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Prolonged Use of Herbicides Increased the Resistance of Soil Aerobic Heterotrophic Bacteria and Fungi to Antibiotics

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A B S T R A C T

Increase in the use of herbicides in agricultural soil can cause multiple antibiotic resistance in potential pathogens that can undermine antibiotic therapy. The study shows the antimicrobial susceptibility of aerobic heterotrophic bacteria and fungi isolated from soils polluted with different herbicides. A total of sixteen (16) bacterial and twenty-three (23) fungal isolates from herbicides polluted soils were subjected to different antimicrobial sensitivity test (AST) using Kirby-Bauer disc diffusion method. Both Gram positive and Gram-negative antibacterial agents were used for the sensitivity test of bacteria, and a four of antifungal agents were used for the sensitivity test of fungi. The result showed that resistance to antimicrobial agents were higher in soils polluted with herbicides especially where two different herbicides (paraquat and glyphosate) were combined compared to the control soil without history of herbicides usage.This work demonstrates that prolonged usage of herbicides increased the rate at which aerobic heterotrophic bacteria and fungi in soil acquire antibiotic resistance.

Introduction

Soil is a complex and sensitive biomaterial that is strongly affected by anthropogenic activities, especially agriculture activities. Agriculture requirements especially the use of herbicides to control weeds lead to the soil pollution by agrochemicals. The soil is a habitat for majority of microbial communities i.e., soil actinomycetes, bacteria and fungi (Zain *et al.*, 2013). The microbial population in soil is an efficient measure of soil health and environmental fluctuations (Avidano *et al.*, 2005). Herbicides have been a suitable, economical, effective and perceived immediate weed control in agricultural lands (Santín-Montanyá *et al.*, 2013; MacDonald *et al.*, 2013). The initial form of herbicides was used during mass clearing of land to destroy all the plants (Hassall, 1982). In the past seven decades, several research and development programmes have created hundreds of active ingredients of herbicides and many are now in agrochemical market. These herbicides affect plants only, it is effective even at lower concentration, perceived to be less harmful to environment, and have lower cost of production (Duke, 2012).

The herbicides used on soil may affect non-target soil microorganisms. This includes; decreased organisms growth rate, changes in behavior and/or even death (Bukowska, 2006). Lately, there are increased concern regarding the safety of agrochemicals especially herbicides. There are contradictory views regarding the safety of herbicides in the soil. However, agrochemical companies and some monitoring agencies consider herbicides to be environmentally friendly (Shehata *et al.,* 2013). But recent studies have reported likely fatal effects of these herbicides on non-target organisms. 2,4-Dinitrophenylhydrazine (2,4-D) has been reported to inhibits the growth of *Escherichia coli* (Balague *et al.,* 2001). In another study by Botelho *et al.,* (2012) glyphosate and 2,4-D are toxic to *E. coli* by causing growth reduction at a concentration of 0.09 and 0.23, respectively. Glyphosate is one of the most widely use herbicide in all continents of the world (Coupe *et al.,* 2012). Herbicides especially glyphosate that binds to soil particles are inactive and is irreversible and as a result, the uptake of glyphosate through the plant roots rarely occurs (Macdonald *et al.,* 2013). The glyphosate inhibitsenol-pyruvyl-shikimate synthase (EPSP) via chelation of manganese (a cofactor for synthesis of EPSP), in the shikimate pathway of the target plant and it can also inhibit EPSPs in fungi and bacteria (Kishore and Shah, 1988).

Neither the reduction in use of antibiotics nor detection of new antibiotics may be enough approaches to circumvent the post-antibiotic period. This is because microorganisms may be exposed to other chemical compounds that subject them to become more rapidly resistance to antibiotics.

Herbicides are examples of the common agrochemicals that are frequently used globally.New studies discovered that microorganisms develop resistance to antibiotic drugs faster when exposed to commonly used herbicides (Kurenbach *et al.,* 2018). Therefore, this study aimed to determine the antimicrobial sensitivity of microorganism isolated from soil polluted with herbicides.

Materials and Methods

Sample collection

Sixteen bacteria (*Staphylococcus lugdunerisis, Corynebacterium flavescens, Corynebacterium jeikeium, Staphylococcus arlettae, Staphylococcus aureus, Corynebacterium mycetoides, Corynebacterium boris, Corynebacterium minutissinum, Corynebacterium paurometabolum, Staphylococcus saccharolyticus* and *Coryne-bacterium cystitis, Alcaligens faecalis, Enterobacter hafnia, Enterobacter aerogenes, Enterobacter agglomerans* and *Klebsiella pneumonia*) and twenty-three (23) fungi (*Aspergillus oryzae, Alternaria arborescens, Wallemia sebi, Chrysonilia sitophila, Zygosaccharomyces bailii, Ulocladium alternariae, Saccharomyces cerevisiae, Neoscytalidium dimidiatum, Microsporum persicolor, Aspergillus flavus, Trichoderma longibrachiatum, Candida glabrata, Candida parapsilosis, Candida tropicalis, Aspergillus flavus, Aspergillus acidus, Penicillium chrysogenum, Acremonium strictum, Syncephalastrum racemosum, Cryptococcus spp, Epicocum nigrum, Rhodotorula minuta and Pichia membranifaciens*) isolated from soil polluted with herbicides and soil without history of herbicides usage were tested for antimicrobial susceptibility.

The bacteria and fungi were isolated from soil sample collected from different plots at Ayepe, Iwaro-Oka Akoko, Ondo State with known prior herbicides treatment and soil without prior herbicides treatment (control).

The bacteria and fungi were isolated and identified in Microbiology Laboratory, Adekunle Ajasin University, Akungba Akoko. Plot 1: Soil without history of herbicide usage; Plot 2: Paraquat and glyphosate simultaneously used for less than 1 year; Plot 3: Paraquat has been used as herbicide constantly for 4 years; Plot 4: Glyphosate has been used as herbicides constantly for 5years; plot 5: Paraquat herbicide constantly used for 8 years (Bello, 2021).

Antibiotics susceptibility test

A modified method of Bauer-Kirby method (1966) was used during the study. Mueller Hinton agar (MHA) (Oxoid, UK) and potato dextrose agar (PDA) (Oxoid, UK) were prepared according to manufacturer's instruction. The bacterial species initially preserved on the nutrient agar and PDA at 4 ^{o}C. All bacteria used were standardized to 0.5 McFarland standard according to Oyeleke *et al.*, (2008). A sterile swab stick was dipped into the standardized broth culture and excess liquid was drained from the swab stick by pressing it gently to the inner side of the test tube containing the broth culture. Thereafter, the surface of the MHA in Petri-dish was streaked with the swab stick, while the antibiotics sensitivity of the fungi was done according to NCCLS, (2002). Antibacterial discs (amoxicillin (30µg), Augmentin (30µg), gentamycin (10µg), pefloxacin (10µg), tarvid (30µg), streptomycin (30µg), septrin (30µg), chloramphenicol (10µg), pefloxacin (10µg), ciprofloxacin (10µg), rocephin (25µg), erythromycin (10µg), ampiclox (30µg) and zinnacef (25µg)) and antifungal discs(terbinafine (25µg), griseofulvin (50µg), ketoconazole (20µg) and fluconazole (20µg)) (Sigma Aldrich, UK)used were mounted aseptically onto the surface of the inoculated agar plates. Each disc was firmly pressed to ensure complete contact with the agar surface.

Inoculated plates were incubated at 37 ^{o}C for 24 h (bacteria) and 25 ^{o}C for 72 h(fungi). Susceptibility was determined by measuring the diameter of the zone of inhibition and the results were expressed as the mean value of triplicate values.

Results and Discussion

A total of sixteen (16) bacterial and twenty-three (23) fungi isolated from soil polluted with herbicides were tested for antimicrobial sensitivity. Eleven of the bacteria tested Gram-positive bacteria (*Staphylococcus lugdunerisis*, *Corynebacterium flavescens, Corynebacterium jeikeium, Staphylococcus arlettae, Staphylococcus aureus, Corynebacterium mycetoides, Corynebacterium boris, Corynebacterium minutissinum, Corynebacterium paurometabolum*, *Staphylococcus saccharolyticus*and *Corynebacterium cystitis*), while only five were Gram-negative bacteria (*Alcaligens faecalis, Enterobacter hafnia, Enterobacter aerogenes*, *Enterobacter agglomerans* and *Klebsiella pneumoniae*). All Gram-positive bacteria tested were resistant to ampiclox and zinnacef (Figure 2(d and e)). Gram-positive bacteria isolated from plot 1(control soil without history of herbicides), plot 3 (Paraquat has been used as herbicide constantly for 4 years), plot 4 (Glyphosate has been used as herbicides constantly for the 5years) and plot 5 (Paraquat herbicide constantly used for 8 years) are all sensitive to the remaining eight (8) antibiotics tested. However, Gram-positive bacteria isolated from plot 2 (soil where Paraquat and glyphosate are simultaneously used for less than 1 year) (*Corynebacterium jeikeium*, *Staphylococcus arlettae* and *Staphylococcus aureus*) showed resistance toamoxicillin and erythromycin, *Staphylococcus aureus* showed resistance to rocephinand streptomycin, *Corynebacterium jeikeium* and *Staphylococcus arlettae* both showed resistance to septrin, while both *Corynebacterium jeikeium* and *Staphylococcus aureus* showed resistance to pefloxacin and gentamycin (figure 1 and figure 2).

There were no Gram-negative bacteria isolate from plot 1 and plot 3. Bacterium (*Alcaligens faecalis*) was sensitive to all antibiotics used except septrin (figure 3 and 4). The two Gram-negative bacteria (*Enterobacter hafnia* and *Enterobacter aerogenes*) isolated from plot 4 were sensitive to all antibiotics used. However, bacteria isolated from plot 5, *Enterobacter agglomerans* showed resistance to amoxicillin, augmentin, tarvid and streptomycin while *Klebsiella pneumoniae* showed resistance to streptomycin only (figure 3 and 4).

Majority of fungi (*Aspergillus oryzae*, *Alternaria arborescens* and *Wallemia sebi*) isolated from plot 1 were sensitive to the four (4) antifungal agents used except *Chrysoniliasitophila* which showed resistance to terbinafine, *Zygosaccharomyces bailii* also showed resistance to terbinafine, griseofulvin and fluconazole while *Ulocladium alternariae* showed resistance to all four antifungal agents used (figure 5).

Saccharomyces cerevisiae isolated from plot 2 showed resistance to all the antifungal agent tested. In plot 3, *Trichoderma longibrachiatum* showed resistance to only fluconazole while *Candida parapsilosis* and *Neoscytalidium dimidiatum* are both sensitive only to fluconazole. However, *Microsporum persicolor* showed resistance to all antifungal agents except griseofulvin, *Candida tropicalis* was resistant to all four antifungal agents while *Penicillium chrysogenum* showed resistance to only fluconazole (figure 5).

Aspergillus flavus and *Aspergillus acidus* were both sensitive to terbinafine and griseofulvin while the remaining fungi isolated from plot 4 (*Syncephalastrum racemosum*, *Candida glabrata*, *Cryptococcus spp (yeast)*, *Aspergillus niger*, *Pichia membranificus, Epicoccum nigrum*, *Acremonium strictum* and *Rhodotorula minuta* were resistant to both the terbinafine and griseofulvin. *Aspergillus flavus*, *Acremonium strictum* and *Aspergillus acidus* were both sensitive to ketoconazole and fluconazole while the remaining fungi isolated from plot 4 (*Syncephalastrum racemosum*, *Candida glabrata*, *Cryptococcus spp (yeast)*, *Aspergillus niger*, *Pichia membranificus, Epicoccum nigrum* and *Rhodotorula minuta* were resistant to both the ketoconazole and fluconazole (figure 5).

The effect of prolonged application of herbicides (paraquat and glyphosate) on the antimicrobial susceptibility of aerobic heterotrophic bacteria and fungi isolated from soils were evaluated.

The result of this study indicates that prolonged usage of herbicides on soil enhanced the resistance of aerobic heterotrophic bacteria and fungi to antibiotics compared to the soil without history of herbicides usage. This observation agrees with previous studies by Kurenbach *et al.,* (2015; 2017; 2018) and Heinemann, (2017), who demonstrated that herbicides such as dicamba, 2,4-D and glyphosate, as well as a common surfactant can change the susceptibility of bacteria to a diverse range of antibiotics upon concurrent exposure. The observed response of bacteria and fungi to antibiotics varied with different herbicides and combination of herbicides.

The bacteria and fungi isolated from plot 2 where both paraquat and glyphosate have been used for one year showed increased tolerance to antibiotics (both antibacterial and antifungal agents) when compared to other plots where a single species of herbicides was used and control.

The variation in responses of agree with previous studies by Rosner (1985) and Wang *et al.,* (2003) who reported significant differences in response of microorganisms to various biocides in their independent studies (Rosner, 1985; Wang *et al.,* 2003).

Therefore, the combination of herbicides on soil increases the antimicrobial resistance by microorganisms (aerobic heterotrophic bacteria and fungi) in soils compared to soil where only single type of herbicides is used.

The pattern of responses by aerobic heterotrophic bacteria and fungi isolated from herbicides polluted soil to antibiotics are suggestive of a change in target exposure to antimicrobial agents; this may be achieved by either an increase/decrease in efflux, permeability, alteration of the targeted enzymes, structures or pathways (McMurry *et al.,* 1998; Alekshun and Levy, 1999; Renau *et al.,* 1999; Webber and Piddock, 2003; Keeney *et al.,* 2008; Chubiz and Rao, 2011). The effect of the herbicides either must be biophysical or must be able to induce gene expression (Webber and Piddock, 2003; Keeney *et al.,* 2008; Kurenbach *et al.,* 2018).

Fig.1 Antibiotics (Amoxicillin (a), Rocephin (b) ciprofloxacin (c) streptomycin (d) and septrin (e)) sensitivity test against Gram-positive bacteria isolated from soil polluted with herbicides. Data represent mean of a triplicate sample. Plot 1: Control soil without herbicides. Plot 2: Soil treated with glyphosate and Paraquat for 1 year. Plot 3: Soil treated with paraquat for 4 years. Plot 4: Soil treated with glyphosate for 5 years. Plot 5: Soil treated with paraquat for 8 years.

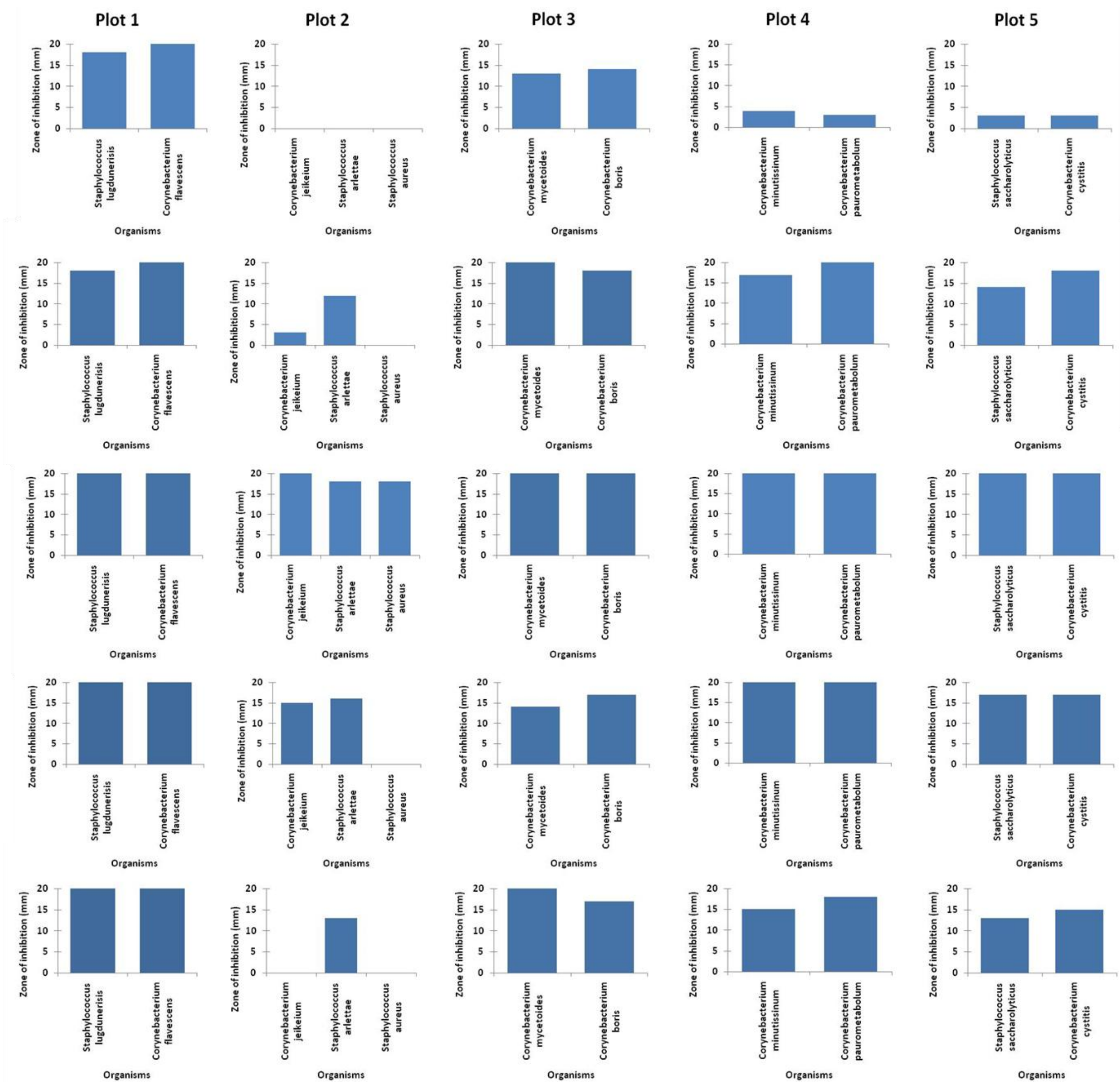


Fig.2 Antibiotics (erythromycin (a), pefloxacin (b) gentamycin (c) ampiclox (d) and zinnacef (e)) sensitivity test against Gram-positive bacteria isolated from soil polluted with herbicides. Data represent mean of a triplicate sample. Plot 1: Control soil without herbicides. Plot 2: Soil treated with glyphosate and paraquat for 1 year. Plot 3: Soil treated with paraquat for 4 years. Plot 4: Soil treated with glyphosate for 5 years. Plot 5: Soil treated with paraquat for 8 years.

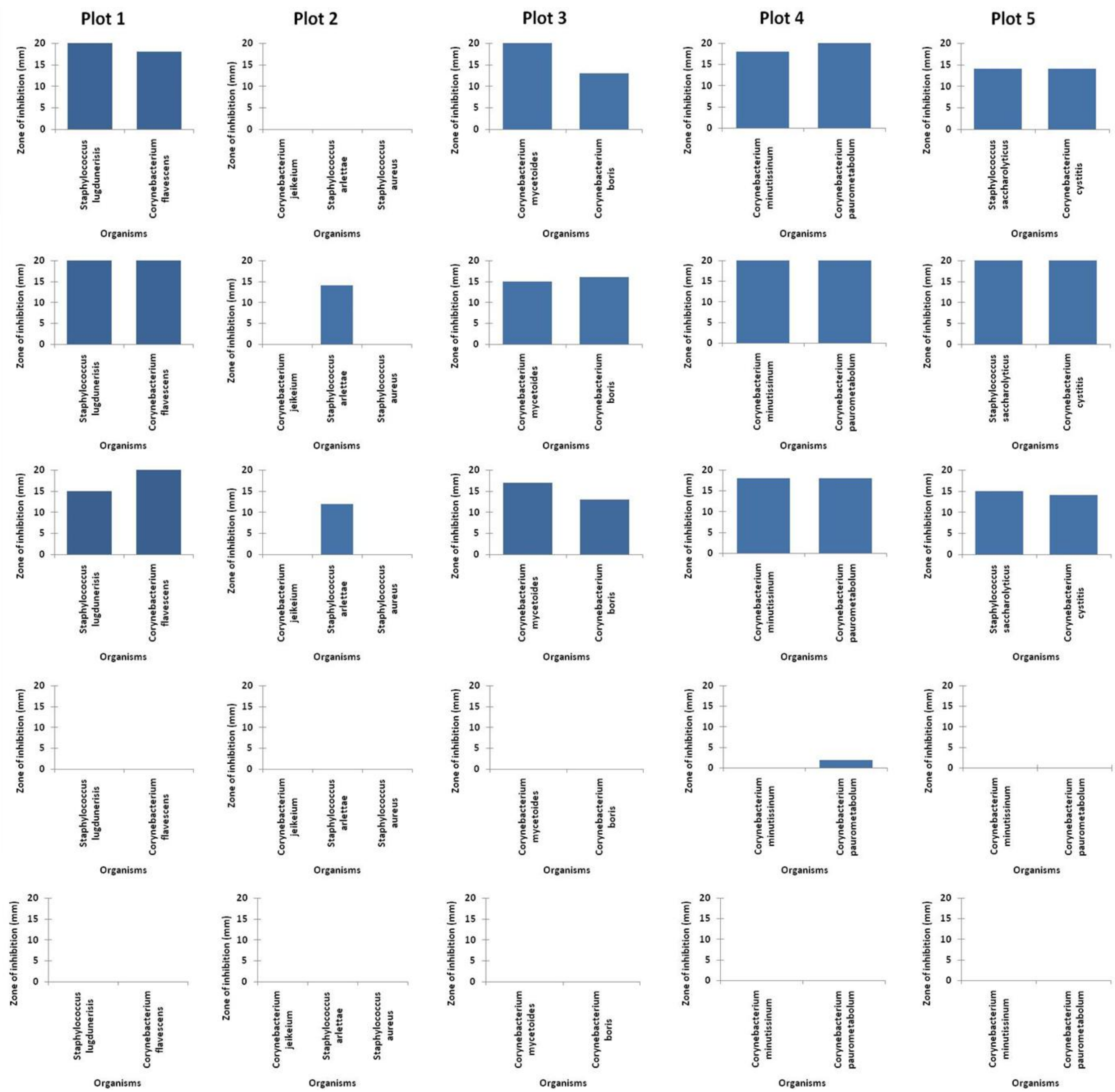


Fig.3 Antibiotics (amoxicillin (a), augmentin (b) gentamycin (c) pefloxacin (d) and tarvid (e)) sensitivity test against Gram-negative bacteria isolated from soil polluted with herbicides. Data represent mean of a triplicate sample. Plot 2: Soil treated with glyphosate and paraquat for 1 year. Plot 4: Soil treated with glyphosate for 5 years. Plot 5: Soil treated with paraquat for 8 years.

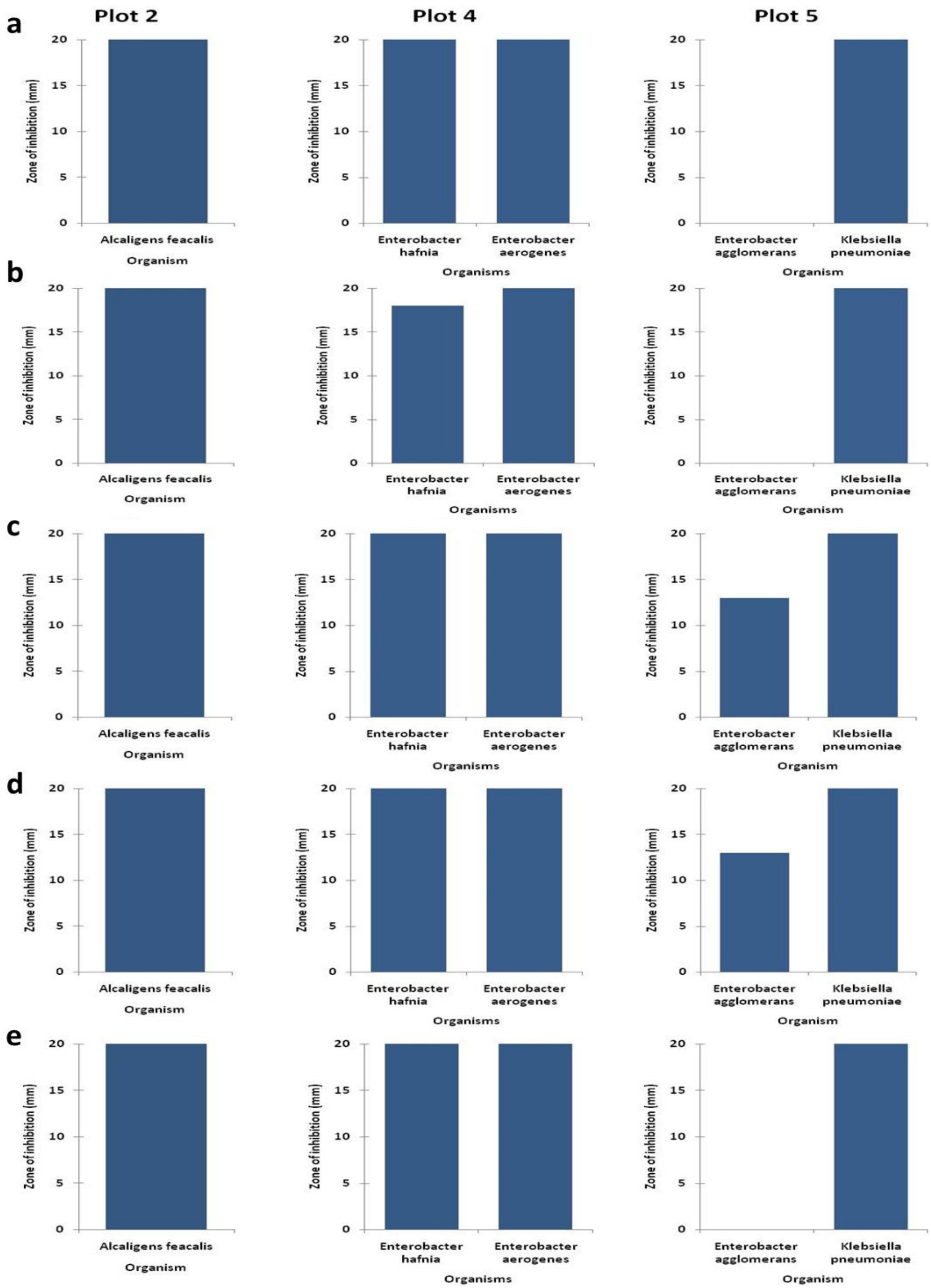


Fig.4 Antibiotics (streptomycin (a), septrin (b) chloramphenicol (c) pefloxacin (d) and ciprofloxacin (e)) sensitivity test against the Gram-negative bacteria isolated from soil polluted with herbicides. Data represent mean of a triplicate sample. Plot 2: Soil treated with glyphosate and paraquat for 1 year. Plot 4: Soil treated with glyphosate for 5 years. Plot 5: Soil treated with paraquat for 8 years.

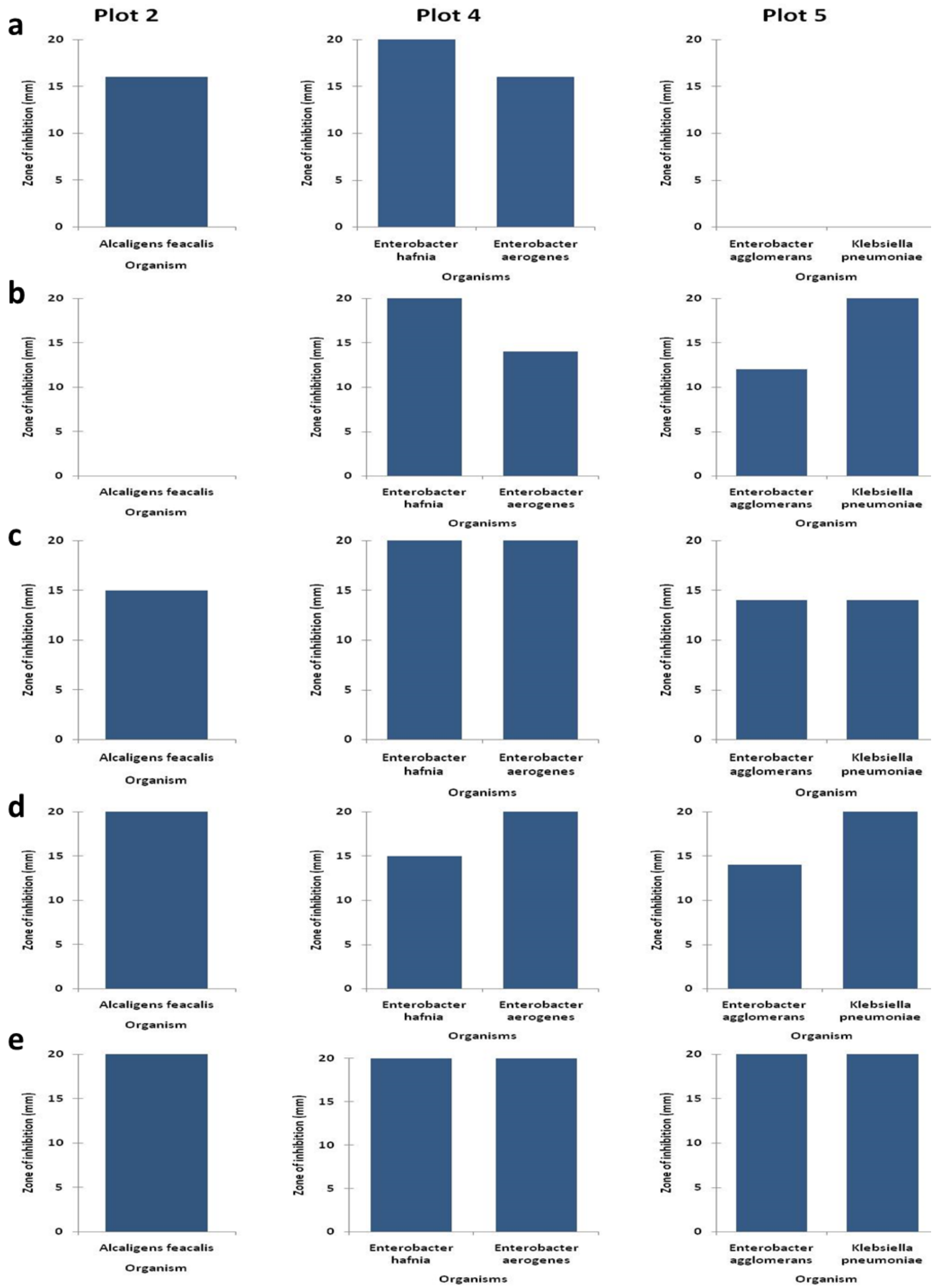
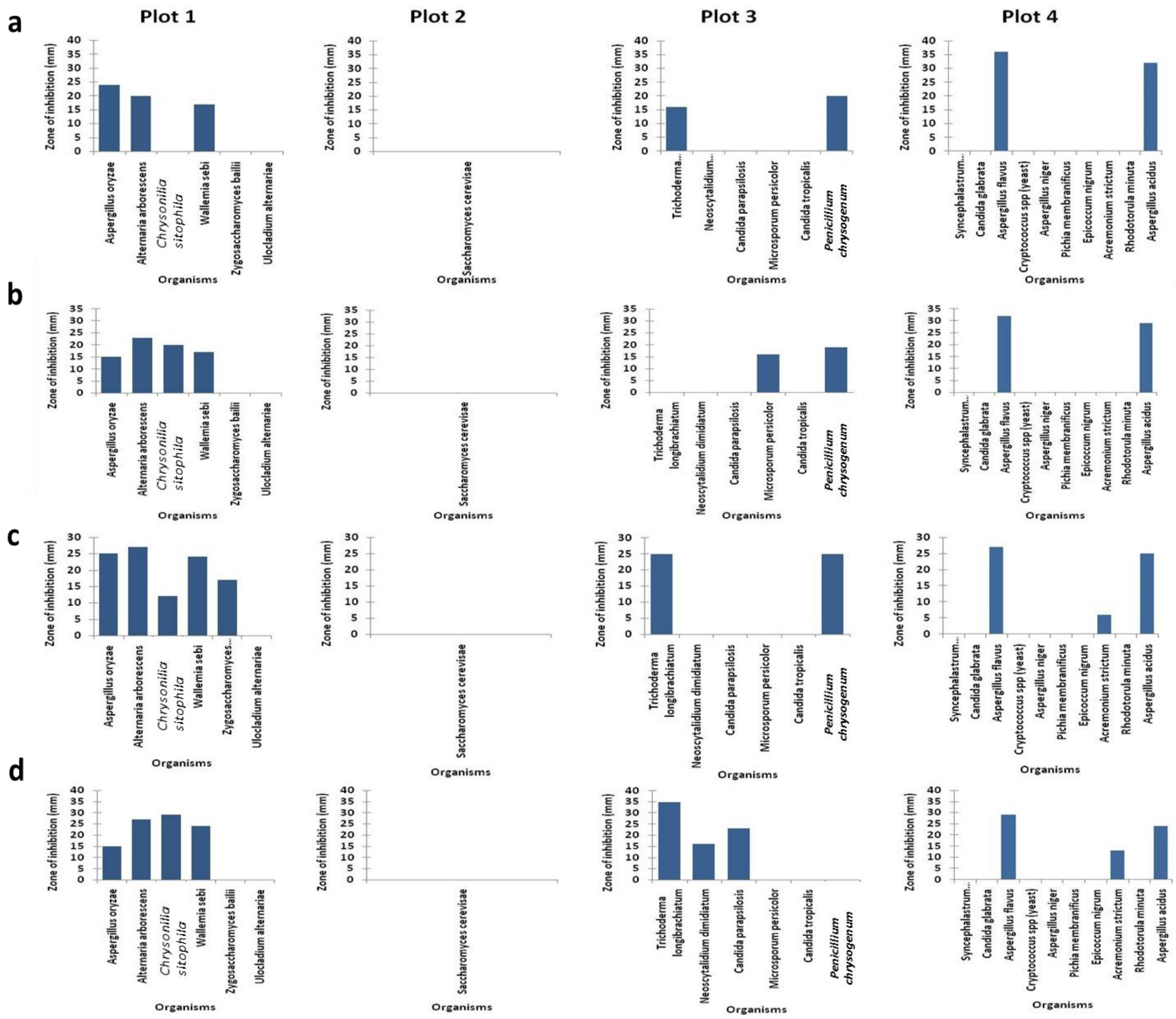


Fig.5 Antifungal (Terbinafine (a), Griseofulvin (b), Ketoconazole (c) and Fluconazole (d)) sensitivity test against the fungi isolated from soils polluted with herbicides. Data represent mean of a triplicate sample. Plot 1: Control soil without herbicides. Plot 2: Soil treated with glyphosate and paraquat for 1 year. Plot 3: Soil treated with paraquat for 4 years. Plot 4: Soil treated with glyphosate for 5 years.



The prolonged use of herbicides increased the resistance of aerobic heterotrophic bacteria and fungi to antibiotics. Hence, more research works are recommended to further establish the mechanisms of antibiotics resistance of microorganisms in soil polluted with herbicides and to compare the antimicrobial susceptibility and growth pattern of microbes isolated from herbicides polluted soils with both clinical and isolates obtained from non-herbicides polluted soil at different antibiotic concentrations.

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