SOME GENETIC EFFECTS OF AGROCHEMICALS ON NITROGEN FIXING BACTERIA

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university

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ABSTRACT

Ambush (Cypermethrin), Delan (Dithianon) and Dithane M-45 (Nabam) are widely used agrochemicals (pesticides). Their lethal doses (LD_{50}) are well known. The three induce C-metaphases, micronuclei, anaphase bridges, condensation and mitostasis in *V. faba* but information is lacking on their effects on nitrogen fixing bacteria. This study aimed at assessing the genetic effects of the three agrochemicals on nitrogen fixing bacteria. This was done by investigating the induction of forward and reverse mutations in the laboratory and field strains of *Rhizobium phaseoli* strain 445, 446 & CC-511, *Derxia gummosa* and *Azotobacter chroococcum*. The effect on nodulation and dry weight was also investigated with *Phaseolus vulgaris*.

The spot and direct tests were conducted. In the spot test sterile filter discs, 6 mm in diameter, soaked in solutions of each agrochemical at concentrations 10^{-6} to 10^{4} ppm were placed at 9 separate points on media on which separate strains had previously been plated. Control samples of discs soaked in sterile water were used. Genetic activity was assessed in terms of proportion of discs around which growth occurred out of the total number used. Toxicity was assessed by scoring zones of inhibition of growth. In the direct test, killing effect (toxic) was assessed by plating approximately 400 cells suspended in the respective agrochemical on complete medium and assessing the emergent colonies out of the total number expected.

In an attempt to map the genome of *R.phaseoli* 445, 446 and CC-511, the bacteria were grown minimal media supplemented with amino acids eliminated one at a time. Antibiotic resistance/senstivity was assessed by introducing discs of antibiotics to bacterial cultures inoculated on complete media.

The effects of Ambush, Delan and Dithane M-45 on nodulation by *P. vulgaris* were investigated by applying the agrochemicals at 10^3 ppm (the recommended rate), 10^5 ppm and 10^1 ppm every 10 days for 56 days. Every week for four consecutive weeks two plants per replicate were selected and the number of nodules was scored and the dry weight was determined. No agrochemical was applied in the controls.

Ambush, Delan and Dithane M-45 reduced colony emergence and inhibited growth of *R. phaseoli, D.gummosa* and *A.chroococcum* at high agrochemical concentration (10^4 ppm). At low concentration (10^{-6} to 10^0 ppm) the 3 agrochemicals induced selective growth of *R. phaseoli* around filter discs but had no observable effect on the growth of *A. chroococcum* and *D. gummosa*. Experiments with amino acids did not elucidate the nature of growth probably due to auxotrophic pre-emption. Dithane M-45 induced antibiotic resistance and/or sensitivity in *R. phaseoli* 445, *R. phaseoli* CC-511, and in *A. chroococcum* in the antibiotic resistance test. High concentration (10^5 ppm) of Ambush, Delan and Dithane M-45 significantly ($P<0.05$) reduced the dry weight and number of nodules of *P. vulgaris* especially during the sixth to eighth week of growth.

Overall the results indicate that Ambush, Delan and Dithane M-45 at high concentration are toxic to nitrogen fixing bacteria. Selective growth of *R. phaseoli* suggests genetic activity of the three agrochemicals. Dithane M-45 at low concentration induces antibiotic resistance and/or sensitivity in *R. phaseoli* 445, *R. phaseoli* CC-511, and in *A. chroococcum*. There is also evidence for reduction of nodulation and dry weight of *P. vulgaris* by Ambush , Delan and Dithane M-45

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

A large number of agrochemicals have been found to be genotoxic in bacterial, yeast, higher plant and mammalian systems (Grant, 1978; Nagao *et al.*, 1981; Klopman *et al.*, 1985). The agrochemicals, Ambush, Delan and Dithane M-45, which are widely used in agricultural systems are known to induce chromosome aberrations in *Vicia faba* meristematic cells (Budambula, 1991).

About 5 x 10^6 tons of agrochemicals are used at the cost of US $ 16.3 billion annually. Out of the total agrochemicals used, about 52, 17 and 26% herbicides, fungicides and insecticides, respectively, are applied annually world wide (Helsel, 1987).

Application of agrochemicals such as herbicides, fungicides, insecticides and so forth is intended to reduce populations of target pests to below economic or injurious threshold. According to Pigmentel and Levitan (1986) 99% of agrochemicals applied are widely dispersed in the environment and affect non-target organisms (NTOs) .

There is high possibility that important organisms including nitrogen fixing bacteria may be targets of lethal and sub-lethal effects of agrochemicals. Factors that have adverse effects on activities of nitrogen fixing bacteria are an added limiting factor to food production.

Fifty percent of food in the world is being lost despite application of pest control procedures (Pigmentel and Pigmentel, 1979)

Food production that is increasing at 2.7% per year can not meet nutritional demand of more than 5 billion world population (Austin, 1986; FAO, 1987). It is estimated that by 2000 A.D. food production will have gone up by 3% against a food demand of 3.5% annually (FAO, 1987). To be able to boost food production, use of appropriate husbandry practices, disease and pest management practices, and optimal activities of nitrogen fixing bacteria are essential.

Effects of agrochemicals on nitrogen fixing bacteria have not been documented. Because of the important role nitrogen fixing bacteria play in food production there is need to investigate effects of environmental pollutants including agrochemicals on these important microorganisms. This study was thus designed to investigate effects of, Ambush, Delan and Dithane M-45, commonly used agrochemicals in many agricultural systems, on nitrogen fixing bacteria.

1.2 LITERATURE REVIEW

1.2.1 GENOTOXINS

Genotoxins are agents that induce genetic changes. They mainly induce deleterious effects on the genetic material. Genotoxins comprise carcinogens, teratogens and mutagens that cause cancer, teratogeny and mutations respectively (Brusick 1980). Examples of carcinogens are steroid hormones, mycotoxins such as aflatoxin B1, metal asbestos fibres and drugs like treosulphur (Pagano and Zeiger, 1989; Groopman *et al.*, 1991; Weinstein and Groopman 1991). Carcinogenic mutagens were 88%, and carcinogenic nonmutagens 11.95% (Nagao *et al.*, 1978). Teratogens include thalidomide, dithiocarmates, ethlenthiurea and propineb (Larson *et al.*, 1976; Tomatis *et al.*, 1978; Rolandi *et al.*, 1984). Most (85-90%) animal carcinogens have been shown to be mutagens (Nagao *et al.*, 1978). Examples of mutagens are radiation and chemicals.

1.2.1.1 Radiation

1.2.1.1.1 Ionizing radiation

Ionizing radiation are fast moving rays that induce ionization of atoms resulting in higher positive ions. They include beta rays, gamma and cosmic rays (Hutterman *et al.*, 1978). The genotoxic effects of ionizing radiation were first demonstrated by Muller (1927). Muller showed that mutation rates in *Drosophila* could be greatly enhanced by irradiation with X-rays. X-rays were also shown to induce mutations in barley and maize (Stadler, 1928; Stadler and Roman, 1948). Low oxygen concentration, enzyme poisons like potassium cyanide, high temperature, centrifugation and vibrations enhance induction of chromosome aberrations following irradiation (Hutterman *et al.*, 1978).

The biological effects of ionizing radiation include induction of chromosome aberrations such as terminal deletions, duplications, inversions, sister chromatid exchanges (SCEs) and recombination.

At early interphase prior to DNA synthesis, ionising radiation mainly induces chromosomal type of breakages. Chromatid and isochromatid breakages are mainly induced in cells irradiated during and/or after DNA synthesis (Hutterman *et al.*, 1978). Indirect effects of ionizing radiation include the ionization of water leading to free radicals from which hydrogen peroxide is formed. Hydrogen peroxide is highly mutagenic (Hutterman *et al.*, 1978).

1.2.1.1.2 Non-ionizing radiation

Non-ioinizing radiations are radiowaves, infrared, visible light and ultraviolet light (UV) (Hutterman *et al.*, 1978) with wavelength 10^4 to 10^6 angstrom units. The best studied effect of non-ionising radiation is that of UV. Its mutagenic effect was first discovered by Altenburg, (1933) in the irradiation of *Drosophila* eggs. Although UV can induce chromosomal aberrations, its mutagenic effects are milder than those of X-rays. UV appears to act by affecting compounds with benzene ring structure such as purines and pyrimidines. The pyrimidines, thymine and cytosine, absorb UV. Cytosine is hydrated following irradiation. This results in the insertion of a water molecule into the C=C double bond. The double carbon bonds of thymine bases may form a thymine dimer. Thymine dimerization is the primary mutagenic lesion induced by UV irradiation.

Indirect effects of UV on DNA through absorption by intermediate compounds have been observed.

Stone *et al.*, (1974) demonstrated an increase in mutation rates of non UV irradiated *Staphylococcus aureus* cultured in media with UV shortly before inoculation. The pretreatment of bacterial cells with DNA bases prior to UV irradiation increases mutation rates significantly (Witkin, 1958). These observations suggest that UV mutagenesis may be mediated indirectly. The effects of UV on DNA are lesions and not mutations.

1.2.1.2 Chemicals

Chemical mutagenesis was first demonstrated in *Aspergillus* using nitrous acid by Thom and Steinberg (1939). Other chemicals such as formaldehyde, diethylsulphate, deazomethane, nitrogen and sulphur mustards were soon shown to be mutagenic (Rapaport , 1946; Auerbach, 1949). Chemicals such as methyl methanesulphonate, (MMS) ethyl methanosuphonate (EMS) nitrosoguanidine (NTG), 4-nitrosoquinoline (4NQO) and mitomycin-C were later shown to be mutagenic.

Today chemical genotoxins include industrial compounds, medicinal plant products, food and feed additives, viruses, cosmetics, laboratory chemical and agrochemicals, (Malling and Wassom, 1977; Rinkus and Legator, 1980).

1.2.1.2.1 Agrochemicals

A large number of agrochemicals have been found to be genotoxic in bacterial, yeast, higher plant and mammalian systems (Grant, 1978; Nagao *et al.*, 1981; Klopman *et al.*, 1985). Their genotoxic effects including mutations have been associated with development of resistance by microorganisms to selection pressure (Brown, 1992). The resistance leads to enhanced pesticide degradation by microorganisms which in turn leads to application of higher concentrations of the agrochemicals and thus possing a greater threat to the environment (Head and Cain, 1991; Morley, 1992).

The mutagenicity of agrochemicals including pesticides is of great concern for they are widely used in agriculture, household pest control and public health (Brown, 1992).

The agrochemicals Alanap-3, atrazine, Banuel D, Endrine, Phospamidon, Metapa, Cytrol, Embotox E and Hyvar X were found to be clastogenic. They induced chromosome fragments and anaphase bridges in mitotic cells of barley (Wuu and Grant, 1967). The insecticides Summithion, Lannate, Carbicron and Thiodon, and the herbicides, Ramrod and Grammaxone, induced acute mitostatic effects in root meristematic cells of *Vicia faba* (Njagi and Gopalan 1981a). Benomyl has been shown to induce aberrant mitoses in *Allium cepa* (Njagi and Gopalan 1981b). Majeeth *et al.*, (1989), found that Nivan and Ekalux induced a high frequency of micronuclei in erthrocytes. Ekatin, Methyparathion and Phorate were shown to be mutagenic in rat bone marrow cells. They induced chromatid gaps, ring chromosomes, terminal deletions, chromosome fragments and chromatid breakages (Malhi and Grover, 1987). Paraquat, Alachlor, Butachlor and Monocrotophos were found to be clastogenic in Chinese hamster ovary cells (CHO) (Lin *et al.*, 1987). Captafol, Vomidothion, DDVP and CBN were found to be base pair mutagens (Shirasu *et al.*, 1976). Gene conversion at *trp* locus in *Saccharomyces cerevisiae* D-7 was induced by polyram, Zineb, Maneb and Dithane M-45 (Warren *et al.*, 1976). Captafol, Captan and Dichlorvos induced point mutations and crossingover in *Aspergillus nidulans* (Bignami *et al.*, 1977). Dexon a fungicide, increased the rate of forward mutation to D-cycloserine and streptomycin resistance in *E. coli,* reversion in *Salmonella typhimurium* and that of gene conversion at *trp* locus in *S. cerevisiae* strain D-7 (Rogers *et al.*, 1978). Davis *et al.*, (1987) reported Bis(tri-n-butytin) oxide (TBTO) to be mutagenic in *S. typhimurium* TA 100.

The effect on soil microorganisms is unknown yet soil is major destination of agrochemicals after application. Agents that induce genetic changes are not limited to agrochemicals.

1.2.1.2.2 Industrial compounds

Industrial compounds pose genetic risks at sites of production and in the environment where they are released. Industrial compounds such as formaldehyde, ethylene oxide, hydrogen peroxide, calcium chloride, mercuric chloride, chromium salts, nickel benzene, lead and tin salts are mutagens (Rapaport, 1946; Kallins, 1967; Robinson *et al.*; 1982; Han, 1989; Moutschen *et al.*; 1975, 1976; Helmi *et al.*, 1989).

Kallins, (1967) showed that ethylene oxide, an epoxide, increases the frequency of leukaemia. The induction of histidine reversions in *S. typhimurium* strain TA102 and TA 104 by hydrogen peroxide has been reported by Han (1989). Mercuric and calcium chloride induced cytological aberrations in *V. faba* and CHO respectively (Kochlar *et al.*, 1989; Helmi *et al.*, 1989). Benzene, an organic solvent was shown to increase the frequency of micronuclei in male *ddy* in mice and in human lymphocytes *in vitro* (Shimada *et al.*, 1989; Anwar *et al.*, 1992).
The induction of aneuploidy in *Drosophila* by colchicine, sodium fluoride and mitomycin-C was demonstrated by Haiyi *et al.*, (1989).

Nickel induces chromosome aberrations and micronuclei in erythrocyctes (Robinson *et al.*, 1982, Zeng *et al.*, 1989). Other metals like chromium, lead and tin have been shown to induce genetic effects in *S. cerevisiae* and SCEs in human lymphocytes (Robinshon, 1982; Bockhov, 1989).

Chromosome aberrations were induced in lymphocytes of workers exposed to carbon black. Coal and coal based fuels were mutagenic in *S. typhimurium* and induced SCEs in Chinese hamster lung (V79) cells (Keane *et al.*, 1991).

1.2.1.2.3.Food and feed additives

A variety of chemicals that constitute the human diet have been identified as toxicants with diverse biological effects. Sodium benzoate and sodium sulphite, widely used feed additives, induce chromosome aberrations in *V. faba* and *A. Cepa* mitotic root cells and point mutations in *S. typhimurium* (Njagi, 1978).

Major sources of proteins contain trace quantities of mutagens. Heated protein, especially meat, contains 2-amino-3-methylimidazo (4,5-f) quinoline (IQ) which was shown to be mutagenic in *Drosophila* and cultured mammalian cells. Imidazoquino axalines that are IQ derivatives were genotoxic to *S. typhimurium* (Wild *et al.*, 1985; Hatch *et al.*, 1985). Smoked lamb was found to be mutagenic to *S. typhimurium* strain TA 100 and TA 98 (Eyfjord, 1985). Hot air roasted and charcoal roasted coffee beans were shown to contain at least six amino mutagens generated during roasting (Kikugawa *et al.*, 1989). Caffeine was found to inhibit DNA repair and photoreactivation in *E. coli in vivo* (Selby and Sancar, 1989).

1.2.1.2.4 Lifestyle

Habits such as cigarette smoking are other sources of genotoxins. Cigarette smoking plays a major role in the induction of human lung cancer (Doll and Petro, 1981). Chemicals found in cigarette smoke have been associated with cancer induction (de Martini, 1983). The presence of mutagens in urine of cigarette smokers is widely documented (Yamasaki and Ames 1977; Kado *et al.*, 1985). Cigarette smoke condensates induced SCEs and micronuclei in mouse bone marrow cells and causes chromosome aberrations in the blood lymphocytes of smokers (Ghosh and Ghosh, 1987; Reidy *et al.*, 1989; Channarayappa and Ong, 1991). Chewing tobacco extracts treated with sodium nitrite were shown to be mutagenic to *S. typhimurium* (Whong *et al.*, 1985). Betel nut chewing was shown to lead to the development of cancer of the soft palate and upper oesophagus (Seedat and Van, 1988). Amyl and butyl nitrite inhaled for their physiological properties of enhanced sexual activities were found to be mutagenic to *S. typhimurium* (Dunkel *et al.*, 1989).

1.2.1.2.5 Cosmetics

Cosmetics constitute another class of substances that have been shown to be genotoxic. Hair dyes, especially those containing aromatic amines have been shown to induce urinary cancer (Clemmensen, 1981). A number of cosmetics especially those containing mercury and bleaches are also genotoxic (Njagi, personal communication).

1.2.1.2.6 Hormones

Hormones such as oral contraceptives that are now widely used, constitute another class of genotoxins. The oral contraceptive, 19-norprogesterone, induced ovarian cancers in mice. Lyndol and anovlar were found to be clastogenic (Lipsuchutz *et al.*, 1966; Carr, 1967). Progesterone administered alone induced ovarian, mammary and uterine tumours (IARC, 1980). Ethinyloestradiol, a pill containing both progesterone and estradiol, increased the incidence of both pituitary and malignant mammary tumours in both male and female mice (IARC, 1980). According to Seety and Hayez, (1992) Nordette and Norminest two oral contraceptives induced C-metaphases and chromosome heterochromatization in *V. faba* and *A. cepa*.

1.2.1.2.7 Viruses

Genotoxins are not limited to man-made substances like industrial chemicals, food additives, cosmetics and hormonal contraceptives but also include natural agents like viruses. During the malignant transformation of cultured cells infected by Rauscher leukemia virus or Simian virus, an increased frequency of SCEs was observed (Brown and Crossen, 1976). Chronic infection with hepatitis B virus (BBV) has been shown to account for 1-10% of hepatocellular carcinoma (HCC) (Bosch and Munoz, 1988). Epstein-Bar virus is associated with all histological forms of nasopharynges carcinoma (Henderson, 1988; Goldstein and Bernstein, 1990).

The human cytomegalovirus (HCMV) induced a significant increase in the frequency of chromosome aberrations in human fibrobrasts and peripheral blood lymphocytes (Abubaker and Deng, 1991; Deng *et al.*, 1991). Horner-Nielsen and Logar, (1991) demonstrated that certain types of human papilloviruses (HPV) are associated with human cancer especially cervical carcinoma. The AIDs virus, HIV, has been shown to cause Karposi's sarcoma, a rare cancer of the skin, mouth and eyes.

1.2.1.2.8 Medicinal plants

Medicinal plant extracts are becoming more popular as an alternative source of medicine. Medicinal plant extracts are also known to be genotoxic. Water extracts from *Astragalus membranceus, Sophora japonica* and *Euconmia* were positive in Ames/*Salmonella* test. *Ajuga remota, Rumex usambarensis, Catharanthus roseus, Euphorbia hirta* and *Achyranthes aspera* were shown to be mutagenic in *S. cerevisae* strains rad 7-1, D7 and D61M. The plant extracts also induced anaphase bridges, mitostasis, micronuclei, chromosome fragments and chromatid exchanges in *V. faba* (Mwangi, 1990). *Petiveria alliacea* induced SCEs in human lymphocytes, centric fragments and bridges in metaphase and anaphase cells of *A. cepa* (Hoyos and Au, 1991). It is not only medicinal plants that are genotoxic, conventional drugs have also been shown to be genotoxic.

1.2.1.2.9 Pharmaceuticals

Most of the therapeutic agents used in the treatment of cancer are inducers of DNA damage (Onsanto *et al.*, 1991). Cis-diammine-dichloroplatinum has mutagenic effects in bacteria and Chinese hamster v79 cells, clastogenic effects in haemoporetic tissues *in vivo* and induced SCEs *in vitro* in human and rabbit cells (Anderson, 1979; Beck and Fisch, 1980; Morrison *et al.*, 1981). Nitrogen mustard was shown to induce chromosome and chromatid breakages in patients and cytogenetic damage *in vitro* in isolated cells and cell lines including human ones (Sen *et al.*, 1990). Recombination and point mutations were induced in yeast by 5-azacytidine (Aza-C) which is used in the treatment of leukaemia (Harrison *et al.*, 1983). Botran and bleomycin, anti-cancer drugs, induced crossing-over, point mutations and aneuploidy in *A. nidulans* (Demapoulos *et al.*, 1982; Hannes *et al.*, 1991). Antibiotics that are antitumour drugs show a mutagenic effect in mammalian cells. Mitomycin C induced chromosomal aberrations in *V. faba* root tips and SCEs and crossing modifications in *D. melanogaster* (Nowell, 1964; Morad *et al.*, 1973). Gabridge *et al.*, (1969) showed that streptomycin is mutagenic in *S. typhimurium*. Phleomycin induced chromosomal aberrations in *V. faba* and leucocytes, and meiotic aberrations in Mouse ovarian cells (Kilman *et al.*, 1967; Jagiello, 1968). Both tetracycline and chloramphenical increased the frequency of mutant colonies of strain TA102 of *S. typhimurium*.

Point mutations were induced in bacteria and germ cells of *D. melanogaster* by Ariamycine (Seino *et al.*, 1978; Albertini, 1989). Antihelminthic drugs have also been reported to have mutagenic activity. Nitroscante, Nitrosohyxylamine were mutagenic in *S. typhimurium* (Gupta *et al.*, 1989). Chlroroquine a drug used for the treatment of malaria induced frameshift mutations in 24 *S. typhimurium* and chromatid breakages in human lymphocytes *in vitro* (Runne and Orfanos, 1975; Espinosa-Aguirre *et al.*, 1987). Brunny *et al.*, (1989) demonstrated the carcinogenicity of anti-histamine, methapyridene hydrochloride (MP) in cultured CHO cells.

1.2.2 Induction of mutagenesis

There are at least three major mechanisms of altering the structure of DNA. They are base substitutions during replication, base changes resulting from chemical instability of the N-glycosidic bond and alterations resulting from the action of other chemicals, radiation and other environmental agents (Freifelder, 1987). Alteration of DNA structure can result from lack of base pairing, mispairing, strand breaks and crosslinkages (Freifelder, 1987).

Mutagens can induce primary lesions (premutations) that are not mutations (Bridges, 1977; Lehman and Bridges, 1977). They however can promote errors during DNA repair (Boiteux *et al.*, 1978; Radman *et al.*, 1979; Condra and Pauling, 1982). Premutations are not heritable.

There are three possible ways by which DNA repair may occur. The lesion may be repaired correctly prior to replication. This is termed error free repair (Loeb 1974; Kornberg, 1974; Banks and Yarranton, 1976). Secondly the lesion may be repaired but in the process an error is made, mainly postreplicatively, hence a mutation is induced. This is termed as error prone repair (Boiteux *et al.*, 1978; Condra and Pauling, 1982). Thirdly the lesion may not be repaired at all. The most likely fate is cell death. Error prone repair of DNA is therefore the primary cause of mutations.

The effects of repair on UV damage to DNA is well documented. In bacteriophage T_4 three alternative pathways for repair of damaged DNA have been identified. Photoreactivation, is a process where a pyrimidine photodimer can be repaired by a photoreactivating enzyme (PRE), alkyltransferase which removes certain alkyl groups added to the oxygen at six positions of guanine. Another pathway, excision repair removes dimers by hydrolysis of the N-glucosylic bond followed by cleavage and resynthesis of the gap left behind (Radany and Friedberg, 1980; Demple and Linn 1980; Seawell *et al.*, 1980). Excision is not only effective for UV but also for chemically induced damage (Williams 1977). Recombinational repair pathways recognise errors after replication. DNA replication stalls at a lesion and restarts past the block leaving a single strand gap on the daughter strand. A homologous parental strand is nicked and used to repair the gap and the newly created gap on parental strand using the other daughter strand (Howard-Flanders, 1975).

The repair pathways are among cellular functions that are induced by exposure to a mutagen. These cellular functions are also termed as 'SOS' functions. Other SOS functions include filamentous growth. In *E. coli* SOS aims at promoting survival of the cell or its phages (Radman, 1974, 1975).

Exposure to a genotoxin does not necessarily result in heritable change due to the existence of methods of avoiding errors and repairing DNA (Loeb, 1974; Kornberg, 1974; Banks and Yarranton, 1976). In spite of activity of these processes that function to preserve the integrity of the genome, mutations do arise spontaneously through faulty editing, or through the action of mutagens, at frequencies that exceed normal spontaneous rates (Hastings *et al.*, 1976; Radman *et al.*, 1979).

1.2.3. NITROGEN FIXING BACTERIA

Nitrogen fixing bacteria include free living bacteria and those with symbiotic associations. Symbiotic nitrogen fixing bacteria include members of the genera *Rhizobium, Bradyrhizobium, Franchia and Azolla* (Vincent, 1970; Chun, 1978). Free living bacteria include aerobic and anaerobic heterotrophs, photosynthetic bacteria, and cyanobacteria (Buchanan and Gibbons, 1974; Mulder and Brotonegoro, 1974; Burns and Hardy, 1975). *Azotobacter, Derxia, Azomanas, Beijerinckia, Clostridium*, and *Klebsiella pneumoniae* are examples of free living nitrogen fixing bacteria (Becking, 1981; Gordon, 1981).

The various genotoxins, all end up in the soil. If these agents are genotoxic in other microbial systems there is no reason to believe they are not harmful to soil bacteria.

1.2.3.1 *Azotobacter*

Azotobacter belongs to the class Schizomycetes, and the family Azotobacteraceae. The *Azotobacter* are found in neutral and alkaline soil, water, rhizosphere and phyllosphere (Becking, 1981). *Azotobacter* are strict aerobes, but can grow and fix nitrogen under reduced oxygen conditions. They are highly sensitive to acid conditions and only grow and fix nitrogen at pH above 6 (Becking, 1981). *Azotobacter* do not form endospores but some form thick walled cysts. They move by peritrichous flagella or are non motile. *Azotobacter* are gram negative or gram variable (Becking, 1981). Members of the genus *Azotobacter* are difficult to mutagenize. Auxotophic mutants are rare, often unstable and difficult to obtain with classical procedures that work well with *E. coli* or rhizobia. *Azotobacter vinelandii* contains between 10 and 40 times DNA per cell as *E. coli* because of the presence of multiple chromosomes in the cell. Lethal consequences of certain recessive mutations are usually masked by the multiple chromosomes (Kennedy and Toukdarin, 1987).

A. vinelandii mutants that have been isolated include chlorate resistant strains that fix nitrogen with little or no nitrate reductase activity, methylanine resistant strains that fix nitrogen in the presence of NH_4^+ and L-methionine-D<-sulfox-mine (MSX), resistant strains with altered glutamate synthetase (Sorger, 1968, 1969; Gordon and Jacobson, 1983; Laugue *et al.* 1986; Santero *et al.*, 1986). Auxotrophic mutants of *A. vinelandii* that require methionine, uracil, hyposanthine, and adenine strains of *A. vinelandii* have been isolated. Adenine and leucine auxotrophs of *A. beijerinckii* have also been isolated (Mishra and Wyss 1968; Page and Sadoff 1976).

1.2.3.2 *Derxia*

Derxia is another member of the family Azotobactereaceae. *Derxia* are gram negative or gram variable, aerobic, heterotropic, free living nitrogen fixing organisms (Becking 1981). *Derxia* has only one species, *Derxia gummosa* which was first isolated by Jensen *et al.*, (1960). *Derxia* is found in soils with pH 4.5-6.5. It is found in Brazil, China, tropical Africa and South Africa (Becking 1981). *Derxia* appear as mass nitrogen fixing colonies among many thin white non nitrogen fixing colonies. The presence of oxygen affects colony morphology. At 0.2 atm only mass colony type predominates (Becking, 1981).

1.2.3.3 *Azomonas*

Azomonas belongs to the family Azotobactereaceae. Members of this genus are aerobic, heterotrophic nitrogen fixing bacteria (Becking, 1981). They are gram negative or gram variable. *Azomonas* have relatively large cells which frequently occur singly with flagella which gives them high motility. The cells exhibit a high degree of pleomorphism and produce a water soluble flourescent pigment in medium (Becking, 1981).

1.2.3.4 *Berjerinckia*

Berjerinckia consists of aerobic, heterotrophic free living nitrogen fixing bacteria. *Berjerinckia* belongs to the family Azobacteraceae. The genus forms two polar lipoid bodies whose cells are gram negative or gram variable (Becking, 1981). On agar medium, *Berjerinckia* develops highly raised glistening colonies of very elastic and tough slime (Becking, 1981). The genus is highly acid tolerant, tolerating upto pH of 4.5. Though primarily found in acid soils, can be isolated from neutral and slightly acidic soils by applying an acid (pH 5) enriched medium (Becking, 1981). *Berjerinckia* is resistant to extremely high iron levels. In the tropics it is present mainly in eluvial, lateritic and absent in illuvial soils. Outside the tropics, the distribution is sporadic. Sugarcane rhizosphere favours proliferation of *Berjerinckia* (Becking, 1981).

1.2.3.5 *Clostridium*

Clostridium Spp are anaerobic nitrogen fixing bacteria found in soils and muds all over the world. Clostridia are capable of utilising a variety of carbohydrates thus the global distribution. They are capable of tolerating a wide pH (4.5-8.5) range with optimum nitrogen fixation at a pH 5.5-6.5 (Gordon, 1981). Nitrogen fixation by *Clostridium* is significant only when population density is greater than 10^6 per gram of soil (Gordon, 1981).

1.2.3.6 *Klebsiella pneumoniae*

Klebsiella pneumoniae is a free living bacterium and a facultative anaerobe that is a member of the family Enterobacteriaceae (Gordon, 1981). *K. pneumoniae* inhabit the rhizophere of nodulated legumes and leaf nodules on the plant *Psychotria*. It is also found in association with fungi in decaying wood, marine waters and sediments (Gordon, 1981).

K. pneumoniae is the only organism for which there is detailed information on genes required for nitrogen fixation. The nitrogen fixation genes are designated *nif* genes. The *nif* genes are clustered together on the genome (Gordon, 1981). Thirteen of the fourteen genes (*nifQ, nifB, nifA, nifL, nifF, nifM, nifV, nifS, nifN, nifE, nifK, nifD, nifH, nifJ*) present in a cluster are required for nitrogen fixation (MacNeil *et al.*, 1978). The *nifA* and *nifL* are involved in regulation of all other nif genes. The *nifH, nifD* and *nifK* encode the nitrogenase subunits.

The *nifQ, nifB, nifE, nifN* and *nifV* are involved in the biosynthesis of the iron-molybdenum cofactor (FeMoCo) of nitrogenase. The *nifF* and *nifJ* are involved in electron transport to nitrogenase (Appelbaum, 1990). The roles of several other nif genes are less well defined. Regulatory sites control whether or not an enzyme is made and properly modified. These sites respond to the presence of oxygen and fixed nitrogen. The control of nitrogen fixation in rhizobia may be different from that in free-living nitrogen fixing bacteria (Gordon, 1981).

Other free living nitrogen fixing bacteria include *Methobacterium, Desulfovibrio, Enterebacter cloacae, Rhodospirillum, Chromatium, Nostoc, Calothrix* and *Cylindrospenum* (Buchanan and Gibbons, 1974; Burns and Hardy, 1975).

1.2.3.7 Rhizobia

Symbiotic nitrogen fixers belong to the genera *Rhizobium* (fast growers) and *Bradyrhizobium* (slow growers) and the family Rhizobiaceae (Vincent, 1970). The two genera are commonly referred to as rhizobia (Appelbaum, 1990). Members of the genus, *Rhizobium* that fix nitrogen include *Rhizobium leguminosarum, R. trifolii, R. phaseoli* and *R. meliloti*. *Bradyrhizobium* that fix nitrogen are *Bradyrhizobium japonicum, B. lupinus, B. vigna* and *B. Lotus* (Appelbaum, 1990).

The growth of rhizobia is affected by various factors. Fast growing rhizobia have been shown to be susceptible to antibiotics.

Slow growing rhizobia are less susceptible to antibiotics. Seventeen strains of fast growing rhizobia including *R. frifolii*, tested against 15 antibiotics were found to be generally sensitive to antibiotics including penicillin (Schwinghamer, 1967). Rhizobia were inhibited by the amino acids, glycine, histidine, alanine, L-arginine and L-glutamic acid. All amino acids produced some distorted cells and the proportion of distorted cells was least with those which caused little or no inhibition of growth (Skinner, 1977).

The growth of *R. trifolii* in the nutrient broth was significantly retarded by the herbicides diquat, paraquat, glyphosate and chlorosulfuron (Eberbach abd Douglas, 1989). Methyl bromide, Zinet and manet inhibited nitrification by *Rhizobium* (Kreutzer, 1968; Koike, 1961).

The presence of an appropriate host is a major factor determining the abundance of specific rhizobia. Conditions that favour the host also favour the associated rhizobia (Vincent, 1974). The occurance of particular rhizobia varies according to their relative sensitivity to acidity. *R. trifolii* has its numbers reduced at pH 4.8-5.0. *R. meliloti* is absent at this pH. *R. leguminosorum* and *R. trifolii* survive in saline - alkaline soils with pH less than 10 (Vincent, 1974). Water shortage reduced survival of rhizobia (Vincent, 1974). Fast growers were found to be more susceptible to dessication than slow growers. Alternate wetting and drying was shown to be detrimental to rhizobia (Bushy and Marshall, 1977). Increased soil temperature may reduce rhizobial populations.

R. meliloti was found to be less sensitive to elevate temperature than *R. leguminosarum* (Vincent, 1974).

Predatory protozoa, myxobacteria, Bdellovibrio and bacteriophage are antagonistic to rhizobia and can reduce rhizobial populations. Mycorrhizal fungi and saprozoic nematodes may enhance survival of rhizobia (Vincent, 1977; Jatala *et al* 1974).

The genetics of rhizobia have been studied in *Rhizobium phaseoli*, the symbiont of *phaseolus vulgaris* (the common bean). *R. phaseoli* is a heterogenous group on the basis of protein patterns. *R. phaseoli* strain CFN-42 has three non-contigous regions that contain nitrogen fixation structural genes characterised by the presence of DNA reiterations (Palacious *et al.*, 1985). Structural genes are known to exist with regulatory genes forming clusters but this is not clear from the information given about *R. phaseoli*.

The significance of *nif* genes reiteration is related to the general organization and dynamics of the *Rhizobium* genome (Palacious *et al.*, 1985). *R. phaseoli* strain CFN-42 has six large plasmids. Plasmid p42-d behaves as a symbiotic plasmid since nodulation ability is completely lost when the plasmid is removed (Palacious *et al.*, 1985). DNA reiteration is a common feature of the eukaryotic genome. Reiteration is not expected in prokarytes.

In *R. phaseoli* two events of symbiotic instability were found at high frequency, the deletion of symbiotic plasmid or loss of whole symbiotic plasmid (Martinez-Salazar *et al.*, 1991).

Intensive genetic analysis has led to the identification of *nod* genes essential for nodulation and, *nif* and *fix* genes for nitrogen fixation. In all fast growing *Rhizobium* species these genes are carried on large plasmids (Martinez *et al.*, 1990).

R. phaseoli recA gene has been cloned and it restored recombination proficiency and conferred resistance to DNA damaging agents, MMS and nitrofuration in an *E. coli recA* mutant as expected. The *R. phaseoli recA* mutants were more sensitive to DNA damaging agents and exhibited 100-fold reduction in recombination frequency (Martinez *et al.*, 1991).

Frequent genomic rearrangements have been detected in different rhizobia including *R. trifolii, R. phaseoli, B. japonicum*. These rearrangements mainly constitute deletions, that may affect symbiotic properties either for nodulation or nitrogen fixation of the strain (Djordjevic *et al.*, 1982; Romero *et al.*, 1988). High frequency amplification, an increase in the copy number of 120-kb region carrying *nod* and *nif* genes and deletions, were detected (Romero *et al.*, 1991).

Most mutagens described for other microorganisms have been reported to be capable of inducing mutations in fast growing rhizobia (Cunningham, 1980). The use of mutagens to induce an increase in symbiotic ability of rhizobia has been limited by difficulties of detecting the desired mutants (Schwinghammer, 1977).

According to Kondorosi and Johnstone (1982) the genetic maps show no representatives of mutations leading to Ala$^-$, Asp$^-$, Asp$^-$, Hom$^-$,lle$^-$, lys$^-$, Pro$^-$, Thr$^-$ or Val$^-$ auxotrophic mutants.

Possibly, some classes of mutants are more readily obtained than others. Tryptophan requiring mutants are among the most readily available amino acid auxotrophs of *R. leguminosarum*. Eleven trytophan auxotrophs of *Bradyrhizobium japonicum* I-110 ARS were isolated by Wells and Kuykendall (1983). Three of them had the ability to produce indole and were capable of nodulating the host plant. The other 8 mutants were only capable of nodulating host plants following reversion to prototrophy (Kuykendall, 1987). Amino acid auxotrophs of *R. leguminosarum* and *B. Japonicum* have been obtained using chemical mutagenesis by nitrous acid (Wells and Kuykendall, 1983). Nitrous acid induced mutations due to its deaminating activity on cytosine and adenine (Kuykendall, 1987).

Mutants of *R. meliloti* 1028 that require isoleucine and valine for growth on minimal medium were unable to induce nodules on alfalfa. DNA sequence analysis shows that the region has a coding sequence homologous to those of *S. cerevisiae* I/V 5 (Anguilar and Grasso, 1991). Most auxotrophic mutants still produce effective nodules implying the relevant factor can be provided by the host plant for the growth of infecting rhizobia (Schwinghammer, 1977). Adenine and leucine auxotrophs form ineffective nodules unless exogenous adenine or leucine is provided (Denarie *et al.,* 1976).

Reversion from purine auxotrophy resulted in increased symbiotic effectiveness of *R. meliloti* (Schwinghammer, 1977). Restoration of effectiveness of *R. meliloti* in some auxotrophs offers a potential means of improving symbiotic activity of a strain of *rhizobia* (Schwinghammer, 1977).

Antibiotic resistant mutants of *Rhizobium* are easy to obtain (Kuykendall, 1987). Streptomycin and spectinomycin resitant mutants are likely to retain normal symbiotic ability. Viomycin and neomycin resistance may be associated with loss of effectiveness of *R. trifolii, R. Leguminosarum* and *R. meliloti* (Vincent, 1977; Levin and Montgomery, 1974). Symbiotic effectiveness of resistant mutants is influenced by characteristics of both rhizobial strain and host plant (Pakhurst, 1977).

Other types of mutants have been isolated. Mutants of *R. trifolii* unable to utilise glucose, fructose or sucrose have been isolated. Mutants resistant to some herbicides and fungicides have been described but the effect on symbiotic ability was not investigated (Schwinghamer, 1977). In soybean several non-nodulating and supernodulating mutants have been isolated after EMS mutagenesis and M_2 family selection (Carrol *et al.*, 1986).

1.2.4 Nodulation

Nodulation is the development of gall-like structures called nodules on plant roots. Nodule formation involves specific recognition between prokaryotic microsymbiont and eukaryotic macrosymbiont.

Nodulation results from effective symbiosis between rhizobia and a host plant (Schwinghamer, 1977).

Nodulation is affected by various factors including temperature, moisture, pH, nutrient composition and host specificity. Low temperatures, less than 10°C, inhibit nodulation in *Trifolium subterranean* and *V. faba*. Nodulation is favoured by 18-19°C (Roughley *et al.*, 1970; Tyson and Sprent, 1982).

Low soil moisture -36 to -360 pKa inhibits nodulation in *T. Subterranean* and can retard nodule development in *V.faba* and accelerate senesence in *Trifolium repens* (Worral and Roughley, 1976; Gallacher and Sprent, 1978). It is evident that poor plant growth has an inhibitory effect on nodulation (Gordon *et al.*, 1989).

High solute or low solute concentration may inhibit nodulation. High NaCl concentration inhibits nodulation by *V. faba* while $NaHCO_3$ or Na_2CO_3 inhibits nodulation by *Medicago sativa* (Singh *et al.*, 1973; Yousef and Sprent, 1983).

Low pH, reduce nodulation, perhaps due to reduced growth and multiplication of rhizobia in the soil, increased numbers of ineffective rhizobia or sensitivity of the infection process (Rice, *et al.*, 1977; Mulder *et al.*, 1977; Date, 1981).

The presence of high levels of combined nitrogen, reduced molybdenum, low iron and large amounts of nickel inhibit nodulation (Becana and Sprent, 1977; Dalton,*et al.*, 1988).

There is high specificity between individual host and rhizobia. Lack of compatibility between rhizobia and host strain when introducing crops into new areas can inhibit nodulation (Sprent and Sprent, 1990).

1.2.4.1 Nodule assessment

The colour of nodules provides evidence for nitrogen fixation (FAO, 1983). Nitrogen fixation is associated with pink mature nodules but not with white and young or brown old nodules. The pink colour is due to presence of leghaemoglobin which is essential for nitrogen fixation. The brown colour is due to the presence of inactive verdhaemoglobin (FAO,1983).

Nodule abundance is used for specific determination of nitrogenase activity, because variation is directly related to nitrogenase activity and therefore to nitrogen fixation (FAO, 1983; Danso, 1984).

1.2.5 Dry matter yield

Dry matter yield can give indirect evidence for nitrogen fixation. Differences in dry matter may be attributed to differences in nitrogen amounts. Nitrogen is required for superior plant performance (Danso, 1984)

1.2.6 Biochemistry of nitrogen fixation

In order to investigate the possible genetic effects of agrochemicals on nitrogen fixing bacteria, it is essential to understand the biochemistry of nitrogen fixation.

Nitrogen fixation involves the activity of the enzyme nitrogenase which catalyses the reduction of nitrogen to ammonia (Postgate, 1982). Nitrogenase consists of

iron-sulfur protein and iron protein (Paul and Clark, 1989). The iron-sulfur protein contains molybdenum (Paul and Clark, 1989).

The iron-protein accepts electrons from metabolic routes via ferrodoxin. The protein then forms a complex with adenosine triphosphate (ATP) which in turn reduces the iron-sulphur protein (Paul and Clark, 1989). This process releases inorganic phosphate and electrons. The electrons are then passed to nitrogen or any of the following, cyanide, allene, cyanogen azide, nitrous oxide and hydrogen ion (Postgate, 1982; Paul and Clark, 1989). Because of molybdenum iron in the iron-sulfur protein, the protein plays a major role in the reduction of nitrogen ions to nitrogen gas. Products are either hydrogen gas or nitrogen gas. Hydrogen gas is the major product at low electron flux. Nitrogen gas is the main product at high electron flux and high ATP content (Paul and Clark, 1989).

1.2.7 Ambush, Delan and Dithane M-45

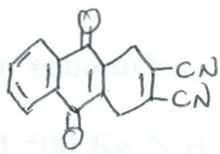
Ambush, Delan and Dithane M-45 that were used in this study are widely used agrochemicals. Ambush (propionaldehyde, 2 methyl (Methylthio)-O-(Methylcarbomonyl) is commonly called Cypermethrin. Ambush has the structural formula

$$H_3C-\overset{\displaystyle CH_3}{\underset{\displaystyle CH_3}{C}}-CH = N\text{-}O\text{-}CO\text{-}NH\text{-}CH_3$$

(Buchell, 1983)

Ambush is a broad spectrum insecticide used on vegetables, tobacco, beans, fruit trees, ornamentals and other agricultural and horticultural crops. The acute LD_{50} in rats is 0.93mg/Kg (Martin and Worthing, 1977). Ambush is a systemic insecticide that interferes with the synthesis of cholinesterase (Martin and Worthing 1976).

Delan (Naptho (2,3-b)-p-Dithin-2, 3- Dicarbonitrile, 5, 10-dihydro-5, 10 Dioxo) commonly called Dithianon has the structural formula



(Buchell, 1983)

Delan is a protective fungicide against powdery mildews, coffee berry disease, coffee rust, scab and melanose of oranges (Buchell, 1983). The acute oral LD is 638 mg/Kg in rats. Delan reacts with mercapto and amino groups in biological systems (Buchell, 1983).

Dithane M-45 (Manganese [[1,2-ethanediylbis-carbamadithioato]] (2-)] in combination with [[1,2 ethanediylbis [carbamadithioato]]-2-] zinc (8018-01-

7), whose common name is Nabam consists of 62% ethyLene bisdithiocarbamate ion has the structural formula

$$[\ .S\overset{S}{\overset{\|}{C}}NHCH_2CH_2NH\overset{S}{\overset{\|}{C}}S.Mn]\ (zn)y$$

(Martin and Worthing, 1977)

Dithane M-45 is an organic fungicide used against early and late blight, rust, septoria blight, downey mildew and leaf spot. The acute LD_{50} is greater than 8000 mg/Kg in rats (Martin and Worthing, 1977). Dithane M-45 inhibits glutamic acid dehydrogenase, aminoxidase and polynoloxine (lvanova-chemishanska *et al.*, 1974; Buchell, 1983). Dithane M-45 is readily degraded by soil microorganisms releasing C atoms as CO_2 (Lyman and Lactoste, 1974). Breakdown products can effect enzyme activities respiration and nutrification at 10 mg/Kg.

1.2.8 The relevance of genotoxicity testing to Kenya

Between 40 and 500 Kg N/Ha/Yr are estimated to be fixed by trees, forage and grain legumes (Keya, 1985). This nitrogen source is a significant supplement to the amendment of soils with nitrogen containing fertilizers. Nitrogen is essential for superior plant performance and high production.

Fertilizers are too expensive for poor resource farmers who are the majority in the Kenyan agricultural based economy. Besides being a source of food security, agriculture contributes two thirds to the Kenyan foreign exchange earnings.

Nitrogen fixing bacteria are therefore important in food production and therefore, genotoxic effects, if any, resulting from environmental pollutants including agrochemicals need to be documented. No data are available on the genotoxic effects of agrochemicals on nitrogen fixing bacteria.

This study was designed to investigate the genotoxic effects of the agrochemicals Ambush, Delan and Dithane M-45 that are known to induce cytological aberrations, mitostasis, C-metaphases, pulverization, anaphase, bridges, condensation of chromosomes, binucleates, micronuclei and/or stickiness in *V. faba* (Budambula, 1991). The genotoxicity of these agrochemicals may not be limited to *V. faba*. In absence of data the possibility that even important organisms in agriculture such as nitrogen fixing bacteria may be vulnerable cannot be ruled out.

1.3 Objectives of this study were

1. To determine genotoxic effects of Ambush, Delan and Dithane M-45 in laboratory strains of nitrogen fixing bacteria, *Rhizobium, Azotobacter* and *Derxia*.
2. To investigate effects of Ambush, Delan and Dithane M-45 on nodulation by *Phaseolus vulgaris* (common bean).
3. To investigate effects of the three agrochemicals on nitrogen fixing bacteria from three localities in different ecological zones namely Kenyatta University, Kisumu and Kericho, Kenya.

CHAPTER II

MATERIALS AND METHODS

2.1 Bacteria strains

Laboratory strains of nitrogen fixing bacteria, *Rhizobium phaseoli* 445, 446 and CC-511 were obtained from MIRCEN, University of Nairobi, and *Azotobacter chroococcum* and *Derxia gummosa* from The University of Sussex (U.K). Field strains (Table I) of the same bacteria were isolated from soil samples from Kenyatta University, Kericho and Kisumu.

2.2. Soil sampling techniques

Topographically uniform areas in each localty were selected for sampling. Twenty separate soil cores were taken randomly from each area. The twenty soil cores were bulked to form a 500 cm^3 soil sample and placed in a plastic bag. The bags were labelled indicating the sample number, locality and crop history (Iowa State University, 1983-84).

2.3.1 Isolation of *Rhizobium* (Vincent, 1970)

Nodulated bean roots were thoroughly washed before nodules were severed. Undamaged nodules were immersed in 95% ethanol for 5-10 seconds before transferring to 3% calcium hypochlorite for sterilization for 3-4 minutes. The sterilized nodules were rinsed in sterilized water thoroughly.

TABLE I. Field strains of nitrogen fixing bacteria isolated from Kericho, Kisumu and Kenyatta University.

Location	Sample Number	Locality	Strains Isolated	Previous crop History
Kericho	1	Belgut	A,D	Maize, cabbage beans
	2	Belgut	R,D	Maize, beans
	3	Belgut	A,D	Cabbage
	4	Kericho town	A,D	grass
	5	Kericho town	D	grass
	6	Kericho town	D	grass
	7	Chepalungu	R,A,D	maize
	8	Chepalungu	R,D	cabbage, beans
	9	Chepalungu	R,A,D	maize
Kisumu	10	Kano plains	R,D	sugarcane
	11	Kano Plains	A,D	sugarcane
	12	Kano plains	R,A,D	rice
	13	Kisumu town	R,A,D	millet
	14	Kisumu town	R,A,D	millet
	15	Kisumu town	R,A,D	millet

TABLE I CONTINUED...

	16	Nyahera	R,A,D	maize, bananas
	17	Nyahera	A,D	maize, bananas
	18	Nyahera	R,A,D	cassava
Kenyatta University	19	Kenyatta Uni.	R,A,D	maize, beans
	20	Kenyatta Uni.	R,A,D	maize, beans cowpeas
	21	Kenyatta Uni.	R,A,D	cowpeas,bananas,cassava

R = *Rhizobium phaseoli*

A = *Azotobacter chroococcum*

D = *Derxia gummosa*

The nodules were aseptically crushed in sterilized water and a loopful of the crushed nodules was streaked on yeast mannitol agar (YMA) (Table II). After a 3-5 day incubation period at 26°C, *Rhizobium* colonies characterised by being watery, translucent or whitely opaque were aseptically picked and streaked on a slant of YMA. Enrichment and subculturing was done on YMA.

2.3.2 Isolation and enrichment of *Derxia gummosa*

A suspension of 2 grams of soil in 10ml of distilled water was streaked on the solid medium (Campello and Dobereiner, 1970) for *D. gummosa* (Table III). After a 3 day incubation period at 26°C, *D. gummosa* appeared as mass colonies among many thin white colonies. One of the larger colonies was subcultured on solid medium.

2.3.3 Isolation of *Azotobacter chroococcum*

To 100 ml of nutrient solution (Table IVa) as described by Beijerinck, (1901), 0.5g of soil was added and flasks incubated at 29°C for 3 days. An *A. chroococcum* pellicle was formed on the liquid surface. The pellicle was subcultured on agar medium (Table IVb) described by Beijerinck (1901).

2.4 Sterilization

Media and Glassware were sterilized at 121°C, 15lb/in^2 for 30 and 20 minutes respectively in an Oldhams 225 E Model autoclave. Test agents were sterilized with a 0.45 millipore filters and a 1425 PPM suction pump.

TABLE II. Yeast mannital agar (YMA) used for the isolation and growth of *Rhizobium* (Vincent, 1970).

Constituent	Quantity
Mannitol	10 g
K_2HPO_4	0.5 g
$MgSO_4.7H_2O$	0.2 g
NaCl	0.1 g
Yeast extract	0.5 g
Agar	15 g
Distilled water	1000 ml
pH	6.8

Yeast mannitol liquid had the same composition as YMA without agar.

TABLE III. Medium for isolation and enrichment of *Derxia gummosa* (Campello and Dobereiner, 1970, modified from Lipman 1903).

Constituent	Quantity
Starch	20 g
K_2HPO_4	0.05 g
KH_2PO_4	0.15 g
$MgSO_4.7H_2O$	0.20 g
CaCl	0.02 g
$NaHCO_3$	0.1 g
$FeCl_3$ (10% wt/vol	1 drop
$NaMoO_4.2H_2O$	0.002 g
C_2H_5OH	5 ml
Agar	20 g
Distilled water	1000 ml

TABLE IVa. Nutrient solution used for the isolation of *Azotobacter chroococcum* (Berjerinck, 1901)

Constituent	**Quantity**
Glucose	20.0 g
K_2HPO_4	1.0 g
$MgSO_4.7H_20$	0.5 g
$NaMoO_4.2H_2O$	0.005g
Distilled water	1000 ml

TABLE IVb. Agar medium for growth of *Azotobacter chroococcum* (Beijerinck 1901)

Constituent	Quantity
Glucose	20.0 g
K_2HPO_4	0.8 g
KH_2PO_4	0.2 g
$MgSO_4.7H_2O$	0.5 g
$FeCl_3.6H_2O$	0.1 g
$CaCl.2H_2O$	0.05 g
$NaMO_4.2H_20$	0.05 g
Agar	20 g
Distilled water	1000 ml
pH	7.4-7.6.

2.5.1 Genotoxic tests

Preparation of strains for genotoxic tests was done as illustrated in figure 1. Spot test and direct test (Fig. 2 and 3) were used in investigating the genotoxicity of the chosen agrochemicals.

2.5.2 Amino acid supplements

Following the results obtained with *R. phaseoli* 445, 446 and CC-511, an attempt was made to map their genome using minimal medium (Table V) supplemented with amino acids which were eliminated one at a time (Table VI).

2.5.3 Antibiotic resistance test

The susceptibility of different genera of nitrogen fixing bacteria to antibiotics was tested using the antibiotics listed in table VII. The antibiotic discs were placed on freshly inoculated cultures on complete media and incubated at 26^{oC} for 3-5 days.

2.6 The nodulation test

A field test was set up at Kenyatta University in 1992 to investigate effects of Ambush, Delan and Dithane M-45 on nodulation by *Phaseolus vulgaris* (common bean). Effect on dry weight was also investigated. A completely randomised design with 5 replicates was used. *Phaseolus vulgaris L. CV* Canadian wonder seeds were planted 10 cm apart within a row. Rows were 40 cm apart.

Fig. 1. General scheme of preparing strains for tests.

1. Bacteria strains were grown on complete solid medium on slants.

2. Streaked on complete medium

3. Two colonies were transfered to complete liquid medium and incubated at recommended temperature for 24 hours.

4. The medium was decanted, supernatant centrifuged, pellet washed in distilled water.

5. 10 ml of distilled water were added to pellet and shaking done. 0.1ml of the suspension were used for plating.

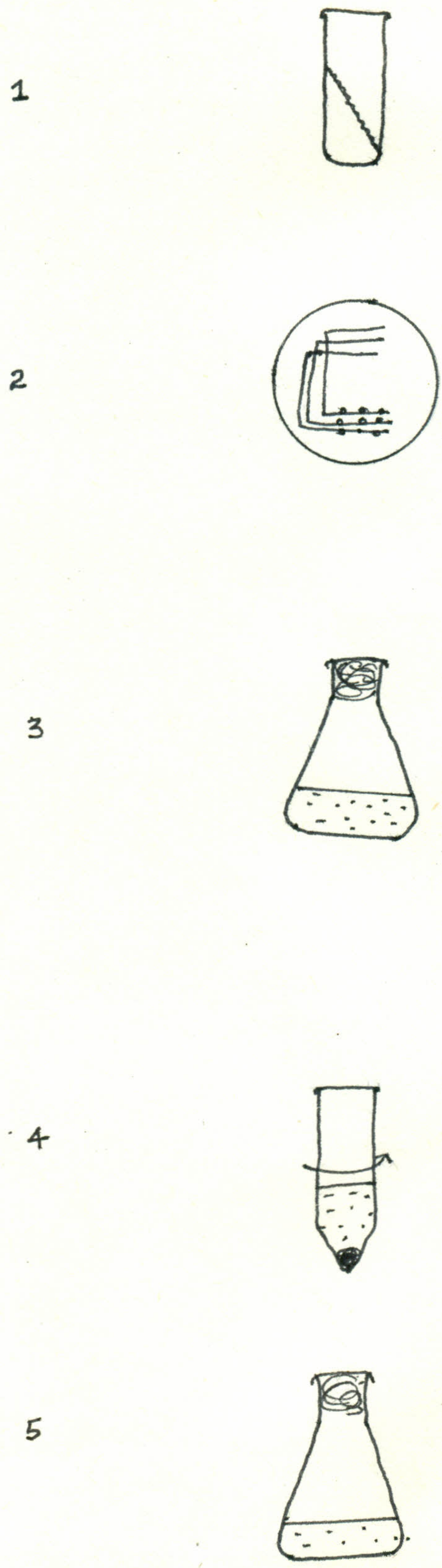


Fig.1

Fig. 2. The spot test.

1. Sterile filter paper discs were soaked in the different solutions of varying concentration of the agrochemicals.

2. 0.1 ml (approximately 400 cells) aliquots of bacterial cell suspension were inoculated onto apropriate media and spread evenly with a sterile bent rod.

3. Soaked discs (9 per plate) were transferred onto appropriate media using sterile forceps.

4. The plates were incubated at the recommended temperature for each strain for 3-7 days.

5. Positive results were scored as the growth of colonies under and around the test discs in experimental plates.

Agrochemicals were omitted in the controls.

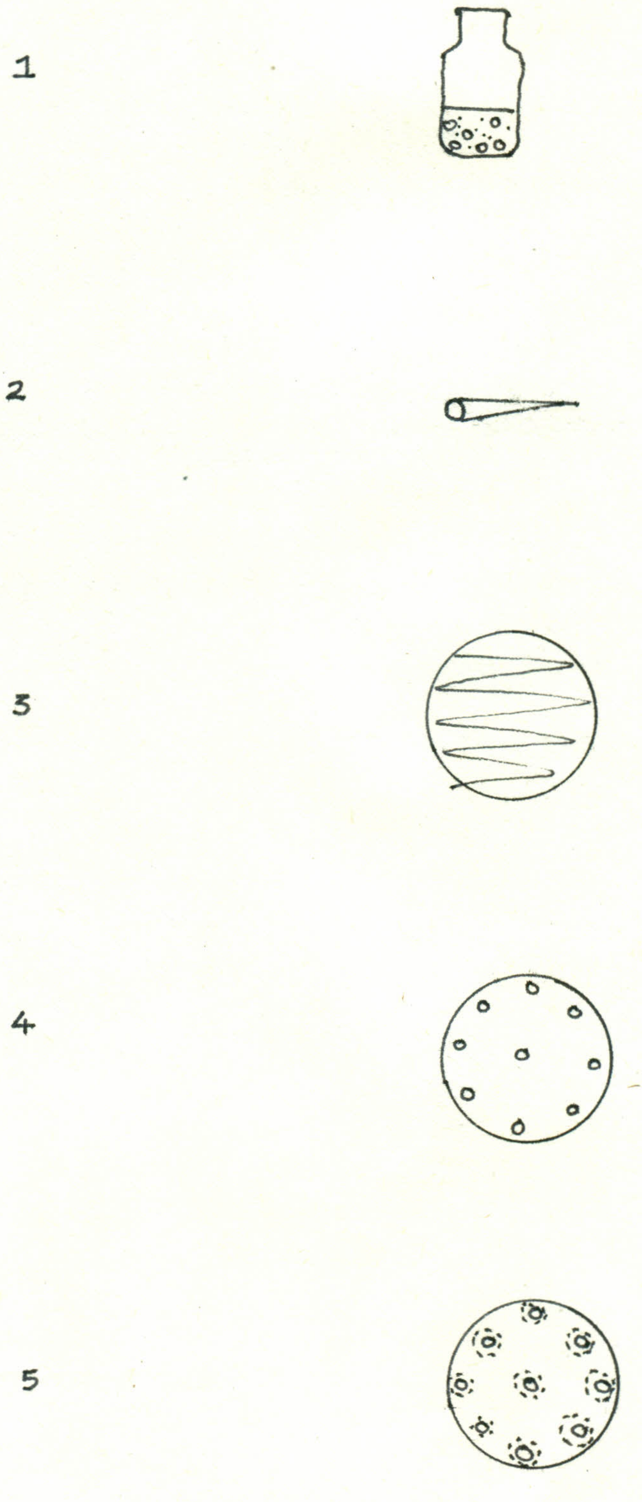


Fig. 2

Fig. 3. The direct test

1. Filter columns were sterilised by autoclaving at 121^{oC,} 15lb/In^2 for 20 minutes.

2. The agrochemicals were filter sterilized and serially diluted using sterile distilled water and sterile universal bottles.

3. About 400 cells of bacterial strain and 0.1 ml of the agrochemical were pipetted to respective complete media and spread with a sterile bent glass rod.

4. The petridishes were incubated for 3 days at the temperature recommended for each genus.

5. Direct killing was assessed by scoring the number of emergent colonies out of the number expected per petridish.

Agrochemicals were omitted in the control

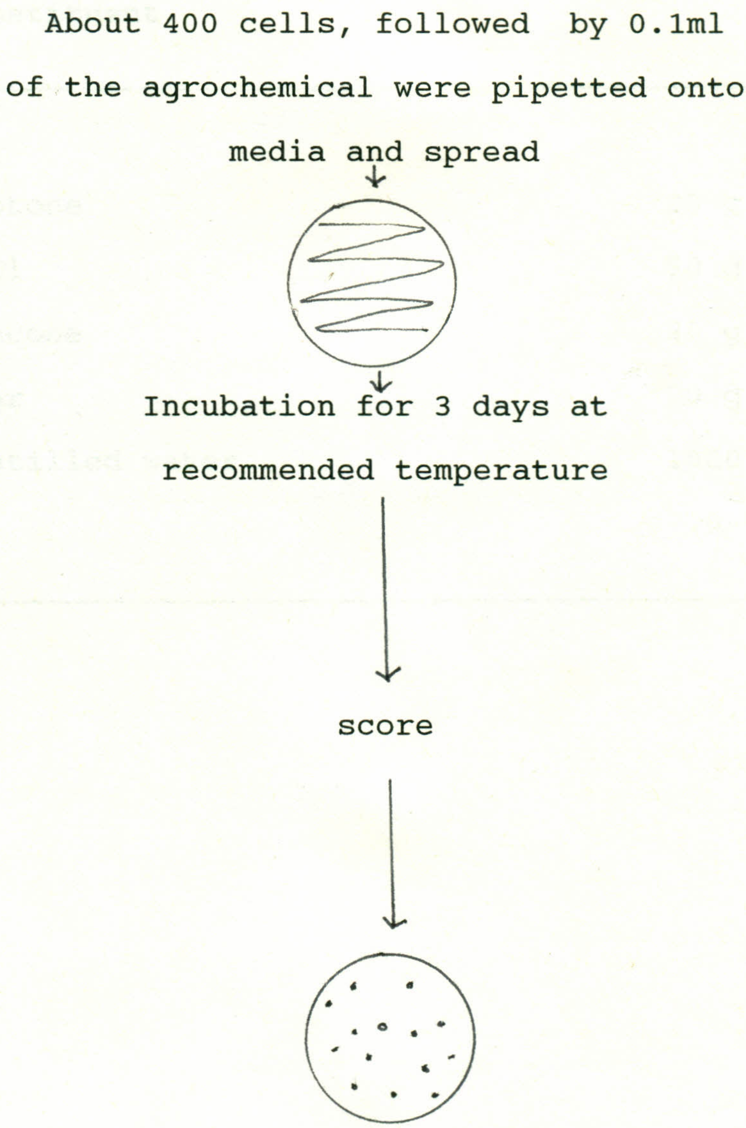

About 400 cells, followed by 0.1ml
of the agrochemical were pipetted onto
media and spread
Incubation for 3 days at
recommended temperature
score

Fig. 3

TABLE V. Minimal medium for bacteria.

Constituent	Quantity
Peptone	20 g
NaCl	50 g
Glucose	20 g
Agar	20 g
Distilled water	1000 ml
pH	7.0-7.2

TABLE VI . Amino acids used to supplement minimal medium.

Expt 1. The 20 amino acids that were used present.
2-(minus) Lys
3-Lys,tyr
4-Lys,tyr,pro
5-Lys,tyr,pro,asp
6-Lys,tyr,pro,asp,ser
7-Lys,tyr,pro,asp,ser,his
8-Lys,tyr,pro,asp,ser,his,arg
9-Lys,tyr,pro,asp,ser,his,arg,ala
10-Lus,tyr,pro,asp,ser,his,arg, ala,try
11-Lys,tyr,pro,asp,ser,his,arg,ala,try,val
12-Lys,tyr,pro,asp,ser,his,arg,ala,try,val,phe
13-Lys,tyr,pro,asp,ser,his,arg,ala,try,val,phe,met
14-Lys,tyr,pro,asp,ser,his,arg,ala,try,val,phe,met,leu
15-Lys,tyr,pro,asp,ser,his,arg,ala,try,val,phe,met,leu,gly
16-Lys,tyr,pro,asp,ser,his,arg,ala,try,val,phe,met,leu,gly,asp acid,
17-Lys,tyr,pro,asp,ser,his,arg,ala,try,val,phe,met,leu,gly,asp acid,thr
18-Lys,tyr,pro,asp,ser,his,arg,ala,try,val,phe,met,leu,gly,asp acid,thr,cys
19-Lys,tyr,pro,asp,ser,his,arg,ala,try,val,phe,met,leu,gly,asp acid,thr,cys,Hyd.pro
20-Lys,tyr,pro,asp,ser,his,arg,ala,try,val,phe,met,leu,gly,asp acid,thr,cys,Hyd.pro,glu acid.

L-Lysine (Lys), L-tyrosine (Tyr), L-Proline(Pro), L-Asparagine (Asp), DL-Serine (Ser), L-Histidine (His), L-Arginine (Arg), DL-Alanine (Ala), DL-Tryptophan (Try), DL-Valine (Val), L-Phenylalanine (Phe), L-Methionine (Met), L-Leucine (Leu), Glycine (Gly), DL-Aspartic acid (Asp.acid), DL-Threonine (Thr), L-cystein (Cys) Hydroxy-L-proline (Hyd.Pro), L-Glutamic acid (Glu.acid). Stock solutions of 1g/100 ml of distilled water were prepared and 2ml was added per 1000ml of medium.

Glutamine and isoleucine were not available.

TABLE VII. Antibiotics used in the antibiotic resistance test.

Antibiotic	Concentration levels
Single discs	
Miraxid	100 µg
Amoxyllin (amoxil)	25 µg
Spiramycine	100 µg
Ofloxacin (Tarivid)	5 µg
Minocycline	30 µg
Trimethoprim	5 µg
Combined discs I	
Ampicillin	25 µg
Tetracycline	100 µg
Nitrofurantoin	200 µg
Naladixic acid	30 µg
Sulphamethoxazole	200 µg
Gentamicin	10 µg
Cotrimoxazole	25 µg
Combined discs II	
Ampicillin	25 µg
Tetracycline	25 µg
Cotrimoxazole	25 g
Streptomycin	10 µg

Table VII cont...

Kanamycin	30 µg
Gentamicin	10 µg
Sulphamethaxazole	20 µg
Chloramphenicol	10 µg
Combined discs III	
Penicillin	1 unit
Minocycline	30 µg
Erythromycin	15 µg
Methicillin	5 µg
Cotrimoxazole	25 µg
Ampicillin	10 µg
Lincomycin	2 µg

Agrochemicals Ambush, Delan and Dithane M-45 were applied at different concentrations (Table VIII) and application was repeated every 10 days as recommended by manufacturers.

2.6.1 Sampling techniques

Sampling started when plants were four weeks old. For every concentration two plants per replicate, selected randomly, were uprooted. The dry weight of the whole plant, and, colour, size and the number of nodules, were assessed. This was repeated every week for 4 consecutive weeks.

TABLE VIII. Concentration levels of agrochemicals that in the nodulation test.

Agrochemical	Concentration Levels ppm 1	2	3
Ambush	$1.5x10^5$	$1.5x10^3$	$1.5x10^1$
Delan	$3.3x10^5$	$3.3x10^3$	$3.3x10^1$
Dithane M-45	$3.0x10^5$	$3.0x10^3$	$3.0x10^1$

1. 100 x recommended rate
2. recommended rate
3. 1/100 recommended rate

CHAPTER III

RESULTS

3.1 Genetic effects of agrochemicals on nitrogen fixing bacteria

3.1.1 Laboratory strains

3.1.1.1 Direct test

High concentration (10^4 ppm) of Ambush significantly ($P<0.01$) inhibited the growth of *R. phaseoli* (Figs.4 to 6) and *A. chroococcum* (Fig. 7). Moderate concentration (10^0 ppm) of Ambush tended to enhance growth of *R. phaseoli*

(Figs.4 to 6). Ambush did not have any observable effect on the growth of *D. gummosa* (Fig. 8).

Delan at high concentration (10^4 ppm) inhibited growth of *A. chroococcum* (Fig. 7) and *D. gummosa* (Fig. 8). Moderate concentration (10^0 ppm) of Delan enhanced growth of *R. phaseoli*. At concentration of 10^2 ppm and below, the growth of *A. chroococcum* and *D. gummosa* was, above 50%.

High concentration of Dithane M-45 (10^4 ppm) inhibited the growth of *R. phaseoli* (Fig. 4 to 6), *A. chroococcum* (Fig. 7) and *D. gummosa* (Fig. 8) significantly ($P<0.01$). The number of growing cells of *D. gummosa* increased with decreasing concentration of Dithane M-45 (Fig. 8).

R. phaseoli 445, 446 and CC-511 were most susceptible to Delan, Ambush and Dithane M-45 respectively (Figs. 4 to 6). *D. gummosa* and *A. chroococcum* were most susceptible to Ambush and Dithane M-45 respectively (Figs. 7 and Fig. 8)

Fig. 4. Number of colonies (growing cells) per petri dish after treatment of *R. phaseoli* 445 (laboratory strain) with Ambush, Delan and Dithane M-45.

Four Petri dishes (replications) were used for each of the different concentrations of the 3 agrochemicals. The bacteria were grown on complete medium for *Rhizobium*.

1, 2 and 3 represent Ambush, Delan and Dithane M-45 respectively.

The reduction of growth of *R.phaseoli* 445 by the three agrochemicals was significant ($P<0.01$) in the analysis of variance (ANOVA) test.

Fig. 5. Number of colonies (growing cells) per petri dish after treatment of *R. Phaseoli* 446 (laboratory strain) with Ambush, Delan and Dithane M-45.

Four Petri dishes (replications) were used for each of the different concentrations of the 3 agrochemicals. The bacteria were grown on complete medium for *Rhizobium*.

1, 2 and 3 represent Ambush, Delan and Dithane M-45 respectively

The reduction of growth of *R.phaseoli* 446 was significant ($P<0.01$).

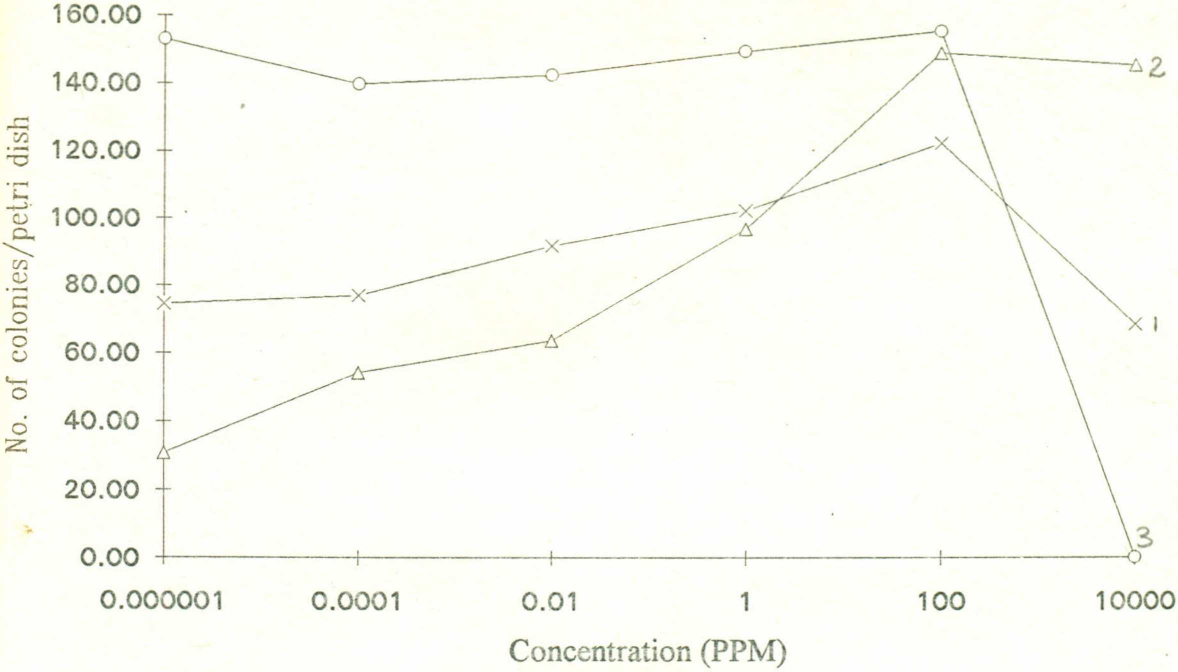

160.00
140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
No. of colonies/petri dish
0.000001
0.0001
0.01
1
100
10000
Concentration (PPM)
1
2
3

Fig. 4

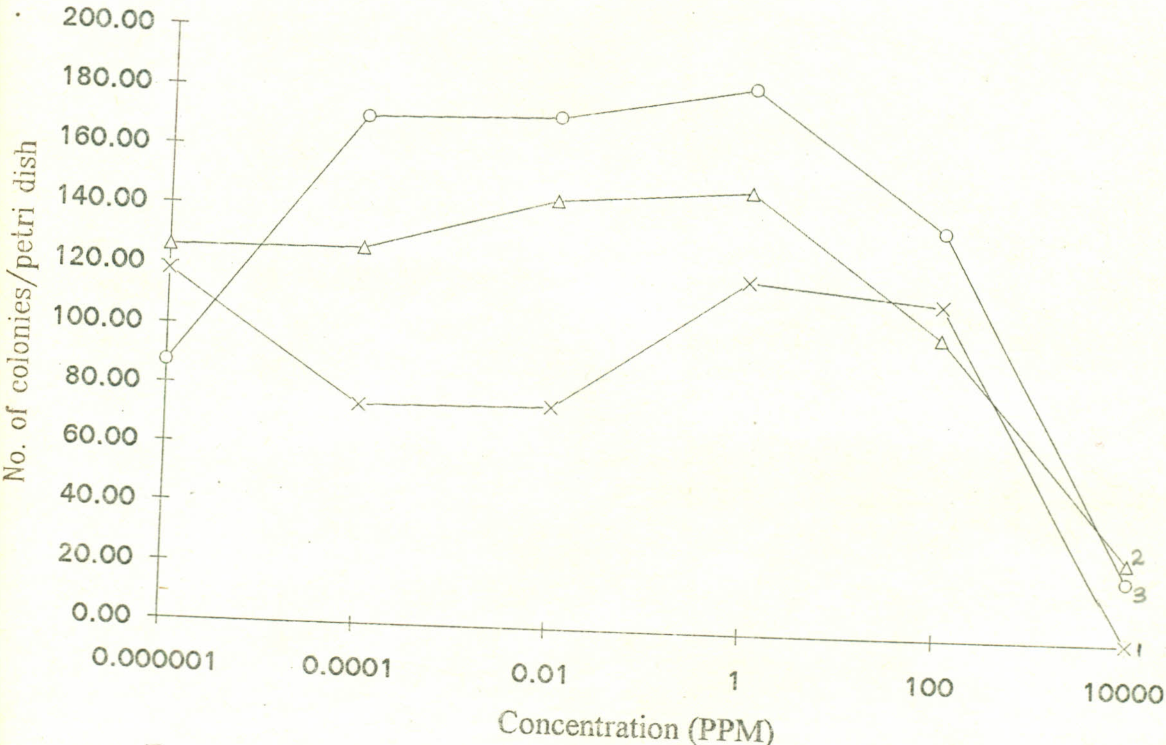

200.00
180.00
160.00
140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
No. of colonies/petri dish
0.000001
0.0001
0.01
1
100
10000
Concentration (PPM)
1
2
3

Fig 5

Fig. 6. Number of colonies (growing cells) per petri dish after treament of *R. phaseoli* CC-511 (laboratory strain) with Ambush, Delan and Dithane M-45. Four Petri dishes (replications) were used for each of the different concentrations of the 3 agrochemicals. The bacteria were grown on complete medium for *Rhizobium*.

1, 2 and 3 represent Ambush, Delan and Dithane M-45 respectively.

The reduction of growth of *R.phaseoli* CC-511 was significant ($P<0.01$).

Fig. 7. Number of colonies (growing cells) per petri dish after treatment of *A chroococcum* (laboratory strain) with Ambush, Delan and Dithane M-45. Four petridishes (replications) were used for each of the different concentrations of the agrochemicals. The bacteria were grown on complete media for *A. chroococcum.*

1, 2 and 3 represent Ambush, Delan and Dithane M-45 respectively.

Population reduction of *A.chroococcum* was significant ($P<0.01$)

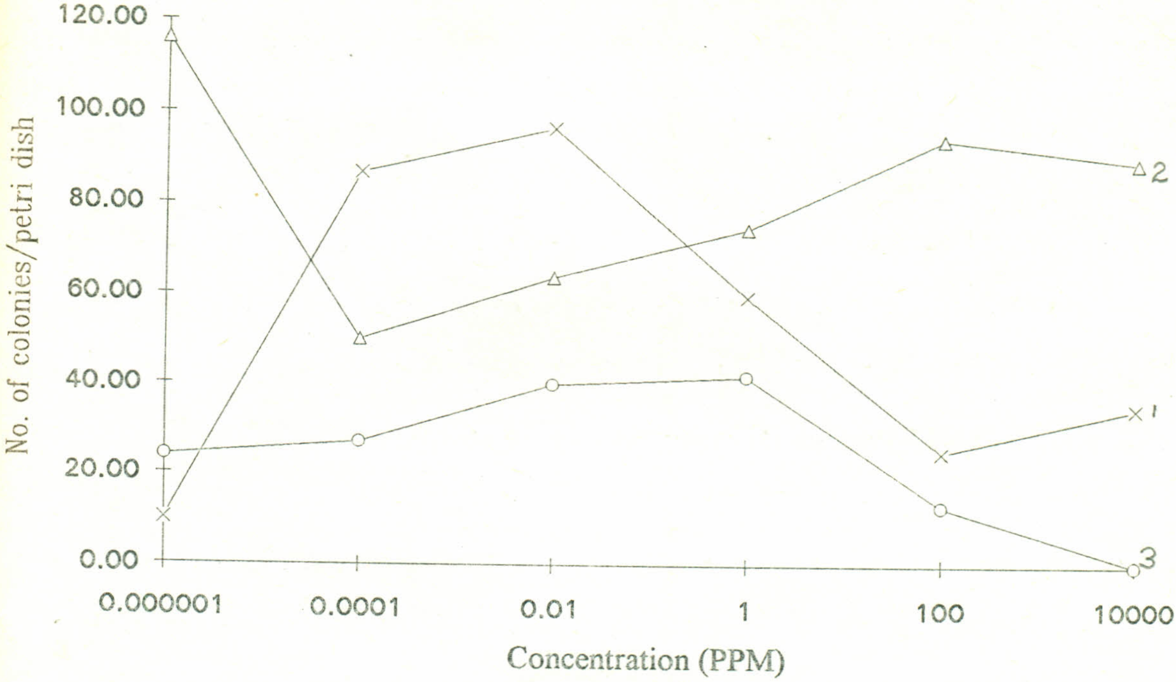

120.00
100.00
80.00
60.00
40.00
20.00
0.00
No. of colonies/petri dish
0.000001
0.0001
0.01
1
100
10000
Concentration (PPM)
1
2
3

Fig 6

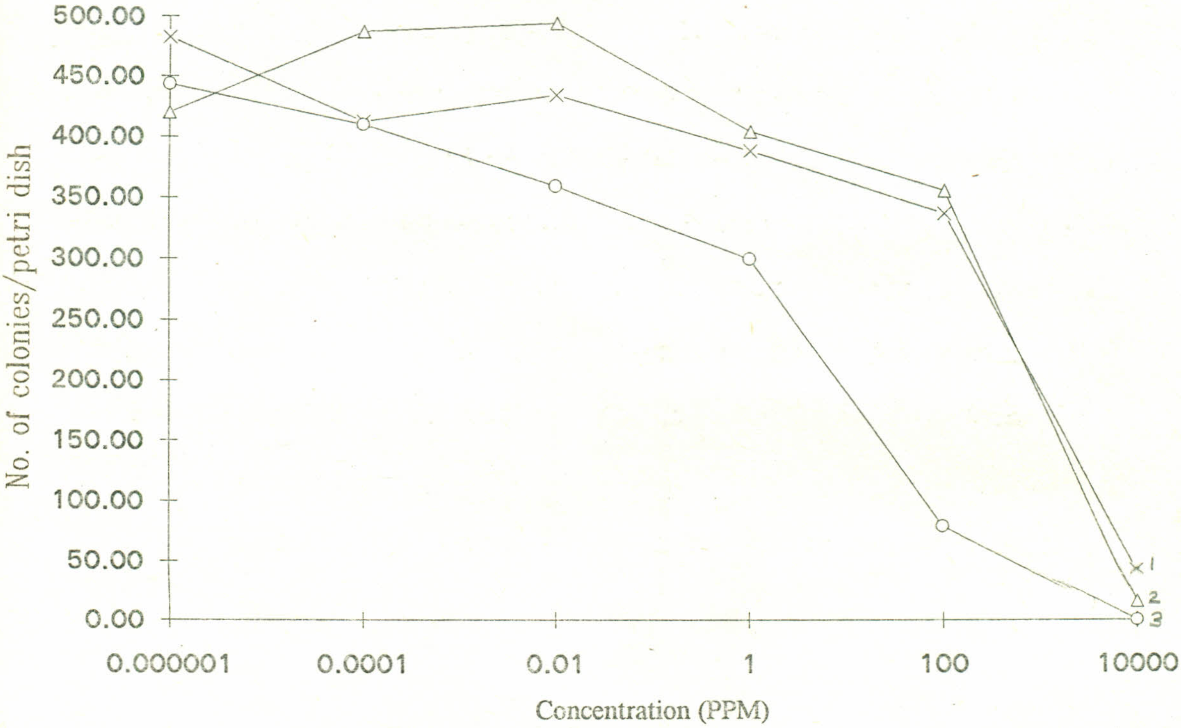

500.00
450.00
400.00
350.00
300.00
250.00
200.00
150.00
100.00
50.00
0.00
No. of colonies/petri dish
0.000001
0.0001
0.01
1
100
10000
Concentration (PPM)
1
2
3

Fig 7

Fig. 8. Number of colonies (growing cells) per petri dish after treatment of *D. gummosa* (laboratory strain) with Ambush, Delan and Dithane M-45.

Four petri dishes (replications) were used for each of the different concentrations of the 3 agrochemicals.

The bacteria were grown on complete media for *Derxia*.

1, 2 and 3 represent Ambush, Delan and Dithane M-45 respectively

The reduction of growing cells of *D.gummosa* by the three agrochemicals was significant ($P<0.01$).

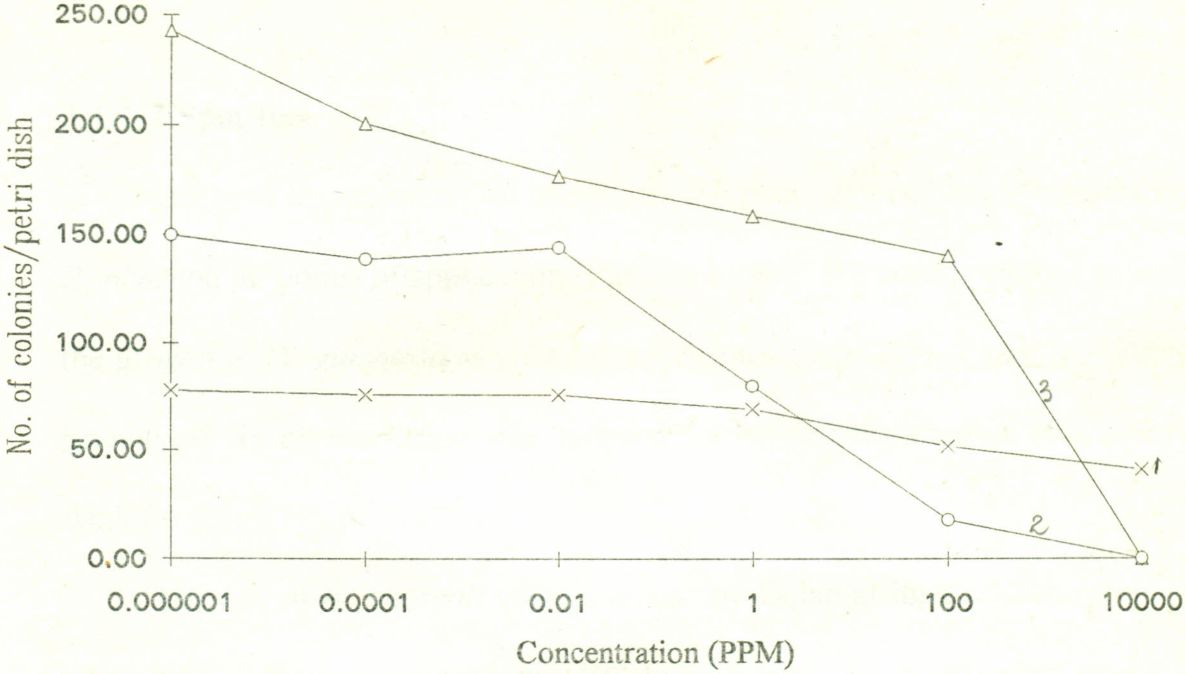

250.00
200.00
150.00
100.00
50.00
0.00
No. of colonies/petri dish
0.000001
0.0001
0.01
1
100
10000
Concentration (PPM)
1
2
3

Fig. 8

3.1.1.2 Spot test

Ambush at concentration of 10^{-6} to 10^{0} ppm induced selective growth of *R. phaseoli* at points of application (Figs. 9a to 9b). All concentrations enhanced the growth of *D. gummosa* at points of application (Fig. 10). Zonal inhibition of growth of *A. chroococcum* was induced by high concentration (10^{4} ppm) of Ambush (Figs.11a to 11b).

Growth of *R. phaseoli* was inhibited by Delan at high concentration (10^{4} ppm) while lower concentrations (10^{-6} to 10^{-2} ppm) favoured selective growth around filter discs (Figs. 12a to 12d). There was zonal inhibition of growth of *A. chroococcum* and *D. gummosa* at high concentration (10^{4} ppm) of Delan.

High concentration (10^{2} to 10^{4} ppm) of Dithane M-45 inhibited growth of *R. phaseoli*. Low concentration (10^{-6} to 10^{0} ppm) induced selective growth around filter discs (Fig.13). Dithane M-45 at high concentration (10^{4} ppm)induced zonal inhibition of growth of *A. chroococcum* (Fig. 14) and *D. gummosa*.

Results of the spot test with laboratory strains are shown in tables IXa, IXb, IXc, X and XI.

Figs. 9 to 11

Selective growth of *R. Phaseoli* 445, 446, CC-511 around filter disks soaked in Ambush, enhanced growth of *D.gummosa* and inhibition of growth of *A. chroococcum.*

Fig. 9a. Selective growth of *R.phaseoli* 445 (laboratory strain) around filter disks soaked in Ambush (10^{-6} to 10^{0} ppm).

Fig. 9b. Selective growth of *R. phaseoli* CC-511 (laboratory strain) around discs soaked in Ambush (10^{-6} to 10^{0} ppm).

Fig. 9c. Selective growth of *R.phaseoli* 446 (laboratory strain) around filter discs soaked in Ambush (10^{-6} to 10^{-0} ppm)

Fig. 10 . Enhanced growth of *D. gummosa* (laboratry strain) around filter discs soaked in Ambush (10^{-6} to 10^{4} ppm).

Fig. 11a. Zonal inhibition of *A.chroococcum* (laboratory strain) by Ambush (10^{0} to 10^{4} ppm).

Fig. 11b. Zonal inhibition of *A. Chroococcum* (laboratorystrain) by Ambush (10^{0} to 10^{4} ppm).

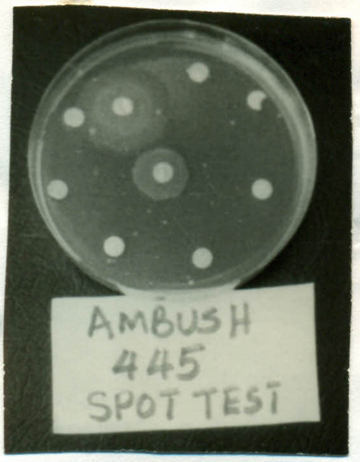


Fig 9a

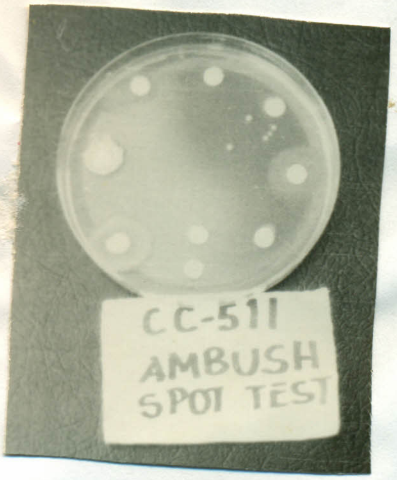


Fig 9b

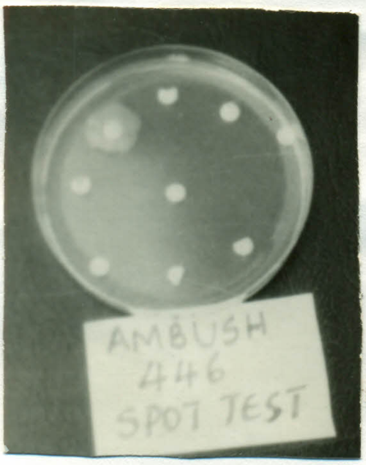


Fig 9c

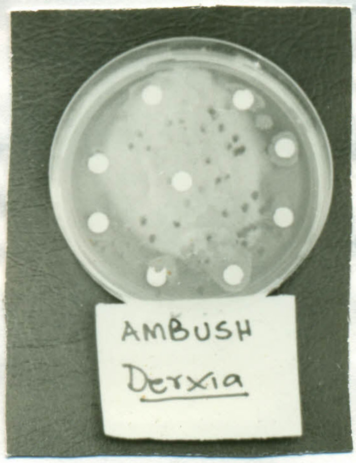


Fig 10

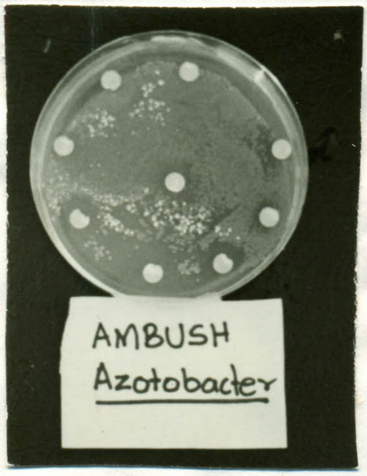


Fig 11a

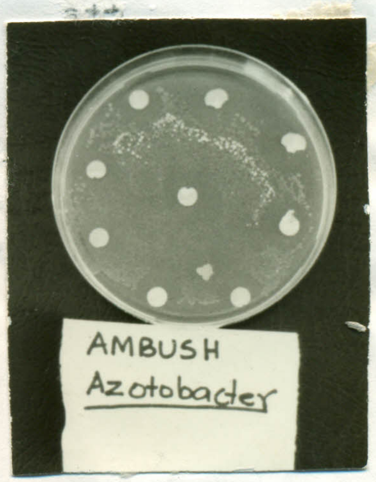


Fig 11b

Fig. 12. Zonal growth of *R. phaseoli* (laboratory strains) and inhibition of *R. phaseoli* with varying concentrations of Delan.

Fig. 12a. Selective growth of *R. phaseoli* CC-511 around filter discs soaked in Delan (10^{-6} to 10^{-4} PPM)

Fig. 12b. Selective growth of *R. phaseoli* 446 by Delan (10^{-6} to 10^{-2} ppm) around filter discs.

Fig. 12c. Total inhibition of *R.phaseoli* 445 by high concentration (10^{4} ppm) of Delan.

Fig 12a

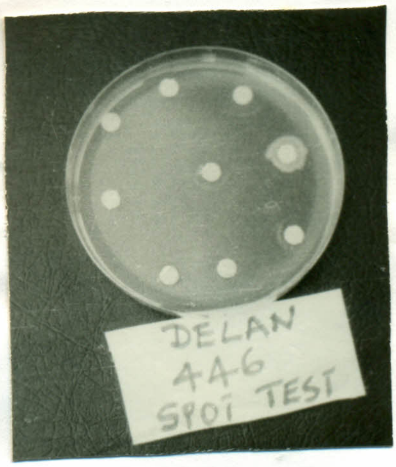


Fig 12b

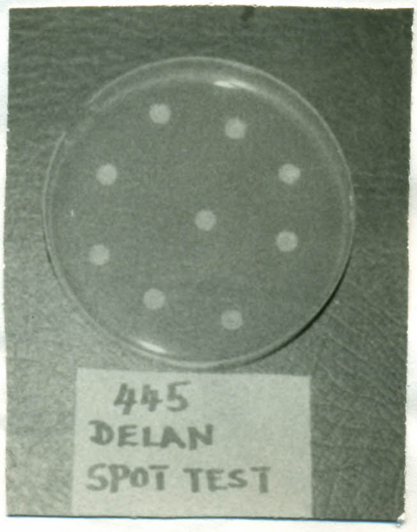


Fig 12c

Fig. 13 and 14

Selective growth of *R. phaseoli* CC-511 (laboratory strain) around filter discs soaked in Dithane M-45 and zonal inhibition of growth of *A.chroococcum* (laboratory strain)

Fig. 13. Selective growth of *R. phaseoli* CC-511 around filter discs saoked in Dithane M-45 (10^{-6} to 10^{0} ppm).

Fig 14a. Zonal inhibition of *A. chroococcum* by high concentrations of Dithane M-45 ($10^{-2}$to 10^{4}ppm).

Fig. 14b. Zonal inhibtion of *A. chroococcum* by high concentrations of Dithane M-45 (10^{-2} to 10^{4} ppm).

Fig 13

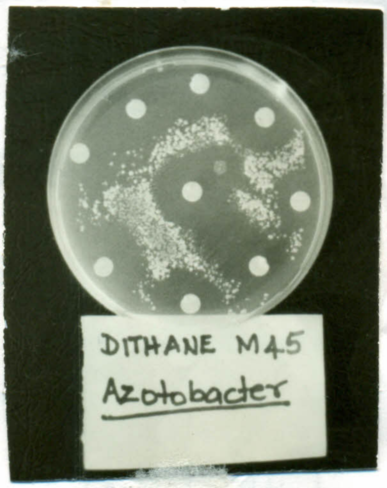


Fig 14a

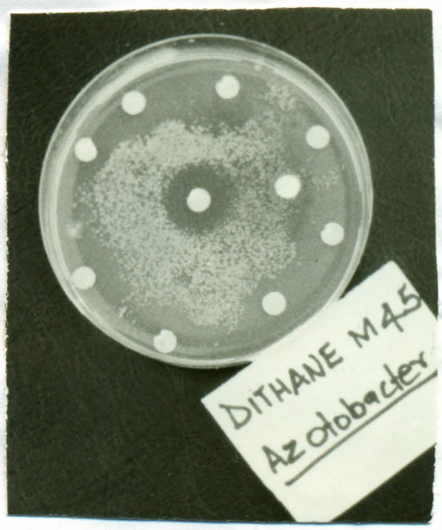


Fig 14b

TABLE IXa. Effect of Ambush, Delan and Dithane M-45 on *R.phaseoli* 445 (lab. strain)

G = Growth I = Inhibition
1 = Number of discs out of 9 with selective growth/zones of inhibition
C = Continous growth
- = Zones of inhibition of growth
-- = Total inhibition of growth
+ = Zones of selective (induction of growth)
++ = Growth on all background with no distinct borders

Agroc. (PPM)	Expt I		II		III		IV.	
	G or I	$X^1/9$	G or I	$X^1/9$	G or I	$X^1/9$	G or I	$X^1/9$
Ambush								
10^{-6}	++	C	-	2	+	2	++	C
10^{-4}	-	1	+	2	++	C	+	2
10^{-2}	+	1	-	1	+	2	+	1
10^{0}	+	3	++	C	++	C	++	C
10^{2}	++	C	+	2	-	3	-	3
10^{4}	++	C	++	C	+	1	+	1
Delan								
10^{-6}	+	1	-	1	+	3	+	1
10^{-4}	-	7	-	4	+	2	-	4
10^{-2}	-	6	+	1	-	2	+	1
10^{0}	+	1	+	1	+	2	+	2
10^{2}	+	5	+	4	--	0	+	1
10^{4}	--	0	--	0	--	0	--	0
Dithane M-45								
10^{-6}	-	7	-	4	-	4	-	7
10^{-4}	-	7	-	9	-	2	-	3
10^{-2}	-	8	--	0	-	1	-	5
10^{0}	-	3	-	5	-	6	-	6
10^{2}	-	2	-	4	-	3	-	2
10^{4}	-	6	-	6	-	1	-	4

TABLE IXb. Effect of Ambush, Delan and Dithane M-45 on *R.phaseoli* CC-511 (lab. strain)

G = Growth I = Inhibition
1 = Number of discs out of 9 with selective growth/zones of inhibition
C = Continous growth
- =Zones of inhibition of growth
-- = Total inhibition of growth
+ = Zones of selective growth (induction of growth)
++ = Growth on all background with no distinct borders

Agroc. (PPM)	Expt I		II		III		IV.	
	G orI	$X^1/9$	G or I	$X^1/9$	G or I	$X^1/9$	G or I	$X^1/9$
Ambush								
10^{-6}	+	5	+	4	+	4	+	2
10^{-4}	+	2	+	4	+	3	+	4
10^{-2}	+	5	+	5	+	5	+	1
10^{0}	+	1	+	7	+	5	+	4
10^{2}	--	0	--	0	--	0	--	0
10^{4}	--	0	--	0	--	0	--	0
Delan								
10^{-6}	+	1	+	1	+	2	+	3
10^{-4}	+	2	+	5	+	1	+	2
10^{-2}	--	0	--	0	+	1	+	2
10^{0}	+	2	--	0	--	0	--	0
10^{2}	--	0	--	0	--	0	--	0
10^{4}	--	0	--	0	--	0	--	0
Dithane M-45								
10^{-6}	+	1	+	1	+	1	++	C
10^{-4}	+	2	++	C	+	1	+	1
10^{-2}	+	1	++	C	++	C	++	C
10^{0}	+	2	+	1	++	C	++	C
10^{2}	++	C	++	C	++	C	++	C
10^{4}	++	C	++	C	++	C	++	C

TABLE IXc. Effect of Ambush, Delan and Dithane M-45 on *R.phaseoli* 446 (lab. strain)

G = Growth I = Inhibition
1 = Number of discs out of 9 with selective growth/zones of inhibition
C = Continous growth
- = Zones of inhibition of growth
-- = Total inhibition of growth
+ = Zones of selective (induction of growth)
++ = Growth on all background with no distinct borders

Agroc. (PPM)	Expt I		II		III		IV.	
	G or I	$X^1/9$	G or I	$X^1/9$	G or I	$X^1/9$	G or I	$X^1/9$
Ambush								
10^{-6}	--	0	--	0	++	0	++	0
10^{-4}	-	1	-	3	-	3	-	4
10^{-2}	+	2	-	2	-	2	-	1
10^{0}	+	2	+	1	--	0	--	0
10^{2}	--	0	--	0	--	0	--	0
10^{4}	--	0	--	0	--	0	--	0
Delan								
10^{-6}	-	2	-	6	+	1	+	2
10^{-4}	+	1	+	1	-	1	-	1
10^{-2}	+	1	--	0	--	0	--	0
10^{0}	--	0	--	0	--	0	--	0
10^{2}	--	0	--	0	--	0	--	0
10^{4}	--	0	--	0	--	0	--	0
Dithane M-45								
10^{-6}	--	0	-	5	-	3	-	4
10^{-4}	-	1	-	4	+	1	-	4
10^{-2}	++	C	++	C	++	C	-	3
10^{0}	--	0	--	0	--	C	--	0
10^{2}	--	0	--	0	--	0	--	0
10^{4}	--	0	--	0	--	0	--	0

TABLE X. Effect of Ambush, Delan and Dithane M-45 on *A. chroococcum* (lab. strain)

G = Growth I = Inhibition
1 = Number of discs out of 9 with selective growth/zones of inhibition
C = Continous growth
\- = Zones of inhibition of growth
\-- = Total inhibition of growth
\+ = Zones of selective (induction of growth)
++ = Growth on all background with no distinct borders

Agroc. (PPM)	Expt I		II		III		IV.	
	G or I	$X^1/9$	G or I	$X^1/9$	G or I	$X^1/9$	G or I	$X^1/9$
Ambush								
10^{-6}	++	C	++	C	++	C	++	C
10^{-4}	++	C	++	C	++	C	++	C
10^{-2}	+	9	+	C	++	C	++	C
10^{0}	-	9	-	9	-	9	-	9
10^{2}	++	C	++	C	-	C	-	9
10^{4}	-	9	-	9	--	0	-	9
Delan								
10^{-6}	++	C	-	9	++	0	++	C
10^{-4}	-	9	-	9	-	9	-	9
10^{-2}	-	9	-	9	-	9	-	9
10^{0}	++	C	++	C	-	9	-	9
10^{2}	++	C	++	C	-	9	-	9
10^{4}	-	9	-	9	-	9	-	9
Dithane M-45								
10^{-6}	++	C	++	C	++	C	++	C
10^{-4}	++	C	++	C	++	C	++	C
10^{-2}	++	C	-	9	-	9	++	C
10^{0}	-	9	-	9	-	9	-	9
10^{2}	-	9	-	9	-	9	-	9
10^{4}	--	0	--	0	--	0	--	0

TABLE XI. Effect of Ambush, Delan and Dithane M-45 on *D. gummosa* (lab. strai)

G = Growth I = Inhibition
1 = Number of discs out of 9 with selective growth/zones of inhibition
C = Continous growth
- = Zones of inhibition growth
-- = Total inhibition of growth
+ = Zones of selective growth (induction of growth)
++ = Growth on all background with no distinct borders

Agroc. (PPM)	Expt I		II		III		IV.	
	G or I	$X^1/9$	G or I	$X^1/9$	G or I	$X^1/9$	G or I	$X^1/9$
Ambush								
10^{-6}	+	7	+	9	+	9	+	8
10^{-4}	+	1	+	3	+	5	+	6
10^{-2}	+	6	+	5	+	3	+	8
10^{0}	+	3	+	4	+	4	+	5
10^{2}	+	8	+	5	+	1	+	3
10^{4}	-	9	+	5	+	5	-	9
Delan								
10^{-6}	++	C	++	C	++	C	++	C
10^{-4}	++	C	++	C	++	C	++	C
10^{-2}	++	C	++	C	++	C	++	C
10^{0}	-	9	++	C	-	9	-	9
10^{2}	-	9	-	9	-	9	-	9
10^{4}	-	9	-	9	-	9	-	9
Dithane M-45								
10^{-6}	++	C	++	C	++	C	++	C
10^{-4}	++	C	+	1	++	C	++	C
10^{-2}	++	C	++	C	++	C	++	C
10^{0}	-	9	-	9	++	C	-	9
10^{2}	++	C	-	9	++	C	-	9
10^{4}	--	0	--	0	-	0	-	9

3.1.2 Field strains

3.1.2.1 Direct test

High concentrations (10^4 ppm) of Delan and Ambush reduced the growth of field strains of *R. phaseoli* to below 50% (Fig. 15). The growth of *R. phaseoli* was reduced by Dithane M-45 at high concentration (10^4 ppm) to below 60% (Fig. 17).

The growth of *A. chroococcum* was reduced by high concentration (10^4 ppm) of Delan and Ambush to below 40% (Fig.18) and 10% (Fig. 19) respectively. High concentration (10^4 ppm) of Dithane M-45 caused total inhibition of growth of *A. chroococcum* (Fig. 20)

Over 50% of (Figs. 21 to 22) *D. gummosa* grew at concentration range (10^{-6} to 10^4ppm) of Delan and Ambush. High concentration (10^4 ppm) of Dithane M-45 inhibited the growth of *D. gummosa* to below 10% (Fig. 23).

3.1.2.2 Spot test

As was the case with laboratory strains, Ambush at high concentration (10^4 ppm) induced zonal killing of field strains of *R. phaseoli*. A strain from Kenyatta University (Ku) and another from Chepalungu (C) showed consistent selective growth ground filter discs soaked in Ambush.

Fig. 15. Number of colonies (growing cells) after treatment of *R. Phaseoli* from different areas of Kenya with different concentrations of Delan

Area	**Code**
Belgut	B
Kericho	Ke
Chepalangu	C
Kano Plains	Ka
Kisumu town	Ki
Nyahera	N
Kenyatta University	Ku

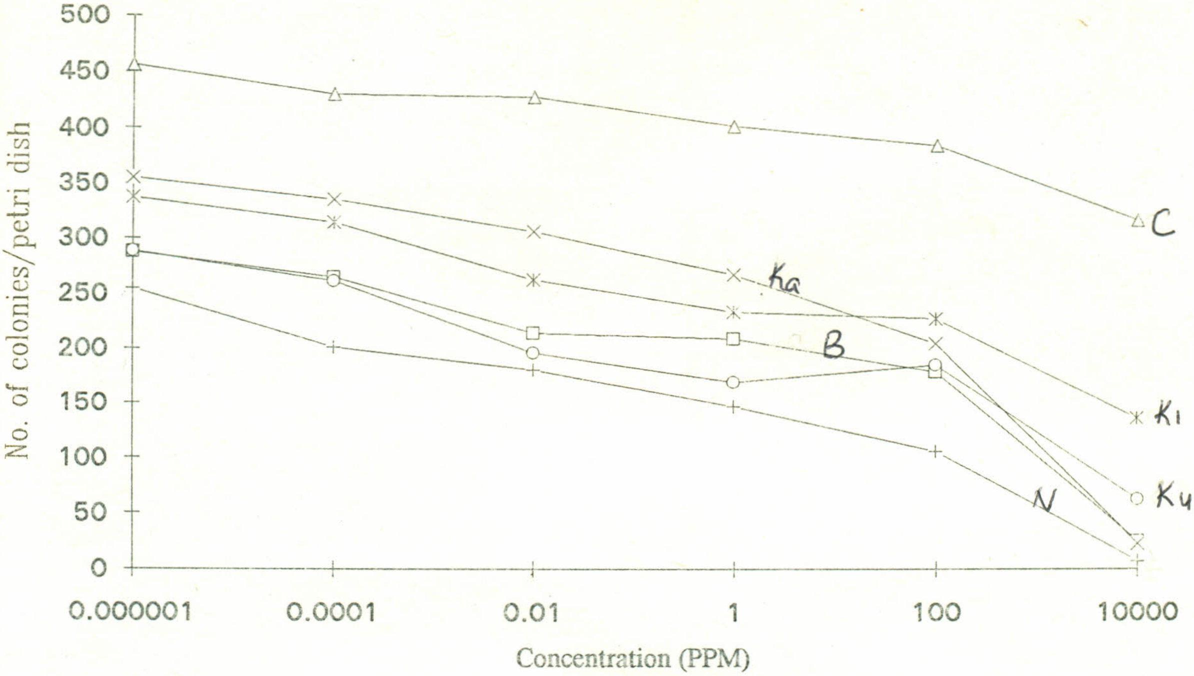
No *R. phaseoli* was isolated form Ke. Three replicates were used for each concentration. *R. phaseoli* was grown complete medium for *Rhizobium.*

Reduction of growth of *R.phaseoli* from the field was significant ($P<0.01$)

Fig. 16. Number of colonies (growing cells) after treatment of *R. phaseoli* from different areas of Kenya with different concentrations of Ambush.

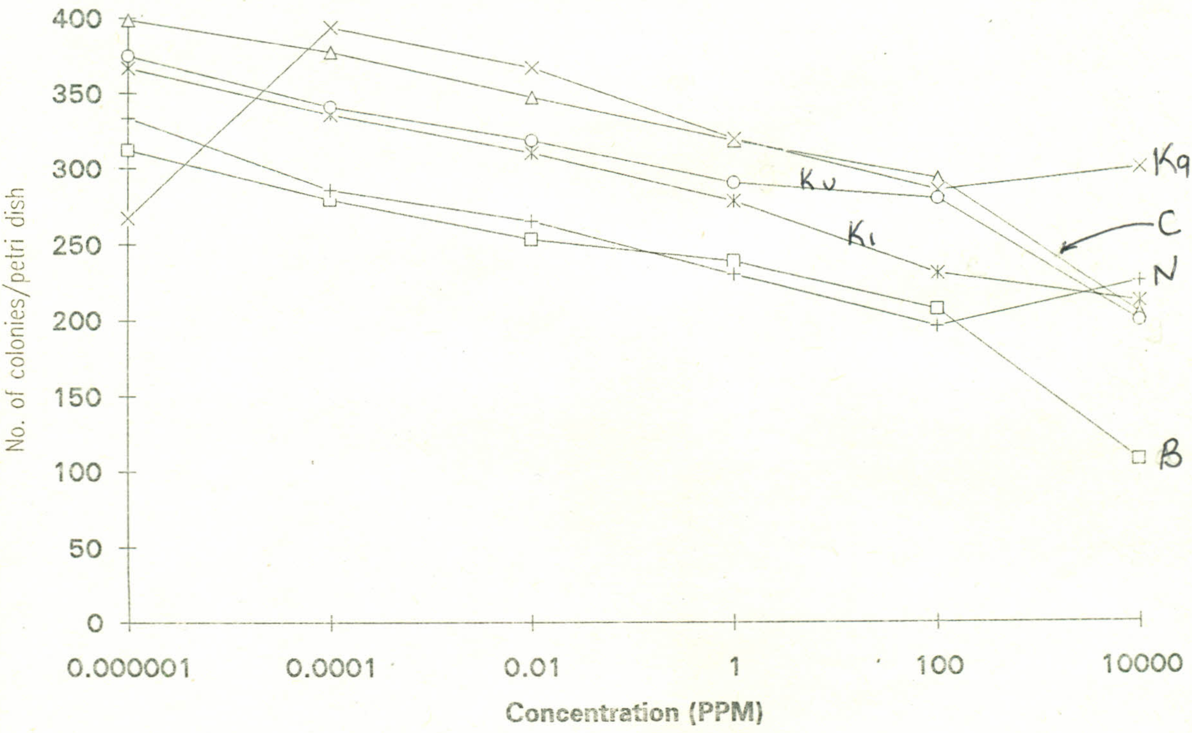
Three replicates were used for each concentration. *R. phaseoli* was grown complete medium for *Rhizobium.*

Reduction of growth was significant ($P<0.01$)



500
450
400
350
300
250
200
150
100
50
0
No. of colonies/petri dish
0.000001
0.0001
0.01
1
100
10000
Concentration (PPM)
C
Ka
B
K1
K4
N

Fig 15



400
350
300
250
200
150
100
50
0
No. of colonies/petri dish
0.000001
0.0001
0.01
1
100
10000
Concentration (PPM)
K9
Ku
K1
C
N
B

Fig 16

Fig. 17. Number of colonies (growing cells) after treatment of *R. phaseoli* from different areas of Kenya with different concentrations of Dithane M-45. Three replicates were used for each concentration.

R. phaseoli was grown complete medium for *Rhizobium.*

Population reduction by Dithane M-45 was significant ($P<0.01$)

Fig. 18. Number of colonies (growing cells) after treatment of *A. chroococcum* from different areas of Kenya with different concentrations of Delan. Three replicates were used for each concentration.

A. chroococcum was grown on its complete medium.

The reduction of growth of *A. chroococcum* from the field by Delan was by significant ($P<0.05$).

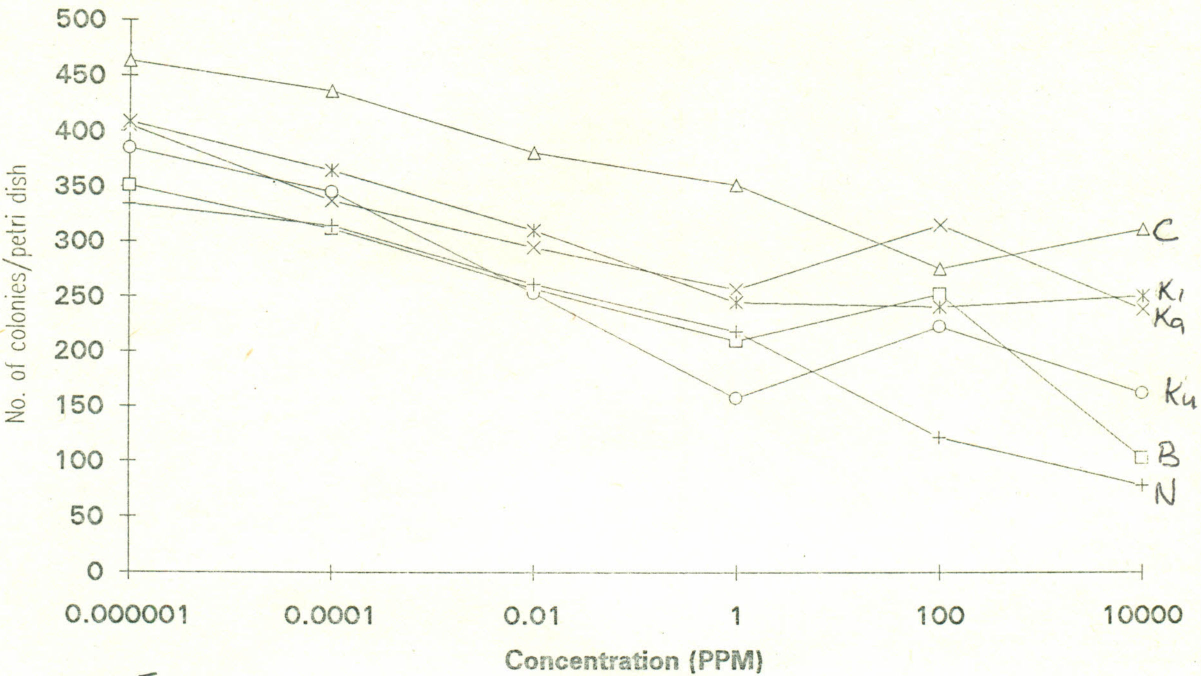

500
450
400
350
300
250
200
150
100
50
0
No. of colonies/petri dish
0.000001
0.0001
0.01
1
100
10000
Concentration (PPM)
C
K1
Ka
Ku
B
N

Fig 17

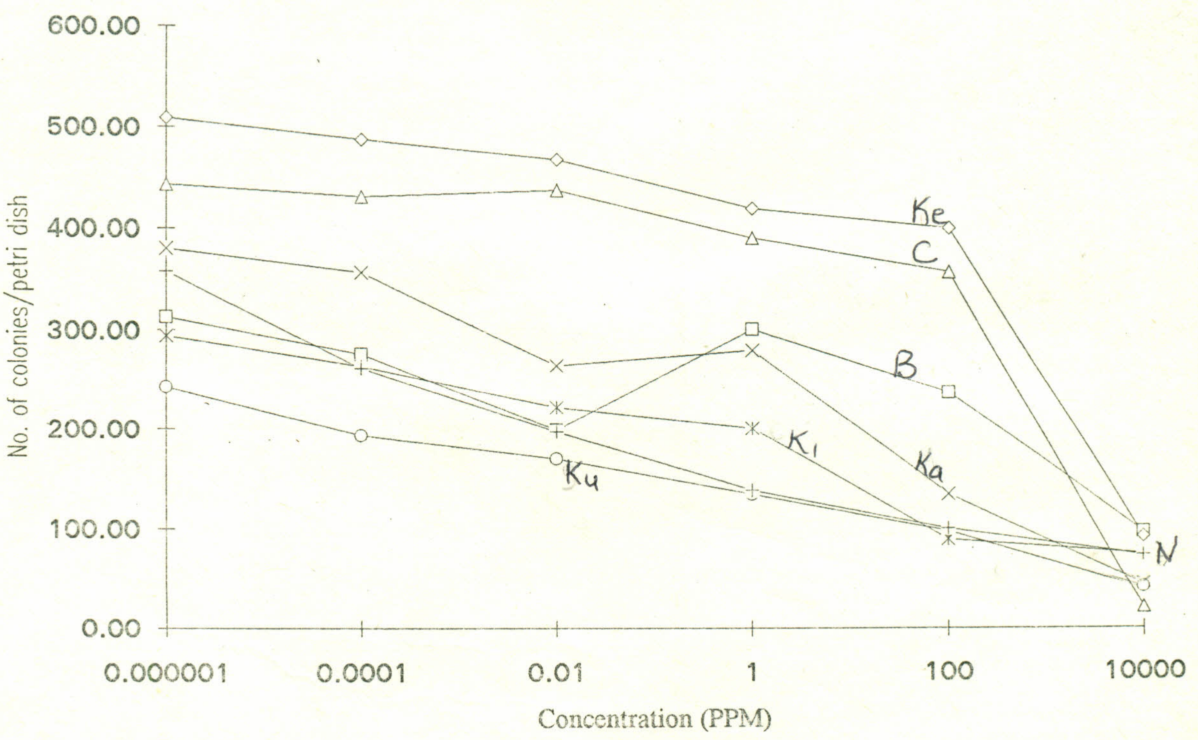

600.00
500.00
400.00
300.00
200.00
100.00
0.00
No. of colonies/petri dish
0.000001
0.0001
0.01
1
100
10000
Concentration (PPM)
Ke
C
B
K1
Ka
Ku
N

Fig 18

Fig. 19. Number of colonies (growing cells) after treatment of *A. chroococcum* from different areas of Kenya with different concentrations of Ambush.

Three replicates were used for each concentration.

A. chroococcum was grown on its complete medium.

The reduction of growth of *A.chroococcum* by Ambush was significant ($P<0.05$)

Fig. 20. Number of colonies (growing cells) after treatment of *A. chroococcum* from different areas of Kenya with different concentrations of Dithane M-45.

Three replicates were used for each concentration.

A. chroococcum was grown on its complete medium.

Population reduction by Dithane M-45 was significant ($P<0.05$)

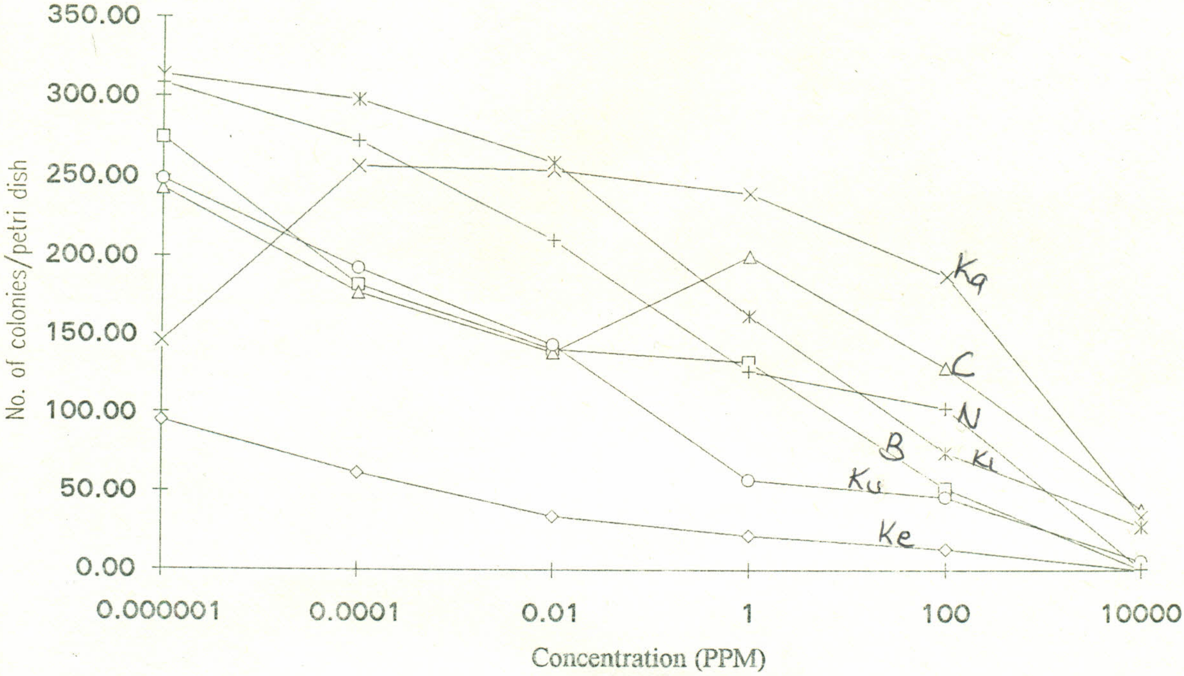

350.00
300.00
250.00
200.00
150.00
100.00
50.00
0.00
No. of colonies/petri dish
0.000001
0.0001
0.01
1
100
10000
Concentration (PPM)
Ka
C
N
B
Ki
Ku
Ke

Fig 19

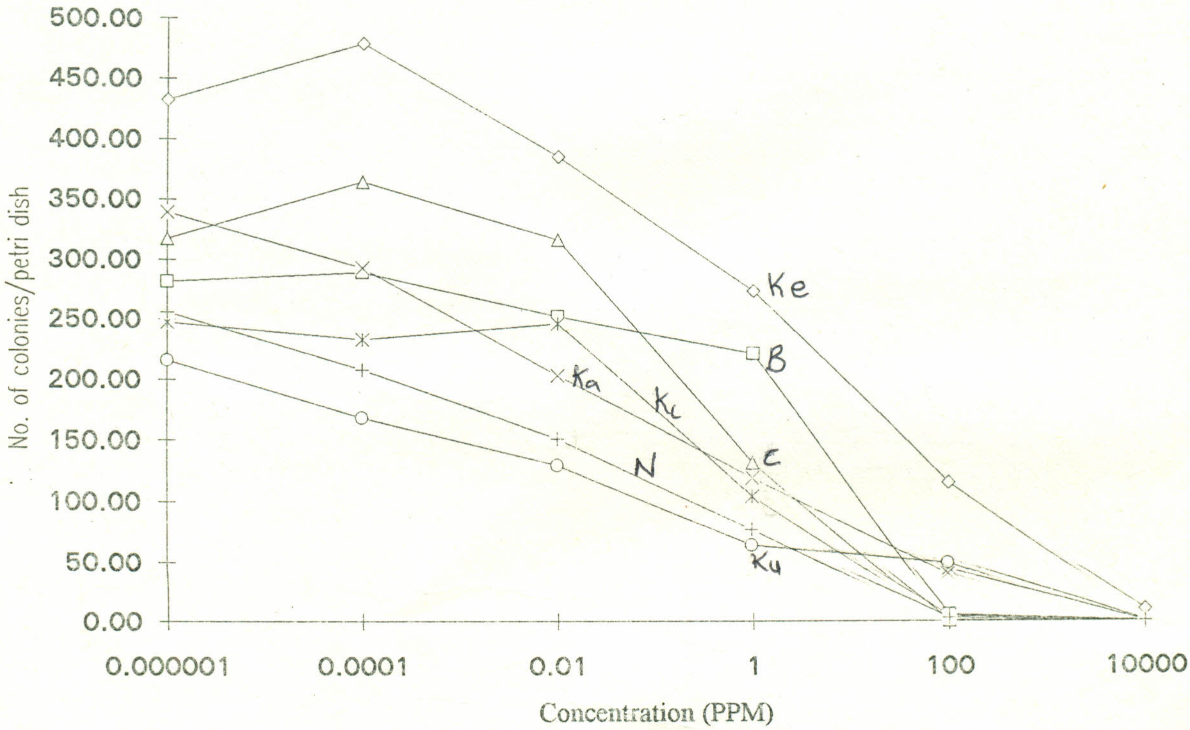

500.00
450.00
400.00
350.00
300.00
250.00
200.00
150.00
100.00
50.00
0.00
No. of colonies/petri dish
0.000001
0.0001
0.01
1
100
10000
Concentration (PPM)
Ke
B
Ka
Ki
C
N
Ku

Fig 20

Fig. 21. Number of colonies (growing cells) after treatment of *D. gummosa* from different areas of Kenya with different concentrations of Delan. Three replicates were used for each concentration.

D. gummosa was grown on complete medium for *Derxia*.

The reduction of growth of *D.gummosa* from the field by Delan was significant ($P < 0.05$)

Figure. 22. Number of colonies (growing cells) after treatment of *D. gummosa* from different areas of Kenya with different concentrations of Ambush. Three replicates were used for each concentration.

D. gummosa was grown on complete medium for *Derxia*.

The reduction of growth of *D.gummosa* from the field by Ambush was significant ($P < 0.05$)

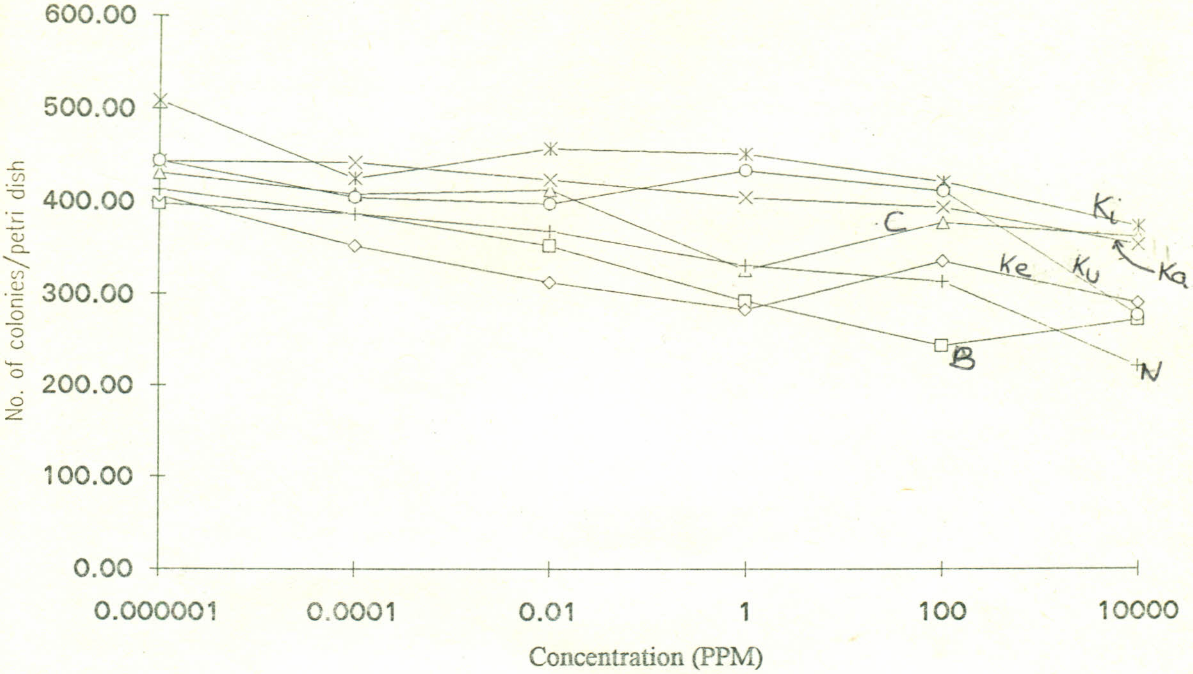

600.00
500.00
400.00
300.00
200.00
100.00
0.00
No. of colonies/petri dish
0.000001
0.0001
0.01
1
100
10000
Concentration (PPM)
Ki
C
Ke
Ku
Ka
B
N

Fig 21

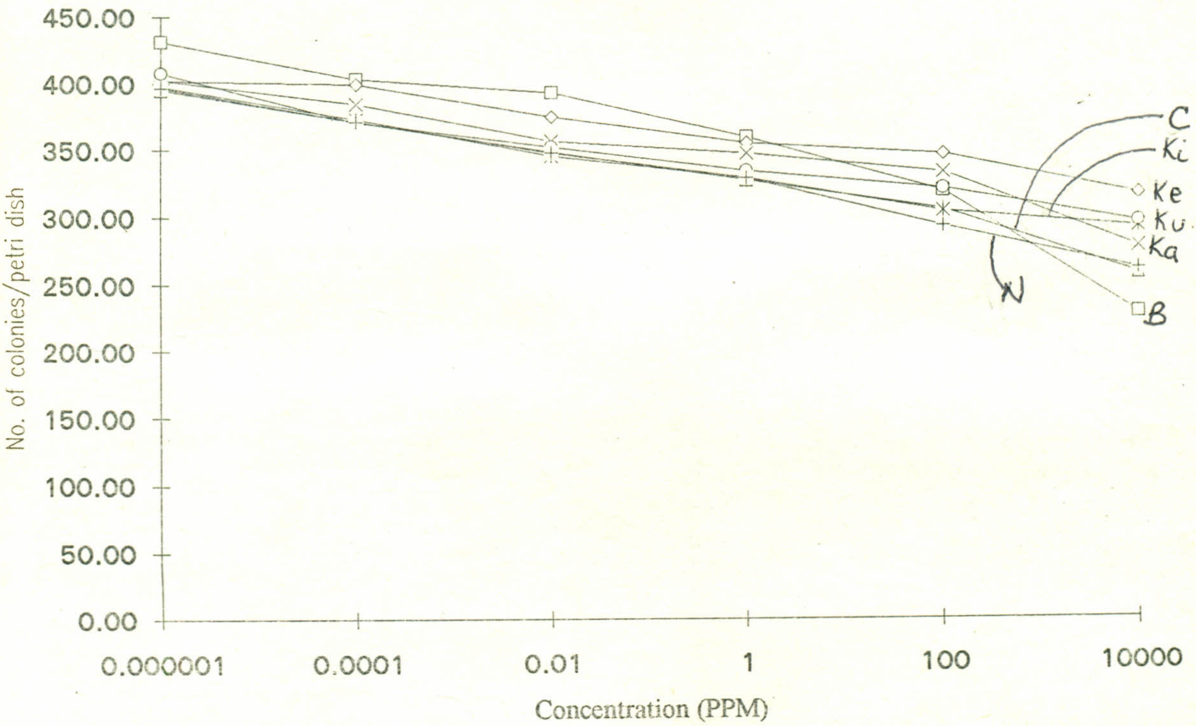

450.00
400.00
350.00
300.00
250.00
200.00
150.00
100.00
50.00
0.00
No. of colonies/petri dish
0.000001
0.0001
0.01
1
100
10000
Concentration (PPM)
C
Ki
Ke
Ku
Ka
N
B

Fig 22

Fig. 23. Number of colonies (growing cells) after treatment of *D. gummosa* from different areas of Kenya with different concentrations of Dithane M-45. Three replicates were used for each concentration.

D. gummosa was grown on complete medium for *Derxia.*

The reduction of growth of *D.gummosa* from the field by Dithane M-45 was significant ($P<0.05$).

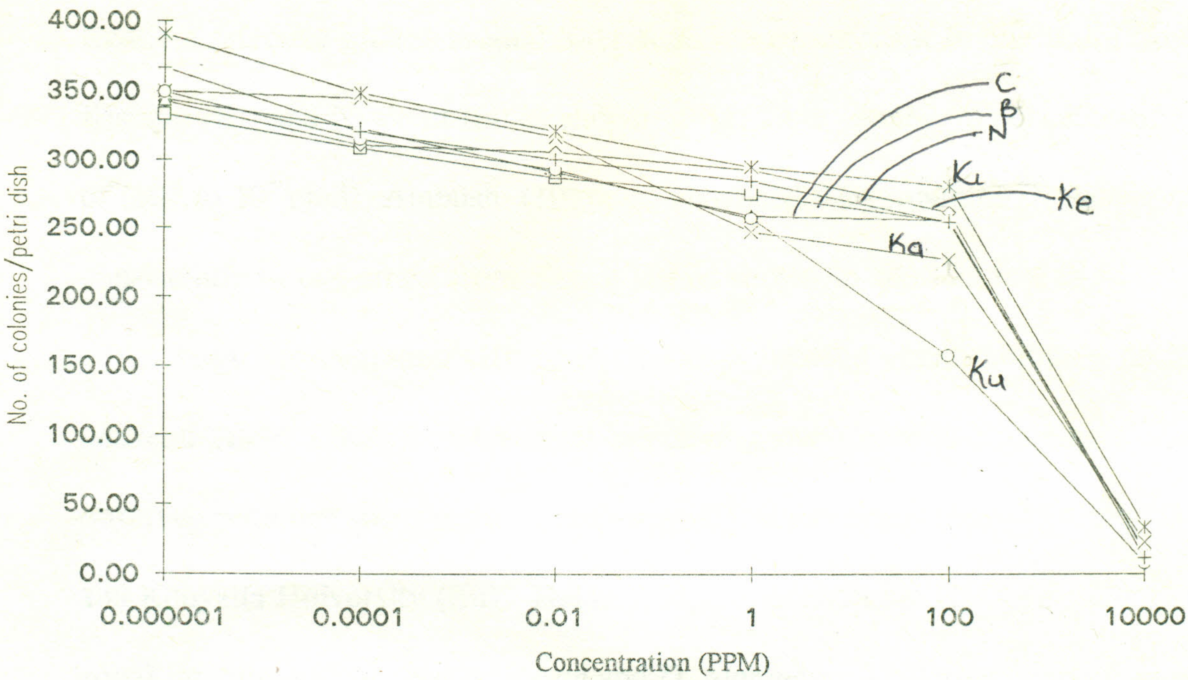

400.00
350.00
300.00
250.00
200.00
150.00
100.00
50.00
0.00
No. of colonies/petri dish
0.000001
0.0001
0.01
1
100
10000
Concentration (PPM)
C
β
N
Ki
Ke
Ka
Ku

Fig 23

Random selective growth around filter discs was observed with one strain from Kisumu (Ki) and Nyahera (N) as shown in fig. 24 at Ambush concentrations of (10^{-6} to 10^{2} ppm). Ambush (10^{0} ppm) enhanced the growth of *D. gummosa* consistently in one strain from Belgut (B) as shown in figs. 25 to 25b.

High concentration (10^{4} ppm) of Delan induced zonal inhibition of *R. phaseoli* (field strains). Consistent selective growth around filter discs was observed with one strain from Chepalungu (C), Kano plains (Ka), Nyahera (N) and Kenyatta University (Ku). Delan at high concentration (10^{4} ppm) induced zonal inhibition of *A. chroococcum* and *D. gummosa*. Moderate concentration (10^{-2} ppm) of Delan induced selective growth of *D. gummosa* around filter discs (Fig. 26a to 26b).

Dithane M-45 (10^{2} to 10^{4} ppm) induced zonal killing of *R. phaseoli* (field strains) and totally inhibited growth of *A. chroococcum*. Zonal killing of *D. gummosa* was induced by Dithane M-45 at concentrations $10^{2}$to 10^{4} ppm (Fig. 27a to 27b). At lower concentration (10^{-2} to 10^{0} ppm), there was random induction of zonal killing (Fig.28).

The details of the results of the spot test with field strains are shown in tables XII, XIII and XIV

3.1.2.3 Soil analysis.

The nitrogen content and pH of soil samples were analysed by the National Agricultural Research Laboratories (Table XV). The results obtained did not show any direct relationship with the nitrogen content or the pH.

Figs. 24 to 26. Selective growth of *A. chroococcum* and *D. gummosa* (field strains) around filter disc soaked in Ambush and Delan.

Fig. 24. Zonal growth of *A. chroococcum* (Ny) around filter discs soaked in Ambush (10^{-6} to 10^{2} ppm).

Fig. 25a. Enhanced growth of *D.gummosa* (Ker) soaked in Ambush (10^{2}) and growth of a few colonies in background lawn.

Fig. 25 b. Enhanced growth of *D. gummosa* (Be) around filter discs soaked in Ambush (10^{0} ppm) and normal growth in background lawn.

Fig. 26a . Selective growth of *D. gummosa* (Ker) around filter discs soaked in Delan (10^{-2} ppm) and a few colonies in background lawn.

Fig. 26b. Induction of selective growth of *D. gummosa* (Ka) around filter discs soaked in Delan (10^{0} to 10^{4} ppm) with normal growth in the background lawn.

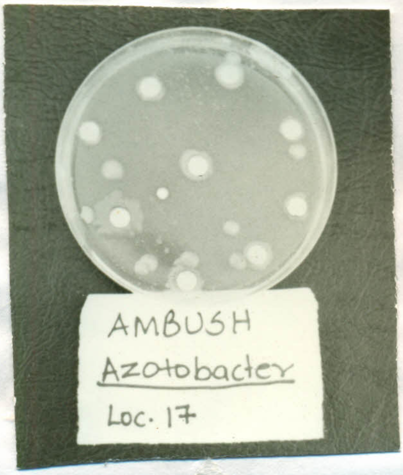


Fig 24

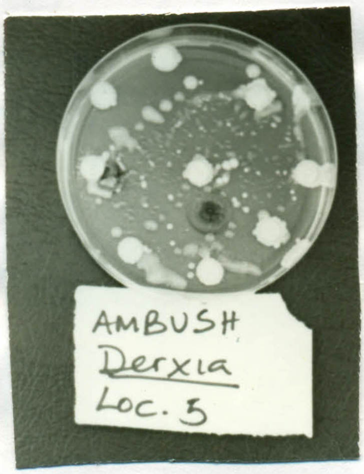


Fig 25a

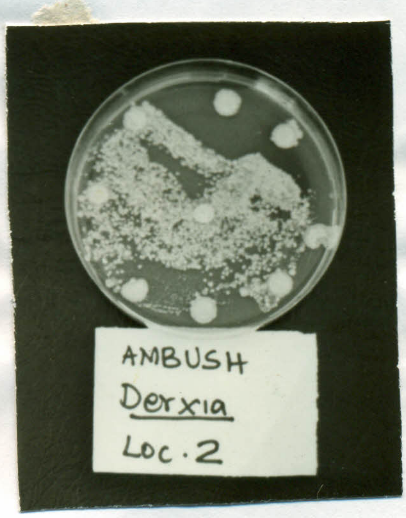


Fig 25b

Fig 26a

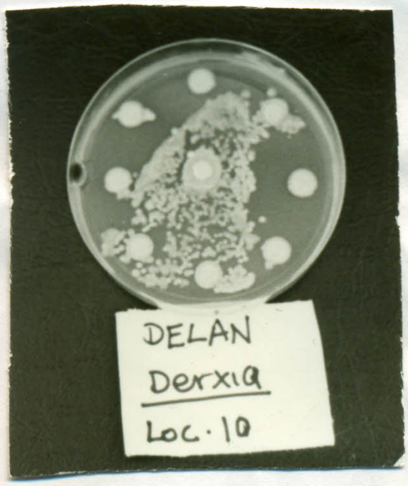


Fig 26b

Figs. 27 and 28. Zonal inhibition of *D. gummosa* (field strains) and zonal induction of growth by high and low concentrations of Dithane M-45 respectively.

Fig 27a. Zonal inhibition of growth of *D.gummosa* (C) by Dithane M-45 (10^4 ppm).

Fig. 27b. Inhibition of growth of *D. gummosa* by Dithane M-45 (10^4 ppm).

Fig. 28. The induction of growth of *D.gummosa* (B) around filter discs soaked in Dithane M-45 (10^{-2} to10^0 ppm)

Fig 27a

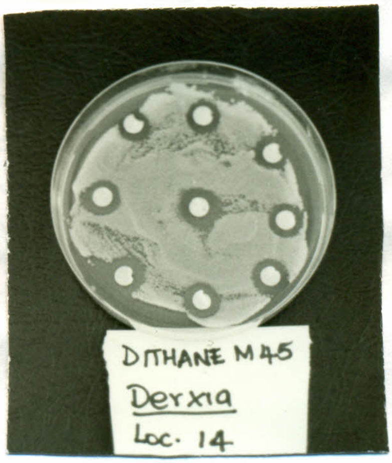


Fig 27b

Fig 28

TABLE XII. Effect of Delan, Ambush and Dithane M-45 on field strains of *R. phaseoli*

G = Growth or I= Inhibition
* = Number ofdiscs out of 9 with selective growth
\- = Zones of inhibition of growth.
-- = Total inhibition of growth
\+ = Zone of induction of growth
++ = Growth on all background with no distinct borders.
N = No *R.phaseoli* was isolated
C = Continous growth on all background.

Agroc		area	B					Ke						C						Ka						Ki								N					Ku			
conc	1		2		3		4		5		6		7		8		9		10		11		12		13		14		15		16		17		18		19		20		21	
	GorI	X*/9	G/I	X/9	G/I	X/9	G/I	X/9	G/I	X/9	G/I	X/9	G/I	X/9	G/I	X/9	G/I	X/9	G/I	X/9	G/I	X/9	G/I	X/9	G/I	X/9	G/I	X/9	G/I	X/9	G/I	X/9	G/I	X/9	G/I	X/9	G/I	X/9	G/I	X/9	G/I	X/9
Delan																																										
10^{-6}	N	N	++	C	N	N	N	N	N	N	N	N	++	C	++	C	+	7	+	3	N	N	++	C	+	5	++	C	++	C	+	2	N	N	++	C	++	C	+	C	+	1
10^{-4}	N	N	++	C	N	N	N	N	N	N	N	N	++	C	++	C	+	7	+	2	N	N	++	C	+	4	++	C	++	C	+	2	N	N	++	C	++	C	++	C	+	2
10^{-2}	N	N	++	C	N	N	N	N	N	N	N	N	++	C	++	C	++	C	+	9	N	N	++	C	++	C	++	C	++	C	-	9	N	N	++	C	++	C	++	C	+	3
10^{0}	N	N	++	C	N	N	N	N	N	N	N	N	++	C	++	C	+	7	+	1	N	N	++	C	++	C	++	C	++	C	-	9	N	N	++	C	++	C	++	C	+	2
10^{2}	N	N	++	C	N	N	N	N	N	N	N	N	++	C	++	C	++	C	--	0	N	N	++	C	++	C	++	C	-	9	-	9	N	N	++	C	++	C	++	C	+	2
10^{4}	N	N	++	C	N	N	N	N	N	N	N	N	++	C	-	9	++	C	--	0	N	N	++	C	++	C	++	C	-	9	-	9	N	N	++	C	-	9	-	9	-	9
Ambush																																										
10^{-6}	N	N	++	C	N	N	N	N	N	N	N	N	++	C	++	C	+	4	+	1	N	N	++	C	++	C	++	C	+	1	++	C	N	N	++	C	++	C	++	C	++	C
10^{-4}	N	N	+	C	N	N	N	N	N	N	N	N	++	C	++	C	+	5	+	2	N	N	+	2	++	C	++	C	++	C	++	C	N	N	++	C	++	C	++	C	++	C
10^{-2}	N	N	++	C	N	N	N	N	N	N	N	N	-	9	-	9	+	9	++	C	N	N	++	C	++	C	++	C	++	C	++	C	N	N	++	C	++	C	++	C	++	C
10^{0}	N	N	-	9	N	N	N	N	N	N	N	N	++	C	-	9	+	9	--	0	N	N	-	9	++	C	++	C	+	2	++	C	N	N	++	C	++	C	++	C	++	C
10^{2}	N	N	-	9	N	N	N	N	N	N	N	N	++	C	-	9	+	9	++	C	N	N	-	9	-	9	-	9	-	9	+	C	N	N	++	C	++	C	++	C	+	2
10^{4}	N	N	-	9	N	N	N	N	N	N	N	N	-	9	-	9	++	C	--	0	N	N	-	9	-	9	++	C	-	9	++	C	N	N	--	0	++	C	++	C	-	2
Dithane M-45																																										
10^{-6}	N	N	++	C	N	N	N	N	N	N	N	N	++	C	++	C	+	9	++	C	N	N	++	C	++	C	++	C	++	C	+	2	N	N	++	C	++	C	++	C	+	2
10^{-4}	N	N	++	C	N	N	N	N	N	N	N	N	++	C	-	9	+	9	++	C	N	N	++	C	+	2	+	C	++	C	+	1	N	N	++	C	++	C	++	C	+	1
10^{-2}	N	N	++	C	N	N	N	N	N	N	N	N	++	C	++	C	+	9	+	2	N	N	++	C	++	C	++	C	++	C	++	C	N	N	+	9	++	C	+	3	+	2
10^{0}	N	N	++	C	N	N	N	N	N	N	N	N	++	C	++	C	+	9	+	1	N	N	-	9	++	C	++	C	++	C	++	C	N	N	++	C	++	C	++	C	+	1
10^{2}	N	N	++	C	N	N	N	N	N	N	N	N	++	C	++	C	+	9	--	0	N	N	-	9	-	9	-	9	++	C	+	3	N	N	+	9	++	C	+	1	+	2
10^{4}	N	N	-	9	N	N	N	N	N	N	N	N	-	9	-	9	-	9	-	9	N	N	-	9	-	9	-	9	-	9	-	9	N	N-	-	9	-	9	-	9	-	9

TABLE XIII. Effect of Delan, Ambush and Dithane M-45 on field strains of *A. chroococcum*

G = Growth or I= Inhibition
* = Number ofdiscs out of 9 with selective growth
\- = Zones of inhibition of growth.
-- = Total inhibition of growth
\+ = Zone of induction of growth
++ = Growth on all background with no distinct borders.
N = No *A. chroococcum* was isolated
C = Continous growth on all background.

Agroc conc	area 1 GorI	1 ^{x}*/9	B 2 G/I	2 X/9	3 G/I	3 X/9	4 G/I	4 X/9	Ke 5 G/I	5 X/9	6 G/I	6 X/9	7 G/I	C 7 X/9	8 G/I	8 X/9	9 G/I	9 X/9	10 G/I	Ka 10 X/9	11 G/I	11 X/9
Delan																						
10^{-6}	++	C	N	N	++	C	++	C	N	N	N	N	++	C	N	N	++	C	N	N	++	C
10^{-4}	++	C	N	N	++	C	++	C	N	N	N	N	++	C	N	N	++	C	N	N	++	C
10^{2}	++	C	N	N	++	C	++	C	N	N	N	N	++	C	N	N	++	C	N	N	++	C
10^{0}	++	C	N	N	++	C	++	C	N	N	N	N	++	C	N	N	++	C	N	N	++	C
10^{2}	++	C	N	N	++	C	++	C	N	N	N	N	-	9	N	N	++	C	N	N	++	C
10^{4}	++	C	N	N	-	9	++	C	N	N	N	N	-	9	N	N	-	9	N	N	-	9
Ambush																						
10^{-6}	++	C	N	N	+	3	++	C	N	N	N	N	+	7	N	N	++	C	N	N	++	C
10^{-4}	++	C	N	N	+	4	++	C	N	N	N	N	+	2	N	N	++	C	N	N	++	C
10^{2}	++	C	N	N	++	C	++	C	N	N	N	N	++	C	N	N	++	C	N	N	++	C
10^{0}	++	C	N	N	+	6	++	C	N	N	N	N	+	9	N	N	++	C	N	N	++	C
10^{2}	++	C	N	N	+	1	-	9	N	N	N	N	+	4	N	N	++	C	N	N	++	C
10^{4}	++	C	N	N	+	1	--	9	N	N	N	N	++	C	N	N	++	C	N	N	++	C
Dithane M-45																						
10^{-6}	++	C	N	N	++	C	++	C	N	N	N	N	++	C	N	N	++	C	N	N	++	C
10^{-4}	++	C	N	N	++	C	++	C	N	N	N	N	++	C	N	N	++	C	N	N	++	C
$10-^{2}$	++	C	N	N	-	9	++	C	N	N	N	N	++	C	N	N	++	C	N	N	++	C
10^{0}	++	C	N	N	++	C	++	C	N	N	N	N	++	C	N	N	++	C	N	N	++	C
10^{2}	--	0	N	N	--	9	++	C	N	N	N	N	--	0	N	N	--	0	N	N	--	0
10^{4}	--	0	N	N	--	9	--	9	N	N	N	N	--	0	N	N	--	0	N	N	--	

Agroc conc	12 G/I	12 X/9	Ki 13 G/I	13 X/9	14 G/I	14 X/9	15 G/I	15 X/9	16 G/I	16 X/9	N 17 G/I	17 X/9	18 G/I	18 X/9	19 G/I	19 X/9	Ku 20 G/I	20 X/9	21 G/I	21 X/9
Delan																				
10^{-6}	++	C	+	5	++	C	++	C	++	C	++	C	++	C	++	C	++	C	+	1
10^{-4}	++	C	+	4	++	C	++	C	++	C	++	C	++	C	++	C	++	C	+	2
10^{2}	++	C	++	C	++	C	++	C	++	C	++	C	++	C	++	C	++	C	+	3
10^{0}	++	C	++	C	++	C	++	C	++	C	++	C	++	C	++	C	++	C	+	2
10^{2}	++	C	--	0	--	9	-	9	++	C	++	C	++	C	-	9	-	9	+	2
10^{4}	-	9	--	0	--	9	-	9	-	9	++	C	-	9	-	9	-	9	-	9
Ambush																				
10^{-6}	++	C	++	C	++	C	++	C	++	C	+	9	++	C	++	C	++	C	++	C
10^{-4}	++	C	++	C	+	9	++	C	++	C	+	9	++	C	++	C	++	C	++	C
10^{2}	++	C	++	C	++	C	++	C	++	C	++	C	++	C	++	C	++	C	++	C
10^{0}	++	C	++	C	++	C	++	C	++	C	+	8	++	C	++	C	++	C	++	C
10^{2}	++	C	++	C	+	9	++	C	++	C	+	5	++	C	-	9	-	9	-	9
10^{4}	++	C	++	C	+	9	++	C	++	C	--	0	--	0	++	C	-	9	--	0
Dithane M-45																				
10^{-6}	++	C	++	C	++	C	++	C	++	C	++	C	++	C	++	C	++	C	++	C
10^{-4}	++	C	++	C	++	C	++	C	++	C	++	C	++	C	++	C	++	C	++	C
$10-^{2}$	++	C	++	C	++	C	++	C	++	C	++	C	++	C	++	C	++	C	++	C
10^{0}	++	C	++	C	++	C	++	C	++	C	++	C	++	C	++	C	++	C	++	C
10^{2}	--	0	-	9	-	9	-	9	-	9	--	0	--	0	--	0	--	0	--	0
10^{4}	--	0	-	9	-	9	-	9	-	9	--	0	--	0	--	0	--	0	--	0

TABLE XIV. Effect of Delan, Ambush and Dithane M-45 on field strains of *D. gummosa*

* = Number of discs out of 9 with selective growth
G = Growth or I= Inhibition
* = Number ofdiscs out of 9 with selective growth
\- = Zones of inhibition of growth.
-- = Total inhibition of growth
\+ = Zone of induction of growth
++ = Growth on all background with no distinct borders.
N = No *D. gummosa* was isolated
C = Continous growth on all background.

Agroc conc	area 1 GorI	1 X*/9	B 2 G/I	2 X/9	3 G/I	3 X/9	Ke 4 G/I	4 X/9	5 G/I	5 X/9	6 G/I	6 X/9	C 7 G/I	7 X/9	8 G/I	8 X/9	9 G/I	9 X/9	Ka 10 G/I	10 X/9	11 G/I	11 X/9
Delan																						
10^{-6}	++	C	++	C	++	C	++	C	++	C	++	C	++	C	++	C	++	C	++	C	++	C
10^{-4}	++	C	++	C	++	C	++	C	++	C	++	C	++	C	++	C	++	C	++	C	++	C
$10\ ^{2}$	++	C	++	C	++	C	++	C	++	C	++	C	++	C	++	C	++	C	++	C	+	9
$10\ ^{0}$	++	C	+	3	++	C	++	C	--	0	++	C	++	C	++	C	++	C	+	9	+	9
10^{2}	++	C	++	C	++	C	++	C	--	0	++	C	-	9	++	C	++	C	+	9	++	C
10^{4}	-	9	+	4	-	9	-	9	--	0	++	C	-	9	++	C	++	C	+	9	++	C
Ambush																						
10^{-6}	++	C	++	C	++	C	++	C	++	C	++	C	++	C	++	C	++	C	++	C	++	C
10^{-4}	++	C	++	C	+	9	++	C	++	C	++	C	++	C	++	C	++	C	+	8	++	C
$10\ ^{2}$	+	9	++	C	+	9	++	C	++	C	++	C	++	C	++	C	++	C	+	8	++	9
$10\ ^{0}$	+	9	+	9	+	9	+	9	++	C	+	9	++	C	++	C	++	C	++	C	++	C
10^{2}	++	C	++	C	++	C	++	C	+	9	++	C	++	C	++	C	++	C	++	C	+	3
10^{4}	-	9	+	9	++	C	++	C	+	9	++	C	++	C	-	9	-	9	++	C	++	C
Dithane M-45																						
10^{-6}	++	C	++	C	++	C	++	C	++	C	++	C	++	C	++	C	++	C	++	C	++	C
10^{-4}	++	C	++	C	++	C	++	C	++	C	++	C	++	C	++	C	++	C	+	4	++	C
$10-^{2}$	++	C	+	8	++	C	++	C	++	C	++	C	++	C	++	C	++	C	+	4	++	C
$10\ ^{0}$	++	C	+	9	++	C	++	C	++	C	++	C	++	C	++	C	++	C	++	C	++	C
10^{2}	+	2	+	5	-	9	-	9	--	0	+	3	++	C	++	C	-	9	++	C	++	C
10^{4}	-	9	-	9	-	9	-	9	--	0	-	9	-	9	-	9	-	9	+	9	-	9

Agroc conc	12 G/I	12 X/9	Ki 13 G/I	13 X/9	14 G/I	14 X/9	15 G/I	15 X/9	16 G/I	16 X/9	N 17 G/I	17 X/9	18 G/I	18 X/9	19 G/I	19 X/9	Ku 20 G/I	20 X/9	21 G/I	21 X/9
Delan																				
10^{-6}	++	C	++	C	++	C	++	C	++	C	++	C	++	C	++	C	++	C	++	C
10^{-4}	++	C	++	C	++	C	++	C	++	C	++	C	++	C	++	C	++	C	++	C
$10\ ^{2}$	++	C	-	9	++	C	++	C	-	9	++	C	++	C	+	9	++	C	++	C
$10\ ^{0}$	++	C	-	9	++	C	++	C	++	C	++	C	++	C	++	C	++	C	++	C
10^{2}	++	C	++	C	++	C	++	C	+	1	++	C	++	C	++	C	++	C	++	C
10^{4}	-	9	++	C	++	C	-	9	-	9	-	9	++	C	-	9	-	9	-	9
Ambush																				
10^{-6}	++	C	++	C	++	C	++	C	++	C	++	C	++	C	++	C	++	C	++	C
10^{-4}	++	C	++	C	++	9	++	C	++	C	++	C	++	C	++	C	++	C	++	C
$10\ ^{2}$	++	C	++	C	++	C	+	2	-	9	++	C	++	C	++	C	++	C	++	C
$10\ ^{0}$	+	8	+	3	++	C	++	C	++	C	+	4	+	9	++	C	++	C	++	C
10^{2}	+	6	++	C	+	9	+	9	++	C	++	C	++	C	++	C	++	C	++	C
10^{4}	+	3	++	C	++	C	-	9	-	9	++	C	++	C	++	C	++	C	++	C
Dithane M-45																				
10^{-6}	++	C	++	C	++	C	++	C	++	C	++	C	++	C	++	C	++	C	++	C
10^{-4}	++	C	++	C	++	C	++	C	++	C	++	C	++	C	++	C	++	C	++	C
$10-^{2}$	++	C	++	C	++	C	+	2	-	9	++	C	++	C	++	C	++	C	++	C
$10\ ^{0}$	+	8	+	3	++	C	++	C	++	C	+	4	+	9	++	C	++	C	++	C
10^{2}	-	9	++	C	++	C	++	C	-	9	-	9	++	C	++	C	++	C	++	9
10^{4}	-	9	-	9	-	9	-	9	-	9	-	9	-	9	-	9	-	9	-	9

TABLE XV. The nitrogen content and pH of different soil samples

Sample Number	1	2	3	4	5	6	7	8	9	10	11	12	13	14
pH	5.6	6.0	6.2	6.0	5.6	5.6	5.7	6.0	6.4	6.4	6.8	6.9	8.2	6.4
Nitrogen %	0.40	0.49	0.52	0.30	0.36	0.37	0.19	0.27	0.27	0.33	0.15	0.13	0.15	0.19

Sample Number	15	16	17	18	19	20	21
pH	6.8	6.5	7.2	7.3	7.2	7.8	8.4
Nitrogen %	0.22	0.16	0.11	0.21	1.27	1.31	1.38

3.1.3 Attempts to map the genome of *R. phaseoli*

Experiments with the spot test showed that there was selective growth of *R. phaseoli* 445, 446 and CC-511 around filter discs soaked in the agrochemicals Ambush, Delan and Dithane M-45. Experiments with amino acids and antibiotics to determine the nature of genetic activity gave the following results.

3.1.3.1 Amino acid supplemented minimal media

There was normal growth of *R. phaseoli* CC-511 on partially supplemented media and on medium supplemented with all amino acids (Fig. 29a and 29d). *R. phaseoli* 445 and 446 did not grow at all on both completely and partially supplemented media (Fig. 29b to 29f). There was selective growth of *R. phaseoli* 445, 446 and CC-511 on minimal media around filter discs soaked in agrochemicals (Table XVI).

3.1.3.2 Antibiotic resistance test

R. phaseoli 445, 446 and CC-511 were tested against ampicillin, tetracycline, cotrimoxazole, streptomycin, kanamycin, sulphamethaxazole, chloramphenicol, minocycline, trimethoprim, miraxid, spiramycine, amoxyllin (amoxil) and afloxacin. In absence of the agrochemicals Ambush, Delan and Dithane M-45 *R. phaseoli* 445 was sensitive to all antibiotics as shown by zonal killing and resistant to trimethoprim (marked by growth).

Fig. 29. Growth/lack of growth of diffferent lab. strains of *R. phaseoli* plated on media supplemented with amino acids.

Fig. 29a. *R. phaseoli* CC-511 grew on media from which tyrosine (-tyr) was eliminated following treatment with Ambush.

Fig. 29b. *R. phaseoli* 446 treated with Ambush did not grow on minimal medium after elimination of serine (-ser)

Fig. 29c. *R. phaseoli* 445 that was treated with Delan did not grow on minimal meidium after elimination of histidine (-his)

Fig. 29d. Minimal medium without alanine (-ala) allowed *R.phaseoli* CC-511 to grow after treatedment with Delan.

Fig. 29e. *R. phaseoli* 445, treted with Dithane M-45 did not grow on minimal medium without asparagine (-asp).

Fig. 29f. Minimal medium without proline (-pro) did not allow *R. phaseoli* 446 treated with Dithane M-45 to grow.

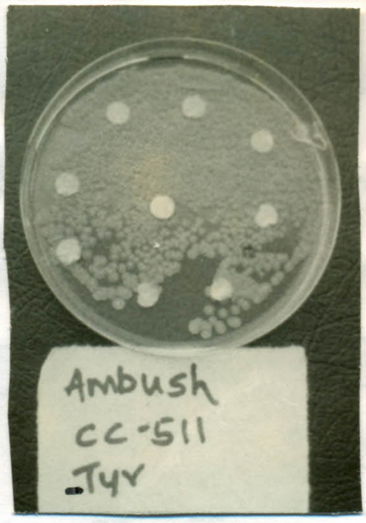


Fig. 29a

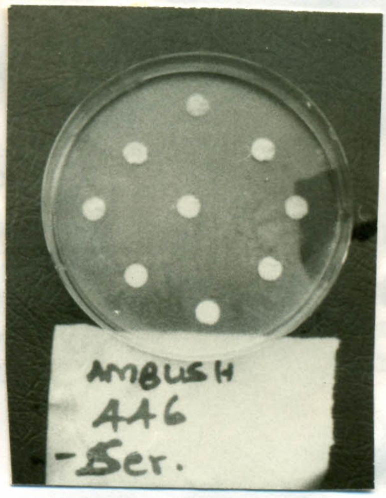


Fig 29b

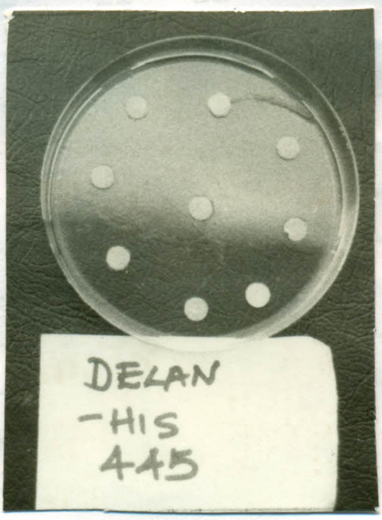


Fig 29c

Fig. 29d

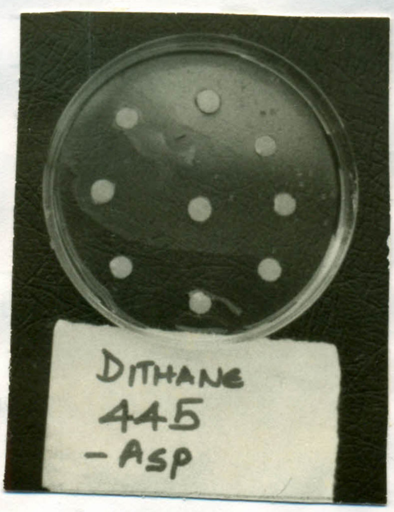


Fig. 29e

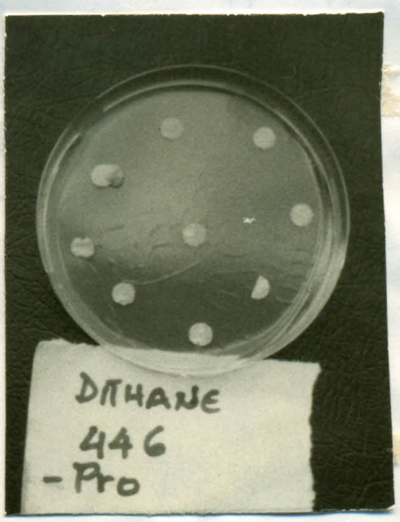


Fig. 29f

TABLE XVI

Results obtained with amino acid supplemented media

	R. phaseoli 445			446			CC-511		
Media	Amb	Del	Dit	Amb	Del	Dit	Amb	Del	Dit
Minimal	+	+	+	+	+	+	+	+	+
-Lysine	--	--	--	--	--	--	++	++	++
-Tyrosine	--	--	--	--	--	--	++	++	++
-Proline	--	--	--	--	--	--	++	++	++
-Asparagine	--	--	--	--	--	--	++	++	++
-Serine	--	--	--	--	--	--	++	++	++
-Histidine	--	--	--	--	--	--	++	++	++
-Arginine	--	--	--	--	--	--	++	++	++
-Alanine	--	--	--	--	--	--	++	++	++
-Tryptophan	--	--	--	--	--	--	++	++	++
-Valine	--	--	--	--	--	--	++	++	++
-Phenylal.	--	--	--	--	--	--	++	++	++
-Methionine	--	--	--	--	--	--	++	++	++
-Leucine	--	--	--	--	--	--	++	++	++
-Glycine	--	--	--	--	--	--	++	++	++
-Aspartic A.	--	--	--	--	--	--	++	++	++
-Threonine	--	--	--	--	--	--	++	++	++
-Cysteine	--	--	--	--	--	--	++	++	++
-Hydroxypro.	--	--	--	--	--	--	++	++	++
-Glutamic A.	--	--	--	--	--	--	++	++	++
All amino A. present.	--	--	--	--	--	--	++	++	++

++ = normal growth, + = zones of growth,

-- = no growth.

R. phaseoli 446 was sensitive to all the antibiotics and CC-511 was sensitive to the antibiotics except to trimethoprim, ampicillin and cotrimoxazole (Figs.30a to 30b). *R. phaseoli* CC-511 was sensitive to ampicillin and cotrimoxazole after treatment with Dithane M-45 and, sensitive to ampicillin cotrimoxazole and trimethoprim, after treatment with the three agrochemicals combined (Fig. 30c). *R. phaseoli* 446 remained sensitive to all agrochemicals (Fig.30e). Dithane M-45 induced resistance in *R. phaseoli* 445 which was sensitive to become resistant to ampillicin, tetracycline,minocycline, afloxacin, spiramycine, amoxycillin and miraxid.

A. chroococcum, in absence of agrochemicals, was sensitive to ampicillin, tetracycline, nitrofurantoin naladixic acid, sulphamethoxazole, streptomycin, gentamicin cotrimoxazole, miraxid amoxycillin, ofloxacin and minocycline, and resistant to trimethoprim and spiramycine. After treatment with Dithane M-45, *A. chroococcum* became resistant to nitrogurantoin and minocycline, and sensitive to trimethoprim. In absence of agrochemicals, *D. gummosa* was sensitive to penicillin, minocycline, erthromycin, methicillin, cotrimoxazole, ampillicin, linomycin, minocycline, trimethoprim, miraxid, spiramycine, amoxycillin and afloxacin. *D. gummosa* remained senstitive even after treatment with agrochemicals. The details of these results are shown in tables XVII to XIX .

Fig. 30. Inhibition of growth of *R. phaseoli* (lab strains) by antibiotics.

Fig. 30a. Resistance (evidenced by growth) of *R. phaseoli* CC-511 to ampicillin (A) and Cotrimoxazole (Co).

Fig. 30b. Resistance of *R. phaseoli* CC-511 to trimethoprim (T).

Fig. 30c. Sensitivity (kill) of *R.phaseoli* CC-511 to ampicillin (A) and cotrimoxazole(C) after treatment with with the 3 agrochemicals combined.

Fig. 30d. Sensitivity of *R. phaseoli* 445 to antibiotics after treatment with the 3 agrochemicals.

Fig. 30e. Sensitivity of *R.phaseoli* 446 to all antibiotics tested before treatment with agrochemicals.

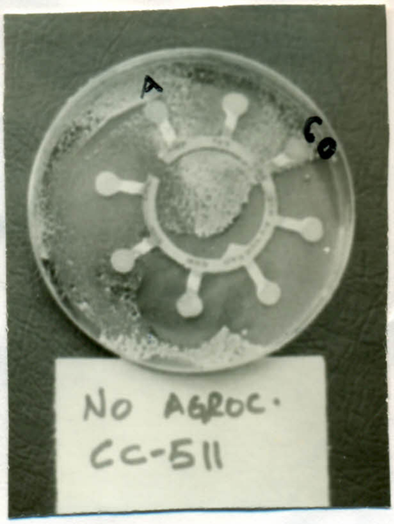


Fig. 30a

Fig. 30b

Fig. 30c

Fig. 30d

Fig. 30c

Figs. 31 to 33. Photographs showing sensitivity of *A.chroococcum* (lab. strain) and *D.gummosa* (lab. strain) to antibiotics.

Fig. 31a and 31b. *A. chroococcum* was sensitive to all antibiotics tested after treatment with all the 3 agrochemicals in combination.

Fig. 32. Sensitivity of *A. chroococcum* to all antibiotics after treatment wtih Delan.

Fig. 33. Sensitivity of *D.gummosa* to all antbiotics after treatment with all the 3 agrochemicals.

Fig 31a

Fig 31b

Fig. 32

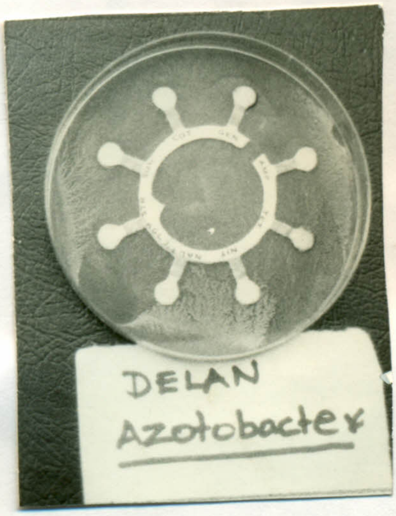


Fig. 33

TABLE XII. The sensitivity/resistance of *R. phaseoli* to different antibiotics after treament with Ambush, Delan and Dithane M-45.

R = resistant

S = susceptible

***R.phaseoli* 445**

Antibiotics	No agroc.	Ambush	Delan	Dithane	All
Minocycline	S	S	S	R	S
Trimethoprim	R	R	S	R	S
Miraxid	S	S	S	R	S
Spiramycine	S	S	S	R	S
Amoxycillin	S	S	S	R	S
Ofloxacin	S	S	S	R	S
Ampicillin	S	S	S	R	S
Tetracycline	S	S	S	R	S
Cotrimoxazole	S	S	S	S	S
Streptomycin	S	S	S	S	S
Kanamycin	S	S	S	S	S
Sulphamethaxazole	S	S	S	S	S
Cloramphenicol	S	S	S	S	S
***R.phaseoli* 446**					
Minocycline	S	S	S	S	S
Trimethoprim	S	S	S	S	S
Miraxid	S	S	S	S	S
Spiramycine	S	S	S	S	S
Amoxycillin	S	S	S	S	S
Ofloxacin	S	S	S	S	S
Ampicillin	S	S	S	S	S
Tetracycline	S	S	S	S	S
Cotrimoxazole	S	S	S	S	S
Streptomycin	S	S	S	S	S
Kanamycin	S	S	S	S	S
Sulphamethaxazole	S	S	S	S	S
Cloramphenicol	S	S	S	S	S

TABLE XVII CONT...

***R.phaseoli* CC-511**

Antibiotics	No agroc.	Ambush	Delan	Dithane	All
Minocycline	S	S	S	S	S
Trimethoprim	R	S	S	R	S
Miraxid	S	S	S	S	S
Spiramycine	S	S	S	S	S
Amoxycillin	S	S	S	S	S
Ofloxacin	S	S	S	S	S
Ampicillin	R	S	S	S	S
Tetracycline	S	S	S	S	S
Cotrimoxazole	R	S	S	S	S
Streptomycin	S	S	S	S	S
Kanamycin	S	S	S	S	S
Sulphamethaxazole	S	S	S	S	S
Cloramphenicol	S	S	S	S	S

TABLE XVIII. The sensitivity/resistance of *D. gummosa* to different antibiotics after treament with Ambush, Delan and Dithane M-45.

R = resistant

S = susceptible

Antibiotics	No agroc.	Ambush	Delan	Dithane	All
Pencillin	S	S	S	S	S
Minocyline	S	S	S	S	S
Erythromycin	S	S	S	S	S
Methicillin	S	S	S	S	S
Cotrimoxazole	S	S	S	S	S
Ampicillin	S	S	S	S	S
Lincomycin	S	S	S	S	S
Trimethoprim	S	S	S	S	S
Miraxid	S	S	S	S	S
Spiramycine	S	S	S	S	S
Amoxycillin	S	S	S	S	S
Ofloxacin	S	S	S	S	S

TABLE XIX. The sensitivity/resistance of *A. chroococcum* to different antibiotics after treament with Ambush, Delan and Dithane M-45.

R = resistant

S = susceptible

Antibiotics	No agroc.	Ambush	Delan	Dithane	All
Ampicillin	S	S	S	S	S
Tetracycline	S	R	S	S	S
Nitrofurantoin	S	S	S	R	S
Naladixic acid	S	S	S	S	S
Sulphamethoxazole	S	S	S	S	S
Minocycline	S	S	S	R	S
Trimethoprim	R	S	S	S	S
Miraxid	S	S	S	S	S
Spiramycine	R	S	S	S	S
Amoxycillin	S	S	S	S	S
Ofloxacin	S	S	S	S	S

3.1.4.1 The effect of agrochemicals on nodulation by *P. vulgaris*

The agrochemicals Ambush, Delan and Dithane M-45 significantly (P<0.05) reduced nodulation by *P. vulgaris* as shown in the ANOVA test only during the 6th, 7th and 8th week after application (Figs. 34 to 36) The other effects of the agrochemicals were the presence of greenish nodules and very small nodules compared to the negative control. The occurence of greenish and very small nodules was not repeatable making it difficult to quantify.

3.1.4.2 The effect of agrochemicals on the dry weight of *P. vulgaris*

During weeks 4-7 there was a general increase in dryweight with increasing concentration of the agrochemicals applied as shown in figs. 37 to 39. Between week 7 and 8 there was a general decrease in dry weight with increasing concentration of the agrochemicals (Figs.37 to 39). The reduction in dry weight was not significant (P>0.05) during weeks 4-6. Between week 7 and 8 the reduction was significant (P<0.05)

Fig. 34. Number of nodules per plant following treatment of *P. vulgaris* with different levels (concentrations) of Ambush.

The different levels of Ambush that were used are

level **1** = 1.5×10^5 ppm (100 X recommended rate)

level **2** = 1.5×10^3 ppm (recommended rate)

level **3** = 1.5×10^1 ppm (1/100 X recommended rate

No Ambush was applied in the control.

Two plants were uprooted from each of the 5 replicates and the mean number nodules was recorded.

Nodulation reduction by Ambush was not significant ($P>0.05$) between weeks 4-5 and significant ($P<0.05$) between weeks 6-8.

Fig. 35. Number of nodules per plant following treatment of *P. vulgaris* with different levels (concentrations) of Delan.

The different levels of Delan that were used are

level **1** = 3.3×10^5 ppm (100 X recommended rate)

level **2** = 3.3×10^3 ppm (recommended rate)

level **3** = 3.3×10^1 ppm (1/100 X recommended rate)

No Delan was applied in the control.

Two plants were uprooted from each of the 5 replicates and the mean number nodules was recorded.

Nodulation reduction by Delan was not significant ($P>0.05$) between weeks 4-5 and significant ($P<0.05$) between weeks 6-8.

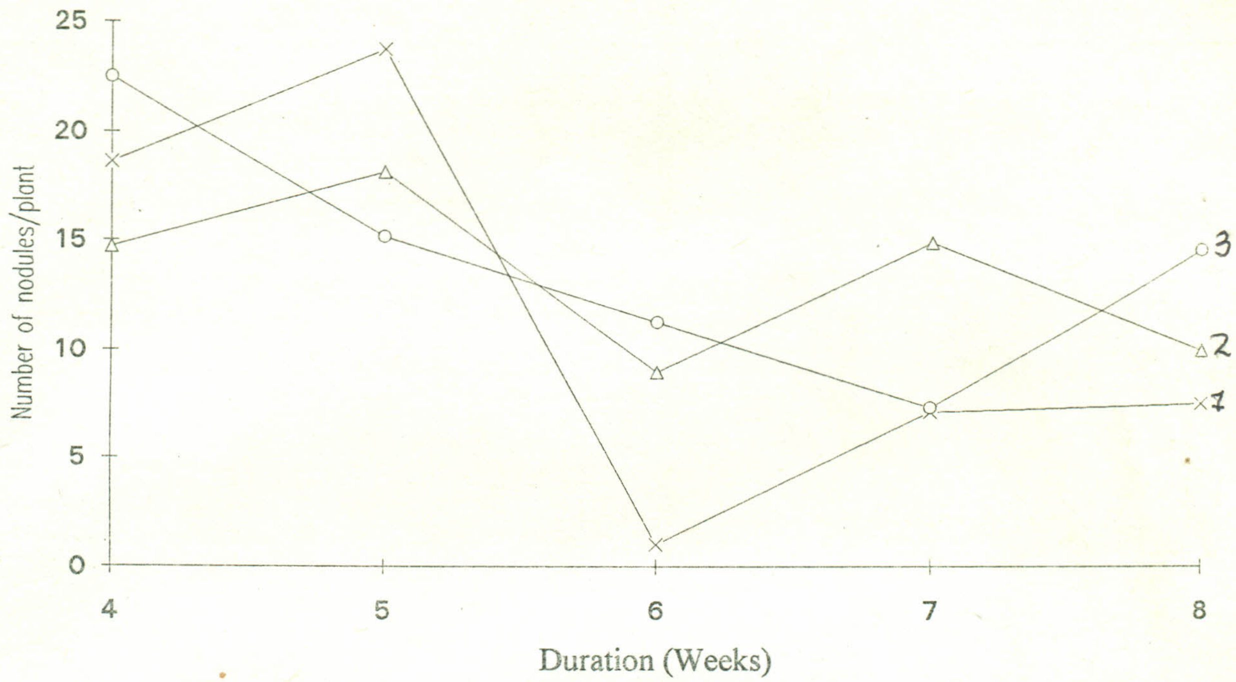

25
20
15
10
5
0
Number of nodules/plant
4
5
6
7
8
Duration (Weeks)
3
2
1

Fig. 34

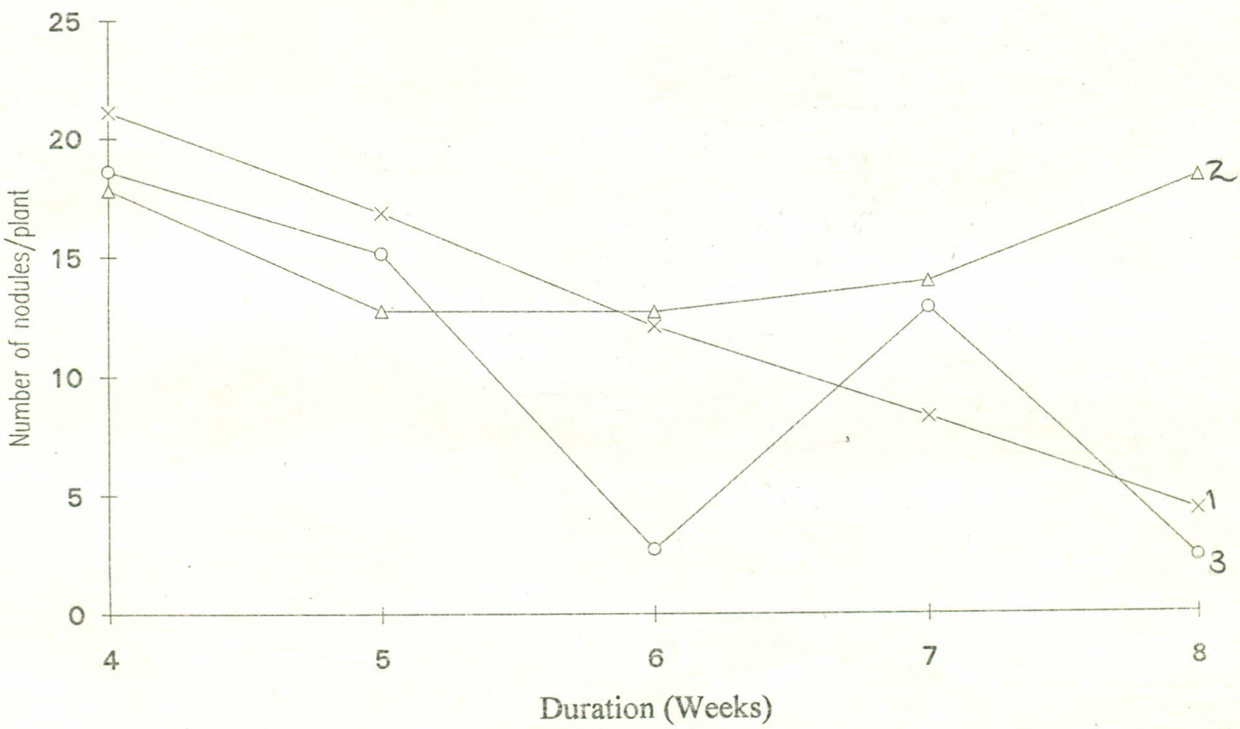

25
20
15
10
5
0
Number of nodules/plant
4
5
6
7
8
Duration (Weeks)
2
1
3

Fig. 35

Fig. 36. Number of nodules per plant following treatment *P.vulgaris* with different levels (concentrations) of Dithane M-45

The different levels ofDithane M-45 that were used are

level **1** = 3.0 X 10^5 ppm (100 X recommended rate)

level **2** = 3.0 X 10^3 ppm (recommended rate)

level **3** = 3.0 X 10^1 ppm (1/100 X recommended rate)

No Dithane M-45 was applied in the control.

Two plants were uprooted from each of the 5 replicates and the mean number nodules was recorded.

Nodulation reduction by Dithane M-45 was not significant ($P>0.05$) between weeks 4-5 and was significant ($P<0.05$) between weeks 6-8.

Fig. 37. Dry weight per plant after treatment of *P.vulgaris* with different levels of Ambush.

Ambush levels **1, 2** and **3** were used.

Two plants were uprooted from each of the 5 replicates and the dry weight assessed.

The dry weight of *P. vulgaris* decreased with increasing concentration of Ambush. This decrease was not significant ($P>0.05$) between weeks 4-5 and was significant ($P>0.05$) between weeks 6-8.

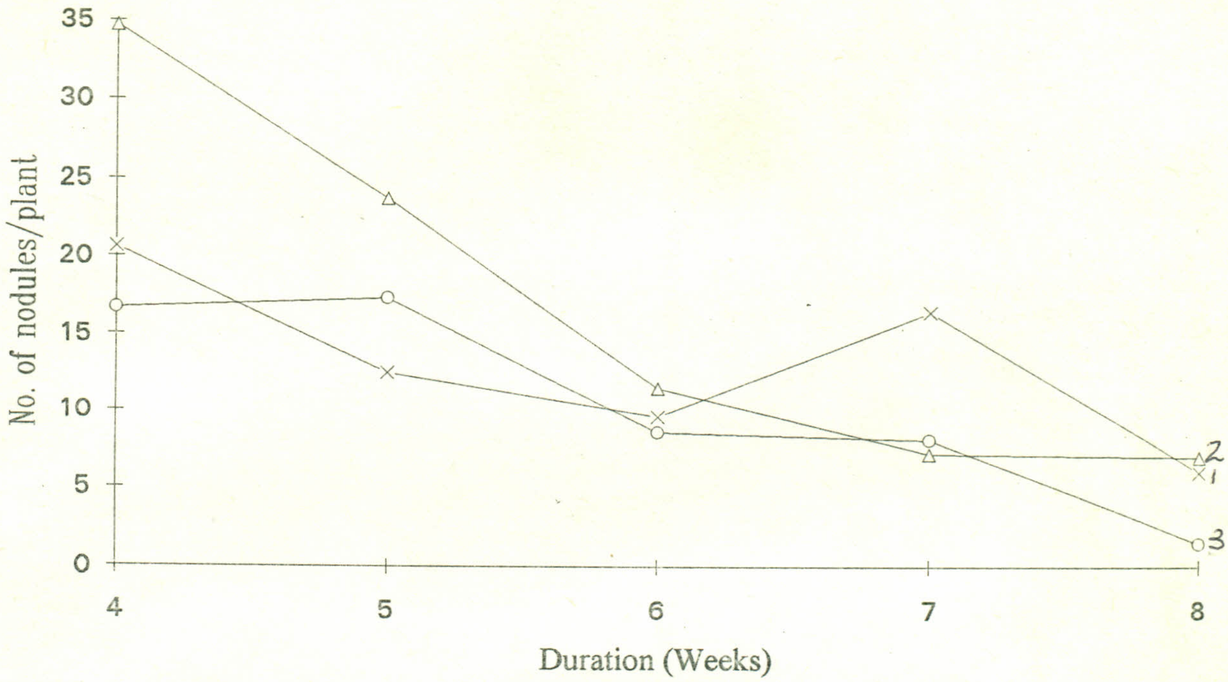

No. of nodules/plant
35
30
25
20
15
10
5
0
4
5
6
7
8
Duration (Weeks)
2
1
3

Fig 36

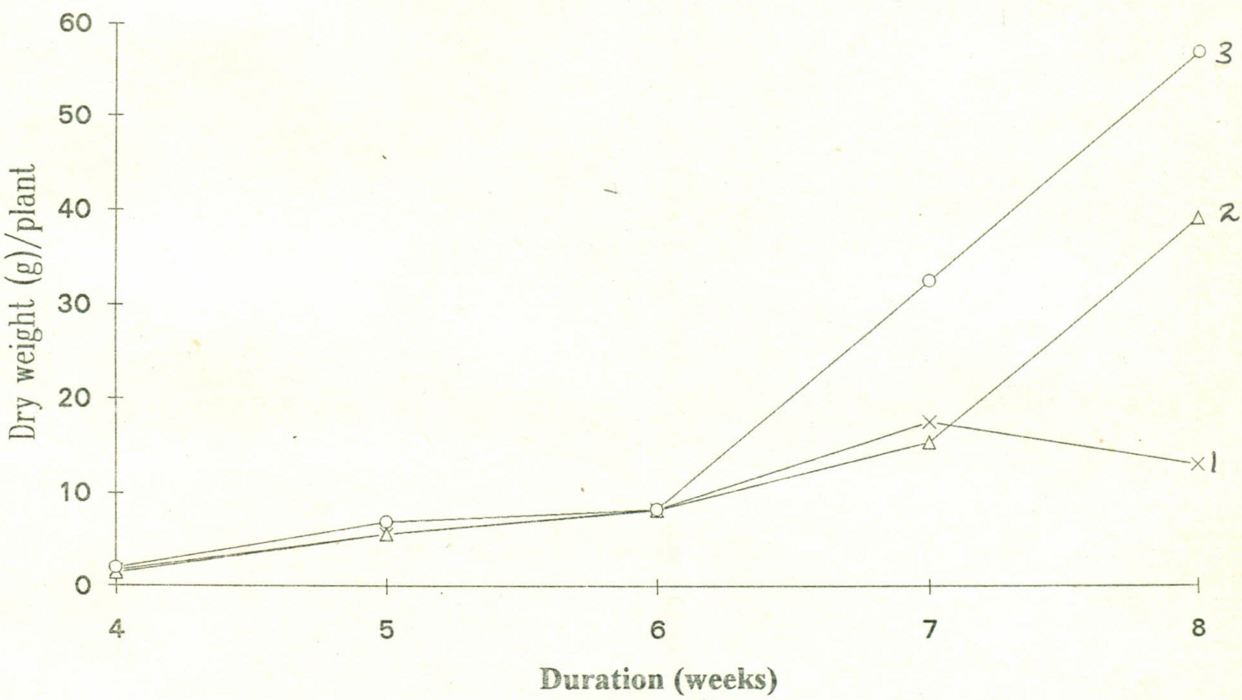

Dry weight (g)/plant
60
50
40
30
20
10
0
4
5
6
7
8
Duration (weeks)
3
2
1

Fig 37

Fig. 38. Dry weight per plant after treatment of *P.vulgaris* with different levels of Delan

Delan levels **1, 2** and **3** were used.

Two plants were uprooted from each of the 5 replicates and the dry weight assessed.

The dry weight of *P. vulgaris* decreased with increasing concentration of Delan. This decrease was not significant $(P>0.05)$ between weeks 4-5 and was significant $(P>0.05)$ between weeks 6-8.

Fig. 39. Dry weight per plant after treatment of *P.vulgaris* with different levels of Dithane M-45.

Delan levels **1, 2** and **3** were used.

Two plants were uprooted from each of the 5 replicates and the dry weight assessed.

The dry weight of *P. vulgaris* decreased with increasing concentration of DithaneM-45. This decrease was not significant $(P>0.05)$ between weeks 4-5 and was significant $(P>0.05)$ between weeks 6-8.

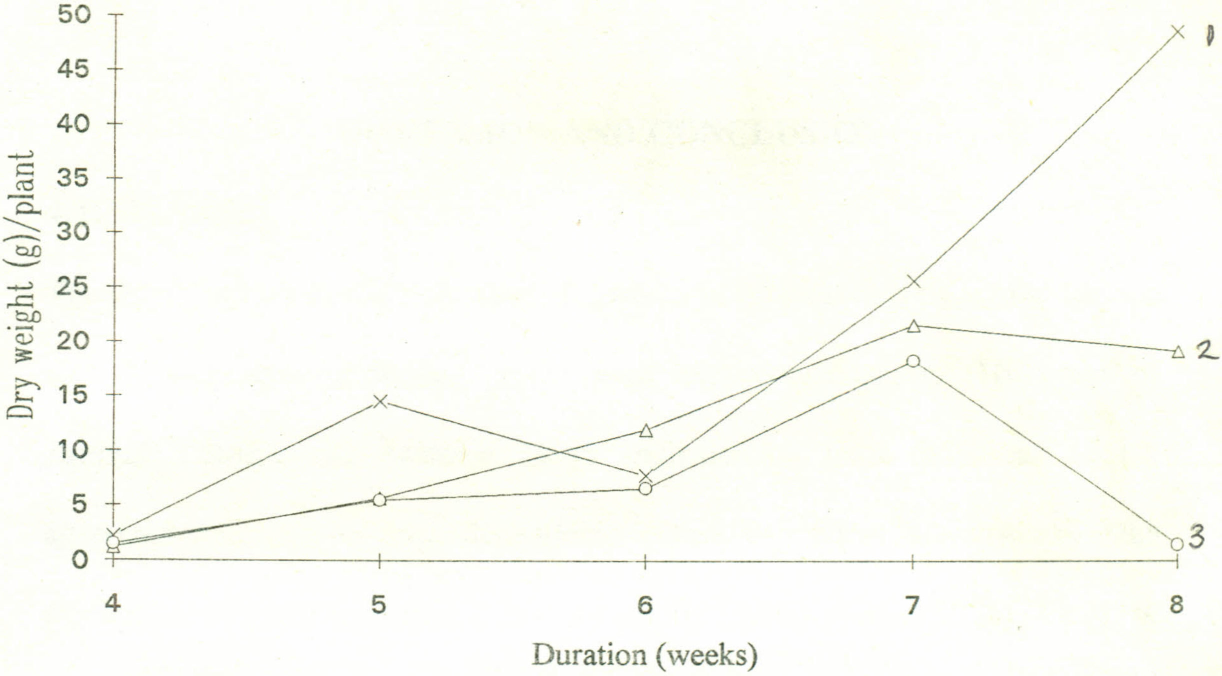

50
45
40
35
30
25
20
15
10
5
0
Dry weight (g)/plant
4
5
6
7
8
Duration (weeks)
1
2
3

Fig 38

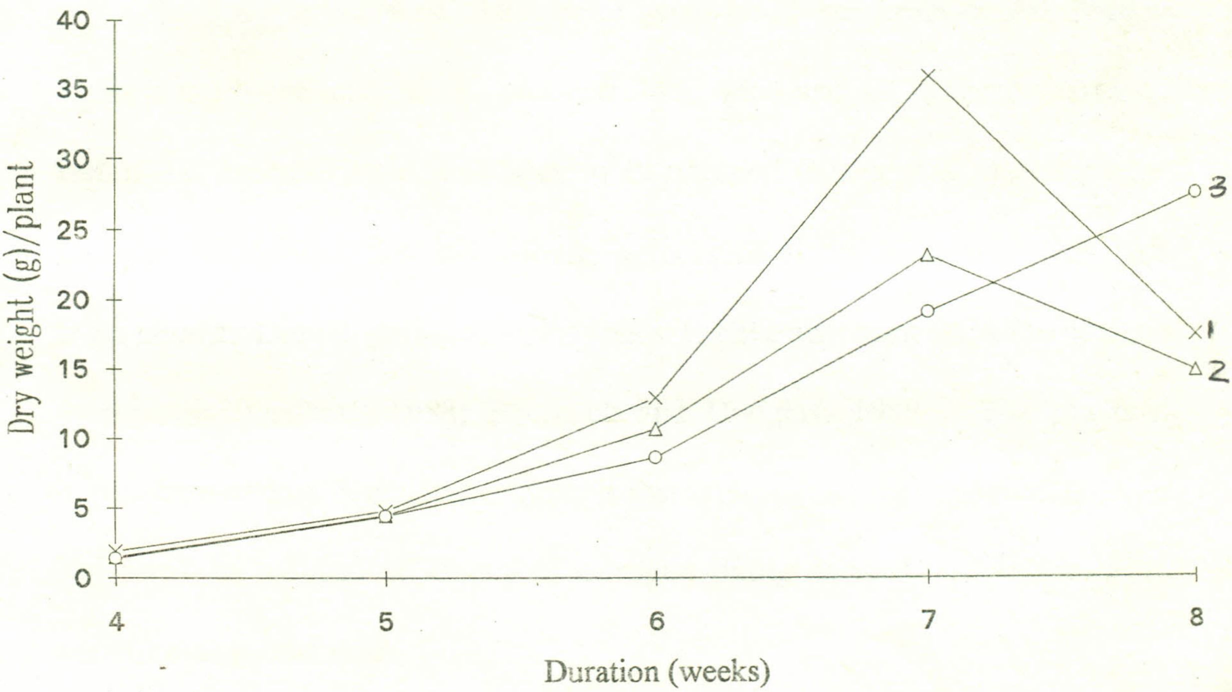

40
35
30
25
20
15
10
5
0
Dry weight (g)/plant
4
5
6
7
8
Duration (weeks)
3
1
2

Fig 39

CHAPTER IV

DISCUSSION AND CONCLUSION

4.1. Discussion

The zonal inhibition and reduction of growth of *R. phaseoli*, *A. chroococcum* and *D. gummosa* in media treated with high concentration (10^4 ppm) of Ambush, Delan and Dithane M-45 indicate the toxic potential of the agrochemicals that is dose dependent. Dose dependent toxicity has been implicated in zonal inhibition (kill) of bacteria (Ames *et al* ., 1975). Death of bacteria could also be due to the inability of *uvrB*⁻ strains to repair DNA damage (Ames *et al*., 1975). The inability to repair DNA by the strains used in this study needs to be verified.

Toxicity varied with the type of bacteria. Delan Ambush and Dithane M-45 were most toxic to *R. phaseoli* 445, 446, and CC-511 respectively. Delan and Ambush were most toxic to *R. phaseoli* (Ku) and (C) respectively. Dithane M-45 was the most toxic agrochemical to *A.chroococcum* and *D.gummosa*. Diquat, paraquat and glyphosate have also been shown to be toxic to rhizobia (Gadkari, 1988; Eberbach and Douglas, 1989). The practical implication of dose dependent toxicity is that improper use of the agrochemicals can result in reduced efficiency of nitrogen fixing bacteria and reduction in agricultural production.

Induction of selective growth of *R. phaseoli* around filter discs soaked in low concentrations (10^{-6} to 10^0 ppm) of Ambush, Delan and Dithane M-45 suggests genetic activity of the agrochemicals. Such selective growth of microorganisms is usually associated with genetic activity viz reversion mutation. (Zimmerman, 1977). Ambush, Delan and Dithane M-45 are known to be clastogenic in *V. faba* (Budambula, 1991). Cunningham, (1980) showed that most mutagens can induce mutations is rhizobia.

The failure to yield reversion mutations in selective media might have been due to depletion of supplements by auxotrophic strains. Selective growth might have been due to lysis of bacterial cells which in turn causes proliferation of surviving cells a condition called auxotrophic pre-emption. Auxotrophic pre-emption has been demonstrated in *B. subtilis, S. pombe and E. coli* (Clarke, 1963,1965; Chopra, 1967:Corran; 1969) Therefore mutagenicity of Ambush, Delan and Dithane M-45 to *R. phaseoli* can not be completely ruled out, in spite of the fact that the nature of genetic activity was not elucidated.

The lack of consistent selective growth of *A. chroococcum* around points of application might have been due to the probable stability of *A. chroococcum*. *A. vinelandii* which belongs to same genus as *A. chroococcum* contains between 10 and 40 times as much DNA as *E. coli* (Kennedy and Toukarin, 1987). Lack of information on the genetics of *D. gummosa* made it difficult to estimate the consequences of the agrochemicals on the bacterium.

Change to antibiotic resistance or sensitivity is widely attributed to induction of forward and reversion mutations respectively (Pai and Marcus-Roberts, 1981).

R. phaseoli 445 which is sensitive to all antibiotics except trimethoprim became resistant to ampicillin, tetracycline, minocyline, afloxacin, spiramycine, amoxycillin and miraxid after treatment with Dithane M-45. *R. phaseoli* CC-511 which is resistant to ampicillin and cotrimoxazole became sensitive to the two antibioitcs after treatment with Dithane M-45. *A.chroococcum* is sensitive to nitrogurantoin minocycline and resistant to trimethoprim. On treatment with Dithane M-45 the same strain turned resistant to nitrogurantoin and minocycline and sensitive to trimethoprim. *R. phaseoli* 446 and *D.gummosa* were sensitive to all antibiotics with or without treatment. Verification that the induction of antibiotic resistance/sensitivity observed here is due to mutations awaits further investigation.

Mutation from antibiotic sensitive to resistance is associated with loss of effectiveness of symbiosis of *R.trifolii, R. leguminosarum* and *R. meliloti* (Schwinghamer, 1977). Full loss of effectiveness accompanies resistance to viomycin, neomycin, and kanamycin. Resistance to streptomycin, spiramycine and tetracycline is associated with little or no effect on symbiosis (Schwinghamer,1977). The effect of resistance to ampicillin, minocycline, afloxacin, amoxycillin, miraxid and nitrogurantoin on effectiveness of symbiosis that was induced by Dithane M-45 is yet to be documented.

Should such resistance be accompanied by loss of effectiveness, the effects on agriculture are no doubt detrimental.

Tetracyclines in particular have been associated with antibiotic resistance due to wide exposure (Timoney, 1984). In the present study the induction of antibiotic resistance and/or sensitivtiy to tetracyclines and penicillins was notably observed. Induction of antibiotic resistance or sensitivity was also observed with a wide range of other antibiotics. Therefore such inductions can not be reasonably confined to a particular group(s) of antibiotics.

Resistance genes have been shown to be of extranuclear origin, mainly, R plasmids (Watanabe, 1971; Suzuki *et al* ., 1989). These consist of transposons for example ampicillin resistance gene. In *Shigella* which causes dysentery, multiple resistance to penicillin, tetracyline, sulfanilamide, streptomycin and chloramphenicol is inherited as a single genetic package and transmitted in an infectious manner as identified by Watanabe, (1971). This implies that sequences of genetic material responsible for such resistance are highly linked. The induction of multiple antibiotic resistance was demonstrated in this study by Dithane M-45. Transposons have insertion sequences, enhance recombination and are also highly mobile genetic elements, thus the possibility of multiple antibiotic resistance (Suzuki *et al* ., 1989).

There is no adequate evidence from the present study that this is the manner in which Dithane M-45 induces multiple antibiotic resistance.

There is no evidence for gain in function due to antibiotic resistant mutants. Resistance to streptomycin, chloramphenicol or spiramycine provides a useful marker system since it is stable, free from cross-resistance and does not impair symbiosis (Schwinghamer, 1967). Resistance might provide clues relating to metabolic steps involved in symbiosis affected by mutatiom (Schwinghamer, 1963). It is speculated that mutation to antibiotic resistance may play a significant role in elucidating the origin of ineffective rhizobia observed in nature.

The reduction of nodulation of *P. vulgaris* by Ambush, Delan and Dithane M-45 indicates how agricultural production can be vulnerable to improper use of agrochemicals. The agrochemicals amitrole, atrazine and glyphosate decreased nodulation by sub-clover, implying that the agrochemicals may interefere with nodulating potential of certain strains of rhizobia (Eberbach and Douglas, 1989). Induction of antibiotic resistance and toxicity of Ambush, Delan and Dithane M-45 observed with laboratory strains, may contribute to the reduction in nodulation observed in this study.

The dry weight of *P. vulgaris* was reduced by Ambush, Delan and Dithane M-45. The largest reduction was by Dithane M-45. Such reduction suggests poor plant performance which might have been a result of reduction in nitrogen fixation (Danso, 1984). Reduction in nitrogen fixation implies a further drawback to improving food production.

Evidence from the present study indicates that Ambush, Delan and Dithane M-45 at high concentration are toxic to the bacterial strains used. Dithane M-45 at low concentration induced antibiotic resistance and/or sensitivity in *R. phaseoli* 445, *R. phaseoli* CC-511 and *A. chroococcum*, which has possible effects on the integrity of genetic material of nitrogen fixing bacteria. There is also evidence for the reduction of nodulation and dry weight which may have adverse consequences on food production. Earlier work by the author showed that Ambush, Delan and Dithane M-45 are clastogenic in *V. faba*.

At the moment there is a global call to ban the use of such agrochemicals. This may not be a feasible solution because pest resistant crop strains are not adequately available. Safer alternatives especially for Dithane M-45 and the related dithocarbamates need to be sought urgently. Efforts to identify/generate crop strains resistant to pests and other forms of biological control should be intensified. Further there is need to intensify mapping of the genome of nitrogen fixing bacteria to facilitate research on their genetics. The effect of resistance to ampicillin, minocycline, afloxacin, amoxycllin, miraxid and nitrogurantoin on effectiveness of symbiosis need also be documented.

4.2. Conclusion

Results from the present study indicate that :

the three agrochemicals, Ambush, Delan and Dithane M-45 at high concentration (10^4 ppm) are toxic to *R. phaseoli*, *A. chroococcum* and *D. gummosa*

the three agrochemicals reduced nodulation of *P. vulgaris* significantly ($P<0.05$) and also reduced plant performance as indicated by dry weight.

Dithane M-45 induced antibiotic resistance and/or sensitivity in *R. phaseoli* 445, *R. phaseoli* CC-511 and *A. chroococcum*.

CHAPTER V

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