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Hydrolysis of Organic Phosphate Forms by Phosphatases and Phytase Producing Fungi of Arid and Semi Arid Soils of India

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Abstract: Phytase and phosphatase producing fungi were isolated from the soil's of arid and semi arid regions of India and tested for their ability to hydrolyze two important organic P compounds: phytin and glycerophosphate. The identified organisms belong to the four genera: *Aspergillus, Penicillium, Pseudeurotium* and *Trichoderma.* A significant negative correlation ($r = -0.386$, $n = 28$, $p<0.05$) was observed between the development of fungal mats and the pH of the media. The extracellular phosphatases (E) released by different fungi were less than that released intracellularly (I), but the trend was reversed in case of phytase production. The E:I ratio of different fungi ranged from 0.45-0.61 for acid phosphatase, 0.41-0.54 for alkaline phosphatase and 44.9-71.9 for phytase. The efficiency of hydrolysis of different organic P compounds by different fungi varied from 0.98-3.54 μg min^{-1} g^{-1} for phytin to 1.82-5.89 μg min^{-1} g^{-1} for glycerophosphate. The trend of efficiency was as follows: *Trichoderma* sp.>*Aspergillus* sp.>*Pseudeurotium* sp.>*Penicillium* sp. *T. harzianum* was found to be most efficient organic P mobilizer as compared to other fungi tested. In this paper, we investigate the relative abilities of intra-and extracellular fungal acid and alkaline phosphatase respectively and of phytase to hydrolyze different organic P compounds normally present in soil.

Key words: *Trichoderma* · *Aspergillus* · *Penicillium* · Organic P

INTRODUCTION

A large proportion of P that is applied to soil as fertilizer rapidly becomes unavailable to plants, accumulating in inorganic P fractions that are fixed by chemical adsorption and precipitation, and organic P fractions that are immobilized in soil organic matter [1]. Consequently, fertilized soils contain a significant amount of total soil P, of which some 50-80% may exist in organic forms [2]. Inositol penta-and hexaphosphates (phytates) and their derivatives account for a major component of the soil organic P. The abundance of myco-inositol phosphates in the soil seems to be due to their low solubility, their firm association with the solid phase and to their high stability [3].

The importance of soil organic P as a source of plant available P depends on its rate of solubilization and the rate of inorganic P release. Several types of phosphatases, such as phytases, are able to increase the rate of the dephosphorylation (hydrolysis) of organic P. Phosphatases in the rhizosphere may arise from plant roots [4, 5] or from soil microorganisms [6, 7]. In soil, the hydrolysis of organic P is predominantly mediated by the activity of soil microorganisms, although plant roots also possess phosphatase and phytase activity [8, 9]. Moreover, microbial acid phosphatase was found to be more efficient in hydrolysis of organic P compounds than plant sources [10]. The potential role of phytase in increasing the availability of P from phytate in soils remains to be established. The additions of phytase increased the P content of maize seedlings when supplied with phytate and it was concluded that the utilization of phytate by plant was limited by low rates of hydrolysis [11]. The potential role of soil microorganisms for increasing the P availability from phytate through phytase activity is well defined [12]. The comparative efficiency of fungal intra-and extracellular phytase activity demonstrates that both fractions are efficient in the hydrolysis of organic P compounds [13]. In this study we isolated both phosphatase and phytase producing fungi from arid and semi arid soils of Rajasthan, India and examine their efficiency to hydrolyze different organic P compounds so that the amount of P mobilized by different fungi could be quantified and the most efficient fungus

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could be used as inoculum to exploit native organic phosphorus for plant growth.

MATERIALS AND METHODS

Survey for Phosphatase and Phytase Producing Fungi: A survey for both phosphatase and phytase producing fungi was undertaken in five arid and semi-arid districts of Western Rajasthan, India (1,11,681 km^2 area) during March 2004. The upper layers of soil were scrapped off to remove foreign particles and litter before taking samples. Soil samples were collected from furrow layer in 15 replications. The collected soil samples were stored in self sealing polythene bags and placed in an insulated carrier for transport during field trip and then immediately refrigerated at 4°C. All the soil samples were sieved (<2 mm mesh size) to remove foreign particles before processing. Sub sample of each soil was air dried and used for estimation of various physico-chemical properties.

Soil Parameters: Soil samples were analyzed for pH and electrical conductivity (EC) on 1:2.5, soil: water suspension, organic matter, particle size distribution and phosphorus following standard methods [14]. Esterase (EC 3.1.1.1) activity, indicates the catabolic activity in the soil and is directly correlated with microbial activity [15], was determined by measuring the hydrolysis of fluorescein diacetate (FDA) and the fluorescein released was quantified spectrophotometrically [16]. The enzyme activity was expressed as enzyme unit (EU = μ kat fluorescein g^{-1} min^{-1}).

Isolation of Fungi: Fungi were isolated in pure culture from the 60 soil samples, using dilution plate technique employing Martin's Rose Bengal agar medium with streptomycin sulphate. Thirty different fungi (on the basis of colour, shape, growth, spore formation, etc.) were isolated and purified from the single spore on medium slants. Isolated fungi were identified up to species level by Agharkar Research Institute, Pune, India and their pure cultures were maintained on a potato dextrose agar (PDA) medium. Based on their intra and extracellular acid phosphatase, alkaline phosphatase and phytase (data not shown), ten fungi were selected and their growth, reaction, activity and efficiency were compared.

Extracellular Enzyme Extraction: Fungi were grown in 125 ml Czapek-Dox broth in 250 ml Erlenmeyer Flasks. The medium was inoculated with 8 mm discs of 5 day-old fungal growth (on PDA medium) and the flasks were incubated at 30±1°C. There were twelve flasks of each fungal species started. At the end of 7, 14, 21 and 28 days of incubation, pH of the broth was measured and three flasks of each fungal culture were chilled in ice and the contents were filtered through Whatman No. 1 filter paper into another flask, kept in ice. The final volume of each filtrate was made up to a known volume using sterilized cold distilled water. The filtrate was used for assaying the extracellular acid phosphatase, alkaline phosphatase and phytase activity.

Intracellular Enzyme Extraction: For determination of intracellular activity, fungal mats at each sampling time were washed at least 10 times with ice-cold distilled water (10 ml each time) to remove traces of extracellular enzymes. Washed fungal mat were weighed after lightly pressing them between sheets of filter paper. A small part of the mat was dried to express the results on dry weight basis. The remaining part was ground with acid-washed quartz sand in a mortar. Ice-cold sterilized distilled water was added to obtain a fine suspension of fungal mat. The extract obtained was centrifuged at 12,000 rpm for 20 min to settle the fungal debris. A clear supernatant containing the intracellular enzymes was obtained and made up to a known volume and used for assaying the intracellular acid phosphatase, alkaline phosphatase and phytase activity.

Estimation of Phosphatase and Phytase: Acid and alkaline phosphatases were assayed by adopting the standard procedure of Tabatabai and Bremner [17] using acetate buffer (pH 5.4) and sodium tetra borate-NaOH buffer (pH 9.4), respectively. The enzyme substrate (p-nitrophenyl phosphate) mixture was incubated at 37°C for 1 h and the enzyme activity was expressed as enzyme unit (EU). One unit is the amount of enzyme, which hydrolyze 1.0 μ kat of p-nitrophenyl phosphate min^{-1} at pH 5.4 (acid phosphatase) or pH 9.4 (alkaline phosphatase) at 37°C. Phytase activity was assayed by measuring the amount of inorganic phosphate (Pi) released by hydrolysis using sodium phytate. The reaction was initiated by addition of 100 ml phytase preparation to an assay mixture (1.0 ml) containing 0.2 M sodium acetate buffer (pH 4.5) and 1mM sodium phytate and incubated at 37°C for 1 h. The reaction was terminated by the addition of 0.5 ml 10% trichloro acetic acid. Proteins precipitated by TCA were removed by centrifugation at 10,000 rpm for 10 min and the

supernatant was analyzed for liberated Pi [18]. One unit of phytase activity was defined as the amount of enzyme, which liberated 1 µ kat Pi min^{-1}.

Quantification of Organic P Hydrolysis: To test the efficiency of fungi towards the hydrolysis of different organic P compounds, 1g of fungal mat, in triplicate for all the fungi, was crushed in acid washed quartz sand in a mortar with 30 ml of ice-cold sterilized water. The extract was centrifuged as described earlier. Five hundred ppm of phytin and Na-glycerophosphate was added to the clear supernatant and incubated at 37°C for 24h. The released P was quantified colorimetrically as described by Jackson [14] and expressed as µg P released min^{-1} g^{-1} of fungal mat.

Statistical Analysis: The data were subjected to analysis of variance and the least significant differences (LSD) were used to separate means [19].

RESULTS

The soil characteristics of surveyed area, vegetation and rainfall are presented in Table 1. The mean maximum temperature of the surveyed area varied from 43.0-46.0°C and mean minimum temperature varied from 3.0-4.5°C. The ten greatest phosphatases and phytases producing fungi identified are listed in Table 1. They belong to the four genera: *Aspergillus*, *Penicillium*, *Pseudeurotium* and *Trichoderma*. The acidification of the media with time was comparable among isolates (Fig. 1). A gradual decline in pH of the media with time was observed. *T. harzianum* exhibited marked decline in pH after 4 weeks (7.3-2.4) closely followed by *T. viride* (7.3-3.0). *Penicillium simplicissimum* released least acid in the media (7.3-5.5) followed by *P. rubrum* (7.3-5.4). In general, the acid secretion in the medium followed in the order: *Trichoderma* sp.>*Aspergillus* sp.>*Pseudeurotium* sp.>*Penicillium* sp. The change in pH is an important criterion as it regulates the P release.

A. candidus accumulated the most biomass; closely followed by *Penicillium* sp. (Fig. 2). A significantly negative correlation ($r = -0.386$, $n = 28$, $p<0.05$) was observed, with the development of fungal mat and pH of the media.

Intra-and extracellular acid phosphatase activities of various fungi at different time intervals are presented in Table 2. In general, all the fungi had higher intracellular acid phosphatase activity (10.4-97.3 EU × 10^{-6} g^{-1}) than the enzyme released by the fungi extracellularly (4.3-42.7 EU × 10^{-3} g^{-1}) but the ratio of extra/intra cellular (E:I) acid phosphatase activity varies with the fungal species. The mean E:I ratio of *Aspergillus* sp., *Penicillium* sp., *Pseudeurotium* sp. and *Trichoderma* sp. were 0.54, 0.47, 0.45 and 0.61 respectively. The average intracellular acid phosphatase activity was two times higher than extracellular acid phosphatase secretion per unit of dry fungal mat. In general, the maximum acid phosphatase activity was observed at 21 day and *Trichoderma* sp. showed greatest activity at the same day.

The intra-and extra cellular alkaline phosphatase activity of different fungi (Table 3) revealed, 2.35 times less production of alkaline phosphatase as compare to acid phosphatase. The intracellular alkaline phosphatase activity was 48.87 % higher than extracellular release. The mean E:I ratios for alkaline phosphatase of different fungi were as follows: *Penicillium* sp.

Table 1: Characteristics of the soil, vegetation and rainfall in the survey area and list of efficient fungi identified from the soil

Parameters	Barmer	Hanumangarh	Jaisalmer	Jodhpur	Pali
pH	8.6-8.7	8.6-8.8	7.8-8.1	8.4-9.5	8.4-8.7
Organic matter (%)	0.41-0.46	0.43-0.45	0.65-0.67	0.44-0.65	0.39-0.41
Esterase (EU × 10^{-3})	92-107	90-112	381-421	140-421	88-112
Total-P (mg kg^{-1})	128-169	163-186	879-918	242-686	381-401
Mineral P (mg kg^{-1})	68-75	91-104	653-673	90-582	108-124
Olsen-P (mg kg^{-1})	2.6-2.9	5.7-6.9	7.0-18.7	8.6-18.9	3.3-3.9
Soil texture	Loamy sand	Sandy	Sandy	Loamy sand	Clay loam
Rain fall (mm)	280-286	210-222	217-230	389-460	415-420
Vegetation	Pearl millet, Sesame	Cotton, Wheat	Chickpea, Wheat	Cotton, Sesame, *Vigna* sp., Pearl millet, Cumin	Cluster bean, *Lowsonia*, Wheat
Organisms	*Penicillium rubrum*, *Aspergillus niger*	*Penicillium simplicissimum*, *Aspergillus terreus*	*Pseudeurotium zonatum*, *Trichoderma viride*	*Aspergillus candidus*, *A. parasiticus*, *T. harzianum*	*Aspergillusrugulosus*

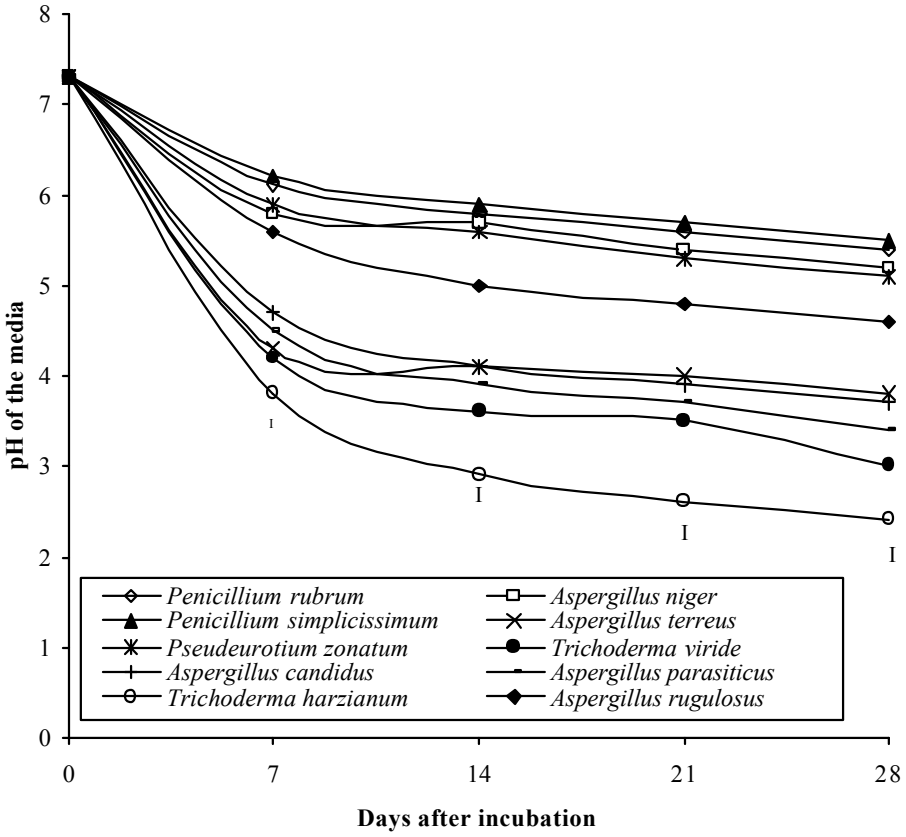

Fig. 1: Changes in pH of the medium due to different fungi at different intervals
Vertical bars are the LSD ($p < 0.05$). The initial pH of the media was 7.3

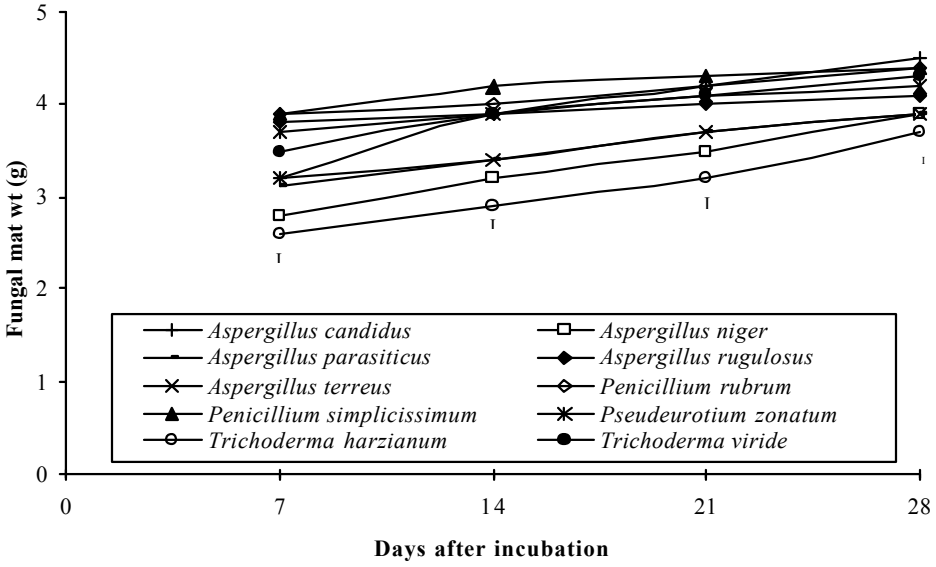

Fig. 2: Development of fungal mat (g^{-1}) at different intervals. Vertical bars are the LSD ($p<0.05$)

Table 2: Secretion of acid phosphatase by fungi at different time intervals

	Acid phosphatases activity (EU × 10^{-6}) g^{-1} dry fungal mat							
	Days after incubation							
	7		14		21		28	
Species	I	E	I	E	I	E	I	E
Aspergillus candidus	11.7	7.9	32.3	12.4	68.7	28.3	29.8	8.7
Aspergillus niger	13.6	8.7	39.8	29.3	65.6	19.3	14.7	12.3
Aspergillus parasiticus	19.3	7.9	56.3	43.8	79.6	25.7	50.3	24.3
Aspergillus rugulosus	14.9	11.4	16.3	13.5	27.9	11.1	14.3	7.9
Aspergillus terreus	34.6	20.7	57.3	37.2	72.3	25.7	26.9	13.6
Penicillium rubrum	10.4	4.3	21.3	12.9	43.6	18.9	13.4	6.9
Penicillium simplicissimum	20.4	12.3	39.6	18.7	59.8	21.3	21.3	10.5
Pseudeurotium zonatum	16.3	8.9	31.6	17.4	53.8	24.3	22.4	6.7
Trichoderma harzianum	22.9	16.8	39.6	21.7	97.3	42.7	26.3	17.4
Trichoderma viride	21.7	17.3	38.3	20.6	89.4	40.6	21.7	16.3
LSD ($p<0.05$)	1.2	0.6	2.1	1.0	3.9	2.1	0.8	0.4

Table 3: Secretion of alkaline phosphatase by fungi at different time intervals

	Acid phosphatases activity (EU × 10^{-6}) g^{-1} dry fungal mat							
	Days after incubation							
	7		14		21		28	
Species	I	E	I	E	I	E	I	E
Aspergillus candidus	17.3	10.6	32.6	12.7	13.7	5.8	4.8	3.1
Aspergillus niger	5.4	3.2	8.9	5.4	6.6	3.4	4.9	2.6
Aspergillus parasiticus	21.6	12.9	38.2	14.3	12.3	6.2	6.9	2.8
Aspergillus rugulosus	5.6	3.7	9.1	5.6	6.9	3.2	4.6	2.9
Aspergillus terreus	20.6	11.9	30.6	14.6	10.0	6.1	6.8	3.2
Penicillium rubrum	4.6	2.8	7.2	4.9	6.1	3.4	3.9	1.2
Penicillium simplicissimum	17.9	12.3	29.7	13.9	18.6	8.2	7.2	4.6
Pseudeurotium zonatum	18.9	12.8	36.7	9.8	11.3	4.9	6.9	2.1
Trichoderma harzianum	28.6	18.3	45.7	18.4	16.2	7.3	8.9	3.6
Trichoderma viride	21.9	16.3	42.8	12.8	14.9	7.8	7.6	4.9
LSD ($p<0.05$)	0.5	0.8	1.1	1.3	1.0	0.2	0.3	0.2

Table 4: Secretion of phytase by fungi at different time intervals

	Acid phosphatases activity (EU × 10^{-6}) g^{-1} dry fungal mat							
	Days after incubation							
	7		14		21		28	
Species	I	E	I	E	I	E	I	E
Aspergillus candidus	0.16	10.1	0.11	6.2	0.04	3.6	0.02	0.9
Aspergillus niger	0.16	6.2	0.12	5.2	0.08	4.1	0.04	1.2
Aspergillus parasiticus	0.20	10.4	0.12	7.1	0.08	4.2	0.03	1.0
Aspergillus rugulosus	0.11	6.1	0.09	3.2	0.02	2.1	0.01	0.3
Aspergillus terreus	0.19	10.2	0.10	6.9	0.06	4.1	0.01	0.9
Penicillium rubrum	0.12	7.2	0.09	4.1	0.06	3.2	0.02	0.8
Penicillium simplicissimum	0.14	6.9	0.09	4.0	0.04	2.1	0.02	0.3
Pseudeurotium zonatum	0.19	7.0	0.06	3.9	0.02	1.9	0.01	0.4
Trichoderma harzianum	0.16	12.8	0.12	8.9	0.08	4.3	0.06	1.2
Trichoderma viride	0.15	11.9	0.12	7.6	0.06	3.9	0.01	1.4
LSD ($p<0.05$)	0.02	0.4	0.01	0.3	0.01	0.1	0.001	0.1

0.54>*Aspergillus* sp. 0.52>*Trichoderma* sp. 0.50>*Pseudeurotium* sp. 0.41. In general, the maximum alkaline phosphatase activity was observed at 14 day and *Trichoderma* sp. showed maximum activity at the same day.

In general, the maximum phytase activity was observed at 7 day (Table 4). The mean E:I ratio of different species were as follows: *Trichoderma* sp. 71.9>*Pseudeurotium* sp. 58.7>*Aspergillus* sp. 51.3>*Penicillium* sp. 44.9. The extracellular phytase

Table 5: Efficiency of fungal mat of different fungi to hydrolyze different organic P compounds

	Efficiency µg P release* min^{-1}	
Fungal species	Phytin	Glycerophosphate
Aspergillus candidus	2.72	4.72
Aspergillus niger	1.72	2.46
Aspergillus parasiticus	3.21	5.12
Aspergillus rugulosus	0.98	1.82
Aspergillus terreus	3.09	4.98
Penicillium rubrum	2.16	2.90
Penicillium simplicissimum	2.21	2.99
Pseudeurotium zonatum	2.39	3.82
Trichoderma harzianum	3.54	5.89
Trichoderma viride	3.32	5.46
LSD ($p<0.05$)	0.64	0.71

* Initial P added 500 ppm either as phytic acid or as Na-glycerophosphate in all the replication

activity was 55.7 times greater than intracellular phytase activity.

Table 5 shows the capacity per EU to hydrolyze different organic P compounds (mono or hexa phosphate). Considerable variation in efficiency of different fungi to hydrolyze glycerophosphate (1.82-5.89 µg P release min^{-1}) and phytin (0.98-3.54 µg P release min^{-1}) was observed. In general *T. harzianum* was found to be the most efficient fungi in hydrolyzing glycerophosphate (mono phosphate) and phytin (hexa phosphate). The trend of efficiency among the different fungal species was as follows *T. harzianum>T. viride>A. parasiticus>A. terreus> A. candidus>P. zonatum>P. simplicissimum> P. rubrum>A. niger>A. rugulosus*.

DISCUSSION

The reduction in pH of medium with time may be due to release of different organic acids by different fungi. The production of organic acids such as malate, citrate and oxalate by different microorganisms is well known [20]. The negative correlation between development of fungal biomass and pH of medium may be due to the affinity of the fungi towards acidity.

The decline in activity of enzymes after 21 day (acid phosphatase) or 14 day (alkaline phosphatase) might be due to the on set of stationary phase in fungal culture [21]. The differences in the amount of extra-and intracellular phosphatases or phytase between different fungi may be due to differences in the fungal structure [10]. The higher extracellular than intracellular phytase activity suggest increased fungal membrane permeability for phytase than for phosphatases. The other reason may be that part of the phosphatases might be present in the vacuole whereas phytase may be mainly located at/near the cell surface, which results in their enhanced release as extracellular enzyme [22].

When the same amount of fungal mat (g^{-1}) was compared, all the ten fungi had differences in their capability to hydrolyze phytin and glycerophosphate. Isoenzymes released by different fungi were different and therefore, their efficiency per unit of enzyme to hydrolyze different organic P compounds may also different. The efficiency per unit of enzyme (phosphatases and phytase) released was greatest with *T. harzianum*. Our results are in consistence with the earlier reports [21].

CONCLUSION

The present experiments highlight the potential of *Trichoderma* sp. regarding hydrolysis of organic P compounds by phosphatases and phytase secretion. Therefore, its culture may be used as efficient inoculum to exploit native soil organic P for plant nutrition. However, the extent to which such microorganisms release P in soil for subsequent uptake by plant roots remains to be determined.

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