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Biodegradation of glyphosate herbicide by bacterial isolates from Banana (*Musa spp*.) Plantation soil

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ABSTRACT

Glyphosate (N-phosphonomethyl Glycine) is an organophosphorus pesticide with dangerous effects by enters the food chain of Ecosystem. In this study, the biodegradation of glyphosate herbicide by native bacteria isolated from glyphosate sprayed banana plantation soil was investigated. The isolated bacterial strains were *Bacillus subtilis* (NCBT-008), *Citrobacter koseri* (NCBT-042), *Escherichia coli* (NCBT-001) and *Pseudomonas fluorescens* (NCBT-046). After sampling and bacterial isolation from the native banana plantation soil the strains grown in the presence of glyphosate in nutrient broth was tested at a wavelength of 660 nm and also the degradation of glyphosate at a wavelength of 220 nm. The chromatogram, FTIR, GC-MS, and SEM-EDX analysis confirmed the biodegradation process of glyphosate by these bacterial strains. Thus these native banana plantation soil bacteria can be used for biodegradation of glyphosate herbicide to keep the environment green.

Key Words: Glyphosate, Biodegradation, Native bacterial strains

INTRODUCTION

The human population increase demanded in the increase in agricultural products. It led human to search for more efficient methods for increasing agricultural products. Weed control is normally performed to increase the agricultural products. To control weed broad-spectrum herbicide glyphosate is more frequently used from 1970 onwards[1]. The sales of herbicides in 2006 were about 3 billion liters. One of these herbicides is glyphosate (N-phosphonomethyl Glycine), commercially known as roundup. Glyphosate is utilized extensively for control of plants including grass, sedge, broad-leaved weeds and even woody plants. It can be used on non-cropland as well as on a great variety of crops. Demand for glyphosate has increased substantially in the world. WHO has been reported a 15-fold increase in usage of glyphosate on major crops between 1994 and 2005. Glyphosate itself is an acid, but it is commonly used in salt form, most commonly isopropyl-amine salt[2,3] or trimethyl sulfonium salts as water-soluble concentrates and powders. This herbicide is from the phosphoric acid group, and blocks the EPSPS enzyme by disturbing the plant's synthesis of amino acids and enzyme activity, and blocks its shikimate pathways.[2] Considering the fact that most of the agricultural soil of banana cultivation become saline soil, the environmental problems derived from harmful chemicals especially organophosphorus chemicals in saline environments have been known for long, but the information about the contaminants of these environments is still not convening, because the glyphosate sprayed banana plantation soil microorganisms that degrade these toxic chemicals have not been yet evaluated. Due to the wide range of used pesticides, it is difficult to produce a single method

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for the removal of pesticide from water and wastewater. Thus, there are several treatment processes for the removal of pesticides – e.g. biodegradation, photodegradation, oxidation (with air, chlorine, permanganate or ozone), flocculation and filtration, adsorption and membrane techniques. As a general rule, biological treatment is more economical than physicochemical remediation methods as it can be cost-effective and achieve the complete degradation of organic pollutants.[4]

Microorganisms that degrade glyphosate act in two ways. One path leads to the intermediate formation of sarcosine and glycine, and the other path leads to the formation of 2-amino-3 (5-methyl-3-oxo-1,2-oxazol-4-yl) propanoic acid (AMPA).[5,6] The indicator microorganisms of glyphosate sprayed banana plantation soil are the ones that are capable of living in environments with high and low temperatures, with acidic and alkaline pH, high hydrostatic pressure, and high salt concentrations.[7]

The aim of this study was to evaluate the degradation of glyphosate herbicide by the glyphosate sprayed banana plantations indicator bacteria as bioremediation process for green environment.

MATERIAL AND METHODS

Isolation of bacteria

In order to isolate the bacteria, Nutrient Broth and Agar medium with 50 mg/l glyphosate herbicide was used. After autoclaving the medium, about 1 gram of soil in 250 ml Erlenmeyer flask containing 100 ml of culture medium was incubated at 34°C shaking at 150 rpm for 7 days. Then it was inoculated and incubated in the agar medium at 30°C for 48 hours. After incubation (in 30°C for 48 hours), the bacteria were detected with diagnostic tests. Eventually the isolating and purifying processes were done.

Diagnosing the bacteria

After Gram staining the bacteria and spores staining, the initial identification and biochemical tests including motivation tests, oxidation tests, catalase tests, gelatin-starch-caseine hydrolysis tests, Tween 80 hydrolysis tests, Bile Esculin hydrolysis tests, Nitrate reduction test, H_2S production, acid production from sugar (glucose, lactose, raffinose, arabinose, mannitol) and tolerance and sensitivity to antibiotic tests including chloramphenicol, penicillin G, tetracycline, nalidixic acid, cephalothin, ampicilin, rifampin, erythromycin, and streptomycin were performed.[8]

Evaluating the logarithmic growth of bacteria in the presence of herbicide

In order to evaluate the degradation ability of bacterial isolates different concentrations, i.e., 10, 20, 30, 40 and 50 mg/l of glyphosate were used. Control was maintained without the herbicide. One ml of bacterial culture were added to the flask and the flasks were placed in a shaker incubator in 30°C and 150 rpm, and after 48 hours, their absorption was measured by the spectrophotometer in 660 nm wave lengths for evaluating growth and in 220 nm for evaluating degradation of the herbicide.[8,9] The best isolates with maximum absorption at 660 nm and minimum absorption at 220 nm were chosen.

In order to choose the best isolates based on optimum growth in the presence of herbicide, their growth during different hours of incubation in wave lengths of 660 nm were measured.

Analysis of glyphosate degradation by Thin Layer Chromatography (TLC)

In order to do this experiment, cellulose chromatography paper and solutions of ethanol, water, ammonium hydroxide (17 molar), trichloroacetic acid and acetic acid (15 molar) with proportions of (2:3.5:2.5:35:55 v/w/v/v/v) were used.[10,11]

After 48 hours the best isolated grown bacteria were taken from the top of the solution in the flask and were placed on chromatography paper. Also glyphosate herbicide was used as control and was placed on chromatography paper. Then, the chromatography paper was placed in a dish including a prepared solvent. After the elution the chromatogram was viewed by UV light in 220 nanometers.[7]

Analysis of glyphosate degradation by FTIR - Spectroscopy

FTIR spectroscopy was performed by standard method[12]; 2.5 mg of sample was mixed and ground with 75 mg of KBr in an agate mortar. The translucent discs were prepared by pressing the ground material with the aid of 8 tonnes of pressure bench press. The tablet was immediately analysed with a spectrophotometer in the range of 1000-4000 cm^{-1} with a resolution of 5 cm^{-1}.

Analysis of glyphosate degradation by GC-MS

GC-MS was performed using thermo GC-Trace Vibra ver.5.0, Thermo MS DSQ II. Gas chromatography was conducted in the temperature programming mode with a 100-250°C, Rate: 8/min, holding time: 10 min at 250°C. The initial column temperature was held at 100°C for 8 min, then increased linearly to 250°C at 10°C/min, and held for 4 min at 270°C. The temperate at the injection port was 275°C and the GC-MS interface was maintained at 300°C. Helium was used as carrier gas with a flow rate at 1.0 ml/min. The injection was split less to increase sensitivity. Identification at degradation products was made by comparing the retention time and fragmentation pattern with known reference compounds as well as with mass spectra in the library search results stored in the computer software (Version 1.10, Beta Shimadzu) of the GC-MS.[13]

Scanning Electron Microscopy

The surface structure of biosorbent was analysed by scanning electron microscopy (SEM-TESCON) and the element analysis by SEM-EDX (Energy Dispersive X-ray analysis).[14] The dried bacterial biomass samples were mounted on aluminum stab sequenced by sputter coating with a thin layer at gold under vacuum to increase the electron conduction and to improve the quality at the micrographs.

RESULTS AND DISCUSSION

Results from bacteria logarithmic growth and toxin degradation

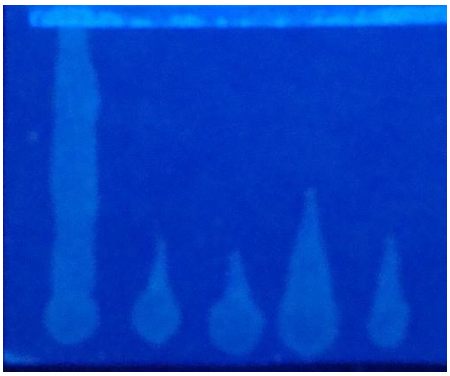
Diagnostic tests revealed that bacteria are Gram-positive, catalase-positive, oxidase-positive, non-spore forming bacteria, and non-motile and has the ability to grow in glyphosate sprayer soil. The *Bacillus subtilis* (NCBT-008), *Citrobacter koseri* (NCBT-042), *Escherichia coli* (NCBT-001) *and Pseudomonas fluorescens* (NCBT-046) isolates from the banana field soil native bacteria showed good activity in degrading glyphosate herbicide which is valuable, and the *P. fluorescens* (NCBT-046) isolate had the highest growth rate among the isolates of banana plantation soil.

Eventually the consortium of all four isolates had a higher activity in degrading the glyphosate herbicide in comparison to individual isolates.

Thin layer chromatography

The four isolates which are capable of degrading the glyphosate herbicide after 48 hours incubation. In the picture below, the result of the experiment are seen. The column (column 1) is the control (glyphosate herbicide), and its band is seen.

Figure: 1 Glyphosate degradation study by TLC



C – Glyphosate (control); 1-Glyphosate degradation by *B. subtilis*; 2-Glyphosate degradation by *C. koseri*; 3-Glyphosate degradation by *E. coli;* 4-Glyphosate degradation by *P. fluorescens*

The other columns (columns 2-5) are the 48-hour result in which the herbicide is degraded by the isolates and the bands are very much reduced, which ensures the degradation of glyphosate by the bacterial isolates (Fig. 1).

Fourier Transform-Infrared Spectroscopy (FTIR) Analysis

One important characteristic of degradation is the surface functional groups present in the degradation process were characterized by the FTIR spectroscopy method, a qualitative description. In addition transmission spectra shift at certain wave numbers confirm that several surface functional groups are involved in the degradation process. The FTIR spectra of untreated glyphosate before degradation the bands observed were at 2421.28, 2924.92, 2289.31, 1647.45, 1282.00, 1034.08 and 592.22 cm^{-1}. In *Bacillus subtilis* (NCBT-012) remediated samples the bands observed were at 3395.12, 2857.38, 2922.84, 2301.13, 1233.28, 1617.61, 1408.45, 1368.33, 1136.14 and 252.36 cm^{-1} (Fig. 2).

Whereas the *Citrobacter koseri* (NCBT-016) bacterial remediated glyphosate sample have shown the bands at 3783.33, 3418.31, 2932.26, 2110.97, 1650.91, 1308.42, 1237.03, 1062.73 and 612.29 cm^{-1} in remediated samples the bands observed were at 3919.52, 3769.54, 3400.17, 2935.62, 2339.60, 1641.89, 1388.84, 1248.05, 1105.28 and 656.12 cm^{-1}.

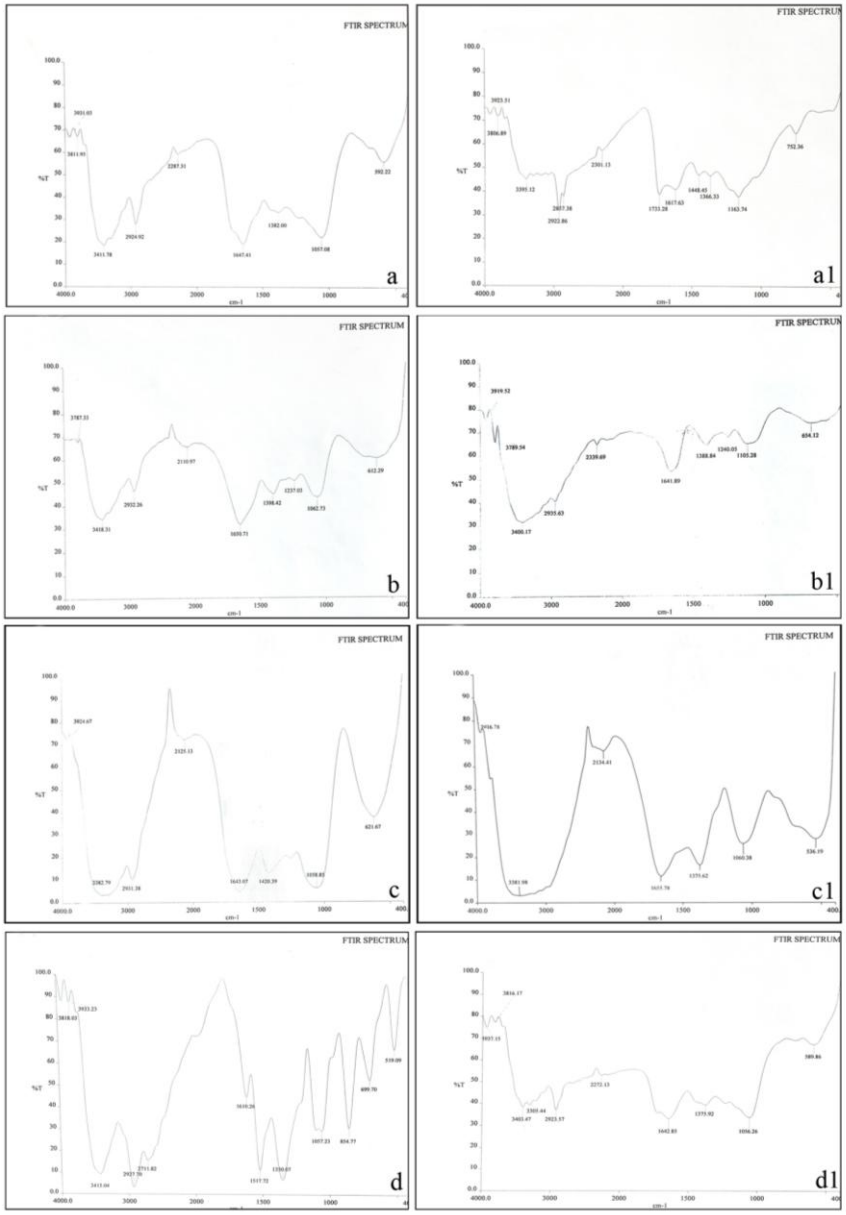
The glyphosate sample, the bands observed were at 3924.67, 3382.39, 2955.38, 2425.13, 1633.67, 1420.38, 1058.85 and 621.67 cm^{-1} whereas the *Escherichia coli* (NCBT-001) remediated sample the bands observed were at 3914.78, 3381.98, 2434.41, 1655.78, 1325.62, 1060.36 and 515.19 cm^{-1}.

The glyphosate sample the bands observed were at 3933.23, 3818.03, 3415.04, 2927.10, 2311.42, 1610.26, 1517.72, 1350.67, 1057.23, 854.77, 699.70 and 519.09 cm^{-1}, whereas the *Pseudomonas fluorescence* (NCBT-046) remediated sample, and the bands observed were at 3931.15, 3816.17, 3001.47, 3205.44, 2923.57, 2272.13, 1642.85, 1375.20, 1056.26 and 589.30 cm^{-1}.

GC-MS Analysis

The GC-MS analysis of control soil sample revealed the presence of chlorozotocin, 1-Nitro-2-acetamido-1,2, dideoxy-d-glucitol, Deoxyspergualin, perhydroindene-4-carboxylic acid, 6-acetoxy-2,3-epoxy-1, 1-epoxy Benzene-ethanamine, Phenol, 4-bromo-2-chloro-phenol-2-bromonaphthalene, diethylphthalate, phthalic acid, benzaldehyde, cyclohexanecarboxylic acid, and oleic acid whereas the glyphosate sprayed banana plantation soil revealed the presence of 1-Nitro-β-d-arabinofuranose, tetraacetate, d-glycero-d-ido-heptose, 1,2,3,4,5-cyclopentanephenol, 3-Ethylheptanoic acid, 1,1-dimethylethyl ester, [1,1-Bicyclopropyl]-2-octanoic acid, 2'-hexyl-methylester perhydroindene-4-carboxylic acid, 6-acetoxy-2,3-epoxy-1,1-epoxy 3-(2,5,8,11,14-pentaoxacyclohexadecyl), 4-bromo-2-chlorophenol, 2,4-Dichlorophenethylamine, Diethylphthalate, Heptanediamide, Benzoic acid, n-Hexadecanoic acid and z-e-methyl-9-tetradecenoic acid (Fig. 3).

Figure-2. Dioremediation of glyphosate herbicide by bacterial Isolates : FTIR analysis



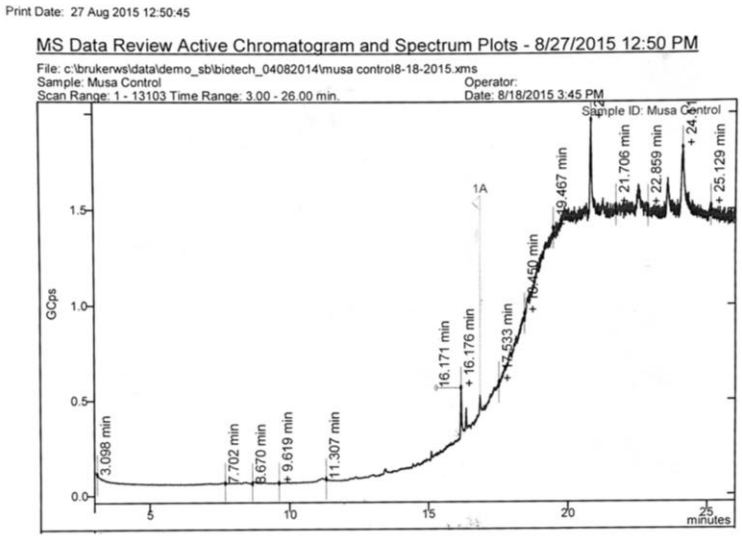
a) Before remediation, a1) after remediation by *Bacillus subtilis* (NCBT-012)
b) Before remediation, b1) after remediation by *Citrobacter koseri* (NCBT-016)
c) Before remediation, c1) after remediation by *Eschericia coli* (NCBT-002)
d) Before remediation, d1) after remediation by *Pseudomonas fluorescens* (NCBT-046)

Discussion

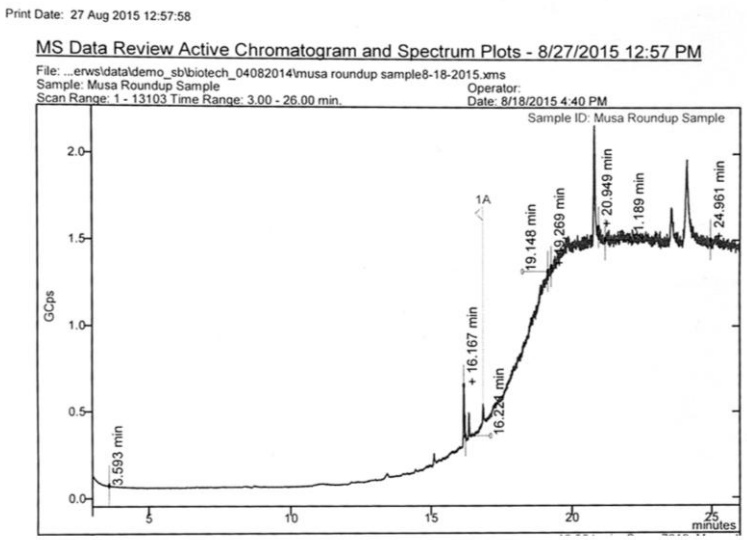
Herbicides are chemicals that are used for weed control. When herbicides are added to soil, the consequences of their presence might be critical. Their long presence in soil can lead to elimination of the beneficial microorganism in soil. Therefore their biodegradation is important and is the best alternative for preventing their accumulation and environmental stability. To achieve this goal by isolating bacteria that can degrade this herbicide. Usually microbial ways for eliminating glyphosates are more effective than chemical ways, because herbicides have a strong carbon-phosphorus bond that can be degraded by these bacteria. [9]

According to the result of these experiments, the dominant or indicator bacteria *B. subtilis* (NCBT-008), *Citrobacter koseri* (NCBT-042), *E. coli* (NCBT-001) and *P. fluorescens* (NCBT-046) which are able to degrade effectively this herbicide up to 50 mg/l concentration.

Figure-3. GC-MS spectrum of glyphosate amended *Musa sp*. Plantation soil



GC-MS spectrum of glyphosate remediated *Musa sp*. Plantation soil by bacterial consrotium



Monke *et al*. evaluated the effect of different concentrations of the glyphosate herbicide in non-saline environments and in regular conditions in the *Acetobacter* sp. and *P. fluorescens* bacteria and found out that the best growth was in concentrations equal to 7200 ppm, and these two bacteria could tolerate herbicides up to 10,000 ppm. [11]

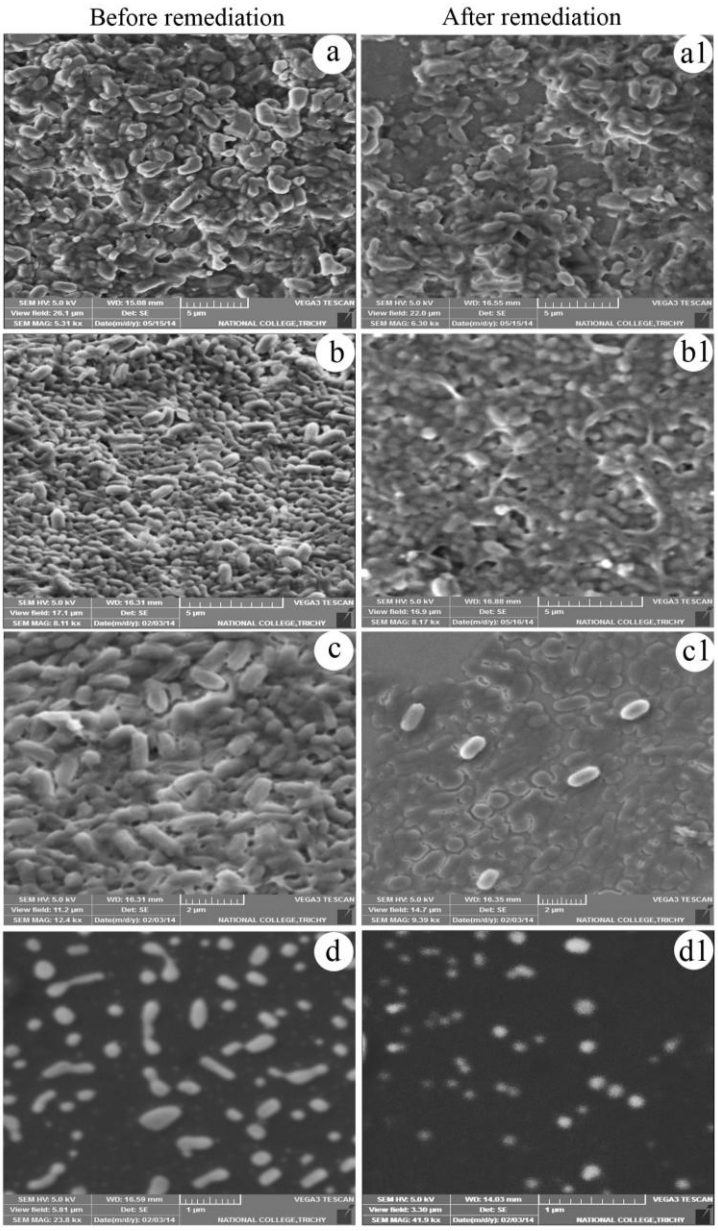
Ermakova *et al.* claimed that none of the two *Achromobacter* sp and *Ochrobactrum anthropi* bacteria could use glyphosate as a source of carbon and phosphorus. Both bacteria grew in an environment that the herbicide and methyl phosphonic were added as a source of phosphorus; and were able to break the C-P bond in glyphosate[4].

Other bacteria that have been identified in degrading glyphosates are *Arthrobacter atrocyaneus* and *Flavobacterium* sp [1, 5]. The bacteria in soil are the only organisms that degrade glyphosate[17]. Also *Pseudomonas* sp. isolate LBr[18] *Acetobacter atrocyaneus* [19], and *Flavobacterium* sp. bacteria were able to degrade the glyphosate herbicide. Adelowo *et al*. discovered this first fungal degradation of glyphosate by *Penicillium citrinum* . [20, 21]

The SEM micrography of bacteria before and after bioremediation process has shown a different morphology. The morphology had undergone remarkable physical disintegradation, the matrix layer of cell wall of bioremediated samples were seen to shrink and stick. The structural change was attributed to the strong cross linking of glyposate by negatively changed chemical groups in the cell wall polymers as reported (Fig. 4). [15, 16]

The SEM-EDX analysis confirms the presence of elements such as C, O, Na, Mg, Al, Si, P, S, Cl, K, Ca,

Figure-4. Glyphosate herbicide bioremediation : SEM pictures of Bacteria



a,a1) *Bacillus subtilis* (NCBT-008)
b,b1) *Citrobacter koseri* (NCBT-042)
c,c1) *Eschericia coli* (NCBT-001)
d,d1) *Pseudomonas fluorescens* (NCBT-046)

Fas, Cu and Zn in the control fruit samples of *Musa* sp. whereas the glyphosate sprayed samples have shown the elements such as C, O, Na, Mg, Al, Si, Cl, K, Ca, Ti and Fe. These results have shown that the glyphosate sprayed soil the nutrients like P, S, Cu and Zn were missing (Fig 5).

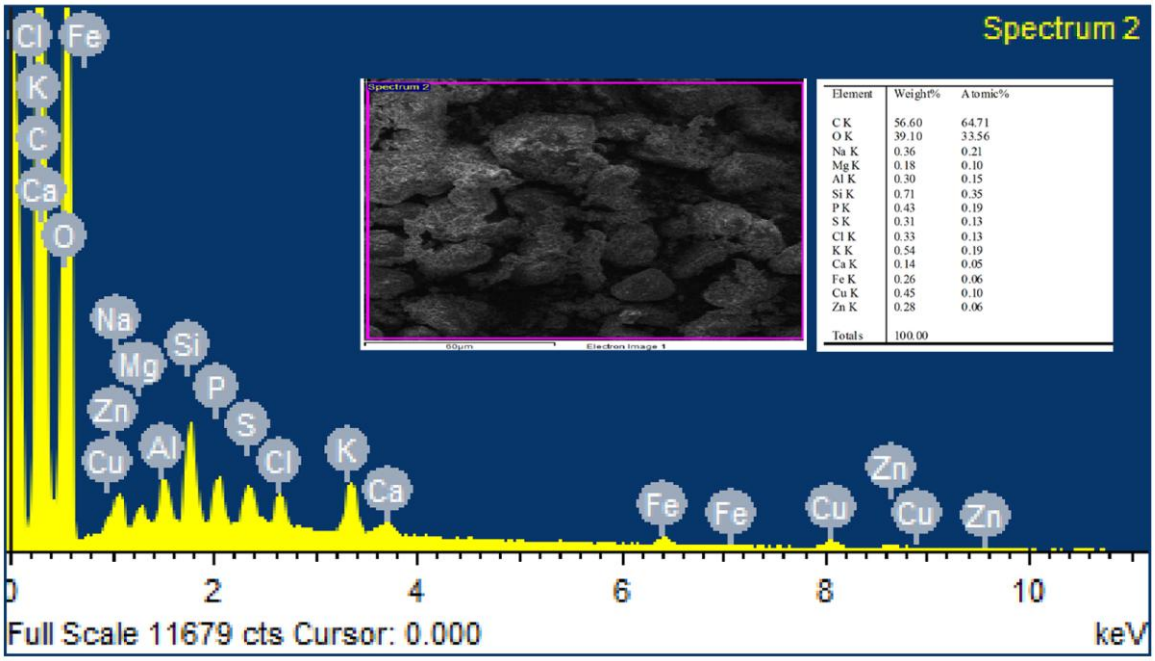
Conclusion

Glyphosate can stimulate certain microbial variables in the soil, the indicator bacteria isolated from glyphosate sprayed banana plantation soil capable of utilize glyphosate effectively and can grow in the presence of herbicide and can metabolize this environmental contaminant, thereby protect the non-target organism including mycorrhizal fungi and nitrogen fixing bacteria.

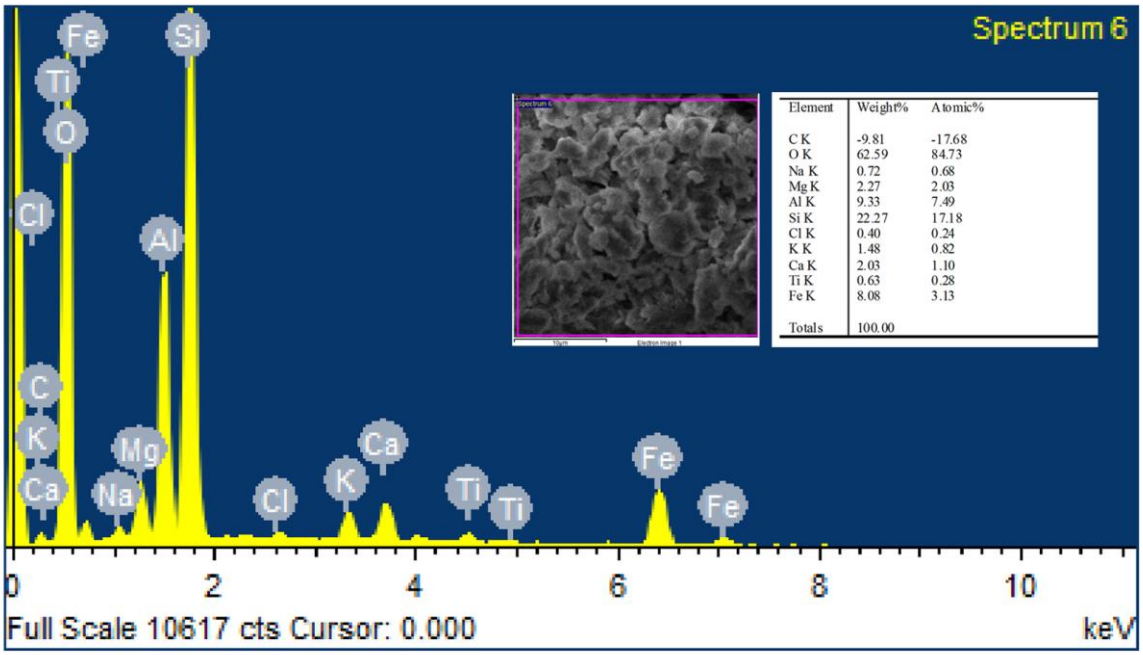
Conflict of Interests

Authors declare that there is no conflict of interests regarding the publication of this paper.

Figure-5. SEM – EDX Elemental analysis of Musa sp. Fruit in normal soil



SEM – EDX Elemental analysis of Musa sp. fruit in glyphosate amended soil



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