ATTEMPTS TO RELEASE AND UTILIZE SOIL NUTRIENTS BY MEANS OF CHELATING AGENTS

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CONTENTS

SUMMARY

The first chapter is a review in four parts. Part one considers, firstly, the forms in which trace metals appear in soil, methods of extraction, and the relation between the quantity of metal extracted by reagents and the quantity naturally available to the plant, and, secondly, the nature of soil organic matter, its extraction and fractionation. Part two reviews both the evidence for and studies into the nature of trace metal-organic matter interactions. Part three discusses various methods of applying trace metals to soil. Finally, in the last part, consideration is given to the influence on trace metal levels in plants of chelating and complexing agents (trace metal free).

Chapter 2 presents the results of the initial pot and field experiments. Small quantities (0 - 150 μg/100 g soil) of the chelating agents EDDHA, EDTA, and 2-ketogluconic acid, were soil-applied to acid and limed soil. There was no significant effect on trace metal content or upon yield of the pot-grown bean Phaseolus vulgaris var. Canadian Wonder. On comparing results from the two experiments it was noted that plants grown on limed soil were taller, thinner, more easily broken and less green than plants grown on acid soil. Most trace metals become less available as soil pH increases and this was reflected in lowered manganese and zinc contents in the lime-grown plants. Stem iron levels, however, were higher than, or comparable with, those of acid-grown plants, which suggests the chlorosis occurred not because there was insufficient iron in the plant but because the iron present was in an unusable form.

In field experiments, bicarbonate, citrate, EDDHA and 2-ketogluconic acid, all soil-applied, in the same concentration range, to the same species, also had no significant effect on yield or upon trace metal content.

Subsequent pot experiments using the same species are discussed in Chapter 3. Applications in the range 0 - 1000 μg/100 g soil showed the chelating agents citrate, EDTA, and 2-ketogluconic acid to have little significant effect upon trace metal levels or upon yield.

Some trends were observed, however, and, while not statistically significant, it was felt that these indicated that further research might be worthwhile.

Iron, manganese and zinc chelates, soil-applied, in the same range, were rather more successful but their effects were not straightforward. For example, increasing iron EDTA applications raised iron and also manganese levels. Manganese EDTA not only raised manganese levels but gave the highest iron levels while producing the lowest zinc levels. On the other hand, changing the application level of zinc EDTA had no significant effect in decreasing or increasing zinc levels although zinc EDTA did produce the highest levels of zinc in the plant root.

In Chapter 4, a series of autoradiographic experiments were conducted to determine if a radioactive label incorporated in some of the chelating agents used in both pot and field experiments could be absorbed by the bean plants. Using ^{14}C-labelled bicarbonate, citrate and EDTA, it was observed that ^{14}C was absorbed into the root and translocated to the stem with all three agents, although translocation seemed less pronounced with citrate than with EDTA and bicarbonate. Examination of the control plant negatives suggested that the $^{14}C\text{-}HCO_3^-$ supplied to the treated plant was breaking down with the release of $^{14}CO_2$ (g) which then became available to the control during photosynthesis. Investigations into the possibility that this breakdown occurred in the soil are described in the Appendix. Manometric experiments using sucrose solutions indicated that manometry would be unsuitable for the detection of the small volume changes to be expected from the quantities of $^{14}C\text{-}HCO_3^-$ involved in the autoradiographic experiments. β-scintillation, however, was found to be capable of detecting small volumes of labelled gas and, being highly specific, pinpointed the source of the $^{14}CO_2$ (g) evolved. The problems of incomplete incorporation of sample in scintillant and of phase separation require to be overcome.

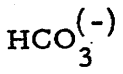
Chapter 5 reviews the work of the thesis and suggests possible future work.

ABBREVIATIONS USED IN THIS THESIS

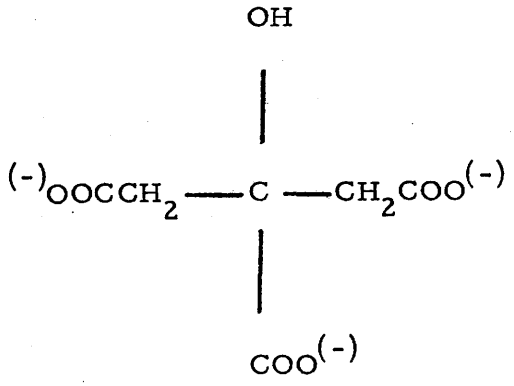
CDTA	($C_{14}H_{22}O_8N_2$)	Cyclohexanediaminetetra-acetic acid
DTPA	($C_{14}H_{23}O_{10}N_3$)	Diethylenetriaminepenta-acetic acid
EDDHA	($C_{18}H_{20}O_6N_2$)	Ethylenediamine di (o-hydroxy-phenylacetic acid)
EDTA	($C_{10}H_{16}O_8N_2$)	Ethylenediaminetetraacetic acid
EGTA	($C_{14}H_{24}O_{10}N_2$)	Ethyleneglycol-bis (2-amino-ethylether) tetraacetic acid
HEDTA/HEEDTA	($C_{10}H_{18}O_7N_2$)	Hydroxyethylethylenediamine-triacetic acid
NTA	($C_6H_9O_6N$)	Nitrilotriacetic acid

SOME CHELATING AGENT STRUCTURES

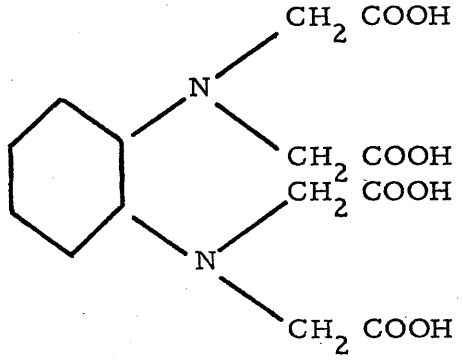
1. Bicarbonate*



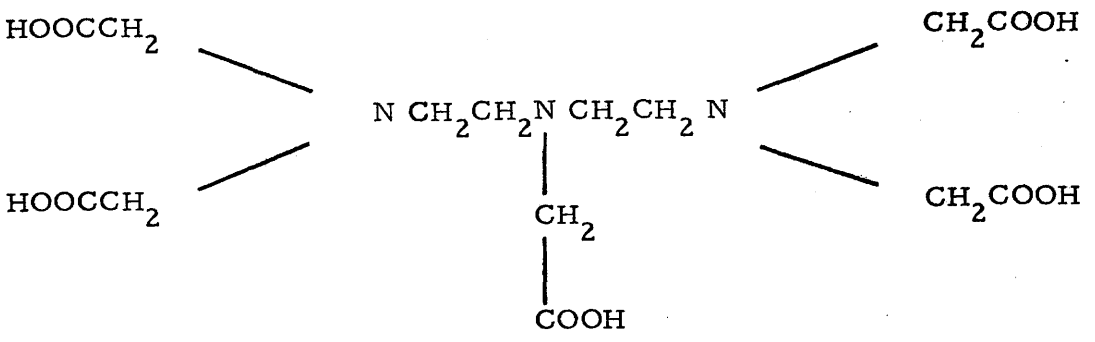
2. Citrate*



3. CDTA

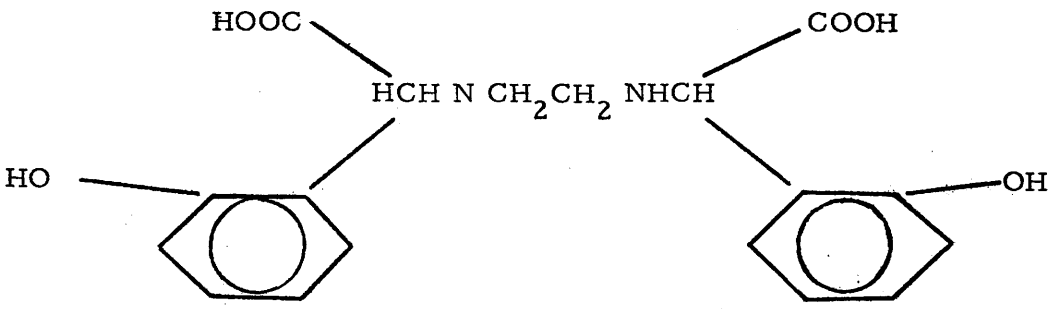


4. DTPA

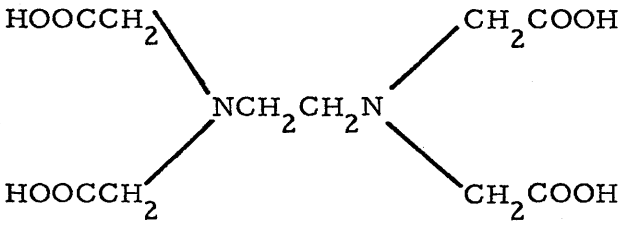


* Used in experimental work

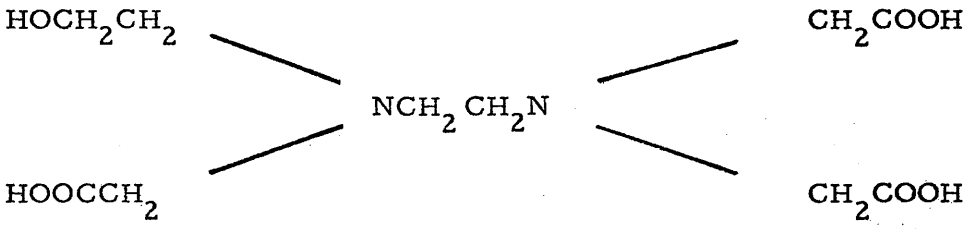
5. EDDHA*



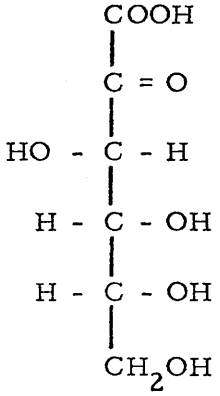
6. EDTA*



7. HEDTA



8. 2-ketogluconic acid*



* Used in experimental work

NOTE ON NOMENCLATURE

The term chelating agent has been used in this thesis although in some cases (e. g. where bicarbonate is mentioned) the term complexing agent would be more correct.

CHAPTER 1

INTRODUCTION

1.1 TRACE METALS

Trace metals in soil may be defined as those metals which are present at such low levels that their concentrations are usually measured in parts per million. The following table indicates concentration ranges for a few of the trace metals commonly found in soil.

Table 1.1.1 The range in micronutrient content commonly found in soils

Nutrient	Normal Range	
	%	parts per million
Fe	0.5 - 5.0	5,000 - 50,000
Mn	0.020 - 1.0	200 - 10,000
Zn	0.001 - 0.025	10 - 250
B	0.005 - 0.015	5 - 150
Cu	0.0005 - 0.015	5 - 150
Co	0.0001 - 0.005	1 - 50
Mo	0.00002 - 0.0005	0.2 - 5

From Buckman and Brady, 1969, p. 23.

Interest in trace metals in soil has increased over the last twenty to thirty years largely as a result of a greater awareness of their important role in many aspects of plant and animal nutrition. Techniques, such as Atomic Absorption Spectrophotometry, which allow the quantitative analysis of metals at very low levels have also contributed to this growth of interest. Initially there was concentration upon toxicity and deficiency problems. More recently, however, attention has tended to focus upon the specific roles of trace metals in the biochemical and physiological processes of plant and soil. Because the trace metals involved in these processes originated in the soil many researchers have been concerned to determine the levels at which they occur, the forms

in which they appear and to isolate those factors which influence their availability. For example, organic matter, indigenous and added, has been shown to be one of these influences, being able both to increase and decrease trace metal availability.

This thesis will review aspects of the indigenous trace metals and organic matter of soil, the interactions between them and methods of increasing trace metal availability by the addition of various types of fertilizer. The practical work described in Chapters 2, 3 and 4 considers in more detail the direct addition of complexing and chelating agents to soil and their effects on the plant availability of certain trace metals.

1.2 INDIGENOUS TRACE METALS

Trace metals occur in soils in a variety of forms. Copper, for example, has been demonstrated to exist in five different soil fractions - in soil solution, weakly bound to specific sites, organically bound, occluded in soil oxide material and as a residue in clay lattice structures (McLaren and Crawford, 1973).

A few of the more common forms in which trace metals appear will be considered briefly.

1.2.1 FORMS IN WHICH TRACE METALS APPEAR IN SOIL

(1) Trace metals in primary minerals

Trace metals have been found in varying quantities in igneous rocks. Iron and manganese, for example, are prominent in occupying structural positions in some original silicate minerals while cobalt is capable of replacing manganese (II) ions in the crystal lattices of a number of synthetic and naturally occurring manganese minerals (McKenzie, 1970; Taylor and McKenzie, 1966). Trace metals present in crystalline form or within lattice structures are not immediately available to plants. However, they may be released, slowly, over a period of time, by a variety of weathering processes.

(2) Trace metals held in adsorbed forms

Many readily available trace metals are held in soil by adsorption

either onto clay minerals themselves or onto metal oxide coatings on the clay minerals. Manganese oxides, for example, appear to contain high concentrations of Fe, Co, Ni, Zn, Ba and Pb,while iron oxides retain V, Mn, Ni, Co, Zn, As, Se and Mo (Norrish, 1975).

Le Riche (1973), after finding that nearly all the cobalt could be extracted from a variety of soils by hydrogen peroxide and ammonium oxalate (under ultra-violet light), concluded that the metal had been associated with the sesquioxide fraction. Vinogradov (1959) and Mitchell (1964) found molybdenum in association with iron oxides and suggested it was present as an adsorbed phase. Jones (1956, 1957) noted a serious decrease in molybdenum retention when iron oxides were removed from soils which usually strongly adsorbed it. Harder (1961) concluded that boron was adsorbed by illite clay when he was unable to recover added boron while others have suggested that its incorporation in silicate minerals may involve solid state diffusion (Ellis and Knezek, 1972).

Adsorption of trace metals may occur via specific covalent bonds or by electrostatic interactions. Metals having a high affinity for a surface are able to displace other, already adsorbed, metals of lower affinity. Mitchell (1964) gives the following displacement order:

Cu > Pb > Ni > Co > Zn > Ba > Rb > Sr > Ca > Mg > Na > Li.

(3) Trace metals in soil solution

As far as the plant is concerned the most important source of trace metals (and other nutrient species) is probably the soil solution. Soil solution may act as a transport medium bringing trace metals to the root surface where they may be passively absorbed with the soil solution in the course of the normal functioning of the plant. They may also arrive at the plant root as the result of diffusion through the soil solution.

The Soil Society of America (1965) defined soil solution to be the aqueous liquid phase of soil and its solutes, consisting of ions dissociated from the surfaces of the soil particles and other soluble materials. A simpler definition would be the aqueous component of a soil at field moisture conditions.

Because the volume of soil solution in a soil is very small and the concentration of trace elements in it extremely so, it is usual to extract the solution before attempting to determine the concentrations of the various trace metals in it. This assumes, of course, that extraction does not modify the soil solution in any way. Using a flameless Atomic Absorption technique Yamasaki et al. (1975) obtained the following trace element concentrations in soil solutions from a variety of soils.

Table 1.2.1.1 Concentration of trace metals in soil solution (ppb)

Elements	Soil		
	Alluvial	Pumice	Volcanic ash
Al	460	211	1,400
Cd	5.64	0.098	0.61
Co	2.88	0.74	8.34
Cr	0.35	0.53	1.25
Cu	36.8	22.8	52.7
Fe	16.3	12.3	16.7
Mn	243	10	700
Mo	2	N.D.	<0.5
Ni	150	60.5	555
Pb	8.34	14.9	20.0
Zn	351	127	541

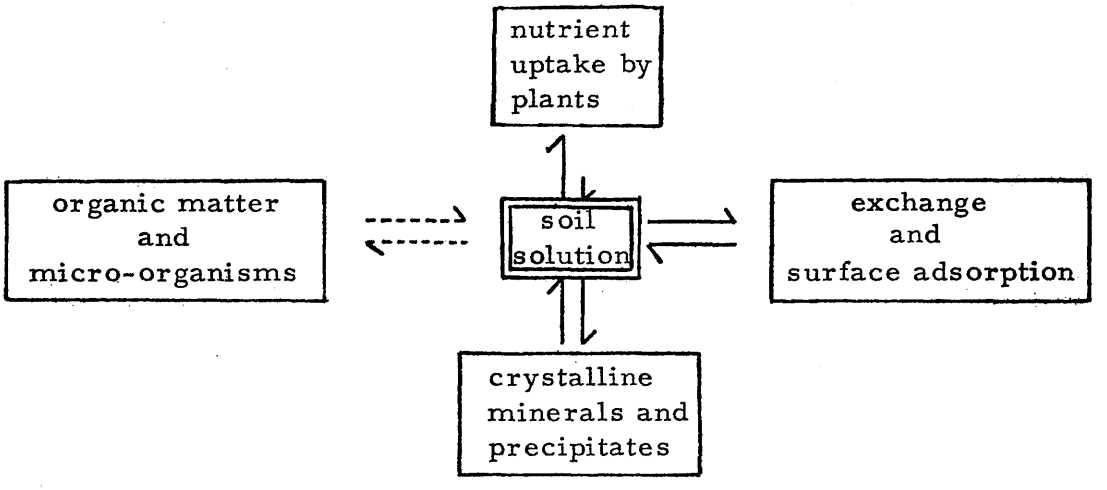
It should be noted that although these results were stated in ppb it is not clear whether the authors intended the American or British 'billion'. The zinc results suggest the American convention was used in which 'billion' signifies one thousand million.

Various factors affect the concentration of trace metals in soil solution:

(i) Plants: plants are continuously removing trace metals from soil solution as a normal part of their life cycles. Total depletion of the soil solution does not occur, however, because these losses are

made good from other sources. For example, the solid phases in a soil tend to have a buffering effect upon the concentration of soluble nutrient species within soil solution. Figure 1.2.1.1 illustrates the situation.

Figure 1.2.1.1 Diagrammatic representation of the dynamic equilibria occurring in soils.



From Lindsay, 1972a, p. 42.

It is unlikely that true equilibrium is ever achieved due to the intervention of factors such as fluctuating temperature, growing roots and changing moisture, oxygen and carbon dioxide levels.

(ii) Soil pH: generally an inverse relationship exists between trace metal concentrations in soil solution, and soil pH, with trace metal concentrations decreasing as pH increases. Molybdenum, in which the opposite occurs, is a notable exception. In consequence, deficiency problems may arise at higher pH's while at lower pH's toxic metal levels may occur. Magistad (1925) seems to have been the first to correlate poor plant growth in soils at high and low pH with the high (and he suggests toxic) concentrations of aluminium found at such pH's.

(iii) Soil organic matter: it appears that soil organic matter plays a very important role in keeping trace metals in a soluble form in soil solution. Hodgson et al. (1965) found a possible association between the soluble organic fraction of a soil and certain cations. In addition, metal complexing was observed to be greater in soil solution

from surface than from sub-surface horizons. Hodgson et al. (1966) suggested that,on average,99% of the copper and 60% of the zinc in soil solution displaced from calcareous soil was complexed with soil organic matter. Geering and Hodgson (1969) also considered copper and zinc in soil solution to be largely present as metal - organic matter complexes and attempted to characterise the materials responsible.

Norvell (1972) approached the idea of trace metal - organic matter complexes in soil solution in a slightly different way by considering the effects of synthetic chelating and complexing agents on the solubilities and concentrations of trace metals in soil solution. He concluded that manganese (II) ions were less able to compete for chelating agents in soil solution than copper (II) and zinc (II) ions and that copper (II) chelates were generally more stable than those of zinc (II). Also, he showed that chelation of iron (III) by certain chelating agents such as EGTA and HEDTA could be affected by calcium and aluminium (III) ions which competed successfully for the agents. The chelating ability of other agents (for example, EDDHA) was not seriously affected. A similar competitive situation probably exists between trace metals and naturally occurring chelating agents in natural soil solution.

This discussion has considered only the role of soluble and not of total organic matter. The latter does have a part to play in supplying trace metals to plants and this will be considered later. Additionally, only the role of organic matter in increasing or maintaining trace metal availability has been considered. Organic matter may also reduce the amount available by competition with plant roots and/or by the formation of insoluble organic matter - metal complexes (Kirkham, 1977; Miller and Ohlrogge, 1958).

(iv) Microbial activity: Duff and Webley (1959) have shown that rhizosphere populations excrete 2-ketogluconic acid which is capable of bringing ferric ions into solution. Subsequent reduction would allow plant uptake to occur. Geering et al. (1969) have attempted to show that microbial activity is more important than physical - chemical factors in determining manganese concentration in soil solution.

(v) Restricted drainage and waterlogging: increasing the water content of a soil decreases its oxygen content leading to reduction of the metals previously present in an oxidised form. Iron (III) and manganese (IV) are probably most affected. Because the reduced forms of both these metals are more soluble than the oxidised forms it would be expected that their concentrations in soil solution would increase under conditions of restricted drainage. A consequence of this reduction would be the dissolution of iron (III) and manganese (IV) hydrous oxides and the possible release, into the soil solution, of metals like cobalt, copper and zinc which may have been associated with them. Mitchell's observation (1971) that uptake of a number of trace elements was increased when plants were grown on poorly drained soils appears to confirm these suppositions. Similar conclusions could be drawn from a greenhouse experiment conducted at high moisture levels by Kubota et al. (1963) in which they noted increased cobalt in soil solution and also, to some extent, in the plant.

1.2.2 EXTRACTION OF INDIGENOUS TRACE METALS

(1) Total metal

The correlation between the total metal content of a soil and plant availability is very poor. For this reason little will be said about methods for extracting total metal from a soil. Generally, the first step involves bringing the metal into solution either by acid digestion of the soil or by fusion of the soil followed by acid digestion of the melt. The second step is to measure, by appropriate analytical techniques, the metal concentrations in the solutions so produced.

(2) Metals associated with particular soil fractions

It was mentioned earlier (see page 2) that metals are associated with different fractions within a soil. Over a long period a number of extractants have been developed which will selectively remove metals from individual fractions. One reason for developing these extractants was to attempt to relate the quantity of metal extracted to the availability of the metal and to subsequent uptake by the plant. Generally, it has been accepted that sesquioxide-associated metal is extracted by oxalate

or other buffered reagents, that readily exchangeable metal is removed by neutral ammonium acetate and that chelating agents may be used to extract organically bound metals. Any remaining, relatively insoluble, metal may then be extracted using strong acid (Mitchell, 1964; Mitchell, 1971; McLaren and Crawford, 1973).

McLaren and Crawford (1973) distinguished five separate fractions when they investigated the copper content of twenty-four different soils. Different extractants were used to obtain the metal from each fraction and these are listed below:

(i)	soil solution and exchangeable copper	- 0.5 M calcium chloride.
(ii)	copper weakly bound to specific sites	- 2.5% acetic acid.
(iii)	organically bound copper	- 0.1 M potassium pyrophosphate.
(iv)	copper occluded by soil material	- acid oxalate.
(v)	residual copper found mainly in clay lattice structures	- hydrofluoric acid.

Correlation studies by the same authors appeared to confirm that these extractants did, in fact, remove copper from the relevant fractions. For example, there was a good correlation between the amount of copper in the pyrophosphate extract and the organic matter content of the soil.

It should be noted that this paper only correlates metal extracted with specific soil fractions and makes no attempt to correlate the metal from a particular fraction with its availability to the plant.

(3) Relating extractable trace metal to plant availability

A lot of research has been directed towards finding extractants which will give the best correlations between soil content and plant availability. Availability is usually determined by measuring plant uptake, plant content or plant response. The large number of soil types existing world-wide makes it unlikely that a universal extractant is to be found giving good correlation for every metal.

Mitchell (1971) has listed the following extractants as being of use in Scottish soils:

(i) 0.5 M or 2.5% acetic acid, the most widely used extractant, correlates well with copper uptake by herbage. In addition, it has been used to diagnose high levels of zinc and nickel,

(ii) neutral, molar ammonium acetate appears to be the best in the diagnosis of high molybdenum levels and for manganese,

(iii) 0.05 M EDTA appears to give the best correlation between soil content and crop response in copper deficient soils, although the correlation is only reasonable for clovers and not for grasses or cereals,

(iv) hot water has been suggested as the best reagent for boron extraction.

This list is by no means complete and other workers have found other extractants giving good correlations with some of the above-mentioned metals. For example, Lowe and Massey (1965) found that the amount of molybdenum extracted by hot water correlated highly with plant uptake. Mishra et al. (1973), on testing the ability of nine extractants to estimate copper availability in red and black soils, noted that acid ammonium oxalate extracted the highest quantity of copper from both and, furthermore, the amounts obtained were correlated significantly with wheat uptake. Vijay et al. (1973), however, found that, in pot experiments, copper uptake by wheat was most highly correlated with copper extracted by neutral ammonium acetate. Different soils were used in the two experiments which probably explains the difference in results. Differences may also occur because different plants have been used to measure metal uptake or content. Rastogi and Rai (1975) found a significant correlation between soil zinc and total uptake by wheat using molar ammonium acetate + 0.01% dithizone whereas Stewart and Berger (1965) discovered that 1 M magnesium chloride gave a better measure of the zinc absorbed by millet than an ammonium acetate-dithizone mixture. However, as different soils were probably used in each case it is not possible to say definitely that the difference was due solely to plant factors although these would obviously be important.

It is also the case that the extractant which removes the largest quantity of a particular metal does not necessarily give the best correlation with plant uptake. Vittal and Gangwar (1975) found that decreasing amounts of zinc were extracted from the plough layer of ten different soils in the order, (1) hydrochloric acid, (2) chelating agents (EDTA, DTPA, dithizone), (3) ammonium acetate, (4) water. Only two, dithizone and EDTA (in that order), gave a significant correlation with the zinc uptake of potted maize and wheat.

Another interesting observation is that sometimes an extractant removes more metal from a soil than does a plant and yet still gives a good correlation with plant uptake. For example, Mitchell (1971) noted that although EDTA removed more copper from soil than did either acetic acid or a plant, the amount extracted was better correlated with the plant level of removal. Φien (1966) discovered that 0.02 M sodium EDTA solution and 0.043 M nitric acid solution extracted more copper from soil than did plants in a complete growing season but that, despite this, both showed good correlation with copper uptake.

This has indicated, briefly, a few of the areas in which research into metal availability in soils has been concentrated. Two main problems have emerged from such studies. Firstly, the great variability which exists in soils and, secondly, the equally great variation in the amount of a particular metal absorbed by different plants. Environmental factors, plant species, density of sowing and length and stage of growth all affect the latter. Correlations will obtain between quantity extracted and plant uptake using a particular extractant, a particular soil and a single species but it is not possible to guarantee that the correlations will hold under other conditions.

1.3 INDIGENOUS ORGANIC MATTER

Previous sections have shown that the trace metals and the organic matter of a soil are associated with one another in some manner. Soil organic matter also affects trace metal supply to plants. Historically it was considered that a knowledge of the structure and functional group content of indigenous soil organic matter would allow one to predict

which metals would be associated with the organic matter, which organic matter groupings and types of bond might be involved and finally, how this might affect availability of trace metals to the plants. Before any of this could be attempted it was necessary to extract the organic matter from the soil.

1.3.1 EXTRACTION OF INDIGENOUS ORGANIC MATTER

The first step in the extraction process is the ultrasonic dispersal of the soil in a fairly dense liquid (specific gravity about 2) to separate unhumified from humified material. The negatively-charged, colloidal, humic material remaining can associate with the mineral matter of the soil in three ways:

(i) by binding to the clay fraction via polyvalent cations,

(ii) by binding through hydrous oxides, mainly of iron and aluminium. These hydrous oxides, which often form coatings on clays, have a pH-dependent charge which is positive at the pH of most soils. Bonding between these oxides and organic matter will tend to stabilize them and help to retain their high surface area,

(iii) by binding through van der Waals or hydrogen bonds. These bonds, which individually are quite weak, will only form if the organic matter can get very close to the clay surface. If soil organic matter were considered to be a flexible polymer, the formation of bonds at several points on the polymer surface would be possible and would result in an overall increase in bond stability.

To extract the organic matter it is necessary to overcome these attractions.

1.3.2 METHODS OF EXTRACTING INDIGENOUS ORGANIC MATTER

(1) Alkali extraction

0.1 - 0.5 M sodium hydroxide and sodium bicarbonate have been widely used and, in some soils, repeated extraction with them has

resulted in solubilization of almost all the organic matter (Mortensen, 1965). There are several reasons for the use of these two reagents:

(i) they replace polyvalent, bridging cations with sodium ions which are unable to bridge,

(ii) they produce alkaline pH's at which most metals will form hydroxides and precipitate,

(iii) they increase ionization of functional groups in organic matter, increasing negative charges which, in turn, cause the organic colloids to repel one another,

(iv) they decrease the pH-dependent positive charges on clay edges and hydrous oxides which reduces their ability to attract oppositely charged organic colloids.

A major criticism of alkaline extractants is that they can cause serious changes in the structure of the organic matter, for example, as a result of hydrolysis (Tinsley and Salam, 1961) or autoxidation (Swift and Posner, 1972a; Choudhri and Stevenson, 1957).

(2) Acid extraction

Although organic matter is not particularly soluble in dilute mineral acids, with the possible exception of HF-HCl mixtures (Schnitzer and Wright, 1956, 1957), it is usually more soluble in them than in water. The acids have an ability greater than water to protonate oxygen and nitrogen-containing compounds thereby producing soluble species. As with alkaline extraction hydrolysis may occur, the acid acting as a catalyst. Sulphuric acid extraction is often accompanied by sulphonation, polymerization and dehydration (Mortensen, 1965).

(3) Neutral salt extraction

Di-sodium pyrophosphate has become the most commonly used extractant of this type since Heintze and Mann (1949) observed that it dissolved large quantities of organic matter. Although less effective than alkaline extractants in terms of the quantity of organic matter extracted, it does have certain advantages over them in that it does not add carbon to the system, probably reduces hydrolysis and autoxidation

and is known to extract organic matter more rapidly than sodium hydroxide. The pyrophosphate anion is thought to chelate metal cations formerly in chelation with soil organic matter thereby increasing solubility of organic matter.

(4) Organic reagent extraction

Various reagents such as EDTA, acetylacetone, cupferron and 8-hydroxyquinoline have been used to extract organic matter from the B horizons of podzols (Martin and Reeve, 1957; Schnitzer et al., 1958). They probably operate in a similar manner to di-sodium pyrophosphate (see (3) above).

(5) Sequential extraction

Soil organic matter is composed of a whole series of compounds of varying molecular weights and differing physical and chemical properties. Many of these compounds are associated with the inorganic components of soil. Because the associations vary with the inorganic species and the precise nature of the organic matter, it follows that no single extractant is able to extract more than a small fraction of the total organic matter. To increase the percentage of organic matter extracted from a soil a number of extractants has been employed, used in series. Kononova (1967) presents one such sequence (a modification of Tyurin's method) which involves preliminary extraction with alcohol-benzene followed by decalcification with 0.05 M sulphuric acid, treatment with 0.1 M sodium hydroxide, digestion with hot 0.5 M sulphuric acid and re-extraction with cold 0.1 M sodium hydroxide.

This scheme illustrates one of the problems of sequential extractions - their complexity. This problem is encountered not only in the extraction of total organic matter but also in the extraction of particular fractions of the organic matter. For example, Swincer et al. (1968), after extracting ten soil groups with 1 M hydrochloric acid followed by 0.5 M sodium hydroxide, found that only 50% of the soil carbohydrates were removed in all but two of the cases. A third treatment was necessary with acetic anhydride containing 2.5% concentrated

sulphuric acid to increase this figure to 80%.

Sequential extractions to obtain a reasonably high percentage of total soil content are also necessary with other organic matter fractions.

Section (5) above illustrates very clearly that it is not possible to extract all the organic matter in one step because the major part of the soil organic matter is composed of high molecular weight species (e.g. waxes, polysaccharides, proteins and lignins) which are not equally soluble in any one extractant. Pre-treatments have been tried to increase extraction efficiency. Grinding and ball-milling, for example, break up soil aggregates thereby increasing the surface area of the soil available to the extractant. HF-HCl mixtures in water have been used to dissolve silicate minerals prior to extraction (Choudhri and Stevenson, 1957). The pre-treatments to increase extraction efficiency, while effective, may cause irreversible changes in the soil organic matter and this has to be borne in mind.

1.3.3 FRACTIONATION OF SOIL ORGANIC MATTER

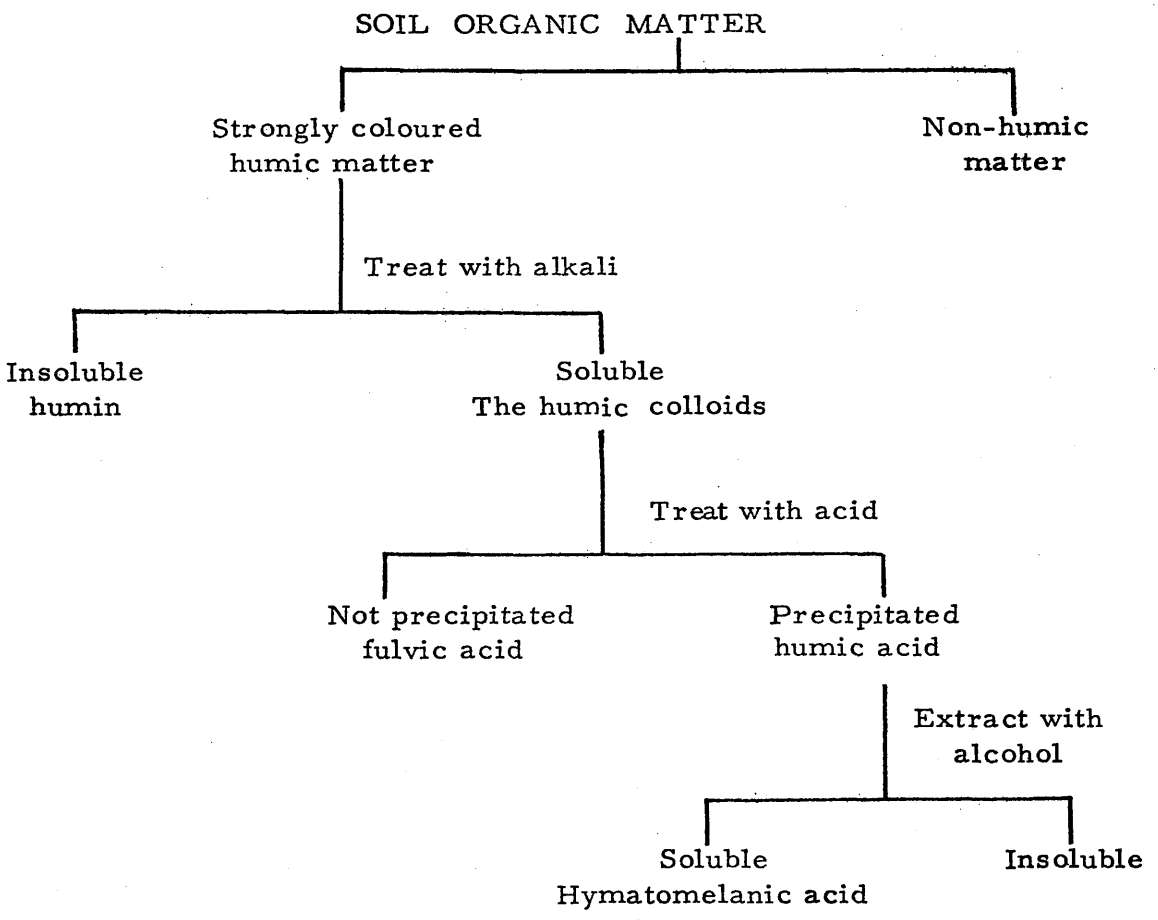
(1) Classical method

The classical method of extracting soil organic matter uses 0.1 - 0.5 M sodium hydroxide. A gross separation of the humic material so extracted may be effected by acidifying the extract. Part of the organic matter (the humic acid) precipitates while the remainder (the fulvic acid) remains in solution. A further fractionation is obtained by treating the humic acid with ethanol. Figure 1.3.3.1 illustrates the situation.

(2) Fractionation according to molecular weight

Total soil organic matter, fulvic acid and humic acid all have high polydispersity, i.e. they contain a mixture of compounds having a wide range of molecular weights. Gel filtration techniques have been widely employed in attempts to reduce this polydispersity. Sephadex gel (an α - 1, 6 polyglucose polymer cross-linked by 3C paraffinic chains) is frequently used. Behaving rather like a sieve, Sephadex separates substances according to their molecular weights. This ability is closely related to the swelling properties of the gel and a variety of Sephadex

Figure 1. 3. 3. 1 Classical fractionation of soil organic matter



From Russell, 1973, p. 284.

gels exist which differ in this respect. Those gels with greater swelling capacities are used to fractionate high molecular weight substances while those with lesser swelling capacities are used with substances of lower molecular weight.

Using a gel filtration method Dell 'Agnola et al. (1964) were able to distinguish four groups of compounds in extracted organic matter with molecular weights in the ranges 0 - 4,000; 4,000 - 9,000; 9,000 - 100,000 and 100,000 - 200,000. Khan and Friesen (1972) found that passing humic acid from the Ah horizon of various soils through Sephadex G-75, G-100 and G-150 produced a number of molecular weight fractions corresponding to weights around 50,000, weights around 100,000 and to weights around 150,000 respectively. Karpukhim and Fokin (1969), on the other hand, found that separating fulvic acid mixtures from Ao and Ai horizons of podzolic soils gave five fractions with molecular weights ranging from several hundreds to tens of thousands.

The separation obtained obviously depends upon the particular gel in use and the material being fractioned. It also seems to depend upon the extractant originally used to obtain the material to be fractioned. Dell 'Agnola et al. (1965) after extracting humic material with 0.5 M sodium hydroxide, sodium carbonate + sodium bicarbonate, sodium fluoride and sodium pyrophosphate, passed the extracts through various Sephadex gels and analysed the filtrates by electrophoresis. The results indicated that the pyrophosphate extracts differed from the others and the authors attributed this to differences in the alkalinities of the various extractants. Jacquin et al. (1971), using a G-25 to G-150 technique, found that resin extracted humic acids were more highly polymerised than those extracted with sodium hydroxide or sodium pyrophosphate.

A major problem in the use of Sephadex gels is caused by interaction between the gel and molecules in the extracts. This prevents true separation on a molecular weight basis because those molecules which interact with the gel are retained regardless of whether their molecular weights are high or low. Coulombic and/or adsorption (or van der Waals) forces are probably responsible. Coulombic forces

resulting from attraction between charges on gel and solute have been overcome by adding electrolytes to the eluant to suppress these charges (Posner, 1963). Adsorption most often occurs between Sephadex and aromatic, heterocyclic andphenolic compounds (Gelotte, 1960; Demetriou et al., 1968; Brook and Housley, 1969) and is probably due to interaction between solute molecules and the cross-linkages in the gel. Swift and Posner (1971) have suggested using an alkaline buffer containing a large amino cation to overcome both types of interaction. The most commonly used buffer system is tris (2 amino-2 (hydroxylmethyl) - propane - 1, 3-diol). Other alkaline buffers have been used but have been only partially successful in removing adsorption effects (Dubach et al., 1964; Dell'Agnola et al., 1964 quoted by Swift and Posner, 1971).

Further analyses of fractions obtained by gel filtration have been conducted by a number of workers using a variety of techniques. For example, Swift and Posner (1972b) assayed for total and amino nitrogen, phosphorus and sulphur; Karpukhim and Fokin (1969) used paper chromatography and Dell 'Agnola et al. (1965), as previously mentioned, used electrophoresis.

Although these fractionation methods may ultimately allow one to collect a lot of information about extracted organic matter a major difficulty arises when one attempts to interpret this data. It has been noted (see page12) that sodium hydroxide is capable of causing changes in the organic matter during extraction and it is more than likely that some or all of the other types of extractant also do this. The question, therefore, arises of whether or not it is valid to extrapolate from the results of any analyses carried out on this extracted material to the situation in the soil system in which the material originated. For example, Himes and Barber (1957) found that the organic matter which they extracted from a soil amounted to only 15% of the total organic matter content and yet accounted for 70% of the zinc complexing capacity. This seems disproportionately large and while it may suggest that the most active fraction of the organic matter had been extracted it could also mean that new complexing sites had been created. Consequently, care is necessary when extrapolating from results obtained using

extracted organic matter to a natural soil situation.

1.4 ORGANIC MATTER-METAL INTERACTIONS

1.4.1 EVIDENCE FOR ORGANIC MATTER-METAL INTERACTIONS

Much of the evidence for organic matter-metal interactions, though principally in relation to podzolization, has been reviewed by Petersen (1976).

As early as 1946 Bremner et al. found that sodium and potassium pyrophosphate solutions extracted both organic matter and metals from soils. Their explanation for this apparent association was that the pyrophosphate was able to complex metal previously present in the soil as part of an organic matter-metal complex. Consequently, metal extraction and solubilization of the organic matter occurred. Duchaufour (1963) made similar assumptions but, since 0.1 M sodium pyrophosphate has been shown to be capable of extracting iron from biotite and hydroxides (Titova, 1962), doubt has been cast upon the validity of these assumptions. It may be that the correlation between the extracted organic matter and metal was incidental and that the metal released came, at least in part, from other sources. Himes and Barber (1957), however, observed that the ability of soil to chelate zinc was destroyed by removing the organic matter but not by removing the hydrous silicates. This certainly suggests a close association between this particular metal and organic matter. Broadbent (1957) used an adsorption technique to show that soil organic matter preparations were able to retain copper and calcium by complexation and to show that the groups involved were different in each case. A thin layer of copper (or calcium) saturated sample, was leached, with successively increasing concentrations of hydrochloric acid, through a column containing the soil organic matter preparation and the effluent was analysed for cations. Graphs of cation concentration against effluent volume revealed four peaks for copper but only two for calcium. The first peak was common to both and was assumed to be the result of metal retention by carboxyl groups, being the only peak obtained when synthetic carboxyl resin was substituted for the organic matter preparations. It appears that more functional groups are capable of complexing copper

than calcium.

Metal complexing by organic matter has also been used to explain the process of podzolization. In a series of papers Bloomfield (1953a, b; 1954a, b; 1955) reported that aqueous extracts of Scots pine and larch needles, of kauri leaves and bark and of rimu litter were able to dissolve ferric and aluminium hydroxides. Even under aerobic conditions the reduction of ferric to ferrous ions was involved and Bloomfield postulated the formation of soluble metal-organic matter complexes of high stability which transported iron down the profile. The metal could then be released to form the illuvial horizon characteristic of a podzol. Wright and Schnitzer (1963) suggested that the fulvic acid fraction of soil organic matter was most important in solubilizing and transporting metals. They explained the formation of an illuvial horizon by assuming, firstly, that the fulvic acid had formed from humic acid and, secondly, that as this change progressed, water solubility increased and the dissolved material moved down the profile. During this downward movement functional groups would react with metals in the soil, until, metal bonds having formed in each group, precipitation would occur.

Various authors have noticed that flocculation occurs when ferric iron and aluminium solutions are added to dilute solutions of organic matter (Schnitzer and Skinner, 1964; Martin and Reeve, 1960; Wright and Schnitzer, 1963). Wright and Schnitzer (1963) used as a measure of the complexing power of organic matter the minimum amount required to prevent flocculation at a particular pH.

As a consequence of such experiments it became widely accepted that organic matter-metal complexes existed in soils although the mechanisms by which such complexes formed were not fully understood. Efforts to understand these mechanisms have involved a wide variety of techniques from analyses of functional groups which might be involved to attempts to make and study 'synthetic' complexes with a view to relating their properties to those of natural complexes.

1.4.2 STUDIES ON THE NATURE OF ORGANIC MATTER-METAL INTERACTIONS

(1) General

Hildebrand and Blum (1974) using infra-red spectroscopy to study humic acids found, after lead fixation, that the carbonyl band at 1710 cm^{-1} and the broad carboxylic shoulder at 2700 - 2300 cm^{-1} had disappeared while absorbance increased in the region 1400 - 1380 cm^{-1}. This, they concluded, was the result of salt formation with acid carboxyl groups.

Schnitzer and Skinner (1965) used a different approach to the problem of which functional groups are involved in bonding with metals. They compared the ability of organic matter to hold metals before and after selectively blocking certain of its functional groups. Using iron (III), aluminium (III) and copper (II) ions they found that blocking acidic carboxyl or phenolic hydroxyl groups significantly decreased metal retention. Alcoholic hydroxyl groups did not appear to participate. Himes and Barber (1957), in a similar experiment, found, after methylating organic matter from a sandy loam soil, that carboxyl groups did not appear to be important in the complexing of metals.

Doubts have been expressed as to the validity of the conclusions drawn from experiments such as these. One objection which applies to all experiments using extracted organic matter is the very fact that extraction is involved. It is extremely unlikely that naturally occurring functional groups will be totally unaffected by the extractants employed, so that, although the experiments may accurately determine the functional groups in the extracted material, there is no guarantee that these groups exist naturally in the soil situation. Another objection applies specifically to blocking experiments. Soil organic matter is highly heterogeneous, consisting of a large number of molecules and an even larger number of functional groups, and it seems rather simplistic to assume that blocking agents will react only with one particular type of functional group. On the contrary, it seems more reasonable to assume that they will react with a large number of similar types. Therefore, conclusions based on the assumption that only one type of functional group was involved could

give a false impression of the actual soil situation.

(2) Potentiometric titrations

Despite the doubts expressed in (1) above it is generally accepted that organic matter-metal complexes do exist in soil and that the organic matter groups most involved are amino, carboxyl and phenolic hydroxyl. All these groups are of an acidic character and ought,on reaction with metal ions, to dissociate, with the release of hydrogen ions and an accompanying drop in pH. These facts have been used in a variety of potentiometric experiments to provide additional evidence for the formation of organic matter-metal complexes. The following equations have been used to describe the situation:

$$M^{n+} + HA \rightleftharpoons MA^{n-1} + H^{+}$$

$$MA^{n-1} + HA \rightleftharpoons MA_2^{n-2} + H^{+}$$

$$M^{n+} + HmA \rightleftharpoons MA^{n-m} + mH^{+}$$

(from Schnitzer and Khan, 1972, p. 207).

The pH drop has been used both to indicate the formation of complexes and to obtain their stability constants:

$$M^{n+} + HmA \rightleftharpoons MA^{n-m} + mH^{+}$$

$$K = \frac{(MA^{n-m})\ (H^{+})^{m}}{(M^{n+})\ (HmA)}$$

where K is the stability constant and ( ) denotes activity.

Schnitzer and Skinner (1963) potentiometrically titrated various metal ion solutions against standard base in the presence and absence of organic matter. Inflections in the curves obtained during the titration of ferric and aluminium ions were taken to indicate formation of ferric and aluminium hydroxides. The same ions titrated in the presence of organic matter did not produce such inflections and this was taken as an indication of complex formation. Complex and hydroxide formation were also indicated during the titration of nickel (II) and copper (II) ions in the presence and absence (respectively) of organic matter.

The authors then went on to calculate the number of protons released by the base during the titration of the organic matter. In consequence, they decided that at pH 3, one carboxyl group had been titrated, while at pH 6, the number was five and at pH8, it was six. Furthermore, at pH 10, they stated that, in addition to six carboxyl groups, two phenolic hydroxyl groups had also been titrated. These latter conclusions seem rather dubious, based as they are upon a molecular weight of 670 and a molecular formula of $C_{21} H_{12} (COOH)_6 (OH)_5 (CO)_2$ for the organic matter used. As this organic matter was obtained by extraction with 0. 5 M sodium hydroxide which could be expected to break down larger organic molecules and to cause alterations in smaller ones, it seems unlikely that a single formula would adequately describe the resulting mixture of organic molecules. At best it would be an approximation of the situation. Similar objections could be made to the molecular weight quoted and, in fact, in a later paper it was shown to be subject to considerable error (Hanson and Schnitzer, 1969). In view of this it seems rather unreasonable to attempt to obtain information of a quantitative nature from such experiments.

Potentiometric titrations using a variety of soils, humic preparations and cations, have been conducted by Khanna and Bajwa (1967), Khanna and Stevenson (1962) and Stevenson (1977) and all have interpreted their results as confirming the formation of organic matter-metal complexes. Martin and Reeve (1958), however, reached the opposite conclusion as a result of their experiments.

(3) Spectroscopic techniques (Job plots)

This spectroscopic technique has been used both to demonstrate complex formation and to determine the metal to organic matter molar ratio within the complexes. The technique, originally developed by Vosburgh and Cooper (1941), is based on the assumption that if the ratio of metal ion and complexing agent in a solution is varied while keeping the total concentration constant then a change in the optical density of the solution will be observed. Assume the following equation for complex formation:

$M + nX \rightleftharpoons MXn$ (where M denotes metal ion and X ligand). .

It should be possible to obtain a value for n from measurement of the optical density resulting from mixing, in varying proportions, solutions of M and X of the same concentration. A plot of composition against optical density (corrected for the optical density which would have resulted if no complex had formed) should give a maximum or minimum at a composition corresponding to that of the metal complex. From this a formula for the metal complex may be determined.

Using this method Schnitzer and Skinner (1963) concluded that organic matter from a podzol Bh horizon formed 1 : 1 molar complexes with copper (II), iron (III) and aluminium (III) ions at pH3. At pH 5, 2 : 1 molar complexes were detected with copper (II) and iron (III) ions and 1 : 1 complexes with aluminium (III) ions. During these experiments the authors assumed that they were mixing aliquots of a 1.8×10^{-2} M organic matter solution with aliquots of metal ion solutions of the same molarity. This molarity was calculated using the molecular formula and molecular weight mentioned earlier (see (2) above). In view of the previously noted objections to both of these it seems unrealistic to assign such a molarity to the organic matter solution and, perhaps, would lead one to consider with caution their conclusions regarding the formation of metal-organic matter complexes.

It appears, therefore, that this spectroscopic technique may only be used to indicate whether or not metal-organic matter complexes are formed and not to give definite molar ratios. In addition it should only be used where one type of complex is formed (Vosburgh and Cooper, 1941) and when interferences caused by light scattering during optical density determinations have been reduced to a minimum.

(4) Conclusion

Evidence presented in preceding sections would seem to suggest that organic matter-metal complexes do exist naturally in soil and that it is possible to synthesise organic matter-metal complexes in the laboratory. The question of whether the two types of complex are the same

remains to be answered.

1.5 ADDITION OF TRACE METALS TO SOIL

The foregoing discussion has been concerned with the forms and concentrations in which trace metals appear in soils, methods of extracting them and methods of studying the organic matter with which they may be associated. Mention has also been made of the fact that although a particular metal is present in a soil it does not necessarily follow that it is available to plants.

Trace metal deficiencies may be treated by applying trace metal-containing fertilizers. The trace metal may be applied as a purely micro-element fertilizer or in conjunction with normal macro-element fertilizers.

1.5.1 TRACE METALS APPLIED WITH MACRO-ELEMENT FERTILIZERS

Applying trace elements along with macro-element fertilizers is of advantage to the farmer because the number of operations involved in fertilizer spreading are reduced, saving both time and money. The crop and soil type will determine the quantities of trace elements which must be added. Whatever the quantities, however, it is essential to thoroughly mix the components of the macro-element fertilizer to prevent uneven distribution and plant response. Trace metals tend to become unavailable at high pH and to avoid this any highly alkaline fertilizer components, e. g. limestone, must be thoroughly mixed in before trace metals are added. The technology of mixing micronutrients in macronutrient fertilizers is reviewed by Silverberg et al. (1972) and chemical reactions between micronutrients and macronutrients in mixed fertilizers are reviewed by Lehr (1972). Neither aspect, therefore, will be considered in this brief discussion.

To be of maximum use these fertilizers, as with any other, must be applied at just the right time. For example, Nikitin (1954) quotes Rademacher as finding that the copper requirement for oats is greatest in the early stages of growth, before flowering. Therefore, to be of

most value, copper-containing fertilizers should be applied early in the season. As a result of Steenbjerg's observation (1950) that higher concentrations of soil manganese are available in spring and summer than in autumn and winter, Nikitin (1954) suggested that manganese could be applied in greater concentrations to autumn and winter than to spring and summer crops.

Animal or farmyard manure could be considered to be natural macro-element/micro-element fertilizer. Large applications have been shown to be capable of providing sufficient trace elements to overcome deficiencies. Unfortunately, however, the composition of this natural fertilizer varies considerably so that one would never be absolutely certain that sufficient of a particular element had been applied.

1.5.2 TRACE METALS APPLIED ALONE

(1) Trace metals applied as metal salts

The use of fertilizers to correct micronutrient deficiencies has been reviewed by Murphy and Walsh (1972).

A number of the methods by which trace metal salts are applied will be considered:

(i) Soil application: soil-applied trace metals will be most readily available for plant uptake if they are in close proximity to the roots. For this reason readily soluble salts are most often used for soil applications. If, however, the trace metal is supplied in too soluble a form, losses by leaching down through the soil and/or run-off from the soil surface can occur. To overcome this problem some elements have been supplied in various insoluble silicates which will release the trace elements only very slowly during weathering. This ensures that, at any one time, only small quantities of trace elements will be present in forms which are easily lost from the soil. Again, the amount applied and the application method depend very much upon the plant species and the soil type. Plants growing on calcareous soils are frequently iron deficient. Iron, under alkaline conditions, is readily

immobilized and made unavailable to plants. Consequently, to overcome iron deficiencies by soil application of iron very high application rates must be used. Mathers (1970), for example, found rates of 112 and 560 kg iron/ha as iron sulphate were necessary to produce significant yield increases in grain sorghum.

Soil-applied, soluble manganese also rapidly assumes an unavailable form and it is found that the effectiveness of a manganese fertilizer depends very much upon the application method. Generally, lower application rates are necessary when band, as opposed to broadcast, methods are used. For example, manganese sulphate, manganese oxide (MnO), manganese frits and manganese sulphate-carbonate, at levels of 56 kg manganese/ ha, gave consistently higher onion yields with banded, as against broadcast, application (Shepherd et al., 1960).

The effectiveness of zinc fertilizer is also affected by the application method, with band-applied zinc sulphate being shown to have a greater availability than granular zinc oxide or zinc carbonate. Zinc availability from granular sources can be increased by crushing the granules and by mixing them with the soil (Lindsay, 1972b).

(ii) Foliar application: several of the difficulties encountered with soil-applied trace element fertilizers are overcome when foliar sprays are used. For example, soil reactions are eliminated such as those which render elements unavailable (e. g. iron and manganese). The need for irrigation to move the fertilizer from the soil surface to the root zone is also eliminated. Against these advantages, however, is the increased likelihood of toxicity when nutrient containing solutions come into contact with crop leaves. Benson (1967) suggested adding lime to sprays containing inorganic copper as one way of preventing such toxicity. Lindsay (1972b) notes that hydrated lime is often added to zinc sprays to neutralise acidity and reduce damage to leaves and fruit. Complete leaf coverage can be ensured by using very fine sprays.

Although the cost of applying foliar sprays may be higher than for soil applications, because several treatments are usually required in a growing season, the increased cost is somewhat offset by a reduction

in the quantity of material necessary. For example, Shepherd et al. (1960) found that 3.8 kg manganese/ha spray-applied to onions was equivalent to 56 kg manganese/ha applied broadcast to the soil and Withee and Carlson (1959) showed that, while soil applications of iron to grain sorghum were not economic, three sprays with 4% ferrous sulphate solution at a rate of 280 litres/ha resulted in increased grain yields.

An additional advantage is that foliar-applied micronutrients should be able to enter the translocation stream more rapidly than those from soil applications. In the former, the only barrier to absorption is the leaf cuticle, while in the latter, micronutrients must first be washed down the soil profile to the root zone, with the possibility of all kinds of soil interactions before absorption can occur. It would seem, therefore, that foliar methods of application will allow micronutrients more rapidly to reach the plant centres where they are required.

(iii) Seed treatments: attempts have been made to overcome trace metal deficiencies by treating seeds with powders or solutions prior to planting. This treatment mode, which ensures a more even distribution than soil treatments, has been commonly used to rectify molybdenum deficiency. Hagstrom and Berger (1965) noted that wet seed treatments of peas and vines were more effective than applications of talc and anhydrous sodium molybdate dust. Common treatment rates lie in the range 50 - 100 g molybdenum/ha. Above 400 g molybdenum/ha seed viability appears to be reduced (Reisenhauer, 1963). The success or otherwise of seed treatments appears to depend very much upon the species and the metal being considered. For example, under normal and dry conditions, corn seeds dusted with copper (II) sulphate at a rate of 300 mg copper/kg grain have shown increased yields (Gnilitskaya, 1967) while bean seeds treated with zinc appear to have been less successful (Rasmussen and Boawn, 1969).

(iv) Injection treatment: deficiencies have been treated both by placing dry, neutral, trace element salts in holes bored into the sapwood of trees and by forcing water suspensions of trace metals into the

sapwood from a syringe. Zinc-coated nails have even been driven into trees in an attempt to overcome zinc deficiency. Large scale use of such methods, however, involves much manpower and is very time-consuming. In consequence they are used infrequently.

(2) Trace metals applied as metal chelates

In the early 1950's a considerable amount of work was carried out using metal chelates to overcome trace metal deficiencies. It has been shown that rhizosphere organisms can produce a variety of compounds (e. g. amino acids, organic acids (Duff and Webley, 1959; Rovira, 1962; Vancura and Hovadik, 1965)) some of which are capable of complexing or chelating metals (Duff and Webley, 1959). The hypothesis is that such compounds are capable of solubilizing trace metals, thus making otherwise unavailable metals available to the plants by enabling their transport to the roots in the soil solution.

It was thought that applications of synthetic metal chelates could achieve the same result in situations where the natural mechanisms of the plant had been unable to cope and deficiencies had resulted.

A large part of earlier and more recent work has been concerned with the correction and/or control of iron chlorosis occurring on calcareous and limed soils. Consequently, there is very much more data available concerning the use of iron chelates than of other metal chelates.

Holmes and Brown (1955) compared the effectiveness of five iron chelates - EDTA, HEDTA, CDTA, DTPA and EDDHA - in eliminating iron chlorosis in soybeans growing on seventeen calcareous soils and found only DTPA and EDDHA to be effective. Kuykendall et al. (1957) found rapid and almost complete re-greening of chlorotic orange and lemon trees with iron EDDHA applied at rates of 12 and 24 g iron/tree. However, there was less response with iron DTPA and iron HEDTA. Stewart and Leonard (1957) also found that soil applications of iron EDDHA caused rapid re-greening of chlorotic leaves on citrus grown on calcareous soils. Iron DTPA gave good but less consistent results.

Zinc chelates have also been used. They are generally soil-applied and zinc EDTA appears to be the most popular. Holden and Brown (1965) while comparing various inorganic zinc sources also included zinc EDTA and found that in comparison with zinc sulphate on a neutral soil there was a two-fold increase and on a calcareous soil there was up to a six-fold increase in the zinc content of alfalfa. However, Leonard et al. (1956) found that, in citrus, on an acid sandy soil, chelated zinc was no more effective than zinc sulphate in increasing the zinc content of the leaves. Butler and Bray (1956) observed that the effect of soil-applied zinc EDTA depended upon the clay content of the soil. There was an increase in the zinc and copper content of perennial ryegrass on sandy soil. On a heavy silt loam there was no such increase although up to 50 ppm zinc was added. On the other hand, Benson et al. (1956) found that not only was zinc deficiency in peach and sweet cherry trees corrected by soil applications of zinc chelates but also that the effect persisted for well over a year. Wallace and Romney (1970), in a greenhouse experiment with sweetcorn, observed that zinc EDTA increased the zinc content of the corn more than zinc NTA which, in turn, increased it more than zinc sulphate.

Manganese chelates appear to have been used with rather less success than iron and zinc chelates. Wallace and Mueller (1959) found that manganese HEEDTA decreased the manganese content of Lincoln soybeans and suggested that it could be used to overcome manganese toxicity. Knezek and Greinert (1971) observed that soil-applied manganese EDTA also decreased manganese uptake and increased visual symptoms of manganese deficiency. They thought this may have been caused by substitution of soil iron for the manganese of the chelate, leading to an increase in iron uptake and a corresponding decrease in manganese uptake. Wilcox and Cantliffe (1969) discovered that soil-applied manganese EDTA was sometimes less effective than manganese sulphate where the soil was high in organic matter. Beattie (1954), however, reported that in grapes known to be suffering from manganese and potassium deficiencies, yield and sugar content could be increased by the application of chelated manganese in conjunction with potassium sulphate.

As with inorganic trace metal fertilizers, metal chelates may also be applied directly to soil or as foliar spray.

(i) Spray application: the metal chelate chosen must not be toxic to either fruit or leaves and must be capable of penetrating the leaf cuticle and entering the translocation stream. Once the metal chelate has been transported in the translocation stream to the point where the metal is required it must be readily released from the chelate to allow it to participate freely in various plant reactions.

Application rates of 1 - 5 lb chelate/100 gallons of water have been observed to give favourable results on many occasions (Wallace, 1956, pp. 4 - 23). The time for which a particular treatment is effective varies considerably from species to species. Wallace (1956, pp. 4 - 23) notes that 1 lb of iron EDTA in 100 gallons of water corrected chlorosis in St. Augustine grass but was effective for only 6 weeks after application. Some citrus, however, has remained green for $1\frac{1}{2}$ years after a chelate application

(ii) Soil application: it was rapidly discovered that the correction of trace metal deficiencies involved more than merely adding the appropriate metal chelate to the soil in the vicinity of plant roots. A few of the many problems which were encountered are mentioned below:

(a) Stability: two aspects of stability - pH stability and stability with respect to various metals - will be considered.

Some metal chelates are more stable at some pH's than at others. Ferric EDTA, for example, is most stable between pH 3 and pH 8. Above pH 8 it is readily hydrolysed to insoluble ferric hydroxide and some salt of EDTA (Haertl, 1956). Consequently, one would expect ferric EDTA to be ineffective in the treatment of iron chlorosis in highly limed or calcareous soils. Ferric EDDHA, on the other hand, is stable up to pH 10 (Kroll, 1957) and would be suitable under these conditions.

Some metals are more stable with a particular chelating agent than with others. For example, ferric iron forms a more stable chelate with EDTA than does manganese or zinc (Kroll, 1957). It would be possible, therefore,for soil iron to displace manganese or zinc from their soil-applied EDTA chelates. Ferric EDTA would form and the displaced metal could then become unavailable to plants by precipitation or by adsorption onto soil constituents. Where zinc and manganese chelates have been used successfully (see page 29) their effectiveness may have resulted because the ferric iron in the soil was held in such an insoluble and/or unavailable form that it was unable to displace either the manganese or the zinc from their respective chelates.

(b) Clay fixation: ferric EDTA and divalent manganese and zinc EDDHA have been found to be fixed irreversibly on clay surfaces while divalent manganese and zinc EDTA and ferric EDDHA are not (Lunt et al., 1956; Wallace and Lunt, 1956). Wallace and Lunt (1956) have suggested that metal-oxygen-clay linkages are responsible for the fixation and have concluded from consideration of X-ray diffraction studies that fixation occurred primarily at clay edges.

(c) Microbiological attack: if the chelating agent associated with the metal were susceptible to attack and decomposition by soil micro-organisms it is possible that the metal could be released into the soil after the destruction of the chelating agent and could, by precipitation or adsorption, become unavailable. Tiedje (1977) showed that ^{14}C-EDTA was slowly degraded to $^{14}CO_2$ by a variety of soils differing in use, pH and texture. Hale and Wallace (1961) applied ^{14}C-labelled iron EDDHA to soil in which a small, fruit-bearing avocado was growing. Subsequent analysis of the fruits, leaves, soil, material removed by leaching and of bush beans grown in the soil around the tree, accounted for only one-sixth of the added ^{14}C. From this they inferred that the labelled EDDHA had been metabolized by soil micro-organisms. The results of other experiments involving incubation of soil with ^{14}C-EDDHA and ^{14}C-DTPA have been more ambiguous. There was loss of ^{14}C from soil incubated with ^{14}C - EDDHA but no change, or at most a slight gain, in ^{14}C with ^{14}C-DTPA (Hale et al., 1962). This loss, as the authors note, could

be the result of isotopic exchange and need not indicate changes in the chelating agent molecule.

(d) Competition between chelating agents and roots: once a metal chelate reaches the root it is possible that the metal will not be released to the root if the association between metal and chelating agent is greater than that between metal and root. Brown et al. (1960) noticed an apparent competition for iron between root and chelating agent when they grew several species in nutrient solution containing various concentrations of EDTA, DTPA, CDTA and EDDHA (the iron supplied as ferric chloride). Not only did the concentration of chelating agent in the nutrient solution affect the ability of the roots to absorb iron but it also affected the total iron content of the plant tops. In 1961 Brown et al. observed that the capacity of roots to absorb iron (and copper) from nutrient solutions containing 1×10^{-5}M iron and $0.16 - 18 \times 10^{-5}$M DTPA decreased as the chelating agent concentration increased. Also the roots' ability to compete with the chelating agent was seen to vary with the species considered. Kidney beans, for example, were unable to compete and developed chlorosis when the molar concentration of DTPA was greater than that of iron. Lupins, however, did not develop chlorosis until the DTPA concentration reached 6×10^{-5}M. Wallace and Hale (1961), on the other hand, found only slight evidence of competition for iron between plant roots and excess EDDHA when they grew soybean seedlings for 24 hours in a nutrient solution containing both ^{59}Fe and ^{14}C-EDDHA. Hale (1963), also using soybeans, did find evidence of competition between roots and chelating agents, noting that not only was the plants' ability to absorb iron decreased by increasing chelating agent concentration but also,by increasing chelate stability. However, he also observed that the proportion of the absorbed iron reaching the leaves was increased under these circumstances.

Despite these problems soil application of metal chelates has been successful in supplying trace metals to plants. Benson et al. (1956), for example, found that 2 lb zinc EDTA or zinc HEEDTA per tree increased the zinc content of peach tree foliage while Leonard and Stewart (1952, 1953) reported great success using iron EDTA to correct

chlorosis in citrus on acid sandy soil. Wallace (1956, p. 44) found that soil-applied iron EDTA increased iron, manganese and zinc content of soybeans in comparison with those of a control.

Increased growth and yields have also been noted after metal chelate applications. Wallace et al. (1957) quote studies in which soybeans were grown in sand culture with a nutrient solution, complete, but lacking iron. The addition of EDDHA with or without iron appeared to increase yields. Ford et al. (1954) obtained an increase in feeder root growth on treating iron deficient citrus with soil-applied iron EDTA and Moraghan and Freeman (1978) noticed increased yields in flax treated with 2 ppm iron EDDHA.

So far, only the ability of metal chelates to increase plant metal contents has been considered. However, in some instances they decrease metal levels and this property has also been used - to overcome trace metal toxicities. Batjer and Benson (1956) used zinc EDTA to overcome arsenic toxicity in peach trees while Wallace and North (1956) showed that, in pot experiments, against controls, iron EDDHA applied to chlorotic soybeans eliminated the chlorosis, increased growth and, surprisingly, at low application levels, decreased the iron content of plant tops. DeKock (1956) noted that chlorosis and stunting in mustard, caused by nickel and copper in the culture solution, were eliminated in the presence of ferric, nickel or copper EDTA and that there was a marked decrease in the uptake of nickel and copper. More recently, in flax, Moraghan and Freeman (1978) corrected lower leaf scorch symptoms, similar to those caused by manganese toxicity, by the application of 2 ppm iron EDDHA.

As a consequence of these successes metal chelates have been widely used in some agricultural areas, the most notable being the citrus fruit industry.

1.6 APPLICATION OF CHELATING OR COMPLEXING AGENTS ALONE TO INFLUENCE TRACE METAL LEVELS IN PLANTS

Two main objections may be made to the use of trace metal chelates in the treatment of plant deficiency and toxicity. Firstly, metal chelates are still expensive to produce because, apart from the chemical processes involved, there is less demand for them than for more conventional treatments such as N, P, K fertilizers. Some people have successfully overcome this difficulty by applying chelating agents as part of a physical mixture with trace metal salts. This method, however, increases both the likelihood of uneven application and of uneven plant response. Secondly, in many cases, deficiencies in plants are a result not of the lack of a particular metal in the soil but of an insufficiency of metal in a form readily available to the plant. By applying metal chelates one ignores this trace metal reservoir and makes use only of the applied metal. It appears likely that previously unavailable metal could be made available by adding metal-free chelating agents to the soil. Wallace (1956, p. 44) found that acid and salt forms of chelating agents were capable of chelating micronutrients from soil and earlier, in 1955, Holmes and Brown indicated that as little as 0.01 g EDDHA/7 lb soil (3 ppm) partially corrected chlorosis in soybeans (PI - 54619-5-1) while 0.03 g prevented it developing. Higher levels of chelating agent, however, decreased manganese and copper contents. Guinn and Joham (1962) observed similar effects in cotton after applying the sodium salts of EDTA and EDDHA in the range 0 - 200 μmole/litre nutrient solution. They also observed that treatment levels near the high end of the range decreased the iron content of the cotton leaves compared to the 25 and 50 μmole treatments.

Despite these indications that chelating agents alone can mobilize soil metal and alter trace metal levels in plants there appears to have been comparitively little research into this aspect of trace metal supply. More recently, however, Trevett et al. (1972) showed that EDTA alone at 400 and 600 lb/acre (448 and 672 kg/ha) significantly increased leaf manganese, zinc and iron in lowbush blueberries. The chelating agent effect probably depends upon such factors as plant species, soil type, the metal under consideration and, of course, the chelating agent being used. In view of this, it was decided, with a particular species, to

investigate the effects of some chelating agents, applied at relatively low levels, upon the concentrations of a few trace elements in the plant. Pot and field experiments designed with this purpose in mind are described and discussed in Chapters 2 and 3.

CHAPTER 2

INITIAL POT AND FIELD EXPERIMENTS

2.1 AIMS OF THE THESIS

A large body of evidence exists in the literature to show that soil-applied, trace metal chelates can influence the trace metal content of vegetation. In many instances the researchers were attempting to correct trace metal deficiencies using chelates to supply the deficient metal. This approach, however, overlooks the fact that deficiencies can occur where the metal content of the soil would be sufficient to meet plant requirements but where the metal does not exist in a form in which it is available to the plant. A number of workers have successfully demonstrated that additions of metal-free chelating agents to soils low in available iron, manganese and zinc, have often resulted in increased uptake of these metals (Abdulla and Smith, 1963; Gonzalez et al., 1972). It has been suggested that chelating agents accomplish this by chelating indigenous soil metals, hitherto unavailable, forming soluble chelates which present the metals in a form available to plants (Holmes and Brown, 1955; Abdulla and Smith, 1963). Application rates typical of such studies are: 10 - 100 mg EDTA, DTPA/100 g soil (Gonzalez et al., 1972); 250 lb/acre (120 ppm) DTPA (Holmes and Brown, 1955) and 25 ppm EDTA, EDDHA, DTPA in soil (Wallace and Mueller, 1968).

These latter observations are the basis for this thesis, the aims of which are: firstly, to determine whether or not uncomplexed chelating agents influence the trace metal content of a particular plant species; secondly, to ascertain whether application rates lower than the usual would be effective and thirdly, to see if such a regime could be applied successfully in a practical agricultural situation.

The following experiments were set up to reach conclusions under each of these headings.

2.2 EXPERIMENTAL

2.2.1 CHARACTERIZATION OF THE SOIL USED IN THE POT EXPERIMENTS

The soil, a well-drained brown earth, was collected from a north-east facing, bracken covered slope at Carbeth (Map reference NS 522797).

Its characteristics are detailed in Tables 2.2.1.1, 2.2.1.2 and 2.2.1.3.

2.2.2 POT EXPERIMENTS INVOLVING APPLICATION OF SMALL QUANTITIES OF CHELATING AGENT TO AN ACID SOIL

2.2.2.1 Introduction

The purpose was to determine whether the application to the soil of low levels of chelating agent (trace metal free) influenced the levels of trace metal present in a particular species of bean.

2.2.2.2 Materials and Methods

(1) Treatments

The chelating agents used were EDTA (as the di-sodium salt), EDDHA and 2-ketogluconic acid. The former are examples of commonly used, synthetic, commercial treatments. The latter, known to occur in plant exudates, was included as an example of a naturally occurring chelator.

Treatment solutions containing 0.02 (1.0), 0.20 (10.0), 0.50 (25.0), 1.00 (50.0), 1.50 (75.0), 2.00 (100.0) and 3.00 (150.0) ppm chelating agent made up in deionised water were applied in the experiment. Figures in parenthesis refer to the total quantity (expressed as μg/100 g soil) of chelating agent supplied in each treatment. EDDHA was converted to its sodium salt on account of the low solubility of its acid form. A deionised water control was also included in the experiment.

Table 2.2.1.1 Some physical characteristics of the soil used in the pot experiments[a]

pH[b]	% L.O.I.[c]	% O.M.[d]	Mechanical Analysis[e]		
			% Sand	% Silt	% Clay
4.5	23.23	18.70	33.13	38.48	26.72

a All data given as mean of 2 replicates.

b Determined at 1 : 2.5 soil/water ratio.

c Determined at 700°C.

d By the Walkley-Black wet combustion method (Walkley, 1947). Conversion factor 1.72.

e By a modified pipette method. Primary soil particles separated according to the International scheme.

Table 2.2.1.2 Some chemical characteristics of the soil used in the pot experiments[a]

C. E. C.[b]	Exchangeable Bases[c]				Tamm acid oxalate extractable metal[d]			Available Nutrient[e]	
(meq/100 g)	(meq/100 g)				(mg/100 g)			(mg/100 g)	
	K	Na	Ca	Mg	Fe	Al	Mn	P	K
75.71	0.50	0.44	0.34	0.33	1192.89	2316.54	23.37	0.18	9.11

a All data given as mean of 2 replicates.

b Determined after leaching with 1 M potassium acetate (pH 7.0) followed by 1 M ammonium acetate (pH 7.0).

c Determined after leaching with 1 M ammonium acetate (pH 7.0).

d Extracted 16 hours on end-over-end shaker; 1 : 10 soil/acid ratio; extracts analysed by Atomic Absorption Spectrophotometry.

e Extracted 16 hours with 0.5 M acetic acid on end-over-end shaker; 1 : 10 soil/acid ratio; in the extract potassium determined by flame photometry and phosphorous determined by the method described by Olsen and Dean, 1965, p. 1045.

Table 2.2.1.3 Some characteristics of the soil used in the pot experiments: Total metal analysis[a, b, c]

Fe (%)	Al (%)	Cu ($\mu g.g^{-1}$ soil)	Mn ($\mu g.g^{-1}$ soil)	Zn ($\mu g.g^{-1}$ soil)	K ($\mu g.g^{-1}$ soil)	Na ($\mu g.g^{-1}$ soil)	Ca ($\mu g.g^{-1}$ soil)	Mg ($\mu g.g^{-1}$ soil)
3.68	3.54	572.84	140.30	24.00	505.00	305.00	3294.50	3921.00

a All data given as mean of 2 replicates.

b Method taken from Hesse (1971, p. 377).

c Metals determined by Atomic Absorption Spectrophotometry. Potassium, sodium, calcium and magnesium were determined first in the samples. Appropriate concentrations of these metals were then added to the A.A. standards before determining iron, copper, manganese, zinc and aluminium.

(2) Method

Bean plants (Phaseolus vulgaris var. Canadian Wonder) were planted (one per pot) in 63 mm diameter plastic pots containing 100 g, 2 mm soil in field moist condition. The pots were kept in a growth chamber adjusted to a 16 hour day-length and a temperature of 24°C. Deionised water was supplied from below as required throughout the period of the experiment. On the 21st day after planting (planting counting as day 1) treatment was initiated. On each of 5 successive days 10 cm^3 of chelating agent solution were applied to the soil in the pots. Each treatment was replicated 4 times. On the 4th day after treatment was completed the plants were harvested, washed with deionised water, separated into above-ground (STEM) and below-ground (ROOT) parts and dried overnight in a forced-draught oven at 100°C. Dry matter yields were noted.

(3) Analytical technique

After drying and obtaining the yield each replicate was analysed separately as follows: quantities of dried material (approximately 0.1 g per sample) were accurately weighed and then digested for 15 minutes with 5 cm^3 ARISTAR nitric acid in a 250 cm^3 tall-form beaker covered by a watch-glass. After cooling, 5 cm^3 ARISTAR perchloric acid were added and digestion continued for a further 15 minutes. After a final cooling period the contents of the beaker were transferred, with washings from both beaker and watch-glass, to a 25 cm^3 volumetric flask and made to the mark with distilled water. The sample solutions were analysed for iron, manganese and zinc using a Perkin-Elmer 370A Atomic Absorption Spectrophotometer. An air/acetylene flame mixture was used in every case and matrix interferences were minimised by including in the standards, appropriate volumes of ARISTAR nitric, and perchloric, acids.

(4) Precision of the analytical technique

Because insufficient material was produced in the pot experiments, material collected during the field experiment (see section 2.2.4) was used in determination of the analytical errors. From one of the sub-

plots, randomly chosen, five subsamples (of approximately 0.1 g each) were taken, accurately weighed, and then analysed as described above. Errors on each metal were calculated and are presented in Table 2.2.2.1.

2.2.2.3 Results and Discussion

The results are presented in Tables 2.2.2.2, 2.2.2.3 and 2.2.2.4.

One-way analysis of variance shows both EDTA and 2-ketogluconic acid to have no significant effect on yield or upon the iron, manganese or zinc contents of the plants. On the other hand, EDDHA, while having no significant effect upon metal levels and stem yield, did increase root yield. The effect was slight and only just significant at the 5% level.

Growth effects of this sort have been observed with other chelating agents. For example, Majunder and Dunn (1958) found that in nutrient solutions, in greenhouse conditions, low concentrations of EDTA (10 μmole and below) were extremely beneficial to plant growth, especially to root development. Weinstein et al. (1956) also recorded substantial increases in the length of hypocotyl sections of etiolated white lupin seedlings floated in solutions of di-sodium EDTA (at concentrations of 3×10^{-4}M, 1×10^{-4}M and 5×10^{-5}M). They suggested that the free chelating agent promoted growth by complexing and removing calcium from the cell wall structure, permitting expansion. Other suggestions as to the cause of growth promotion by chelating agents have been, stimulation of enzyme activity and improved micro-nutrient balance (quoted by Wallace, 1962).

It is possible that EDDHA operates in one or more of these ways.

2.2.3 POT EXPERIMENTS INVOLVING APPLICATION OF SMALL QUANTITIES OF CHELATING AGENT TO A LIMED SOIL

2.2.3.1 Introduction

Table 2.2.2.1 Precision of the analytical technique

		Mean[a]	Standard Deviation	Standard error on mean	Standard error as % of mean
Fe ($mg.kg^{-1}$)	S[b]	365.757	10.292	4.406	1.205
	R[b]	460.127	36.825	15.764	3.426
Mn ($mg.kg^{-1}$)	S	56.621	7.747	3.316	5.857
	R*				
Zn ($mg.kg^{-1}$)	S	73.776	6.315	2.703	3.664
	R	33.322	22.664	9.702	29.115

a All data given as mean of 5 replicates.

b R and S signify root and stem respectively.

* Manganese concentrations in the root were too low to be measured by Flame Atomic Absorption Spectrophotometry.

Table 2.2.2.2 The effect of EDTA treatments on the subsequent yield and trace metal content of beans growing on acid soil (Cultivar - Phaseolus vulgaris var. Canadian Wonder) (Application level 0 - 150 μg/100 g soil)

		Treatment level (μg/100 g soil)[a]								ANOVA[b]
		0.0	1.0	10.0	25.0	50.0	75.0	100.0	150.0	
Yield (g)	S[c]	0.208 (0.012)[d]	0.314 (0.037)	0.242 (0.013)	0.284 (0.036)	0.254 (0.023)	0.247 (0.029)	0.215 (0.034)	0.580 (0.363)	N.S.[e]
	R[c]	0.099 (0.008)	0.112 (0.014)	0.080 (0.008)	0.130 (0.015)	0.087 (0.010)	0.115 (0.003)	0.096 (0.019)	0.264 (0.168)	N.S.
Fe ($mg.kg^{-1}$)	S	110.5 (45.9)	100.4 (8.3)	89.9 (18.8)	119.6 (3.2)	151.6 (26.2)	162.6 (15.5)	131.2 (20.9)	119.1 (15.9)	N.S.
	R	339.0 (88.0)	329.0 (75.5)	405.0 (30.5)	678.0 (180.5)	352.0 (54.5)	363.0 (97.0)	288.0 (56.5)	472.0 (115.0)	N.S.
Mn ($mg.kg^{-1}$)	S	405.0 (48.1)	328.0 (41.5)	346.0 (54.5)	378.0 (25.5)	366.0 (72.0)	630.0 (236.0)	409.0 (27.5)	336.0 (37.0)	N.S.
	R	367.1 (11.5)	405.9 (8.6)	409.0 (29.1)	316.3 (30.1)	372.4 (29.8)	476.8 (105.5)	371.0 (27.6)	382.1 (17.6)	N.S.
Zn ($mg.kg^{-1}$)	S	408.0 (267.5)	155.0 (29.5)	129.0 (30.0)	142.0 (46.0)	158.0 (59.0)	136.0 (64.5)	177.0 (14.0)	196.0 (34.0)	N.S.
	R	476.0 (148.0)	646.0 (59.0)	676.0 (54.0)	584.0 (26.0)	723.0 (150.5)	605.0 (47.0)	605.0 (21.0)	679.0 (44.5)	N.S.

a All data given as mean of 4 replicates.
b Analysis of variance - significance of treatment effect based on F statistic (5%).
c R and S signify root and stem respectively.
d Standard error on mean.
e Not significant.

Table 2.2.2.3 The effect of 2-ketogluconic acid treatments on subsequent yield and trace metal content of beans growing on acid soil (Cultivar - Phaseolus vulgaris var. Canadian Wonder) (Application level 0 - 150 μg/100 g soil)

		Treatment level (μg/100 g soil)[a]								ANOVA[b]
		0.0	1.0	10.0	25.0	50.0	75.0	100.0	150.0	
Yield (g)	S[c]	0.2687 (0.0138)[d]	0.2767 (0.0133)	0.2363 (0.0237)	0.2350 (0.0091)	0.1979 (0.0381)	0.1905 (0.0298)	0.2298 (0.0239)	0.2262 (0.0221)	N.S.[e]
	R[c]	0.1401 (0.0131)	0.1177 (0.0120)	0.0882 (0.0222)	0.1204 (0.0142)	0.0541 (0.0218)	0.0911 (0.0174)	0.0925 (0.0127)	0.0802 (0.0218)	N.S.
Fe ($mg.kg^{-1}$)	S	187.4 (38.5)	76.7 (5.5)	100.0 (10.9)	136.0 (31.1)	96.1 (21.7)	114.9 (23.9)	124.8 (24.7)	110.9 (17.4)	N.S.
	R	756.9 (212.4)	637.0 (137.5)	879.0 (160.0)	655.0 (106.0)	743.0 (217.0)	531.0 (107.0)	576.0 (76.0)	1028.0 (207.5)	N.S.
Mn ($mg.kg^{-1}$)	S	387.0 (64.0)	483.0 (38.5)	391.0 (95.5)	374.0 (17.0)	388.0 (30.0)	489.0 (124.0)	444.0 (43.0)	367.0 (49.0)	N.S.
	R	371.1 (21.1)	460.2 (45.5)	442.3 (10.7)	382.6 (35.7)	456.2 (27.2)	474.5 (70.9)	521.0 (32.1)	452.0 (43.0)	N.S.
Zn ($mg.kg^{-1}$)	S	169.0 (32.9)	261.5 (14.0)	105.0 (24.5)	301.0 (45.0)	134.0 (28.5)	191.0 (29.5)	240.0 (68.0)	176.0 (47.0)	N.S.
	R	755.7 (69.2)	1071.0 (72.5)	958.0 (35.5)	891.0 (81.5)	1012.0 (161.5)	1048.0 (89.0)	1103.0 (75.5)	993.0 (48.0)	N.S.

a All data given as mean of 4 replicates.
b Analysis of variance - significance of treatment effect based on F statistic (5%).
c R and S signify root and stem respectively.
d Standard error on mean.
e Not significant

Table 2.2.2.4 The effect of EDDHA treatments on subsequent yield and trace metal content of beans growing on acid soil (Cultivar - Phaseolus vulgaris var. Canadian Wonder) (Application level 0 - 150 μg/100g soil)

		Treatment levels (μg/100 g soil)[a]								ANOVA[b]
		0.0	1.0	10.0	25.0	50.0	75.0	100.0	150.0	
Yield (g)	S[c]	0.2078 (0.0119)[d]	0.2051 (0.0167)	0.2223 (0.0403)	0.1876 (0.0203)	0.2101 (0.0431)	0.2387 (0.0215)	0.2627 (0.0227)	0.2726 (0.0416)	N.S.[e]
	R[c]	0.0994 (0.0082)	0.0764 (0.0136)	0.0681 (0.0086)	0.0755 (0.0058)	0.0612 (0.0097)	0.0872 (0.0027)	0.0932 (0.0090)	0.1015 (0.0051)	SIG.[f]
Fe ($mg.kg^{-1}$)	S	110.5 (45.9)	96.4 (16.6)	113.7 (19.9)	121.2 (11.4)	152.9 (54.0)	136.3 (37.0)	107.5 (17.6)	118.2 (21.2)	N.S.
	R	339.0 (88.0)	272.0 (44.5)	378.0 (109.0)	281.0 (44.0)	275.0 (79.5)	368.0 (77.0)	228.0 (32.5)	297.0 (49.5)	N.S.
Mn ($mg.kg^{-1}$)	S	405.5 (48.1)	361.3 (28.1)	469.6 (97.2)	377.1 (17.2)	405.7 (19.0)	404.4 (27.8)	346.8 (26.4)	392.3 (38.4)	N.S.
	R	367.0 (11.5)	388.0 (51.0)	443.0 (54.0)	392.0 (15.0)	428.0 (18.0)	451.0 (50.5)	488.0 (102.0)	405.0 (38.0)	N.S.
Zn ($mg.kg^{-1}$)	S	408.0 (267.5)	238.0 (43.0)	221.0 (52.0)	231.0 (53.0)	199.0 (28.0)	242.0 (48.0)	140.0 (24.5)	249.0 (74.5)	N.S.
	R	476.0 (148.0)	783.0 (105.5)	919.0 (92.5)	746.0 (34.0)	748.0 (18.5)	807.0 (47.0)	709.0 (87.0)	722.0 (43.0)	N.S.

a All data given as mean of 4 replicates.
b Analysis of variance - significance of treatment effect based on F statistic (5%).
c R and S signify root and stem respectively.
d Standard error on mean.
e Not significant.
f Significant at the 5% level.

The last series of experiments showed that none of the chelating agents significantly affected trace metal levels in plants. One possible explanation for this is that chelating agents only influence metal levels in plants growing on metal-deficient soils. Therefore, a further set of experiments was conducted using the same chelating agents, application rates and cultivar, but on a limed soil, as a deficiency situation.

2.2.3.2 Materials and Methods

There were two modifications to the procedure described in 2.2.2 above:

(1) the soil pH was raised to 7.1 by incorporating powdered calcium carbonate,

(2) the 1 ppm (50 μg) and 2 ppm (100 μg) treatments were omitted.

2.2.3.3 Results and Discussion

The results are given in Tables 2.2.3.1, 2.2.3.2 and 2.2.3.3.

One-way analysis of variance shows that none of the chelating agents has a significant effect on yield or upon trace metal content.

The following are the observations on comparison of the results on a limed soil with an acid soil:

(1) stem yields were increased under treatment with EDDHA and 2-ketogluconic acid. Had the yield figures been available for EDTA treated beans the same effect might have been noted because, regardless of treatment, plants grown on the limed soil were taller than those grown on the acid soil, although stems and petioles were thinner and more easily broken,

(2) zinc and manganese were reduced in root and stem in all chelating agent treatments. This result was not unexpected because these metals are known to become less soluble and less available as soil pH increases,

(3) iron levels in the stem were increased in the EDTA and

Table 2.2.3.1 The effect of EDTA treatments on the subsequent trace metal content* of beans growing on limed soil (Cultivar - Phaseolus vulgaris var. Canadian Wonder) (Application level 0 - 150 μg/100 g soil)

		Treatment level (μg/100 g soil)[a]						ANOVA[b]
		0.0	1.0	10.0	25.0	75.0	150.0	
Fe ($mg.kg^{-1}$)	S[c]	182.0 (44.0)[d]	170.0 (29.5)	163.0 (43.0)	217.0 (79.5)	178.0 (67.0)	101.0 (26.5)	N.S.[e]
	R[c]	260.0 (67.5)	276.0 (90.5)	331.0 (68.0)	297.0 (72.0)	180.0 (40.5)	171.0 (22.0)	N.S.
Mn ($mg.kg^{-1}$)	S	140.8 (13.5)	174.6 (18.4)	155.9 (23.7)	137.9 (26.6)	147.2 (24.8)	141.0 (22.2)	N.S.
	R	575.0 (96.5)	854.0 (82.0)	725.0 (71.0)	486.0 (83.5)	633.0 (49.5)	566.0 (120.0)	N.S.
Zn ($mg.kg^{-1}$)	S	30.0 (3.5)	926.0 (899.5)	40.0 (7.5)	20.0 (11.0)	24.0 (8.0)	17.0 (8.0)	N.S.
	R	13.0 (6.0)	532.0 (526.5)	22.0 (7.5)	65.0 (49.0)	10.0 (2.5)	3.0 (2.5)	N.S.

* Yield figures were not determined.
a All data given as mean of 4 replicates.
b Analysis of variance - significance of treatment effect based on F statistic (5%).
c R and S signify root and stem respectively.
d Standard error on mean.
e Not significant.

Table 2.2.3.2 The effect of 2-ketogluconic acid treatments on the subsequent yield and trace metal content of beans growing on limed soil (Cultivar - Phaseolus vulgaris var. Canadian Wonder) (Application level 0 - 150 μg/100 g soil)

		Treatment level (μg/100 g soil)[a]						ANOVA[b]
		0.0	1.0	10.0	25.0	75.0	150.0	
Yield (g)	S[c]	0.4282 (0.0113)[d]	0.4120 (0.0236)	0.4108 (0.0503)	0.3620 (0.0699)	0.3876 (0.0504)	0.4001 (0.0125)	N.S.[e]
	R[c]	0.0759 (0.0113)	0.1016 (0.0168)	0.0827 (0.0118)	0.0861 (0.0160)	0.0989 (0.0055)	0.1063 (0.0098)	N.S.
Fe ($mg.kg^{-1}$)	S	157.2 (33.7)	189.2 (22.8)	217.5 (53.6)	141.5 (42.5)	185.6 (26.2)	277.5 (56.0)	N.S.
	R	219.0 (16.5)	357.0 (72.5)	668.0 (340.5)	335.0 (6.5)	385.0 (64.5)	514.0 (103.5)	N.S.
Mn ($mg.kg^{-1}$)	S	91.0 (14.4)	152.1 (10.9)	97.9 (26.7)	107.5 (16.0)	151.7 (40.1)	131.3 (29.8)	N.S.
	R	138.0 (84.0)	305.0 (70.0)	150.0 (75.0)	130.0 (57.5)	443.0 (133.0)	195.0 (98.0)	N.S.
Zn ($mg.kg^{-1}$)	S	49.4 (16.4)	111.0 (48.5)	101.0 (78.5)	95.0 (64.0)	148.0 (37.0)	232.0 (32.0)	N.S.
	R	56.3 (18.7)	92.7 (46.7)	36.6 (24.8)	104.1 (36.2)	36.6 (21.3)	109.7 (27.4)	N.S.

a All data given as mean of 4 replicates.
b Analysis of variance - significance of treatment effect based on F statistic (5%).
c R and S signify root and stem respectively.
d Standard error on mean.
e Not significant

Table 2.2.3.3 The effect of EDDHA treatments on the subsequent yield and trace metal content of beans growing on limed soil (Cultivar - Phaseolus vulgaris var. Canadian Wonder) (Application level 0 - 150 μg/100 g soil)

		Treatment level (μg/100 g soil)[a]						ANOVA[b]
		0.0	1.0	10.0	25.0	75.0	150.0	
Yield (g)	S[c]	0.4282 (0.0113)[d]	0.4470 (0.0260)	0.3528 (0.0294)	0.4217 (0.0225)	0.3876 (0.0533)	0.3916 (0.0185)	N.S.[e]
	R[c]	0.0759 (0.0113)	0.0733 (0.0047)	0.0574 (0.0089)	0.0986 (0.0150)	0.0989 (0.0156)	0.0892 (0.0076)	N.S.
Fe ($mg.kg^{-1}$)	S	157.2 (33.7)	154.6 (34.3)	96.3 (50.9)	107.8 (19.2)	114.0 (45.6)	189.4 (44.6)	N.S.
	R	219.0 (16.5)	328.0 (89.0)	320.0 (93.0)	376.0 (76.0)	330.0 (39.0)	323.0 (56.5)	N.S.
Mn ($mg.kg^{-1}$)	S	91.0 (14.4)	92.5 (31.2)	122.6 (21.5)	78.7 (10.5)	123.1 (32.1)	92.0 (11.6)	N.S.
	R	138.0 (84.0)	140.0 (80.5)	190.0 (70.0)	196.0 (131.0)	306.0 (131.5)	184.0 (121.0)	N.S.
Zn ($mg.kg^{-1}$)	S	49.4 (16.4)	65.1 (17.2)	53.2 (18.8)	49.3 (14.3)	62.1 (32.2)	93.4 (28.1)	N.S.
	R	56.3 (18.7)	163.3 (92.7)	164.5 (36.8)	107.9 (46.8)	60.8 (22.2)	74.8 (35.1)	N.S.

a All data given as mean of 4 replicates.
b Analysis of variance - significance of treatment effect based on F statistic (5%).
c R and S signify root and stem respectively.
d Standard error on mean
e Not significant

2-ketogluconic acid treated plants. Despite this the plant leaves were of a lighter green than similarly treated plants grown on the acid soil, indicating slight chlorosis. The results with EDDHA were rather less clear. In comparison with plants in acid soil, in half the treatments, stem iron levels were increased and in half, decreased. A slight loss in green colouration was still apparent.

In comparison with green plants, increased iron levels in chlorotic plants have been observed by others. DeKock (1955), for example, states that "the percentage iron in the ash of chlorotic plants has frequently been found to be higher than in the ash of healthy plants." Shannon (1956) appears to compliment this by stating that "foliage from chlorotic trees growing in lime-soil orchards contains nearly the same concentrations of iron as foliage from adjacent green trees."

(4) when applied under these conditions, these chelating agents were unable to prevent chlorosis occurring.

2.2.4 FIELD EXPERIMENT INVOLVING THE APPLICATION OF SMALL QUANTITIES OF CHELATING AGENT

2.2.4.1 Introduction

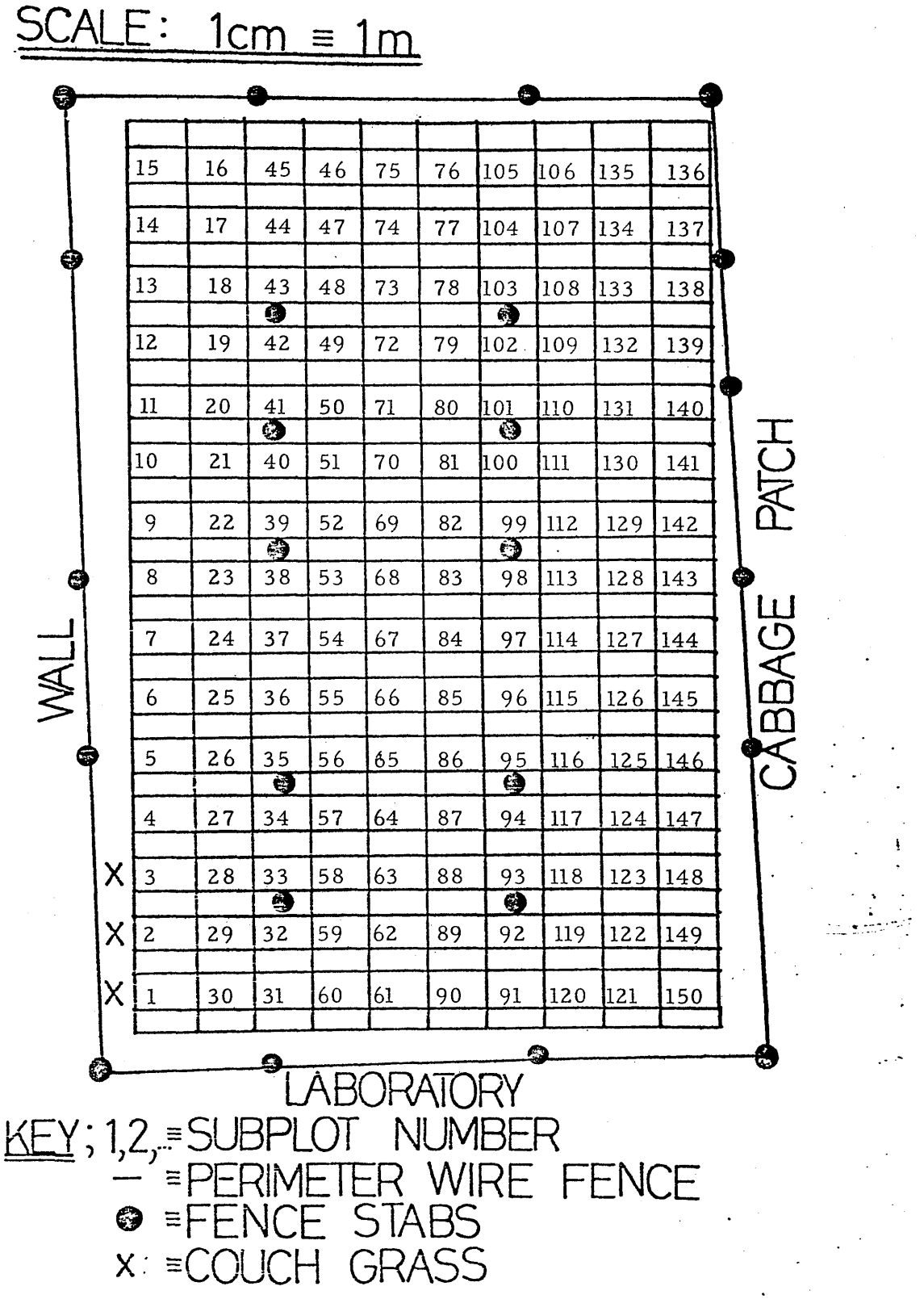
A field trial was undertaken concurrently with the pot experiments to determine whether these low levels of chelating agent could influence the trace metal content of plants grown in circumstances more nearly approximating those found in an agricultural situation.

2.2.4.2 Materials and Methods

(1) Description and preparation of the plot

The experimental plot, situated at Garscube Estate, Glasgow (Map reference NS 550705), occupied an area approximately 15 m x 11 m. The area was fenced off with plastic netting stapled to creozoted, wooden stabs and secured to the ground. In addition, the area was roofed with the same material supported on posts within the plot. 150 sub-plots, 100 cm long by 60 cm wide, were marked out. A plan of the plot, given in Figure 2.2.4.1, shows the arrangement of sub-

Figure 2.2.4.1 Plan of the experimental plot (Field experiment)



plots, separating paths, netting, netting supports and the salient features of the ground around the fenced area.

Three rows of sub-plots (marked X in Figure 2.2.4.1) were badly affected by couch grass persisting from an experiment of the previous year. Frequent weeding was required to reduce competition with the experimental cultivar. The soil was also shallower and more stony here than elsewhere. Cultivar performance was noticeably poorer in these sub-plots.

Soil samples from twelve, randomly selected sub-plots were sieved to pass a 2 mm, round-holed, brass sieve, air-dried at 30°C, and characterized with respect to pH and percentage loss on ignition (see Table 2.2.4.1 and N. B.).

(2) Planting

Beans (Phaseolus vulgaris var. Canadian Wonder) were germinated in vermicullite and laboratory-grown for 12 days in this medium. On 30th May, 1978 the seedlings were planted out, 12 per sub-plot, in two rows of six, along the 100 cm sides. The plants were spaced 10 cm apart and the rows started and finished 25 cm from the ends of sub-plots to ensure separation between beans in adjoining sub-plots. Plants were watered immediately after planting and those dying within a week were replaced by others, germinated and laboratory-grown in vermicullite, and kept in a nearby shed until required.

(3) Husbandry

Dry weather immediately prior to, and following, planting made hand-watering necessary for the first week. Thereafter, precipitation was sufficient to satisfy plant requirements.

Hand-weeding was carried out as necessary throughout the experiment.

(4) Treatments

Two chelating agents used in the pot experiments - EDDHA and

Table 2.2.4.1 Field experiment - pH and percentage loss on ignition in 12 randomly selected sub-plots

Sub-plot number	pH[a]	% Loss on ignition[b]
3	6.98	13.20
6	7.07	12.28
18	6.87	11.55
24	7.11	11.87
61	6.71	11.19
70	7.20	13.05
93	6.91	12.61
105	6.57	11.97
133	7.08	13.56
145	7.04	13.68
148	6.81	12.28
149	6.69	13.06

a 1 : 2.5 soil/water ratio.

b Determined at 820°C.

N.B. Because pH and percentage loss on ignition values did not fluctuate widely it was assumed that the soil throughout the plot was reasonably homogeneous.

2-ketogluconic acid - were chosen to allow a direct comparison to be made between their effects in the two situations. Citrate and bicarbonate, which, like 2-ketogluconic acid, have been detected in plant root exudates and are thought to be natural chelators, were also included.

Treatments, calculated on the basis of 2,500 metric tonnes soil/hectare, and corresponding to the 0, 10, 50, 75, 100 and 150 μg levels of the pot experiments, were made up in deionised water. The treatment levels were therefore equivalent to applying in the respective sub-plots 0, 25, 125, 188, 250 or 375 mg of chelating agent (as citrate, bicarbonate, EDDHA or 2-ketogluconic acid). This corresponds to the addition per hectare of 0, 0.42, 2.1, 3.2, 4.2 or 6.3 kg.

The sub-plots were divided into three groups, the first of which was treated on 13th June, the second on 22nd June and the third on 7th August (i.e. the 15th, 24th and 70th days after planting). Six sub-plots (numbers 13, 29, 56, 76, 85 and 134) remained untreated throughout.

(5) Experimental design

Two independently randomised blocks were set up each containing all 72 treatments.

(6) Application of treatments

1.25 litres of appropriately diluted chelating agent solution was applied to the designated sub-plot from a plastic watering can fitted with a plastic dribble bar. Approximately one half of this volume was applied to the soil adjacent to each of the rows of beans, taking care to avoid spillage onto the leaves. Treatments were applied sequentially, starting with the control. This procedure was repeated with each of the chelating agents in turn. The can and attachments were well washed between agents.

(7) Harvesting

There were two harvest dates. The first, on 27th June, 1978 (the 29th day after planting), corresponded approximately to the harvest

time in the pot experiments and was chosen to allow comparison of field and pot results. The second, on 15th August, 1978 (the 78th day after planting), was designed to determine whether early or late treatments influenced trace metal content.

At the first harvest half the surviving bean plants were removed, with roots, from the sub-plots included in Treatments 1 and 2, taking alternate beans from each row. At the second harvest the remaining plants were taken from these sub-plots. Half the beans from sub-plots treated in Treatment 3 and half the beans from the totally untreated sub-plots were also harvested, again taking alternate beans from each row.

(8) Material handling

After harvesting, plants were divided into above-ground (STEM) and below-ground (ROOT) parts. Material from each sub-plot was bulked, thoroughly washed with deionised water and dried at 100°C in a forced-draught oven. Some stem material required 36 to 48 hours but, generally, 24 hours sufficed. Dried material was crushed in a mortar and sieved to pass a 30 mesh stainless steel sieve.

Material from Treatment 2, Harvest 1 sub-plots could not be dealt with immediately upon arrival in the laboratory. When finally removed from storage for drying and analysis, decomposition had begun and the material had to be discarded.

Because a large quantity of material was collected in Harvest 2, only the middle leaf and petiole of each trifoliate was retained, and it was found sufficient to crush the dried leaf material by hand without recourse to the mortar.

(9) Analysis

Dried material was digested and analysed as described in section 2.2.2.

2.2.4.3 Results and Discussion

The results are presented in Tables 2.2.4.2, 2.2.4.3, 2.2.4.4 and 2.2.4.5

One-way analysis of variance showed that, regardless of treatment or harvest date, none of the chelating agents was having a significant effect upon yields or upon trace metal levels. Although the few significant F statistics which did occur could have been explained in other ways (see footnotes to the tables) further examination by regression analysis confirmed them as spurious.

2.3 CONCLUSIONS

The following may be concluded from the experiments described above:

(1) low levels (0 - 150 μg/100 g soil) of soil-applied EDTA, EDDHA and 2-ketogluconic acid, have no significant effect upon the yield or trace metal content of the bean Phaseolus vulgaris var. Canadian Wonder, pot grown, in either acid (pH 4.5) or limed (pH 7.1) soil,

(2) correspondingly low levels of soil-applied EDDHA, 2-ketogluconic acid, citrate and bicarbonate, in the field (soil pH approximately 7.0), have no significant effect upon the yield or trace metal content of the same species.

Table 2.2.4.2 The effect of chelating agent treatments on subsequent yield and trace metal content of beans growing in the field (Treatment 1, Harvest 1) (Cultivar - Phaseolus vulgaris var. Canadian Wonder) (Chelating agents - bicarbonate, citrate, EDDHA, 2-ketogluconic acid)

			Treatment level (μg/100 g soil)[a]						ANOVA[b]
			0.0	10.0	50.0	75.0	100.0	150.0	
1.	Bicarbonate								
	Fe	S[c]	370.3	482.8	277.6	336.9	316.1	340.1	SIG.[d]
	($mg.kg^{-1}$)	R[c]	433.0	490.0	340.0	348.0	371.0	933.0	N.S.[e]
	Mn	S	43.9	56.8	29.8	45.4	33.2	36.6	N.S.
	($mg.kg^{-1}$)	R*							
	Zn	S	73.8	77.8	40.0	117.4	48.7	57.0	N.S.
	($mg.kg^{-1}$)	R	31.6	46.2	27.6	35.7	18.9	52.8	N.S.
2.	Citrate								
	Fe	S	423.6	313.5	351.8	315.8	331.2	292.5	N.S.
	($mg.kg^{-1}$)	R	618.0	730.0	370.0	288.0	310.0	429.0	N.S.
	Mn	S	48.2	35.2	37.1	40.5	30.2	37.2	N.S.
	($mg.kg^{-1}$)	R*							
	Zn	S	64.5	51.5	64.0	57.8	56.8	55.7	N.S.
	($mg.kg^{-1}$)	R	31.9	35.2	27.5	32.7	21.2	46.4	N.S.
3.	EDDHA								
	Fe	S	350.3	341.4	403.1	393.4	421.4	371.5	N.S.
	($mg.kg^{-1}$)	R	424.0	488.0	585.0	457.0	597.0	234.0	N.S.
	Mn	S	33.7	32.3	34.6	32.2	31.9	32.3	N.S.
	($mg.kg^{-1}$)	R*							
	Zn	S	62.4	57.4	55.7	47.3	55.8	65.4	N.S.
	($mg.kg^{-1}$)	R	21.3	33.3	21.3	93.2	27.0	18.1	N.S.
4.	2-ketogluconic acid								
	Fe	S	280.0	316.9	400.4	428.8	348.0	295.5	N.S.
	($mg.kg^{-1}$)	R	663.0	472.0	332.0	372.0	341.0	502.0	N.S.
	Mn	S	33.8	38.7	36.6	39.3	31.8	37.1	N.S.
	($mg.kg^{-1}$)	R*							
	Zn	S	53.0	63.4	58.4	59.6	53.5	59.2	SIG.[f]
	($mg.kg^{-1}$)	R	17.8	21.6	24.0	25.9	53.6	11.7	N.S.

a All data given as mean of 2 replicates.

b Analysis of variance - significance of treatment effect based on F statistic (5%).

c R and S signify root and stem respectively.

d Significant at the 5% level. Probably a spurious figure resulting from replicates of the 50 μg treatment which are low in comparison with the others.

e Not significant.

f Significant at the 5% level. Probably a spurious figure resulting from replicates of the 10 μg treatment which are close to one another but distant from the grand mean.

* Manganese concentrations in the root were too low to measure by Flame Atomic Absorption Spectrophotometry.

Table 2.2.4.3 The effect of chelating agent treatments on subsequent yield and trace metal content of beans growing in the field. (Treatment 1, Harvest 2) (Cultivar - Phaseolus vulgaris var. Canadian Wonder) (Chelating agents - bicarbonate, citrate, EDDHA, 2-ketogluconic acid)

			Treatment levels (μg/100 g soil)[a]						ANOVA[b]
			0.0	10.0	50.0	75.0	100.0	150.0	
1.	Bicarbonate								
	Fe	S[c]	532.0	867.0	317.0	545.0	512.0	596.0	N.S.[d]
	($mg.kg^{-1}$)	R[c]	4898.0	2134.0	2830.0	3046.0	3179.0	2881.0	N.S.
	Mn	S	49.9	63.9	46.7	48.4	50.6	55.4	N.S.
	($mg.kg^{-1}$)	R	94.1	57.6	66.0	71.2	62.4	62.6	N.S.
	Zn	S	53.6	51.3	57.4	47.0	59.1	51.4	N.S.
	($mg.kg^{-1}$)	R	73.1	50.8	51.3	50.0	57.2	53.3	N.S.
2.	Citrate								
	Fe	S	425.0	727.0	460.0	471.0	570.0	558.0	N.S.
	($mg.kg^{-1}$)	R	5008.0	1968.0	3423.0	2382.0	7056.0	5588.0	N.S.
	Mn	S	46.6	55.8	45.2	49.9	46.7	55.2	N.S.
	($mg.kg^{-1}$)	R	72.2	49.2	76.0	52.1	138.6	103.7	N.S.
	Zn	S	58.5	46.3	66.2	49.1	51.9	56.1	N.S.
	($mg.kg^{-1}$)	R	55.1	46.3	77.9	62.6	80.5	75.3	N.S.
3.	EDDHA								
	Fe	S	711.0	574.0	534.0	464.0	1002.0	624.0	N.S.
	($mg.kg^{-1}$)	R	3121.0	5931.0	2598.0	3661.0	2141.0	6644.0	N.S.
	Mn	S	51.4	47.9	44.2	42.1	52.1	45.1	N.S.
	($mg.kg^{-1}$)	R	49.3	117.6	60.0	76.1	45.7	127.5	N.S.
	Zn	S	62.4	50.1	56.1	51.7	57.6	65.7	N.S.
	($mg.kg^{-1}$)	R	44.0	90.8	49.2	41.4	41.3	72.3	N.S.
4.	2-ketogluconic acid								
	Fe	S	470.0	372.0	574.0	693.0	458.0	998.0	N.S.
	($mg.kg^{-1}$)	R	7133.0	2339.0	4301.0	2836.0	1841.0	3363.0	N.S.
	Mn	S	43.8	50.5	58.4	55.1	47.1	59.5	N.S.
	($mg.kg^{-1}$)	R	131.5	52.8	102.7	59.4	57.7	70.4	N.S.
	Zn	S	40.5	52.8	54.5	54.6	53.2	68.3	N.S.
	($mg.kg^{-1}$)	R	74.8	48.2	60.6	58.1	181.3	53.2	N.S.

a All data given as mean of 2 replicates.

b Analysis of variance - significance of treatment effect based on F statistic (5%).

c R and S signify root and stem respectively.

d Not significant.

Table 2.2.4.4 The effect of chelating agent treatments on subsequent yield and trace metal content of beans growing in the field (Treatment 2, Harvest 2) (Cultivar - Phaseolus vulgaris var. Canadian Wonder) (Chelating agents - bicarbonate, citrate, EDDHA, 2-ketogluconic acid)

			Treatment level (μg/100 g soil)[a]						ANOVA[b]
			0.0	10.0	50.0	75.0	100.0	150.0	
1.	Bicarbonate								
	Fe	S[c]	967.1	433.0	604.0	624.0	780.0	456.0	N.S.[d]
	($mg.kg^{-1}$)	R[c]	3561.5	3256.0	1752.0	2108.0	7883.0	2240.0	SIG.[e]
	Mn	S	61.0	59.0	57.4	54.0	63.6	53.6	N.S.
	($mg.kg^{-1}$)	R	87.8	70.5	45.6	59.6	154.5	46.2	N.S.
	Zn	S	50.5	58.1	49.1	56.8	60.7	44.4	N.S.
	($mg.kg^{-1}$)	R	58.3	66.7	55.2	51.4	78.6	45.2	N.S.
2.	Citrate								
	Fe	S	944.0	836.0	519.0	620.0	547.0	1632.0	N.S.
	($mg.kg^{-1}$)	R	5203.0	2995.0	2337.0	2770.0	2429.0	3265.0	N.S.
	Mn	S	70.5	64.5	48.9	61.4	58.3	87.7	N.S.
	($mg.kg^{-1}$)	R	94.6	67.5	54.4	73.0	69.7	78.6	N.S.
	Zn	S	52.8	54.5	50.8	66.5	55.0	76.5	N.S.
	($mg.kg^{-1}$)	R	62.3	67.0	53.1	62.9	53.9	70.2	N.S.
3.	EDDHA								
	Fe	S	555.0	425.0	1278.0	278.0	550.0	573.0	N.S.[f]
	($mg.kg^{-1}$)	R	2238.0	3209.0	2537.0	6055.0[g]	5374.0[g]	3374.0	SIG.[f]
	Mn	S	57.2	47.9	70.7	39.6	57.2	43.4	N.S.
	($mg.kg^{-1}$)	R	51.3	72.1	52.8	113.1[g]	143.2[g]	71.7	SIG.[f]
	Zn	S	56.9	53.5	55.0	53.7	56.9	59.0	N.S.
	($mg.kg^{-1}$)	R	49.2	74.2	48.3	63.4	63.8	59.6	N.S.
4.	2-ketogluconic acid								
	Fe	S	420.0	518.0	1191.0	468.0	625.0	737.0	N.S.
	($mg.kg^{-1}$)	R	3441.0	3408.0	2191.0	3405.0	5608.0	2000.0	N.S.
	Mn	S	54.8	47.7	78.2	45.6	58.7	54.3	N.S.
	($mg.kg^{-1}$)	R	81.8	59.7	55.0	81.3	114.2	50.7	N.S.
	Zn	S	49.9	57.8	74.2	50.1	87.5	60.3	N.S.
	($mg.kg^{-1}$)	R	57.2	57.7	48.3	53.4	84.2	48.3	N.S.

a All data given as mean of 2 replicates.

b Analysis of variance - significance of treatment effect based on F statistic (5%).

c R and S signify root and stem respectively.

d Not significant

e Significant at the 5% level. Probably a spurious figure resulting from replicates of the 100 μg treatment which are close to each other but distant from the grand mean.

f Significant at the 5% level. Probably caused by the abnormally high values for the 75 and 100 μg treatments.

g Based on a single replicate.

Table 2.2.4.5 The effect of chelating agent treatments on subsequent yield and trace metal content of beans growing in the field (Treatment 3, Harvest 2) (Cultivar - Phaseolus vulgaris var. Canadian Wonder) (Chelating agents - bicarbonate, citrate, EDDHA, 2-ketogluconic acid)

			Treatment level (μg/100 g soil)[a]						ANOVA[b]
			0.0	10.0	50.0	75.0	100.0	150.0	
1.	Bicarbonate								
	Fe	S[c]	421.0	812.0	661.0	505.0	423.0	532.0	N.S.[d]
	($mg.kg^{-1}$)	R[c]	4115.0	3557.0	2589.0	1534.0	4902.0	3177.0	N.S.
	Mn	S	42.5	50.2	60.0	40.4	51.0	42.6	N.S.
	($mg.kg^{-1}$)	R	103.4	77.3	56.1	53.4	105.3	67.4	N.S.
	Zn	S	48.4	64.3	44.6	55.6	54.3	38.4	N.S.
	($mg.kg^{-1}$)	R	93.9	61.5	49.0	50.1	55.6	52.3	N.S.
2.	Citrate								
	Fe	S	721.0	680.0	355.0	355.0	709.0	451.0	N.S.
	($mg.kg^{-1}$)	R	4931.0	5443.0	2435.0	3374.0	3062.0	4264.0	N.S.
	Mn	S	67.4	45.3	34.1	41.5	39.8	41.4	N.S.
	($mg.kg^{-1}$)	R	80.3	108.3	49.3	87.9	60.3	95.6	N.S.
	Zn	S	42.5	45.4	52.8	45.9	53.7	73.0	N.S.
	($mg.kg^{-1}$)	R	49.0	296.0	41.0	55.0	51.0	53.0	N.S.
3.	EDDHA								
	Fe	S	594.0	596.0	889.0	888.0	447.0	329.0	N.S.
	($mg.kg^{-1}$)	R	2174.0	2177.0	3544.0	2315.0	3811.0	3777.0	N.S.
	Mn	S	56.0	60.9	48.9	51.8	48.6	43.0	N.S.
	($mg.kg^{-1}$)	R	43.2	53.8	81.7	55.6	71.3	81.2	N.S.
	Zn	S	50.5	56.2	47.2	50.1	48.6	36.7	N.S.
	($mg.kg^{-1}$)	R	121.9	67.2	71.5	49.6	56.9	60.8	N.S.
4.	2-ketogluconic acid								
	Fe	S	461.0	747.0	1173.0	841.0	495.0	1285.0	N.S.
	($mg.kg^{-1}$)	R	4058.0	2016.0	2919.0	2179.0	9823.0	6541.0	N.S.
	Mn	S	53.0	46.6	57.2	49.1	54.3	69.4	N.S.
	($mg.kg^{-1}$)	R	86.3	43.7	65.7	49.1	216.5	122.8	N.S.
	Zn	S	55.0	45.4	40.7	63.9	42.9	68.9	N.S.
	($mg.kg^{-1}$)	R	61.4	35.8	59.2	59.3	90.5	88.0	N.S.

a All data given as mean of 2 replicates.

b Analysis of variance - significance of treatment effect based on F statistic (5%).

c R and S signify root and stem respectively.

d Not significant.

CHAPTER 3

SUBSEQUENT POT EXPERIMENTS

3.1 INTRODUCTION

Previous experiments (see Chapter 2) show that the application of low levels of chelating agent (trace metal free) has no significant effect upon yield and trace metal (iron, manganese and zinc) content of the bean Phaseolus vulgaris var. Canadian Wonder. This suggests that, either chelating agents alone, are unable to affect growth and trace metal content, or, that the applications rates (0 - 150 μg chelating agent/100 g soil) have been too low.

Two series of pot experiments are considered in this chapter. The first was designed to answer the question, will higher levels (up to 1000 μg/100 g soil) of chelating agent significantly affect trace metal levels and yields. The second was included to allow comparison to be made of the relative effectiveness of metal chelate and chelating agent treatments.

3.2 EXPERIMENTAL

3.2.1 POT EXPERIMENTS INVOLVING APPLICATION OF HIGHER LEVELS OF CHELATING AGENT TO AN ACID SOIL

3.2.1.1 Materials and Methods

(1) Soil

The soil was that described and characterized in Chapter 2, section 2.2.1.

(2) Treatments

The chelating agents used were citrate (as the sodium salt), EDTA (as the di-sodium salt) and 2-ketogluconic acid.

Treatment solutions containing 5.0 (250.0), 10.0 (500.0), 15.0 (750.0) and 20.0 (1000.0) ppm chelating agent were made up in deionised

water. The figures in parenthesis refer to the total quantity (expressed as μg/100 g soil) of chelating agent supplied in each treatment. A deionised water control was also included in the experiment.

(3) Method

The method is described in Chapter 2, section 2. 2. 2.

(4) Analytical technique

For the analytical method, the precision and errors associated with it, see Chapter 2, section 2. 2. 2.

3. 2. 1. 2 Results and Discussion

The results are presented in Tables 3. 2. 1. 1, 3. 2. 1. 2 and 3. 2. 1. 3.

One-way analysis of variance, using these data, showed citrate, EDTA and 2-ketogluconic acid to have little significant effect on yield or upon trace metal levels. In discussing the few significant effects (five in all), reference is made to the trend diagrams appearing in Figure 3. 2. 1. 1. (The diagrams are considered in greater detail later in the chapter).

Root manganese levels decreased slightly as the level of citrate applied increased. Possibly, competition between chelating agent and root inhibits manganese absorption. Both citrate and 2-ketogluconic acid appeared to increase stem zinc levels. In both cases, however, the effect was small, being only just significant at the 5% level. The trend diagram (Figure 3. 2. 1. 1) for EDTA indicates that this chelating agent also tended to increase zinc in the stem, but not significantly.

The effect of 2-ketogluconic acid in decreasing root zinc levels was small and only just significant at the 5% level. Root-chelating agent competition may, once again, be the cause. Interestingly, trend diagrams (Figure 3. 2. 1. 1) imply that EDTA also depressed zinc levels in the root (although the trend is not statistically significant), while the opposite is true of citrate.

Table 3.2.1.1 The effect of citrate treatments on the subsequent yield and trace metal content of beans growing on acid soil (Cultivar - Phaseolus vulgaris var. Canadian Wonder) (Application level 0 - 1000 μg/100 g soil)

		Treatment level (μg/100 g soil)[a]					ANOVA[b]
		0.0	250.0	500.0	750.0	1000.0	
Yield (g)	S[c]	0.2330 (0.0033)[d]	0.3355 (0.0124)	0.2539 (0.0204)	0.2276 (0.0290)	0.2631 (0.0242)	SIG.[e]
	R[c]	0.0971 (0.0027)	0.0846 (0.0190)	0.0898 (0.0144)	0.0780 (0.0088)	0.0876 (0.0075)	N.S.[f]
Fe ($mg \cdot kg^{-1}$)	S	117.0 (10.7)	500.0 (59.0)	421.0 (136.0)	81.0 (6.5)	443.0 (49.5)	SIG.[g]
	R	328.2 (42.5)	587.0 (98.0)	371.0 (116.5)	369.0 (180.0)	512.0 (150.5)	N.S.
Mn ($mg.kg^{-1}$)	S	392.6 (15.8)	305.8 (40.6)	350.0 (36.7)	364.2 (30.7)	372.7 (61.7)	N.S.
	R	352.0 (11.2)	282.3 (36.1)	260.9 (16.2)	287.1 (14.7)	263.6 (15.6)	SIG.[h]
Zn ($mg.kg^{-1}$)	S	137.0 (8.7)	289.2 (64.6)	254.1 (66.5)	354.1 (20.4)	275.1 (31.8)	SIG.[h]
	R	705.2 (38.8)	726.4 (29.7)	716.1 (52.7)	775.6 (7.9)	754.0 (49.8)	N.S.

a All data given as mean of 4 replicates.

b Analysis of variance - significance of treatment effect based on F statistic (5%).

c R and S signify root and stem respectively.

d Standard error on mean.

e Significant at the 5% level. Probably caused by replicates of the 250.0 μg treatment which are close to one another but are distant from the grand mean.

f Not significant.

g Significant at the 1% level. Probably caused by replicates of the 750.0 μg treatment which are close to one another but are distant from the grand mean.

h Significant at the 5% level.

Table 3.2.1.2 The effect of EDTA treatments on the subsequent yield and trace metal content of beans growing on acid soil (Cultivar - Phaseolus vulgaris var. Canadian Wonder) (Application level 0 - 1000 μg/100 g soil)

		Treatment level (μg/100 g soil)[a]					ANOVA[b]
		0.0	250.0	500.0	750.0	1000.0	
Yield (g)	S[c]	0.2330 (0.0033)[d]	0.2226 (0.0177)	0.2298 (0.0217)	0.2252 (0.0234)	0.1869 (0.0217)	N.S.[e]
	R[c]	0.0971 (0.0027)	0.1028 (0.0063)	0.1041 (0.0081)	0.1110 (0.0102)	0.1015 (0.0104)	N.S.
Fe ($mg.kg^{-1}$)	S	117.0 (10.7)	107.0 (10.4)	99.6 (13.8)	140.0 (37.1)	219.7 (43.0)	SIG.[f]
	R	328.2 (42.5)	335.1 (28.1)	309.8 (23.1)	446.1 (40.2)	405.2 (57.3)	N.S.
Mn ($mg.kg^{-1}$)	S	392.6 (15.8)	446.8 (44.8)	489.0 (45.7)	402.6 (60.8)	449.9 (33.7)	N.S.
	R	352.0 (11.2)	339.3 (8.8)	346.0 (18.5)	326.4 (13.0)	390.1 (21.1)	N.S.
Zn ($mg.kg^{-1}$)	S	137.0 (8.7)	281.0 (82.0)	240.0 (47.5)	253.0 (49.5)	213.0 (47.0)	N.S.
	R	705.2 (38.8)	548.5 (44.0)	539.7 (39.9)	593.2 (31.1)	606.8 (44.5)	N.S.

a All data given as mean of 4 replicates.

b Analysis of variance - significance of treatment effect based on F statistic (5%).

c R and S signify root and stem respectively.

d Standard error on mean.

e Not significant.

f Significant at the 5% level.

Table 3.2.1.3 The effect of 2-ketogluconic acid on the subsequent yield and trace metal content of beans growing on acid soil (Cultivar - Phaseolus vulgaris var. Canadian Wonder) (Application level 0 - 1000 μg/100 g soil)

		Treatment level (μg/100 g soil)[a]					ANOVA[b]
		0.0	250.0	500.0	750.0	1000.0	
Yield (g)	S[c]	0.2330 (0.0033)[d]	0.2571 (0.0196)	0.2166 (0.0223)	0.2451 (0.0102)	0.2827 (0.0287)	N.S.[e]
	R[c]	0.0971 (0.0027)	0.0920 (0.0075)	0.0878 (0.0086)	0.1089 (0.0116)	0.1125 (0.0070)	N.S.
Fe ($mg.kg^{-1}$)	S	117.0 (10.7)	126.2 (12.4)	115.2 (23.6)	107.3 (16.4)	117.5 (5.2)	N.S.
	R	328.2 (42.5)	240.2 (7.0)	247.4 (34.7)	341.0 (42.3)	259.4 (48.5)	N.S.
Mn ($mg.kg^{-1}$)	S	392.6 (15.8)	457.5 (43.1)	489.2 (33.5)	461.4 (55.0)	474.1 (21.2)	N.S.
	R	352.0 (11.2)	355.7 (32.0)	360.6 (29.1)	397.1 (53.3)	324.0 (22.2)	N.S.
Zn ($mg.kg^{-1}$)	S	137.0 (8.7)	207.7 (39.2)	271.5 (31.1)	227.6 (21.1)	219.8 (17.1)	SIG.[f]
	R	705.2 (38.8)	594.1 (48.2)	551.6 (28.1)	593.0 (10.8)	560.9 (24.1)	SIG.[f]

a All data given as mean of 4 replicates.

b Analysis of variance - significance of treatment effect based on F statistic (5%).

c R and S signify root and stem respectively.

d Standard error on mean.

e Not significant.

f Significant at the 5.0% level.

Increasing the level of EDTA resulted in a significant increase in stem iron level without, however, a marked increase in total iron in the plant and it would appear that metal transport within the plant is enhanced by the chelating agent. While the trend diagram (Figure 3.2.1.1) shows some increase in iron in the root with increasing levels of EDTA there appears to be no statistical correlation.

A correlation matrix for each of the chelating agents is included in Table 3.2.1.4. Those correlations, significant at the 5% level, are listed (along with the sign of the correlation coefficient) in Table 3.2.1.5.

Correlations with citrate are considered first. The negative relationship between citrate level and manganese in the root re-inforces the suggestion that citrate inhibits manganese absorption by the root (see page63). It might be inferred from the positive relationship observed between zinc in the stem and the level of citrate that citrate facilitates translocation of zinc within the plant.

Correlations with EDTA are considered next. The correlation between EDTA and stem iron was discussed earlier (see page 67). The highly significant correlation between root and stem yield may be explained thus; as the root system extends into a larger volume of soil a larger quantity of nutrient becomes available. Increased nutrient uptake may increase vegetative growth and increase stem yields. A similar effect was observed with 2-ketogluconic acid but not with citrate.

The simplest explanation for the correlation between iron in stem and in root is that a constant proportion of iron is transported from root to stem. Therefore, as the quantity of root iron increases (or decreases) so does the stem iron content.

Generally, in plants, the relationship between iron and manganese is one of antagonism (Moraghan and Freeman, 1978; Moraghan, 1980). The direct correlation between stem iron and root manganese is, therefore, somewhat unexpected but may result from a reduction in antagonism between the two metals as iron moves from root to stem.

Table 3.2.1.4 Matrices of correlation coefficients[c] for three chelating agents - citrate, EDTA, 2-ketogluconic acid - applied to acid soil at levels up to 1000 μg/100 g soil (Cultivar - Phaseolus vulgaris var. Canadian Wonder)

	Level[a]	Yield (S)[b]	Yield (R)[b]	Fe (S)	Fe (R)	Mn (S)	Mn (R)	Zn (S)
1. Citrate								
Yield(S)	-0.130							
Yield(R)	-0.168	0.390						
Fe(S)	0.154	0.520	0.094					
Fe(R)	0.088	0.049	0.021	0.051				
Mn(S)	0.035	-0.645	-0.112	-0.233	0.443			
Mn(R)	-0.499	-0.206	0.210	-0.354	0.054	0.408		
Zn(S)	0.459	-0.011	-0.183	-0.039	0.408	0.214	-0.398	
Zn(R)	0.287	-0.193	-0.135	-0.073	0.050	0.271	0.265	0.172
2. EDTA								
Yield(S)	-0.343							
Yield(R)	0.164	0.598						
Fe(S)	0.527	-0.656	-0.492					
Fe(R)	0.432	-0.286	-0.164	0.566				
Mn(S)	0.121	-0.139	0.201	-0.120	-0.376			
Mn(R)	0.264	-0.597	-0.334	0.449	0.139	0.244		
Zn(S)	0.169	0.017	0.032	-0.271	-0.022	0.113	0.002	
Zn(R)	-0.237	0.318	0.114	-0.132	-0.028	-0.046	0.285	-0.057

Table 3.2.1.4 continued

	Level[a]	Yield (S)[b]	Yield (R)[b]	Fe (S)	Fe (R)	Mn (S)	Mn (R)	Zn (S)
3. 2-ketogluconic acid								
Yield(S)	0.310							
Yield(R)	0.402	0.588						
Fe(S)	-0.095	0.407	0.514					
Fe(R)	-0.066	0.285	0.372	0.073				
Mn(S)	0.330	0.077	-0.435	-0.117	-0.106			
Mn(R)	-0.033	-0.246	-0.408	-0.063	-0.005	0.576		
Zn(S)	0.422	0.151	0.005	0.378	-0.116	0.614	0.286	
Zn(R)	-0.521	-0.190	-0.144	0.101	0.453	-0.094	0.382	-0.201

a Level refers to the quantity of chelating agent applied (0 - 1000 μg/100 g soil).

b (S) and (R) refer to stem and root respectively.

c Least significant values of correlation coefficient (n = 20).

5% level	1% level
0.4445	0.5629

Table 3.2.1.5 Correlations significant at the 5% level

Citrate		EDTA		2-ketogluconic Acid	
Correlation	Sign	Correlation	Sign	Correlation	Sign
level vs Mn (R)[a]	neg.[b]	level vs Fe (S)	pos.	level vs Zn (R)	neg.
level vs Zn (S)[a]	pos.[b]	yield (S) vs yield (R)	pos.*	yield (S) vs yield (R)	pos.*
yield (S) vs Fe (S)	pos.	yield (S) vs Fe (S)	neg.*	yield (R) vs Fe (S)	pos.
yield (S) vs Mn (S)	neg.*	yield (S) vs Mn (R)	neg.*	Fe (R) vs Zn (R)	pos.
		yield (R) vs Fe (S)	neg.	Mn (S) vs Mn (R)	pos.*
		Fe (S) vs Fe (R)	pos.*	Mn (S) vs Zn (S)	pos.*
		Fe (S) vs Mn (R)	pos.		

a (R) indicates root; (S) indicates stem.

b Neg. indicates a negative correlation; pos. indicates a positive correlation.

* These correlations are also significant at the 1% level.

Finally, the correlations obtained with 2-ketogluconic acid are considered. The effect of the chelating agent on root zinc was mentioned on page 63. Again, the relationship between root and stem yield might be explained as for EDTA (see page 67). It is interesting that positive relationships should occur between manganese and zinc in the stem and between iron and zinc in the root because other workers have observed both manganese and iron to have inhibitory effects upon zinc absorption (Reddy et al., 1978; Hawf and Schmid, 1967). Reddy and his associates, noting a reduction in root absorption of ^{65}Zn as nutrient iron was increased, attributed this to competition for absorption sites in Donnan-free-space. They suggested that "manganese interferes with absorption of zinc, perhaps by competing for the same absorption sites as well as for translocation involving physiological inhibition of transport from roots to shoots." Hawf and Schmid, on the other hand, concluded from experiments on bush beans in which different ions were added to nutrient solutions containing ^{65}Zn that, rather than by competing with translocation mechanisms, the added ions (including manganese) inhibit ^{65}Zn movement to tops by immobilizing it in the roots. In addition, they noted that ^{65}Zn uptake was inhibited only at high manganese concentrations. Manganese, however, did not alter the internal distribution of ^{65}Zn.

Positive correlations between manganese root and stem levels are explicable in much the same manner as the relationship between stem and root iron following application of EDTA (see page 67).

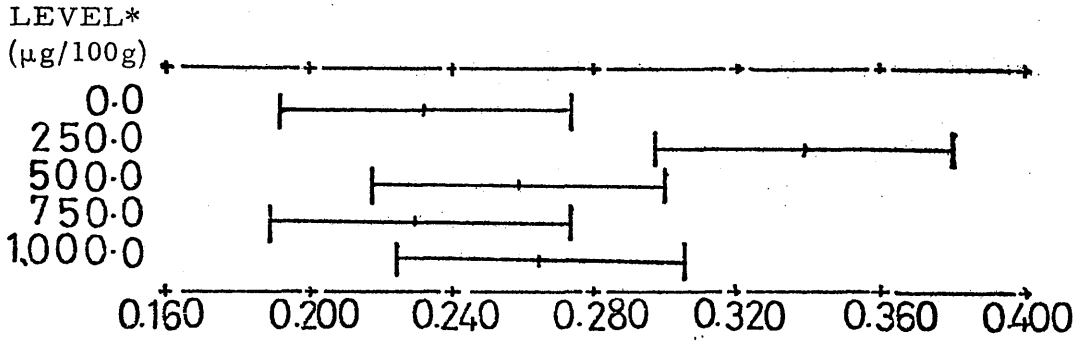
As stated earlier, one-way analysis of variance indicated citrate, EDTA and 2-ketogluconic acid to have few significant effects on yield or upon trace metal levels. However, some trends were observed when graphs were constructed of chelating agent level against trace metal content/yield. These are illustrated in the trend diagrams in Figure 3.2.1.1. Although not statistically significant, further research could be fruitful.

Increasing levels of citrate appear to slightly increase iron and zinc in the stem and zinc in the root while slightly decreasing root manganese levels. Increased levels of root zinc infer that citrate

Figure 3.2.1.1 TREND DIAGRAM - the effects of five application levels of three chelating agents on trace metal content and yield. Treatment means with pooled standard deviation from one-way analysis of variance

A. CITRATE

1. YIELD (STEM)



2. YIELD (ROOT)

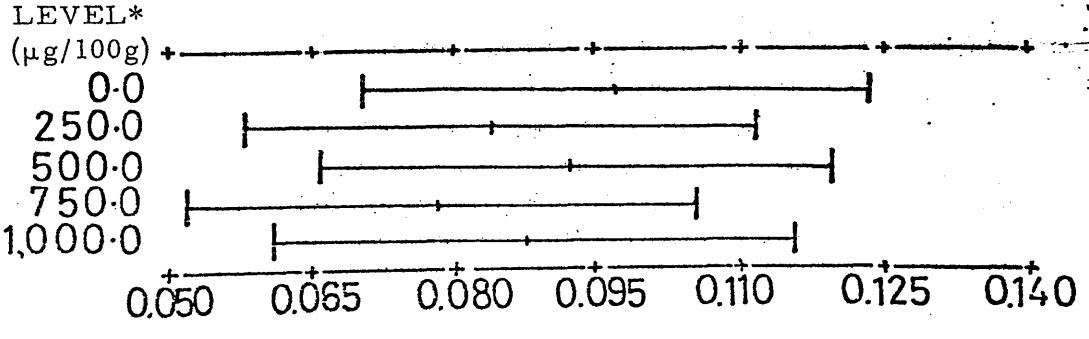
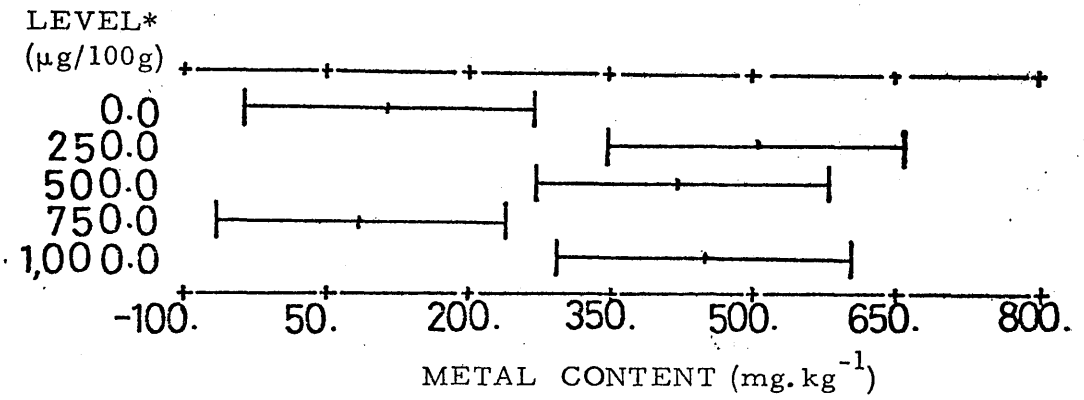


Figure 3.2.1.1 continued

A. CITRATE

3. IRON (STEM)



4. IRON (ROOT)

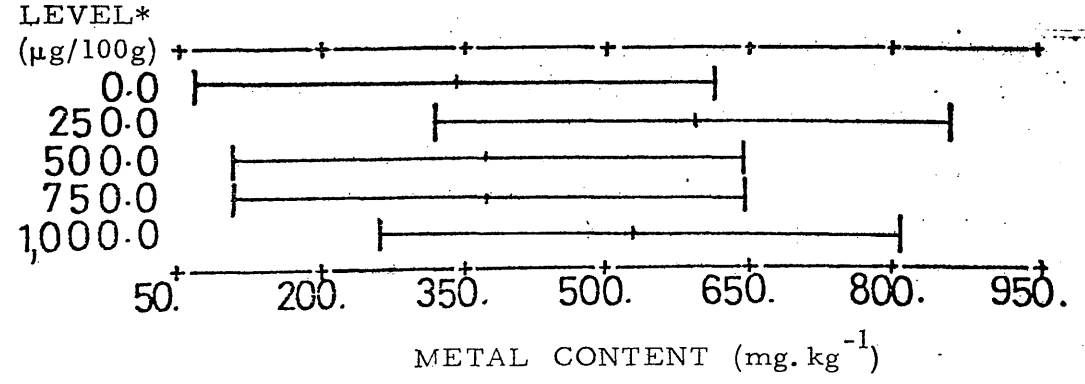
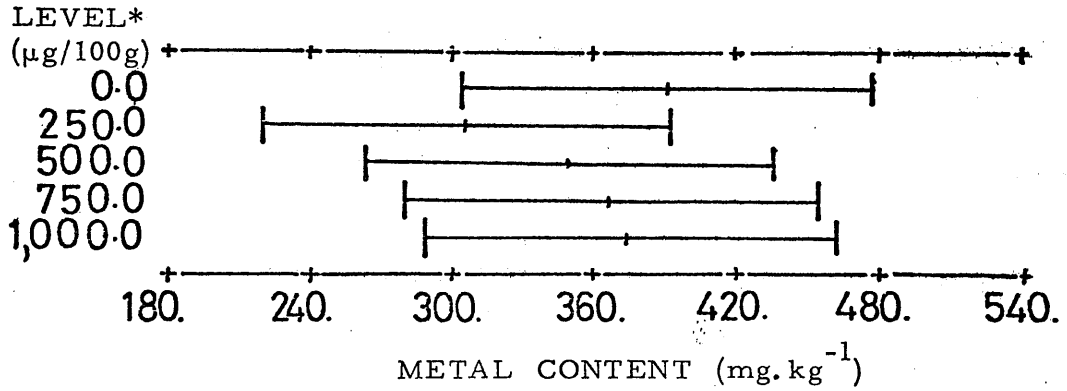


Figure 3.2.1.1 continued

A. CITRATE

5. MANGANESE (STEM)



6. MANGANESE (ROOT)

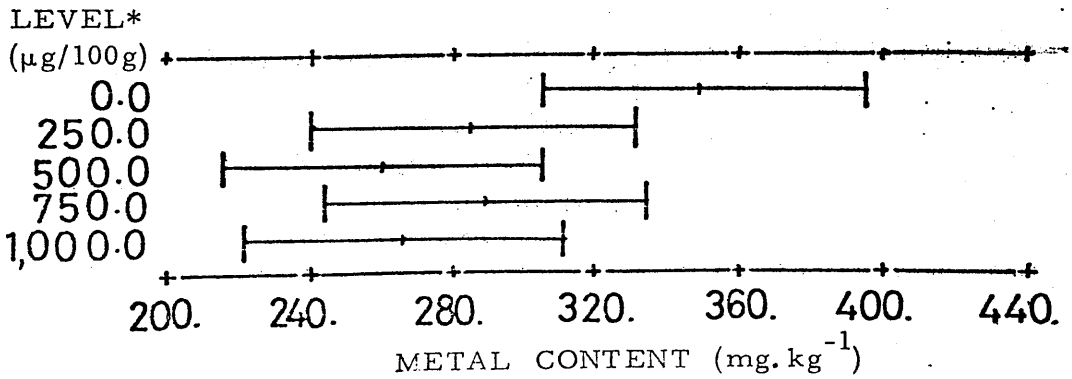
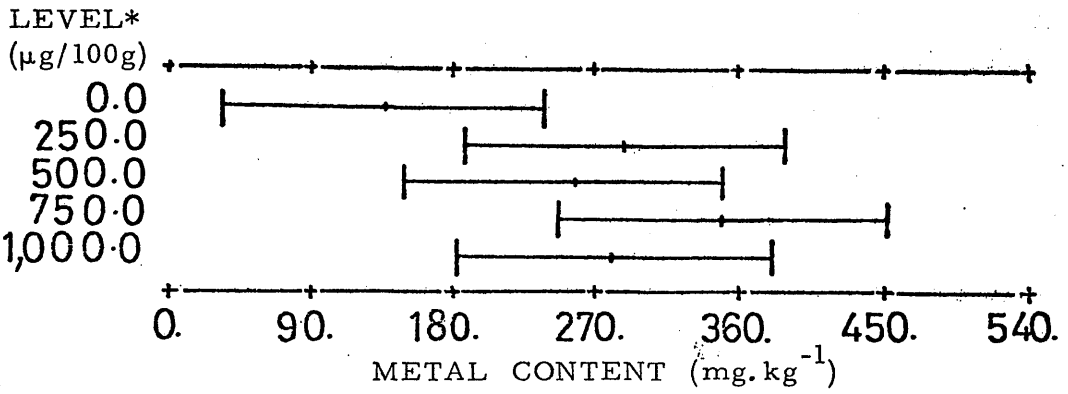


Figure 3.2.1.1 continued

A. CITRATE

7. ZIŃC (STEM)



8. ZINC (ROOT)

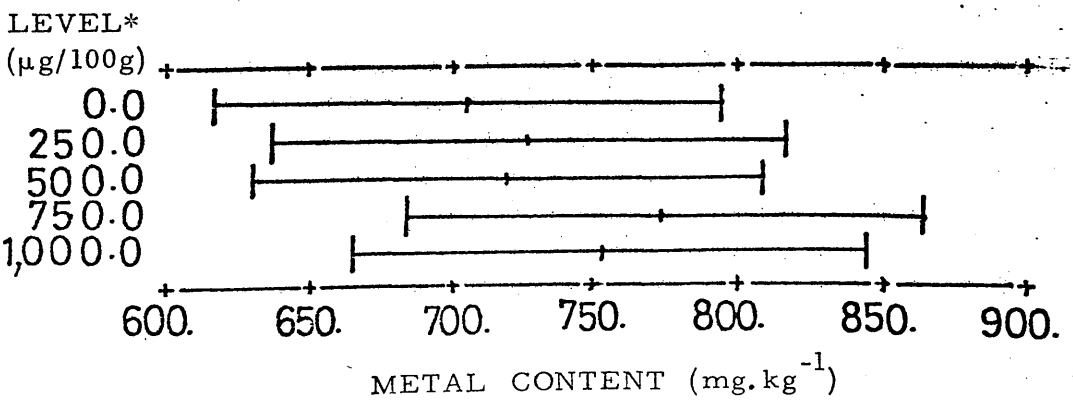
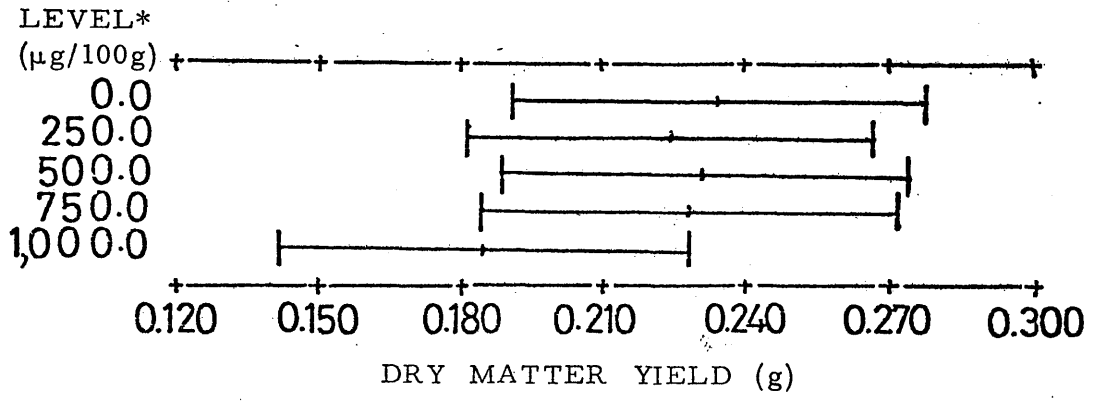


Figure 3.2.1.1 continued

B. EDTA

1. YIELD (STEM)



2. YIELD (ROOT)

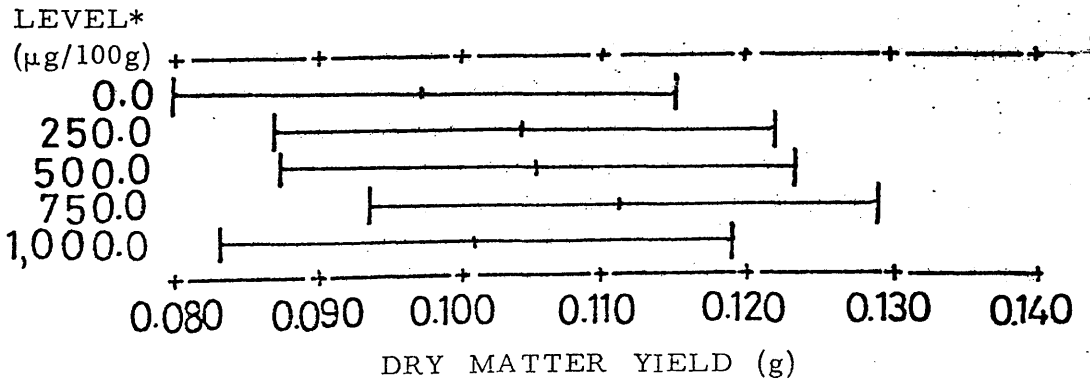
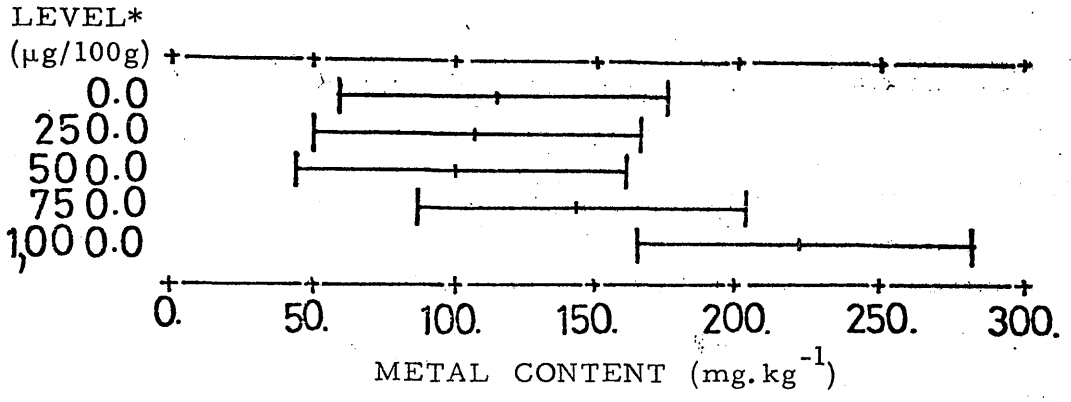


Figure 3.2.1.1 continued

B. EDTA

3. IRON (STEM)



4. IRON (ROOT)

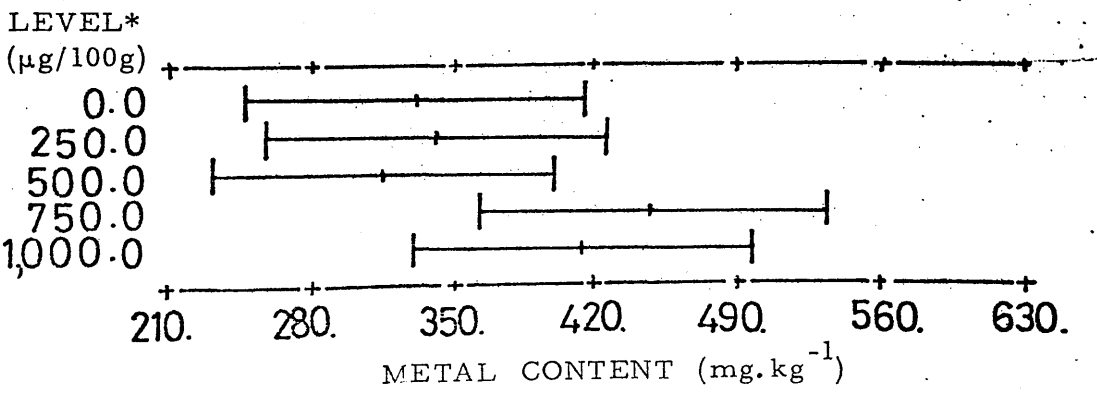
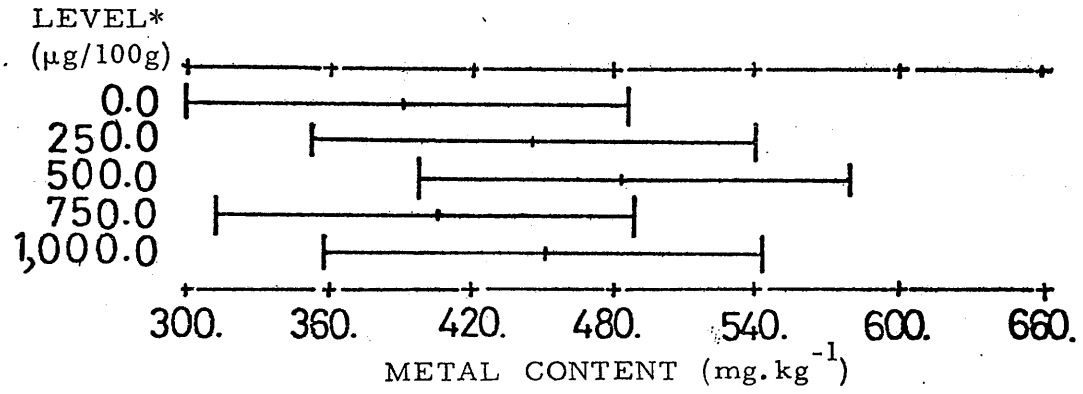


Figure 3.2.1.1 continued

B. EDTA

5. MANGANESE (STEM)



6. MANGANESE (ROOT)

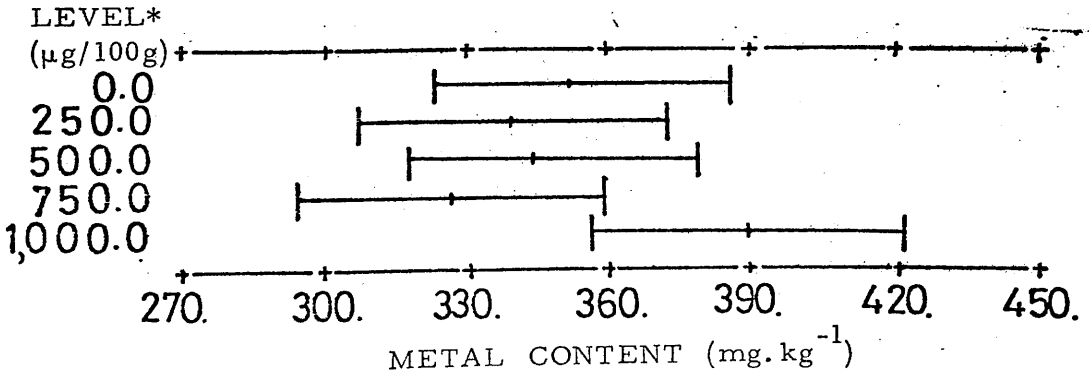
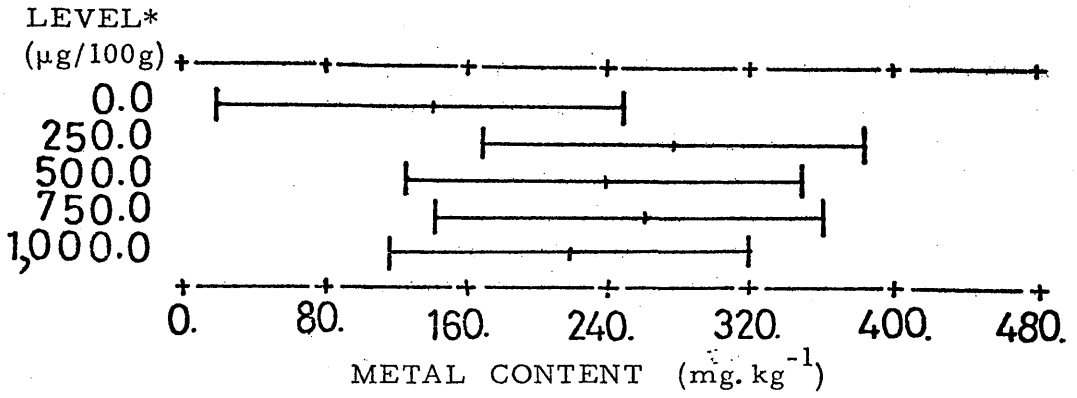


Figure 3.2.1.1 continued

B. EDTA

7. ZINC (STEM)



8. ZINC (ROOT)

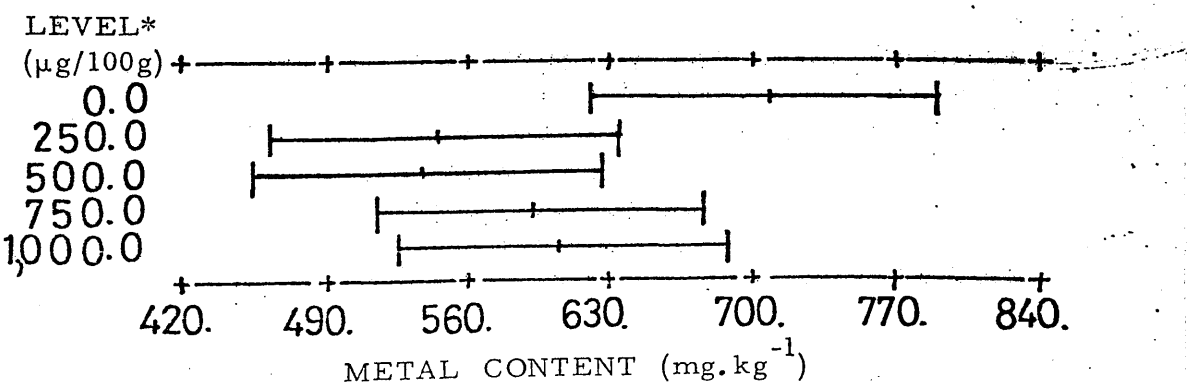
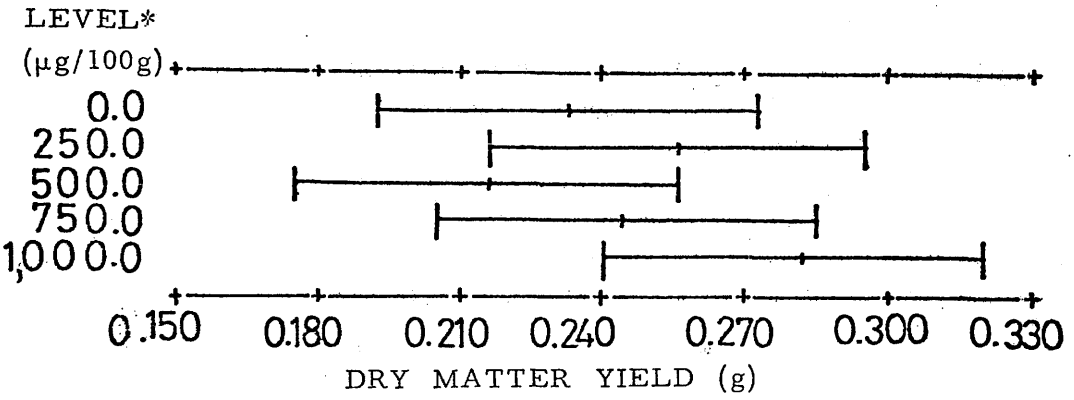


Figure 3.2.1.1 continued

C. 2-KETOGLUCONIC ACID

1. YIELD (STEM)



2. YIELD (ROOT)

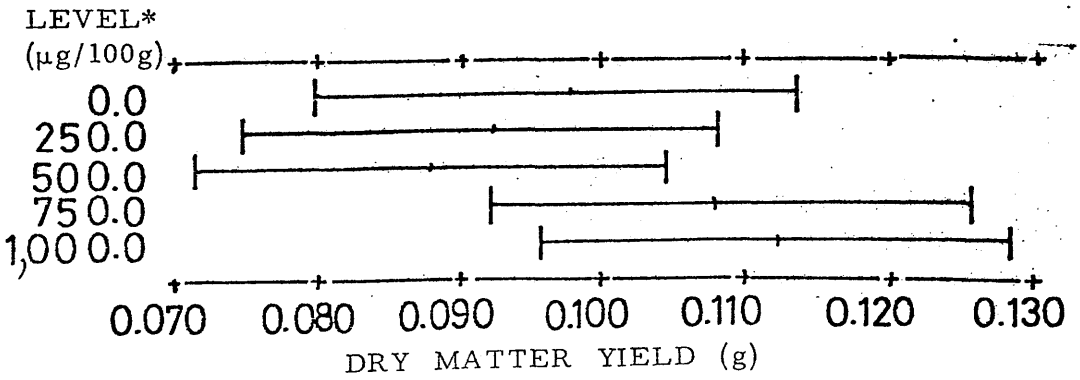
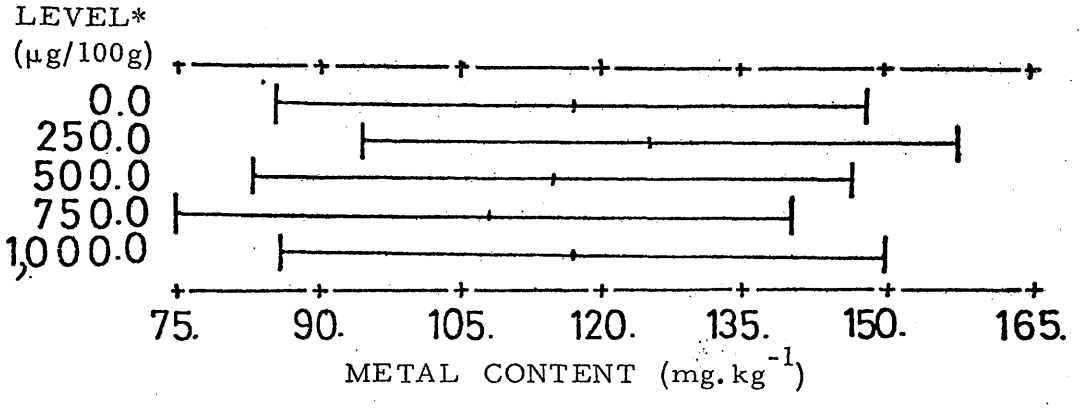


Figure 3.2.1.1 continued

C. 2-KETOGLUCONIC ACID

3. IRON (STEM)



4. IRON (ROOT)

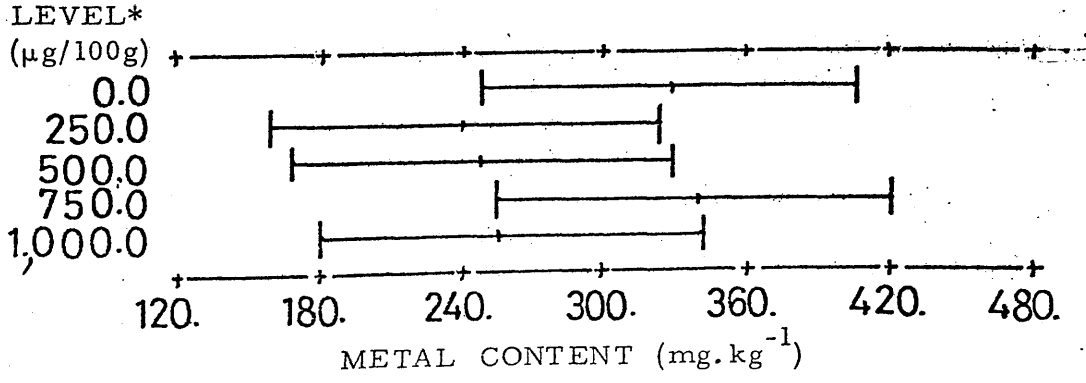
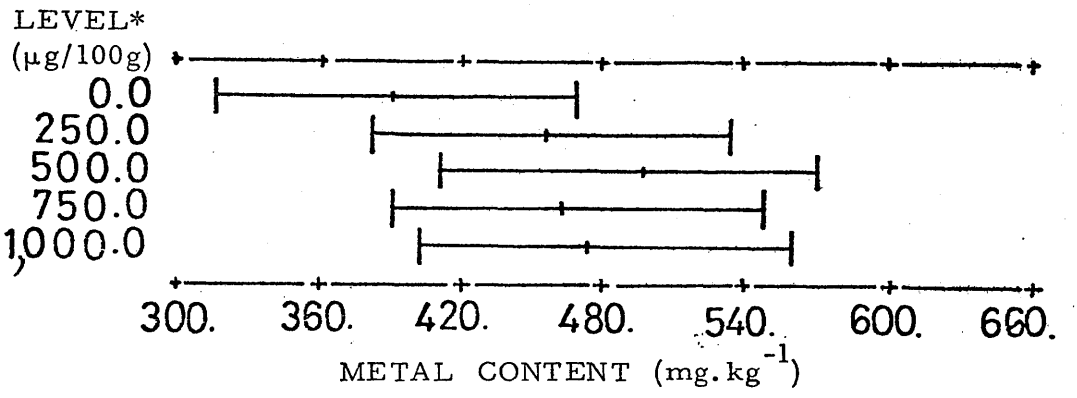


Figure 3.2.1.1 continued

C. 2-KETOGLUCONIC ACID

5. MANGANESE (STEM)



6. MANGANESE (ROOT)

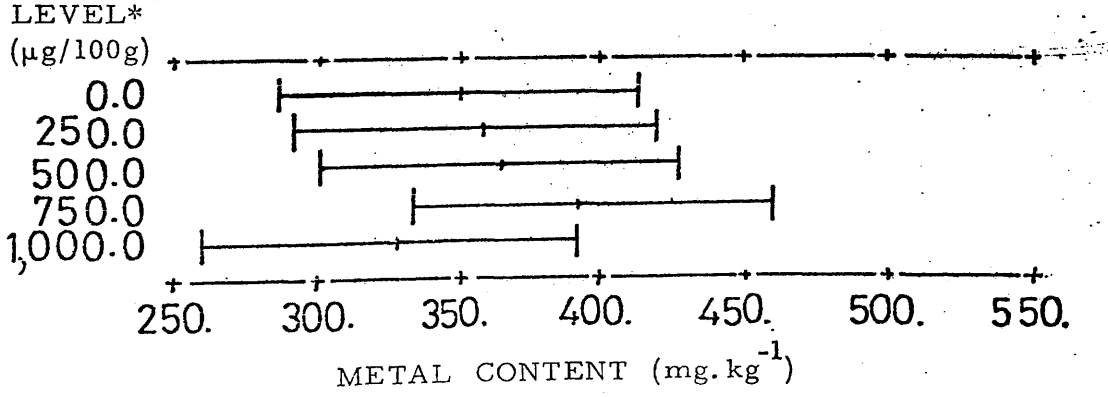
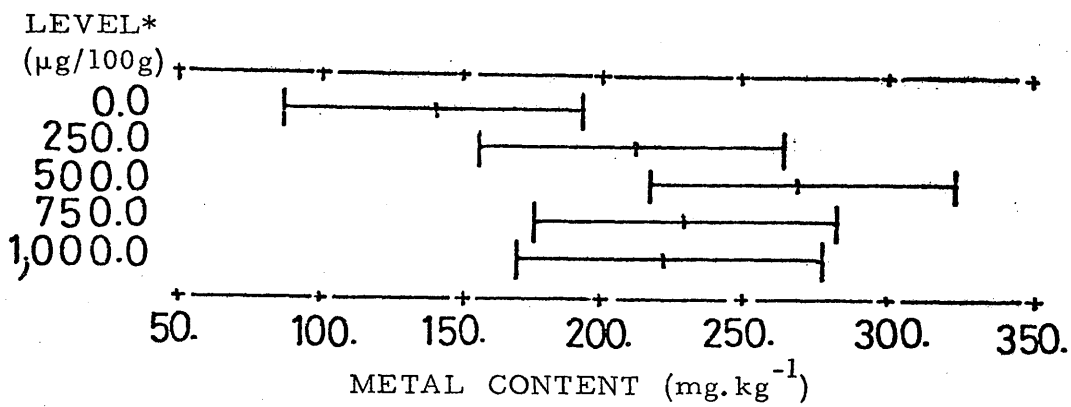


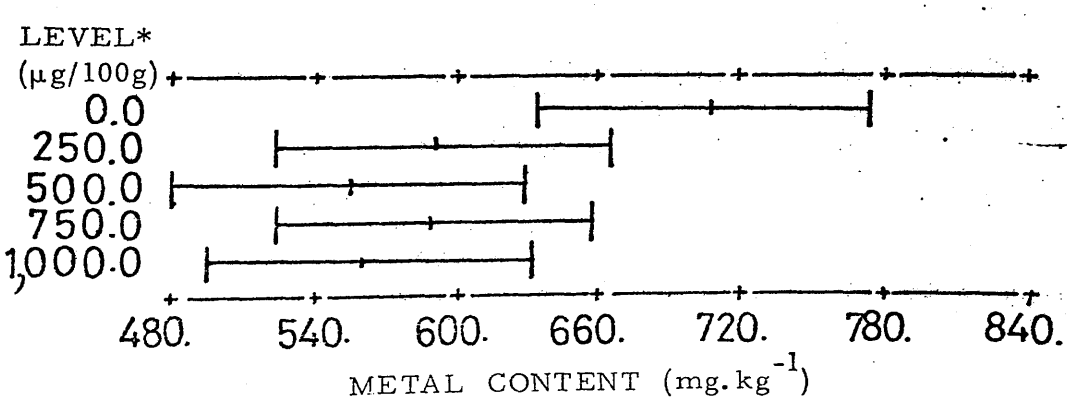
Figure 3.2.1.1 continued

C. 2-KETOGLUCONIC ACID

7. ZINC (STEM)



8. ZINC (ROOT)



* Levels of chelating agent supplied (µg/100g soil).

chelates zinc in the soil and transports it to be absorbed at the root, while decreased manganese levels suggest chelating agent-root competition. The increase in stem iron levels with increased chelating agent application, might be by the formation of iron-citrate complexes in the soil, absorption of these by the plant, and subsequent transfer through xylem to stem without significant release of metal to root. Tiffin (1966) conducted an experiment in which the stem exudate from sunflower plants was examined electrophoretically. The results suggested that the iron was bound to citrate and the author considered the experiments confirmed citrate as the principal agent involved in iron transport. However, Tiffin (1967), again on the basis of electrophoretic evidence, discounts the possibility of zinc-citrate complexes in the xylem (which could explain the increase in stem zinc with increasing levels of citrate). Synthetically prepared zinc-citrate complexes were anodic, while zinc in xylem exudates from tomatoes was slightly cathodic, implying that the metal moved as an inorganic cation. In addition, the zinc-citrate complex has a relatively low equilibrium constant (Tiffin (1971) quotes 70, 800) which also makes it likely that significant dissociation of zinc-citrate complexes occurs in xylem fluid and that zinc moves as an inorganic cation. Such experimental considerations also discount an alternative explanation that, on entering the root, metal-free citrate chelates already present, but inactivated, zinc and then transports it to the stem.

Several trends were noted with EDTA. Increasing the level of application resulted in slight increases in root yield, in root iron and in stem zinc and slight decreases in root manganese and root zinc.

The yield increase may result because EDTA acts as a nitrogen source or because it removes calcium from the root cells, allowing elongation to occur (Weinstein et al., 1956). The observed decreases suggest competition between chelating agent and root and, since EDTA forms a more stable chelate with iron than with zinc or manganese, a pronounced decrease in root iron might have been expected.

The increase observed is, therefore, interesting and contrary

to results of Brown et al. (1960). These authors suggested competitive reactions were involved, noting that, if the concentration of chelating agent was increased beyond that of iron, the amount of iron absorbed by the plant decreased.

Stem zinc levels may increase because EDTA affects translocation within the plant.

2-ketogluconic acid applications slightly increased stem manganese and stem zinc and slightly reduced root zinc. The increases might be explained by improved translocation and the reduction by chelating agent-root competition.

In summary, therefore, it appears that these three chelating agents (citrate, EDTA and 2-ketogluconic acid) have few significant effects upon trace metal levels or upon yield. Some trends, however, do appear which suggest further investigation could be fruitful.

3.2.2 POT EXPERIMENTS INVOLVING APPLICATION OF METAL CHELATES TO AN ACID SOIL

3.2.2.1 Introduction

In the previous section the trends observed suggest that metal-free chelating agents at higher levels (up to 1000 μg/100 g soil) may influence trace metal levels and yields in the bean Phaseolus vulgaris var. Canadian Wonder.

To compare the relative effectiveness of metal-free chelating agents and metal-containing chelates in changing metal levels and yields in this particular species, a further series of experiments was conducted using iron, manganese and zinc chelates of EDTA.

3.2.2.2 Materials and Methods

(1) Soil

The soil was that described and characterized in Chapter 2, section 2.2.1.

(2) Treatment solutions

Stock solutions of iron, manganese and zinc EDTA (1 : 1 metal/chelating agent ratio) were prepared by mixing stoichiometric quantities of reagent grade $FeCl_3$, $MnCl_2.4H_2O$ and $ZnCl_2$, respectively, with Analar EDTA (as the di-sodium salt). Treatment solutions containing 0.2, 1.0, 2.0, 5.0, 10.0, 15.0 and 20.0 ppm chelating agent/metal ion were obtained from stock solutions by dilution with deionised water. The corresponding figures for the total quantity of chelating agent/metal ion in each of these treatments was 10.0, 50.0, 100.0, 250.0, 500.0, 750.0 and 1000.0 (expressed as μg/100 g soil). A deionised water control was also included in the experiment.

(3) Method

The method is described in Chapter 2, section 2.2.2.

(4) Analytical technique

The technique, precision and associated errors are discussed in Chapter 2, section 2.2.2.

3.2.2.3 Results and Discussion

The results are presented in Tables 3.2.2.1, 3.2.2.2 and 3.2.2.3

One-way analysis of variance shows iron EDTA to have a highly significant effect upon iron content of the stem and a lesser, but still significant, effect upon manganese levels in both root and stem. In each case the metal content increased as the level of chelate applied was increased. Highest treatment levels had the greatest effect.

Manganese EDTA had a significant effect upon manganese and zinc levels in the root and a highly significant effect upon manganese in the stem. As the level of chelate applied was increased, gradual increases in stem and root manganese levels and a slight decrease in root zinc levels were observed.

Zinc EDTA appeared to have no significant effect on yield or upon metal content.

Table 3.2.2.1 The effect of iron EDTA treatments on the subsequent yield and trace metal content of beans growing on acid soil (Cultivar - Phaseolus vulgaris var. Canadian Wonder) (Application level 0 - 1000 μg/100 g soil)

		Treatment levels (μg/100 g soil)[a]								ANOVA[b]
		0.0	10.0	50.0	100.0	250.0	500.0	750.0	1000.0	
Yield (g)	S[c]	0.2276 (0.0218)[d]	0.2416 (0.0246)	0.2182 (0.0328)	0.2557 (0.0347)	0.2412 (0.0276)	0.2712 (0.0097)	0.2609 (0.0409)	0.2234 (0.0155)	N.S.[e]
	R[c]	0.0947 (0.0105)	0.1118 (0.0111)	0.0894 (0.0182)	0.1016 (0.0122)	0.0911 (0.0210)	0.0995 (0.0110)	0.1139 (0.0209)	0.0904 (0.0065)	N.S.
Fe ($mg.kg^{-1}$)	S	37.2 (17.1)	48.0 (17.4)	45.5 (11.4)	48.9 (5.8)	79.9 (7.8)	67.3 (5.2)	109.4 (31.8)	202.4 (39.7)	SIG.[f]
	R	215.0 (26.0)	213.0 (41.0)	149.0 (40.0)	130.0 (42.0)	205.0 (83.0)	291.0 (58.0)	295.0 (63.0)	257.0 (23.5)	N.S.
Mn ($mg.kg^{-1}$)	S	318.1 (17.5)	307.8 (26.3)	241.0 (16.1)	328.7 (51.3)	312.8 (14.4)	274.6 (33.7)	284.9 (17.8)	422.9 (39.3)	SIG.[g]
	R	338.4 (43.9)	265.1 (9.7)	243.8 (14.0)	343.9 (36.3)	250.0 (22.2)	272.2 (17.4)	268.9 (23.1)	373.3 (45.6)	SIG.[g]
Zn ($mg.kg^{-1}$)	S	256.1 (15.2)	558.0 (417.0)	183.0 (13.0)	223.0 (34.5)	242.0 (32.5)	221.0 (20.5)	299.0 (31.0)	255.0 (33.0)	N.S.
	R	722.0 (48.0)	672.0 (75.5)	672.0 (59.5)	774.0 (52.5)	638.0 (84.0)	752.0 (51.0)	731.0 (81.0)	556.0 (24.0)	N.S.

a All data given as mean of 4 replicates.
b Analysis of variance - significance of treatment effect based on F statistic (5%).
c R and S signify root and stem respectively.
d Standard error on mean.
e Not significant.
f Significant at 0.1% level.
g Significant at 5.0% level.

Table 3.2.2.2 The effect of manganese EDTA treatments on the subsequent yield and trace metal content of beans growing on acid soil (Cultivar - Phaseolus vulgaris var. Canadian Wonder) (Application level 0 - 1000 μg/100 g soil)

		Treatment levels (μg/100 g soil)[a]								ANOVA[b]
		0.0	10.0	50.0	100.0	250.0	500.0	750.0	1000.0	
Yield (g)	S[c]	0.2276 (0.0218)[d]	0.2505 (0.0339)	0.2538 (0.0321)	0.2389 (0.0473)	0.2395 (0.0202)	0.2404 (0.0259)	0.2197 (0.0353)	0.2327 (0.0104)	N.S.[e]
	R[c]	0.0947 (0.0105)	0.1244 (0.0119)	0.1296 (0.0159)	0.1002 (0.0137)	0.1453 (0.0092)	0.1317 (0.0144)	0.1351 (0.0135)	0.1304 (0.0091)	N.S.
Fe ($mg.kg^{-1}$)	S	37.2 (17.1)	171.0 (71.0)	212.0 (76.0)	70.0 (33.0)	160.0 (69.0)	125.0 (59.5)	108.0 (14.0)	229.0 (52.0)	N.S.
	R	215.0 (26.0)	200.0 (17.0)	349.0 (54.0)	311.0 (125.0)	283.0 (43.0)	343.0 (78.5)	267.0 (68.5)	407.0 (115.0)	N.S.
Mn ($mg.kg^{-1}$)	S	318.1 (17.5)	431.6 (54.9)	406.0 (27.4)	412.9 (36.6)	500.1 (52.0)	462.3 (33.9)	480.7 (23.9)	564.8 (34.9)	SIG.[f]
	R	338.4 (43.9)	320.4 (31.4)	296.0 (19.1)	356.1 (39.3)	347.4 (18.5)	363.5 (39.7)	368.2 (16.4)	464.0 (9.1)	SIG.[g]
Zn ($mg.kg^{-1}$)	S	256.1 (15.2)	149.4 (74.0)	185.9 (47.2)	195.8 (16.7)	217.7 (24.1)	226.8 (36.1)	249.1 (52.2)	233.7 (47.6)	N.S.
	R	722.0 (48.0)	594.5 (61.4)	563.1 (41.9)	590.4 (63.5)	546.2 (58.6)	547.5 (10.2)	431.2 (20.4)	542.8 (35.1)	SIG.[g]

a All data given as mean of 4 replicates.
b Analysis of variance - significance of treatment effect based on F statistic (5%).
c R and S signify root and stem respectively.
d Standard error on mean.
e Not significant.
f Significant at 1.0% level.
g Significant at 5.0% level.

Table 3.2.2.3 The effect of zinc EDTA treatments on the subsequent yield and trace metal content of beans growing on acid soil (Cultivar - Phaseolus vulgaris var. Canadian Wonder) (Application level 0 - 1000 μg/100 g soil)

		Treatment levels (μg/100 g soil)[a]								ANOVA[b]
		0.0	10.0	50.0	100.0	250.0	500.0	750.0	1000.0	
Yield (g)	S[c]	0.2276 (0.0218)[d]	0.2527 (0.0171)	0.2889 (0.0405)	0.2482 (0.0198)	0.2798 (0.0190)	0.2630 (0.0304)	0.2461 (0.0087)	0.2628 (0.0285)	N.S.[e]
	R[c]	0.0947 (0.0105)	0.1123 (0.0069)	0.1146 (0.0081)	0.0997 (0.0081)	0.1035 (0.0089)	0.1003 (0.0151)	0.0967 (0.0077)	0.1058 (0.0081)	N.S.
Fe ($mg.kg^{-1}$)	S	37.2 (17.1)	64.7 (29.9)	188.4 (90.6)	64.8 (15.0)	71.1 (20.2)	53.3 (13.2)	185.9 (58.5)	96.8 (21.5)	N.S.
	R	215.0 (26.0)	266.0 (92.0)	199.0 (24.0)	169.0 (50.0)	396.0 (134.5)	228.0 (42.5)	242.0 (74.0)	250.0 (63.0)	N.S.
Mn ($mg.kg^{-1}$)	S	318.1 (17.5)	354.0 (20.0)	366.0 (19.5)	584.0 (97.5)	362.0 (88.5)	320.0 (6.5)	382.0 (56.5)	287.0 (18.0)	SIG.[f]
	R	338.4 (43.9)	307.0 (33.0)	346.0 (14.0)	581.0 (111.5)	364.0 (83.0)	328.0 (8.5)	347.0 (47.0)	259.0 (18.5)	SIG.[f]
Zn ($mg.kg^{-1}$)	S	256.1 (15.2)	301.8 (58.8)	264.7 (22.3)	225.7 (23.8)	230.2 (5.9)	293.8 (70.2)	223.0 (19.1)	341.1 (68.2)	N.S.
	R	722.0 (48.0)	809.0 (130.5)	793.0 (33.0)	867.0 (14.0)	890.0 (70.5)	806.0 (122.0)	628.0 (30.0)	763.0 (58.0)	N.S.

a All data given as mean of 4 replicates.
b Analysis of variance - significance of treatment effect based on F statistic (5%).
c R and S signify root and stem respectively.
d Standard error on the mean.
e Not significant.
f Significant at the 5.0% level. Probably caused by abnormally high values for the 100 μg treatment.

These trends are illustrated in Figure 3. 2. 2. 1.

It is not appreciated why applications of iron EDTA should increase the manganese content of root and stem. It has been shown that, generally, iron chelates reduce the manganese content of plants (Wallace, 1956, pp. 44 - 48; Moraghan and Freeman, 1978) and, indeed, they have been used to overcome manganese toxicity in flax (Moraghan and Freeman, 1978).

From consideration of the stability constants for manganese and iron EDTA (respectively 14. 1 and 25. 1 (Martell, 1957)) it appears unlikely that soil manganese should displace iron from iron EDTA to form the manganese chelate and, therefore, there should be no increase in the manganese available to the plant and no increase in its manganese content.

It is, therefore, interesting that, in previous pot experiments, when metal-free EDTA was applied, a similar positive correlation between iron and manganese was observed (see Tables 3. 2. 1. 4 and 3. 2. 1. 5 and page 67.

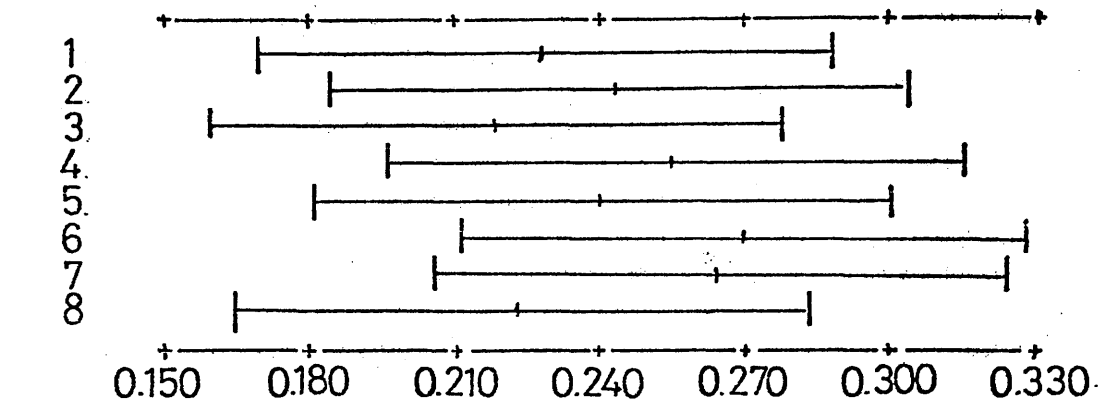
The slight decrease observed in root zinc levels as increasing quantities of manganese EDTA were applied suggests that zinc uptake is inhibited by manganese. Similar trends have been observed by other workers. For example, Hawf and Schmid (1967) noted, in experiments with bush beans, that high concentrations of added manganese reduced uptake of radiozinc from nutrient solutions but did not alter the internal distribution of the radioactive species. Reddy et al. (1978), however, concluded, from short-term experiments with soybean seedlings in nutrient solution, that manganese had an inhibitory effect not only on ^{65}Zn absorption but also on its translocation to shoots.

There is a further comparison to be made with the results of previous pot experiments (see Chapter 3, section 3. 2. 1), where, under treatment with 2-ketogluconic acid, there was a positive correlation between zinc and manganese. This infers that in translocation, if not during uptake, there is no antagonism between the metals and it is the

Figure 3.2.2.1 TREND DIAGRAM - the effects of eight application levels of three metal chelates on trace metal content and yield. Treatment means with pooled standard deviation from one-way analysis of variance

A. FeEDTA

1. YIELD (STEM)



2. YIELD (ROOT)

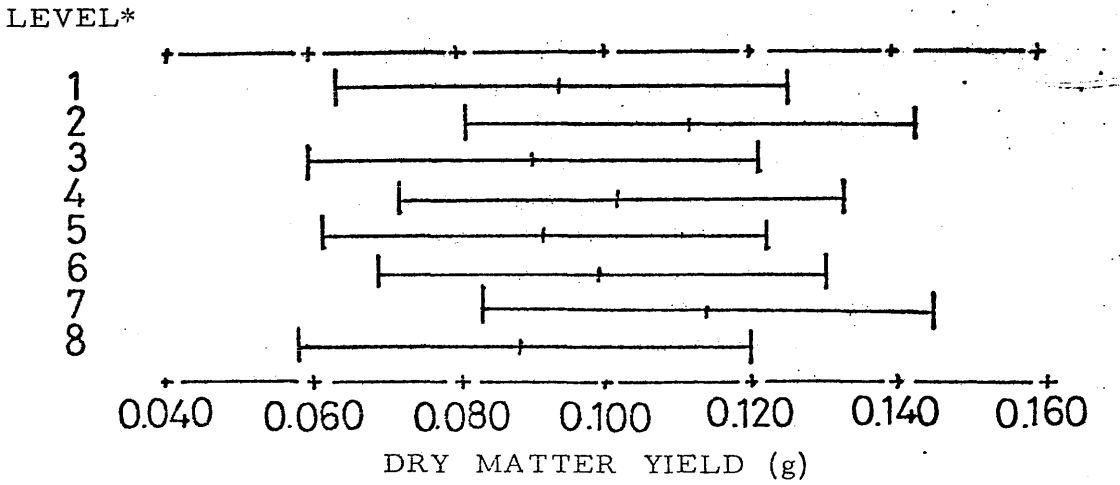
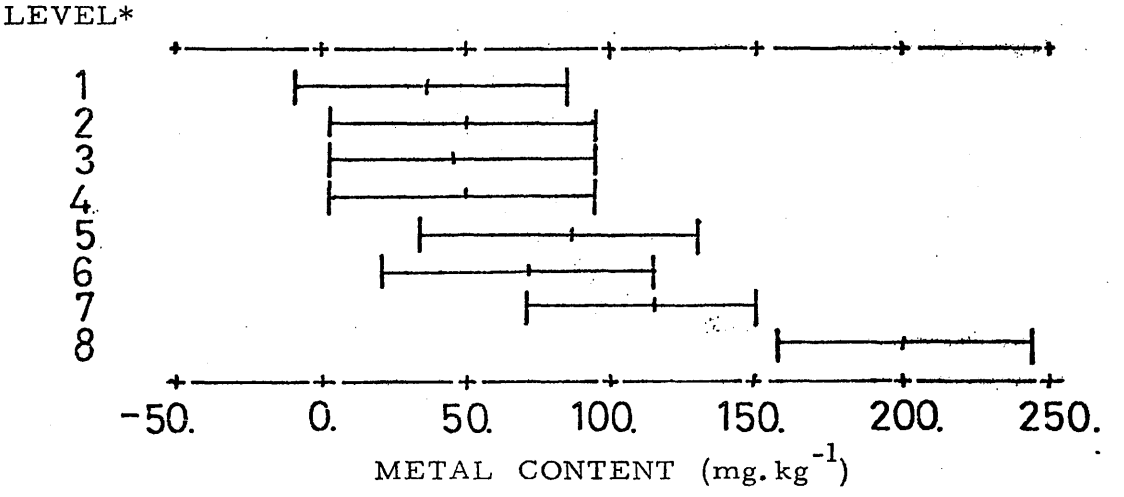


Figure 3.2.2.1 continued

A. FeEDTA

3. IRON (STEM)



4. IRON (ROOT)

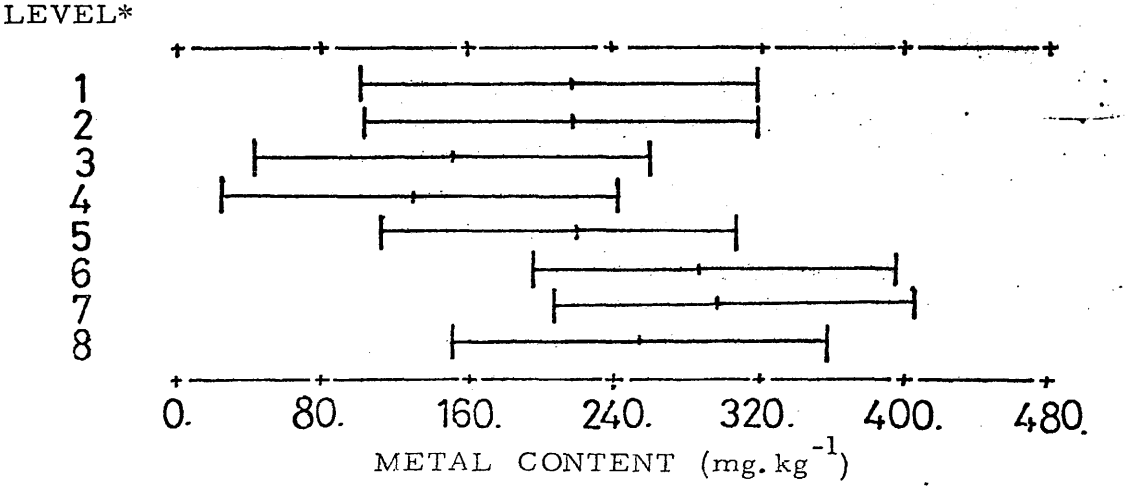
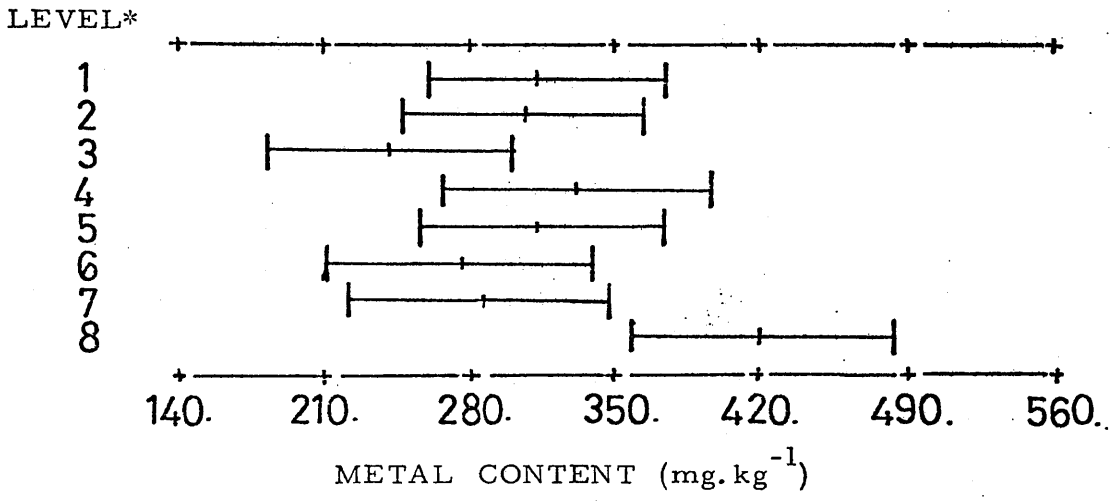


Figure 3.2.2.1 continued

A. FeEDTA

5 MANGANESE (STEM)



6. MANGANESE (ROOT)

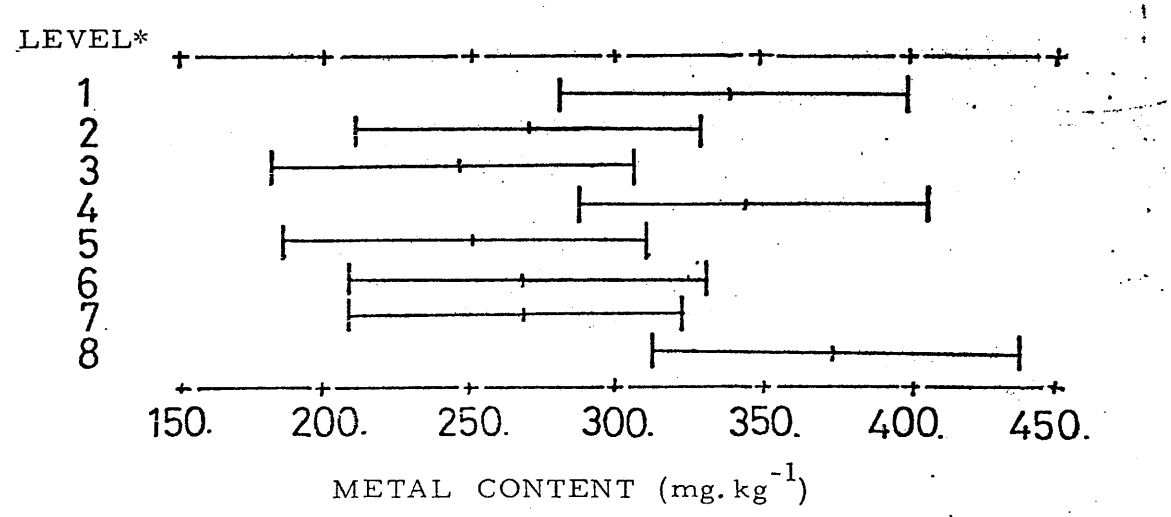
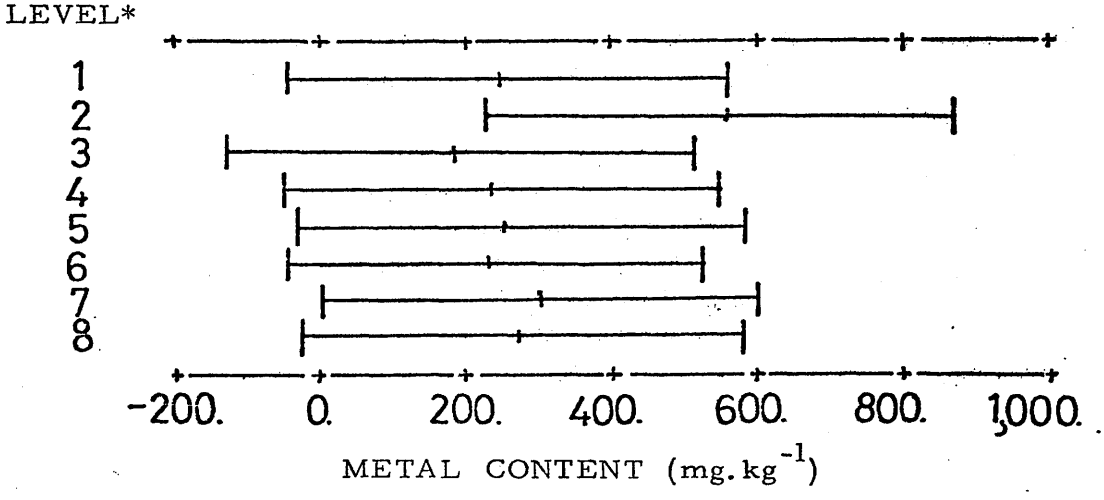


Figure 3.2.2.1 continued

A. FeEDTA

7. ZINC (STEM)



8. ZINC (ROOT)

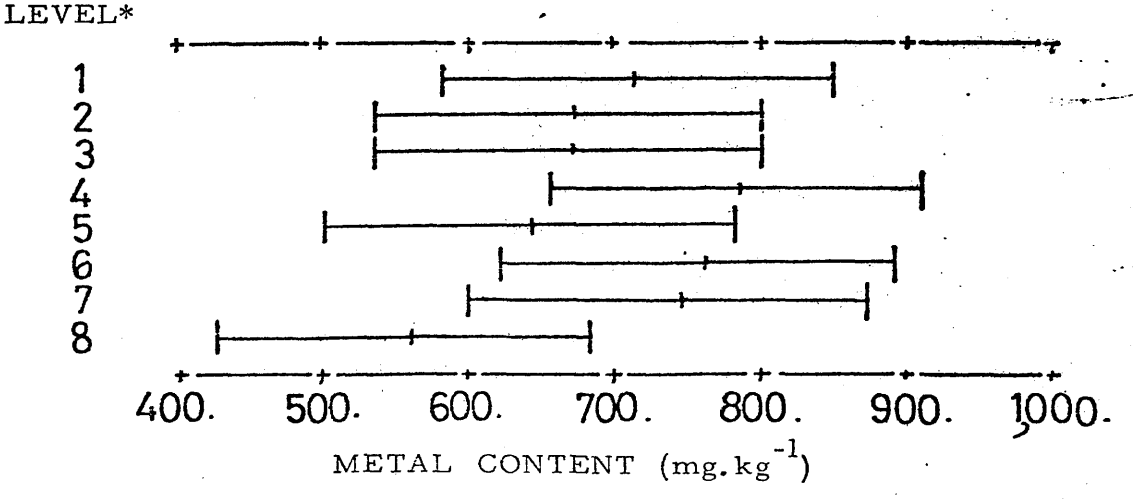
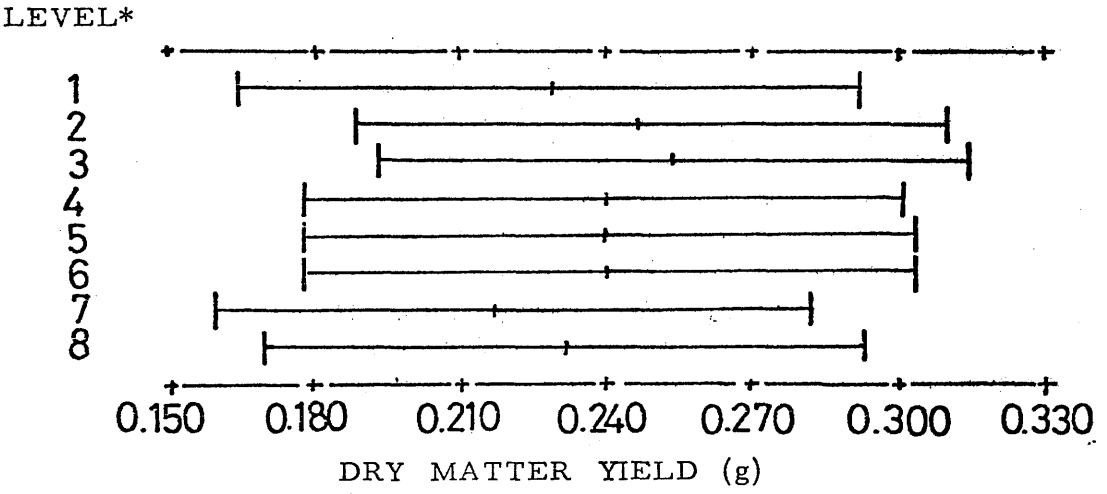


Figure 3.2.2.1 continued

B. MnEDTA

1. YIELD (STEM)



2. YIELD (ROOT)

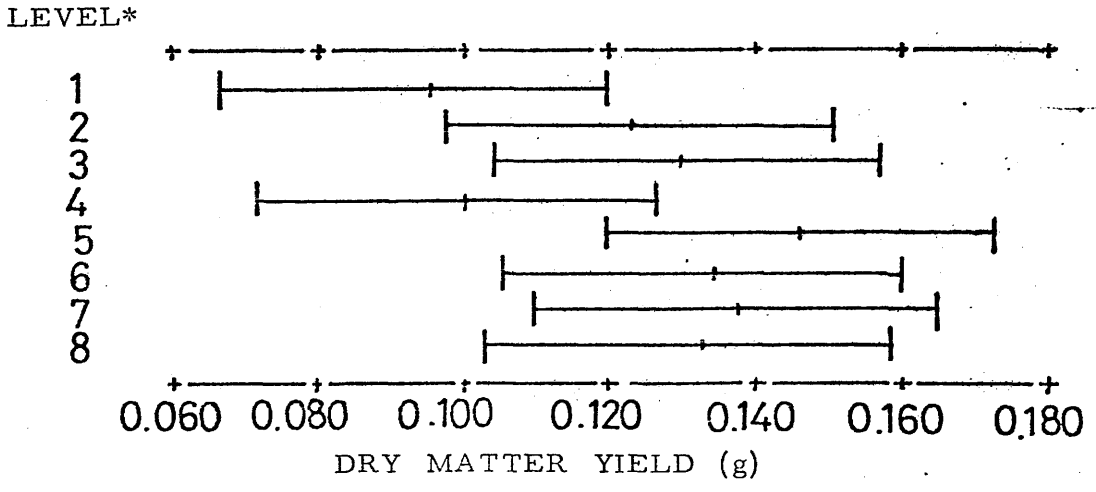
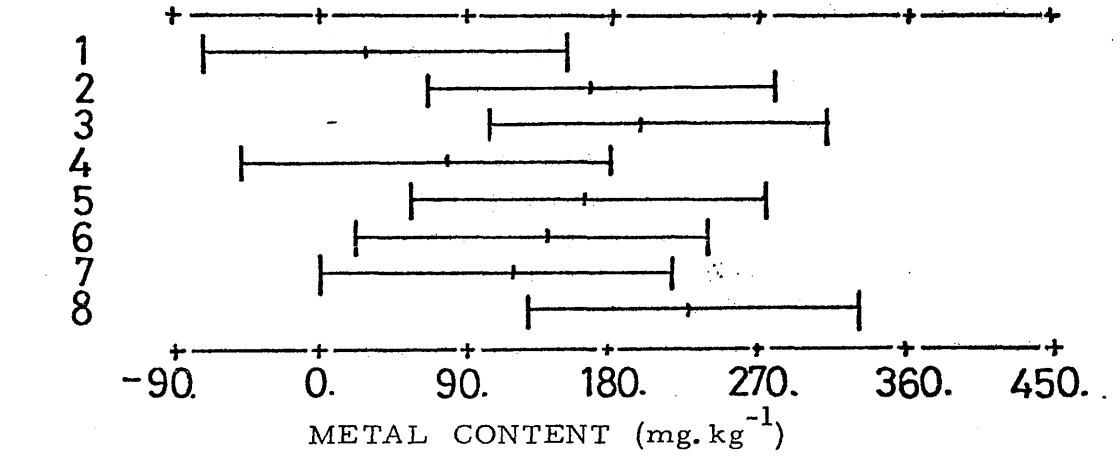


Figure 3.2.2.1 continued

B. MnEDTA

3. IRON (STEM)



4. IRON (ROOT)

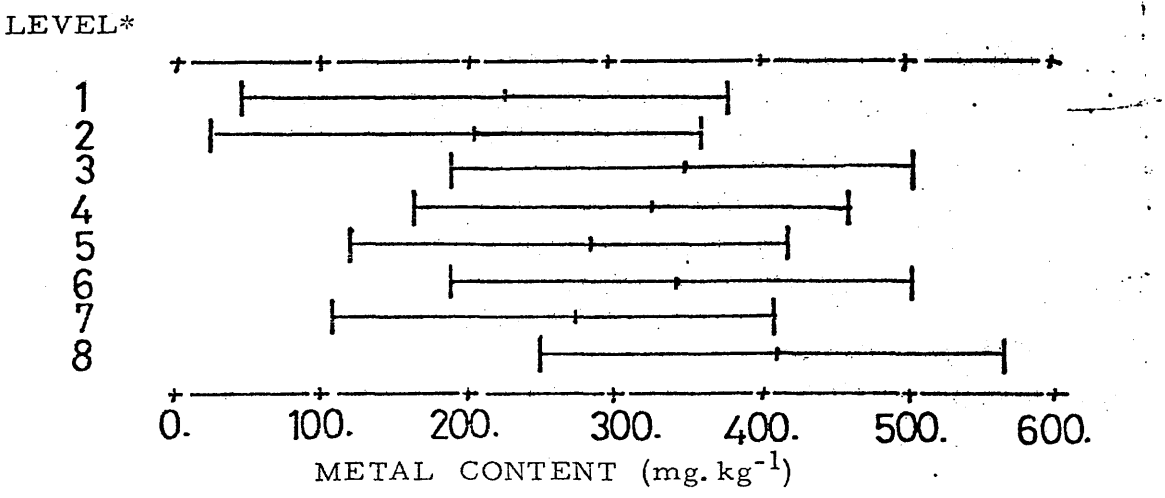
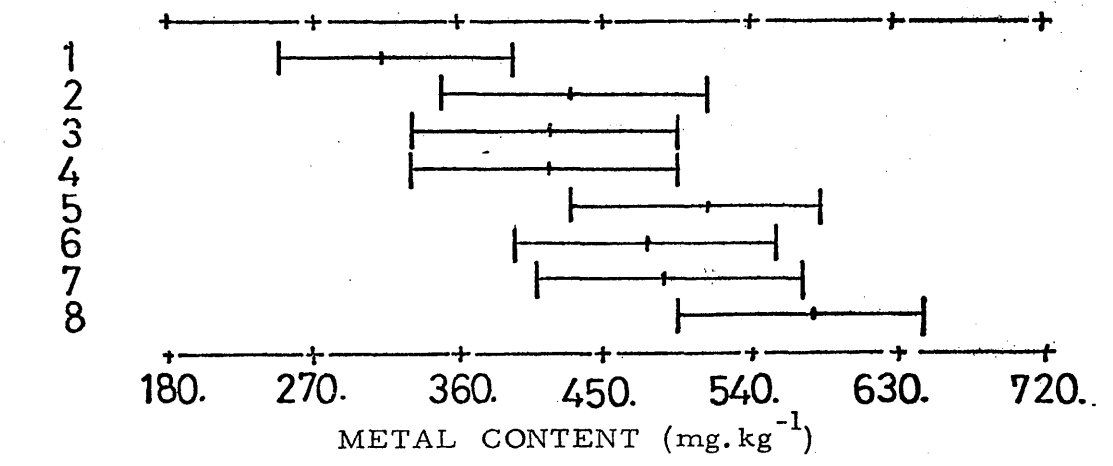


Figure 3.2.2.1 continued

B. MnEDTA

5. MANGANESE (STEM)



6. MANGANESE (ROOT)

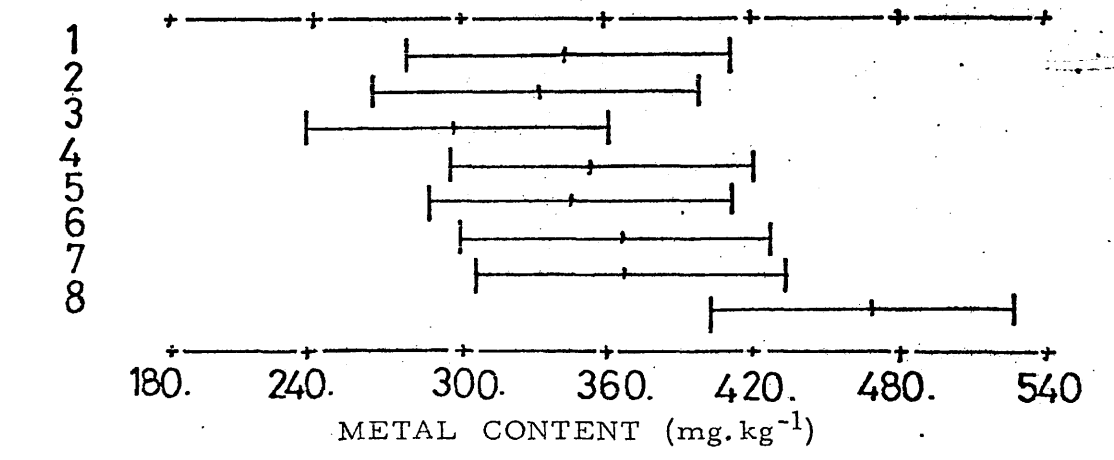
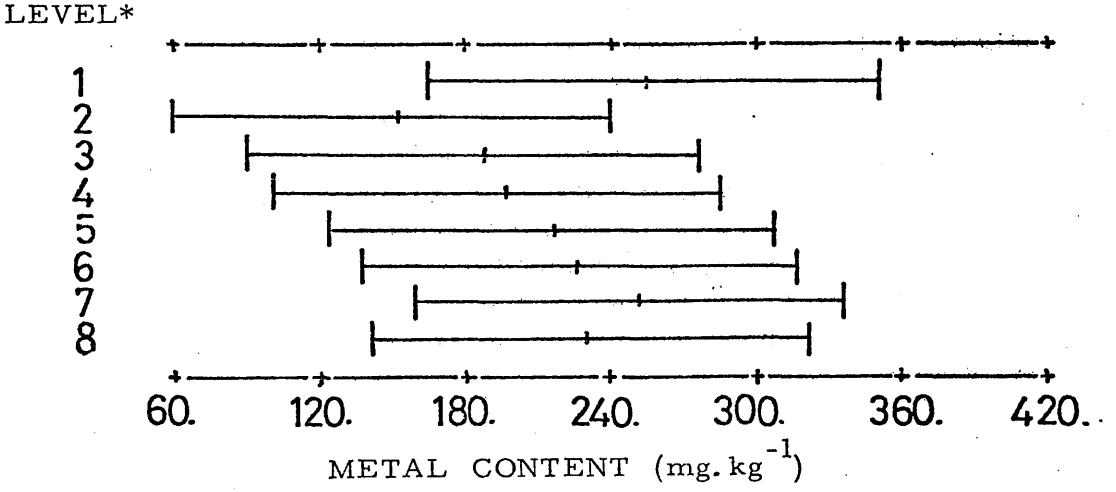


Figure 3.2.2.1 continued

B. MnEDTA

7. ZINC (STEM)



8. ZINC (ROOT)

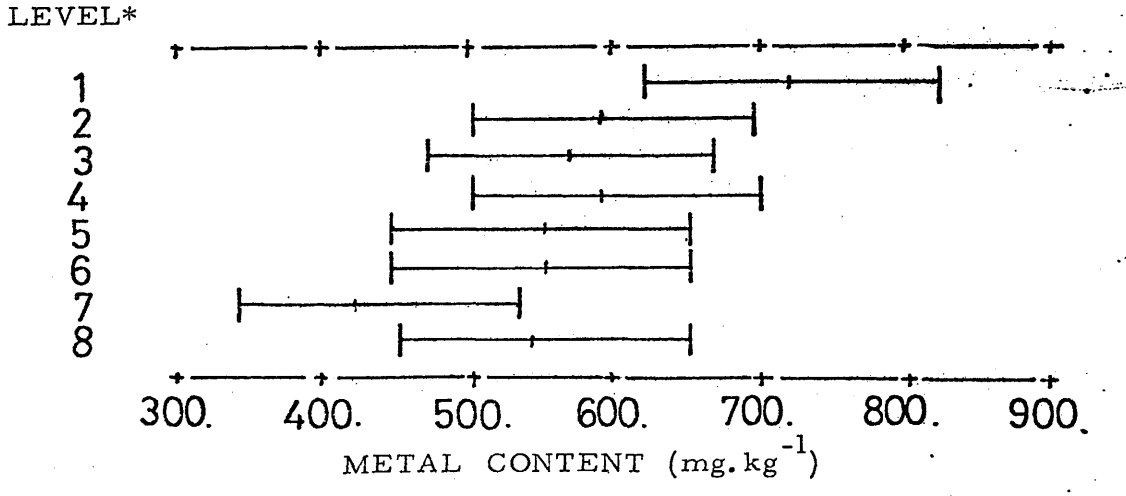
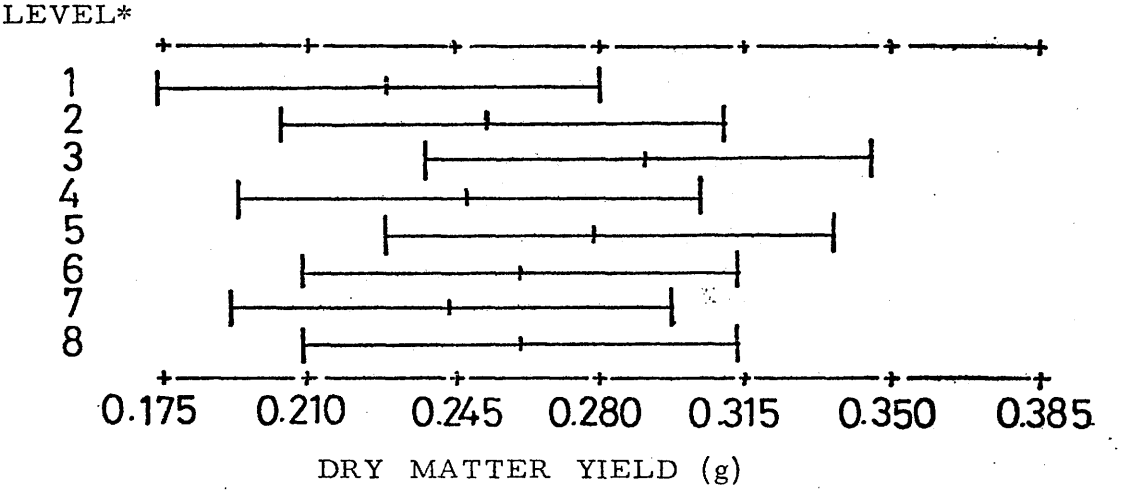


Figure 3.2.2.1 continued

C. ZnEDTA

1. YIELD (STEM)



2. YIELD (ROOT)

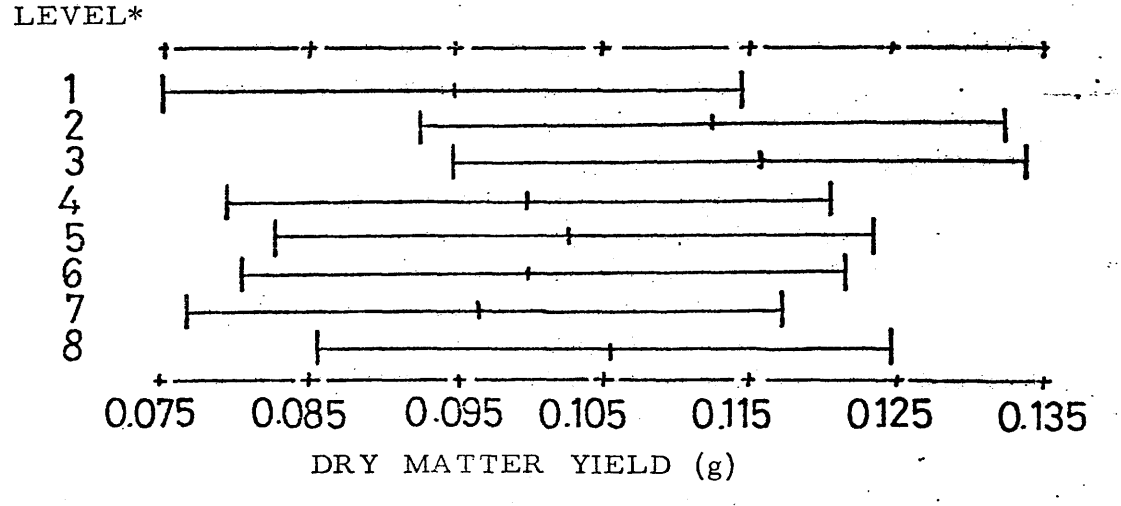
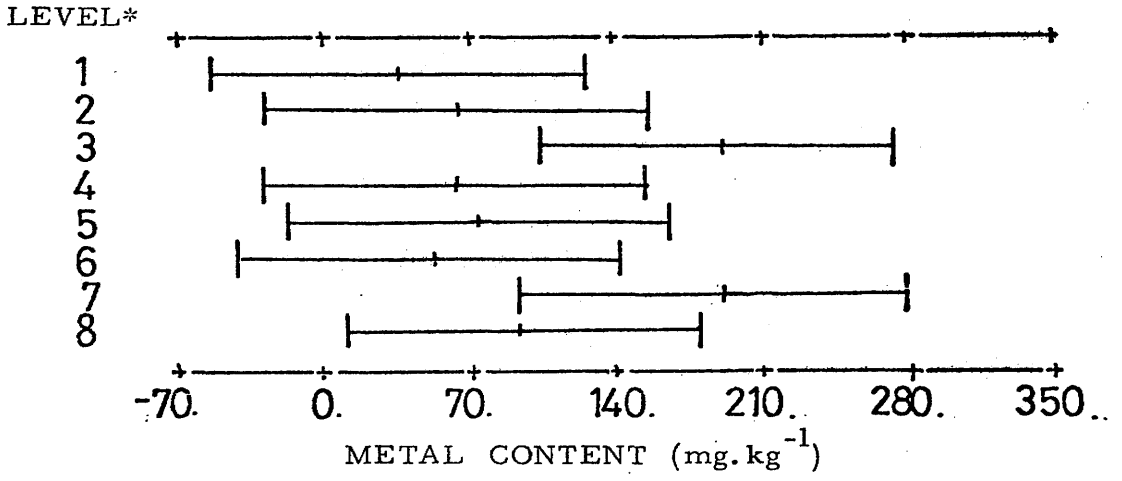


Figure 3.2.2.1 continued

C. ZnEDTA

3. IRON (STEM)



4. IRON (ROOT)

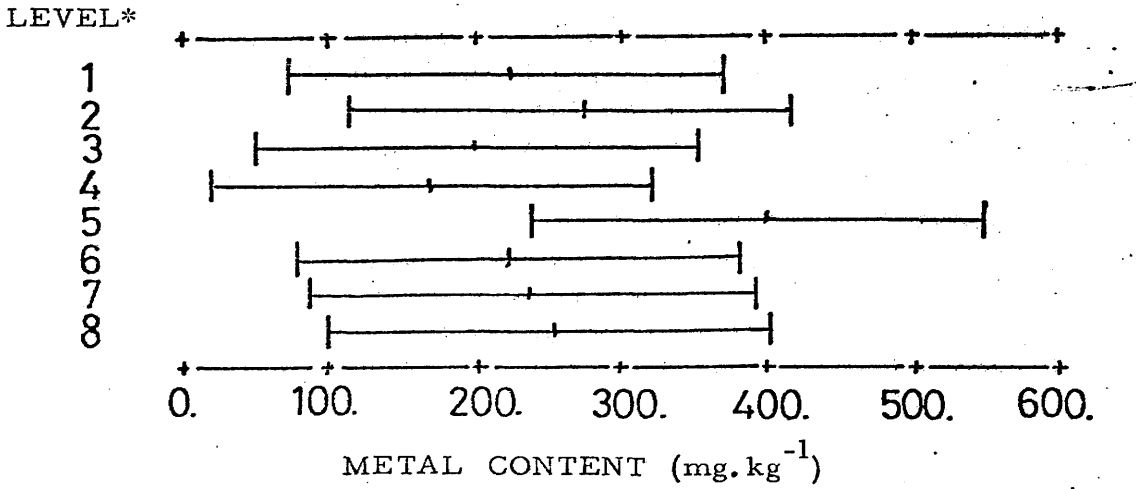
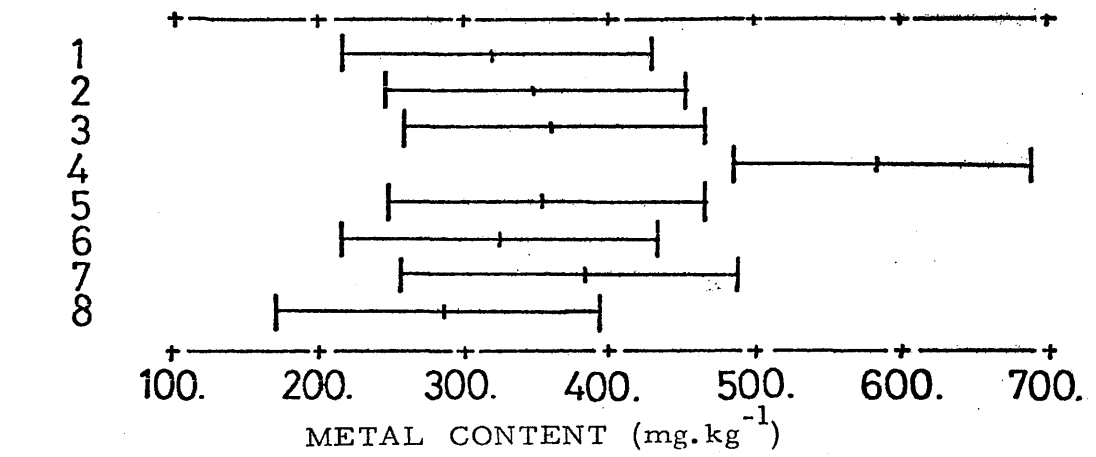


Figure 3.2.2.1 continued

C. ZnEDTA

5. MANGANESE (STEM)



6. MANGANESE (ROOT)

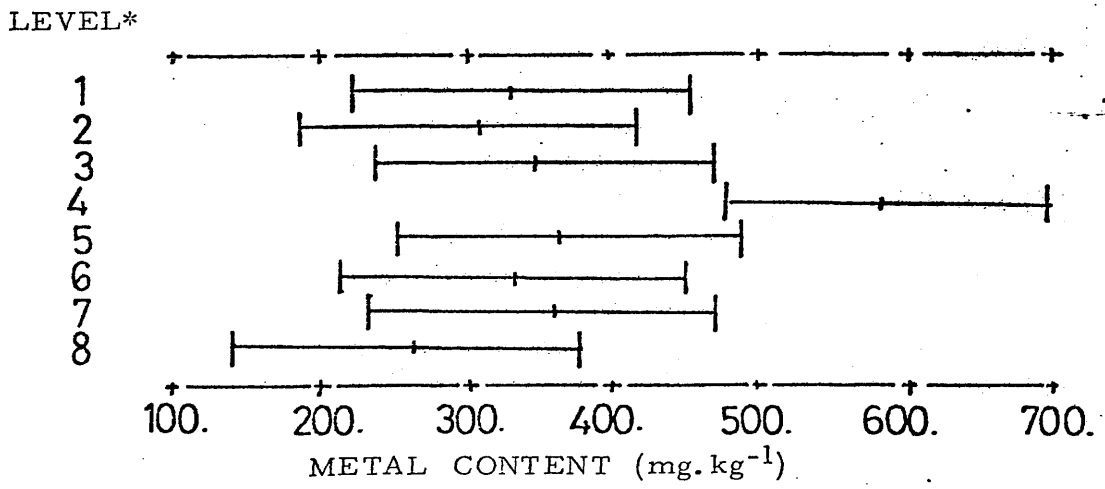
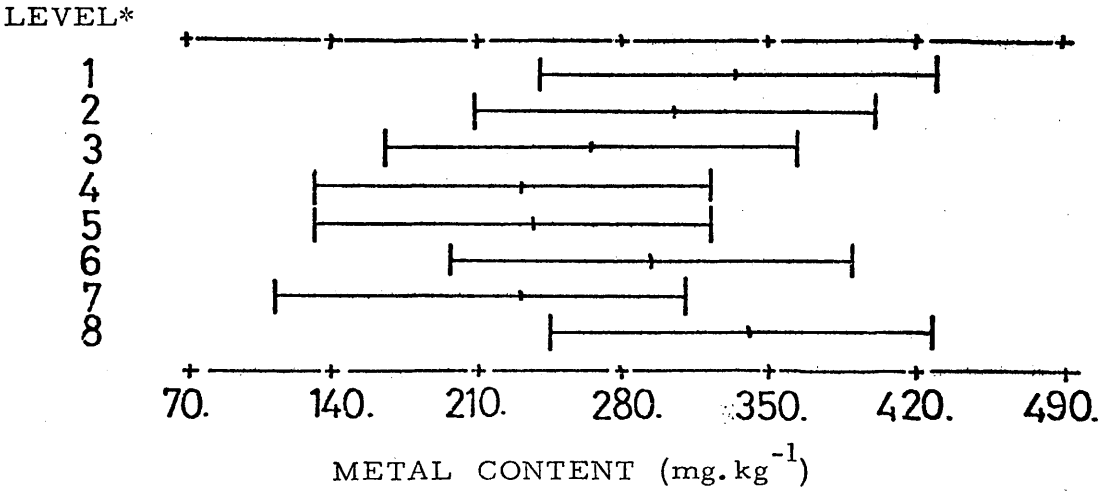


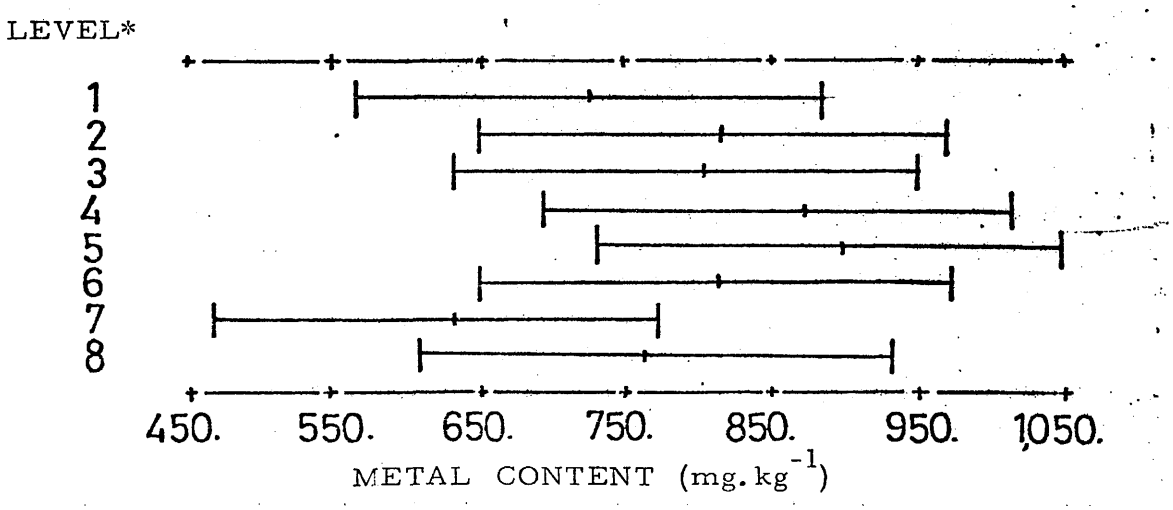
Figure 3.2.2.1 continued

C. ZnEDTA

7. ZINC (STEM)



8. ZINC (ROOT)



* Levels of metal chelate supplied (µg/100g soil).
1 = 0 µg; 2 = 10 µg; 3 = 50 µg; 4 = 100 µg; 5 = 250 µg; 6 = 500 µg;
7 = 750 µg; 8 = 1,000 µg.

reverse of the expected result (see page 71).

Two-way analysis of variance showed individual metal chelates to have the following effects:

(1) manganese EDTA gave the highest values for root yield, for iron and manganese in the stem, for iron in the root and gave the lowest levels for root zinc,

(2) iron EDTA gave the lowest levels of manganese in both root and stem and the lowest levels of stem iron,

(3) zinc EDTA and manganese EDTA gave very similar high values for manganese in the root and this result is thought to be anomolous. It may be explained by the abnormally high value obtained from the 100 μg zinc EDTA treatment (Table 3. 2. 2. 3),

(4) highest values for root zinc were obtained with zinc EDTA treatments.

These trends are illustrated in Figure 3. 2. 2. 2.

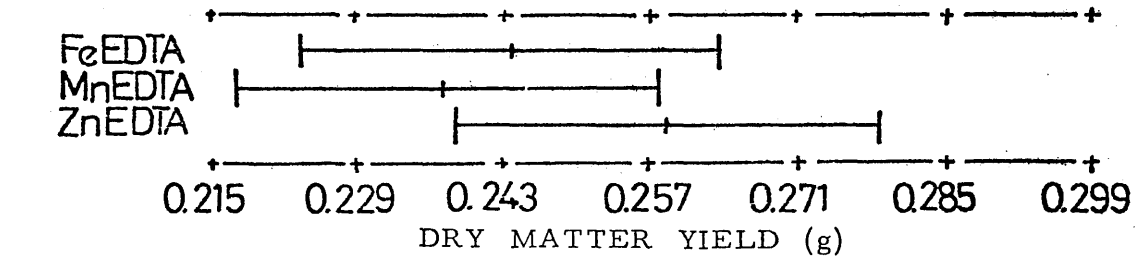
The high iron levels may have occurred with manganese EDTA because soil iron displaced manganese from the chelate forming the more stable iron chelate. Iron in this form should be more available to the plant and its uptake could be expected to increase. Knezek and Greinert (1971) noted similar increases in iron availability after manganese EDTA treatment and attributed this to rapid exchange of soil iron for manganese in the chelate.

Low zinc levels produced by manganese chelates have already been discussed (see page 90). That the highest level of stem manganese should be produced on application of manganese EDTA is not unexpected because one is supplying manganese in a soluble form which the plant could be expected to absorb and translocate. This assumes, however, that, in the soil, manganese is not displaced from the chelate.

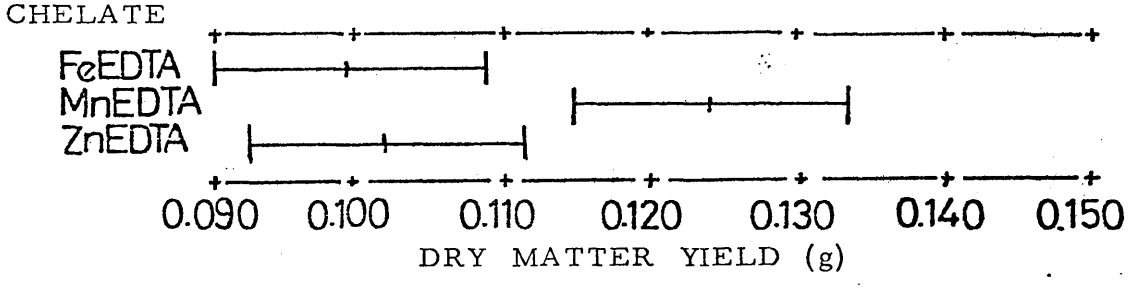
It is not clear why the highest root yield should be obtained with manganese EDTA. If the EDTA were acting as a nitrogen source it

Figure 3.2.2.2 TREND DIAGRAM - the effect of three metal chelates on trace metal content and yield. Means over all application rates with pooled standard deviation from two-way analysis of variance

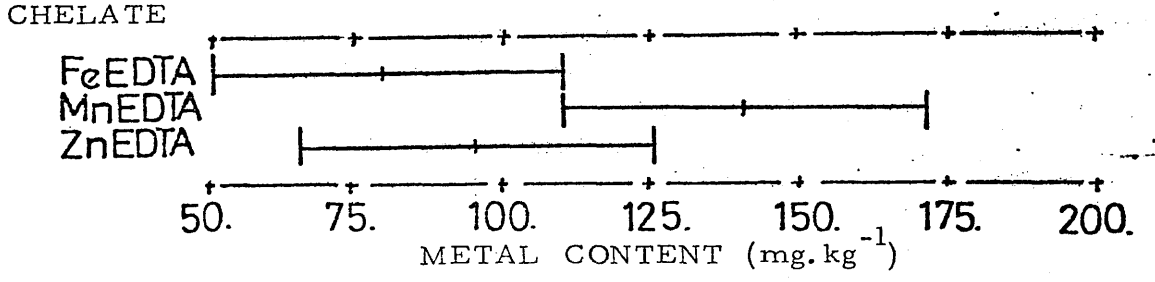
1. YIELD (STEM)



2. YIELD (ROOT)



3. IRON (STEM)



4. IRON (ROOT)

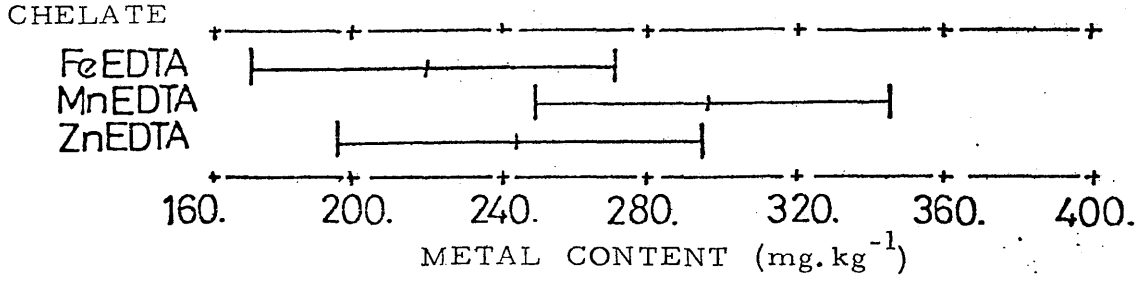
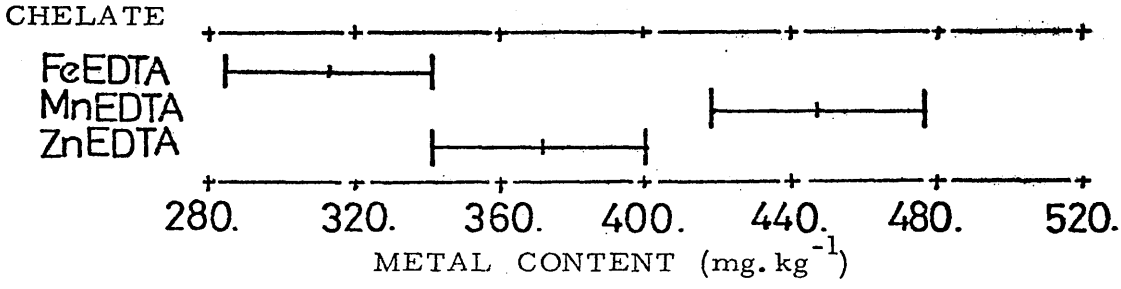
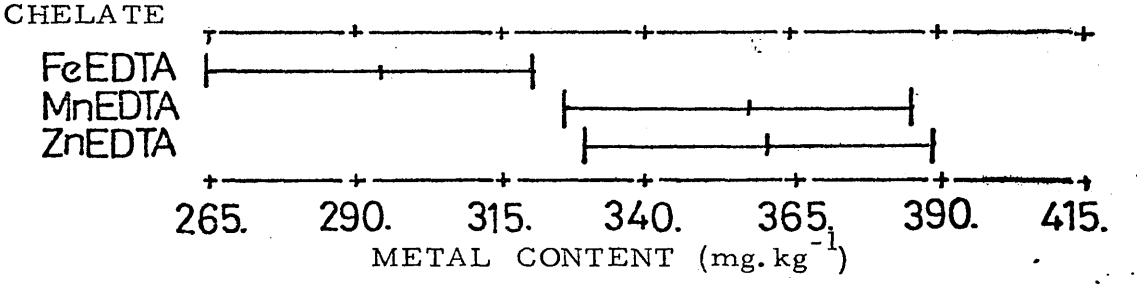


Figure 3.2.2.2 continued

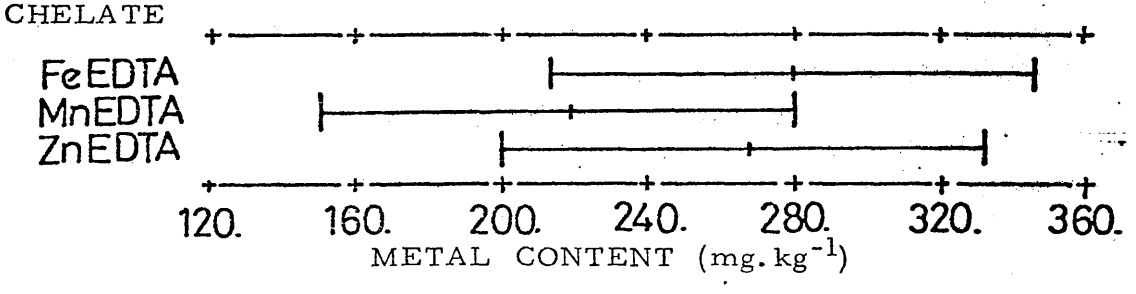
5. MANGANESE (STEM)



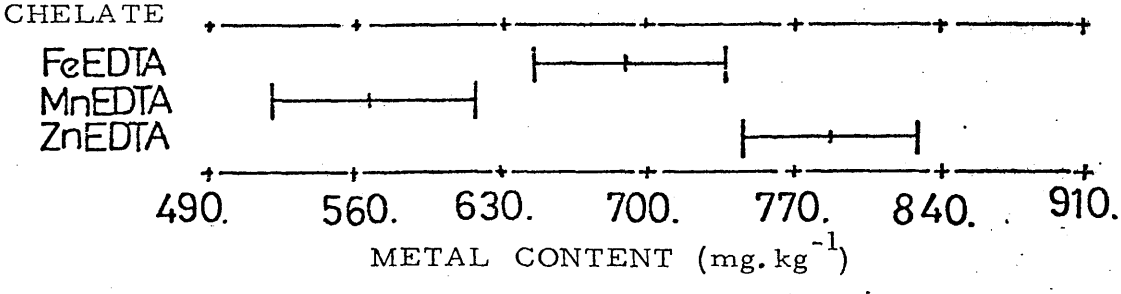
6. MANGANESE (ROOT)



7. ZINC (STEM)



8. ZINC (ROOT)



could be expected that all three chelates would be equally effective. If, on the other hand, root growth (and hence yield increases) were the result of free EDTA complexing and removing calcium from the cell wall structure (Weinstein et al., 1956) one would expect, since it has the lowest stability constant, that manganese EDTA would be most effective.

Low manganese levels after application of iron EDTA suggest that, during uptake, an antagonistic relationship exists between iron and manganese. There is a parallel mentioned by Cheng (1973). It is not clear why iron EDTA gives the lowest stem iron content. The opposite could have been assumed.

The high level of root zinc found following application of zinc EDTA infers that the chelate is acting as a metal carrier transporting zinc to be absorbed at the root.

Two-way analysis of variance also suggested that, overall, metal chelate applications tended, (1) to increase stem levels of manganese and iron, (2) to increase iron in the root (although this is not statistically significant), and (3) to decrease zinc in the root.

These trends are illustrated in Figure 3. 2. 2. 3.

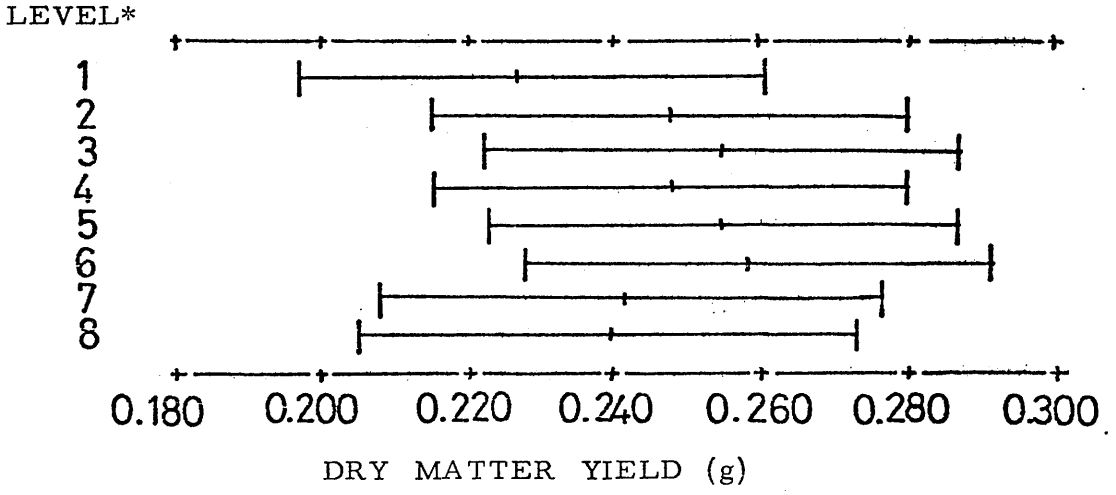
3.3 CONCLUSIONS

It seems from these two series of experiments that metal-free chelating agents (at levels up to 1000 μg/100 g soil) are not particularly effective in changing yields and trace metal contents of this bean species (Phaseolus vulgaris var. Canadian Wonder).

Iron, manganese and zinc chelates (applied in the same range) were rather more successful but their effects were not straightforward. For example, increasing iron EDTA applications raised iron and also manganese levels. Manganese EDTA not only raised manganese levels but gave the highest iron levels while producing the lowest zinc levels. On the other hand, changing the level of application of zinc EDTA had

Figure 3.2.2.3 TREND DIAGRAM - the effect of different application rates of three metal chelates on trace metal content and yield. Means over all three metal chelates with pooled standard deviation from two-way analysis of variance

1. YIELD (STEM)



2. YIELD (ROOT)

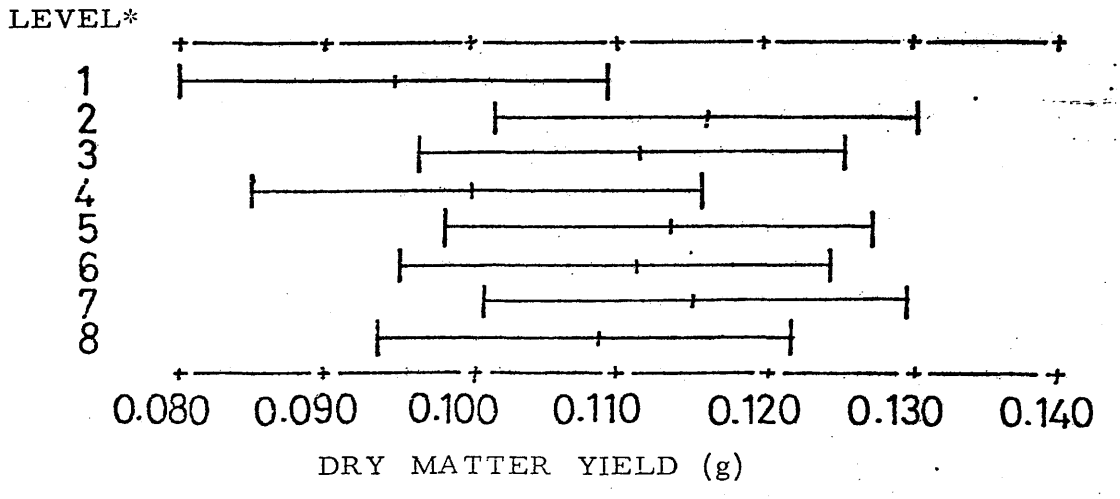
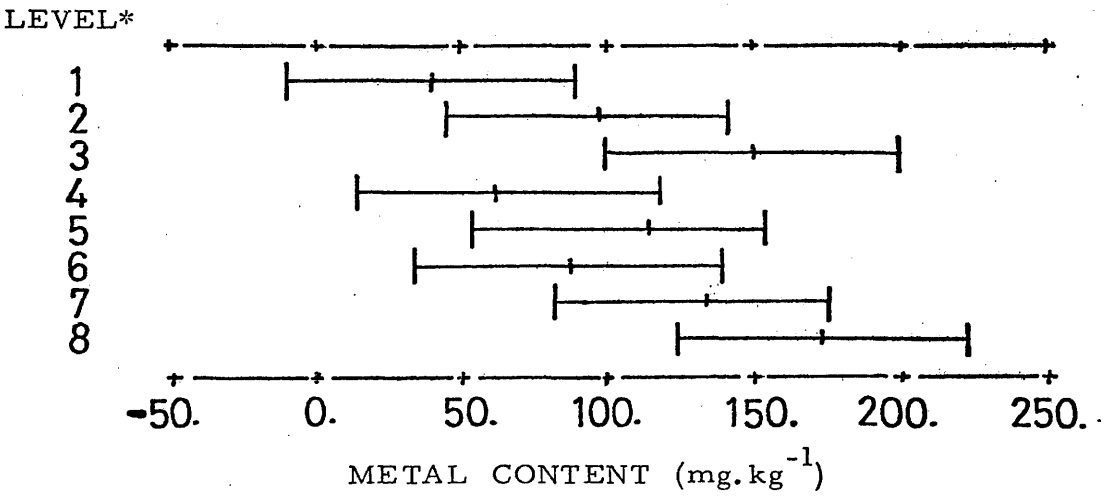


Figure 3.2.2.3 continued

3. IRON (STEM)



4. IRON (ROOT)

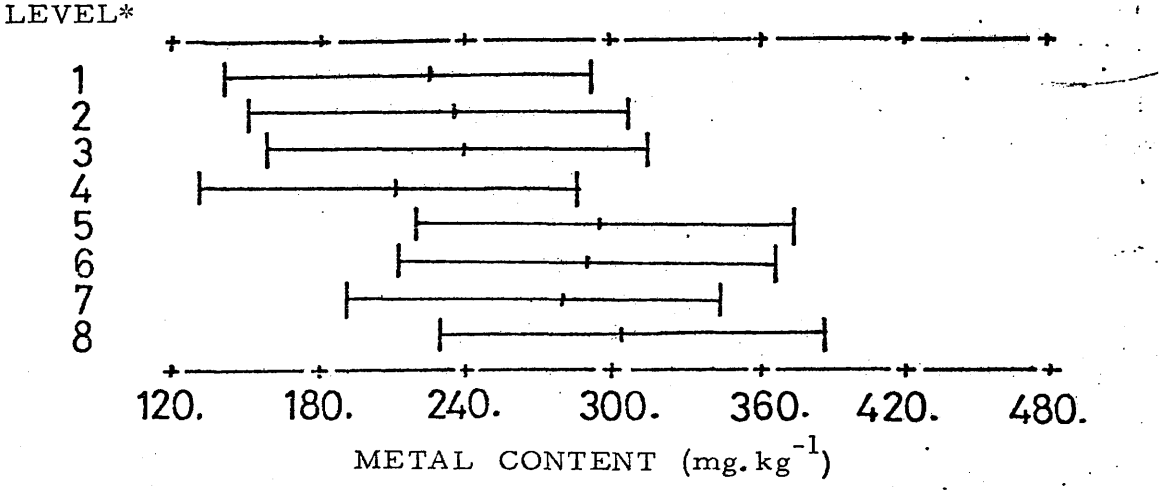
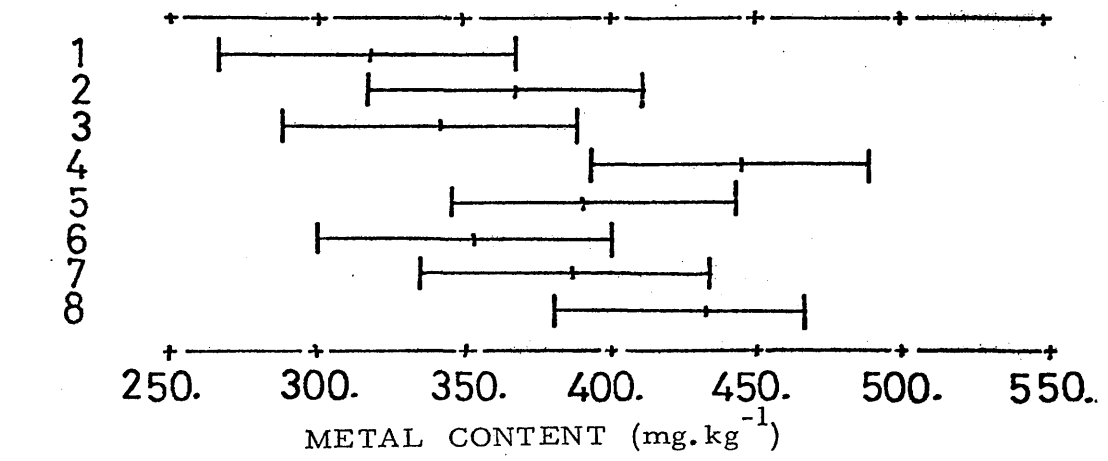


Figure 3.2.2.3 continued

5. MANGANESE (STEM)



6. MANGANESE (ROOT)

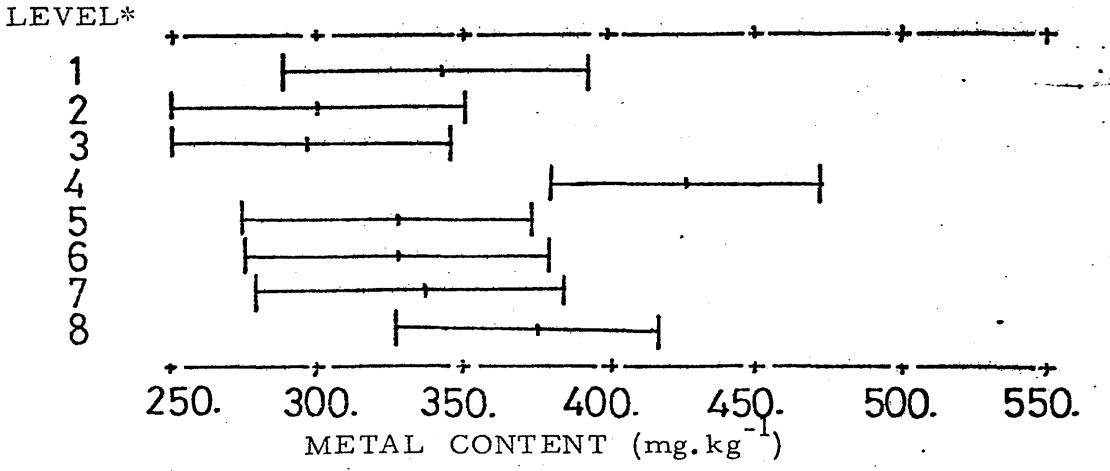
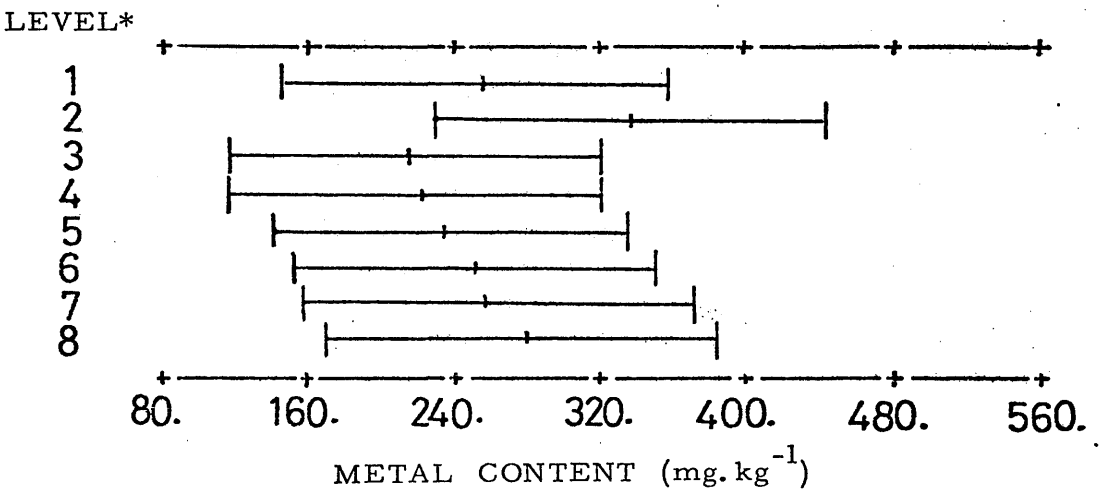
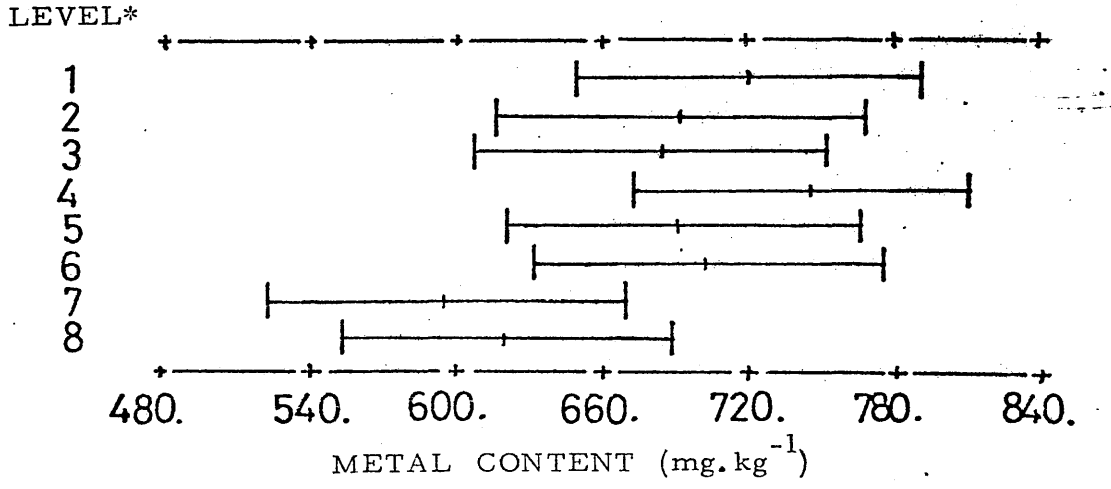


Figure 3.2.2.3 continued

7. ZINC (STEM)



8. ZINC (ROOT)



* Levels of metal chelate supplied (µg/100g soil).
1 = 0 µg; 2 = 10 µg; 3 = 50 µg; 4 = 100 µg; 5 = 250 µg; 6 = 500 µg; 7 = 750 µg; 8 = 1,000 µg.

no significant effect in decreasing or increasing zinc levels although zinc EDTA did produce the highest level of zinc in the plant root.

CHAPTER 4

AUTORADIOGRAPHY

4.1 INTRODUCTION

Although metal chelates have been used for many years to overcome trace metal deficiencies, the exact role of the chelating agent is not completely clear. Stewart and Leonard (1952) observed that a single soil application of iron EDTA resulted, within six weeks, in a complete greening of chlorotic leaves on iron-deficient grapefruit and orange trees and in a higher iron content in treated, as compared with untreated, trees. Consequently, they postulated that the roots removed iron from the chelate by ion exchange. Brown and Tiffin (1960) decided that soybean plants growing in nutrient solutions containing iron EDDHA absorbed the iron and not the EDDHA. They reached this conclusion after they were able to re-form iron EDDHA by the addition of excess iron to the nutrient solution in which the plants had been grown. A similar result was obtained with sunflower plants (Tiffin and Brown, 1959).

As a consequence of such results it was concluded that the chelating agent acted only as a carrier transporting the metal to the roots and was not itself absorbed. Others, however, reached an opposite conclusion. For example, Hill-Cottingham (1957) noted that the concentration of chelated iron in a nutrient solution containing iron EDTA decreased when tomato plants were grown in it. As there was no simultaneous formation of free EDTA he concluded that the iron EDTA had been absorbed intact. Experiments with metal chelates containing radioactively labelled chelating agents suggested that at least part of the chelating agent, or a decomposition product of it, can enter a plant (Wallace and North, 1953; Leonard and Stewart, 1953). Autoradiographs of soybeans grown in soil treated with DTPA labelled with ^{14}C in the carboxyl groupings would seem to confirm this view. Absorption and translocation of the ^{14}C were even throughout the plant and the autoradiograph intensity increased with increasing levels of DTPA application (Holmes and Brown, 1955).

Most recent theory appears to be that both the metal and the

chelating agent components of a metal chelate may be absorbed by the roots but not necessarily in equivalent amounts. Tiffin et al. (1960) examined the stem exudate from plants of three species (zinnia, sunflower and soybean) whose roots had been placed in nutrient solutions containing iron EDDHA. Both the total iron and the iron EDDHA concentrations in the exudate were found to increase as the concentration of iron EDDHA increased in the nutrient solution. However, the total iron concentration was always greater than the concentration of iron as iron EDDHA. Their conclusions were that a large proportion of the iron separated from the iron EDDHA at the root surface and that, because the chelating capacity of the nutrient solution was increased at the end of the experiments, the released EDDHA must have remained in solution. Wallace et al. (1957) found an ^{59}Fe : ^{15}N ratio of 6.13 in the roots of bush beans treated with ^{59}Fe ^{15}N EDTA which might suggest that there was non-equivalent absorption of iron and chelating agent, although the possibility that some of the ^{59}Fe had been adsorbed onto the root surface rather than absorbed by it must be taken into consideration as a cause of the high ratio. ^{59}Fe : ^{15}N ratios for other parts of the plant (entire leaf, 1.41; cell debris, 1.55; stem, 0.98) also indicate that translocation of the ^{59}Fe and the chelating agent may not be equivalent.

It has been suggested that the relative amounts of iron and chelating agent absorbed depend upon the iron status of the plant prior to treatment. Hill-Cottingham and Lloyd-Jones (1965) measured iron uptake from culture solutions by normal and iron-deficient plants and found that in deficient plants iron absorption was rapid until the fourth day. Thereafter the iron content of the plant remained constant. EDDHA absorption, however, was slow and steady throughout, but, even after thirteen days, had not reached a value equivalent to that of the iron uptake after four days. Normal plants, on the other hand, absorbed both iron and EDDHA slowly and steadily and in equivalent amounts. Wallace et al. (1957) considered that the pH of the external solution also affected the relative amounts of iron and EDDHA absorbed from iron EDDHA solutions.

4.2 AIM OF THIS INVESTIGATION

The purpose of this investigation was to determine if a radioactive label incorporated in some of the chelating agents used in pot and field experiments (Chapters 2 and 3) could be absorbed by bean plants. To accomplish this an autoradiographic technique was used.

4.3 EXPERIMENTAL

4.3.1 METHOD

The method described here was based on the suggestions made by Yamaguchi and Crafts (1958).

Bean plants (Phaseolus vulgaris var. Canadian Wonder) were planted (one per pot) in 63 mm diameter plastic flower pots containing 100 g, 2 mm soil in field moist condition. The soil used was that described in Chapter 2. The pots were kept in a growth chamber adjusted to a 16 hour day-length and a temperature of 24°C. After approximately 16 days, the plants, still in the pots, were transferred to a fume cupboard at room temperature to harden off before treatment. Lighting was as before and the fume cupboard fan was run constantly. The plants were treated with 20 cm^3 of a 'doped' chelating agent solution, soil applied, 5 days after transfer to the fume cupboard or on the 21st day after planting, whichever came first (planting day counting as day 1). An uptake time of 44 hours was allowed for both EDTA and citrate while for bicarbonate only 5 hours was necessary. During this time the day-length was adjusted to 24 hours to reduce the possibility of radioactivity losses during transpiration.

After uptake, the plants were harvested and carefully washed in deionised water to remove all traces of soil from both roots and leaves. Each plant was placed on a stiff sheet of filter paper and covered with several sheets of absorbent paper. Plant and paper were then clamped between sheets of thick cardboard and the whole assembly was dried flat for 3 hours in an oven at 80°C. Once dried and cooled, the upper sheet of cardboard and the layers of absorbent paper were removed and an X-ray film was laid directly upon the dried bean plant. To ensure

maximum contact between film and plant, a stiff card was placed over the film and, as a precaution against fogging, the card was covered with black paper. The paper holding the plant, the plant, the film, the card and the covering black paper were now firmly taped to the under sheet of cardboard. The absorbent paper and the upper sheet of cardboard were replaced on top of the pack and the whole arrangement was clamped together and weighted with sheets of glass to maintain flatness and to improve contact. Storage, for exposure of the film, was in a light-tight box.

Control plants, treated with 20 cm^3 deionised water at the same time as the test plants were treated, were handled similarly but stored separately.

4.3.2 NOTES ON THE METHOD

(1) Chelating agents

Those used were (1, 5 - C14) citric acid monohydrate, sodium (C14) bicarbonate and ethylenediaminetetra - (2 - C14) - acetic acid, sodium salt.

(2) 'Doped' chelating agent solutions

These were made as follows:

(i) bicarbonate: 1 cm^3 of sodium bicarbonate solution (5 mCuries/cm^3) was made to 100 cm^3 with distilled water. 0.5 cm^3 of this diluted solution was added to 250 cm^3 of a sodium bicarbonate solution (3 ppm bicarbonate ion) and the 20 cm^3 quantities used in the experiment were taken from this.

(ii) citrate: the chelating agent - supplied as a solid (250 μCuries) - was dissolved in 25 cm^3 distilled water. 1 cm^3 of this radioactive solution was added to 100 cm^3 of a tri-sodium citrate solution (3 ppm citrate). The 20 cm^3 aliquots for the experiment were taken from this.

(iii) EDTA: This, also supplied as a solid (250 μCuries), was dissolved in 25 cm^3 distilled water. 1 cm^3 of this

radioactive solution was added to 100 cm^3 of a di-sodium EDTA solution (3 ppm EDTA) and the required 20 cm^3 came from this stock solution.

(3) Drying the plants

It was necessary to dry the treated plants before placing them in contact with the X-ray film, for two reasons:

(i) to prevent translocation of any radioactive label within the plant after it was harvested and while it was exposed to the film. Translocation after harvesting would result in production of an image which did not reflect the situation within the plant at the instant of harvesting,

(ii) to ensure that no vapours would be produced during exposure which might contain some radioactive label. Such vapours could be expected if the plant were allowed to dry naturally while in contact with the film, with the possibility of fogging.

A 3 hour drying period was found to be most suitable. A shorter time produced a plant which still contained some sap, while a longer time gave one which was too brittle to handle. A temperature of 80°C ensured fairly rapid drying. Higher and lower temperatures were unsuitable - the former because the thinner parts of the plant became very brittle and the latter because this resulted in an unacceptably long drying time with an increased risk of translocation occurring.

(4) X-ray film

Kodak Kodirex film.

(5) Exposure time

The exposure times varied with the chelating agent and are given below:

Table 4.3.2.1 Exposure times for each chelating agent

Chelating agent	Exposure time (days)
Bicarbonate	33
Citrate	29
EDTA	36

Controls corresponding to these treatments were exposed for the same length of time and were developed at the same time.

(6) Development of film

Ilford Phenisol developer and Ilford Hypam rapid fixer were used, prepared according to the manufacturer's instructions, with agitation as recommended. Development was for 10 minutes, the intermediate wash 5 minutes in running water and fixation 10 minutes, all at room temperature. The final wash of 30 minutes was followed by drying in a forced-draught oven for approximately 45 minutes.

4.4 RESULTS AND DISCUSSION

The results are illustrated in the autoradiographs, Figures 4.4.1 and 4.4.2. The autoradiographs of the control plants cannot be shown, nor those of the citrate treated plants, the negatives, in all cases, being too faint to print. From visual examination of these, however, a description is given.

In the ^{14}C - HCO_3^- treated plant the ^{14}C seems to be fairly evenly distributed throughout the stem and leaves except for a slightly greater accumulation in the newer trifoliates. This is indicated by a very slightly higher intensity in these leaves as compared with the older ones. The intensity, and hence the ^{14}C accumulation, is very much greater in the roots than elsewhere in the plant. This may have occurred because the plant roots were very near, or actually on, the soil surface. Contact with the treatment solution would, almost inevitably, result in absorption into the roots.

Figure 4.4.1 Autoradiograph of the bean Phaseolus vulgaris var. Canadian Wonder - radioactive source, soil-applied $^{14}C\text{-}HCO_3^-$

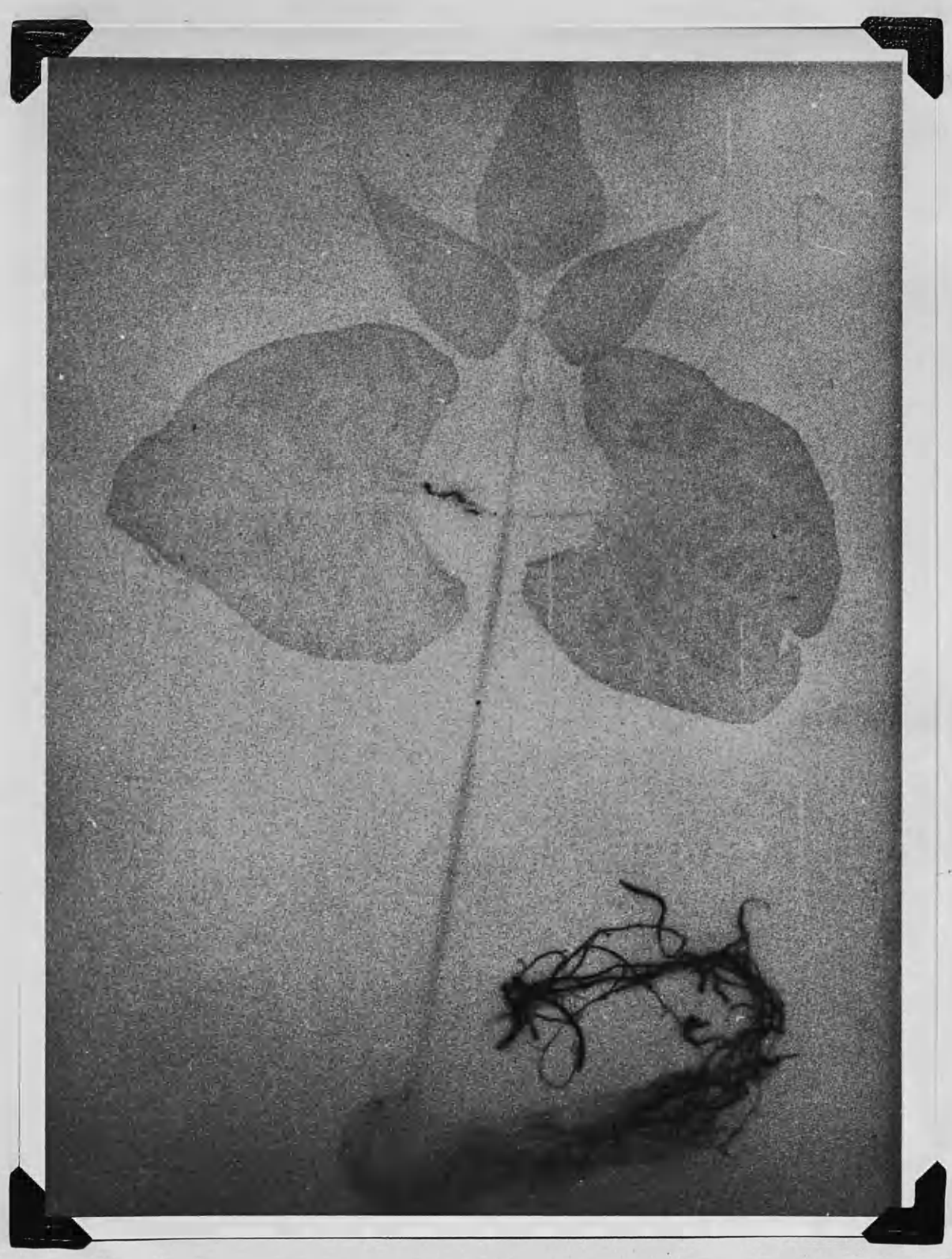


Figure 4.4.2 Autoradiograph of the bean Phaseolus vulgaris var. Canadian Wonder - radioactive source, soil-applied ^{14}C-EDTA

The control plant for this treatment shows some ^{14}C accumulation in the newer leaf growth. Distribution in the other leaf growth is fairly even. No radioactive reagent was supplied to this plant and the source of this ^{14}C would be from the air by photosynthesis. This suggests that, either the ^{14}C - HCO_3^- supplied to the treated plant was being decomposed in the soil with the release of $^{14}C\ O_2$ (g), or that $^{14}C\ O_2$ (g) was produced during the respiration of the treated plant, or as a combination of these. The lower stem and the roots of the control plant indicate only very small quantities of ^{14}C are present and it might be inferred that the ^{14}C in this plant was obtained by photosynthesis and originated in the ^{14}C - HCO_3^- supplied to the test plant.

The ^{14}C - citrate treated plant gave only a very faint darkening on the film. Close examination of the negative showed a very even distribution of ^{14}C throughout leaf and stem with no apparent increase in intensity at any point. Again, as in the ^{14}C - HCO_3^- case, the greatest intensity was found in the root and could probably be explained in the same way.

In this case the control plant gave no image, which suggests that there was no release of $^{14}C\ O_2$ (g) to the environment from the ^{14}C - citrate supplied to the test plant.

The ^{14}C - EDTA treated bean showed very little ^{14}C in the stem. The leaves and the roots were equally darkened, however, and there was some slight indication of ^{14}C accumulation in the newer leaf growth. In individual leaves there appears to be little or no ^{14}C in the veins, but the relatively darker interveinal areas suggest that there has been a migration of ^{14}C towards the leaf edges.

The control plant did not produce an image and thus it appears that there was no release of $^{14}C\ O_2$ (g) from ^{14}C - EDTA during the period of the experiment.

4.5 CONCLUSIONS

(1) Absorption of ^{14}C into the root occurs with all three chelating agents.

(2) Translocation of ^{14}C from the root into the leaves also occurs in all three chelating agents, although this seems less pronounced with citrate than with EDTA and bicarbonate.

(3) Careful examination of the negatives obtained from control plants revealed a faint image of leaves and upper stem in the case of bicarbonate but no detectable image with either EDTA or citrate. The origin of this must have been in $^{14}C\ O_2$ (g) from breakdown of the ^{14}C - HCO_3^- supplied to the treated plant. This $^{14}C\ O_2$ (g), once in the atmosphere, would become available to the controls to be taken up photosynthetically.

(4) Finally, it is recognised that the presence of ^{14}C inside the plant does not imply that the whole chelating agent molecule has been absorbed by the plant and translocated within it. The explanation most likely is that part of the molecule containing the ^{14}C label, or a ^{14}C - containing metabolic product, has been absorbed and translocated.

CHAPTER 5

REVIEW OF THE PROJECT AND POSSIBLE FUTURE WORK

5.1 REVIEW

As stated in Chapter 2 the aims of the thesis were:

(i) to determine whether metal-free chelating agents alter the trace metal content of a particular plant species,

(ii) to determine whether application rates lower than the usual are effective,

(iii) to determine whether low levels of chelating agent, in a field situation, would change the trace metal content and yield of a particular plant species.

The experimental work described in Chapters 2 and 3 has partly satisfied these objectives and a summary of results follows.

In Chapter 2, pot experiments showed that low levels (0 - 150 μg/100 g soil) of EDDHA, EDTA and 2-ketogluconic acid, soil applied to acid and limed soils, had no significant effect upon trace metal content or yield in the bean Phaseolus vulgaris var. Canadian Wonder. Although not presented in this thesis, citrate and bicarbonate gave similar results on acid soil.

Soil applications in the field, at the same rate, to the same species, with the chelating agents bicarbonate, citrate, EDDHA and 2-ketogluconic acid were also found to have no significant effect.

In Chapter 3, further pot experiments on acid soil, using higher levels (0 - 1000 μg/100 g soil) of citrate, EDTA and 2-ketogluconic acid, while not producing statistically significant effects, did show some trends which indicate that chelating agents may be able to influence yield and trace metal content. Increasing the level of citrate applied, tended to increase stem iron levels and stem and root zinc levels while decreasing manganese in the root. EDTA tended to increase root yield, root iron and stem zinc and to decrease root

manganese and zinc. The effects were slight. Finally, with 2-keto-gluconic acid, there was a small increase in stem zinc and stem manganese and a slight decrease of zinc in the root.

In the same chapter, treatment with iron, manganese and zinc EDTA, at rates up to 1000 μg/100 g soil, did have some influence upon trace metal levels. Increasing the level of iron EDTA increased iron and manganese in the stem and manganese in the root. Manganese EDTA increased manganese in the stem and root and decreased zinc in the root. Zinc EDTA had no significant effect upon yield or trace metal content, but it did give the highest content of zinc in the root.

In Chapter 4, autoradiographs obtained using bicarbonate, citrate and EDTA, all ^{14}C-labelled, showed, in each case, that some ^{14}C-containing part (or some ^{14}C-containing metabolic product) of the chelating agent was absorbed into the roots. In the three cases the ^{14}C was also translocated to the leaves, although this seemed less pronounced with citrate than with EDTA and bicarbonate. From consideration of the control plants, it appears that there was some breakdown of the chelating agents (especially bicarbonate), with release of $^{14}CO_2$ gas to the atmosphere. It was not possible to determine from these experiments if breakdown occurred by chemical or microbial activity in the soil, by plant activity or by some combination of all of these.

5.2 MODIFICATIONS TO THE METHOD

In some experiments the degree of control may be such that variations between replicates is virtually eliminated. In experiments involving plants, however, such a measure of control is not possible. In the first place, two plants, even of the same species and under identical experimental conditions, may not respond identically in terms of growth and nutrient uptake. In the second place, in the normal preparation of the soil, composition may vary from replicate to replicate. At the outset, it was recognised that in these experiments there would be variation in results from replicates. In some instances, however, there was an unacceptably large variation. Two probable factors contributing to this excessive variation are, (1) soil con-

tamination (especially in root material) and, (2) deficiencies in sampling method. Modifications to reduce variation from these two sources are proposed as follows.

5.2.1 MODIFICATIONS TO REDUCE CONTAMINATION

In the pot experiments, variation between replicates was greatest, and occurred most frequently, in root samples, suggesting that a major source of contamination may have been from soil adhering to the plant material. In the field experiment, however, leaf samples were as subject to variation as root samples but, again, soil contamination was the most probable cause. Some, but not all, of the leaf contamination could have been prevented by staking and tieing the plants. However, the problems of root contamination and removal of residual soil from leaves remain. Improved washing procedures might reduce contamination from these sources. Wallace et al. (1976) and Wallace and Alexander (1973) used 0.1 M hydrochloric acid and deionised water washes in preparation of leaf material for metal analysis. Labanaukas (1968) washed citrus leaves in detergent and rinsed in 3% hydrochloric acid to reduce contamination from foliar sprays. Jeffreys and Wallace (1968), determining iron EDDHA in plant tissue, washed leaves successively in dilute detergent, running tap water and demineralized water.

Repeated washings might be expected to result in loss of some of the more soluble inorganic material (Piper quoted by Steyn, 1959). The washing regime most effective in reducing contamination, with minimum loss through leaching, can only be determined by experiment.

5.2.2 MODIFICATIONS TO THE SAMPLING TECHNIQUE

Were further pot and field experiments to be conducted the method of sample collection would be changed.

In the pot experiments, each replicate consisted of only one plant and, consequently, the quantity of material collected for analysis was small. For root analysis particularly, it was sometimes necessary to use all the material collected to obtain a sufficiently large sample.

It would be desirable to have more material for analysis but without increasing, to an unmanageable level, the total number of samples. This could be achieved by increasing the number of plants per pot, by bulking the material from each pot and sub-sampling for analysis. This would require a larger pot and the quantity of soil and the volume or concentration of chelating agent would need to be modified. Alternatively, the number of single plant replicates per treatment might be increased, bulked and subsampled.

In the field experiment, the problem, at harvest, was that too much rather than too little material was produced. This made rapid preparation of material for analysis extremely difficult. Some material, stored in field condition, was lost through rotting. The problem was less acute with root material because less was produced and it was easier to clean and less likely to rot when stored in the field condition.

As an alternative to harvesting the whole plant, a reduction in the quantity of leaf material collected might be effected by removing only representative samples (for example, the middle leaf of each trifoliate). Were material so collected to be given an initial wash in the field, there would be a reduction in work and in the time required for sample preparation in the laboratory. Alternatively, the work might be spread over a longer period by staggering treatment and harvest times, while maintaining the sample size at the original level.

5.3 SUGGESTIONS FOR FUTURE WORK

In the course of the work described many areas suggested themselves for future research and some are outlined below.

The work to date suggests that chelating agents (trace metal free) are not particularly effective in changing trace metal levels in plants. However, it may be that there is a critical application level below which agents are not effective. One area of research would be to determine if such a cut-off point exists and, if so, its relationship with chelating agent, plant species and soil type.

The application method is known to influence the effectiveness of certain more conventional fertilizers. For example, Boawn (1973) noted that band-applied zinc sulphate had a lower availability coefficient than zinc sulphate applied broadcast and ploughed in. On the other hand, Murphy and Walsh (1972) stated that the optimum application rate for manganese is generally much lower with band, than with broadcast, application. Similar variations may occur with chelating agent fertilizers and, practically and economically, it would be important to determine the most effective application method. Probably the method will depend not only upon the chelating agent but also upon a number of other variables, such as plant species, soil properties and rainfall. If leaching were likely to be serious, then solid, less soluble, forms of chelating agent might be used. It would be necessary to find by experiment the most effective solid form (powder, granule or pellet). Application rates, times (whether before, during or after planting) and placement would similarly have to be determined. Where spraying is found to be the most effective application mode, suitable solution concentrations, times and frequencies of application would require to be found. Direct injection into plants is unlikely to be commercially viable, being time consuming, labour intensive and expensive. It may be useful in the laboratory, however, since, by varying the injection point and by monitoring the subsequent movement, distribution and re-distribution of metal within the plant tissue, the role of the chelating agent might be determined.

In one study only (see Chapter 2) were chelating agents supplied to plants growing on a soil limed to a pH between 7 and 8 and hence under stress. Chlorosis and lowered trace metal levels were observed and the chelating agents had no significant effect on trace metal content. Further work, with higher levels of application, might allow a categoric statement to be made that chelating agents do not increase trace metal content in conditions likely to cause deficiencies. Again, application modes, rates and placement would require to be studied.

Finally, radiotracers may provide a wide range of information about trace metals in soils, uptake by plants and relationships between

trace metals and chelating agents. For example, they might indicate the forms in which trace metals appear in soils or their function in enzymic reactions and whether the availability of particular metals is affected by chelating agents.

The overall aim in such studies would be to provide as large and as comprehensive a body of information as possible on one plant species and on one soil type. To complete the evaluation of the role of the chelating agent the investigation would require to be widened to include other species and soils.

APPENDIX

MICROBIOLOGICAL STUDIES

A.1 INTRODUCTION

It was noted, in Chapter 4, that controls grown in the vicinity of plants treated with soil-applied ^{14}C - HCO_3^- contained ^{14}C. This radio-active label was confined mainly to the leaves and the upper stem, suggesting that it had been absorbed through the leaves from the atmosphere. A reasonable postulate is that the origin of this label was in $^{14}CO_2$ (g) formed during breakdown of the ^{14}C - HCO_3^- supplied to the treated plant. Degradation of the ^{14}C - HCO_3^- could have occurred in the soil and/or within the plant.

An investigation was conducted into the possibility that the ^{14}C - HCO_3^- was being degraded in the soil, with the release of $^{14}CO_2$ (g). Two experimental techniques were employed, each involving collection and measurement of carbon dioxide gas.

A.2 EXPERIMENTAL

A.2.1 MANOMETRIC DETERMINATION OF CO_2(g) EVOLVED DURING AN INCUBATION EXPERIMENT

(1) Materials and Methods

The apparatus consisted of a 100 cm^3 conical flask with side-arm to which a test-tube was fitted. The outlet from the conical flask was connected by plastic tubing, via a three-way tap for venting, to a manometer containing a dithizone-chloroform mixture. The flask and test-tube were partly immersed in a water bath held at 37°C. The manometer was calibrated in cm. The soil is described in Chapter 2. Sucrose solutions, made up in deionised water, were used in the pilot experiments. Sucrose, a carbohydrate and a good energy source for micro-organisms, should increase carbon dioxide production by increasing microbial respiration. Although a quantitative release of gas was not expected, it was hoped that a sufficiently large volume of gas would be evolved to allow refinements to be made in the technique. The more demanding

situation, involving small quantities of $^{14}C - HCO_3^-$ and small volumes of $^{14}CO_2$ (g), could then be tackled.

10 g, 2 mm soil, in field moist condition, and 5 cm^3 of a 30 ppm sucrose solution were placed in the conical flask and the test-tube respectively. The system was equilibrated for 30 minutes in the water bath and periodically vented to the atmosphere. The sucrose solution was then tipped into the soil and the tap was directed to allow any gaseous products to enter the manometer. Readings were taken immediately before, and immediately after, adding the sucrose. Further readings were taken after 24 and 29 hours.

The experiment was repeated with a 300 ppm sucrose solution.

(2) Results and Discussion

Table A. 2. 1. 1 Manometer readings from incubation experiment

Time after addition	Manometer readings (cm)	
(hours)	30 ppm sucrose solution	300 ppm sucrose solution
0. 0	8. 90	8. 30
0. 016	10. 00	9. 90
24. 0	10. 00	9. 40
29. 0	7. 10	12. 00

It is interesting that, in both cases, the manometer readings increased immediately after mixing. It is unlikely that microbial activity occurred so rapidly and disturbance of the experimental arrangement by the opening of the tap, or by heat production following upon soil wetting, may have been responsible.

The reading obtained with the 30 ppm sucrose solution at 24 hours suggests that some CO_2 (g) was produced. The decrease, 5 hours later, which took the reading to a level below that of the original, may have been a consequence of the apparatus being insufficiently sensitive

to deal with such small volume changes. Other possible explanations are that there was a leak in the system, although this is considered unlikely, or that changes in air pressure affected the manometer readings. Barometric changes were not monitored during the experiment, making it impossible to assess the importance of this factor.

The results with the 300 ppm sucrose solution were less equivocal. Here, the readings continued to increase beyond the 24 hour interval which might suggest that CO_2 (g) had been evolved, but again, barometric influences may have been wholly or partly responsible.

Although a reappraisal, taking account of atmospheric pressure, would be necessary to demonstrate the value of this method, it was considered at the time (because large volumes of sucrose were required before measurable volume changes occurred) that it would be unsuitable for the detection of the small volume changes expected from the quantities of ^{14}C - HCO_3^- involved in the autoradiographic experiment. Consequently, it was decided to consider another method.

A. 2. 2 β-SCINTILLATION TO DETERMINE $^{14}CO_2$ (g) EVOLVED DURING AN INCUBATION EXPERIMENT

(1) Materials and Methods

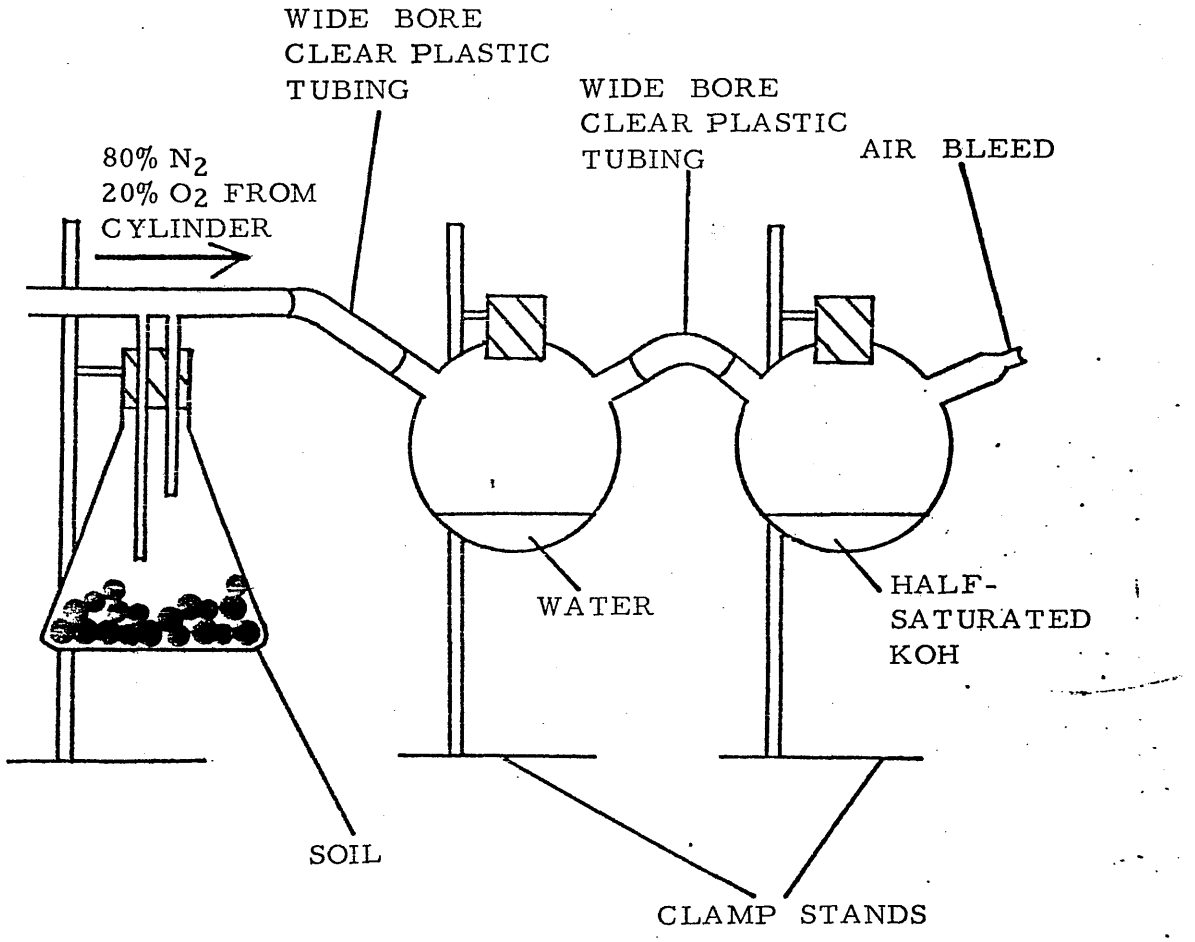
The experimental apparatus is illustrated in Figure A. 2. 2. 1. The following points are noted:

(i) a restrictor was fitted to the gas cylinder to reduce the flow rate,

(ii) the water in the first, three-necked, round-bottomed flask was introduced to increase the humidity of the air prior to its entering the second flask, the purpose being to reduce the rate of evaporation of the potassium hydroxide solution.

The conditions of the autoradiographic experiment were reproduced as closely as possible by placing the apparatus in a fume cupboard with constantly running fan, by lighting for 24 hours a day and by using equivalent treatment levels.

Figure A. 2. 2. 1 Apparatus used to trap CO_2 (g) evolved during an incubation experiment



The soil is described in Chapter 2 and the 'doped' 3 ppm sodium bicarbonate solution in Chapter 4. The half-saturated solution was made by mixing saturated potassium hydroxide with an equal volume of distilled water.

A Phillips Liquid Scintillation Analyzer was used and the scintillant was toluene containing 4 g/litre 2, 5-diphenyloxazole (PPO) and 0.1 g/litre 1,4 -di- (2-(5-phenyloxazolyl)) - benzene (POPOP).

10 cm^3, 'doped', 3 ppm bicarbonate solution were added to the 100 cm^3 conical flask which contained 50 g, 2 mm soil in field moist condition. Air was passed through the system, at a rate of 5 cm^3/ minute, for 44 hours, any $^{14}CO_2$ (g) being trapped in the half-saturated potassium hydroxide solution . Duplicate samples were removed from the gas trap by Epindorf pipette and each sample was mixed with 15 cm^3 of scintillant prior to counting.

A control experiment was undertaken in which distilled water replaced the 'doped' bicarbonate solution.

(2) Results and Discussion

The method demands that the sample be totally incorporated within the scintillant before counting commences. In an attempt to achieve this, Analar methanol was added to the sample-scintillant mixture in the counting phial. The volume of methanol used is critical - too little gives incomplete incorporation while too much raises the quenching to an unacceptably high level. Initially, 0.3 cm^3 methanol was used. However, successive countings of the same sample (Table A.2.2.1) showed a decrease over a period of time, indicating incomplete incorporation and separation of the organic and water layers. Nevertheless, in view of the very low figure obtained with the control, it did appear that $^{14}CO_2$ (g) had formed during degradation of ^{14}C - HCO_3^- in the soil.

To determine the optimum volume of methanol several samples were prepared with varying quantities added. Table A.2.2.2 gives the counts and quenching values obtained. With the highest volume of

Table A. 2. 2. 1 Quenching values and counts obtained from successive countings of the same sample (counting time = 10 minutes in each case)[a]

Sample counting number[b]	Volume methanol[c] (cm^3)	Quenching	counts (d. p. m.)[d]
1	0. 3	0. 674	924. 05
2	0. 3	0. 668	869. 15
3	0. 3	0. 668	856. 45
4	0. 3	0. 666	850. 6
5	0. 3	0. 851	23. 85

a Results given as mean of duplicate samples.

b Numbers 1 - 4 refer to successive countings of the same sample. Number 5 refers to the control.

c Volume of methanol added to each counting phial to improve sample incorporation.

d d. p. m. = disintegrations per minute.

Table A. 2. 2. 2 Effect on counts and quenching of increasing volumes of methanol (counting time = 10 minutes in each case)[a]

Sample number	Volume methanol[b] (cm^3)	Quenching	Counts (d. p. m.)[c]
1	0. 5	0. 666	1002. 5
2	0. 7	0. 584	702. 7
3	0. 9	0. 497	881. 25
4	3. 9	0. 294	1671. 25

a Results given as mean of duplicate samples.

b Volume of methanol added to sample prior to counting.

c d. p. m. = disintegrations per minute.

methanol quenching was unacceptably high. Lower volumes gave quenching within reasonable limits. Once more, however, successive countings in individual samples decreased over a period of time.

A. 3 CONCLUSIONS

From the results of the manometric experiment it appeared that the method was difficult and perhaps unsuitable for the detection of very small quantities of gas. The β-scintillation method, however, is capable of detecting very small quantities and further, being a highly specific method, it may be used to pinpoint the source of the labelled CO_2 (g) evolved in the experiment. If the problems of incomplete incorporation in the scintillant and of phase separation can be overcome it would be possible to obtain quantitative information.

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