 mM NaCl. Dhiab et al., (2007) reported that 250 mM NaCl enhances photosystem II activity compared to photosystem I, photosynthetic efficiency, and light saturated maximum photosynthetic activity. Nevertheless, in our case, dark respiration and compensation points decreased. These contradictory results might be attributed to different genetic and environmental factors.

Changes in the distribution of excitation energy from light harvesting complex to PSII and from PSII to PSI varied depending on the NaCl concentrations and pH. We observed that the dynamism of the photosystems and phycobilisomes, as the main parts of energy and matter production, enhances at low salinity (80 mM) at pH 11. The 80 mM salinity led to a noticeable increase in all parts of phycobilisome, chlorophyll and protein production and PSII activity at pH 11, which caused a more stable structure and better coupling energy by PBS. In other words, our findings show that low salinity (17 and 80 mM NaCl) increased the strength and flexibility of phycobilisomes at pH 11 within 4 days. In contrast, increasing salinity causes regular decline in the coordination between energy production and transfer sectors (PSII and phycobilisome). This regular decrease pattern was similar for pH 9, although no significant differences were observed between 17 and 80 mM NaCl. This effect can be attributed to an increase in the energy transference between the phycobilisomes and PSII; in consequence the PSII fluorescence emission peaks are enhanced (Fig. 29 and 30). The highest rate of energy transfer from phycobilisomes to PSII occurred at pH 11. This should be interpreted as an increased production of ATP and reducing power that can enhance main processes like nitrogen fixation, which is manifested in a significant growth enhancement; similar observations have also been reported by Fernandez-Valiente and Leganes, (1990) and Soltani et al., (2006). Furthermore, our results agree with Ding-ji et al., (1992) who found that in *Nostoc flagelliforme*, a salinity-tolerant strain, photosynthetic activity increases with increasing salinity. However, the role of age of the cultures

would be important. Srivastava et al., (2008) observed a salinity-induced inhibition of *Anabaena doliolum* where PSI, PS II and whole electron chain activities declined after 24 h exposure to 150 mM NaCl and pH 7.5.

The higher growth rate and photosynthesis under 80 mM salinity at pH 11 caused higher EPS production, which may be related to survival and cellular activities under these conditions. Although, there is little evidence of the particular function of cyano-exopolysaccharides (CEPS) in nature, CEPS can play numerous physiological and ecological roles (Cui et al., 2017; Kamennaya et al., 2012). Salinity and alkalinity are the most important factors that influence the dimension, structure, versatility, and release of CEPS production of cyanobacteria (Steele et al., 2014).

Our results show a positive relationship between the increasing CEPS dimensions and growth and photosynthesis at pH 11. Although salinity has a still controversial effect on EPS synthesis, some studies have shown that hyperproduction may be a counteraction against saline stress. Among other functions, CEPS secretion can constitute a metabolic strategy for survival, growth, and protection of cells under stress conditions, and are among the most important compounds synthesized by cyanobacteria (Cruz et al., 2020, Rossi and De Philippis, 2015). The increasing extracellular layer can be due to acclimation of cyanobacteria that occurs in response to physiological-morphological responses. Our results agree with those of Mazor et al., (1996) and Chamizo et al., (2020), who found a similar trend between the increase in chlorophyll a in cyanobacteria-inoculated soils and an increase in CEPS. Bemal & Anil, (2018) found that the soluble and attached CEPS production of *Synechococcus* CCAP1405 increased with salinity and age of the culture, which means they can survive under salinity stress conditions.

The present study indicates that the combination of environmental factors (salinity and alkalinity) plays a key role in the growth process of *Nostoc*. Comparison of growth curves, specific growth rate, and generation time shows that *Nostoc* sp. UAB 206 can significantly acclimatize to survive and grow under extreme alkaline conditions (pH 11)

and relatively high salinity under laboratory conditions. Such a capacity of acclimation may be essential for performance of this *Nostoc* strain under the fluctuating saline-alkaline conditions of the paddy fields in the Ebro delta where sea water intrusion frequently occurs. This extreme alkaliphilic and relatively saline tolerant cyanobacterium is a good candidate for future experiments to evaluate its potential in biotechnological applications.

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Chapter 4: Rice-cyanobacterium interaction under different Nitrogen and salinity conditions.

4.1 Introduction

This chapter aims first to select suitable rice varieties for salinity stress and tolerance studies based on agronomic characteristics and, second, to characterize the rice-cyanobacterium interaction under different Nitrogen and salinity conditions in a salt sensitive variety.

One of the challenges facing agriculture today is producing enough food for a steadily increasing population (Iniesta-Pallarés, 2021). Rice is one of the world's important crops, providing a portion of staple food for nearly half of the global population (FAO, 2004; Lu and Snow, 2005); rice is also highly produced and consumed in Spain (AEE 2020 web.pdf (mercasa.es). Rice yield frequently is limited due to various abiotic stresses, such as drought, salinity, cold, and heat during cultivation (Quin et al., 2020). Among these adverse conditions, salinity is one of the most important abiotic stresses. Salinity affects almost all physiological plant processes by causing both osmotic stress and ionic toxicity, as well as nutrient deficiencies. These adverse factors lead to growth inhibition and hamper rice yield (Khan et al., 2016; Quin et al., 2020). However, rice shows different reactions and mechanisms for salinity tolerance at different life stages. Soil salinity stresses plants in two ways. High concentrations of salts in the soil make it harder for roots to extract water due to the low osmotic potential, and high concentrations of salts within the plant can be toxic, especially due to Na^+ ion interfering with membrane potential and metabolic processes (Khan et al., 2016).

A better understanding of the selection of proper salt-tolerant rice varieties is essential for an effective use of saline land and support sustainable agriculture - economically and environmentally friendly - which can lead to alleviating the world food crisis.

There are several factors to consider choosing a rice variety including (a) adaptability to local climatic conditions (b) duration or maturity (c) tolerance to drought, problem soils, water submergence, and pests. To study salt tolerant rice varieties, the best criteria for proper selection can be agronomic characters based on adaption and growth under salt stress

conditions (Anshori et al., 2018). Therefore, in this hydroponic study we focused our selection of the rice cultivar on several traits that can be considered as a tolerance index (Hariadi et al., 2015; Safitri et al., 2017; Anshori et al., 2018).

The excessive use of chemical fertilizers in paddy field has generated several environmental problems, resulting in degradation of soil structure and acidification of water that, can be tackled by use of biofertilizers (Chittapun et al., 2018). Biofertilization, an inoculation of beneficial soil heterocystous cyanobacteria that are of great relevance in the global carbon and nitrogen cycles, plays an essential role in providing nutrient for plants, regulating the dynamics of organic matter, and enhancing soil biological activities (Chittapun et al., 2018). Consequently, advantages of using cyanobacteria in rice fields include: (a) increase soil porosity and produce adhesive substances; (b) excretion of phytohormones (auxin, gibberellins, etc.), vitamins and amino acids; (c) improve the water holding capacity of soil through their characteristic jelly structure; (d) Increase in soil organic matter after cyanobacterial death and decomposition; (e) decrease in soil salinity; (f) control of weed proliferation; (g) availability of soil phosphate by excretion of organic acids; (h) efficient absorption of heavy metals on the microbial surface (bioremediation) (Saadantia et al., 2009; Chittora et al., 2020).

Most paddy soils have a natural population of cyanobacteria which provides a potential source of nitrogen fixation at no cost for the plant and the farmer (Saadatnia and Riahi, 2009). The paddy field provides a favourable environment for cyanobacteria which are photosynthetic organisms, exhibit abundant growth and limited growth requirements even under stressed environmental conditions. As pointed out by Saadatnia and Riahi (2009), the abundance of cyanobacteria in rice fields has been reported since the beginning of the last century by Fritsch (Fritsch, 1907) and already in 1935, Bannerji, introduced culture studies. The importance of photosynthetic and nitrogen fixing cyanobacteria as a fertility source of rice fields was first recognized by De (1939).

4.2 Material and Methods

4.2.1 Selection experiment

Previously to the rice-algae study, we proceeded to select a suitable, salt sensitive rice cultivar among the varieties grown in the Ebro Delta.

The departing varieties were Cendra, Pinyana, Guars, Bomba, Mare, and Copsemar provided by Cooperativa d'Arrossaires del Delta de l'Ebre (Deltebre, Tarragona, Spain. We further included Nipponbare, kindly provided by Blanca Sansegundo (Centre de Recerca en Agrigenòmica, Bellaterra, Spain) as a reference variety.

4.2.2 Seed germination and hydroponic culture

Rice seeds were disinfected with 1% NaOCl for 15 minutes followed by further washing several times with tap water. Seeds were wrapped in moistened filter paper and kept for 7-10 days at room temperature until small roots and shoots appeared. Uniform 7-10 days old plants -reaching 10-15 cm height were transferred to 2 litre plastic containers covered with aluminium foil paper and filled with Yoshida (Yoshida et al., 1976) solution (Table 10). Three plants per container were used.

Plants were grown under controlled environmental conditions in a growth chamber with light/dark regime of 16/8 h, temperature 23±2°C with constant aeration and a light intensity of 190 µmol photon m^{-2} s^{-1}. Prior to the treatments, plants were acclimatized for 7 days under control conditions (17mM NaCl). Then the different NaCl amounts were added (17, 50 and 100 mM). Nutrient solution was changed weekly.

Growth performance was assessed using root and shoot length as a marker, measured weekly until day 21 after starting the treatment. Collected data were used to select the cultivar employed in the cyanobacteria-plant interaction experiment.

Table 10. Composition of the Yoshida nutrient solution (Yoshida et al., 1976)

Yoshida Solution (Base medium)					
	Bottle #	**Compound**	**Stock solution (prepare 1L)**	**Volume of stock solution in 60 L**	**Final concentration (mM)**
			g/L	**mL**	
Macronutrient	**1**	NH4NO3	114.3	60	10
	2	K2SO4	89.4	60	10
	3	KH2PO4	115.5	60	10
		K2HPO4	21.4		
	4	CaCl2·2H2O	110.8	60	10
	5	MgSO4·7H2O	202.6	120	20
Micronutrient	**6**	MnCl2·4H2O	18.801	6	1
		(NH4)6·Mo7O24·4H2O	0.927		
		H3BO3	11.68		
		ZnSO4 ·7H2O	0.437		
		CuSO4·5H2O	0.39		
	7	Na2EDTA	7.44	106.8	17.8
		FeSO4	5.56		
	pH = 6.8				

	Salt treatments				
Salt	**Bottle #**	**Compound**	**g/L**	**mL in 2.5 L**	**Final concentration**
	8	NaCl	292.2	0	0 mM
				25	50 mM
				50	

4.2.3 Main experiment (rice-algae interaction. Hydroponic culture (Salinity and Nitrogen)

The Yoshida nutrient solution was prepared in two different batches by adding 50 and 100% NH_4NO_3 (referred to Yoshida's listed amount). Culture took place as described in the precedent section. After rice was acclimatized and grown under these conditions, NaCl was added to the Yoshida mediums in increments of 25 mM per 24 hours up to the salinity level of 50 and 100 mM. The control solution contained a low NaCl concentration of 17 mM to facilitate performance of the cyanobacteria. After transferring the seedlings of the selected cultivar (Copsemar), to the

solutions, a very dense and aggregated amount of *Nostoc* sp. (strain UAB 206) was added to the solutions of the +cyano treatment while no bacteria were added in the -cyano treatment. The experimental treatments are outlined in table 11. *Nostoc* sp. (strain UAB 206) was isolated from Mediterranean paddy fields and has been described in detail at chapter 1, 2 and 3.

Table 11: Treatments' outline

Copsemar	- Algae	50%N	17 mM
			50 mM
			100 mM
		100%N	17 mM
			50 mM
			100 mM
	+ Algae	50%N	17 mM
			50 mM
			100 mM
		100%N	17 mM
			50 mM
			100 mM

4.2.4 Analysis of growth and other stress indicators

Shoot land root length were measured weekly with a ruler. After 21 days chlorophyll contents were analysed, and chlorophyll fluorescence measurements were performed (see below). The plants were harvested, and samples were prepared for the analysis of sodium and potassium tissue concentrations.

4.2.5 Chlorophyll fluorescence measurement

Chlorophyll fluorescence measurement is a fast, non-destructive, and relatively simple technique for detecting the energetic/metabolic imbalance of photosynthesis due to environmental stress (Araus et al., 1998). A plant's photosynthetic potential is directly proportional to the quantity of chlorophyll present in the leaf tissue (Schlemmer et al., 2005). Different fluorescence parameters like minimal fluorescence (Fo), maximal fluorescence (Fm), variable fluorescence (Fv = Fm - Fo) and maximum photochemical efficiency of PSII (Fv/Fm) were measured by PAM modulation chlorophyll fluorescence analyser (miniPAM II, ZARGES). Prior to the measurements, the leaves were dark-adapted by aluminium foil paper for 30 min.

4.2.6 Chlorophyll content measurement

The chlorophyll content is a fundamental parameter to understanding a plant's response to the environment in which it resides (Schlemmer et al., 2005). Furthermore, chlorophyll content per unit leaf area is considered to be a good indicator of healthy photosynthetic tissues, photosynthetic rate, and maximum photosynthetic activity (Rosyara et al., 2007). Chlorophyll content was measured with a SPAD (CCM-300 OPTI-SCIENCES) on the same leaves used for chlorophyll fluorescence.

4.2.7 Analysis of mineral content in root and shoots

0.5 g of dried samples were introduced in special digestion flasks and pre-digested overnight with a mixture of 3 mL HNO_3 60% and 1 mL H_2O_2 30% (analytical grade). Then they were digested at 110ºC for 2 h and allowed to cold at room temperature. Volume was set to 15 mL with H_2O (MilliQ, Millipore-Waters) and filtered thru a 0,45 µm filter and diluted to 1/%. Elements were analysed by ICP-OES. For Cl, the digested material obtained as described above was measured with a Chloride selective electrode type 15 213 3000 (Metler-Toledo AG, Switzerlad).

4.3 Results

4.3.1 Preparatory experiment (selection of rice cultivars for salinity tolerance)

Depending on the growth stage, rice shows differences in salt tolerance. It can be quite tolerant during germination and tilling stages but very sensitive at the 1-2 leaf and flowering stages (Akbar and Ponnamperuma, 1982; Jones and Stenhouse, 1983; Yoshida, 1981). There are several selection methods developed for assessing salinity tolerance in rice, such as germination (Pradheeban et al., 2014), hydroponic cultures (Ali et al., 2014; Mondal and Borromeo, 2016), and use of saline soils (Safitri et al., 2016) but although over time rice has developed certain mechanisms of adaptation to salinity (Khan et al., 2016), evaluating the salinity tolerance is a complex task because the above mentioned variation in sensitivity to

salt during the life cycle.

In the present experiment we used several cultivars of rice grown in hydroponic culture and tested for 3 different salinity conditions (17, 50 and 100 mM NaCl). A set of parameters were employed to evaluate the possible tolerance or resistance to salinity (root and shoot length, chlorophyll concentration and chlorophyll fluorescence -Fv/Fm-. As root and shoot length provided best distinction of the differential responses of the rice cultivars to salinity only these data are shown (Figs. 33 and 34).

Among the cultivars, BOMBA and NIPPONBARE exhibited most vigorous shoot growth under control conditions, while Mare was the smallest cultivar (Fig. 33). Root elongation under control conditions was similar in all Spanish cultivars reaching highest length in NIPPONBARE and lowest in PINYANA (Fig. 34).

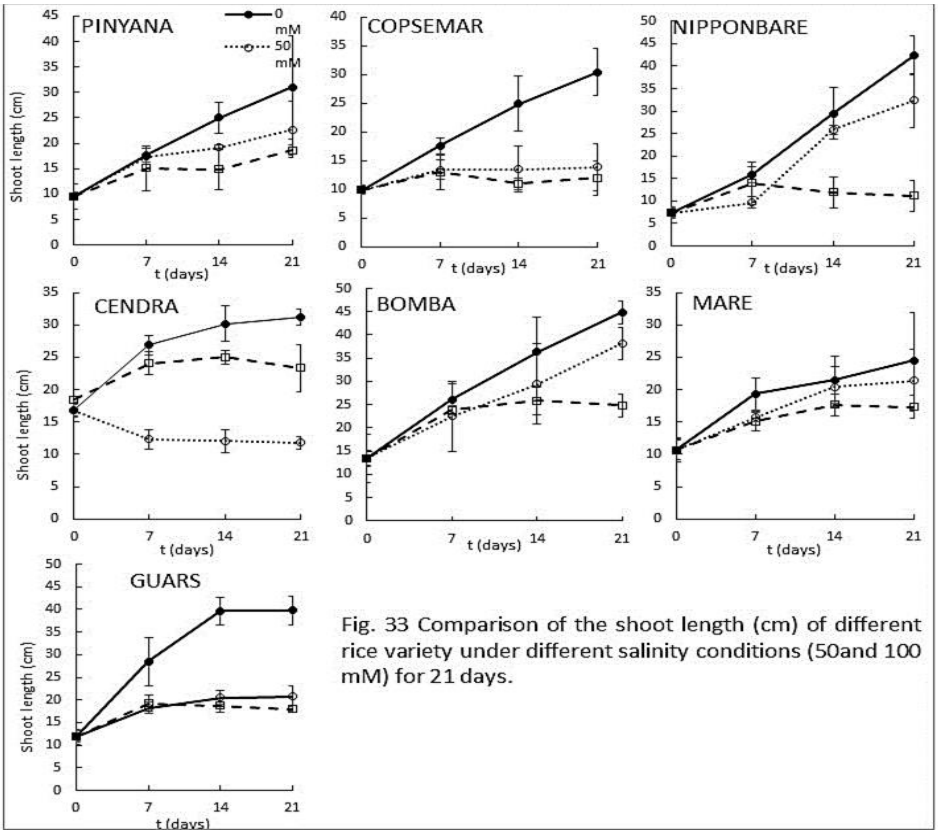

Fig. 33 Comparison of the shoot length (cm) of different rice variety under different salinity conditions (50and 100 mM) for 21 days.

Exposure to salinity reduced shoot and root elongation in all cultivars, excepting in the slow growing cultivar MARE which remained almost unaffected by the 50 mM NaCl treatment. Also, in the slow-growing cultivar PINYANA the salt-induced growth reduction was small. It is well established that slow growth can be a favorable trait in stress tolerance avoiding death and favoring survival. However, fast growing tolerant genotypes are more desirable in crop breeding (Maggio et al., 2018). Among the fast-growing cultivars, BOMBA, followed by NIPPONBARE, were most salt tolerant. Their shoot and root length values were less affected by the 50 mM NaCl treatment than in the other varieties. Contrastingly, 100 mM NaCl stopped shoot elongation after one week of exposure. Shoot elongation in cultivars COPSEMAR and GUARS was already severely affected by 50 mM NaCl and both cultivars can be classified as highly salt sensitive. As COPSEMAR is among the highest yielding rice cultivars (Pla, 2016) we selected this cultivar for studying interaction with *Nostoc* sp. strain UAB 206 in order to see a possible influence on salt tolerance.

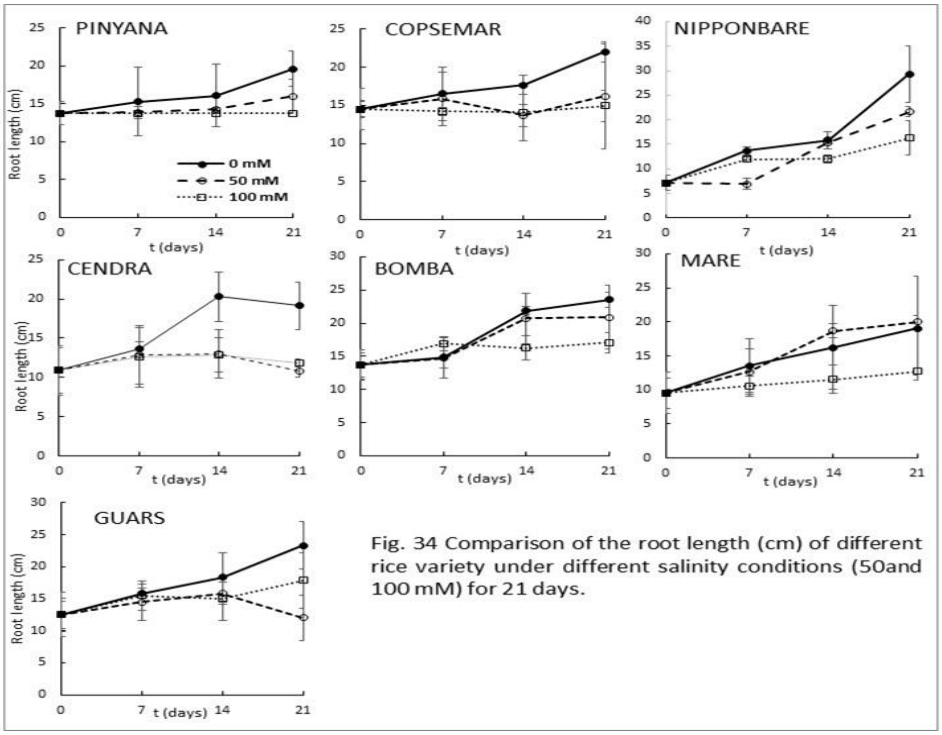

Fig. 34 Comparison of the root length (cm) of different rice variety under different salinity conditions (50and 100 mM) for 21 days.

4.3.2 Main experiment. Influence of *Nostoc* sp. on rice under different nitrogen and NaCl supply

The selected salt sensitive rice cultivar Copsemar was used to analyse the possible influence of the cyanobacterial strain *Nostoc* sp. UAB 206 on its response to salinity using different stress markers such as growth, chlorophyll content, efficiency of photosystem II and Na^+ and K^+ accumulation.

The measured parameters were root and shoot length, total chlorophyll content, and Fv/Fm at days 1, 3, 6, 11 and 14. The Na and K tissue concentrations were analysed in the plant material that was harvested at the end of the experiment on day 14.

Under normal N supply (100% N) and absence of *Nostoc* sp. (-cyano), shoot and root growth curves of cultivar Copsemar (Fig. 35) were similar to those observed in the preliminary screening experiment (Figs. 33 & 34). This confirms the repetitiveness of the conditions and the validity of the measures obtained.

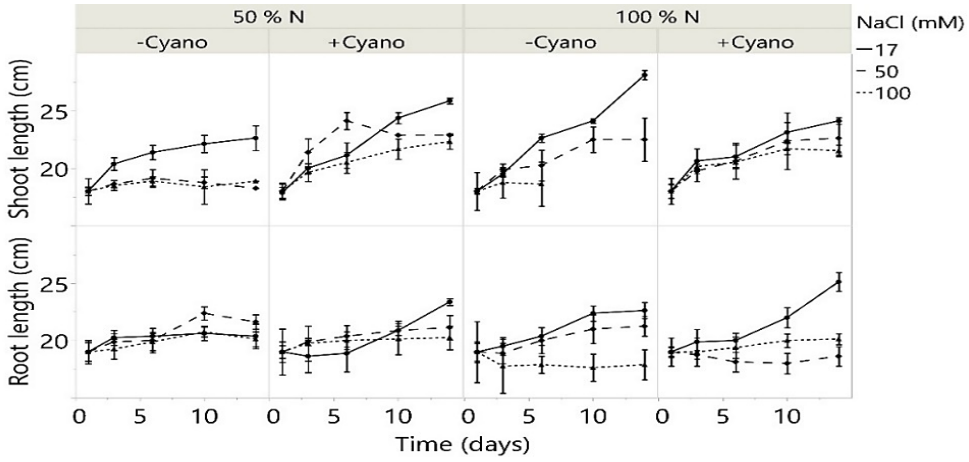

Fig. 35: Shoot and root length of rice cultivar Copsemar exposed to different nitrogen(N) and salt supply in presence or absence of *Nostoc sp*. UAB206

In absence of *Nostoc* sp., plants grew better under full nitrogen supply (100% N of Yoshida solution) than under the 50% N treatment. This was the case both under control and 50 mM NaCl. While full N supply did not

improve growth under strong salinity provided by the 100 mM NaCl treatment.

In the presence of *Nostoc* sp., shoot elongation was favoured in plants with low N supply. This positive effect of the cyanobacteria was observed not only for plants receiving 17 mM NaCl, but also for the higher salinity levels (50 and 100 mM). In plants with the full N supply, comparison of the +cyano and -cyano treatments revealed ameliorated shoot elongation under highest salt exposure (100 mM NaCl, but rather an inhibition under the control treatment (17 mM NaCl).

Salt exposure had a strong negative effect on leaf chlorophyll concentrations in all treatments (Fig. 36). Presence of *Nostoc* sp. slightly improved the chlorophyll concentrations in leaves of plants exposed to salinity under low N supply. No such effect was observed in the 100% N treatment. Under sufficient N supply even a decrease in chlorophyll concentrations occurred in plants exposed to 17 mM NaCl for 11 or 14 days.

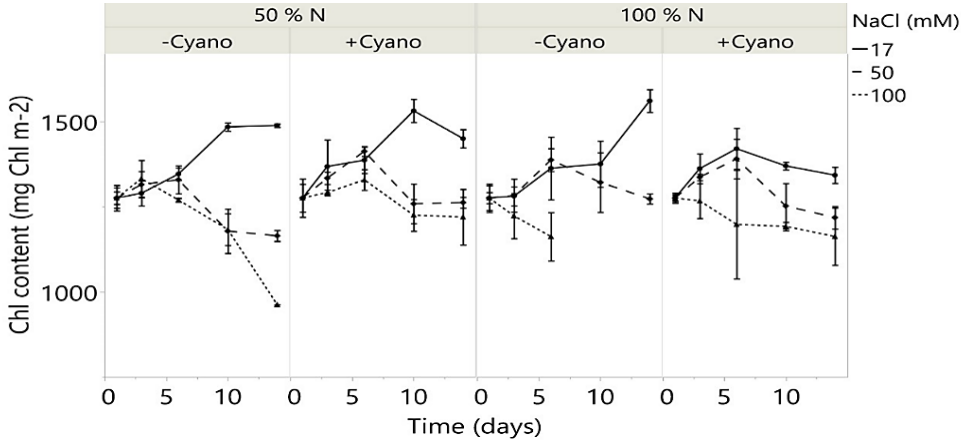

Fig. 36. Evolution of chlorophyll concentrations in rice leaves under different salinity conditions (17, 50 and 100 mM NaCl), N supply (50 and 100%) and presence (+Cyano) or absence (-Cyano) of algae in the nutrient solution.

The maximum quantum yield at photosystem II (Fv/Fm) (Fig. 37) was substantially decreased by salinity. This detrimental influence was observed after more than 6 days exposure to 50 mM NaCl, but after only

1 or 3 days in the 100 mM NaCl treatment. The presence of *Nostoc* sp. UAB 206 (+ cyano) had a small positive influence on Fv/Fm in plants exposed to high salinity. No such effect was observed under the full N supply conditions.

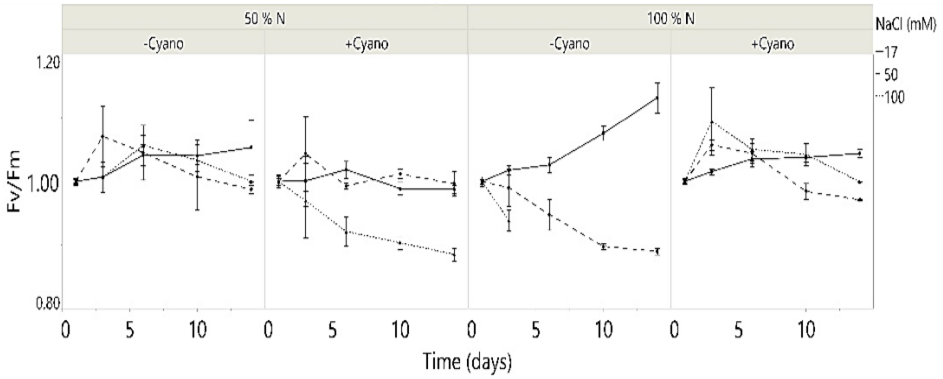

Fig. 37. Evolution of Fv/Fm under different salinity conditions (17, 50 and 100 mM NaCl), N supply (50 and 100%) and presence (+Cyano) or absence (-Cyano) of algae in the nutrient solution.

As expected, salt supply enhanced Na concentrations in both roots and shoots of the rice plants (Fig. 38). Both N supply and cyanobacterial presence had an influence of Na accumulation. Presence of *Nostoc* sp. UAB 206 enhanced the shoot Na concentrations in the 50% N treatment. Contrastingly, under full N supply Na concentrations in shoots of -cyano plants were higher than in +cyano plants. This was especially pronounced in the 100 mM NaCl treatment.

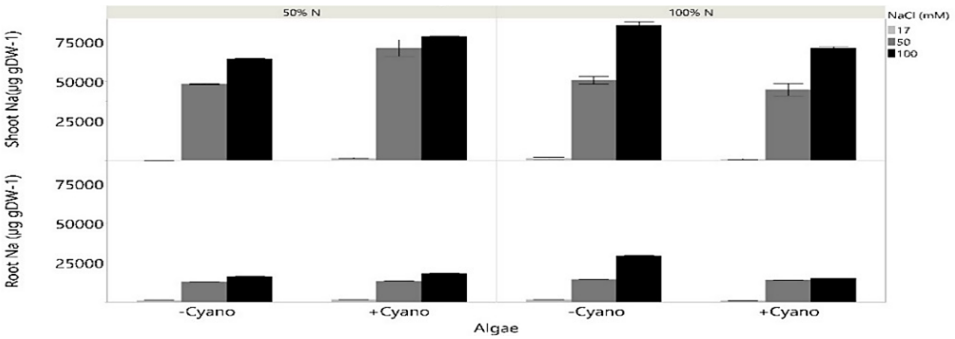

Fig. 38. Na content at day 14 of growth under different salinity conditions (17, 50 and 100 mM NaCl), N supply (50 and 100%) and presence (+Cyano) or absence (-Cyano) of algae in the nutrient solution.

Exposure to high NaCl concentrations had a strong negative influence on the K concentrations both in roots and shoots of all plants (Fig. 39). However, plants receiving high N supply maintained higher K concentrations under salt stress than plants growing in the 50% N solutions. The + cyano treatment slightly enhanced K concentrations in roots and shoots of plants exposed to 100 mM NaCl with 50% N and in the corresponding roots of those receiving 100% N.

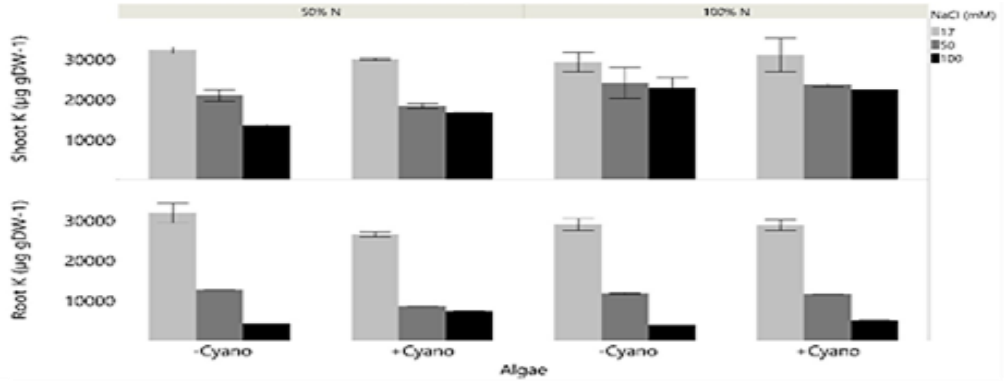

Fig. 39. K content at day 14 of growth under different salinity conditions (17, 50 and 100 mM NaCl), N supply (50 and 100%) and presence (+Cyano) or absence (-Cyano) of algae in the nutrient solution.

The presence of *Nostoc* sp. UAB 206 in the culture medium containing 100 mM NaCl substantially decreased the Na/K ratios in the roots of the rice plants (Fig. 40).

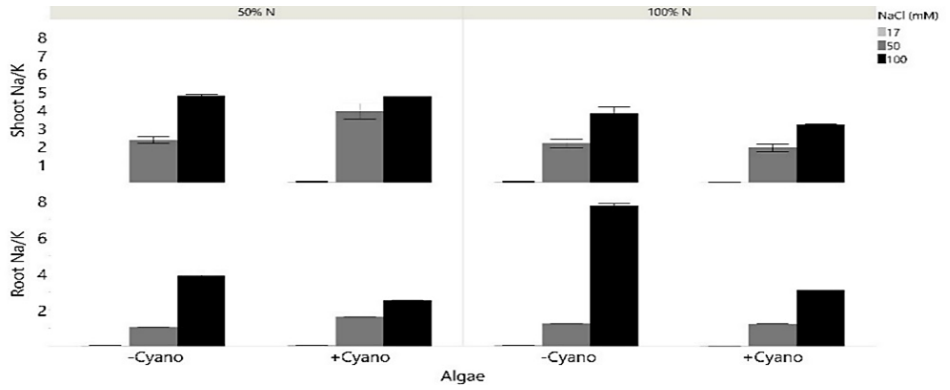

Fig. 40. Na/K ratio at day 14 of growth under different salinity conditions (17, 50 and 100 mM NaCl), N supply (50 and 100%) and presence (+Cyano) or absence (-Cyano) of algae in the nutrient solution.

Besides K, salinity also influenced the root and shoot concentrations of other essential macronutrients such as Ca, Mg, P and S, but no clear influence of the cyanobacterial treatment was observed (data not shown).

Contrastingly, among the micronutrients, Fe and Zn concentrations revealed some specific features (Figs. 41 & 42). Exposure to high salinity increased root and shoot Fe and Zn concentrations under most treatments. The most interesting effect of the +cyano treatment was the lowering of the root Fe concentrations and the enhancement of shoot Fe levels under high salinity in comparison to the -cyano treatment (Fig. 41).

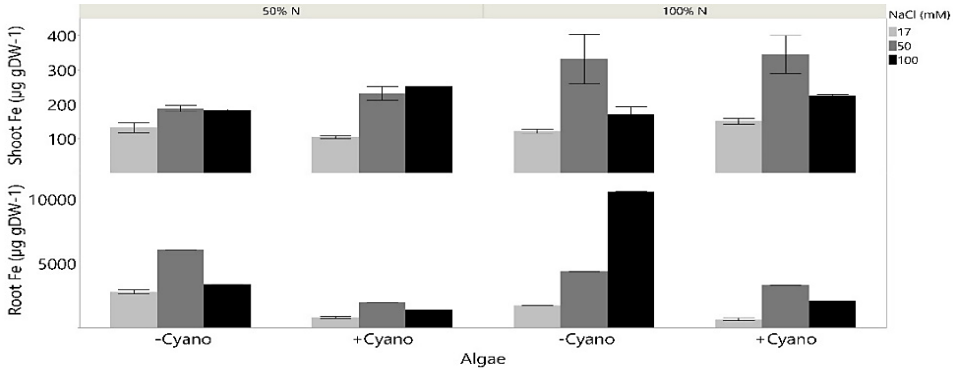

Fig. 41. Fe content at day 14 of growth under different salinity conditions (17, 50 and 100 mM NaCl), N supply (50 and 100%) and presence (+Cyano) or absence (-Cyano) of algae in the nutrient solution.

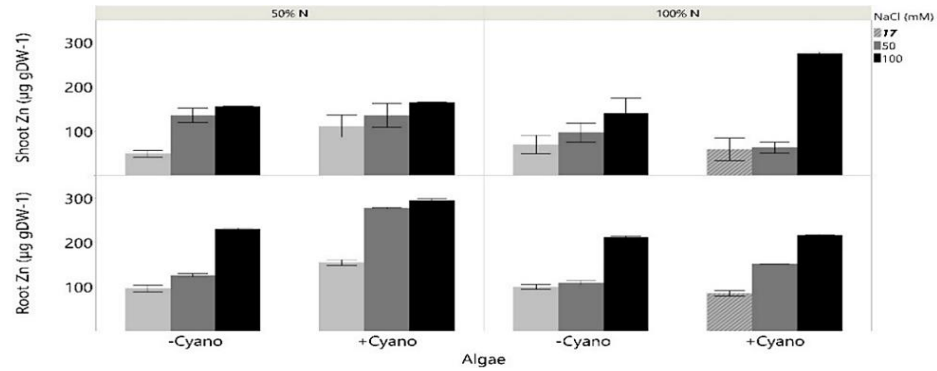

Fig. 42. Zn content at day 14 of growth under different salinity conditions (17, 50 and 100 mM NaCl), N supply (50 and 100%) and presence (+Cyano) or absence (-Cyano) of algae in the nutrient solution.

4.4 Discussion

In the view of more sustainable crop management practices cyanobacteria able to fix atmospheric nitrogen have gained increasing consideration as biofertilizers, especially in the rice crop under paddy conditions (Pathak et al., 2018). Paddy rice fields either conventionally managed or under organic farming practice are natural habitats of diverse cyanobacterial strains and the use of cyanobacteria as biofertilizers in paddies has been adopted especially in low input farming in Asia (FAO, 2004).

Cyanobacteria can either stimulate or inhibit growth of rice plants; also, no significant influence has been reported. These multiple ways of interactions largely depend on the cyanobacterial species and strain, and the culture conditions. In a survey with 133 cyanobacterial strains most inhibited germination of the rice seeds while 21% of the isolated strains promoted rice growth. Among the growth stimulating strains most were from the genus *Anabaena*, while most *Nostoc* strains had a negative effect (Pedurand and Reynaud, 1987).

Here we made a first approach to characterize the initial influence of *Nostoc* sp. UAB 206 on rice cultivar Copsemar grown under saline conditions. The cyanobacterial strain was selected due to its high salt and alkaline tolerance (chapter 3, Fig. 24 & Table 8), while the rice cultivar was selected based on its high yield under optimal condition and its sensitivity to salinity, as seen in the preliminary experiment (Figs. 33 and 34). Salinity has a strong influence on cyanobacterial biodiversity in paddy fields and under saline conditions *Nostoc* sp. has been identified among the most abundant species (Srivastava et al., 2009). Several *Nostoc* species have been found to stimulate rice growth under saline conditions (El-Sheek et al., 2018).

Results obtained here revealed that our *Nostoc* sp. UAB 206 stimulated rice growth under moderate saline conditions (50 mM NaCl) and even ameliorated performance under strong salinity (100 mM NaCl). These positive effects of the +cyano treatment under saline conditions effects

were not only observed in shoot length but also in chlorophyll concentrations (Fig. 36) and the maximum quantum yield at photosystem II (Fig. 37); both parameters were severely affected by salinity in absence of *Nostoc* sp.

Cyanobacteria may improve plant performance under salinity by different mechanisms. Extracellular polysaccharides and proteins produced by *Anabaena sphaerica* have been found to bind Na^+. This Na^+ adsorption reduces Na^+ ion activity in the culture medium thus lowering the ion toxicity and availability to the rice plants and favouring their growth under saline conditions (Manchanda, 2018). Here we observed that *Nostoc* sp. increased the production of extracellular polysaccharides under saline conditions (Chapter 3, Fig. 31). Lowering of Na+ ion activity due to this enhanced binding capacity could be, at least in part, responsible for the amelioration of saline stress without a reduction in Na tissue accumulation observed in rice under the 50% N treatment. The presence of *Nostoc* in the culture medium decreased Na root and shoot concentrations only in plants exposed to 100 mM NaCl in the high nitrogen treatment. Lower Na tissue concentrations in +cyano plants in comparison to -cyano could be responsible for better growth under high salinity and N.

Besides interactions in the nutrient medium, cyanobacteria may further stimulate plant growth under saline conditions due to in planta mechanisms. As other cyanobacteria, *Nostoc* species can produce growth stimulating phytohormones like auxin (Hashtroudi et al., 2013) and cytokinin (Toribio et al., 2020). Moreover, cyanobacteria in the rhizosphere or living as endophytes inside the root tissues can induce signalling substances like salicylic acid (Toribio et al., 2020) and activate defence genes related to enhanced tolerance against abiotic and biotic stresses (Belton et al., 2021). Further chemical characterization of our *Nostoc* sp. UAB 206 strain is required to see whether or not it is able to produce such plant growth promoting substances.

Cyanobacteria may further influence plant growth due to alteration in

mineral nutrition. As a diazotroph, *Nostoc* can fix atmospheric N_2 and this ability can contribute to plant growth stimulation especially under nitrogen deficiency conditions. In fact, in our experiments plant growth was mainly promoted in rice plants cultivated with low N supply (50% of standard Yoshida solution) indicating that the improvement of nitrogen availability may be a main factor in the better salt tolerance of our rice plants. Nitrogen supply under saline conditions is crucial for plant salt tolerance based on the ability to produce both antioxidants and compatible solutes like proline (Sikder et al., 2020). The uptake and transport mechanisms of Na+, K+ and nitrate in plants are strictly coordinated and nitrate supply can stimulate Na+ uptake and transport (Raddatz et al., 2020). Contrastingly $NH4^+$ may reduce Na+ accumulation as observed in salt sensitive rice (Kannan and Ramani, 1987). A mixed supply of ammonium and nitrate was found to decrease Na^+ accumulation under saline conditions in *Nigella sativa* (Bensalem et al., 2020). Here nitrogen was supplied as NH_4NO_3. However, the higher N treatment enhanced rather than decreased the Na^+ accumulation in the salt sensitive rice cultivar Copsemar. Only with a simultaneous supply of high NH_4NO_3 and the presence of *Nostoc* sp. the Na^+ accumulation was somewhat reduced both in roots and shoots. This, in combination with an enhanced K transport from roots to shoots under high N supply, favoured a lower Na/K ratio in the plants with high N supply both with or without *Nostoc* sp. In the rooting medium.

All three factors, salinity, nitrogen supply and *Nostoc* sp. influenced the root and shoot concentrations of Fe and Zn in the rice plants (Figs. 41& 42). In general, salinity negatively affects iron acquisition by plants (Tripathi et al., 2018). Contrastingly, here salinity tended to increase the concentrations of these micronutrients in both roots and shoots. A concentration effect due to salt-induced growth reduction may at least in part be responsible for this. The presence of *Nostoc* lowered root iron accumulation and favoured root to shoot translocation of Fe. To what extend siderophores produced by the cyanobacteria may contribute to this requires further investigation.

From this preliminary experiment on the interaction of *Nostoc* sp. UAB 206 with the salt-sensitive rice cultivar Copsemar we can conclude that this cyanobacterial strain has a beneficial influence on the salt tolerance of this rice cultivar. This positive effect was more pronounced under low N supply. This is a first indicator of the potential usefulness of this strain as a biofertilizer for rice production under saline conditions with lower inorganic fertilizer input. However, much further studies are still required to better characterize both the mechanisms behind this stimulatory effect and the suitability of the strain under field conditions.

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Overall conclusions

- We described, for the first time, a new multidisciplinary approach of a prevailing cyanobacterium from Mediterranean paddy fields in the Ebro Delta under environmental fluctuation (salinity and alkalinity).
- The diagnostic criteria using a combination classical and modern techniques has confirmed that this cyanobacterium belongs to the *Nostoc* genus.
- Statistical analysis revealed the alternation from isomorphism to paramorphism by changing salinity.
- Spectroscopy and growth analysis illustrated that this native strain can be considered alkaliphilic and resistant to mid salinity.
- This strain can adapt through different strategies such as improvement in phycobilisome structure, photosynthetic operation, and oxygen liberation which lead to the highest acclimation ability under saline-alkaline conditions.
- Positive effect of combination of salinity, nitrogen supply, and presence of *Nostoc* sp. UAB 206 was observed in rice growth (shoot length), chlorophyll concentrations, and the maximum quantum yield of photosystem II under mid-levels of salinity.
- In general, the present data reveal the potential usefulness of this cyanobacterium may greatly contribute to alkaline-saline paddy fields -with lower inorganic fertilizer input -as biofertilizers, soil conditioners, and other biotechnological purposes.