Effect of Herbicide (Pendant) Concentration on Arachis Nodular Rhizobial Microbiota Viability

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Abstract

Increasing excessive application herbicides in farming results to accumulation of the chemicals to toxic levels in the soil resulting in harmful effects to microorganisms, plants, wildlife and man. This study investigated the effect of various concentrations of the herbicide pendant on Arachis nodular rhizobial microbiota viability. Soil samples (1.5 kg) in different pouches were treated with different concentrations [x 0.5 %, x 1.0 % (recommended dilution), x 1.5 %, x 2.0 % and x 2.5 % v/v] of the herbicide and population of rhizobia in the root nodules of Arachis hypogea using Most Probable Number (MPN/ml). Rhizobium leguminosarum from crushed A. hypogea nodules was isolated on Rhizobium selective medium. A. hypogea seed germination commenced after 3 days of planting for the recommended (x 1.0 % v/v) as well as other lower herbicide concentrations (x 0.5 % and x 1.5 % v/v) and for the control (without herbicide), but for pouches containing higher herbicide concentrations (x 2. 0 and x 2.5 %), germination commenced only after 5 days. While there was normal growth of Arachis hypogea treated with lower herbicide concentrations, there was stuntedness and discoloration in the leaves for seedlings treated with higher herbicide concentrations. Also, rhizobial population of the root nodules decreased with increased herbicide concentration in the order; control (4600 MPN/ml) > recommended (2100 MPN/ml) > higher concentration (200-360 MPN/ml). Inappropriate herbicide application influences negatively rhizobial microbiota of soil with a cascading effect on crop yield. Strict regulation on the use of herbicide and adequate training of workers and farmers for effective and safe use of the herbicide is necessary.

Keywords: *Arachis hypogea,* herbicide, Most Probable Number, *Rhizobium leguminosorum,* stuntedness, chlorophyll

1. Introduction

Herbicides or "weed killers" are a group of chemicals known as pesticides, which prevent, inhibit, destroy, repel or mitigate or kill weeds and other undesirable plants. Selective herbicides kill specific targets, while leaving the desired crop relatively unharmed. Other familiar pesticides are insecticides, rodenticides and fungicides. There are many different types of herbicides, all of which can be dangerous to humans or the environment if used irresponsibly (Pfeiffer, 2011). They are designed to be biologically active, and while a remarkable degree of selectivity has been achieved in some materials, as in the case of sensitive herbicides and insecticides, it is not surprising that pesticides may produce undesirable effects particularly if they are used at high concentrations (Ayansina and Amusan, 2013)' Herbicides are extensively used in agriculture as a part of unwanted plant control strategies. Owing to their xenobiotic characteristics, herbicides may adversely affect the proliferation of beneficial soil microorganisms and their associated biotransformation in the soil (Sarfraz *et al.,* 2009; Bunce, 1993). Inactivation of nitrogen-fixing and phosphorus solubilizing microorganisms is observed in herbicide-contaminated soils (Burdass, 2002). Recent studies show that some herbicides disturb molecular interactions between plants and N-fixing rhizobacteria and consequently inhibit the vital process of biological nitrogen fixation.[3] Increase in herbicide dose tends to amplify its negative effect on microorganisms.

The herbicide *Pendant* is an emulsifiable concentrate herbicide containing the active ingredient 'Pendimethalin'. Pendimethalin is of the chemical family 'Dintroanilines (EXTONET, 1996). These herbicides inhibit the steps in plant cell division responsible for chromosome separation and cell wall formation (EXTONET, 1996). Pendimethalin acts as a root inhibitor.

Pendimethalin is a selective herbicide used to control most animal grasses and certain broadleaf weeds in field corn, groundnut, potatoes, rice, cotton, soybeans, tobacco and sunflowers (EXTONET, 1996). It is used both pre-emergence, that is before seeds have sprouted, and early post emergence, that is, immediately after seeds have sprouted. Incorporation into the soil by cultivation or irrigation is recommended within 7 days following application. The genus *Arachis* comprises up to 730 species of annual and perennial flowering plants and are generally characterized by the fact that all of its species are geocarpic, that is, they only produce underground fruit (Burdass, 2002). The pea family (Fabaceae) to which *Arachis hypogeae*, commonly called 'peanut' belongs is native to South America (Krapovickas and Gregory, 2007). It is a major food crop species of global importance; some of the other species are cultivated for food. Other species (e.g. *A. pintoi*) are cultivated as forage and soil conditioner plants. The leaves are very important high-protein feed source livestock (Singh and Oswalt, 1995; Krapovickas and Gregory, 2007).

Rhizobia (Greek, *Rhiza*, = 'Root' and *bios* = 'life'; 'singular = Rhizobium') with the first known species, *Rhizobium leguminosarum*, was identified in 1889, with all further species were initially placed in the genus *Rhizobium* (Berge *et al.,* 2009). The organisms are soil bacteria characterized by their unique ability to infect root hairs of legumes and induce effective nitrogen fixing nodules to form on the roots. Rhizobia, rod shaped, live in plants and exist only in the vegetative state, and unlike many other soil microorganisms, produce no spores (FAO, 1984; Celino-Gaminde, 1992). They are Gram negative, aerobic and motile and multiply by simple cell division. The bacteria are not particularly fastidious in their nutritional requirement, and can use sugar, alcohols and acids as sources of energy. Yeast extract provides growth factors and vitamins and usually enhances growth but some species can produce their own growth factors (Lopez-lopez *et al.,* 2010).

Soil fertility often depends on the balance of the different genera of soil fungi and other soil inhabiting microorganisms whose activities determine the efficiencies of the various metabolic cycles (nitrogen, carbon, minerals) (Tu *et al.,* 2001). Therefore the addition to the soil of any potentially toxic herbicide constitutes a threat to this equilibrium and hence to the future fertility of the soil due to selective toxicity for certain groups of microorganisms thus indirectly altering the population equilibrium or by promoting the growth of one or more types of soil organisms. These depressive or stimulatory effects depend upon the kind of chemical and its concentration, possibly moderated by environmental conditions (Gricher, 2006).

In modern agricultural production, herbicide application is a regular practice. While in developed countries weeds and pests reduce yields of agricultural crops from 15 to 20%, reductions increase to 50% in the undeveloped regions (Gricher, 2006). Incorrect and indiscriminate application of herbicides affects negatively the health of humans, plants, animals and microorganisms. Particularly hazardous are the poorly degradable herbicides whose persistence may lead to long-term accumulation (Nada and Mitar, 2002). The problems caused by the increased application of herbicides call for the need to determine the concentration of herbicide that has no or less effect on soil microbiota including that of *Arachis hypogeae* rhizobial microbiota viability. Consequently, this study was carried out to determine the effect of herbicide concentrations on *Arachis* nodular rhizobial microbiota viability.

2. Materials and Methods

2.1 Source of samples

Loamy soil sample was collected around the Microbiology Department building, Modibbo Adama University of Technology, Yola Nigeria, groundnut (*Arachis hypogea*) seed was purchased from Girei market, Girei local Government, Adamawa State, Nigeria and authenticated at the Botany Department of Modibbo Adama University of Technology, Yola Nigeria. Rhizobium agar, nutrient broth and gram reagents were obtained from the Microbiology Laboratory of Microbiology Department, Modibbo Adama University of Technology, Yola, Nigeria, the herbicide Pendant (Multichem industries, Nigeria limited) was purchased from Idris and Sons Agrochemicals, No H262 Moh'd Abubakar Rimi Market, Kano, Kano State, Nigeria.

2.2 Groundnut (*Arachis hypogeae*) seed cultivation

About 1.5 kg of soil sample was weighed into six pouches and an average of two groundnut seeds per pouch were sowed in each of the pouches, watered and monitored until germination. Watering was repeated after every 12 h to prevent drying (Fawole, 2000).

2.3 Soil treatment

The method as described by Ayansina and Oso (2006) and Woomer*et al.* (2011) was used with some modifications where additional concentration of herbicide was employed. The soil samples used had no history of herbicide application. Herbicide was prepared at manufacturer's recommended concentration (x 1.0 % v/v), at a lower concentration of (x 0.5 % v/v) and at higher concentrations of x 1.5, x 2.0 and x 2.5 % (v/v) in order to determine the effects of the herbicide on the rhizobia population at lower than recommended, at recommended and at higher concentrations than the manufacturer's recommended and to compare with untreated soil (no herbicide). To obtain the recommended concentration, 0.001 ml of herbicide was mixed thoroughly with 100 ml of deionized water to obtain a x 1.0 % concentration of herbicide which is then mixed thoroughly with 1.5 kg of soil sample in a pouch. Similar mathematical extrapolations were applied to obtain the other concentrations such that the x 0.5 concentration contain 50 % less of the active ingredient, while the x 1.5, x 2.0 and x 2.5 contain 50 %, 100 % and 150 % more of the active ingredient respectively. Soil with no herbicide application served as control.

2.4 Screening for presence of *Rhizobium leguminosarum*

To detect the presence of *Rhizobium leguminosarum*, the root nodules were stained with carbol fuchsin as earlier described (Chitra *et al.,* 2013). Briefly, using a sterile razor blade, a thin section of nodule was cut. The cut surface was gently rubbed on a clean microscope slide to make a smear. The smear was allowed to air dry and then passed through a flame. The slide was flooded with dilute carbol fuchsin for 10-20 seconds, after which the flooded slide was washed with sterile distilled water, allowed to air dry and then observed under oil immersion objective lens.

2.5 Isolation and identification of *Rhizobium leguminosarum* from *Arachis hypogeae* groundnut) roots

After 6 weeks, the fresh and plump root nodules of groundnut (*Arachis hypogeae*) plant was collected from the plants grown in the pouches. The collected nodules were surface sterilized with 75% ethanol and then 0.1% mercuric chloride solution (10 g/150ml distilled water), then washed thoroughly with distilled water and crushing in a drop of sterile water (Woomer*et al.,* 2011). A loopful of crushed root nodule material (suspension) was transferred to 5 ml of sterile water, of which 0.1 ml sample was spread onto the surface on Rhizobium selective media (RSM). Culture plates were incubated at 35 ºC for 24 - 48 h. Well isolated typical single colonies were restreaked on freshly prepared RSM plates in order to obtain pure cultures. Pure isolates of *Rhizobium leguminosarum* were identified based on colonial morphology such as colour, shape, appearance, colony, diameter, transparency on Rhizobium selective medium, Gram reaction and standard biochemical tests (Shahzad *et al.,* 2012; Deshwal and Chaubey, 2014).

2.6 Biochemical tests

Various biochemical tests such as motility, indole, Voges-Proskaur, citrate and Methyl Red tests, catalase and gelatinase activity, growth in the presence of 2% NaCl, 8% KNO_3 and in glucose peptone agar, hydrolysis of starch and urea, H_2S production and acid production in Yeast extract mannitol (YEM) broth, acid reaction in litmus milk, starch hydrolysis and utilization of different carbon sources were used to identify the isolates as described (Deshwal and Chaubey, 2014).

2.7 Most Probable Number (MPN) estimation of *Rhizobium leguminosarum* population

After 6 weeks, the root nodules of the cultivated *Arachis hypogeae* were collected and crushed in a sterile Petri plate with small amount of distilled water. A ten-fold serial dilution was made and 1 ml sample of 10^{-2}, 10^{-3} and 10^{-4} were inoculated into triplicate broth culture tubes and incubated overnight at 35 ºC. After incubation, the total number of positive cases was counted for each pouch. The experiment was carried out in triplicates and mean values recorded. Based on these scores the most probable number (MPN) of rhizobia in the test soil was calculated from MPN mathematical tables (Martyniuk and Oron, 2008).

3. Results

3.1 Seed germination and growth of *Arachis hypogeae* root nodules

After cultivation of *A. hypogeae* seeds in pouches containing different concentrations of herbicide (x 0.5 %, x 1.0 %, x 1.5 %, x 2.0 % and x 2.5 % v/v) results showed that the seeds commenced germination after 3 days of planting for herbicide concentrations x 0.5, x 1.0, x 1.5 % v/v and for the control (Fig 1a.),

But for pouches containing x 2.0 and x 2.5 % v/v herbicide concentrations, germination commenced only after 5 days (Fig 1b). A close examination of the plant also revealed some differences in the nature of growth of the seeds from the various concentrations with those of control, x 0.5, x 1.0 and x 1.5 % v/v showing normal growth (Fig 2a), while those in pouches containing x 2.0 and x 2.5 % v/v herbicide concentrations showing some evidence of stuntedness and discoloration of the leaves (Fig 2b).

(A) (B)

Fig 1. Germination of seed (a) after 3 days (low concentration of herbicide) and (b) after 5 days (higher concentration of herbicide).

(A) **(B)**

Fig 2. Pouch showing (a) normal growth (control with no herbicide) and (b) discoloration (with higher concentration [x 2.0 % v/v] of herbicide**)**

3.2 Harvesting of root nodule and screening for the presence of *Rhizobium leguminosarum*

After 6 weeks of cultivation, examination of the nodules showed that nodules from pouches containing lower concentrations of herbicide (x 0.5, x 1.0 and x1.5 % v/v) and the control were more numerous and bigger in size compared to those containing x 2.0 and x 2.5 % v/v herbicide concentration. Microscopic examination of carbol fucshin stained root nodules for *R. leguminosarum* showed the presence of clear pinkish bacilli in all the seedlings (Fig 3).

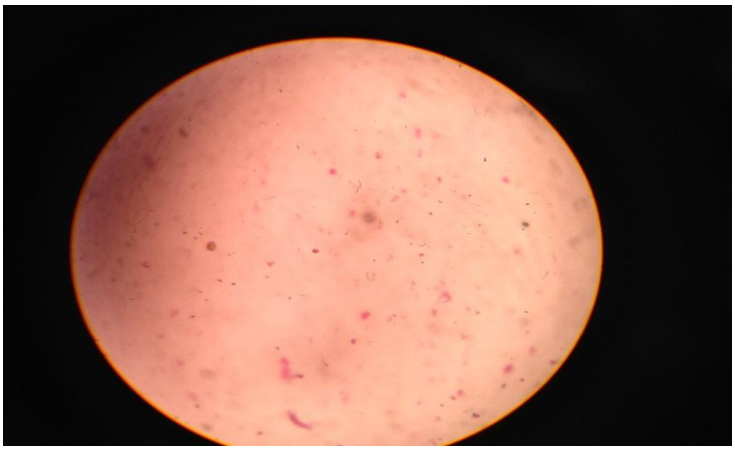

Fig 3. Gram stain reaction (pink rod-like bacilli) of *Rhizobium leguminosarum*

3.3 Isolation and identification of *R. leguminosarum*

Results showed the presence of *R. leguminosarum* in the root nodules after inoculation into Rhizobium selective media and incubation at 35 ^{o}C for 48 h. The colonies were viscous, milky and circular in appearance. Results of gram staining of the isolates showed the presence of rectangular rod shaped Gram negative bacilli (Fig 3). While biochemical test showed that the isolates were catalase positive, oxidase positive, motility, citrate negative and were able to hydrolyze starch and all the sugars tested (mannitol, sucrose, fructose and glucose). Isolates also were able to grow in 2 % NaCl and 8 % KNO_3 (Table 1).

Table 1. Biochemical characteristics *Rhizobium leguminosarum* isolated *Artachis hypogeae*

Biochemical test	Result
Motility	Positive
Indole	Negative
Voges-Proskaur	Negative
Citrate utilization	Negative
Methyl Red	Negative
Catalase	Positive
Gelatin liquefaction	Negative
Growth in 2% NaCl	Positive
Growth in 8% KNO_3	Positive
Growth on glucose peptone agar	Negative
Starch hydrolysis	Negative
Urea hydrolysis	Negative
H_2S production	Negative
Acid production in Yeast extract mannitol (YEM) broth	Negative
Acid reaction in litmus milk	Negative
Gram reaction	Negative
Sugar utilization: D-glucose, Mannitol, D-fructose, Sucrose)	All Positive

3.4 Effect of herbicide (pendant) concentrations on *R. leguminosarum* population

Results of MPN studies carried out to determine the effect of various herbicide (pendant) concentrations on *R. leguminosarum* populations on the nodule revealed that the MPN value for x 0.5 % v/v herbicide concentration was 2900 MPN/ml, while at x 1.0 % v/v concentration the value was 2100 MPN/ml and at x 1.5 % v/v herbicide concentration the MPN value was 360 MPN/ml. Results also showed MPN values of 270 and 200 MPN/ml for x 2.0 and x 2.5 % v/v herbicide concentrations respectively. For the control sample which contains no herbicide the MPN value was 4600 MPN/ml (Table 2).

Table 2. Effect of various herbicide concentrations on *Rhizobium leguminosarum* populations shown as mean Most Probable Number (MPN/ml) values

Herbicide (pendant) concentration	Dilution factors			MPN/ml
	10^{-2}	10^{-3}	10^{-4}	
x 0.5	3	2	3	2900±0.02
x 1.0 (recommended rate)	3	2	2	2100±0.00
x 1.5	2	3	1	360±0.07
x 2.0	2	1	2	270±0.10
x 2.5	2	1	1	200±0.05
Control (no herbicide)	3	3	1	4600±0.11

4. Discussion

Herbicide treatment did not inhibit germination of *Arachis hypogeae* (groundnut) seed, seeds sown germinated and emerged out of soil. However, it was observed that higher doses of the herbicide concentrations resulted in abnormal growth and greater reduction of number of root nodules formed in the groundnut seeds, while lower doses of herbicide concentrations had less pronounced effect on the growth and formation of root nodules. Reduction in the number of nodules formed might account for the abnormalities in growth observed in pouches treated with higher doses of herbicide concentration as they show chlorosis and stunted growth (Willey *et al.*, 2011). Chlorosis is typically caused when leaves do not have enough nutrients including nitrates deficiency for the synthesis of chlorophyll needed for photosynthesis (Willey *et al.,* 2011). It can be brought about by a combination of factors including mineral deficiency in the soil such as Zinc, Magnesium and Nitrogen. Root nodules are vital to the process of conversion of nitrogen to the plant utilizable nitrates in the soil. Pesticides, particularly herbicides may cause chlorosis both to target weeds and occasionally to the crop being treated.

It was earlier reported that Groundnut yields reflect the effect of herbicide injury on plant growth and development as Groundnut yields decreased as herbicide rate increased (Gricher, 2006). It has been proven that plant productivity increases when the Rhizobia are present in rhizosphere. It provides the major biological source of fixed nitrogen in agricultural soils (Shahzad *et al.,* 2012). The reduction in number and size of nodules therefore may mean possible reduction in nitrogen conversion capacity of the plant, since *Rhizobium* spp play that role and consequently reduced plant growth and yield. The identification of the *Rhizobium* sp. is based on the cell colony morphology which has been used by previous researchers to identify the bacteria (Chitra *et al.,* 2013). In addition, the growth media was selective to the *Rhizobium* sp. The *Rhizobium* agar is species specific with yeast extract to provide a source of organic nitrogen and mannitol as the carbon source (Cornish and Burgin, 2005; Martyniuk and Oron, 2008). The colonies obtained were circular, viscous and milky in appearance.

General microscopic view of the isolates showed Gram negative rod shaped cells. The combination of the cell morphology of the Gram stains, the growth of the colonies on the *Rhizobium* selective media, and the colony morphology of the colonies provide sufficient evidence to demonstrate the successful isolation of the *Rhizobium* sp. Gram stain reaction revealed that the *Rhizobium* isolates were Gram negative and further biochemical characterization of isolates showed that the bacteria were catalase positive, oxidase positive and motility positive. Though the isolates were unable to utilize citrate as sole carbon source, their capacity to utilize all the sugars tested and grow on various salt concentrations (2 % NaCl and 8 % KNO_3) explains their potentials in conversion of various compounds such as nitrites, molecular nitrogen and chlorides to plant utilizable forms (e.g. nitrates). On subjecting the culture plates to iodine test, clear zones around the colonies were seen and the colonies turned yellow in appearance, whereas blue colour appeared on areas without *Rhizobium* growth. This indicates that the isolates have potential to hydrolyze starch present in the medium. This was consistent with earlier that also observed that *Rhizobium* isolates were able to utilize starch obtained from different sources (Zabaloy *et al.,* 2011; Chitra *et al.,* 2013). MPN studies showed that the *Rhizobium* spp were present in the soil at different concentrations of herbicides and the population of Rhizobia depended on the herbicide concentrations. Results showed that rhizobial population of the control pouch was significantly greater than those which contain herbicide.

However, the amount of rhizobial population decreases with increase in herbicide concentration. Previous reports indicated that higher concentration of herbicides treatment resulted in much lower microbial counts when compared to soils treated with recommended doses **(**Ayansina and Oso, 2006**).** In this study there was an insignificant difference in the population of *Arachis* nodular *Rhizobium* from soils in pouches treated with the recommended concentration of herbicides compared with that of the control which had no herbicide. This finding corroborates with previous reports which showed that herbicides applied at recommended doses rarely affect microbial population in soils (Fawole, 2000). Also, higher concentrations of herbicide have greater tendencies of toxicity to microbial populations in soils contaminated with high levels of herbicide applications (Stanley *et al.,* 2013**)**. High herbicide concentrations reduce the number of nodules in symbiotic nitrogen-fixing microorganisms, nitrogenase activity microorganisms, dry matter in plants, lysis of bacteroids, and inhibit of energy synthesis of the microorganisms found within the rhizosphere of *Arachis* (Gricher, 2006). Herbicides become incorporated in soil directly, during plant treatment, and indirectly, via water or residues of plant and animal origin. After application, herbicides may evaporate (volatilize), may be washed away through surface run-off, may leach into deep soil strata and ground water, may be inactivated by plants, or may be adsorbed in soil in which case they become subject to chemical or microbiological degradation (Gricher, 2006; Cornish and Burgin, 2005). The misuse of herbicides (as occasioned by high concentration) does result in decrease in microbial counts and elimination of some species (Gricher, 2006).

It is a common practice for local farmers to apply high concentrations of herbicide with the hope to promote effectiveness. They erroneously believe that high dosage of herbicide will lead to total elimination of weed. This motivation makes them to disregard the manufacturer's direction consequently, diluting large quantities of the active ingredient in small volumes of water. This often has grave consequences to the plant and soil microbiota. An adverse effect of herbicide application on legume growth may result from interference of the herbicide with either the plant itself or the *Rhizobium* bacteria (Stanley *et al.,* 2013**)**. It was accepted that an herbicide registered for a particular legume would be relatively non-toxic to the plant at the recommended concentration, and attention was restricted to possible toxic effects on the rhizobia and possible inhibition of nodulation (Willey *et al.,* 2011**).** The effects of these herbicides on plant parts increased gradually with an increase in concentrations of all herbicides (Nada and Mitar, 2002). Herbicides, therefore, affect the viability of rhizobia and thus affect the mechanism involved in rhizobium – legume symbiosis (Zaidi *et al.,* 2005; Denison, 2000).

5. Conclusion

Microorganisms are constitutive elements of the environment and their abundance, enzymatic activity and biodiversity are good indicators of the balance in the agro-ecological system. Herbicides are widely used in Agriculture without their proper investigation of their side effects. In this study higher herbicide concentration decreased root nodular rhizobial microbiota, resulting in abnormal growth of *Arachis hypogea.* To prevent the negative effects of the herbicide on plant growth, plant-soil symbiosis, which ultimately leads to decrease in nitrogen fixation, strict regulation on the use of herbicide with a view to preventing their persistence, bioaccumulation and toxicity (acute and genetic) in agro-ecosystems should be implemented by government. Directions for application of all herbicides should be strictly followed in order to avoid possible abnormal growth and destruction of the soil microbiota. There is also need for operator training to ensure that the herbicide is used safely and effectively. With care, the risk of damage to the soil microbiota will be minimized and result in better weed control. In addition, stakeholders involved in Agriculture such as Agricultural extension workers and farmers should collaborate more closely. Massive awareness and sensitization campaign by trained agricultural workers on the appropriate application of herbicides by farmers should be carried out. Government through regulatory agencies should monitor the activity and practices of farmers on application of herbicides.

6. References

Ayansina, A. & Oso, B.A. (2006) Effect of two commonly used herbicides on soil microflora at two different concentrations. *African Journal of Biotechnology,* 5(2), 129-132.

Ayansina, A. & Amusan, O. (2013). Effect of higher concentrations of herbicides on bacterial populations in tropical soil. *Unique Research Journal of Agricultural Science,* 1(1), 001-005.

Berge, O., Lodhi, A., Brandelet, G., Santaella, C., Roncato, M., Christen, R., Heulin, T. &

Achouak, W. (2009). *Rhizobium alamii* spp: an exopolysaccharide producing species isolated from legume and non-legume rhizospheres. *International Journal of Systemic Evolution and Microbiology,* 59, 367-372.

Bunce, N.J. (1993) *Introduction to Environmental Chemistry.* Wuerz Publishing Ltd.,Winnipeg, Canada, pp. 224-235.

Burdass, D. (2002). *Rhizobium*, root nodules and nitrogen fixation. *Society General of Microbiology*, 1-4.

Celino-Gaminde, E. (1992). Nature of indigenous *Rhjzohia* from *Arachis hypogaea L*. in some bicol soils. *Transactional National Academy of Science,* 4, 455-476.

Chitra, B., Bhagyashree, D. & Bhawana, P. (2013). Isolation and characterization of *Rhizobium* sp. from root of legume plant (*Pisum sativum*) and its antibacterial activity against different bacterial strains. *International Journal of Agriculture and Food Science,* 3(4), 138-141.

Cornish, P. & Burgin, S. (2005). Residual effects of glyphosate herbicide in ecological restoration. *Restorative Ecology,* 13(4), 695–702.

Denison, B.F. (2000). Legume symbiosis and the evolution of symbiotic cooperation by rhizobia. *The American Nature,* 156(6), 567-576.

Deshwal, V.K. & Chaubey, A. (2014). Isolation and Characterization of Rhizobium leguminosarum from Root nodule of Pisum sativum L. *Journal of Academic Industrial Research,* 2(8), 464-467.

EXTONET (1984), *Extension toxicology network, Pesticide Information Profiles.* Oregon, USA, pp. 1-52

FAO, (1996). *Legumes inoculants and their uses.* ISBN 92-5-101441-8.

Fawole, M.B (2000). Effect of pre-emergence herbicide, Galex on fungal and bacterial flora of a cowpea cropped soil. *Bureau for Research Communications,* 12, 195-200.

Gricher W,(2006). Use of Sulfentrazone in a peanut (*Arachis hypogaea*) herbicide program. *The Texas Journal of Agricultural Natural Resources,* 19, 72-79.

Krapovickas A & Gregory WC (2007). Taxonomy of the genus *Arachis* (Leguminosae). *Bonplandia* ,16, 1-205.

Lopez-lopez, A., Marcos A., Ernestos O. & Julio M. (2010). Phaseolus vulgaris seed borne endophytic community with novel bacterial species such as *Rhizobium endophyticum*. *Systemic Applied Microbiology,* 33, 322-327.

Martyniuk, S. & Oron, J. (2008). Populations of rhizobia in some Polish soils not planted with legumes. *Ekologija,* 54(3), 165–168.

Nada, A. & Mitar, M. (2002). Effects of herbicides on microbiological properties of soil. *Proceedings of the National Sciences,*102, 5-21.

Pfeiffer, M. (2011). *Types of herbicides. Pesticide Training Resource*. Arizona,, pp. 1-2.

Sarfraz, H., Tariq, S., Muhammad, S., Muhammad, A. & Azeem, K.. (2009). Impact of pesticides on soil microbial diversity, enzymes, and biochemical reactions. *Advanced Agronomy,* 102, 159-200.

Shahzad, M., Shafee, F., Abbas, S., Babar, M., Tariq, M. & Ahmad, Z. (2012). Isolation and biochemical characterization of *Rhizobium meliloti* from root nodules of alfalfa (*Medico sativa*). F. *The Journal of Animal and Plant Science,* 22(2), 522-524.

Singh, F. & Oswalt, D. (1995). *Groundnut production practices.* International Crops Research Institute for the Semi-Arid Tropics. Andhra Pradesh, India, pp.102-106.

Stanley, H., Maduike, E. & Okerentugba P. (2013). Effect of herbicide (atrazine and paraquat) application on soil bacterial population. *Sky Journal of Soil Scientific Environment and Management,* 29, 101-105.

Tu, E., Li, R., Michael, A., Ware G. & Voos, G. (2001). The *Nature Conservancy*. Weed Control Methods Handbook. Champaign, Illinois, pp. 1-13.

Woomer, P.L., Karanja, N., Kisamuli, S.M., Murwira, M. & Bala, A. (2015). *A revised manual for rhizobium methods and standard protocols* www.N2Africa.org, (accessed 19 January, 2015

Willey, J.M., Sherwood, L.M. & Woolverton. C.J. (2011) *Prescott's Microbiology*. Identification of Microorganisms from Specimen. McGraw Hills. Eight edition. New York..

Zabaloy, M, Graciela, P.Z., Virginia, B., Marisa, A. & Jay., L. (2011). *Herbicides in the soil Environment: Linkage between Bioavailability and Microbial Ecology, Herbicides, Theory and Applications*. Larramendy, M. (Ed.) ISBN: 978-953-307-975-2.

Zaidi, A., Khan, M.S. & Rizvi, P.Q. (2005). Effect of herbicides on growth, nodulation and nitrogen content of greengram. *Agronomy and Sustainable Development,* 25(4), 497-504.