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Prospects of *in vivo* ^{31}P NMR method in glyphosate degradation studies in whole cell system

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ABSTRACT

The degradation of the phosphonate herbicide glyphosate (*N*-phosphonomethylglycine) by four taxonomically distinct microorganisms was studied *in vivo* in whole cell system using phosphorus nuclear magnetic spectroscopy (^{31}P NMR). The time-course of glyphosate metabolization in dense cell cultures was followed by means of ^{31}P NMR up to 21 days after the addition. The results obtained by this non-invasive way confirmed that the cells of *Spirulina platensis* and *Streptomyces lusitanus* biodegrade herbicide. Moreover, phosphorus starvation influenced the rate of glyphosate degradation by *S. platensis*. On the other hand, the results of similar measurements in the cultures of green algae *Chlorella vulgaris* showed that this aquatic plant, however growing in the medium containing 1 mM of *N*-phosphonomethylglycine, did not seem to posses the ability of its biodegradation. Additionally, the use of this method allowed us to find the new fungal strain *Fusarium dimerum*, which is able to biodegrade and utilize the glyphosate as the sole source of phosphorus. The results of our studies on usefulness of *in vivo* ^{31}P NMR for tracing glyphosate degradation in whole cell systems revealed that this non-invasive, one-step method, might be considered as a valuable tool in environmental biotechnology of organophosphonate xenobiotics.

1. Introduction

Because of its widespread and long-term use worldwide, glyphosate (*N*-phosphonomethylglycine), the active ingredient of the popular, non-selective herbicide *Roundup*®, is perhaps the most studied organophosphonate, and it may serve as a model compound when assessing the ability of a new microorganism to cleave the carbon-to-phosphorus bond [1].

Cyanobacteria, a strikingly diverse group of prokaryotes, are the oldest oxygenic photosynthetic organisms sharing the same photosynthetic apparatus of eukaryotic algae and higher plants [2], and comprise a major proportion of the total phytoplankton biomass. These microorganisms show a remarkable tolerance to any kind of stress, including chemical one, and are well known to adapt to contaminated environments [3–7]. A few species have been found to exhibit a remarkable tolerance to glyphosate [8], possessing, however, different biochemical bases of this feature [9]. Being one of the few cyanobacteria that do not produce toxins, *Spirulina platensis* is raising an interest as a source of health food, feed and nutraceuticals, and for this purpose has become of some commercial value, especially in developing countries [10,11]. Such features make it also a good potential candidate for bioremediation purposes, especially considering xenobiotics, which contain a covalent C–P bond. This kind of biodegradation was exhibited by cell culture of mixed blue-green algae *Spirulina* spp., which was found to possess remarkable ability to degrade glyphosate [12].

A few selected strains of *Streptomyces*—another bacterial microorganisms possessing features different to typical bacteria [13,14] utilized glyphosate as phosphorus and nitrogen source, with *Streptomyces lusitanus* being the most effective one. Not only bacteria are able to biodegrade phosphonate compounds. This feature was also determined in lower fungi, however, the number of known fungal species, which are capable to cleave the C–P bond, is significantly lower than bacterial species. Nevertheless some filamentous fungi like: *Trichoderma harzianum*, *Scopulariopsis* sp., *Aspergillus niger* [15], *Cladosporium resinae* [16], *Alternaria alternata* [17], *Mucor* sp. [18], fungi from genus *Penicillium* [19–22] and finally some yeasts as *Kluyveromyces fragilis* [23] and *Candida maltosa* [20] were found as species skilled to biodegrade organophosphonates. In this paper we have added *Fusarium dimerum* to this collection.

Green algae are known to be more sensitive to many chemicals in relation to photosynthetic cyanobacteria, however, their ecological position in most aquatic ecosystems and their essential role in nutrient cycling and oxygen production are comparable with the status of cyanobacteria [24]. Because these plants vary in their response to a variety of toxicants [25], they have been considered as specific indicators of the biocidal activity of industrial and urban wastes. Although some information on the toxicity of pesticides

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towards green algae has been compiled there are no clear data about the ability of these organisms to degrade or transform these chemicals [26].

Previously reported results have already shown the usefulness of NMR spectroscopy in studies on metabolism. For example, Brand et al. have shown the usefulness of ^{13}C NMR for monitoring of neuronal glucose metabolism [27]. Unfortunately the use of ^{13}C NMR to monitor metabolism requires using C^{13} enriched samples, which is not always possible. A technique which does not need isotope enriched sample is ^{31}P NMR and this feature, although limited to phosphorus compounds, broadens its applicability for metabolomic studies of samples of natural origin. *In vivo* phosphorus spectroscopy allows the monitoring of energy status [27–29], observe phosphorus metabolism [28,30,31] including response to drug treatment [30] and metabolic answers to stress conditions, either physical [32] or chemical [33–35]. ^{31}P NMR might be also used as a tool for identification of natural organophosphonate compounds present in the cells of living organisms [29]. Moreover, this method gives the possibility to determine *in vivo* concentration of organophosphorus compounds (phosphocreatine, ATP, phosphoarginine) [31,34]. Additionally, the use of an interrelationship between chemical shift of orthophosphate and pH, permits to estimate intracellular pH. It is also worth noting, that the spectroscopy of nuclear magnetic resonance is the real-time method, thus the results derived from *in vivo* experiments reveal the real-time changes occurring during metabolic processes [28,29,36,37].

Considering, that NMR imaging techniques have been relatively little used for xenobiotic research, the aim of this work was the application of non-invasive nuclear magnetic resonance *in vivo* ^{31}P NMR, as a one-step method allowing the direct detection of microbial biodegradation of phosphonate xenobiotics. The technique applied in this study appeared to be simple and effective and therefore in this paper we describe its more systematic use to study the carbon-to-phosphorus bond cleaving ability of physiologically different cells, namely *S. platensis*, *Streptomyces lusitanus*, *F. dimerum* and *Chlorella vulgaris*. The possibility of using this method as a fast and effective way of finding new microbial strains, which are capable of degrading organophosphonates is also verified and discussed.

2. Materials and methods

Pure *N*-phosphonomethylglycine was obtained from commercial Roundup® 360 SL (Monsanto, MO, USA) formulation by dissolving it in water and maintaining pH of the solution to 1.5–2.0 with hydrochloric acid. This resulted in crystallisation of the glyphosate, which was purified by multistep recrystallisation. The structure and purity of the compound was confirmed using ^{1}H, ^{13}C and ^{31}P NMR spectroscopy. Also the retention time of this substance was examined using HPLC–UV after derivatization with *p*-toluenosulphonyl chloride and was the same as the retention time of pure glyphosate obtained from Monsanto. Additionally the malachite green assay (see Section 2.4) was performed considering the presence of inorganic phosphate contaminations. As the result the level of inorganic phosphate was calculated as maximum 3% of the total amount of phosphorus. Thus, the purity of tested compound was established as 97%.

2.1. Strains and growth conditions

Before the growth in NMR tubes for ^{31}P NMR experiments, all tested microorganisms were pregrown in their appropriate standard media in order to achieve inocula, which were used to growth in the conditions of experiment.

S. platensis strain C1 was grown at 22 ± 1 °C for 16-h days (300 μmol m^{-2} s^{-1} PAR) and 8-h nights in 250 ml Erlenmeyer flasks containing 70 ml of standard medium for *Spirulina* (MSp, ATCC medium 1679). Subcultures were revitalized every 4 weeks in late log-phase by transferring 20 ml aliquots to 50 ml of fresh medium.

For the whole cell NMR measurements, intensive growing 2-week-old *Spirulina* cultures were harvested by centrifugation at $5000 \times g$ for 15 min. Cells were washed three times with phosphate-free MSp, and then transferred to fresh standard medium, generating P-replete cells, or medium lacking any phosphorus generating P-starved cells, for a further 2 weeks of growth. After that time the cells were once again harvested by centrifugation and washed. The P-replete and P-starved cells were separately resuspended 0.5 ml of phosphate-free MSp medium yielding dence cell cultures (DCC). The biomass of *Spirulina* in DCC was based on measurement of chlorophyll levels and was set at 70 ± 5 mg l^{-1} of chlorophyll. The 0.5-ml aliquots were then placed in sterile NMR tubes, and ^{31}P NMR measurements and chlorophyll content examination were done. The experiment with *S. platensis* was carried out in six repetitions.

C. vulgaris was grown at 22 ± 1 °C under 16-h days (300 μmol m^{-2} s^{-1} PAR) and 8-h nights in 250 ml Erlenmayer flasks containing 50 ml of Chlorella medium with 0.50 g K_2HPO_4 per liter as phosphorus source. Subcultures were produced every 4 weeks, in late log-phase, by transferring 15 ml aliquots to 35 ml of fresh medium. The experimental cultures were arranged similar as in the case of *Spirulina* via cell harvesting by centrifugation (5000 rpm for 15 min) and triplicate washing with phosphate-free medium of 4 weeks old *Chlorella* culture. Harvested and washed cells were then resuspended in small volume of phosphate-free Chlorella medium to obtain the dense culture containing 40 ± 5 mg l^{-1} of chlorophyll. The 0.5 ml samples of such dense cell culture were placed in sterile NMR tubes, and ^{31}P NMR and chlorophyll content measurements were performed. After the addition of glyphosate the pH was adjusted to 9.8 with 1 M sodium hydroxide solution This experiment was carried out in six repetitions.

The NMR analysis of cyanobacteria and green algae cultures had been done just before and in time-course mode after addition of the *N*-phosphonomethylglycine as the only phosphorus source at final concentration of 1 mM.

Conidia of *F. dimerum*, were produced in solidified standard Czapek medium [19]. A stock solution was prepared by washing the surface of 10–14 days-old cultures with sterile distilled water containing 0.05% Tween. Spore concentration in the stock suspension was determined using the Thom's camera. Three replicates of 3 ml volumes of liquid Czapek media were inoculated with 10^6 fungal spores and incubated at 25 °C on a rotary shaker (60 rpm). After 7 days of growth the cultures were filtered through sterile Sartorius microbial filter kit, fungal mycelia were washed three times with P-free Czapek medium and then transferred to 0.5 ml of Czapek medium deficient in any phosphorus form and placed in the NMR tube. After the addition of glyphosate the pH was adjusted to 5.5 with 1 M sodium hydroxide solution. The experiment with *F. dimerum* was carried out in four repetitions.

The strain of *Streptomyces lusitanus* was maintained at 30 °C in medium for *Streptomeces* [14] solidified with agar, which provided spores suitable for preparation of inoculum after 10 days of culturing. Spores were then used to inoculate three runs of 3-ml volumes of *Streptomeces* medium, which were incubated at 25 °C on a rotary shaker (60 rpm). After 7 days of incubation the cultures were filtered through sterile Sartorius microbial filter kit, bacterial "hyphae" were washed three times with P-free Czapek medium, resuspended in 0.5 ml volumes of *Streptomeces* media lacking any form of phosphorus, and then transferred to NMR tubes. The pH of the medium was adjusted to 7.0 with 1 M sodium hydroxide solution just after the addition of glyphosate. The experiment with *Streptomyces lusitanus* cell culture was carried out in four repetitions.

The NMR measurements of *Fusarium* and *Streptomyces* had been done just before and in time-course mode after addition of the *N*-phosphonomethylglycine as the only phosphorus source at final concentration of 1 mM.

2.2. ^{31}P NMR analysis

The 0.5-ml aliquots of all experimental cell cultures were placed in sterile NMR tubes and allowed to grow for further 21 days under standard conditions appropriate for each of studied strains as described above. ^{31}P NMR measurements were carried out at: 0, 1, 3, 5, 7, 14 and 21 days in cases of *Spirulina* and *Chlorella*, or 0, 1, 5, 7, 14 and 21 days in cases of *Streptomyces* and *Fusarium*, after the cells were transferred into NMR tubes and supplemented with appropriate amount of glyphosate.

All samples were analyzed by means of ^{31}P NMR using a Bruker Avance DRX 400 spectrometer operating at 161,976 MHz, with 10 mM orthophosphoric acid in DMSO as an internal standard—reference, placed in coaxial insert. All treatments were carried out in triplicate.

2.3. Cells growth determination

The determination of the growth of *S. platensis* and *C. vulgaris* was performed by time-course measurements of total chlorophyll content in experimental samples just after the NMR analysis. Three from among six samples were randomly chosen and the growth was followed by harvest: 20 μl aliquots were withdrawn and tenfold diluted. The resultant 0.2-ml samples were taken and the cells were sedimented by centrifugation for 10 min at $14{,}000 \times g$ in the case of *Spirulina* and $6000 \times g$ in the case of *Chlorella*. Pellets were resuspended with 1.0 ml methanol, and solubilization was allowed to proceed for 30 min in the dark, with occasional mixing. Samples were then centrifuged as above, and total chlorophyll content in the supernatant was determined spectrophotometrically on the basis of the Arnon's formula.

The growth of *Fusarium* and *Streptomyces* was examined by dry mass determination only at the beginning and in the end of experiments. The cultures from appropriate NMR tubes were filtered through Whatman # 2 filter paper, then dried and weighted until the difference between following measured masses was not higher than 5%.