EVALUATION OF PESTICIDE EFFECTS ON BEAN AND SESBANIA RHIZOBIA AND THEIR SYMBIOTIC RELATIONSHIP

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ABSTRACT

Laboratory and glasshouse experiments were conducted at the Sokoine University of Agriculture, Morogoro, to evaluate the effects of kocide 101 (77% cupric hydroxide) a fungicide and oxadiazon [3-(2,4-dichloro-5(1-methylethoxyphenyl)-5-(1,1-dimethylethyl-1,3,4-oxadiazol-2(3H)-one] a herbicide, on the growth and symbiotic performance of bean (*Phaseolus vulgaris* L.) and *Sesbania rostrata* rhizobia, respectively. The growth of the rhizobial strains in the presence of the pesticides was evaluated in Yeast Mannitol Broth (YMB). The legume-*Rhizobium* symbiosis was evaluated in the glasshouse using a sandy loam soil (Typic Rhodustalf) for beans and a clay soil (Udic Ustifluvent) for *Sesbania*.

Kocide 101 in the broth medium inhibited growth of rhizobia. Of the three strains tested for sensitivity to kocide 101, the local isolate was most tolerant followed by the PV_1 strain, and the strain CIAT 899 was least tolerant. The growth of *Sesbania* rhizobia in broth medium was unaffected by the addition of oxadiazon.

The application of kocide 101 at the recommended application rate (equivalent to 1.7 mg/kg soil) had no harmful effect on growth, nodulation and nitrogen fixation of bean plants. Kocide 101 applied at 2x and 4x the recommended application rate (equivalent to 3.4, and 6.8 mg/kg soil) was detrimental to the growth, nodulation and N_2 fixation. The inhibition of nodulation

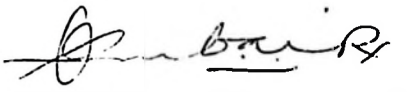
and N_2 fixation in the presence of kocide 101 was mainly due to the direct effect of the fungicide on the bean plants.

The application of oxadiazon at the recommended application rate, at 2x and at 4x the recommended application rate (equivalent to 0.75, 1.50, and 3 mg/kg soil, respectively) adversely affected the growth, nodulation and N_2 fixation by *Sesbania rostrata* plants, the inhibition increasing with increase in oxadiazon concentration. The inhibition in nodulation and N_2 fixation in the presence of oxadiazon was, to a large extent, due to adverse effects of the herbicide on plant growth and development rather than on rhizobial viability.

Nodule formation and plant growth of both beans and *Sesbania* continued to be curtailed by the pesticides residues four months after they were first applied to the soil.

DECLARATION

I, FREDERICK PHILBERT BAIJUKYA, do declare to the Senate of the Sokoine University of Agriculture that this dissertation is my own original work and it has not been submitted for a higher degree award in any other university.

Signature: 

Frederick Philbert Baijukya

Date: 26 July 1996

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I wish to express special tributes to Dr. E. Semu, who supervised this work. His interest and good guidance shall always be remembered. I feel privileged to have had a chance to work under him. Thanks are also due to Dr. G.P. Msumali, for technical assistance, constructive comments, and guidance during the time Dr. Semu was indisposed.

All academic staff members of the Department of Soil Science who contributed in any form to this study are acknowledged. It is not possible to mention them all, but to one and all, I say thank you. The technical staff, especially Mrs. J. Mhaiki, Messrs G.P. Malekela, and M.S Salum, are acknowledged for their assistance and cooperation they rendered in the laboratory analytical phase. I wish to acknowledge Dr. S.F. Nchimbi-Msolla, of the Department of Crop Science for providing the bean seeds for the experiments.

A vote of thanks is also due to the Norwegian Agency for International Development (NORAD) for providing me with the in-country scholarship. I am very grateful to my employer, Ministry of Agriculture, for granting me a study leave. Last but not least I pay tribute to my dear wife Pudensiana for her love and encouragement.

DEDICATION

This dissertation is dedicated to my parents who through tireless efforts I got my education.

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CHAPTER ONE

1.0 INTRODUCTION

Bean (*Phaseolus vulgaris* L.) plants are able to fix atmospheric nitrogen (N_2) in symbiosis with root nodule bacteria (rhizobia). This, if exploited, would reduce demand for industrial N fertilizers for the crop, which would be a relief to the poor farmer. In Tanzania beans are grown predominantly in Arusha, Kilimanjaro, Tanga, Kagera, Kigoma, Mbeya and Rukwa regions, but bean cultivation has now expanded to Mwanza, Iringa, Ruvuma and Shinyanga (Mushi, 1991). Most of the crop is grown by subsistence farmers, normally as an intercrop with coffee, banana, maize or other crops including root crops and trees (Mushi, 1991). In most parts of the country bean production is affected by many constraints among which are insect pests (Karel, 1984), and diseases (Njau *et al.*, 1988). To counter these constraints pesticides are often used.

The increasing use of pesticides in bean production systems in Tanzania is well documented (Karel, 1984). Gondwe (1989) reported the successful use of the fungicide kocide 101 in controlling anthracnose in beans. This fungicide has a broad spectrum and it can control most fungal diseases of common crops in the country. Examples are all fungal diseases of coffee, black sigatoka (banana), early and late blight (potatoes), powdery mildew (grapes), and early blight (tomatoes). The fungicide is reported to be commonly used in coffee and banana plantations,

for example in Kilimanjaro, Arusha, Mbeya and Kagera regions where beans are grown as an intercrop (Mushi, 1991). But such a chemical, being inherently toxic, may not be without adverse effects on different facets of the legume-rhizobia symbiosis which has potential for providing a substantial proportion of the bean plants' N requirement.

Sesbania rostrata, a leguminous plant which thrives in waterlogged soils, and with potential as green manure crop for paddy rice (Roger and Ladha, 1990), also has a large N_2 fixation potential. That large potential is due to the fact that in additional to root nodules, *Sesbania rostrata* also forms effective stem nodules in association with rhizobia of the genus *Azorhizobium caulinodans*. This annual legume is common in East Asia (Bhardwaj *et al.*, 1981; IRRI, 1987), and in parts of West, Central, and Southern Africa (Evans and Roter, 1987). In Tanzania the plant is found growing wildly in some lowland areas with characteristic waterlogging, for example at Mbarali in Mbeya, and parts of Dodoma, Tabora and Morogoro. These areas are famous for paddy rice production. In the Mbarali area, small scale rice farmers appreciate the value of *Sesbania* in their farms (Tarimo, 1993, personal communication).

Becker *et al.* (1990) reported that in areas where *Sesbania rostrata* is found, moderate but stable yields were obtained without adverse effect on soil. This was considered to be due to the manurial value of the *Sesbania* because of its ability

to fix atmospheric nitrogen. Despite this potential, the plant is at present considered to be a weed, and at Mbarali Rice Farm, for example, it is usually controlled using herbicides.

Increasing demand for high bean and rice yields requires, among other things, widespread use of chemical pesticides. But such chemicals may be harmful to efficient nitrogen fixation (De-Felipe *et al.*, 1987; Roberts, 1992).

In many areas of Tanzania N fertilizers are either not available or not affordable. Furthermore, removal of fertilizer subsidies, due to an unfavourable economic climate, has resulted in higher prices and lower supplies (Kamasho *et al.*, 1991), making the situation worse. Another issue of great concern is the sustainability of soil productivity as lands are driven to produce higher yields under intensive cropping systems. The soil organic matter and N levels vital for sustaining crop production often are limiting in most soils of Tanzania. This dictates soil conservation efforts and enhancing the biological alternatives which can augment, and in some cases probably replace, N fertilizers. Biological Nitrogen Fixation (BNF), a microbiological process which converts atmospheric nitrogen into plant usable form, offers this alternative.

Research results obtained from most institutions suggest that soil degradation can be prevented or retarded by maintaining a crop cover or a continuous layer of organic residues on the soil surface (Yost and Evans, 1986). A number of technologies have been successfully tested, including the use of woody plants in alley cropping and cover crops for live and *in situ* mulch (Yost and Evans, 1986). If these species are leguminous, they can also contribute to the system considerable amounts of nitrogen from N_2 fixation.

The use of chemicals in crop production poses a major concern in that the chemicals could be harmful to the environment and hence adversely affect nitrogen fixation. The possibility that the currently used pesticides may have an adverse effect on soil microbial activities, including BNF, should not be ignored.

No studies in Tanzania have focused on the effect of agricultural pesticides on rhizobia *and Rhizobium*-legume symbioses. As cultivated legumes are continuously exposed to agrochemicals, and the fact that the use of pesticides is an accepted practice, the possible effects of chemicals on nitrogen fixing systems need to be studied. While applied initially to foliage, the ultimate sink of most pesticides is the soil where rhizobia are to be found. Therefore products which may get in contact with rhizobia, though initially targeted to plant pests or diseases, require testing in order to assess effects on rhizobia due to their use.

The objectives of the present study therefore were:

1. To investigate the effects of kocide 101 (a copper fungicide) on bean rhizobia population, bean plant growth, nodulation and N_2 fixation.

2. To study the effects of oxadiazon (a herbicide) on *Sesbania rostrata* rhizobia, growth of sesbania plants nodulation and N_2 fixation.

3. To determine the residual effects of the kocide and oxadiazon on plant growth and N_2 fixation in the soil.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1. Effects of pesticides on N-cycling soil microorganisms

Soil microbial ecosystems are maintained under a state of dynamic equilibrium brought about by diverse reactions carried out by various microorganisms. The nitrogen cycle is mediated by microorganisms, for example N_2 fixers, nitrifiers, denitrifiers etc. The use of pesticides in agricultural systems have been reported to affect these soil microbial activities (Alexander, 1977; Graham *et al.*, 1980; Gonzalez-Lopez *et al.*, 1992).

Pesticides may influence soil microbial activities either positively or negatively. Using the gamma-isomer of benzene hexachloride (lindane), Rhaghu and MacRae (1967) reported marked stimulation in growth of indigenous soil algae, when the insecticide was applied at rates up to 50 kg/ha. The stimulation was attributed to the elimination by the insecticide of small animals which feed on the algae. Hiranpradit and Foy (1992) also reported a stimulation of algae growth following application of some triazine herbicides. The stimulation of microbial activities following application of the pesticides was attributed to the pesticides serving as carbon and energy sources for the growth of soil microorganisms.

Yeomans and Bremner (1985) studied the effect of seven insecticides and six fungicides on denitrification of nitrate in soil. Their study revealed that some insecticides (lindane, fonofos and malathion) enhanced denitrification. The fungicide thiram also was found to increase the ratio of N_2:N_2O in the gaseous products of denitrification. Stimulated growth and infective capability of rhizobia following pesticide use have been reported by Ruhloff and Burton (1951), Alexander (1977), and Martennson and Nilsson (1989).

Pesticides are toxic chemicals and often affect non-harmful soil microorganisms by killing or reducing numbers of one or more genera/species (De-Felipe *et al.*, 1987). The extent of harm will vary depending on chemical dosage, soil properties, and environmental factors (Bollen, 1961; Martennson, 1992).

Graham *et al.* (1980) reported reduced survival of *Rhizobium phaseoli* when in contact with Pentachloronitrobenzene (PCNB) and bi-dimethyl thiocarbonyl-disulphide (thiram). Gonzalez-Lopez *et al.* (1992) reported an increase in total viable counts of bacterial and fungal populations in agricultural soils when the acaricide bromopyrate was applied at rates up to 300 mg/kg soil. When assessing individual groups of bacteria, they noted enhanced populations of denitrifiers at concentration of 50-300 mg/kg soil, but decreased populations of nitrifiers even at lower concentrations of the acaricide. The negative impacts that may result from use of pesticides may also involve the inhibition of N_2 fixation.

2.2 The effect of pesticides on N_2 fixation

2.2.1 Effects on rhizobia

The literature on compatibility of rhizobia with pesticides is controversial. Variations in test methods and insufficient concern for quantitative aspects of *Rhizobium* survival may have contributed to these conflicting reports. Because of the interdependence of the bacterium and the host plants, difficulty is encountered in assessing the influence of pesticides on rhizobia in soil. To circumvent this dilemma many researchers have resorted to pure culture studies (Curley and Burton, 1975).

2.2.1.1 Fungicides

It has been reported (Kernkamp, 1948) that of the chemicals used as plant or seed protectants, those which contain mercury, copper, or zinc are extremely toxic to rhizobia. Laboratory studies by Ruhloff and Burton (1951) with arasan (Tetraethyl-thiram disulphide 50% a.i), arasan-SF (as arasan, but with 75% a.i), spergon DDT-SL (95% of tetrachloro-para-benzoquinone and dichloro-diphenyl trichloroethane), and phygon (50% 2,3 dichloro-1,4 naptho-quinone), at the concentration of 0.3 g/g in artificial media, showed varied responses by different rhizobia species to the toxicity of these chemicals. While *R. meliloti*, *R. leguminosarum*, *R. phaseoli*, *R. lupini*, and *B. japonicum*, were affected little by spergon, cowpea and clover rhizobia were susceptible to its toxicity. On the other

hand phygon produced little inhibition zone with all species of *Rhizobium* except *R. phaseoli*.

Working with bean seeds dressed with the fungicide spergon, Odeyemi and Alexander (1977) observed reduced growth of *R. phaseoli*. However, the effects were reversed only when the resistant isolates of the parent *Rhizobium* strain were used. Curley and Burton (1975), using thiram (75% wettable), captan (N,trichloromethio-4 cyclohexane 1,2,dicarboximide), carboxin (5,6 Dihydro 2 methyl-1,4 oxathiin-3 carboxanilide, 75% wettable) and PCNB to study the response of *B. japonicum* to seed protectants, reported reduced *Rhizobium* survival when in contact with PCNB. *B. japonicum* was reported to be tolerant to thiram, captan and carboxin. Compatibility of thiram with *R. phaseoli* was also reported later by Chao and Alexander (1981).

No information is available on the effects of kocide 101 on rhizobia. It is possible that the fungicide may exert similar effects to rhizobia in soil as has been reported for the other fungicides. As bean grows in symbiosis, therefore, the compatibility of kocide 101 with the bean *Rhizobium* microsymbiont and its effect on N_2 fixation need to be evaluated.

2.2.1.2 Herbicides

Reports on herbicide use differ in terms of their effects on the N_2 fixing process in the soil. Singh *et al.* (1978), reported a significant inhibition of the growth of *B. japonicum* and cowpea rhizobia, but little reduction in growth of *R. leguminosarum*, when the herbicide alachlor was applied at rates from 200 to 400 μg/ml in artificial media. While studying the effects of herbicides on the survival of *Bradyrhizobium japonicum* strains, Moorman (1986) reported adverse effects of acifluorfen, bentazon, dinozeb, and fluchloralin when the herbicides were used at rates equal to or higher than ten-fold the recommended rates. Laboratory studies by Martensson and Nilsson (1989) with chlorosalfuron revealed that rates of 0.55 and 5.5 μM in pure culture did not affect the growth of *R. trifolii*.

The herbicide oxadiazon is generally recommended for use in paddy rice production where *Sesbania rostrata*, otherwise a weed, has been found to be a potential green manure crop. However, its influence on nodulation of the *Sesbania* plants and its effect on the growth of associated rhizobia has not been documented. It is possible that the use of this herbicide may impair or retard the N_2 fixation process by *Sesbania*.

2.2.2 Effect on legume-rhizobia symbiosis

Even if not initially targeted and applied to soil, the pesticides, when used, finally find their way into soil. Higher doses of pesticides applied in soil, or their residues, have been reported to inhibit growth of rhizobia, leguminous plants or

affect the nodulation process (Curley and Burton, 1975; Graham *et al.*, 1980; Martennson, 1992).

2.2.2.1 Fungicides

Curley and Burton (1975) reported reduced *Rhizobium* survival on seed of soybean (*Glycine max* L.) when in contact with PCNB at levels of 0.9 g/kg seed and captan (5,6 Dihydro-2 methyl-1,4 oxathiin 3 carboxanilide) at 0.8 g/kg seed. Though captan showed to be less toxic to rhizobia than PCNB, it reduced greatly the tap root nodulation. Poor nodulation of soybean plants was also reported from this study when seeds were planted 24 hours later after the inoculation. Similar results were reported by Graham *et al.* (1980). But when PCNB-treated seeds were inoculated, lime pelleted, and then promptly planted, the nodule numbers subsequently observed were similar to those from inoculated but pesticide-free plants. Lime pelleting of the seeds was considered to have an ameliorating effect, thus enhancing nodulation.

2.2.2.2 Herbicides

Studies on effects of herbicides on the nodulation, nodule development, nodule activity, legume-*Rhizobium* symbiosis, and biomass production by lupin (*Lupinus albus* L.), alfalfa (*Medicago sativa*) and red clover (*Trifolium pratense*) have been reported in Spain (De-Felipe *et al.*, 1987) under field conditions and in Sweden (Martennson and Nilsson, 1989) under glasshouse conditions.

De-felipe *et al.* (1987) studied the influence of lindane and simazine on symbiotic N_2 fixation and the photosynthetic apparatus of lupinus. They reported reduced acetylene reduction assay values (ARA) with increased herbicide application. Herbicide application was also reported to reduce plant development, with the plants becoming chlorotic, the effect being observed more with simazine. These results were in consonance with the finding that herbicides exerted at least a part of their phytotoxicity through interference with the light-dependent phases of the photosynthetic process. Kucey *et al.* (1988) observed reduced yields, but no effect on nodulation of soybean under field conditions, when the herbicides alachlor, fluazifopbutyl and metachlor were applied at their recommended rates.

Laboratory studies by Martensson and Nilsson (1989) showed poor development of alfalfa and red clover plants on agar supplemented with chlorosulfuron. The development of plants in the agar with 55 μM of chlorosulfuron was strongly inhibited. The plants showed turgid root tips and thick roots. The bases of root hairs of uninoculated plants grown in soil treated with this chemical were reported to be swollen. The plants grown in pesticide-treated soil weighed less than the controls. Rates of 4 or 8 g chlorosulfuron a.i/ha under field condition, were toxic to alfalfa and red clover plants. Chlosulfuron was also found to decrease the nitrogenase activity, though it did not affect nodulation. When alfalfa and red clover plants were planted in soil containing 2 g chlosalfuron/ha,

they developed normally only after an initial growth inhibition of 5 to 6 weeks. It was concluded that the herbicides exerted adverse effects more on plants rather than rhizobia.

2.3 The mechanisms by which pesticides affect rhizobia

There is insufficient knowledge on the physiological response of rhizobia to many pesticides to fully assess their mechanisms of action on rhizobia in soils. *Rhizobium* populations may be indirectly affected by pesticides such as by stimulation of microorganisms that are antagonistic or predatory towards *Rhizobium* (Alexander, 1977), or pesticides may be acting directly upon the *Rhizobium* in a bacteriostatic manner (Motorman, 1986; Motorman *et al.*, 1992).

Jaworski (1972) reported inhibition of the growth of *B. japonicum* by glyphosate (N-phosphoromethyl glycine). The fungicide was reported to interfere with the aromatic amino acid synthetic pathway of the *Rhizobium*. Significant reversal of the growth inhibition was achieved by introducing/supplementing phenylamine and tyrosine. Glyphosate was reported to interfere with the biosynthesis of phenylamine and, more specifically, with the metabolism of chorismic acid in the aromatic amino acid biosynthetic pathway. The inhibitory effect of glyphosate on the growth and metabolism of three strains of *B. japonicum* (USDA 110, USDA 123, and USDA 138) was also reported by Motorman (1986) and Motorman *et al.* (1992). The growth of all the strains was affected by

glyphosate at concentrations ranging from 0.5 to 5 mM, with rapid death observed at 10 mM. Accumulation of benzoic acids, especially protocatechuic acid (PCA) which was reported to inhibit the enzyme 5-enolypyruvylshikimate-3 phosphate (EPSP) synthetase (Duke, 1988), was also observed in the culture media of USDA 123 and USDA 138. The high level of PCA produced by *B. japonicum* strains may alter the symbiosis interactions between the rhizobia and the host plant.

2.4 Mechanisms of rhizobia tolerance to pesticides

Assessing the possible effects of pesticides on microorganisms may depend in part on detailed knowledge of microbial tolerance to pesticides (Motorman *et al.*, 1992). Microbial tolerance to pesticides may be due to microbial metabolism of the pesticides, resulting in modification or reduction of their activity. Alexander (1977) reported that a variety of synthetic pesticides were used as substrate by soil microorganisms. Some microorganisms were reported capable of metabolizing pesticides, and the products served as sources of either carbon, energy, and occasionally nitrogen or sulphur. Although little is known on rhizobia specifically, it is probable that their tolerance to pesticides may be based on similar mechanisms.

Furthermore, the ability to reverse the effects of pesticides through utilization of certain amino acids has also been reported as one of the mechanisms of microbial tolerance to pesticides. For example, Lui *et al.* (1991) reported

increased tolerance of *Bradyrhizobium japonicum* to glyphosate in media containing aromatic amino acids than in media free of such amino acids. Bode *et al.* (1984), using the same herbicide, reported the same observation with *Candida maltosa.*

2.5 Persistence of pesticides in soil

The dynamics of pesticides in soils are influenced by various factors and processes operating in the soil system, and also the physico-chemical and biological properties of pesticides. These factors are the ones that determine the differences in the effects of pesticides on plants and microorganisms across different soils.

Pesticides may persist in soil, in the active mode, from a few hours or days to many years, but it is not always that the most persistent chemical has the greatest influence on the numbers of soil micro/macro organisms. Edwards and Thompson (1973) reported that simazine, which is slightly toxic to soil microorganisms, persisted for more than a year. But a chemical D-D mixture (mixture of 1,3-dichloropropene and 1,2-dichlropropane) that is extremely toxic to all soil organisms was reported to persist in soil for only a few weeks, and it may decrease numbers of soil organisms greatly within that short time period.

Differences in persistence of pesticides among soils have often been observed (Moyer *et al.*, 1990; Royuela *et al.*, 1990; Loux and Rees, 1992; Pusino *et al.*, 1992). Residues of inazaquine were greater in clay soil than in silt texture soil three, six and 11 months after application (Loux and Rees, 1992). Pusino *et al.* (1992), using three different soils, observed the contribution of organic matter to the total metachlor adsorption. Metachlor adsorption was high in soils with high organic matter content. A sequential loss in adsorptive capacity was observed when the soils were treated with hydrogen peroxide to oxidize the organic matter. The same observation was reported by Muellar *et al.* (1991). Friesen and Wall (1991) concluded from studies conducted between 1986-1990 in Canada that soil texture had a greater effect than climate on the carry over of CGA-131036 and chlorsulfuron. Residue carry over was greater on a sandy loam but not on a clay loam soil.

The acidity or alkalinity of the soil has been reported to affect pesticide persistence in the soil. Weber (1970) found that maximum adsorption of several s-triazines occurred in the vicinity of their pKa values. As the pH decreased, the herbicide molecules became protonated and, therefore, more cationic, thus becoming more strongly attracted to soil colloids. Loux and Rees (1992) reported increased adsorption of inazaquine with decrease in soil pH. The pesticide was found to persist more in clay soil with low pH. Increased phytotoxicity of metribuzin with decrease in soil pH has been reported by Ladlie *et al.* (1976).

The soil environment into which pesticides are introduced determines to a large extent the rate of disappearance, and many differences in persistence among soils can be attributed to variations of soil temperature and moisture levels.

Weather and climate are among other factors that influence the rate of disappearance of pesticides through their effect on volatilization, surface removal in run off, and downward movement in the soil profile. For example, the pesticides DDT and alachlor have been reported to dissipate very fast in soils of warm moist climates than in soils of cold dry climates (Samuel *et al.*, 1989; Walker *et al.*, 1992). Weather and climate alter persistence through their effect on degradation (photo-chemical, biological and non-biological). Microbial decomposition depends on favourable soil temperature and moisture conditions, and non-biological decomposition also appears to be moisture and temperature dependent (Harris, 1964).

Plant uptake of pesticides, pesticide formulations, and method of pesticide placement in the soil are also among factors that determine pesticide persistence in soils. There is a general agreement that pesticide residues can affect plant growth. Conversely, the presence of plants can significantly affect pesticide persistence in soil. Talbert and Fletchall (1964) noticed that one year after simazine was applied, weeds were growing primarily on the spots/locations where no roots had been located the previous year. Wicks and Burnside (1965) reported similar

observations after atrazine had been applied to sorghum. This accelerated disappearance in the presence of roots was probably due to absorption and metabolism of herbicide by the plants.

The overall pesticide characteristics and configuration, acidity or basicity, solubility in water, electronic effect (charge distribution) and molecule size have also been reported to influence pesticide persistence in soil (Bailey and White, 1970). Subsurface placement of pesticides often enhanced persistence. Nelson *et al.* (1983) reported that residues of oryzalin which was subsurface-applied but not incorporated into soil were almost twice as toxic 25 weeks after application as residues from the same rate applied to the soil surface.

2.6 Adverse effects of pesticides residues on soil microorganisms

Generally, pesticide residues are concentrated in the top 15 cm of the soil. This is the region of greatest activity of soil micro-and macro-organisms (Alexander, 1977), thus setting the stage for interaction of pesticide residues with the soil organisms.

Herbicides such as amitrole-T-ionynil and dalapon were reported to reduce the number of *Azotobacter chroococcum* two to four weeks after application when applied at normal rates (Van Schreven *et al.*, 1970). Bullery *et al.* (1988) reported reduced numbers of vesicular arbsucular mycorrhiza (VAM) due to presence of

fumigant residues in soil. Residues of cynazine herbicide have also been reported to decrease the VAM populations in soils.

Excess concentrations of residues in the edaphic environment may precipitate shifts in microbial populations to temporarily favour some groups of microorganisms, which may then overpopulate the soil (Gonzalez-Lopez *et al.*, 1992). Beneficial microorganisms may be suppressed, provoking new and more complex interactions from microorganisms which have escaped competition with other suppressed groups. Gonzalez- Lopez *et al.* (1992) observed decreased numbers of nitrifiers and increased denitrifiers following application of bromopropylate in the soil. This was reported to affect the nitrogen economy of the soil.

From the review above, it may be said that many pesticides are, in one way or another, disruptive to the environment, and may adversely affect microbial processes, including biological N_2 fixation. The use of pesticides, particularly those with adverse effects N_2 fixing systems, may result in the need to increase the application rates of N-fertilizers.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Soil sampling

The soils used in this study were sampled from two sites, Mafiga and Magadu, in the University farm. These soils have been classified as Typic Rhodustalf and Udic Ustifluvent, respectively, using the USDA soil taxonomy system (Kaaya, 1989). In the present study, these soils are denoted as soil A and soil B for Typic Rhodustalf (Mafiga) and Udic Ustifluvent (Magadu), respectively.

Soil A (sampled from Mafiga) was used to grow beans and soil B (sampled from Magadu) was used to grow *Sesbania rostrata*. The soils were selected because they have the agronomic characteristics suitable for the growth of these plant species (Halliday, 1984; Norman *et al.*, 1984; Becker *et al.*, 1990).

Bulk surface soils (0-15 cm) were collected. The soils were air-dried and larger soil aggregates were broken into smaller ones, and mixed well. Small portions of the soil samples were ground and passed through a 2 mm sieve and used for laboratory analysis to determine the soils' physico-chemical properties (section 3.2 below).

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3.2 Soil analysis

Particle size analysis was done by the Bouyocos hydrometer method as outlined by Day (1965). The pH of the soils was measured using the glass electrode in a 1:2.5 (w/v) soil:water suspension as outlined by MacLean (1982). The soils' organic carbon was determined by the method of Walkley and Black according to Nelson and Sommers (1982), whereas total N content of the soils was by the macro-Kjeldahl method as outlined by Bremner and Mulvaney (1982). The exchangeable cations were extracted with NH_4OAc (pH 7) solution (Chapman, 1965) and the cations determined by atomic absorption spectrophotometer (Thomas, 1982). Available phosphorus was determined by the Bray and Kurtz No. 1 method as outlined by Olsen and Sommers (1982). The selected physico-chemical properties of the soils are given in Table 1.

The pH of soil A was slightly acidic and that of soil B was neutral. The results indicate relatively low organic matter and nitrogen contents in both soils. Soil A had very low Ca, low Mg, K and base saturation, whereas soil B had high base saturation, high Ca, Mg, K and medium P. Soil A had medium CEC whereas soil B had high CEC. These properties indicate that soil B is relatively more fertile than soil A.

Table 1: Some physical and chemical properties of the experimental soils

Property	Magnitude	
	Soil A	Soil B
pH	6.40	7.10
Organic carbon(%)	1.09	1.64
Total N(%)	0.14	0.26
Available P (ppm)	1.75	14.50
CEC* (me/100g soil)	12.74	31.36
Exchangeable (me/100g soil)		
Ca	4.32	13.00
Mg	1.48	3.70
K	0.81	0.44
Na	0.13	2.35
BS (%)	52.90	65.10
Particle size analysis (%)		
- Clay	30	51
- Silt	6	40
- Sand	64	44
Textural Class	SCL	Clay

*By NH_4OAc at pH 7

BS = Base saturation

SCL = Sandy clay loam

3.3 Pesticides

A commercial formulation of a copper fungicide, kocide 101, was used. This fungicide is basically copper hydroxide, containing 77% cupric hydroxide or 50% copper. A commercial formulation of a herbicide, Ronstar 25E (25% emulsifiable concentrate), which contained 250 g/litre oxadiazon [3-(2,4-dichloro-5(1-methylethoxyphenyl)-5-(1,1-dimethylethyl-1,3,4-oxadiazol-2(3H)-one] wasalso used in the present studies.

3.4 Rhizobia strains used

3.4.1 Source of the rhizobia strains

Bean root nodule bacteria [*Rhizobium leguminosurum* (strains PV_1 and a strain designated as "local isolate" and *Rhizobium tropicii* (strain CIAT 899)] and *Sesbania rostrata* rhizobia (*Azorhizobium caulinodans*) were used in this study. The rhizobia strains CIAT 899 and PV_1 were obtained from the Soil Science Department, SUA. The local bean rhizobial isolate was obtained/isolated from the experimental soil A. The *Sesbania* rhizobia isolate was cultured from *Sesbania rostrata stem* nodules collected from Igunga, Tabora.

3.4.2 Recovery of nodules and isolation of rhizobia from potted soil

Isolation of bean rhizobia was undertaken in the glasshouse. Five plastic pots of two-litre capacity were filled with soil A. Basal application of phosphorus was made in the form of KH_2PO_4 at the rate of 99 mg/pot, equivalent to 30 kg/ha

on field basis. The soil in each pot was thoroughly mixed to ensure uniform distribution of the applied P. Bean seeds of the variety SUA 90 were obtained from the Department of Crop Science and Production, SUA. The seeds were surface sterilized using ethanol and hydrogen peroxide (Vincent, 1970). Five surface-sterilized seeds were sown per pot and the pots were placed in the glasshouse. The seedlings were thinned to two plants seven days after germination. Collection of nodules for rhizobial isolation was done at 28 days after planting.

Rhizobia from bean and sesbania nodules were isolated, cultured, and authenticated (tested for ability to nodulate host plant) following the procedures described by Somasegaran and Hoben (1985). The *Sesbania rostrata* seeds used for the authentication test were collected from the Mbarali rice farm, Mbeya.

3.5 Influence of kocide 101 and oxadiazon on bacterial growth *in vitro*

These experiments were carried out to gain an insight into the effect of the pesticides on rhizobia survival *in vitro* and, presumably, of the performance of the rhizobia in soils. Survival of bean rhizobia and the *Sesbania rostrata* isolate in the presence of kocide 101 and oxadiazon, respectively, was tested using the Yeast Mannitol Broth (YMB) defined by Vincent (1970).

One hundred ml of YMB were prepared in 250-ml erlenmeyer flasks. Filter-sterilized kocide 101 and oxadiazon were added to the broth, giving final concentrations of 0, 0.025, 0.050, 0.100 or 0.200 mg/ml and 0, 5.250, 12.500, 25.000 mg/l for kocide 101 and oxadiazon, respectively, in three replications. The experimental flasks were arranged in a Randomized Complete Block Design (RCBD). The broth in the flasks was inoculated with 1 ml of a log-phase culture of the strains under test. Growth of the rhizobia to establish the log phase was monitored by determining absorbance of the rhizobia at 630 nm.

The choice of the 630 wavelength was arrived at as follows: Three day-old broth cultures of the different rhizobia strains were each placed in a cuvette in the Bausch and Lomb Spectronic 88 spectrophotometer. The wavelength was then varied, in steps of 10 nm, from 450 nm to 860 nm, taking the absorbance reading every time the wavelength was changed. The wavelength at which there was an absorbance peak (i.e. the wavelength of greatest instrument sensitivity) was chosen for rhizobia monitoring in subsequent studies. That wavelength for all rhizobia strains was 630 nm (Figure 1).

The log-phase of rhizobia strains was thus determined by inoculating the YMB with respective rhizobia strains and following their growth after every 12 hours for 72 hours (Figure 2). Thirty (30) hours was chosen as the average log-phase time for all strains.

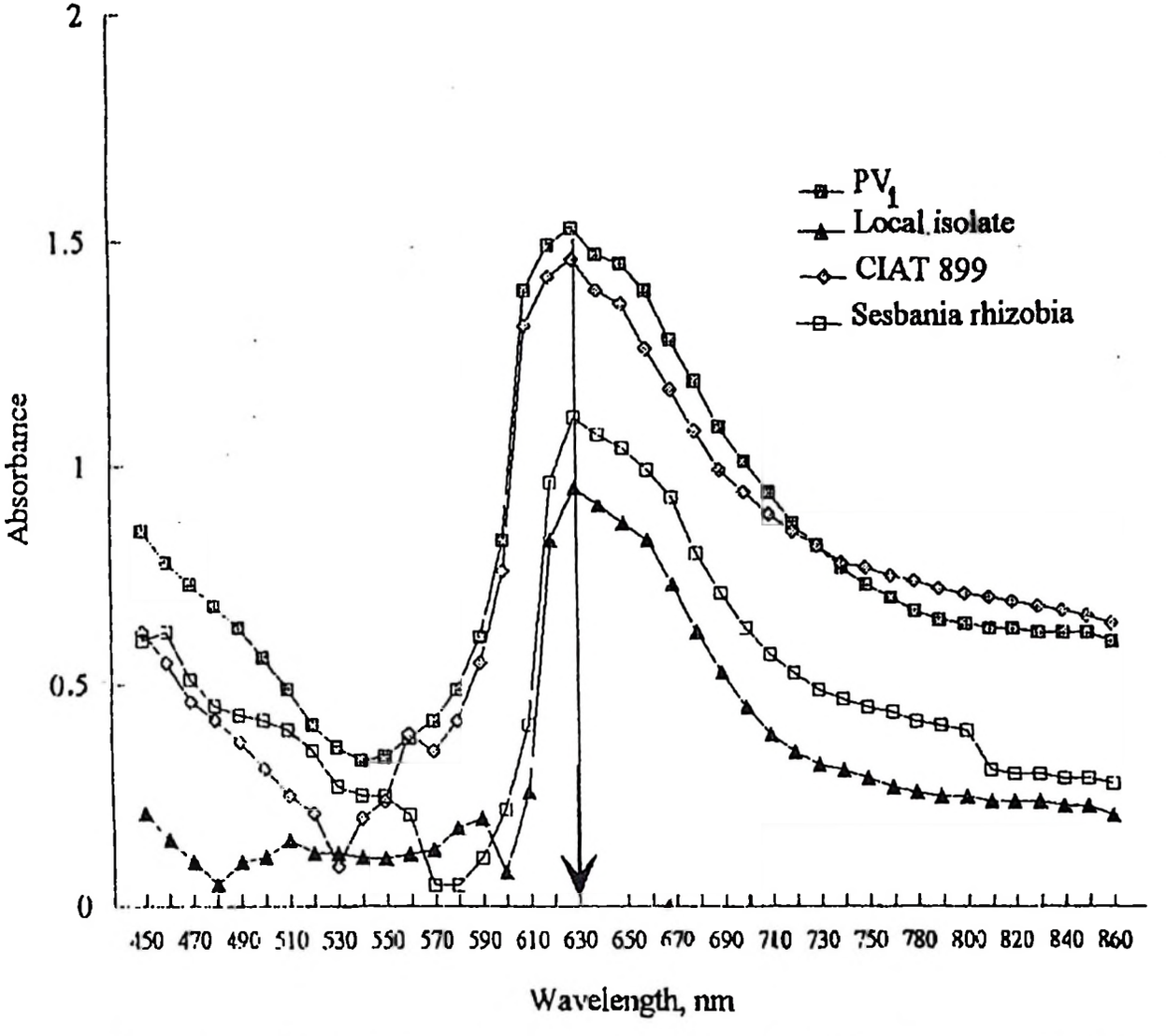

Figure 1. Wavelength of greatest instrument sensitivity for the experimental rhizobial strains.

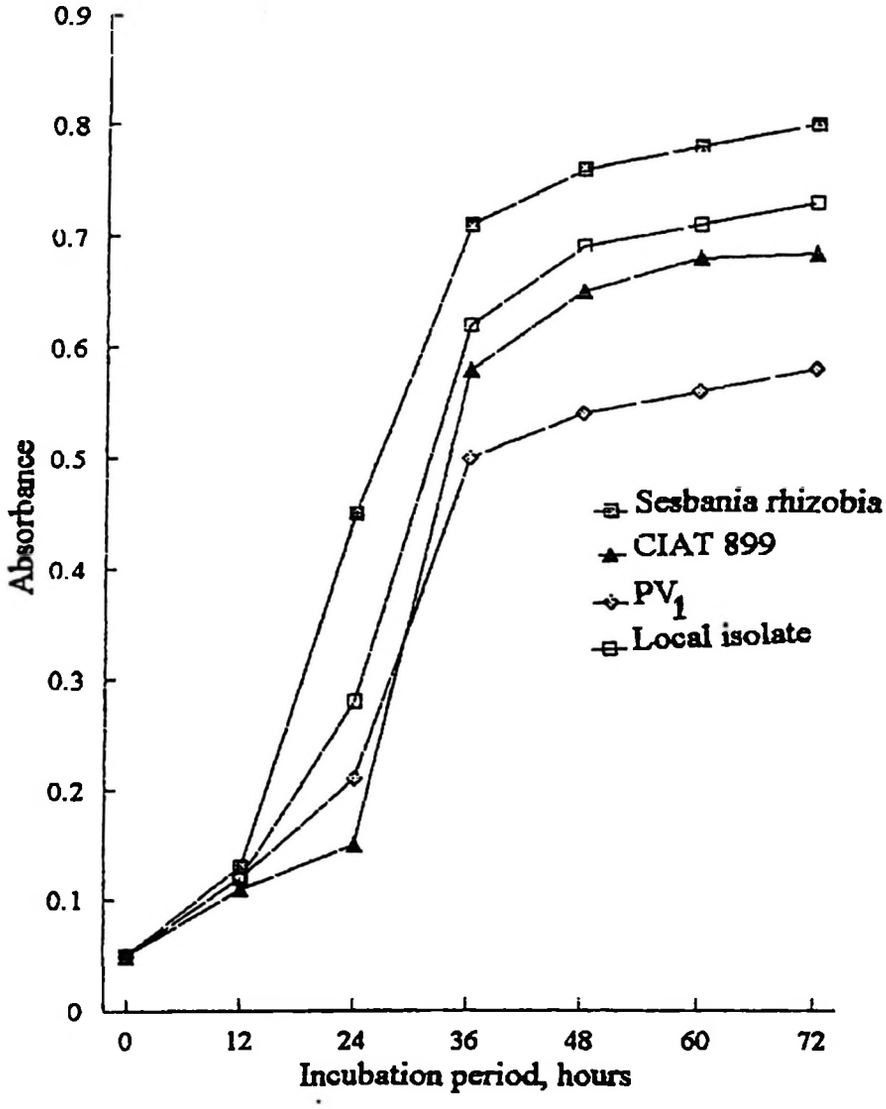

Figure 2. Growth curves of experimental rhizobial strains in YMB, to determine the logarithmic phase.

3.6 Glasshouse experiments on bean-rhizobia symbiosis

Soil A, passed through a 2 mm sieve to remove large particles and plant debris, was sterilized in the autoclave at 121^{0}C and 15 pounds per square inch (psi) for one hour. Kocide 101 at rates 0, 1.7, 3.4, or 6.8 mg/kg soil was aseptically added to two kg of sterile soil in plastic bags. These fungicide concentrations in the soil corresponded to field application rates of 0, 3.4, 6.8, or 13.6 kg/ha (i.e. zero, normal application rate, 2x normal rate, and 4x normal rate), respectively. Although the normal application rate of kocide 101 in beans is 3.4 kg/ha, more than one applications per season may be done depending on the severity of diseases.

The fungicide was thoroughly mixed with soil and the mixture transferred into sterile plastic pots of two litre capacity. Two hundred ml of sterile water were added to each pot to bring the soil to approximately field capacity. The soils in the pots were kept in a cool place for seven days, to allow for equilibration.

Seeds of the bean variety SUA 90 were sterilized first by rinsing in ethanol (95%) for one minute. The seeds were then immersed in (4%) H_2O_2 solution for five minutes, and thereafter rinsed with six changes of sterile water. The sterilized seeds were submerged in sterile water and left in the refrigerator for two hours for the seeds to imbibe water. The seeds were then divided into three portions each of which was inoculated with each of the different bean rhizobia strains. One hundred

ml of a three-day old rhizobia culture in yeast mannitol broth (YMB), containing approximately 10^7 viable rhizobial cells/ml, were used for the inoculation.

Five seeds were sown per pot. The inoculated but fungicide-free treatments served as control. The seedlings were thinned to two plants seven days after emergence. The pots were watered using sterile water, to maintain the soils at about field capacity. The experiment was carried out using the RCBD with three replications. The treatments were a factorial combination of four fungicide levels and three rhizobia strains.

Nodule numbers, nodule dry weights, N-accumulation and shoot dry matter weights were determined at 21, 28 and 35 days after planting. It has been reported (Rennie and Kemp, 1983) that N-accumulation by bean plants is maximum at 50% flowering stage. The bean variety SUA 90 produces 50% of its flowers at 35 days after planting (Nchimbi-Msolla, 1990, personal communication).

At sampling, the above-ground plant material was cut at the soil level, washed to remove adhering soil, and oven-dried at 60^0C to constant weight. The below-ground parts were carefully removed from the soil, and adhering soil particles carefully removed. Nodules were collected, counted, oven-dried and weighed. The dried shoot samples were ground to pass through a 0.5 mm sieve and analyzed for percentage N, using the macro-Kjeldahl method (Bremner and

Mulvaney, 1982). The data obtained from this experiment were subjected to Analysis of Variance (ANOVA). The residual effects of kocide 101 were evaluated four months after the end of this experiment.

3.7 Glasshouse experiment on *Sesbania*-rhizobia symbiosis

Soil B, passed through 5 mm sieve, was sterilized in the autoclave at 121^{0}C and 15 psi for one hour, in portions of 10 kg which were then aseptically transferred into pots of 10-litre capacity.

Two experiments were carried out, one after another. The first experiment was meant to simulate the situation whereby the herbicide is found concentrated on the top soil, as would be following an initial application. The second experiment was meant to simulate the condition whereby the herbicide is found accumulated in soil throughout the root zone, as would happen when the residues of previous applications become mixed with the plow-layer upon repeated cultivation, season after season.

In the first experiment, *Sesbania rostrata* seeds were scarified in concentrated sulphuric acid for 30 minutes. The seeds were then thoroughly rinsed with distilled water and about 100 seeds were planted in a pot. Ten-day old seedlings were transplanted into individual pots, two plants per pot. Two days after transplanting, the seedlings were inoculated with a three-day old culture of

Sesbania rostrata rhizobia containing approximately 10^7 cells per ml. Two days after inoculation, oxadiazon at 0, 0.75, 1.50, or 3.00 mg a.i. /kg soil was sprayed onto soil in the pots. These herbicide concentrations in the potted soils corresponded to field application rates of 0, 6, 12, or 24 l/ha (i.e. zero, normal application rate, 2x application rate, and 4x application rate), respectively.

The second experiment was conducted as the first one, except that in this case the herbicide was thoroughly mixed with the soil before transplanting of the seedlings.

The experiments were arranged in a RCBD. The pots were watered daily using sterile water. Nodule number, nodule dry weight, N-accumulation and shoot dry matter yields were determined at 30, 45, and 60 days after transplanting.

At harvest, shoots were cut at the soil surface, any adhering soil removed, and the shoots oven-dried at 60^0C to constant weight. The nodules were carefully detached, washed, oven-dried, counted and weighed. Dried shoot samples were ground, passed through a 0.5 mm sieve and analyzed for N, using the macro-Kjeldahl method. The data were analyzed statistically by ANOVA. The residual effects of oxadiazon were evaluated four months after the end of this experiment.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Influence of kocide 101 on bean rhizobial growth *in vitro*

The effect of kocide 101 on the growth curve of bean rhizobia is presented in Figure 3. All strains grew equally well in the control treatments, reaching a population of $\log_{10}$= 9/ml (1 billion cells) within 24 to 36 hours. Kocide 101 in the broth medium inhibited rhizobial growth, the inhibition increasing with increase in its concentration. In terms of the rhizobial growth curve shown in Figure 3, the effect of kocide 101 was to flatten the logarithmic phase of the curve, the flattening increasing with increasing kocide 101 concentration in the medium. Given the same kocide 101 concentration dose, the flattening was in the order: local isolate < PV_1 < CIAT 899. These events resulted in lower rhizobial numbers with increasing kocide 101 concentrations. For example the kocide 101 concentrations which resulted in almost total inhibition of growth were 100, 50, and 10 μg kocide 101/ml for the local isolate, the PV_1 strain, and the CIAT 899 strain, respectively. Moreover, to reduce the rhizobial population maximum (plateau) to $\log_{10}$ = 4-5 (i.e. 10,000 to 100,000 cells/ml) required 50, 10, and 8 μg kocide 101/ml for the local isolate, the PV_1 strain, and the CIAT 899 strain, respectively. Thus, the local isolate was most tolerant and the CIAT 899 strain least tolerant to kocide 101.

It should be noted that the growth curves (Figure 3) started off with the logarithmic phase. This was because the cells used to inoculate the media were obtained from rhizobia cultures at the logarithmic phase of growth (section 3.5). The flattening of the logarithmic phase with kocide 101 additions may be explained in terms of the toxicity of kocide 101, thus slowing the rhizobial cell growth and multiplication rates. The eventual effect was to reduce the rhizobia populations in the kocide 101 treatments relative to the controls. The fact that the kocide 101 dose required to effect similar flattening was largest with the local strain, followed by the PV_1, and lastly by the CIAT 899, implies that of the three isolates the local isolate was most tolerant to kocide 101.

It is difficult to extrapolate and predict the effect this fungicide would exert on the growth of rhizobia under field conditions from the results of this *in vitro* culture. The conditions in the soil may be very different from the *in vitro* conditions due to the complexity of soil constituents on the one hand, and media composition on the other (Bird *et al.*, 1985), which would result in adsorption phenomena and other influences on the Cu. Curley and Burton (1975) reported that sub-bacteriostatic doses of PCNB reduced the ability of *B. japonicum* to form a symbiosis with soybean (*G. max* L.), but Graham *et al.* (1980) demonstrated from a field experiment that the fungicide had no effect on nodulation and N_2 fixation.

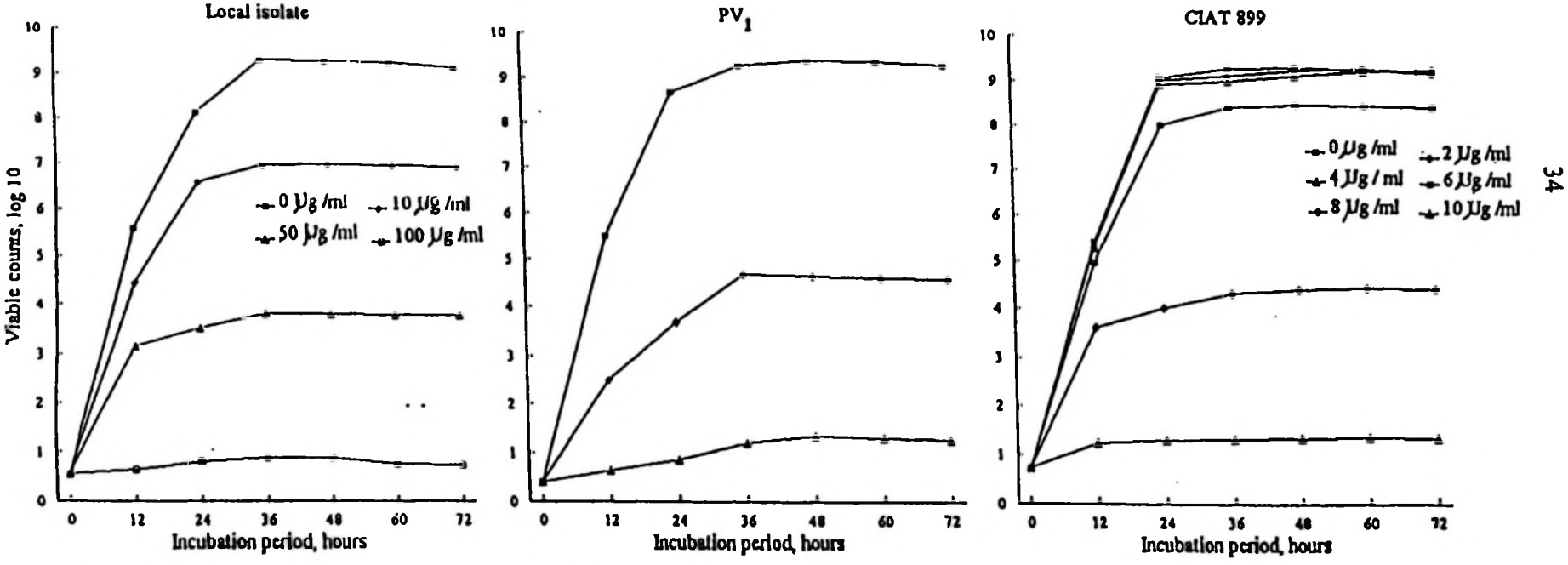

Figure 3. Effect of Kocide 101 at different concentration levels on the growth curves of bean rhizobia *in vitro*.

4.2 Effect of kocide 101 on the bean-rhizobia symbiosis

4.2.1 Growth and dry matter yields

Generally, kocide 101 applied at the recommended rate (equivalent to 1.7 mg/kg soil) had no significant negative effects on the growth of beans, for growth was similar to that of control plants, as was reflected by the shoot dry weights (Figure 4, Appendix 1). Higher levels of kocide 101 reduced plant growth significantly ($P < 0.05$) relative to controls at every sampling stage up to 35 days after planting (DAP), which is the 50% flowering stage of this bean variety. At 28 DAP and longer, those plants that were growing in soil treated with the fungicide at rates greater than the recommended rate had few roots (data not presented) and were chlorotic.

The plants inoculated with different rhizobia strains showed differential growth. Those inoculated with the local isolate had the highest dry matter weights, followed by those inoculated with the PV_1 and CIAT 899 strains, in that order. This differential performance was maintained at all the sampling periods. However, at the higher kocide 101 application rates, this differential performance narrowed. At the recommended kocide 101 application rate, the shoot dry weights of plants inoculated with the local isolate were consistently slightly higher than the control plants, although this difference was not statistically significant. At this recommended application rate, the shoot weights associated with the different strains were significantly ($P < 0.05$) different at all sampling periods.

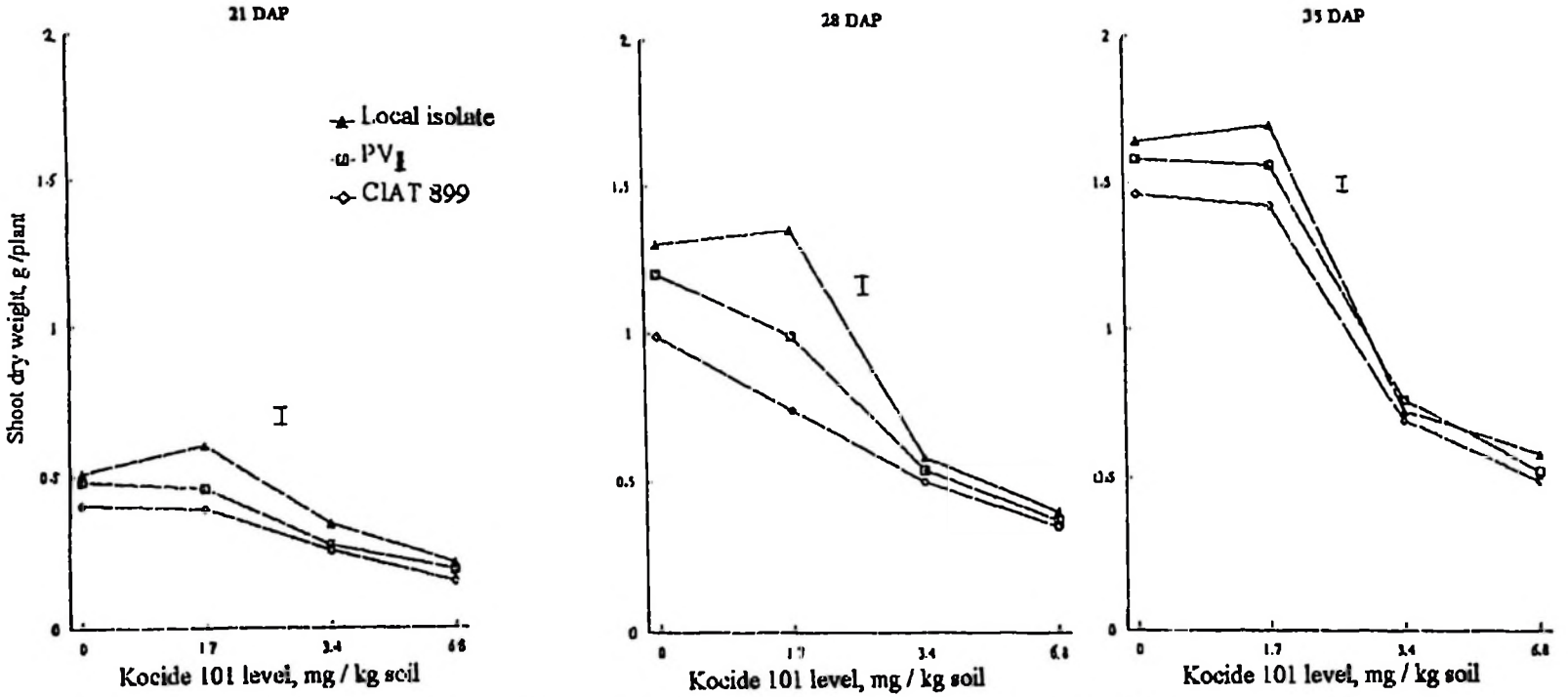

Figure 4. Effect of kocide 101 on shoot dry matter yields of bean plants inoculated with different rhizobial strains, at different sampling periods. Bars indicate one standard error. DAP = Days after planting.

The non-significant effect of kocide 101 on plant growth (shoot dry weights) when this fungicide was applied at the recommended rate implies that the toxicity of this chemical at this dose was not yet intense. The decrease in shoot dry weights that was recorded at the higher doses (2x and 4x the normal dose) implies an intense phytotoxic effect of the fungicide at the higher doses. The mechanism by which kocide 101 depressed bean plant growth cannot be explained by these results. However, it was reported by Kernikamp (1948) that the toxicity of Cu to *P. vulgaris* plants, related in part to the ability of Cu to displace other metal ions, particularly Fe, from the physiologically important centres. Also, the fungicide may have interfered not only with metal ions metabolism but perhaps also with the utilization of soil N, and this may have contributed to the retarded growth and plant chlorosis at the high fungicide application rates. It has been reported (Marschener, 1990) that when the supply of N to plants was sub-optimal, plant tissue development was retarded.

The higher bean dry matter weights associated with the local rhizobial isolate implies that this local isolate was more compatible with the bean variety used than were the other two strains. The fact that the higher shoot dry weight associated with the local isolate strain was consistent with nodulation (section 4.2.2) and N accumulation in plants (section 4.2.3) implies that this strain was more infective and effective than were the other strains. It has been shown (Renie and Kemp, 1983) that considerable differences can occur between rhizobia strains

in their infectiveness and effectiveness on a particular bean cultivar. Although the results of the present studies tend to point to a better performance by the local strain, their scope is narrow because only one strain was evaluated. Therefore they are, strictly, not conclusive. This better performance of the local isolate suggests that there is a need to screen for compatibility of rhizobia strains with their plant symbiont before a firm conclusion can be reached.

4.2.2 Nodulation

Kocide 101 applied at the recommended rate did not affect nodule numbers significantly relative to those in the controls, at either sampling period. However, a significant ($P < 0.01$) reduction in nodule numbers by about 50% and 75% was recorded when the fungicide was applied at 2x and 4x the recommended rate, respectively (Figure 5, Appendix 2). The trend was maintained at every sampling period. More nodules were recovered at 28 DAP, but this was observed only in the controls and at the normal fungicide application rate. At higher fungicide doses, a decrease in nodule numbers with increase in the length of the growth period was observed.

Nodule dry weights per plant showed trends similar to those of nodule numbers (Figure 6). At 21 DAP, the nodules on the plants grown on soil with higher concentrations of kocide 101 were still too small to be detached. Therefore data on nodule weights at that time could not be obtained. Hence, nodule weights

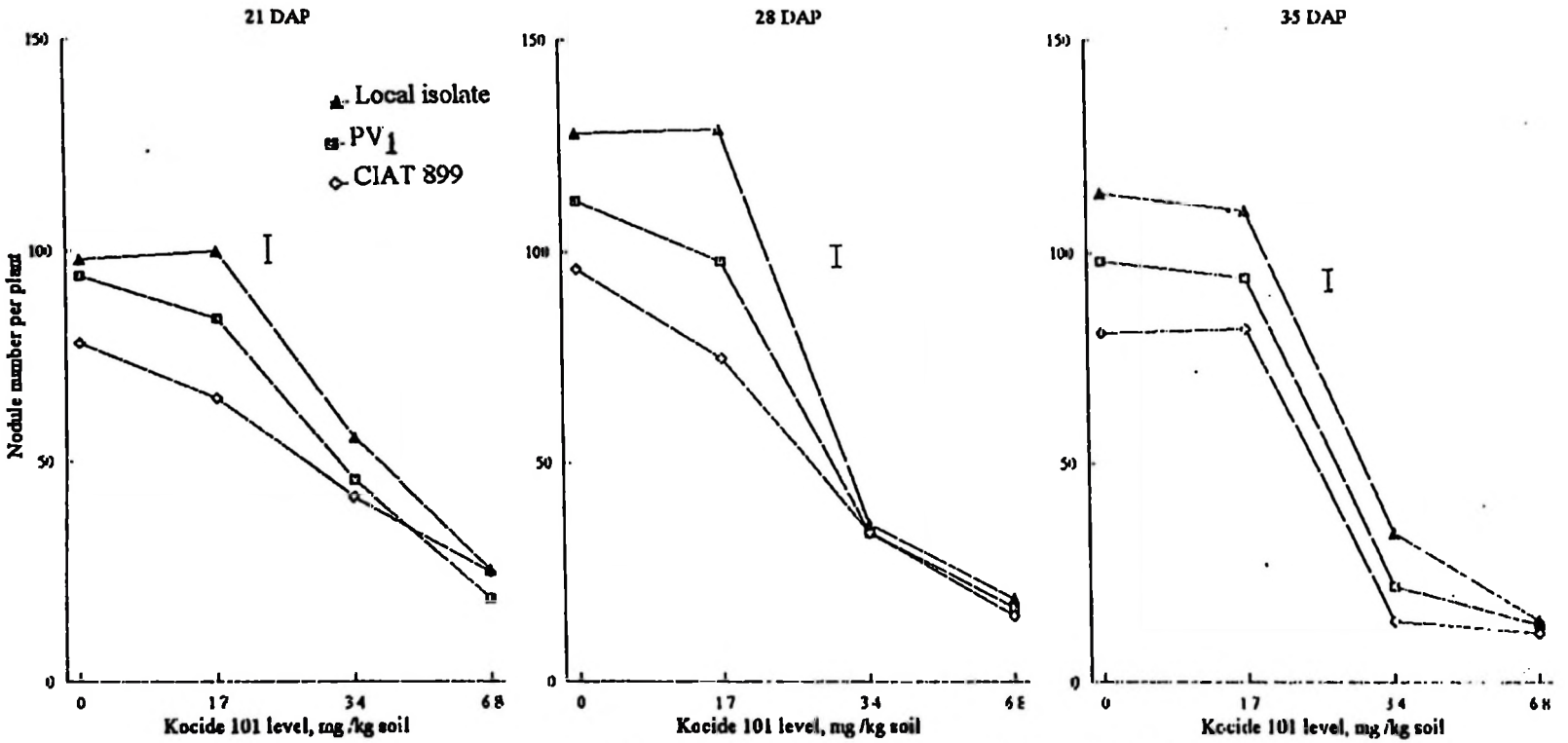

Figure 5: Effect of Kocide 101 on nodule numbers of bean plants, inoculated with different rhizobial strains, at different sampling periods. Bars indicate one standard error. DAP = Days After Planting.

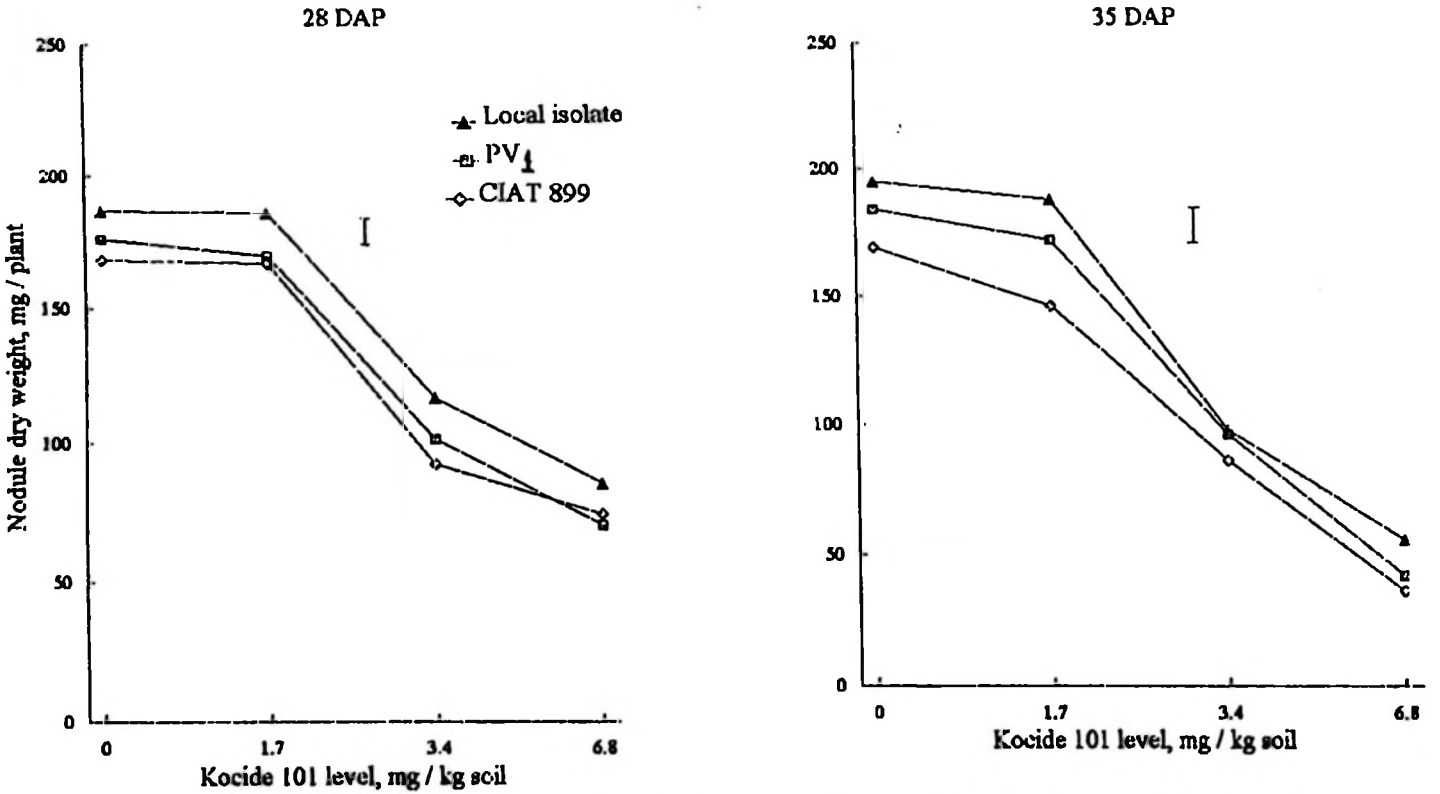

Figure 6: Effect of Kocide 101 on nodule dry weights of bean plants inoculated with different rhizobial strains. Bars indicate one standard error. DAP = Days After Planting

were reported only for the second and third sampling periods (Figure 6).

At the normal fungicide application rate and where no fungicides were applied (i.e. the zero rate) the different rhizobia strains differed in their nodule forming ability. The local isolate resulted in the highest ($P < 0.05$) nodule numbers, followed by the PV_1 strain, and the strain CIAT 899 resulted in the lowest nodule numbers. These observations were also reflected in the nodule dry weights, and were similar to the plant dry matter trends showed in Figure 4.

The lack of significant adverse effects on nodulation when kocide 101 was applied at the normal rate indicates that this fungicide at low levels did not have a significant inhibitory effect on nodulation. The reduction in nodule numbers and dry weights at higher fungicide concentrations is consistence with the toxicity of the fungicide at higher doses to plant growth (section 4.2.1 above). It is difficult to generalize from the results of this study whether the inhibition to nodulation was only due to a direct toxicity of the fungicide to the bean plants. It is possible that rhizobia also were affected by the fungicide. This could be possible because inhibition of rhizobia growth was observed *in vitro* (Figure 3) and was consistent with the reduced nodulation, N accumulation, and plant biomass yields of beans. However, the design of the present studies permits inferences to be drawn relating to the plant. The effect of the kocide 101 on rhizobia in the soil can only be

assumed as they could not be separated from the effect on the plants. This needs further studies.

The results of these experiments may have no direct comparisons presently, because the effect of this fungicide on the bean-rhizobia symbiosis is hardly documented. However, the results are in agreement with the observations by other workers (Curley and Burton, 1975; Chao and Alexander, 1981; Samuel *et al.*, 1989) that when used at higher concentrations, most fungicides impaired nodulation.

The observed higher nodule numbers at the onset of flowering (28 DAP) and the decrease in the numbers at the 50% flowering stage (35 DAP) is in total agreement with the observations made by Peoples *et al.* (1983) using cowpea (*Vigna unguiculata*) and Renie and Kemp (1983) using the common bean (*Phaseolus vulgaris* L.). Such a characteristic pattern of reduction in nodule numbers occurred during ontogenesis, and it was attributed to competition for photosynthates between the developing pods and the root nodules. This resulted in nodule senescence during the later part of the flowering period due to the decreased supply of carbohydrates to the nodules for their growth and respiration for the purposes of prolonging their longevity.

The observed differences in nodule number and nodule dry weights between plants inoculated with different rhizobia strains, which were significant ($P < 0.05$) at the zero and at the recommended fungicide application rates, may imply differences in compatibility of the rhizobia strains with the bean variety that was used. It is worth noting that the presently designated "local isolate" and the strain PV_1, which showed a superior performance in the present study, were isolated from soils in the Morogoro area whereas the strain CIAT 899 is an imported strain. Therefore, it may be concluded that the local rhizobia were more compatible with the SUA 90 bean variety than the foreign CIAT 899 strain was, and hence resulted in increased nodulation. The "local isolate" was obtained from the present experimental soil, in the University farm, the environment in which SUA 90 was bred. Again, this isolate was probably more compatible with SUA 90 bean plants than was the PV_1 strain which was isolated from a more distant soil. These results suggest that before a strain(s) is recommended for use in inoculants, it should be evaluated for compatibility with the intended bean cultivar(s). Strain x cultivar interactions have been reported for soybeans and their rhizobia (Caldwell and Vest, 1968). This phenomenon may exist in the bean-rhizobia system also. Further studies on this are therefore recommended.

Ahmed and Phelps (1990) provided evidence of the presence of variations in nodulation ability of different bean varieties inoculated with different rhizobia strains. Motility, chemotactic differences, the ability to colonise the rhizosphere,

and resistance to environmental stresses were reported to be possible factors leading to differences among strains in their infective ability to result in nodulation (Schroder, 1992).

At higher fungicide application rates, all rhizobia strains resulted in low nodule numbers. This was probably because the fungicide phytotoxicity was too intense that any differences between rhizobia strains may have been masked because the plants were then not functioning normally.

The results of the present study suggest that accumulation in soil of kocide 101 to high levels, as can be achieved after long-term use of the fungicide, may impair the bean plant growth, nodulation and result in reduced N_2 fixation and, ultimately, probably grain yields also.

4.2.3 N_2 fixation

Used at normal application levels, the kocide 101 fungicide had no inhibitory effect on N accumulation by bean plants (Figure 7). However, at higher application rates, kocide 101 reduced the N accumulation. The trend observed for N accumulation was the same as that for nodulation and plant dry matter yields discussed above. The pattern of N accumulation varied between plants inoculated with different rhizobia strains (Figure 7). The plants inoculated with the local isolate accumulated more N, followed by those inoculated with the PV_1 strain. The

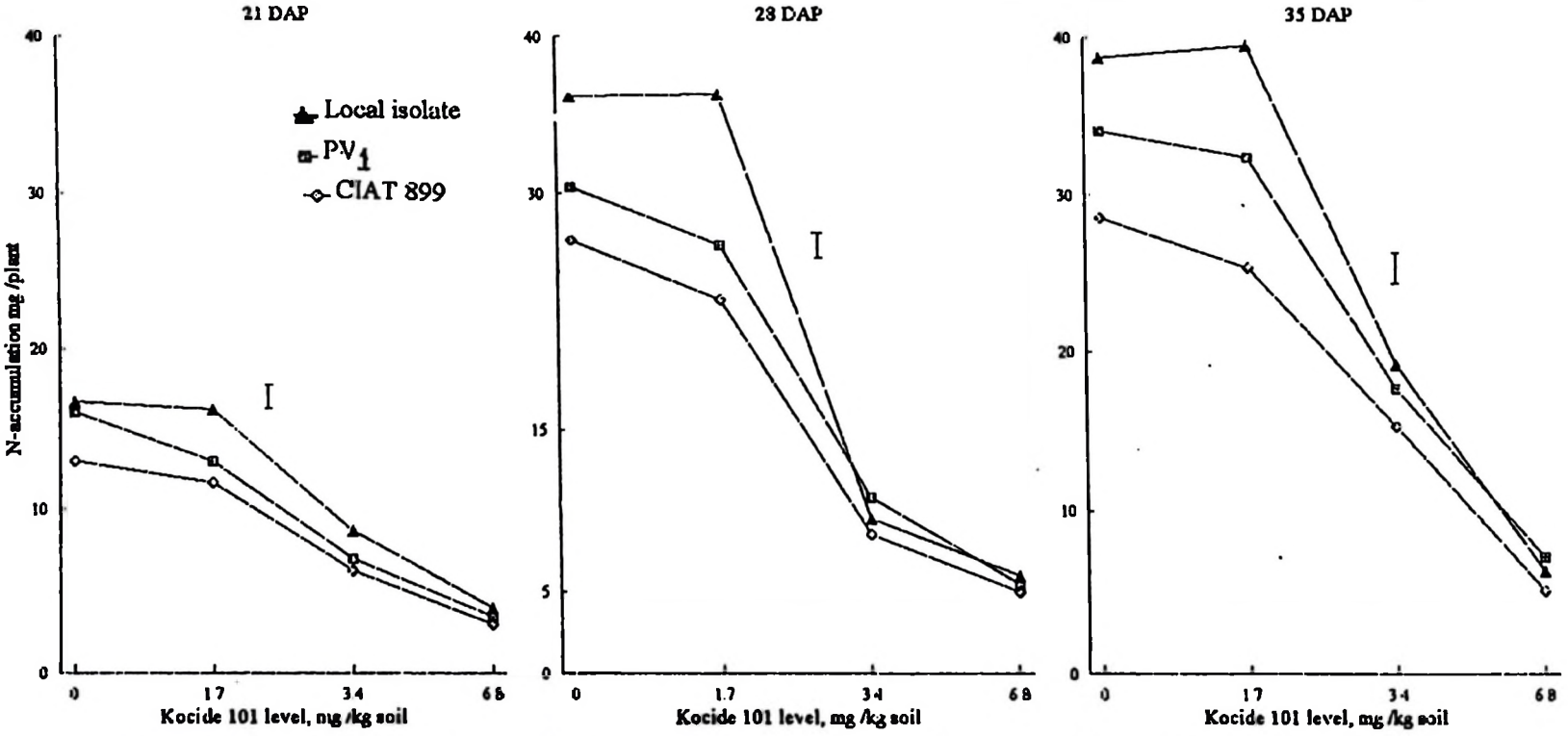

Figure 7. Effect of Kocide 101 on N-accumulation by bean pants, inoculated with different rhizobial strains, at different sampling periods. Bars indicate one standard error. DAP = Days After Planting.

plants that were inoculated with the CIAT 899 strain accumulated the least N. These trends were maintained at all the sampling periods.

The non-significant reduction in N accumulation by bean plants at the normal fungicide application rate indicates that this concentration had no adverse effect on the nodulation and N_2 fixation and probably soil N uptake. The lower N content of shoots that was recorded at higher fungicide concentrations may be attributed to the poor nodulation (section 4.2.2) that was observed at those high fungicide levels. Fisher and Hayes (1981) found that N_2 fixation appeared to be reduced mainly where the vigour and growth of the plant was inhibited by fungicides. This was attributed to reduced nodulation at higher fungicide concentrations which resulted to some degree from loss of photosynthesis necessary to nodule functioning. This finding (Fisher and Hayes), was consistent with the observation in the present study where declines in N accumulation (N_2 fixation) was parallel to decreases in plant growth, indicating the dependence of bean nodulation and N_2 fixation on photosynthesis.

The variations observed among rhizobia strains in the accumulation of N by the plants, probably reflected the rhizobia's ability to fix nitrogen in association with the bean plants. The results were similar and consistent with the trends of dry matter yields (Figure 4) and nodulation (Figures 5 and 6). Similar results were reported by other workers, for example Graham *et al.* (1980), using *Phaseolus*

vulgaris rhizobia; Fisher and Hayes (1981), using *Rhizobium trifolii* and Asanuma (1992) using *Bradyrhizobium japonicum* strains. Differences in competitiveness, effectiveness and compatibility of rhizobia strains with different host cultivars were cited to explain the observed differences in the studies cited. The results of the present study indicate the need to screen cultivars which will amply nodulate with naturally-occurring strains and which will fix significant amounts of nitrogen. Conversely, rhizobia strains should be screened for compatibility and effectiveness with intended bean cultivars, as already mentioned (section 4.2.2).

The *in vitro* rhizobia growth curve studies (section 4.1) were conducted in order to obtain more insight into the reasons behind the growth of bean rhizobia in the presence of kocide 101. While these *in vitro* studies were short-term incubation experiments, it was envisaged that they might shed more light on the effects of kocide 101 on rhizobia, hoping that the trends which emerged were a reflection (or representative) of the effects associated with long-term exposure to the pesticide and/or its residues. It was interesting to note that the inhibition of growth as reflected by the reduction of rhizobia numbers in the presence of kocide 101 (Figure 3) was consistent with the reduced nodulation, N_2 fixation, and plant biomass yields of beans as discussed in this section. These results would appear to suggest that the effect of kocide 101 on rhizobia should be further examined in the soil itself because of the indications given by the results of the *in vitro* growth curve studies.

The fact that the local isolate was the most tolerant to kocide 101 *in vitro* was also consistent with, and might partially explain, its superior nodulation and N_2 fixation which resulted in higher plant dry matter yields as reported in this section. Diatloff (1970) and Sellim *et al.* (1970) also observed differences in rhizobia tolerance to some chemicals (though not copper fungicides). The order of tolerance to kocide 101 *in vitro* in the present studies, namely the local isolate > PV_1 > CIAT 899 was also reflected in the performance of the bean-rhizobia symbiosis. This order may account for the fact that when the strains were evaluated in terms of nodulation, N_2 fixation and bean plant dry matter yields in the pot experiments, the local isolate consistently performed best, followed by the PV_1, and lastly, the CIAT 899 strain. The soil used in pot experiment was sterilized before use. Thus, biotic factors other than the introduced rhizobia were eliminated. It is recommended that further studies be initiated which will separate out the specific effects, if any, of kocide 101 on rhizobia in soil and their ability to fix nitrogen.

4.3 Residual effects of kocide 101 on growth and nodulation of bean plants

The residual effects of kocide 101, four months after it was first applied to the soil, as reflected by the shoot dry weights, nodule numbers, and nodule dry weights, are presented in Figure 8. The results show that the residues associated with the normal fungicide dose (equivalent to 1.7 mg kocide 101/kg soil) continued to have no adverse effects on plant growth and nodulation, as was the case in the

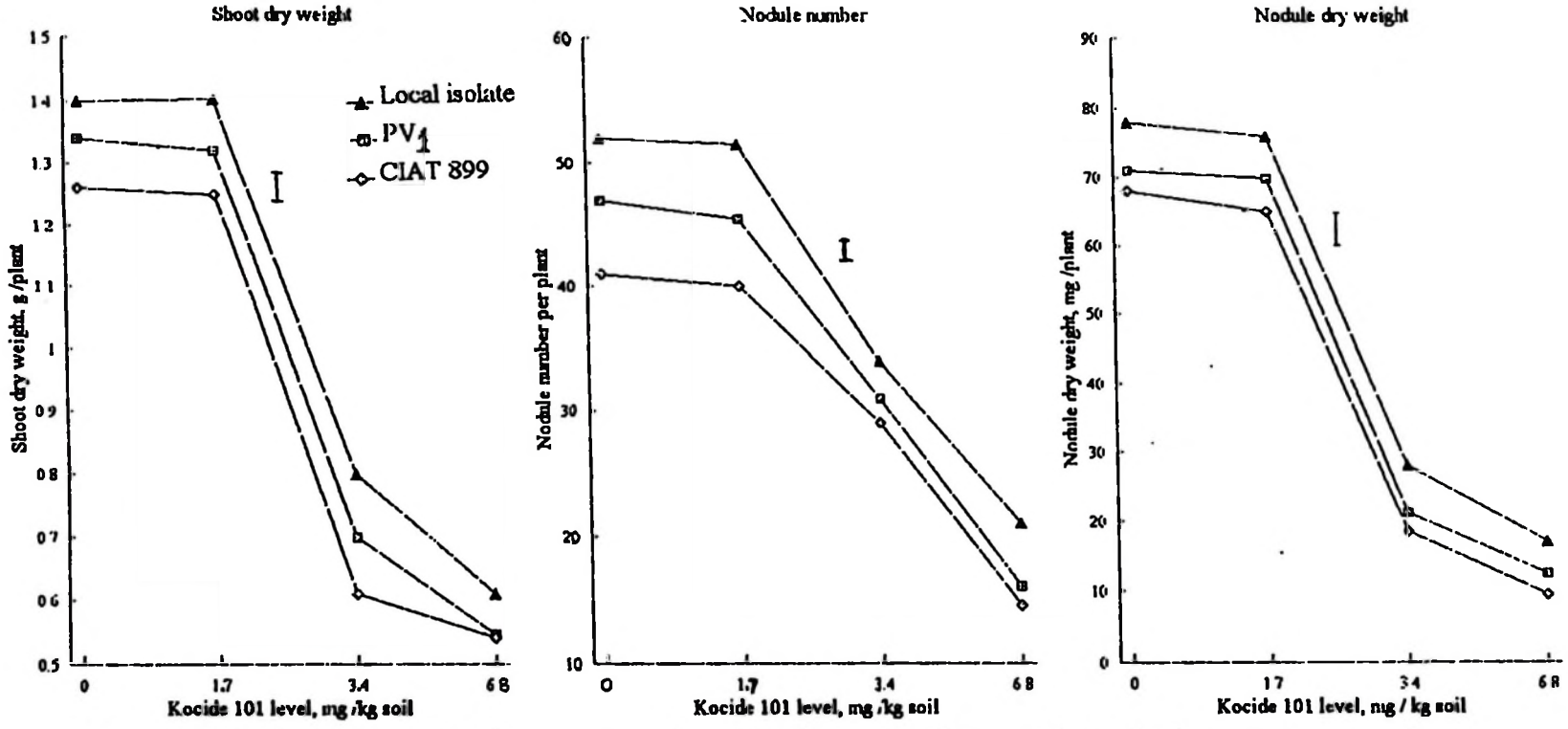

Figure 8. Residual effect of Kocide 101on shoot dry matter yield, and nodulation of bean plants inoculated with different rhizobial strains, at 28 days after planting. Bars indicate one standard error.

initial experiment. However, significant ($P < 0.05$) reductions in shoot dry weights as well as nodulation were observed in the pots which had received higher fungicide doses. Generally, the results were very similar to those of the initial experiment except that in the residual effects experiment, shoot dry weights were slightly higher, while nodule numbers and nodule dry weights were lower by about 50%, when compared to those from the initial experiment. The different rhizobia strains similarly resulted in differences in shoot dry weights and nodulation of bean plants as was observed in the initial experiment (section 4.2).

The lower nodule numbers and weights in the controls of the residual effects experiment as compared to the initial one, may be due to death of some rhizobia because of desiccation during the four months period between the initial experiment and that of the residual effects one when the soil was left dry. Desiccation may also have affected some rhizobia in the kocide 101 treatments so as to contribute, at least partially, to the lower nodulation in those treatments too. But the reduced nodulation in the kocide 101 treatments may be largely explained also by the toxicity of residues of kocide 101 to bean plants because these observations were made on the same pots used for the initial experiment. The same order of performance shown by the different strains, in this experiment when compared to the initial one (i.e. local isolate > PV_1 > CIAT 899 in both cases), further confirms that the kocide 101 residues were still toxic to the bean-rhizobia symbiosis, through their toxicity to bean plants.

4.4 Influence of oxadiazon on *Sesbania* rhizobia growth *in vitro*

The effect of oxadiazon on the growth curve of *Sesbania rostrata* rhizobia is presented in Figure 9. The bacterial growth was not affected by oxadiazon in the range of 0 to 50 μg oxadiazon/ml, as the growth of *Sesbania* rhizobia was abundant, reaching populations of $\log_{10} = 7$ or above (over 10 million cells/ml) within 24-36 hours (Figure 9). At high herbicide concentrations (100 and 200 μg/ml), an initial slow growth of the rhizobia was observed as evidenced by a presence of 24-hour lag phase (Figure 9). After the 24 hours bacterial growth was increasingly improved in the media treated with these higher herbicide concentrations. The growth levels attained by the rhizobia growing at these high concentrations were not significantly($P < 0.05$) different from those growing at lower oxadiazon concentrations and in the control at the termination of the experiment (i.e. 72 hours).

As reported earlier (section 3.3), the cells used to inoculate the media were obtained from rhizobia which were at the logarithmic phase of growth. This explains the logarithmic start-off of the growth curve for the rhizobia growing in media containing low herbicide concentrations (i.e. 0-50 μg/ml). At the higher levels of oxadiazon rhizobial growth may have been suppressed by the toxicity of oxadiazon, hence the prolonged lag phase (Figure 9). The increased growth of the rhizobia after the lag phase may imply that the rhizobia became quickly adapted and then tolerated those high herbicide levels. This observation may suggest that this herbicide may not affect the long term survival of the rhizobia. The symbiotic

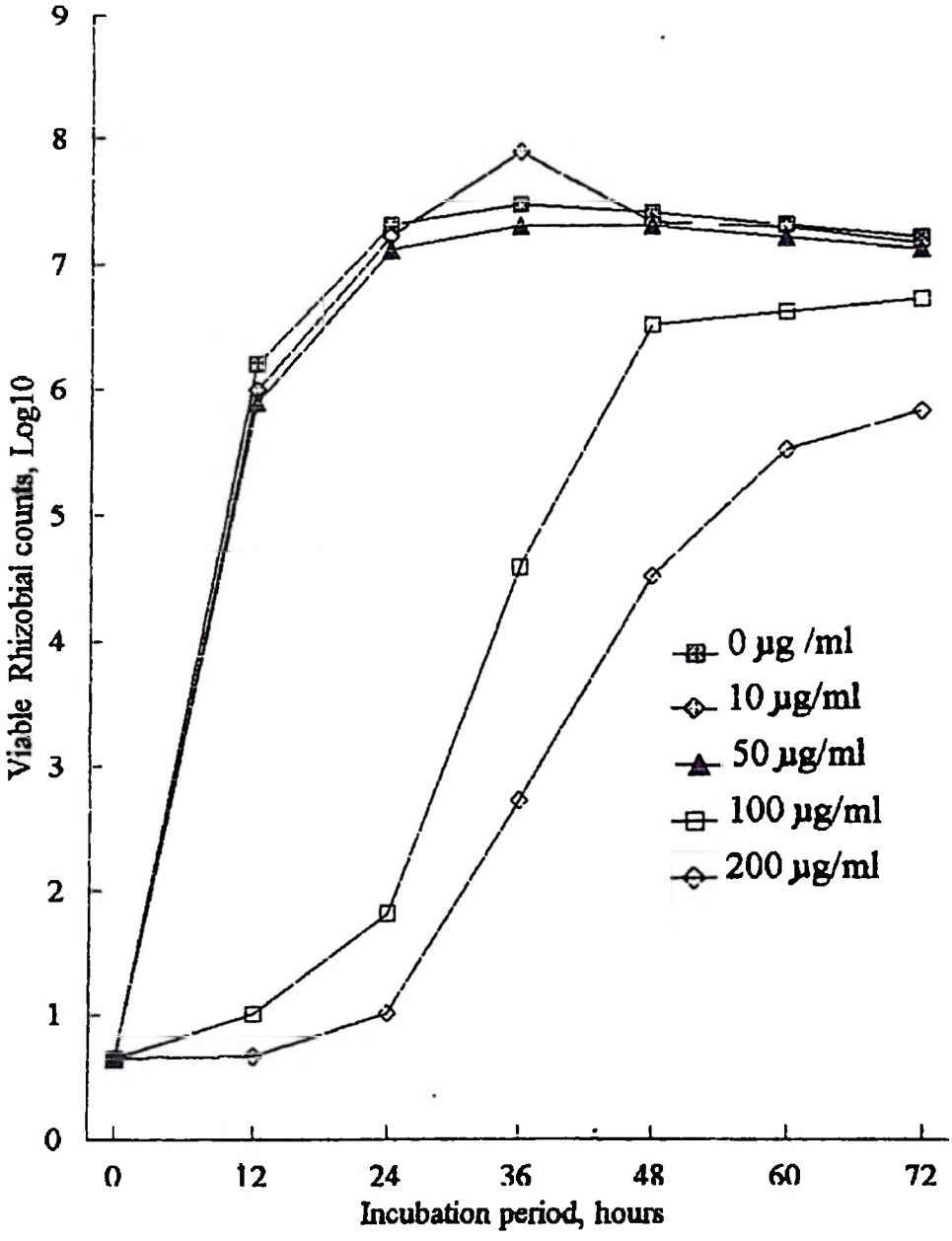

Figure 9. Effect of oxadiazon on growth curves of Sesbania rostrara rhizobia in vitro.

effectiveness of such surviving rhizobia would, however, need to be evaluated.

4.5 Effect of oxadiazon on the *Sesbania*-rhizobia symbiosis.

4.5.1 Growth and dry matter yields

The application of oxadiazon adversely affected the growth of the plants as indicated by a drastic reduction in shoot dry weights (Figure 10, Appendix 5). Growth inhibition was found to increase with increase in the exotoxin application rate. Plants grown on soil where oxadiazon was applied on the surface showed better growth than plants grown in soil in which oxadiazon was incorporated.

The dry matter yields of *Sesbania* plants were found to increase with the period of growth at all oxadiazon treatment levels. However, the biomass accumulated by the plants grown up to 45 days was not significantly ($P < 0.05$) different from those grown up to 60 days (Figure 10). This trend was maintained at all the oxadiazon levels.

The observed differences in performance of plants grown in the same soil, but treated differently with oxadiazon may be explained in terms of the injurious effect of the herbicide in contact with plant roots. The plants grown in the soil where oxadiazon was surface-applied, performed relatively better than those grown in soil in which the herbicide was uniformly incorporated. In the former case, roots were able to proliferate into deeper, less contaminated soil and, thus, escaped

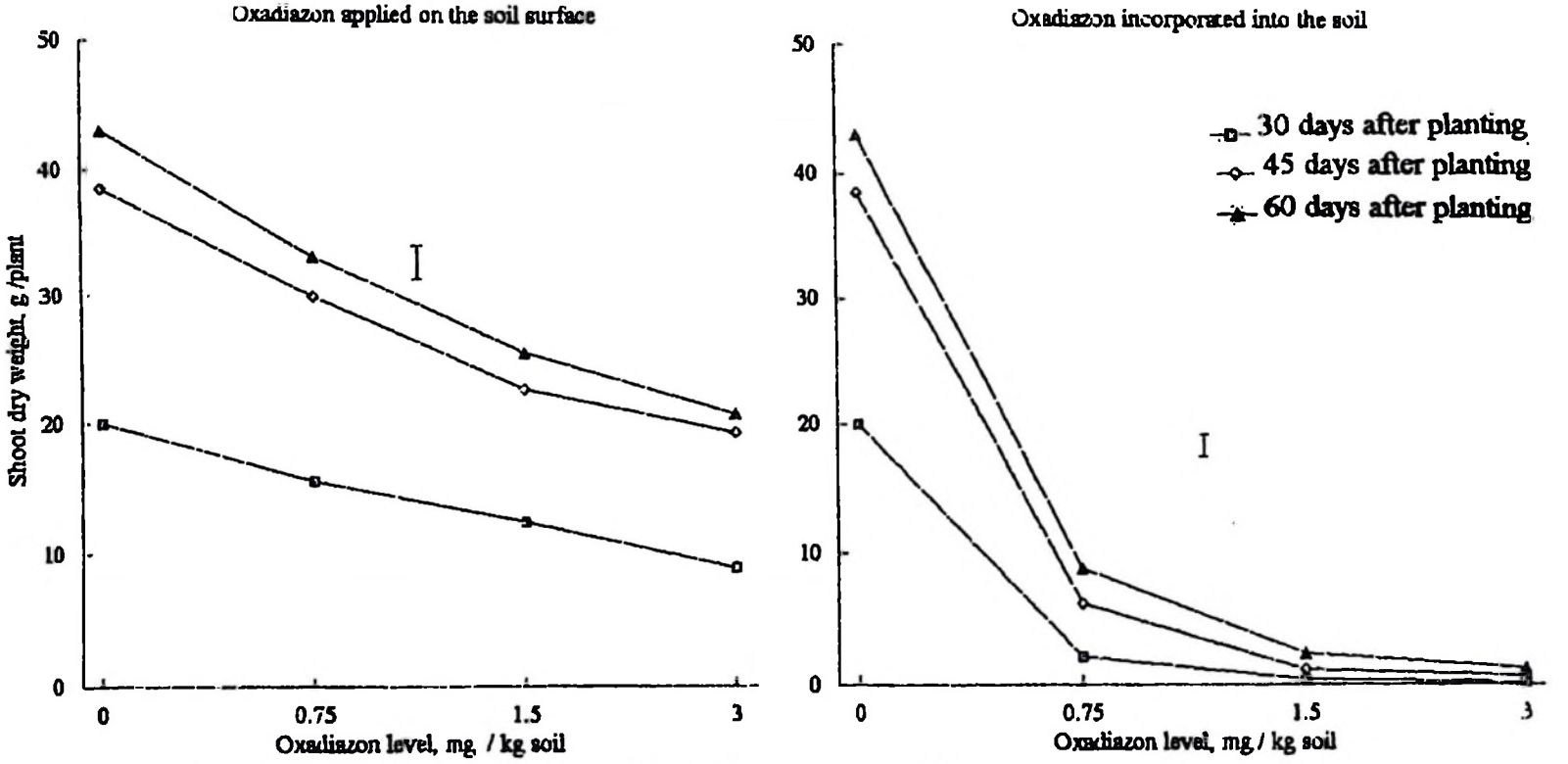

Figure 10. Effect of oxadiazon on shoot dry weights of Sesbania rostrata plants, at different sampling periods. Bars indicate one standard error.

contact with the herbicide which was concentrated on the soil surface. The vertical and lateral movement, and also the degree of leaching of oxadiazon has been reported to be minimum especially in heavy soils (Ambrose *et al.*, 1977), due to its strong adsorption onto the soil colloids and its low solubility in water. It is therefore possible in the present study that the surface-applied oxadiazon was adsorbed by the soil's colloidal matter and remained restricted to the surface soil.

For the plants grown in soil in which oxadiazon was uniformly incorporated, contact of roots with the herbicide was maintained and, therefore, they were continuously exposed to the herbicide's toxicity. The results of these experiments are similar to those by Okafor (1987), who reported higher phytotoxicity from dinitrimine on beans (*Phaseolus vulgaris*) when the seedlings were grown in a zone in which the herbicide was incorporated, and lower phytotoxicity when the herbicide was surface-applied.

The lack of a significant difference in dry matter yields between the plants grown for 45 days and those grown for 60 days indicates that the greatest accumulation of biomass takes place within 45 days. These results may imply that if the *Sesbania* plants were to be incorporated into the soil, the additional growth between the 45th and 60th days would result in insignificant contribution of biomass and N to the soil. The same observation was made on N accumulation (section 4.5.3 below).

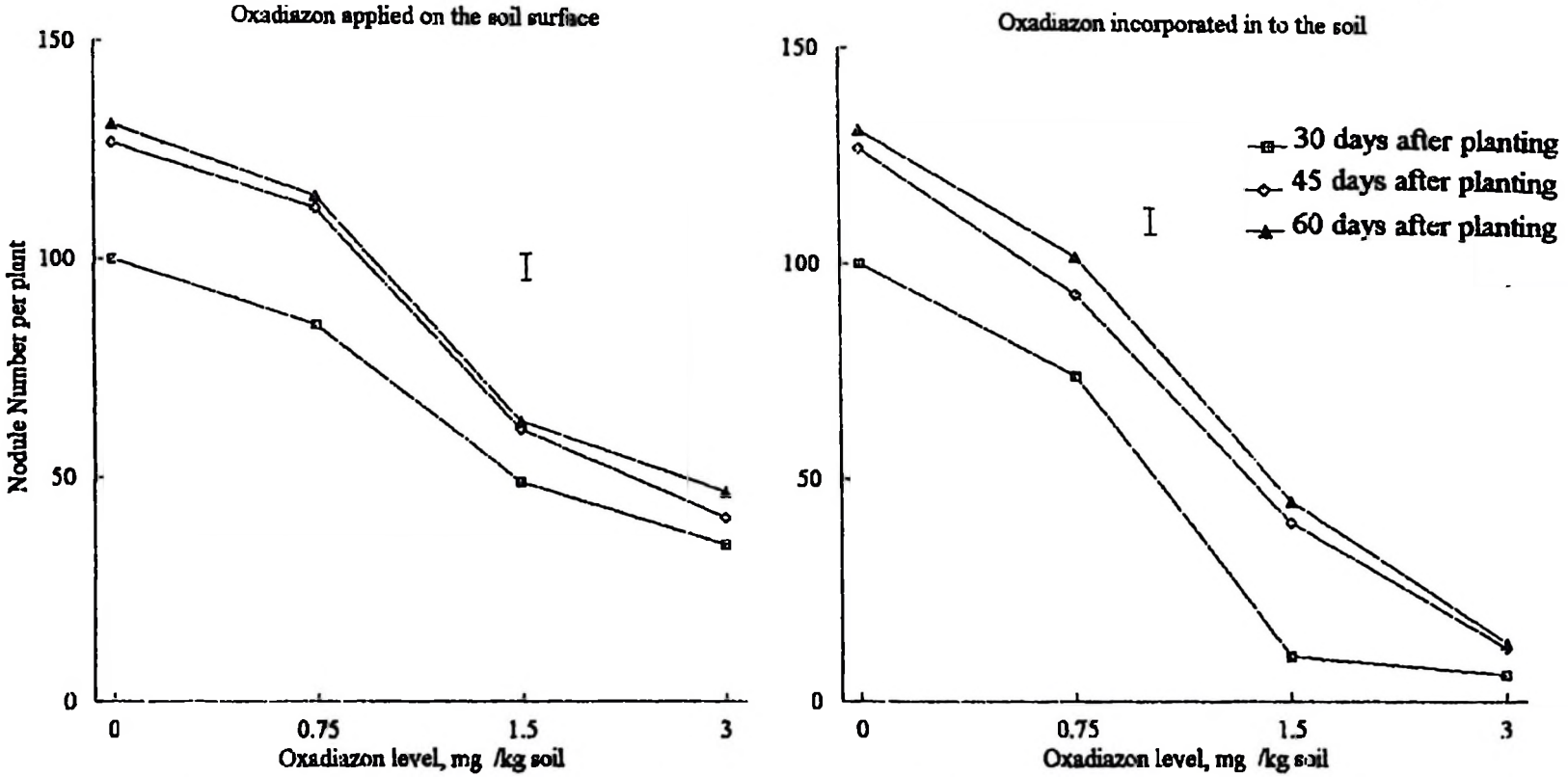

Figure 11. Effect of oxadiazon on nodule numbers of Sesbania rostrata plants at different sampling periods. Bars indicate one standard error.

4.5.2 Nodulation

The effect of oxadiazon on nodule numbers is shown in Figure 11. Oxadiazon significantly ($P < 0.01$) reduced the number of nodules formed per plant. The plants grown in soil in which oxadiazon was incorporated had fewer nodules when compared to those in which oxadiazon was applied on the surface. This trend was even more pronounced for the nodule dry weights (Figure 12, Appendix 6) particularly for the last two treatments (2x and 4x the recommended application rate). For the 2x and 4x the recommended rate, the reductions in nodule dry weights was approximately 50% and 75%, and 75% and 90% for plants grown in the soil where oxadiazon was surface applied, and for those grown in the soil in which oxadiazon was incorporated, respectively.

The nodule numbers and nodule dry weights obtained from plants at 45 days after planting did not differ significantly from those of plants at 60 days after planting at all oxadiazon treatment levels. Similar trends were observed on plant shoot dry matter yields and N-accumulation (sections 4.5.1 and 4.5.3, respectively).

The observed decrease in the nodulation of *Sesbania rostrata* following application of oxadiazon implies a toxic effect of this herbicide on nodulation. Present evidence (from section 4.4 and 4.5.1) indicates that to a large extent, the observed decrease in nodulation was brought about by the injurious effect of the herbicide on plant vigour and root growth rather than on rhizobial viability, for the

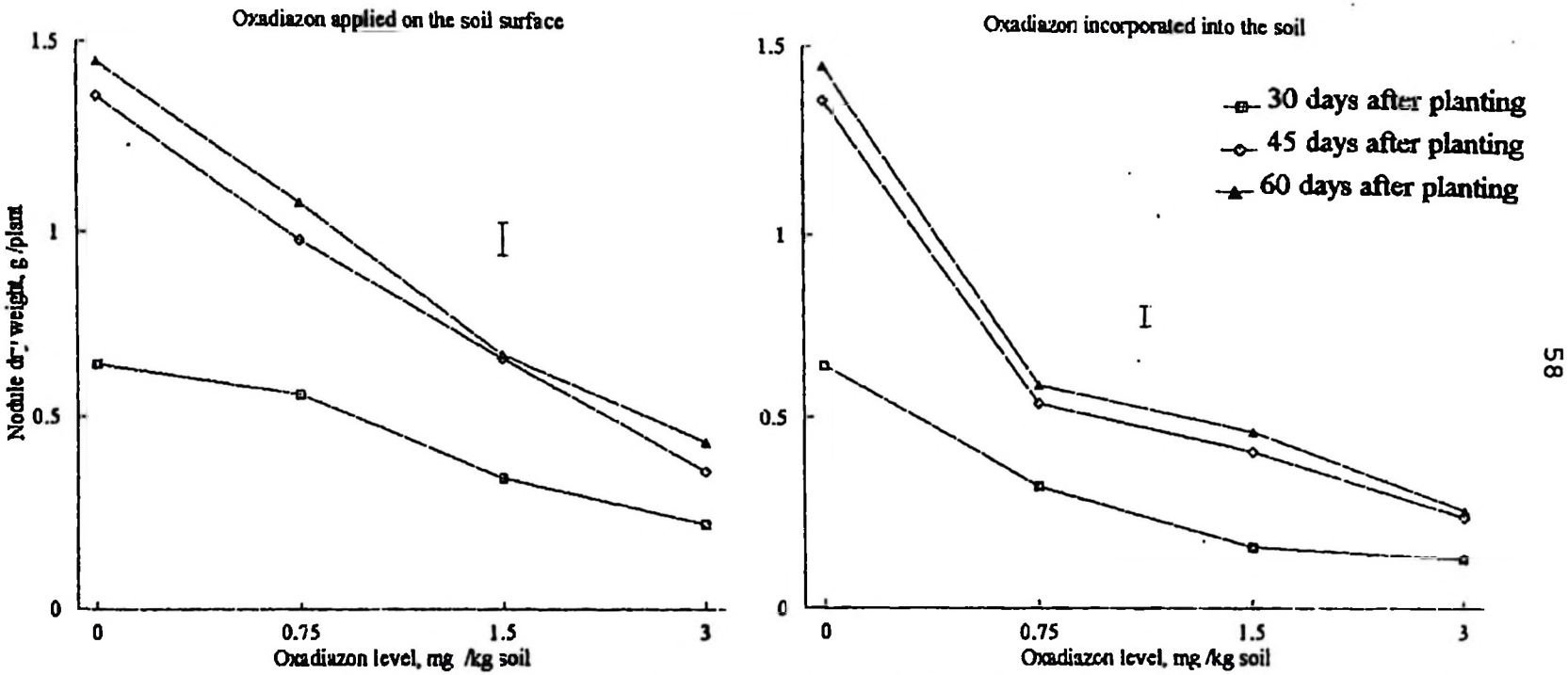

Figure 12 Effect of oxadiazon on nodule dry weights of Sesbania rostrata plants, at different sampling periods. Bars indicate one standard error.

rhizobia were not affected much by oxadiazon *in vitro*. It has previously been observed that the number of nodules was correlated with the extent of lateral root formation (Martensson and Nilsson, 1989). It was observed in the present work that the herbicide induced abnormal lateral root formation, which must have affected nodulation.

The numbers and dry weights of nodules obtained from plants at 45 days after planting did not differ significantly from those of plants at 60 days after planting. These results are not consistent with those reported by Lwekoramu (1993), who observed better nodulation at 60 than at 45 days after planting, using the same soil and under the same climatic conditions as in the present studies. The differences in plant performance from these two different experiments may be attributed to differences in rhizobia strains and plant cultivars that were used. The rhizobia strain that was used in the current study was cultured from *Sesbania rostrata* nodules collected from Igunga, Tabora, and the seeds were obtained from Mbarali, Mbeya, whereas the rhizobia strain and seeds that were used in Lwekoramu's experiment were obtained from Dodoma. These places occur in different agro-ecological zones, and it is possible that they favour the growth of different *Sesbania rostrata* cultivars harbouring different rhizobia strains.

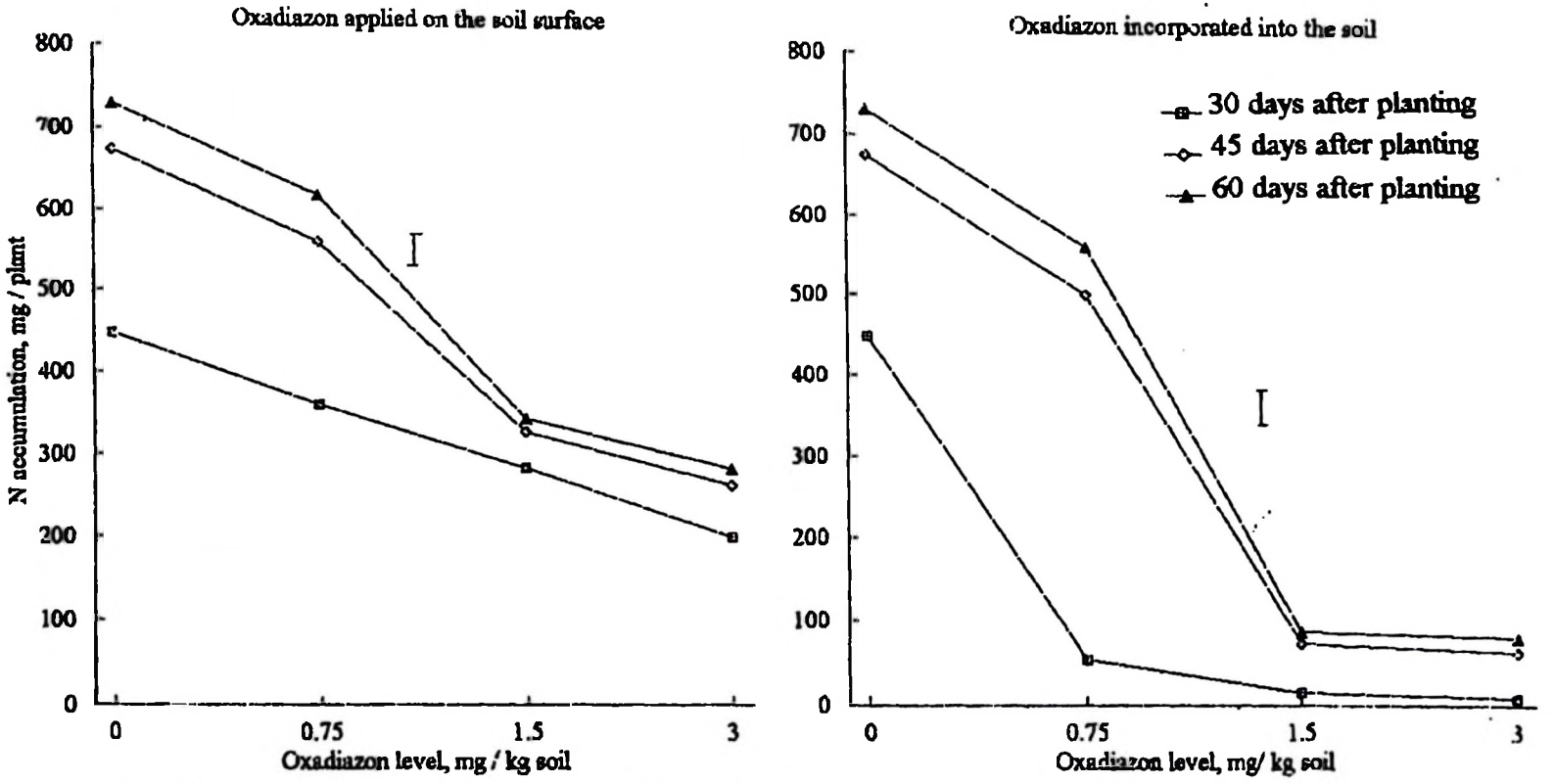

Figure 13. Effect of oxadiazon on N-accumulation by Sesbania rostrata plants at different sampling periods. Bars indicate one standard error.

4.5.3 N_2 fixation

Application of oxadiazon adversely affected N_2 fixation (Figure 13, Appendix 5). The N accumulation by plants was found to decrease with an increase in oxadiazon concentration. The trend for N accumulation was the same as that for nodulation and plant dry matter yields discussed above.

Evidence from the *in vitro* studies (section 4.4) above indicates that the oxadiazon herbicide did not have long-term toxicity effects on rhizobial growth, and probably its subsequent infection of the sesbania plants. The observed reduction in N_2 fixed following application of oxadiazon was brought about by the injurious effect of the herbicide on the plants not to the rhizobia itself. Results from section 4.5.1 indicate that the herbicide inhibited shoot growth, which resulted in a reduction in the quantity of the photosynthetic tissues required to supply the plant with sufficient photosynthates. This may have led to poor nodulation and, eventually, a reduction in N_2 fixation. A close correlation between the supply of photosynthates to roots and N accumulation was reported by Minchin and Pate (1974).

The results of the present study show clearly the adverse effect of oxadiazon on biological nitrogen fixation. Although this herbicide did not have much inhibitory effect to rhizobial growth from the *in vitro* study (section 4.4), it is toxic to the macro-symbiont.

The effect of some environmental factors, physical and biological, on the activity of oxadiazon can not be assessed through this glasshouse experiment. It is possible the effect of herbicide was severe in the glasshouse because of the absence of interacting factors which might have reduced the phytotoxicity of the herbicide. Under the aseptic conditions employed in the glasshouse the effects of soil biotic factors on the herbicide, e.g. microbial degradation, were absent. A field experiment(s) is recommended to obtain information on the effect of environmental factors (both physical and biological) on the activity of oxadiazon under relatively undisturbed/unmodified conditions.

4.6 Residual effects of oxadiazon on growth and nodulation of *Sesbania rostrata* plants

The residual effect of oxadiazon, four months after the herbicide was first applied to the soil, is presented in Figure 13. Plant growth, nodulation and N accumulation were still severely curtailed by the presence of oxadiazon residues in the soil. The nodule numbers and nodule dry weights of plants were reduced by about 35% and 48%; 60% and 70%; and 78% and 75% at the recommended application rate (0.75 mg/kg soil), 2x the recommended rate (1.5 mg/kg soil) and 4x the recommended rate (3 mg/kg soil), respectively. These trends are similar to those of the initial experiment, except that in this residual effects experiment, the shoot dry weights were lower by about 50%, at all oxadiazon application rates.

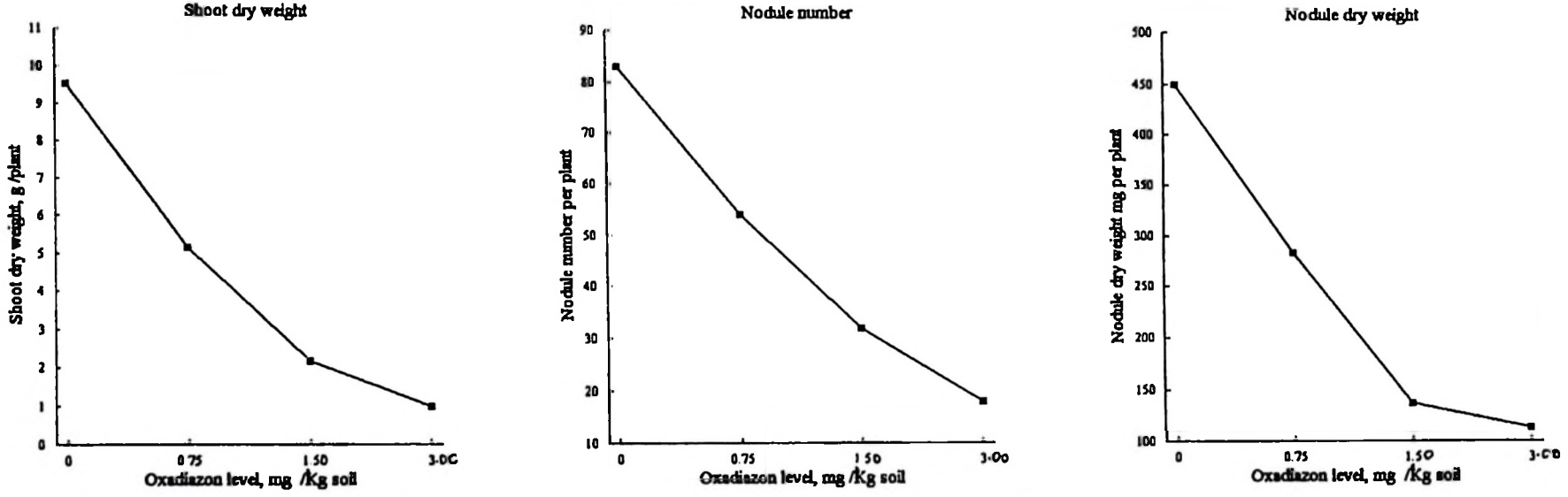

Figure 14. Residual effect of oxadiazon on shoot dry weights, nodule numbers and nodule dry weights of *Sesbania rostrata* plants at 30 days after planting.

The decrease in plant growth reported in this experiment may have been caused by the changes in climatic conditions. The initial experiment was conducted between October and December, the period with high solar radiation whereas this experiment was conducted between May and June, the period with low solar radiation. The biomass accumulation and height of *Sesbania rostrata* plants have been reported to correlate well with solar radiation, whereby the biomass accumulation and plant height increased with increase in solar radiation (Becker *et al.*, 1990).

The observed reduction in nodulation and dry matter yields of *Sesbania* plants indicates that oxadiazon was still toxic to nodulation and, thus, to N_2 fixation. These results suggest that in herbicide testing programmes, especially those involving legumes (as weeds or as crops to be protected from weeds), the effect of the herbicide on the symbiotic behaviour should be an integral part of the programmes. Also, the carryover effects of herbicides applied in previous seasons should be studied before the pesticides are recommended for use in the presence of nitrogen fixing systems. The period of four(4) months studied in the present case is too short, as was dictated by circumstances. Longer periods should be studied for this or other herbicides to gain knowledge as to which herbicide would leave less toxic residues

CHAPTER FIVE

5.0 SUMMARY AND CONCLUSIONS

Kocide 101 in the broth medium inhibited rhizobial growth. The kocide 101 concentrations which resulted in almost total inhibition of growth were 100, 50, and 10 μg/ml for the strain designated as "local isolate", the PV_1 strain, and the CIAT 899 strain, respectively. Thus of the three strains tested for sensitivity against kocide 101, the local isolate strain was most tolerant followed by PV_1 strain, and the strain CIAT 899, least tolerant. The growth of the sesbania rhizobia strain in broth medium was unaffected by the addition of 10 or 50 μg oxadiazon/ml, but a partial/initial inhibition in rhizobial growth was observed with the addition of 100 or 200 μg oxadiazon/ml.

The application of kocide 101 at the recommended application rate (equivalent to 1.7 mg/kg soil) had no harmful effect on growth, nodulation or nitrogen content of bean plants. Kocide 101 applied at 2x and 4x the recommended application rate (equivalent to 3.4 and 6.8 mg/kg soil) proved detrimental to bean plant growth, nodulation and N_2 fixation. The inhibition of nodulation and N_2 fixation in the presence of kocide 101 was to a large extent due to the direct effect of the fungicide on bean plants.

The application of oxadiazon at the recommended application rate, 2x and at 4x the recommended rates (equivalent to 0.75, 1.50, and 3 mg/kg soil, respectively) adversely affected the growth, nodulation and N_2 fixation by *Sesbania rostrata* plants, the inhibition increasing with increase in oxadiazon concentration, and with the extent of herbicide contact with the plant roots (i.e. surface-applied vs soil-incorporated herbicide). The inhibition of nodulation and N_2 fixation in the presence of oxadiazon was, to a large extent, due to adverse effects of the herbicide on plant growth and development rather than on rhizobial growth.

For both beans and *Sesbania* plants, nodule formation and plant growth continued to be curtailed by the pesticides residues 4 months after they were first applied to the soil.

From the results of the present study, it is tentatively concluded that:

1. When used at concentrations higher than the recommended application rate kocide 101 resulted in poor nodulation and low N_2 fixation due to its adverse effects on plant growth rather than on rhizobia.

2. Of the bean rhizobial strains tested, the strain isolated from the experimental soil (local isolate) was more tolerant to kocide 101 *in vitro* when compared to the strain which was isolated from a more distant soil

(PV_1) and the introduced strain (CIAT 899), in that order. This strain was also more compatible with the bean variety used.

3. Oxadiazon had no significant inhibitory effect on the growth of *Sesbania* rhizobia. Its inhibitory effect on N_2 fixation was brought about by its injurious effects on *Sesbania* plant growth rather than on the rhizobia.

4. Kocide 101 and oxadiazon residues "four months after they were first applied" proved detrimental to plant growth and the legume rhizobia symbiosis. From this observation, it is suggested that pesticides be screened for their effects on symbiotic nitrogen fixation, and their carry over effects from previous applications, before being recommended for use in the presence of nitrogen fixing systems.

5. The results of the present glasshouse study suggest that if pesticides which are toxic to leguminous plants and rhizobia are applied to soils, at higher concentrations than recommended, they may affect their symbiotic performance and thus, reduce the benefit of this symbiosis to agricultural production. The higher concentrations may build up due to repeated applications of pesticides. It is recommended that field trials be conducted in order to confirm the result of this laboratory/glasshouse study.

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APPENDICES

Appendix 1. Effect of Kocide 101 application on dry matter yield by bean plants inoculated with different rhizobia strains at 21, 28 and at 35 days after planting

Kocide 101 rate mg/kg soil	Sampling priod (days)														
	21 Rhizobia strain					28 Rhizobia strain					35 Rhizobia strain				
	Local isolate	PV_1	CIAT 899	Mean	LSD (P=0.05)	Local isolate	PV_1	CIAT 899	Mean	LSD (P = 0.05)	Local isolate	PV_1	CAT 899	Mean	LSD (P=0.05)
	---------- mg/plant ----------														
0.0	1.13	0.48	0.40	0.67		2.30	1.37	0.99	1.55		2.64	1.58	1.43	1.88	
1.7	1.15	0.46	0.39	0.66		2.34	1.36	0.74	1.48	± 0.1	2.60	1.56	1.42	1.86	
3.4	0.34	0.27	0.23	0.28	± 0.13	0.67	0.54	0.55	0.58		0.72	0.69	0.69	0.7	±0.01
6.8	0.18	0.19	0.15	0.17		0.40	0.38	0.37	0.38		0.56	0.52	0.49	0.52	
Mean	0.70	0.35	0.29			1.43	0.91	0.66			1.63	1.09	1.01		
LSD(P=0.05)		±0.11					±0.04					±0.01			

Appendix 2: Effect of Kocide 101 on nodule number of bean inoculated with different rhizobia strains at 21, 28 and at 35 days after planting

Kocide 101 rate mg/kg soil	Sampling period (days) 21 Rhizobia strain					28 Rhizobia strain					35 Rhizobia strain				
	Local isolate	PV_1	CIAT 899	Mean	LSD(P=0.05)	Local isolate	PV_1	CIAT 899	Mean	LSD(P=0.05)	Local isolate	PV_1	CIAT 899	Mean	LSD(P=0.05)
	Number per plant														
0.0	94	85	78	86		128	112	96b	112		114	98	81	98	
1.7	100	65	61	76	± 16	130b	98b	75	101	±15.88	110	94	82	95	± 11
3.4	56	46	42	48		36	34	34	34		34	22	14	23	
6.8	25	19	20	21		19	17	15	17		14	13	11	12	
Mean	67	56	50			78	65	55			68	56	47		
LSD (P=0.05)		±12					±14					±10			
CV %		29.21					24.55					20.35			

Appendix 3. Effect of Kocide 101 on nodule dry weights of bean plants inoculated with different rhizobia strains, at 21, 25 and at 35 days after planting

Kocide 101 rate mg/kg soil	Sampling period (days)													
	21 Rhizobia strain					28 Rhizobia strain					35 Rhizobia strain				
	Local isolate	PV_1	CIAT 899	Mean	LSD(P = 0.05)	Local isolate	PV_1	CIAT 899	Mean	LSD(P= 0.05)	Local isolate	PV_1	CIAT 899	Mean	LSD(P= 0.05)
	---------- mg/plant ----------														
0.0	32.33	26.67	12.43	23.81		188.01	176.00	106.35	156.78		195.00	184.00	169.00	182.67	
1.7	30.67	16.00	12.60	19.80	-	186.00	170.00	102.21	152.74		188 33	172.00	146.00	168.78	
3.4	ND	ND	ND			110.03	102.00	94.33	102.22	±25.14	96.00	96.00	55.24	82.41	±16.47
6.8	ND	ND	ND			86.02	71.00	75.00	77.33		56.00	42.00	36.00	44.65	
Mean	-					142.55	129.75	99.67			132.83	123.50	101.56		
LSD(P = 0.05)		-					±25.14					±14.26			
CV (%)		-					23.25					14.08			

ND = Not determined

Appendix 4. Effect of Kocide 101 application on N-accumulation by bean plants inoculated with different rhizobia strains, at 21, 28 and at 35 days after planting

Kocide 101 rate mg/kg soil	Sampling period (days)													
	21					28					35				
	Rhizobia strain					Rhizobia strain					Rhizobia strain				
	Local isolate	PV_1	CIAT 899	Mean	LSD(P=0.05)	Local isolate	PV_1	CIAT 899	Mean	LSD(P=0.05)	Local isolate	PV_1	CIAT 899	Mean	LSD (P=0.05)
						Plant									
0.0	16.67	16.00	13.00	15.22		36.67	34.00	28.30	33.72		36.18	30.37	19.95	28.83	
0.7	16.20	12.56	11.34	13.37	±1.54	39.50	32.33	28.66	33.50	±2.96	36.30	26.70	23.22	28.74	±3.85
3.4	8.73	6.97	6.27	7.32		18.55	17.67	19.93	18.43		9.00	8.47	10.80	9.59	
6.8	4.00	3.50	3.02	3.51		10.38	6.87	5.11	7.45		6.00	5.43	4.00	5.14	
Mean	11.40	9.76	8.41			26.77	22.72	20.34			21.99	18.33	13.91		
LSD(P=0.05)		±1.33					±2.57					±3.34			
CV (%)		15.93					13.01					21.75			

Appendix 5. The effect of oxadiazon application on dry matter yield and N-accumulation by *Sesbania rostrata* plants at 30, 45 and at 60 days after planting

Oxadiazon level mg/kg soil	Dry matter yield g/plant						N-accumulation mg/plant					
	OASS			OIS			OASS			OIS		
	30	45	60	30	45	60	30	45	60	30	45	60
0.00	19.83ef	38.48ab	43.09a	20.00c	38.48ab	43.09a	447.00d	673.33ab	730.00a	447.00d	673.33ab	730.00a
0.75	15.60gh	30.00c	33.21c	2.12f	6.16e	8.87d	360.00e	560.00c	618.00b	53.67ef	500.00c	560.00b
1.50	12.60h	22.74de	25.60d	0.41g	1.12fg	2.30f	283.00g	327.00f	343.00f	14.33f	72.00e	86.00e
3.00	9.00i	16.38fg	20.55e	0.13g	0.64fg	1.20fg	200.00g	263.00g	283.00g	8.30f	6.30f	80.00e
CV (%)		10.95			11.22			6.95			12.59	

Means in a column followed by the same letter do not differ significantly (P = 0.01), according to Duncan's New Multiple Range Test

OASS = Oxadiazon applied on the soil surface

OIS = Oxadiazon incorporated into the soil

Appendix 6 Effect of oxadiazon application on nodulation by *Sesbania rostrata* plants at 30, 45 and at 60 days after planting

Oxadiazon level mg/kg soil	Nodule number/plant						Nodule dry weight, g/plant					
	OASS			OIS			OASS			OIS		
	30	45	60	30	45	60	30	45	60	30	45	60
0.00	100cd	127ab	131a	100b	127a	131a	0.64de	1.36ab	1.40a	0.64b	ab	1.45a
0.75	85d	112bc	115abc	59c	93b	102b	0.56de	0.98bcd	1.08bc	0.32g	0.54d	0.59c
1.50	49ef	61e	63e	10d	40c	45c	0.34e	0.99bcd	0.67cde	0.16i	0.41f	0.47ef
3.05	35f	41e	47ef	6d	12d	13d	0.22e	0.36e	0.26e	0.13i	0.20f	0.26gh
CV (%)		15.05			23.89			42.18			9.21	

Means in a column followed by the same letter do not differ significantly ($P = 0.01$) according to Duncan's New Multiple Range Test

OASS = Oxadiazon applied on the soil surface

OIS = Oxadiazon incorporated into the soil