Novel Approach for the Determination of Nitrogen Fixation in Cyanobacteria

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Summary: Non-heterocystous nitrogen fixing strains of cyanobacteria were screened by their ability to grow in nitrogen deficient media. The selected nitrogen fixing cyanobacterial cells were then cultured in BG11 media supplemented with [^{15}N]-labeled sodium nitrate. Under these growth conditions any organic [^{14}N] found in the cyanobacterial cells would simply come from nitrogen fixation because [^{15}N] was the only available source of nitrogen in the medium. Amino acids extracted after different time periods (after 15, 30, 40, 50 and 60 days of inoculation) were used for the determination of the ^{14}N/^{15}N ratio using GC-MS. Results from the present study support the conclusion that at stationary phase of growth cyanobacterial nitrogen fixation was no longer supplying a significant amount of nitrogen. This approach not only provided a detailed method for the evaluation of the nitrogen fixing potential of the cyanobacteria in culture, but also suggests novel approaches for the assessment of the ability of the strains to provide nitrogen enrichment to plants under co-cultivation conditions.

Key Words: Cyanobacteria, Nitrogen Fixation, GC-MS, Amino acids.

Introduction

Nitrogen is a primary essential element for life on earth to continue and is often limiting, so everything that lives have evolved strategies for obtaining the nitrogen required for their survival [1]. Although it is extensively present in the earth, including accounting for about 78% of the atmosphere's it is nevertheless considered as the second limiting factor for plant growth, after water, as plants are typically unable to use dinitrogengas because of its non-reactive nature [2]. Plants can only exploit reduced forms of nitrogen which they obtain by different mechanisms e.g. decomposition of organic matter, addition of nitrogen fertilizer or manure, alteration of atmospheric nitrogen chiefly by microbial nitrogen fixation, that provide needed forms of nitrogen to the plants [3]. Nitrogen fixation is a vital biological phenomenon performed by prokaryotes, including the cyanobacteria [4-9]. The enzyme nitrogenase, that catalyzes this process, is very sensitive to oxygen [10, 11]. Cyanobacteria have the unique ability to perform both oxygen releasing photosynthesis and oxygen sensitive nitrogen fixation [12, 13]. Mostly cyanobacteria protect nitrogenase from damage by intracellular oxygen by carrying out nitrogen fixation in morphologically and physiologically differentiated cells called heterocysts whereas photosynthesis takes place simultaneously in spatially separated vegetative cells [14-17]. However, there are many unicellular and filamentous, non-heterocystous diazotrophic cyanobacteria which do not have differentiated morphologically distinguishable cells, they overcome the problem of oxygen by fixing dinitrogen either during the dark phase of growth or in light at a time when photosynthesis is inhibited [18-23]. Initially, for our studies, nitrogen fixing strains (*Chroococcidiopsis* sp. SM-04, *Phormidium* sp. SM-14 and *Phormidium* sp. SM-15) were screened by their ability to grow in nitrogen deficient media. Next, the selected nitrogen fixing cyanobacterial cells were cultured in BG11 media supplemented with [^{15}N]-labeled sodium nitrate. Because the medium was essentially devoid of the natural isotope of nitrogen, organic [^{14}N] that was found in the cyanobacterial cells would have come from nitrogen fixation because [^{15}N] was the only available source of reduced nitrogen supplied. Amino acids extracted after different time periods (after 15, 30, 40, 50 and 60 days of inoculation) were used for the determination of the endogenous ^{14}N/^{15}N ratio using GC-MS for analysis.

Experimental

Isolation and purification of Cyanobacteria

One gram of each of the soil samples collected from the rhizosphere and soil surface mat of different crop fields (rice, wheat and maize fields) was shaken in 10 mL sterile distilled water, to

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suspend the sediments, using a Vortex-Mixer model SLV-6. Aliquots of 10 µL of suspension containing the soil microbes were then filtered using aseptic techniques through a 0.45 µm membrane. The filters were incubated on plates of BG11 media [24] containing cyclohexamide (100 µg/mL) for two weeks at 25 ± 2 ºC, illuminated by continuous 18 µmol photons m^{-2} s^{-1} given by a white fluorescent tube light in a controlled environmental growth chamber (Sanyo versatile MLR-350H). Individual colonies from the mixed cultures of cyanobacteria growing on the surface of plates were picked and transferred to fresh BG11 media in order to prepare new suspensions. Purified colonies and filaments were picked and streaked onto fresh BG11 agar plates. This step was repeated several times to obtain an axenic culture. The axenic condition of the cultures was tested by transferring pieces of agar block with the cyanobacterial cultures to LB agar medium plates. Finally purified colonies or filaments were transferred to BG11 broth without cyclohexamide for further experiments.

Characterization and Identification of Cyanobacteria

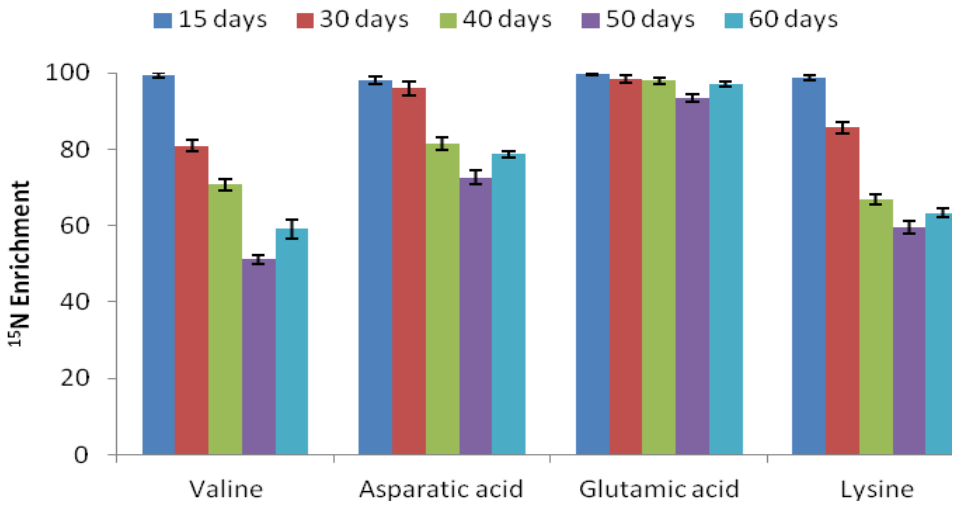
Isolated cyanobacterial cultures were characterized morphologically and physiologically. To study the morphology of cyanobacteria, cell shape, width, color, motility and presence/absence of hormogonial cell, sheath and heterocyst were recorded. For physiological characterization, the effects of different pH and temperature on the growth of cyanobacteria were recorded. Growth of cyanobacteria was measured following the method of Tandeau de Marsac and Houmard, (1988) [25]. Cyanobacterial strains were identified by 16S rRNA sequencing. DNA from 15 days old cyanobacterial cultures was isolated following the method of Srivastava *et al.*, (2006) [26] with minor modifications. Extracted DNA was amplified by using forward primer 27 F1 (5´-TAGTGTAAAACGGCCAGTAGAGTTTGATCCT GGCTCAG-3´) and reverse primer 409R (5´-TTACAACCCAAGGGCCTTCCTCCC-3´) [27]. The amplified and purified DNA was sequenced using an automated sequencer (Applied Biosystem; Model 3100).

Nitrogen fixation

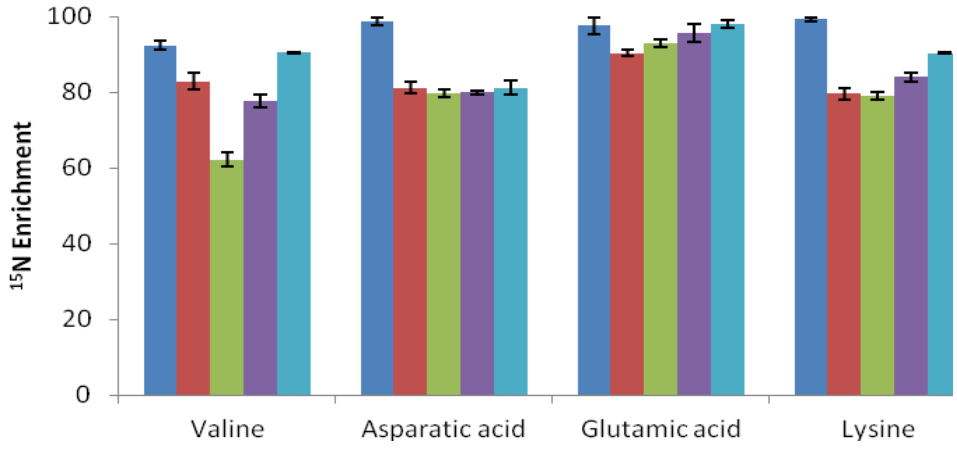
For the screening of nitrogen fixing cyanobacteria, cultures were grown in nitrogen free basal media. After the confirmation of nitrogen fixing strains, cells (1 µg/ml chlorophyll-a) grown in nitrogen free media were shifted to the BG11 media supplemented with [^{15}N]-labeled sodium nitrate (Cambridge Isotopes, Tewksbury, MA, USA, product number NLM-157) such that [^{15}N] was the only available source of nitrogen in the medium. After different time periods (15, 30, 40, 50 and 60 days of inoculation) the free amino acids of the cyanobacterial cultures were extracted for the determination of ^{14}N/^{15}N ratio in the cells using GC-MS amino acid analysis. For the extraction of amino acids, cyanobacterial cultures harvested by centrifugation (1000xg for 10minutes), were then crushed using fine glass beads added to a disposable mortar and pestle (Kimble Chase Kontes, Vineland, NJ, USA) in 1000 µl of 10 mM hydrochloric acid. After vortexing for 15 x minutes at room temperature, samples were centrifuged at 14,000 g for 3 minutes. The obtained supernatant was loaded onto commercial SCX tips (TT2-TWSCX.96, Glygen Corp, Columbia, MD, USA). SCX tips were then washed with 500 µl of 80 % methanol 3 times. Next, the amino acids were eluted from the tips with 100 µl of freshly prepared 1:1 (v/v) 8M ammonium hydroxide:methanol into 250 µl conical glass vials (sold as GC auto sampler inserts).The analyte in 1:1 (v/v) 8M ammonium hydroxide:methanol was dried using nitrogen gas and then 25 µl of methanol was added in the tube and derivatized by using of 2.5 µl of pyridine and 2.5 µl of methyl chloroformate into the 250 µl glass vial [28]. To separate the methyl chloroformate derivatives from the reaction mixture, 50 µl of chloroform and 50 µl of a 50mM sodium bicarbonate solution were added sequentially and mixed well until a clear bottom phase was obtained. The bottom (chloroform) layer was transferred to a new 250 µl GC insert vial containing a few crystals of sodium sulfate, followed by brief vortex mixing. The dry sample was then transferred to a new 50 µl GC insert vial for GC-MS analysis. GC–MS analyses of the derivatized extracted amino acids was performed using a Hewlett-Packard 5890 (GC)/5970 mass selective detector (MSD) in electron impact (EI) mode (70 eV) with a system equipped with a fused silica capillary column (HP-5MS, 30 m×25 mm ID, 0.25 µm film thickness; Agilent J & W Scientific, Folsom, CA, USA). The 2 µl sample was injected using the split/splitless mode. The oven temperature was initially held at 70 ºC for 3 minutes. Thereafter the temperature was raised at 25 ºC/min until 280 ºC and held for 5minutes. Helium was used as carrier gas and delivered at a constant flow rate at 1 ml/min. The injector temperature was set at 240 ºC and the interface temperature was 290 ºC.

Result and Discussion

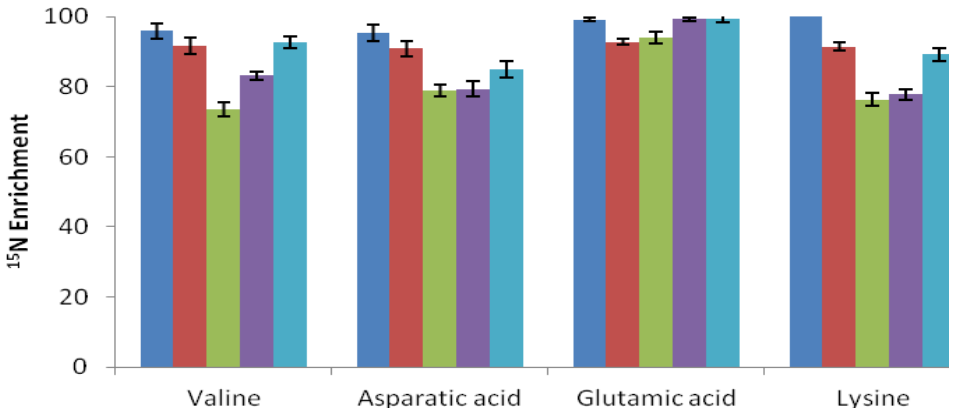
Cyanobacterial strains were cultured in nitrogen free BG11 medium and strains capable to flourish were referred to as nitrogen fixers. Three cyanobacterial strains were found to be nitrogen fixing that include one unicellular strain (*Chroococcidiopsis* sp. SM-04) and two filamentous (*Phormidium* sp. SM-14 and *Phormidium* sp. SM-15). Amino acids extracted from 15 days incubated cultures of cyanobacterial strains showed highly enrichment with [^{15}N]. Whereas after 30 days of incubation reduction was observed in [^{15}N]-enrichment of amino acids and this reduction was continuously recorded up till 40 days in *Chroococcidiopsis* sp. SM-04 and up till 50 days in *Phormidium* sp. SM-14 and *Phormidium* sp. SM-15. After respective days, GC-MS analysis demonstrated that most of the extracted amino acids again become enriched with [^{15}N]. In all three strains maximum enrichment is observed in glutamic acid (above 90 %) throughout all the incubation period whereas significant drop in [^{15}N]-enrichment was recorded in all other three recorded amino acids i.e. valine, aspartic acid and lysine (Fig 1A; 1B and 1C). The drop in [^{15}N]-enrichment of valine and lysine was relatively higher than aspartic acid. In *Chroococcidiopsis* sp. SM-04 [^{15}N]-enrichment recorded in valine after different incubation period was 99.37 % (15 days), 80.92 % (30 days), 70.73 % (40 days), 51.20 % (50 days) and 59.11 % (60 days). [^{15}N]-enrichment recorded in aspartic acid after different incubation period (60 days). 98.71 %, 85.65 %, 66.90 % and 59.56 %, 63.33 %. Mostly the response of *Phormidium* sp. SM-14 and SM-15 was almost same. The [^{15}N]-enrichment recorded in valine of 15 days old cultures of *Phormidium* sp. SM-14 and SM-15 was 92.38 % and 95.96 % which dropped to 82.87 % and 91.58 % respectively after 30 days of incubation. This drop was continuously recorded till 40 days upto 62.09 % and 73.53 % respectively. Afterword rise in [^{15}N] enrichment was recorded. Same pattern was observed with aspartic acid and lysine.



(a)



(b)



(c)

Fig. 1: ^{15}N enrichment in the amino acids of cyanobacterial strains after different incubation time period (15, 30, 40, 50 and 60 days). A) *Synechocystis* sp. SM-10, B) *Phormidium* sp. SM-14 and C) *Phormidium* sp. SM-14 [correct the spelling of aspartic on fig].

For the determination of nitrogen fixation potential of cyanobacterial strains nitrogen deprived isolates were cultured in BG11 media supplemented with the [^{15}N]-labeled sodium nitrate. After different incubation period (15, 30, 40, 50 and 60 days) cultures were taken for the analysis [^{15}N]-enrichment in the amino acid. Amino analysis of cyanobacterial cultures showed that most of amino acids are present in all three strains but glycine, alanine, valine, proline, asparagine, aspartic acid, glutamic acid and lysine were present in appreciable amounts. Therefore focus was given to valine, aspartic acid, glutamic acid and lysine for the determination of the nitrogen fixation potential of cyanobacterial cultures. Although glycine, alanine, proline and asparagine were also present in appreciable amounts but the results were less clear due to problems of overlapping peaks of glycine with alanine and proline with asparagine (Fig 2A, 2B and 2C). Nitrogen starved cells of cyanobacterial cultures were shifted into [^{15}N]-labeled BG11 media. When cyanobacterial strains are cultured in nitrogen free media their capacity for nitrogen assimilation increases dramatically [29, 30]. Therefore, the addition of nitrate salt appeared to induce amino acid synthesis at a rate several fold higher than in non-starved cells [31]. Initially cyanobacterial strains used the readily available nitrogen source from media, therefore all observed amino acids showed high [^{15}N]-enrichment. Typically, cyanobacteria convert nitrogen obtained in the form of nitrate into ammonium and then ammonium compounds are further incorporated for amino acid synthesis through glutamine synthase and glutamine oxoglutarate amino transferase [32]. Once ammonium nitrogen has been incorporated into glutamine, it acts as a source of the amino group for the production of other amino acids [33]. When the nitrogen source became depleted from the medium, cyanobacteria begin to fix atmospheric nitrogen and this was confirmed by the appearance of [^{14}N] in (Fig 3A, 3B and 3C). The appearance of [^{14}N] was higher in valine and lysine as compared to glutamic and aspartic acid. A possible explanation of the slower enrichment of glutamic and aspartic acid is that they are stored in the cells as a reserve nitrogen source. Cyanobacteria synthesizing cyanophycin use it as a source of nitrogen during nitrogen limitation [34] and cyanophycin is typically composed of arginine, aspartic acid and glutamic acid [35]. It is noteworthy that the cyanophycin extracted from nitrogen-starved cells of some cyanobacteria only contained aspartic and glutamic acids with no arginine [36]. It is also confirmed through studies that in most of the cyanobacteria including non-heterocystous forms, cyanophycin acts as a reservoir of nitrogen [37, 38]. When nitrogen deprived cells were transferred to the BG11 media having nitrate salts, these cells likely incorporated nitrate at each cyanophycin nitrogen sites. It is likely that the first response to nitrate depletion under adequate light and other nutrients is that they begin to fix atmospheric nitrogen, which in our system would appear as [^{14}N]-amino acids. However, it would seem likely that as cells became older or at the end of the stationary phase, which was observed in *Chroococcidiopsis* sp. SM-04 after 50 days and in *Phormidium* sp. SM-14 and SM-15 after 40 days, they would begin to use stored nitrogen. Therefore, after the respective days, the appearance of the labeled peak in the free amino acid pool might appear again due to the breakdown of cyanophycin. From the results in this study, we hypothesize that at once the cultures reached the stationary phase of growth; the cyanobacterial nitrogen fixation was likely inhibited at high cell density and no longer supplied a significant amount of nitrogen.

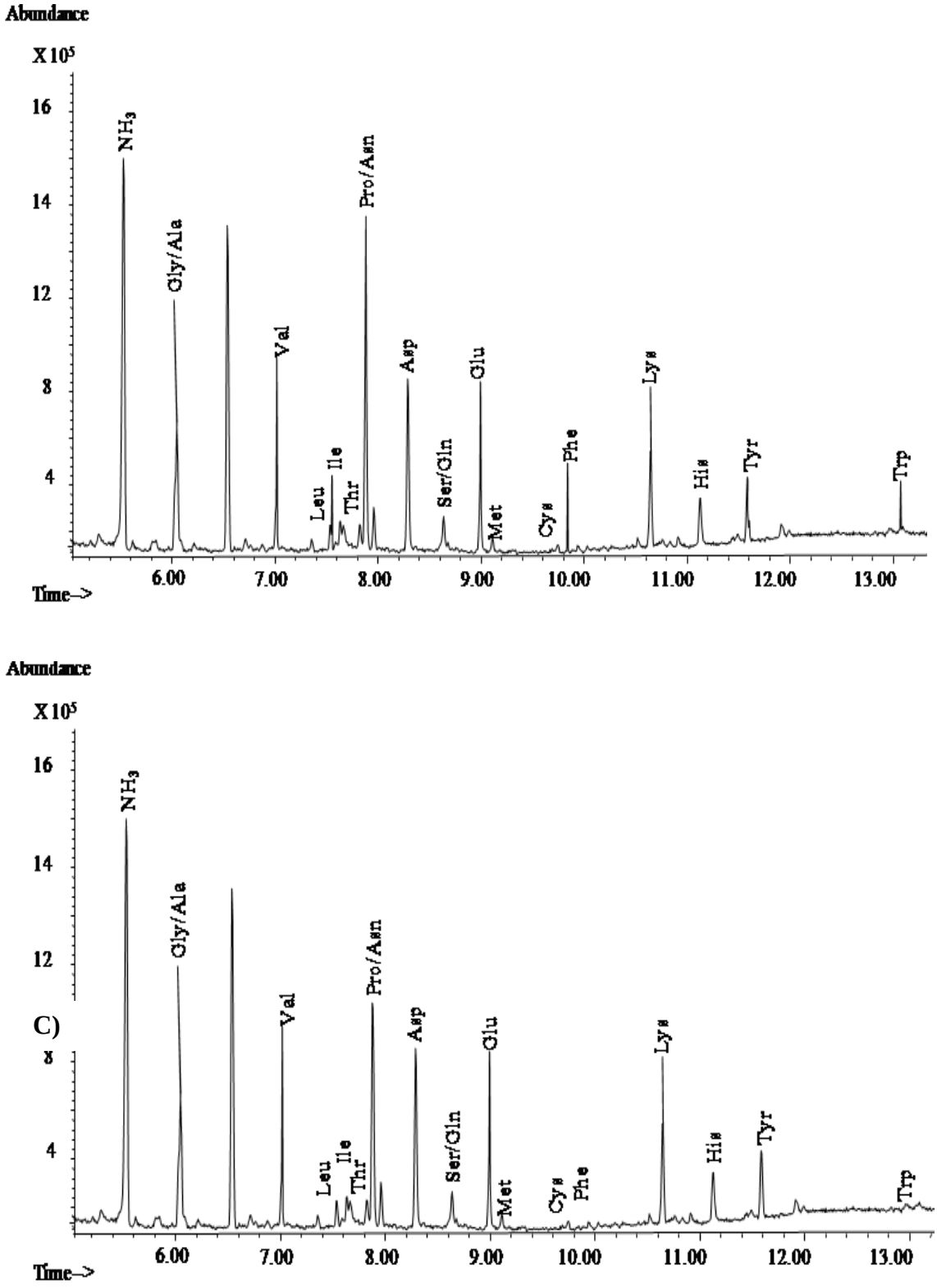


Fig. 2: GC-MS analysis of amino acid A) *Synechocystis* sp. SM-10, B) *Phormidium* sp. SM-14 and C) *Phormidium* sp. SM-14.

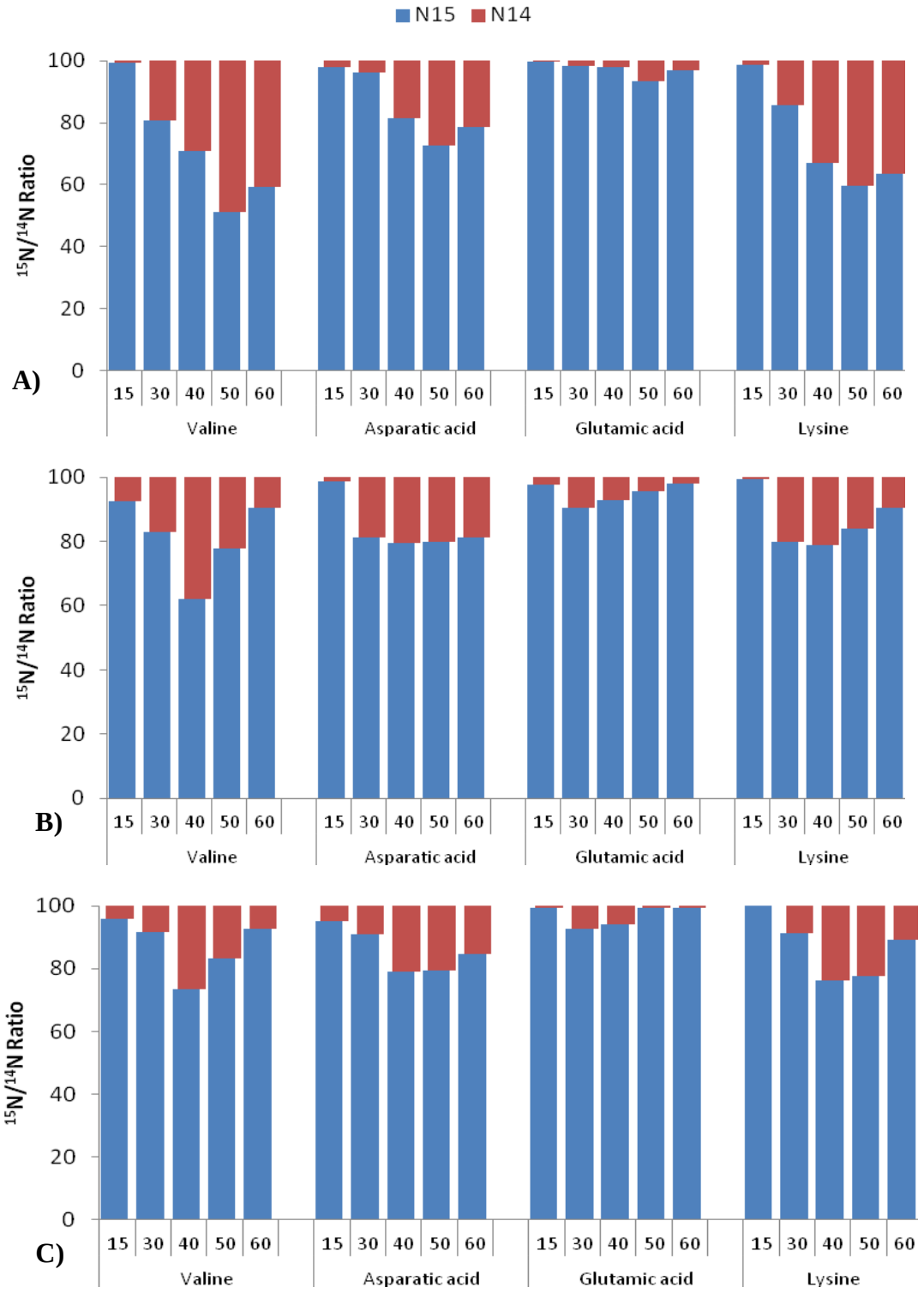


Fig. 3: $^{14}N/^{15}N$ ratio in the amino acid of cyanobacterial strains after different incubation period. A) *Chroococcidiopsis* sp. SM-10, B) *Phormidium* sp. SM-14 and C) *Phormidium* sp. SM-14.

This currently used approach not only provided a detailed method for the evaluation of the nitrogen fixing potential of the cyanobacteria in culture, but also suggests novel approaches for the assessment of the ability of the strains to provide nitrogen enrichment to plants under co-cultivation conditions.

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