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Sustaining Rice Production through Biofertilization with N_2-Fixing Cyanobacteria

Macarena Iniesta-Pallarés [1,2,†], Consolación Álvarez [1,†], Francisco M. Gordillo-Cantón [1], Carmen Ramírez-Moncayo [1], Pilar Alves-Martínez [1,2], Fernando P. Molina-Heredia [1,2] and Vicente Mariscal [1,*]

[1] Instituto de Bioquímica Vegetal y Fotosíntesis, Consejo Superior de Investigaciones Científicas and Universidad de Sevilla, 41092 Seville, Spain; macarena.iniesta@ibvf.csic.es (M.I.-P.); consolacion@ibvf.csic.es (C.Á.); fcomanuelgordilloc@gmail.com (F.M.G.-C.); carmenramirezmoncayo@gmail.com (C.R.-M.); pilar.alvesmartinez@alum.uca.es (P.A.-M.); publio@us.es (F.P.M.-H.)

[2] Departamento de Bioquímica Vegetal y Biología Molecular, Facultad de Biología, Universidad de Sevilla, Avda. Reina Mercedes s/n, 41012 Seville, Spain

* Correspondence: vicente.mariscal@ibvf.csic.es; Tel.: +34-954489534

† These authors contributed equally.

Featured Application: We have designed a cyanobacterial consortium that could be used as a biofertilizer for rice production.

Abstract: Current agricultural productivity depends on an exogenous nutrient supply to crops. This is of special relevance in cereal production, a fundamental part of the trophic chain that plays a vital role in the human diet. However, our agricultural practices entail highly detrimental side-effects from an environmental point of view. Long-term nitrogen fertilization in croplands results in degradation of soil, water, and air quality, producing eutrophication and subsequently contributing to global warming. In accordance with this, there is a biotechnological interest in using nitrogen-fixing microorganisms to enhance crop growth without adding chemically synthesized nitrogen fertilizers. This is particularly beneficial in paddy fields, where about 60% of the synthetic fertilizer that has been applied is dissolved in the water and washed away. In these agricultural systems, N_2-fixing cyanobacteria show a promising biotechnological potential as biofertilizers, improving soil fertility while reducing the environmental impact of the agricultural practice. In the current study, Andalusian paddy fields have been explored to isolate N_2-fixing cyanobacteria. These endogenous microorganisms have been subsequently re-introduced in a field trial in order to enhance rice production. Our results provide valuable insights regarding the use of an alternative natural source of nitrogen for rice production.

Keywords: cyanobacteria; rice; biofertilizer; N_2-fixation; PGPB

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1. Introduction

One of the challenges facing agriculture today is producing enough food for an ever-growing population. Rice is the most important cereal crop in the world and the major food source for millions of households in dozens of countries [1]. In 2019, the world dedicated more than 150 million hectares and almost a third of the Earth's fresh water (http://www.fao.org/faostat/en/#data/QC, accessed on 10 April 2021) to rice cultivation. Rice plants require large amounts of water and mineral nutrients, including N and P, for their growth, development, and grain production [2]. Around 16–17 kg N/ton of N fertilizer, generally urea, are used to meet the crop's demands [3]. Wetland areas provide a perfect place for rice cultivation, but flooded conditions call for the usage of large amounts of inorganic N fertilizers to account for its loss into adjacent water bodies. In fact, only

30–40% of the fertilizer dose, even less in some cases, is utilized by the crop [4]. Large amounts of N fertilizers are lost into surface waters, polluting the environment and causing crop health issues due to ammonia volatilization, denitrification, and leaching [5]. Nitrate that is leached from agricultural systems causes serious eutrophication of ecosystems.

To date, more than 150 eutrophic and hypoxic coastal areas have been identified in Europe, mostly at the delta of important European rivers, including zones where rice is cultivated [6]. One of them occurs in the Guadalquivir marshes, located in the Doñana National Park in southern Spain. This space contains the largest area in Spain that is dedicated to rice cultivation, producing an average of 370,000 tons of this cereal over 40,000 ha divided into plots of 10 and 12 ha. This region is principally located in the municipalities of Isla Mayor and La Puebla del Río, which are economically reliant on the cultivation of this cereal that is farmed by family farmers, most of whom have a few intensive farming plots [7]. At the same time, this region is of great ecological and environmental value due to its rich fauna, especially since it houses migratory birds in their journey between Europe and Africa [8]. However, intensive farming in the region is increasing the levels of N in the water bodies, enhancing the proliferation of phototrophic microorganisms [9]. Some of them that produce hepatotoxins, such as *Microcystis aeruginosa*, are responsible for the cases of massive intoxication of waterfowls in the region [9,10].

The use of plant-growth-promoting bacteria (PGPB) to enhance crop yield and control disease is gaining worldwide acceptance as a sustainable agricultural practice while reducing costs by supplanting the use of expensive (and polluting) agrochemicals. These bacteria can facilitate plant growth either directly, by providing essential nutrients (nitrogen, phosphorus, and essential minerals); modulating plant hormones; and development, or indirectly, by suppressing the inhibitory effects of various plant pathogens, improving soil structure, and bio-remediating polluted soils [3]. The paddy field ecosystem represents a unique aquatic–terrestrial habitat, which provides a favorable environment for the growth of cyanobacteria. From the different groups of cyanobacteria, the N_2-fixing *Nostoc* genera are naturally found in floating assemblages, attached to the root surface, and can colonize the roots of rice [11,12]. These cyanobacteria, which are of great relevance in the global carbon and nitrogen cycles, are the main contributors to the natural deposition of nitrogen in paddy fields [13,14]. Their N_2-fixing ability, which provides a natural nitrogen source for the plant, can potentially be exploited in crop agriculture. However, this biotechnological potential remains utterly unstudied.

In this work, native cyanobacterial strains from the southern Spanish paddies were isolated and used as bioinoculants for rice crops. Our results indicate that this practice enhances plant growth and productivity, thus providing a sustainable alternative for nitrogen fertilization of rice. This poses valuable insights regarding the biofertilizing potential of cyanobacteria in agriculture.

2. Materials and Methods

2.1. Sampling Sites and Isolation of Cyanobacteria

Samples for cyanobacteria enrichment and isolation were obtained from rice fields located in the Guadalquivir marshes in Doñana National Park, Seville, Spain. The locations of the different sampling sites are shown in Figure 1. Soil samples were taken with care from the upper part and immediately transported to the laboratory. For the isolation of N_2-fixing cyanobacteria, samples containing 5 g of soil were suspended in 50 mL $BG11_0$ medium [15] and incubated at 30 °C overnight in continuous light (50 $\mu mol \cdot m^{-2} \cdot s^{-1}$). Subsequently, a series of 10-fold dilutions were made and spread on agarose-solidified $BG11_0$ medium plates in the presence of cycloheximide (0.1 $mg \cdot mL^{-1}$). Plates were incubated for two weeks until the first cyanobacterial colonies appeared. Up to 470 colonies were picked based on differences in color, growth, and morphology. To isolate individual strains, each culture was successively re-streaked in $BG11_0$ medium plates in the presence of cycloheximide until individual colonies were obtained. Cyanobacterial strains were maintained in agar-solidified $BG11_0$ medium plates [15].

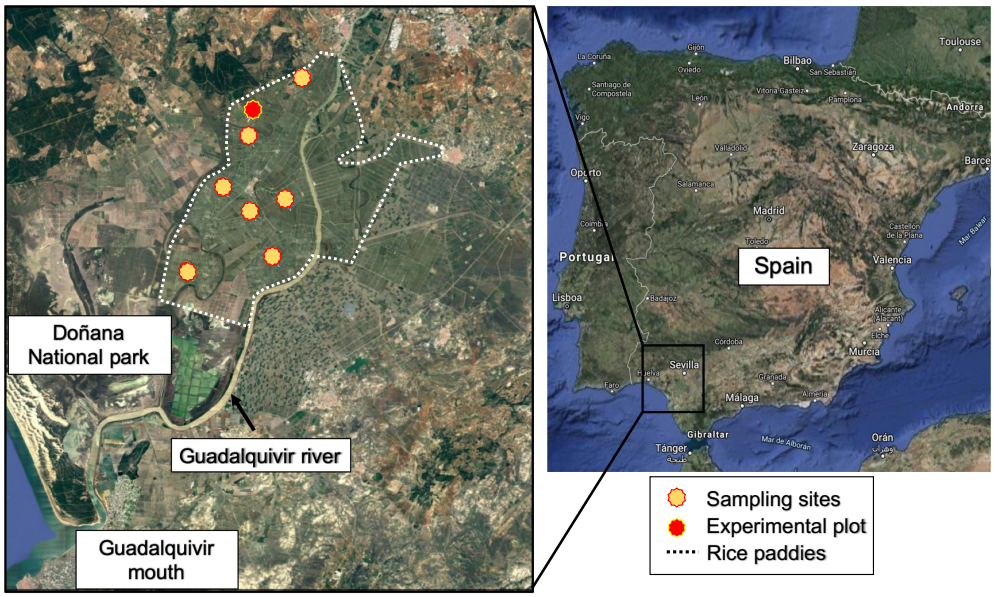

Figure 1. Location of the sampling points selected for this study. In red, location of the experimental plot selected for the field trials.

2.2. DNA Extraction and PCR Amplification

Cyanobacterial genomic DNA was extracted with an NZY Tissue gDNA Isolation Kit (NZYTech) with some modifications. For the genetic characterization of the cyanobacterial strains, a PCR-based approach was used. A hypervariable genomic region conserved in all filamentous cyanobacteria was used as template to design the degenerated primers FsepJ (5′-CAACAYGAHRTBYA-3′) and RsepJ (5′-CAYRTYCAAMCDTCHCT-3′). These primers were used to amplify the genomic region of the isolated cyanobacterial strains with iProof™ High-Fidelity DNA Polymerase (Bio-Rad). PCR products were sequenced in Eurofins Genomics (Ebersberg, Germany). Alternatively, the V4–V5 hypervariable genomic region of the 16S rRNA was amplified in six selected cyanobacterial strains. To that end, we used the primers F16S (5′-GTGYCAGCMGCCGCGGTAA-3′) and R16S (5′-CCGTCAATTCMTTTRAGTTT-3′). PCR products were cloned for subsequent sequencing in the pSpark® I vector (Canvax Biotech, Córdoba, Spain) according to manufacturer's instructions. Ten different transformants from each amplification were selected to be sequenced.

2.3. Bioinformatic Analyses and Phylogenetic Tree Construction

All obtained sequences were blasted against the GenBank nucleotide database, from which the most closely related ones were downloaded and included in the subsequent phylogenetic analyses.

For the bioinformatic analyses, the *sepJ* gene sequences obtained in this study and reference sequences retrieved from GenBank were aligned using MUSCLE algorithm with a maximum of 8 interactions. The phylogenetic tree was constructed with a neighbor-joining algorithm and Tamura–Nei as the distance genetic model, both implemented by the Geneious version 2020.0 created by Biomatters (available at https://www.geneious.com, accessed on 10 April 2021). Tree topology and bootstrap re-sample were also applied with a 50% support threshold and 10,000 as the number of replicates.

2.4. Light Microscopy

For standard light microscopy, cyanobacterial strains were incubated in $BG11_0$ medium for 5 days at 25 °C in the light (75 $\mu E \cdot m^{-2} \cdot s^{-1}$) on a rotary shaker (100 rpm). Samples were taken with care and directly visualized by a Leica microscope with a 40× objective. For trichome length measurement, at least 100 trichomes were counted. Percentage of heterocysts was calculated from at least 1400 cells for each strain in more than 20 different fields. Dividing cells were counted as two cells.

2.5. Batch Cultivation of Cyanobacteria for Field Trials

Strains used for the consortium were G04, G09, G10, G15, G16, and G17. Each strain was cultivated separately. A 100 mL sterile $BG11_0$ medium (in a 250 mL Erlenmeyer flask) was inoculated with the microorganism and incubated for 14 days at 25 °C in the light (75 $\mu E \cdot m^{-2} \cdot s^{-1}$) on a rotary shaker (100 rpm). The culture was then scaled up, inoculating a 700 mL $BG11_0$ medium in a Roux flask supplemented with 10 mM $NaHCO_3$ and bubbled with 1% CO_2 for 7 days at 30 °C in the light (75 $\mu E \cdot m^{-2} \cdot s^{-1}$). The resulting biomass was used to inoculate 20 L tanks, which were cultured for 14 days under the same conditions.

The cyanobacterial biomass was harvested by centrifugation at 4000× *g* for 10 min at room temperature, and each culture was diluted to a chlorophyll concentration of 100 mg/L (calculated according to [16]) in a 3 L final volume. Finally, all the strains were mixed to obtain 18 L of a bacterial consortium that was used for the inoculation of experimental plots at a ratio of 3 L/100 m^2.

3. Results and Discussion

3.1. Isolation and Genetic Characterization of N_2-Fixing Cyanobacteria from the Rice Paddies of the Andalusian Marshes

The Doñana marshes in the south of Spain are one of the most important wetland complexes in Europe and in the Mediterranean region (Figure 1). Part of this region was transformed in the early 1970s from the original natural marshes to one of the most productive and largest areas of rice cultivation in Spain, where specific farming practices and rice varieties are used. Intensive rice cultivation, which is highly dependent on N fertilization, has been associated with the environmental pollution of the area, causing nitrate leaching and emission of nitrous oxide (N_2O) [4]. Because of that, this region has been included in the list of the "nitrate vulnerable zones" by the Andalusian Government (Decree 36/2008), according to the Nitrate Directive (EEC, 1991). Thus, it is a priority to reduce the impact of the ongoing nitrate pollution due to the intensification of agriculture in the watershed.

Natural wetlands provide a favorable environment for the growth of N_2-fixing cyanobacteria, meeting their requirements for light, water, elevated temperature, and nutrient availability [17,18]. The ability of these phototrophic microorganisms to fix nitrogen in the soil makes N_2-fixing cyanobacteria promising biofertilizers for rice paddies. Thus, we aimed to isolate indigenous N_2-fixing cyanobacteria from the Andalusian paddies, then re-introduce these beneficial microorganisms, and restore the health of plants and soil to its natural balance. Soil samples from seven different plots were taken in March 2018, just before initiation of the rice culture (Figure 1). N_2-fixing cyanobacteria strains were isolated by a classical serial dilution technique described by [19] using $BG11_0$ as a growth medium (without combined nitrogen) [15]. A total of 470 different colonies were randomly picked based on their diversity in morphology, color, and micromorphology as observed under a light microscope. The strains were isolated by repeatedly streaking on fresh dishes that were supplemented with cycloheximide in order to inhibit the growth of microalgae and fungi. This strategy has been used in other studies to isolate N_2-fixing cyanobacteria from their natural habitats, including rice paddies from India, Vietnam, and Pakistan [20–22]. However, to our knowledge, this is the first report describing the isolation of N_2-fixing cyanobacteria from the Andalusian rice paddies.

To characterize the isolated cultures, we selected 97 strains to be further studied by a genetic approach. Genetic classification of cyanobacteria has been traditionally performed by PCR-denaturing gradient gel electrophoresis (DGGE). Differential DNA fingerprint patterns are ascribed to different strains [18,23,24]. However, this method does not provide information about the genetic classification of the samples. We designed a new PCR-based approach that was successfully used for the genetic characterization of our samples. It is based on the amplification of a genomic region of the *sepJ* gene, which is required for filament integrity and only found in filamentous cyanobacteria [25]. The *sepJ* gene contains a hypervariable region that is flanked by two conserved regions, based on which we have designed degenerate primers (Figure 2). We set up a colony PCR approach to amplify the genomic region of each of the 152 strains (see Materials and Methods). PCR amplification bands were sequenced and aligned, and identical sequences were grouped. This analysis provided 18 different genotypes, containing 1 to 26 strains in each one. In order to identify these genotypes, they were subjected to a phylogenetic analysis against other reference sequences obtained from the GenBank database (Figure 3). We concluded that the isolated strains could be grouped into five major phylogenetic groups (PG01, PG02, PG03, PG04, and PG05).

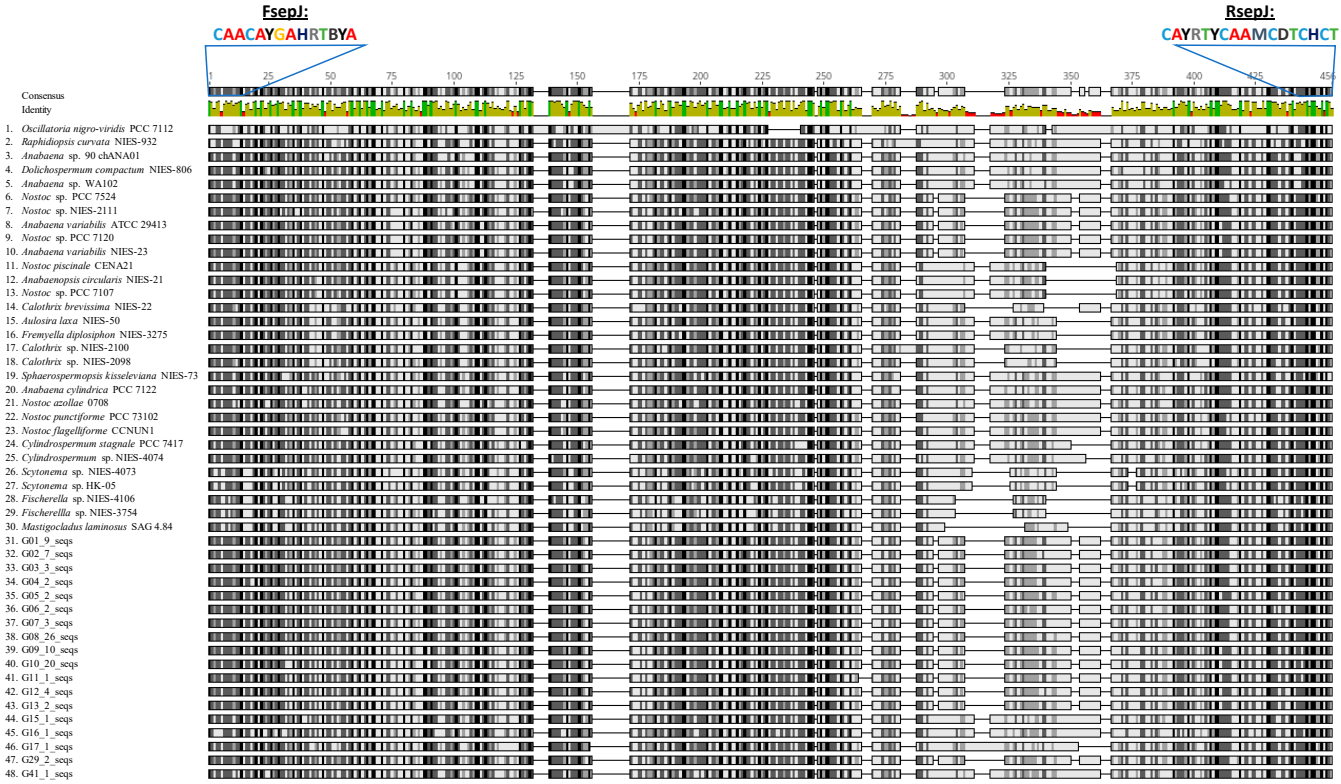

Figure 2. Alignment of the cyanobacterial *sepJ* sequences used for the genetic characterization of the cyanobacterial isolates and primers used for the amplification of the sequences. The genomic region contained a hypervariable region that was used to genetically trace the different cyanobacterial strains.

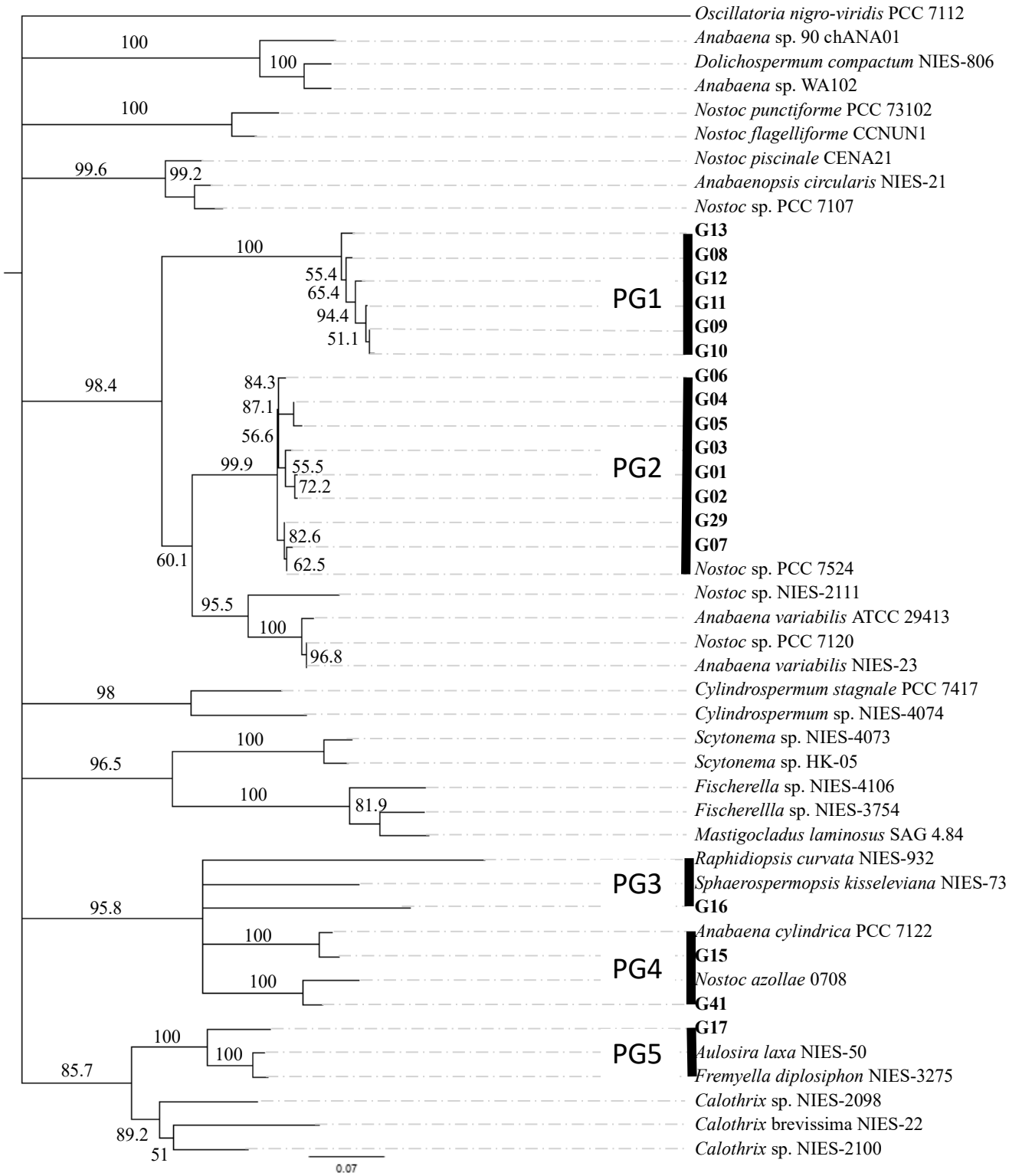

Figure 3. Rooted phylogeny of the *sepJ* genomic sequences, inferred using a neighbor-joining algorithm and Tamura–Nei as the distance genetic model using the bootstrap method (n = 10,000). The bar represents 0.07 substitutions per site. Reference sequences were obtained from the GenBank nucleotide database, using the sequences experimentally amplified as template. Phylogenetic groups are highlighted.

3.2. Morphological Description of the Cyanobacterial Isolates

We selected a representative strain from each of the five phylogenetic groups and further characterized it using morphological traits, such as cell dimensions, shape, color, structure of the trichomes and cell differentiation (Figure 4) [15,26]. We complemented this analysis with a genetic characterization of the 16S of these strains in order to corroborate the previous phylogenetic analysis (Table 1).

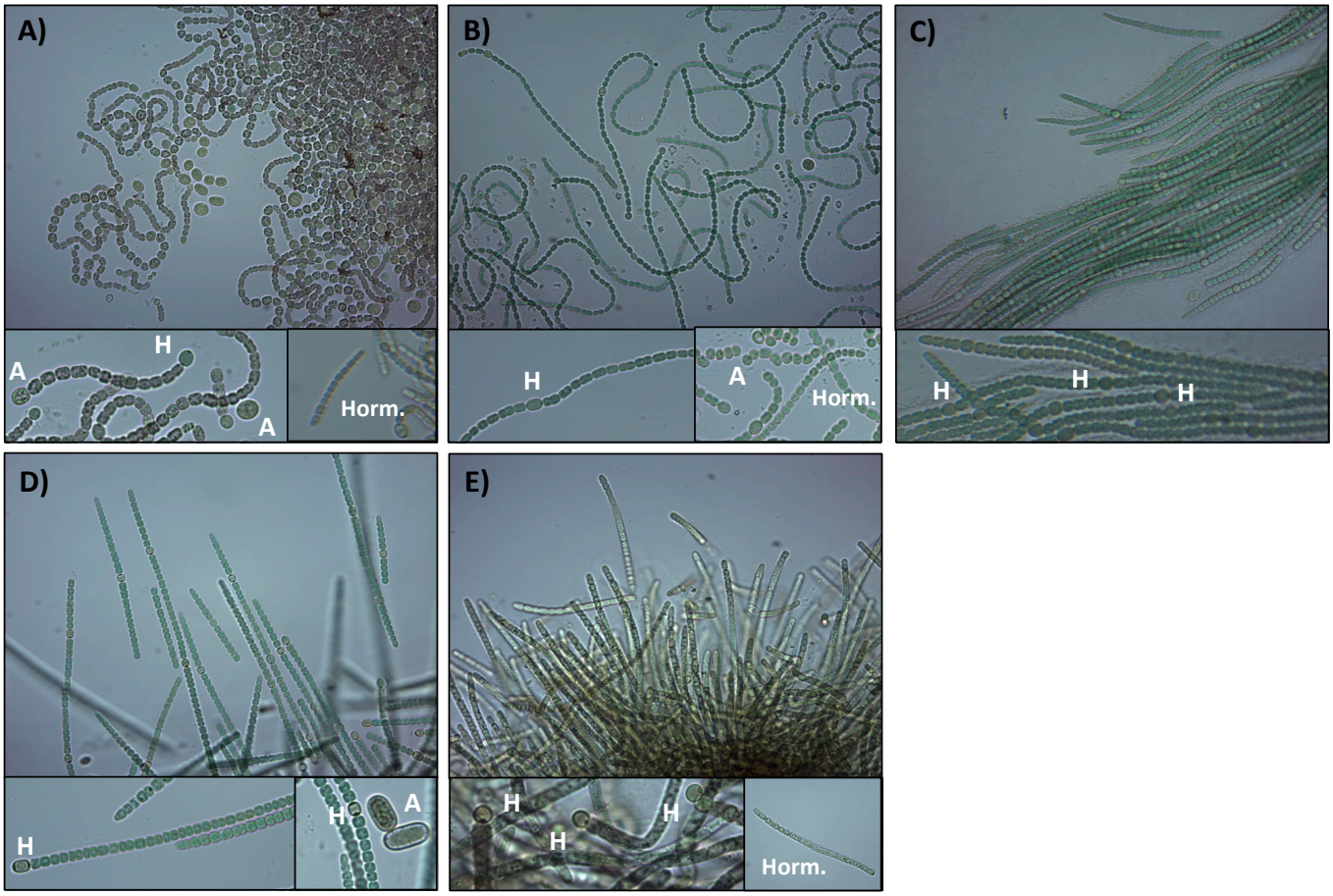

Figure 4. Morphological illustrations of 5 strains used in this work. (**A**) Strain G10 from PG1, (**B**) strain G04 from PG2, (**C**) strain G16 from PG3, (**D**) strain G15 from PG4, and (**E**) strain G17 from PG5. H: Heterocysts; A: Akinetes; and Horm: Hormogonia.

Table 1. Morphological characters of 5 cyanobacterial strains used in this study.

Phylogenetic Group (Strain)	Culture Color	Cells Per Trichome	% Heterocysts	Akinetes	Hormogonia	Closest Related
PG1 (G10)	Brown	24.58 ± 2.17	7.48 ± 0.40	Yes	Yes	*Nostoc punctiforme*
PG2 (G04)	Green	24.31 ± 1.79	9.18 ± 0.35	Yes	Yes	*Nostoc* sp.
PG3 (G16)	Blue-Green	31.62 ± 1.12	5.38 ± 0.25	No	No	*Wollea salina*
PG4 (G15)	Green	41.13 ± 1.53	4.98 ± 0.26	Yes	No	*Anabaena cylindrica*
PG5 (G17)	Dark-Green	12.47 ± 0.59	10.51 ± 0.67	No	Yes	*Calothrix membranacea*

Strain G10 was selected as representative of PG1. A 16S rRNA analysis of this strain revealed a high similarity to *Nostoc punctiforme* MBDU 009. *Nostoc punctiforme* is a terrestrial cyanobacterium with a broad symbiotic competence with fungi and terrestrial plants, including bryophytes, gymnosperms, and angiosperms, providing fixed nitrogen to the plant partner [27]. It has been shown that *Nostoc punctiforme* PCC 73102 enhances rice growth [12]. Strain G10 formed brown colonies on a solid medium and growth forming rough mats in liquid $BG11_0$. The G10 strain showed long curved trichomes (24.58 $\pm$ 2.17 cells per trichome). Intercalary and terminal heterocysts were found at a percentage of 7.48 $\pm$ 0.40 heterocysts per vegetative cell. Akinetes and hormogonia were easily found in the culture (Figure 4A).

Strain G04 was selected as representative of PG2. This group was most closely related to *Nostoc* sp. PCC 7524. They formed green colonies on a solid medium and grew uniformly in liquid $BG11_0$. The trichomes were slightly curved and were composed by rounded vegetative cells with an average of 24.31 $\pm$ 1.79 cells per trichome. However, long trichomes containing more than 100 cells were occasionally observed. Intercalary and terminal heterocysts were found at a percentage of 9.18 $\pm$ 0.35 heterocysts per vegetative cell. Akinetes and hormogonia were easily found in the culture (Figure 4B).

Strain G16 was the only component of PG3. This group was most closely related to *Wollea salina* L38, which was first isolated from Thailand in wet, salty soils [28]. Soil salinization is an environmental stressor that causes a significant reduction in crop productivity [29]. Thus, the use of salt-resistant cyanobacteria in salty paddy fields could have positive effects on rice productivity. G16 formed blue-green colonies on a solid medium and grew with parallel and densely packed trichomes, forming rough mats in liquid $BG11_0$. Trichomes were formed by rounded vegetative cells with conical terminal cells. One intercalary heterocyst was found per trichome at a percentage of 5.38 $\pm$ 0.25 heterocysts per vegetative cell. This strain did not differentiate akinetes or hormogonia (Figure 4C).

Strain G15 was selected as representative of PG4. This group was most closely related to *Anabaena cylindrica* PCC 7122, a planktonic cyanobacterium that has been evaluated previously as a biofertilizer in lettuce crops [30]. G15 formed green colonies on a solid medium and grew uniformly in liquid $BG11_0$. It showed straight trichomes (41.13 $\pm$ 1.53 cells per trichome) arranged in parallel and composed by rounded vegetative cells with rounded and conical terminal cells. Intercalary heterocysts were found at a percentage of 4.98 $\pm$ 0.26 heterocysts per vegetative cell. This strain differentiated akinetes (Figure 4D).

Strain G17 was the only component of PG5. This group was most closely related to *Calothrix membranacea* KLR006. *Calothrix* sp. is commonly found in cultivated soils, being beneficial for rice growth [31]. G17 formed dark-green colonies on a solid medium and grew with densely packed trichomes, forming rough mats in liquid $BG11_0$. G17 formed short heteropolar trichomes with an average of 12.47 $\pm$ 0.59 cells per trichome. Only one terminal heterocyst was found per trichome. Motile hormogonia were easily found in the culture. This strain did not differentiate akinetes (Figure 4E).

The combination of morphological and 16S rRNA molecular characterization confirmed the previous taxonomic identification of the cyanobacterial strains and, in the case of strains G04, G10, and G17, provided more detailed information about the closest related strain. It is important to note that the groups PG01, PG02, PG04, and PG05 contain new strains that had not been described before. This is of particular interest regarding their possible use as commercial byproducts or biofertilizers, since their potential had been, thus far, unexplored. Identification of the 16S sequence of new isolated microorganisms is a well-established strategy for the genetic characterization of new strains [32]. However, obtaining the 16S rRNA amplicon from complex samples requires the construction of clone libraries, a process that is tedious and time-consuming [33]. The genetic selection, which has been designed for the purpose of this work, significantly reduces the number of strains to study, saving time and effort in the subsequent morphological and genetic identification of the strains.

3.3. Plant Growth Promotion in Field Trials

Long-term application of nitrogen fertilizers changes the composition and structure of soil and negatively affects the species richness of plants and soil microbes [34]. In the case of the Andalusian paddy fields, synthetic nitrogen fertilizers are the main factor in producing cropland degradation and decreasing soil microbial biomass and ecosystem services [4]. Novel fertilization approaches involving long-term sustainable farming practices are required for the restoration of these fields. The use of biofertilizers containing soil beneficial microbes has been adopted as an effective method for restoration of degraded soil due to agricultural practices, providing nutrient cycling and improving plant health [3,35]. The application of cyanobacterial biofertilizers in rice paddies would be an eco-friendly approach, contributing to the development of sustainable agriculture.

We studied whether re-inoculation of the cyanobacterial isolates from the five phylogenetic groups influenced plant health and rice productivity in field trials. We used a microbial consortium containing the six representative strains selected upon morphological examination. We chose a representative plot of the Andalusian rice paddies where a conventional mode of rice cultivation was applied (Figure 1). This plot was maintained under continuous flooding and was fertilized with the recommended dose of urea (145 units $N \cdot ha^{-1}$). Within this area, we then selected three smaller sub-plots of 130 m^2, each of which were inoculated with the cyanobacterial consortium containing representatives of all the cyanobacterial isolates from the area. The three sub-plots were managed as the main plot, except for the inoculation with the consortia. We performed two inoculations: 1 month after planting, coinciding with tiller initiation, and 3 months after planting, coinciding with panicle formation. The effect of biofertilization on plant growth was evaluated in plants, 1 month, 2 months and 4 months after seeding, and the yield was measured at the ripening state.

In 2-months-old plants (1 month after the first treatment), we did not observe any significant difference in length (Figure 5A). However, we found a positive significant effect on plant growth in 4 months (1 month after the second treatment), recording a significant increase in plant length of 127% ($p = 1.22 \times 10^{-26}$) (Figure 5A,B). Plants that were treated with the bioinoculant were greener and healthier with respect to the untreated control. At the ripening state, we collected panicles and measured the weight and number of grains per panicle. We recorded a significant increase for both parameters ($p = 3.79 \times 10^{-6}$ and $p = 4.93 \times 10^{-13}$, respectively) (Figure 5B,C).

Taken together, these results point to a promising application of cyanobacterial biofertilizers to sustain rice production. Cyanobacterial bioinoculants have been used previously in Asian countries, such as China, Vietnam, India, etc., for paddy cultivation as an alternative to nitrogen fertilizers. Several cyanobacterial species, such as *Anabaena variabilis*, *Nostoc muscorum*, *Aulosira fertissima*, and *Tolypothrix tenuis*, have been described as effective biofertilizers [3]. Our results were obtained with a group of strains particularly well adapted to local climatic and agronomic conditions. To the best of our knowledge, this is the first report using autochthonous cyanobacteria as biofertilizers for the Andalusian paddy fields, the largest region for rice cultivation in Spain and one of the most productive for the Indica variety in Europe. Under laboratory conditions, it has been shown that the model cyanobacterium *Nostoc punctiforme* enhances the plant shoot/root length and weight of rice plants [12]. The molecular mechanism involved in cyanobacteria-induced plant fortification remains unsolved, yet it is likely that nitrogen fixed in heterocysts and plant phytohormones are involved. To address this, further studies will be carried out in the future.

From a commercial point of view, there is little information regarding commercial byproducts or biofertilizers based on cyanobacterial formulations. Biofertilizers are cost-effective, non-toxic, and easy to apply [36]. If that were the case for cyanobacterial biofertilizers, their use would not only reduce production costs, but would also provide ecosystem goods and services and ensure a more sustainable agricultural system.

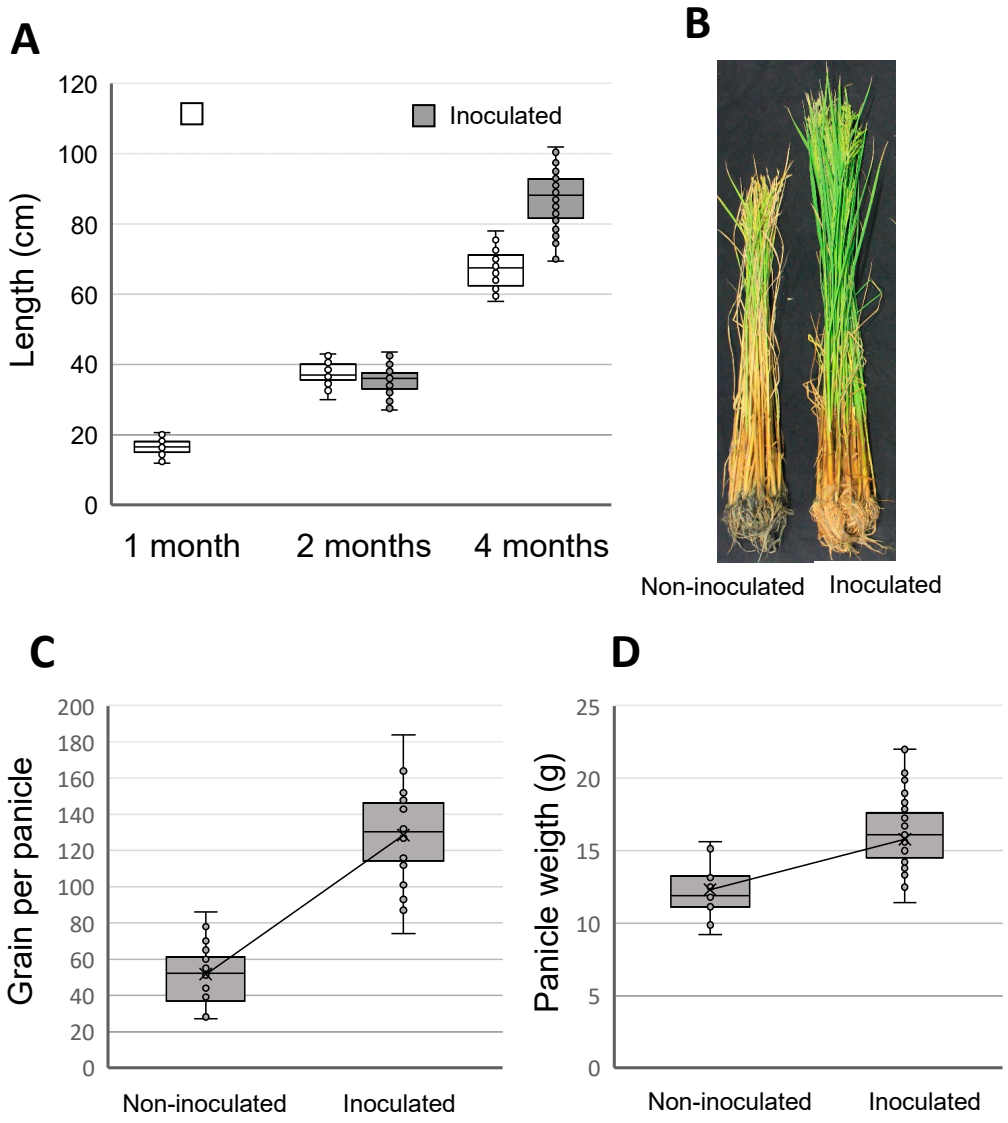

Figure 5. Effect of biofertilization with the cyanobacterial consortia in rice field trials. (**A**) Length of aerial part of rice plants measured at different plant developmental stages. T-Student test revealed a significant difference between treated and non-treated plants at 4 months ($p = 1.22 \times 10^{-26}$). (**B**) Plants collected at 4 months after planting were photographed. The same number of plants are shown in each treatment. (**C**) Effect of the cyanobacterial biofertilizer on the number of grains per panicle. T-Student reveals a significant difference between treated and non-treated plants ($p = 3.79 \times 10^{-6}$). (**D**) Effect of the cyanobacterial biofertilizer on the weight of the panicle. T-Student reveals a significant difference between treated and non-treated plants ($p = 4.93 \times 10^{-13}$).

Author Contributions: Conceptualization, V.M. and F.P.M.-H.; methodology, M.I.-P., C.Á., F.M.G.-C., C.R.-M., and P.A.-M.; data curation, M.I.-P. and C.Á.; writing—original draft preparation, V.M.; writing—review and editing, all authors; funding acquisition, V.M. and F.P.M.-H. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Nagoya protocol for the access to Spanish genetic resources for research use under royal decree 124/2017 of 24 February. Reference: ESNC26.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available upon reasonable request from the corresponding author.

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Conflicts of Interest: The authors declare no conflict of interest.

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