pared with 2 µl of DNA sample with 10X PCR buffer, 2.5 mM $MgCl_2$, 200 µM dNTPs, 20 pmol of each forward and reverse primer and 1.5 U of Taq DNA polymerase (all reagents were from Fermentas, Canada). The PCR conditions include: 95°C (5 min) for initial denaturation, followed by 30 cycles of denaturation at 94°C (1 min), and primer annealing at 59°C (1 min), primer extension at 72°C (1 min). The amplified PCR products were separated on 1.5% agarose containing 4 µl of ethidium bromide, visualized and documented in a Biorad UV Transilluminator.

Results of the PCR analysis are presented in Figure 2 *b*. The expected 141-bp amplified DNA product was detected in all the isolates of *P. azadirachtae* irrespective of geographic location (lanes 1–5). On the other hand, *F. moniliforme* did not show any amplified product, indicating that primers used in the study were genus-specific and could amplify only 5.8S rDNA of *Phomopsis*. Irrespective of the differences in cultural characteristics, different isolates of *P. azadirachtae* are similar in 5.8S rDNA. Similar studies have been conducted based on transcribed spacer regions in *Phomopsis* isolates from fruit trees[8,9].

The current globalized market demands more neem products and pesticides for a variety of applications in future. In neem, the seed is the principal propagating material. As the causative agent of dieback disease of neem is transmitted through seeds[10], sensitive techniques are necessary to detect and control *Phomopsis*. The protocol developed in this study could be used for the detection of the causative agent of dieback disease in infected twigs and seed samples. Further research has to address the disease causing vectors and the possibilities of elimination of pathogen from infected seeds/twigs of neem.

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Ubiquitous presence and activity of sulfur-oxidizing lithoautotrophic microorganisms in the rhizospheres of tropical plants*

Microbiological investigations pertaining to the rhizosphere are largely carried out from the perspectives of biological nitrogen fixation and other nutritional facets of plant growth promotion or biocontrol, but this microhabitat is seldom viewed as a potential seat of microbial sulfur oxidation by aerobic chemolithotrophs and/or anaerobic photolithotrophs. However, a few bacteriological studies involving root-adjacent soils have shown diverse sulfur-oxidizing proteobacteria to be abundant and active in paddy fields[1,2]. While facultatively sulfur-chemolithotrophic bacteria phylogenetically related to species of *Rhizobium* had been previously reported from Indian garden soils[3], in more recent times, bacteriological investigation of the rhizospheric soil of a tropical leguminous herb *Clitoria ternatea* (family Papilionaceae), which occurs in almost every wasteland and village forest of the Lower Gangetic plains of India, has resulted in the isolation and characterization of several taxonomically discrete mesophilic, neutrophilic and facultatively sulfur-chemolithoautotrophic proteobacteria, some of which have even been classified as novel species of *Mesorhizobium* and *Paracoccus*[4,5].

In nature taxonomically diverse species of aerobic chemolithotrophic[6] and anaerobic photolithotrophic[7] sulfur-oxidizing bacteria work in tandem to carry on the oxidative-half of the sulfur cycle which in its turn supplies sulfate, the utilizable form of sulfur, to all soil-dwelling organisms, including plants[8]. Though the number of sulphur-chemolithotrophic species in the bacteriological literature appears to exceed its iron-oxidizing, arsenite-oxidizing, or nitrifying counterparts, little attempt has so far been made to understand the oxidation and cycling of sulfur as *in situ* ecological phenomena and functional survey of rhizospheres *vis-à-vis* microbial sulfur oxidation is all the more wanting.

In the present study, soils adhered to the roots, i.e. immediate root–soil interfaces, were examined *in situ* for the presence and activity of sulfur-oxidizing microorganisms. We have directly surveyed the rhizospheres of a wide variety of tropical plants and observed that mesophilic and neutrophilic sulfur-oxidizing microorganisms are ubiquitous, abundant and active in soils immediately adjacent to roots.

Rhizospheres of more than 50 taxonomically and ecophysiologically diverged angiospermic plant species (some of which are listed in Table 1) having var-

*This paper is only a small part of the wide perspectives and vision of Pradosh Roy whose untimely demise begets his unfortunate student WG to see the publication through on his behalf.

Table 1. Major plants species tested for the *in situ* presence and activity of sulfur-oxidizing microorganisms

Angiosperm families surveyed	Major species Envisaged	Economic importance of the species (if any)	Ecophysiological characters/habitat
Mimosaceae	*Mimosa pudica*	Common place or grass land weed	Waste places or wild grass lands
Caesalpiniaceae	*Senna prostrata*	None	Waste places
Papilionaceae	*Dolichos catjang*	Vegetable	Agricultural crop lands
	Arachis hypogaea	Seeds yield fatty oil	
	Cicer arietinum	Pulses	
	Cytisus cajan		
	Vigna mungo		
	Phaseolus aconitifolius		
	Clitoria ternatea	None	Waste places/village forests
Cucurbitaceae	*Trichosanthes dioica*	Berries eaten as green vegetable	Gardens or agricultural crop lands
Myrtaceae	*Psidium guajava*	Berries are edible fruits	Gardens or common places
Capparidaceae	*Cleome pentaphylla*	Common weed	Waste places
Compositae	*Helianthus annus*	Cultivated ornamentals	Gardens
Chenopodiaceae	*Beta vulgaris*	Edible underground storage roots	Gardens or crop lands
Graminae	*Oryza sativa*	Staple cereal	Agricultural crop lands
	Panicum spicatum		
Liliacea	*Alium sativum*	Spice and flavouring material from underground bulbs	Agricultural crop lands
Tiliacea	*Corchorus capsularis*	Fiber yielding plants	Agricultural crop lands
Malvaceae	*Hibiscus esculentus*	Capsules are edible vegetable	Gardens or crop lands

ied agricultural importance were envisaged for the presence and activity of sulfur-oxidizing lithoautotrophic microrganisms.

Small primary roots in case of herbs and slender but rigid secondary or tertiary roots in case of shrubs having thin films of soil adhered to them were directly used as *in situ* rhizospheric inocula. Plant bodies less than 1 m in height were carefully uprooted causing minimum damage to the root systems. Small but rigid secondary roots of size varying from 2 to 5 inches in length were torn from the primary tap roots while the strongest elements were taken apart from the tufts of fibrous roots, in case of dicotyledonous and monocotyledonous plants respectively. These rootlets, which were still coated by very thin layers of loose soil, were directly stabbed vertically into erect culture tubes having the following thiosulfate-containing semisolid or gel-like medium (MST) based on modified basal and mineral salts solution (MS): NH_4Cl – 1 gl^{-1}; K_2HPO_4 – 4 g l^{-1}; KH_2PO_4 – 1.5 g l^{-1}; $MgSO_4$. $7H_2O$ – 0.5 g l^{-1}; $Na_2S_2O_3$ – 5 g l^{-1}; 0.5% (v/v) trace metals solution[9]; yeast extract – 50 mg l^{-1} (as growth factor) and 0.4% bactoagar (initial pH adjusted to 7.0–7.5). The medium was also supplemented with phenol red indicator (10 µg ml^{-1}) to monitor the production of H_2SO_4. The ratio between the surface area and depth of the total medium always remained less than one and portions of the permanent regions of the roots remained above the surface of the medium while the root tips almost reached the bottom of the tubes. The microaerophilic conditions of the tubes simulated those existing in the rhizospheres. All these procedures were carried out under conditions as aseptic as possible. The tubes were incubated at 30°C and observed every 12 h for microbial chemolithotrophic growth and sulfur-oxidation. Thiosulfate levels in the tubes were estimated by cyanolytic method described earlier[10].

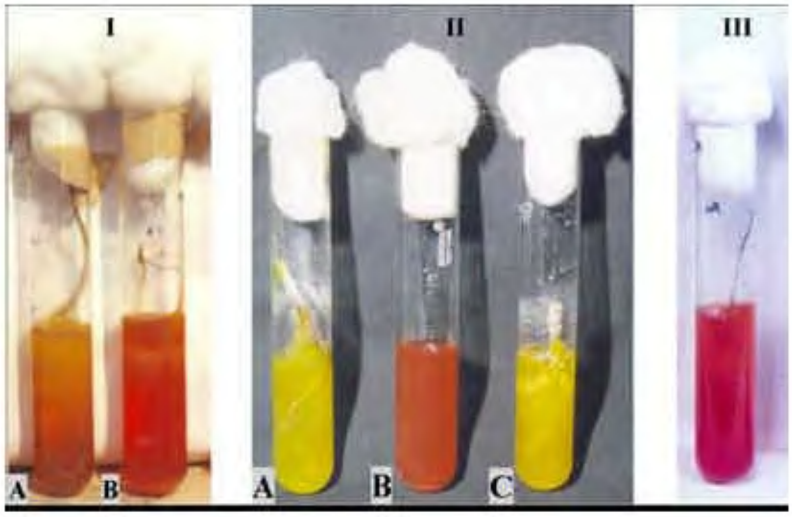

Figure 1. Roots, having thin films of soil adhered to them, used as *in situ* rhizospheric inocula and stabbed into tubes containing semisolid minimal salts thiosulfate (MST) medium. In tubes stabbed with thick and stout roots with greater surface area thiosulfate consumption started within 2 days of insertion of the roots ((I), panel A), involving lowering of the pH of the medium to 6.5 and observable bacterial growth. Tubes stabbed with very slender root specimens of any plant species or major elements of fibrous root systems showed delayed start (after 4 days) in acid production ((I), panel B). Positive results of almost 100% thiosulfate consumption was indicated by the lowering of pH of the medium down to 5.0–5.5 ((II), panels A and C). II, panel B shows a blank uninoculated tube that remained red indefinitely. The negative controls (III) where roots were surface sterilized with $HgCl_2$ (1% in alcohol) did not show any acid production even after indefinite incubation.

Sulphur-lithotrophic microorganisms were found to be present and active on the surface of the roots of all the tested plant species. In the test tubes stabbed with thick and stout roots with greater surface area, irrespective of the plant species, thiosulfate consumption, accompanied by sulfuric acid production indicated by the change in the colour of phenol red, started within 2 days of insertion of the roots (Figure 1(I), panel A). In such test tubes, lowering of the pH of the medium to 6.5, corresponding to 30 to 40% thiosulfate consumption, was registered within 2 to 3 days and observable bacterial growth along the length of, as well as radially around, the inserted roots (accompanied by simultaneous diffusion of acid in these dimensions) started from the 3rd or 4th day. In such tubes stabbed with more robust roots a lowering of the pH to about 6.0, corresponding to around 75% thiosulfate consumption, was observed within 4 to 5 days while total extinction of thiosulfate rendering the pH as low as 5.0–5.5 took 7 to 8 days (Figure 1(II), panels A and C).

Tubes stabbed with very slender root specimens or major elements of fibrous root systems (of monocotyledonous species) showed delayed start (after 4 days) in thiosulfate consumption or acid production and bacterial cell mass generation (Figure 1(I), panel B). Total extinction of thiosulfate in these tubes took 10 to 14 days depending upon the robustness of the root specimens (illustration not shown).

The negative controls, as shown in Figure 1(III), where roots were surface sterilized with $HgCl_2$ (1% in alcohol) and stabbed in the same semisolid MST medium, did not show any consumption of thiosulfate or production of acid even after prolonged incubation. Blank uninoculated MST-containing tubes also remained red indefinitely (Figure 1(II), panel B). Again, root-inoculated tubes containing the above medium formulation minus any reduced sulfur compound (i.e. only MS) were found not to show any lowering of pH or production of acid (illustration not shown).

In general, bacterial cell mass accumulation and intensity of sulfuric acid production in all the tubes was maximum in the longitudinal vicinity of the root hair zones, diffused radially around the same and decreased gradually towards the root tip. The regions of root elongation and root cap showed little or no bacterial growth and acid production in its immediate vicinity. In all the positively testing tubes the uppermost 0.5 to 1 inch portion of the substrate always remained infested with aerophilic fungal mycelia and were devoid of acid (while on the contrary the pH went up in these portions for the first 7–10 days). However, prolonged incubation of the tubes for over two weeks led to the disappearance of the fungal growths and lowering of pH (at par with the other areas of the tube) in these topmost layers of the media also.

The above-mentioned observations for the first time comprehensively demonstrate that active microbial sulfur oxidation is a ubiquitous tropical rhizospheric phenomenon. Our findings also establish mesophilic and neutrophilic lithoautotrophic sulfur-oxidizing microorganisms to be almost certainly represented in the microbial communities inhabiting rhizospheres of tropical plants, both in the wild as well as in agricultural croplands. Given that plants as well as other soil-dwelling microorganisms obtain the sulfur necessary for synthesis of sulfur-containing amino acids through assimilatory sulfate reduction, it can be conjectured that plants in general tend to develop close rhizospheric association with aerobic or microaerophilic species of sulfur-oxidizing chemo- and/or photolithoautotrophic microorganisms in order to ensure the supply of bioavailable sulfate.

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