ALLELOPATHY IN A GRASS-LEGUME ASSOCIATION:

A CASE STUDY WITH

HEMARTHRIA ALTISSIMA AND *DESMODIUM INTORTUM*

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE
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OF THE REQUIREMENTS FOR THE DEGREE OF

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ABSTRACT

In Hawaii, the legume *Desmodium intortum* (Mill.) Urb. could not be established in a pasture of the tetraploid *Hemarthria altissima* (Poir.) Stapf and Hubb cv. bigalta limpograss, but intortum was easily established in a sward of the less vigorous greenalta limpograss. The effects of root residues and root exudates of intortum and the limpograsses on the growth, nodulation and nitrogen fixation of intortum were studied in experiments designed to avoid competition between root systems of the grass and legume for nutrients, water, and space and between aerial plant parts for light. Finally, allelochemicals in the root exudates of bigalta and greenalta limpograsses were collected, isolated and partially characterized.

The growth of intortum in fertilized soil which contained root residue of bigalta limpograss was significantly less than the growth of intortum in the presence of greenalta limpograss. The residue treatments had little or no effect on the mineral nutrient contents of intortum tops. The effects of root exudates of the limpograsses were studied in vermiculite cultures watered with Hoagland's or a dilute nutrient solution. Intortum was grown with each of the limpograsses

in divided pots where the root systems were separated and in pots where the root systems were intermingled. The growth of intortum seedlings and cuttings was inhibited as much as 75% in divided pots and 88% in undivided pots by exudates from bigalta limpograss. Exudates from bigalta limpograss were much more inhibitory than those of greenalta. It is concluded from the data that the inhibition of intortum growth by bigalta limpograss was allelopathic. The inhibition of intortum seedling growth by exudates from established intortum cuttings showed that intortum was autotoxic.

Nodule fresh weight and acetylene reduction per pot of intortum grown with bigalta, greenalta or intortum were significantly lower than the control in pots with and without dividers which were irrigated with Hoagland's nutrient solution. However, the specific nodule activity of intortum was not reduced by root exudates from the two grasses. Specific nodule activity of intortum grown with intortum was significantly less than the control.

A new method was developed for the extraction of hydrophobic allelochemicals from root homogenates and exudates using the resin Amberlite XAD-4. Allelochemicals in aqueous root homogenates of the limpograsses were extracted with equal efficiency by methanol or XAD-4 resin as shown by a lettuce seed germination bioassay. A unique continuous trapping system for the collection of root exudates was developed by connecting a column containing XAD-4 resin to the container used for growing limpograsses in sand culture.

Chemicals trapped by the resin were eluted by organic solvents and separated into neutral, acidic, and basic fractions. The neutral fraction from bigalta limpograss inhibited lettuce seed germination

more than that of greenalta. Allelochemicals were isolated only from the neutral and acidic fractions by paper chromatography. Most zones on the chromatograph containing inhibitors showed a positive color reaction when sprayed with diazotized p-nitroaniline followed by 10% sodium carbonate indicating that the allelochemicals were mainly phenolic compounds.

Root residues of bigalta and greenalta limpograsses and intortum added to soil increased the populations of some fungi and bacteria and the soil levels of the enzymes amylase, cellulase, invertase and dehydrogenase. However, the lowest levels of fungi were found in the pots containing residues from bigalta limpograss. The levels of the four enzymes were highest in soil containing intortum root residues and lowest in soil containing bigalta residues and in the control soil.

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CHAPTER 1

GENERAL INTRODUCTION

Many species of grasses and legumes which provide animal forage in the form of hay or pasture are sown in mixtures. Research on such mixtures has indicated that pasture quality is improved by incorporating a legume with a grass, especially where soil fertility is low. The legume and associated Rhizobium bacteria increase the nutrient quality of the forage mixture because of the legumes characteristicly high nitrogen content. There is some evidence, also, that there may be some nitrogen transfer from the legume to the grass, increasing its productivity and possibly its protein content.

When all of the available space is occupied by the components of the mixture, the growth of one or both plants in a mixture is reduced. The effect of one plant on the growth of another plant in a mixture is very complex and involves competition, allelopathy, or both. Competition refers to those detrimental effects on one or both species which occur as a result of a limiting quantity of essential resources. Nutrients, light, water and space are the common resources which are in limited supply. The existence of competition in grass-legume mixtures has been reported often (Donald, 1963; Hacker and Jones, 1969; Hall, 1971; Jackman and Mouat, 1972). Severe competition between species in an association usually results in the dominance of one species by the other.

Usually dominance is due to the environment being more favorable for the growth of the dominant species, but may also be due to the production of a substance or substances by the dominant species which

inhibits the growth of the associated species. This inhibition is termed allelopathy.

Allelopathy is a direct or indirect biochemical inhibition of one plant or microorganism by another through the production of chemical compounds that are released into the environment (Rice, 1974). Plants in the natural environment are exposed to phytochemical stress that may adversely influence their productivity and usefulness to man. Allelopathy has been extensively studied, and there is now strong evidence that such plant-plant, plant-microbe and microbe-plant interactions do occur. The allelopathic potential of a cultivar is determined by its genetic potential and this potential is manifested in its metabolism. The ecological significance of allelopathy in natural conditions is dependent on climate, particularly rainfall and according to Muller (1966) allelopathy is most likely to be manifested in dry conditions. Allelopathy has been most often studied in natural habitats. However, recently allelopathy has been shown to be significant not only in the natural environment, but also in the applied plant sciences like agronomy, range management, horticulture, and silviculture (Tukey, 1969; Rice, 1974). Since Ahlgren and Aamodt (1939) first suggested that harmful root interactions between grasses and legumes was one mechanism of dominance, there has been little published on allelopathy in temperate grass-legume mixtures and even less data are available for tropical species. The study of allelopathy in tropical grass and legume forage species could improve the understanding of species-species interactions, and suggest approaches to the management and breeding of pasture species that would improve overall productivity.

Both Desmodium intortum (Mill.) Urb. (hereafter referred to as intortum) and Hemarthria altissima (Poir.) Stapf and Hubb cv. bigalta limpograss, a tetraploid cultivar and cv. greenalta limpograss, a diploid cultivar, are tropical forages having high productivity. Intortum has been reported to fix 380 kg N per hectare in 12 months (Whitney, et al., 1967) and has been ranked as one of the most efficient tropical legumes in terms of rate of nitrogen fixation (Graham and Hubbell, 1974). Bigalta limpograss has consistently given high yields in small plot experiments in Hawaii (Whitney, 1975). However, Whitney (1975, personal communication) noted that intortum did not establish well in a pasture with bigalta limpograss, but much better survival of intortum seedlings was obtained in a greenalta limpograss sward. Thus, there is evidence for interference among the above two species although the suppression of intortum by the grass when grown in mixtures probably involves both allelopathy and competition.

The purposes of this study are as follows:

1. To determine whether or not allelopathy in Desmodium intortum - Hemarthria altissima mixtures exists, and if present, its significance.

2. To determine the effect of root exudates from bigalta and greenalta limpograsses and intortum on the growth and nitrogen-fixation potentials of intortum.

3. To develop a simple system for the continuous collection of root exudates which may contain inhibitory substances.

4. To isolate and characterize the allelopathic substances of root exudates from the grasses.

5. To determine the effect of root residues of the two limpograsses and intortum on soil microbial populations and soil enzyme activities.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

A. Meaning and origin of term allelopathy

The term allelopathy was coined by Molish (1937) to refer to biochemical interactions covering detrimental and beneficial reciprocal interactions between all types of plants including microorganisms. However, allelopathy was derived from two Greek words meaning mutual harm. As in all new research fields, semantic problems often exist. The term allelopathy is sometimes given broad and sometimes narrow interpretations. Rice (1974) suggested, in the book "Allelopathy" that the term allelopathy should include any direct or indirect harmful effect by one plant (including microorganisms) on another through the production of chemical compounds that escape into the environment.

B. Terminology for chemical interactions between organisms

Biochemicals that are exuded into the environment by plants have either no effect or have harmful or beneficial effects on other plants or organisms. There is increasing evidence that many biochemicals released by plants are toxic to other plants or to other organisms. These toxic chemicals are called allelopathic substances. There are four distinct types of allelopathic substances identified in the current literature.

(1) Antibiotics - toxic substances formed by microorganisms and toxic to other microorganisms

(2) Kolines - toxic substances formed by higher plants and toxic to other species of higher plants

(3) Marasmins - toxic substances formed by micro-organisms and toxic to higher plants

(4) Phytonicides - toxic substances formed by higher plants and toxic to microorganisms

The interactions among plants involving the above allelopathic substances are summarized in Figure 1. In plant-plant allelopathy, the beginning step is the formation and excretion of active substances into the rhizosphere by the donor plant. These compounds can act directly or indirectly to influence the growth of the acceptor plant. The donor plant may exude kolines which directly inhibit the acceptor plant. It is also possible that the donor plant exudes phytonicides which would inhibit a group of microorganisms which are beneficial to or symbiotic with the acceptor plant. Compounds exuded by the donor plant may be transformed to new toxic compounds by heterotrophic microorganisms. Microorganisms in the rhizoplane or rhizosphere of the donor plant may release marasmins which inhibit the acceptor plant or the donor plant. Antibiotic compounds may also be released by the dominant microorganisms in the rhizoplane or rhizosphere which inhibit microorganisms that are beneficial to or symbiotic with the donor or acceptor plant species. Another source of allelopathic substances is from plant residues.

In soil, many factors interact to influence the production of allelopathic substance, but also decrease the concentration of active substances. Loss of allelopathic substances may occur by the adsorption or fixation of compounds on soil clays or organic matter, evaporation into the air, leaching into deep layers of soil or decomposition by physical, chemical or biochemical reactions. The

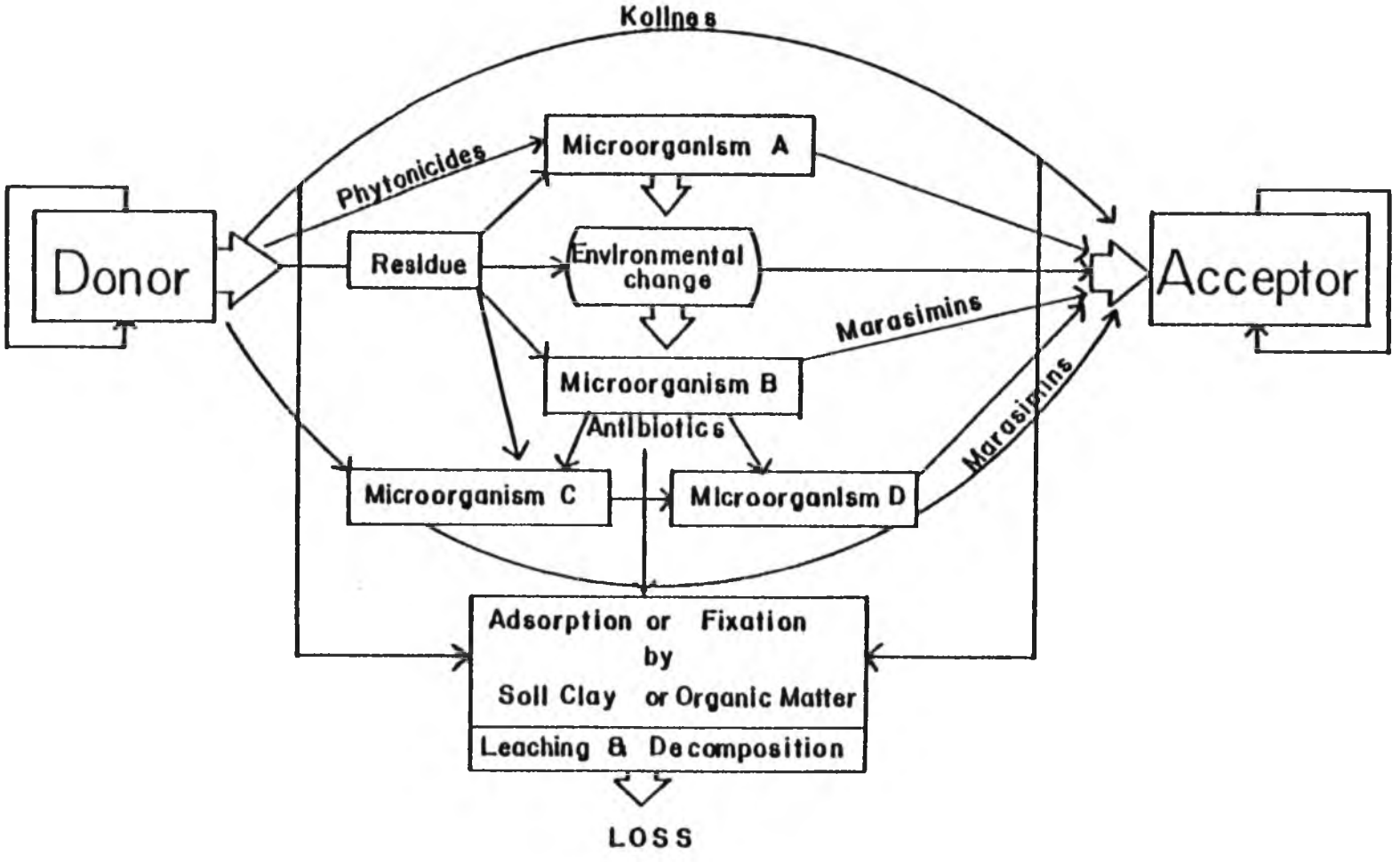

Figure 1. A schematic of allelopathic interaction among plants and microorganisms.

significance of any one process or mechanism is essentially unknown because in nature they act together.

This review will be concerned with allelopathy in grass-legume associations and will deal primarily with kolines, marasmins and phytonicides.

2.2 Allelopathic Interactions in Grasses and Legumes

A. Effects of plant extracts on seed germination and growth

Early workers have shown that extracts of many plants contain germination and growth inhibitors (Evanari, 1949; Bonner, 1950) which may influence the association of different species. In most studies of allelopathic interactions between grasses and legumes, the test plants to which the extracts were applied were chosen for the sake of convenience. Allelopathy can be demonstrated by this method. However, in order to prove the existence of allelopathy in nature it is necessary to demonstrate that the extracted material actually is released to the environment and inhibits the growth of neighboring organisms.

Le Tourneau et al. (1956) demonstrated that germination and growth inhibitors were present in extracts of common weed and crop species. They showed that dilute aqueous extracts of Zea mays var. Minhybrid 504 inhibited growth of the primary root but not the germination percentage of Pisum sativum, Soja max and Phaseolus vulgaris. Nielsen et al. (1960) showed that an aqueous extract of timothy (Phleum pratense) inhibited the germination and early growth of soybean and alfalfa. Grant and Sallans (1964) tried to screen plants which are frequently grown in association to determine their potential

to inhibit growth. Extracts of timothy, brome, orchard and reed canary grasses had very little effect on germination of alfalfa, red, ladino, or birdsfoot trefoil clover seeds. However, dilute extracts of timothy roots, brome tops and reed canary tops significantly reduced the shoot growth of red and ladino clovers. Extracts of roots and tops of timothy and orchardgrass significantly inhibited the shoot growth of birdsfoot trefoil. Shoot growth of alfalfa was not inhibited but shoot and root extracts of timothy, brome and reed canary grasses significantly inhibited root growth of alfalfa. Extracts of roots and tops of all four grass species significantly inhibited the root growth of birdsfoot trefoil clover, a species which is well-known to be difficult to establish. Alfalfa was the species least affected by the extracts, a result similar to that obtained by Nielsen, et al. (1960) and Hoveland (1964). They stated that alfalfa was considered a "very aggressive species" when grown with other forage plants. Bokhari (1978) reported that extracts of fresh material of blue grama, buffalograss and western wheatgrass were more phytotoxic to seed germination than extracts of the plant litter of the grasses.

In the southern United States, poor clover stands are frequently obtained when winter annual clovers are seeded in dormant sods of warm season perennial grasses. Hoveland (1964) found that root extracts of johnsongrass (Sorghum halapense) and Sorghum almum caused the most severe inhibition of clover germination and seedling growth. Extracts from bermudagrass (Cynodon dactylon) were the next most toxic followed by dalligrass (Paspalum dilatatum) and bahiagrass (P. notatum) with tall fescue (Festuca arundinacea) having little or no effect on white, ball, crimson, and arrowleaf clovers. Radicle

growth of white clover was depressed even when the extracts from johnsongrass and bermudagrass were diluted to 12.5% of the original 1:100 root extract.

Bieber and Hoveland (1968) reported that failure of crownvetch (Coronillia varia) seedlings to establish in roadside vegetation tests in Alabama often seemed related to the presence of other crop species. They found 1:15 plant (roots and tops) water extracts of bahiagrass, johnsongrass, and crabgrass (Digitaria sanguinalis) delayed the germination and radicle growth of crownvetch. Weeping lovegrass (Eragrostis curvula) was not toxic while crabgrass was very toxic.

The germination of grass seeds, which are small sized and have small amounts of stored nutrients, usually are more sensitive to plant extracts than legume seeds. Nielsen, et al. (1960) showed that aqueous extracts of alfalfa reduced the germination and early growth of timothy and oat. Grant and Sallans (1964) also showed that the aqueous extracts of red clover (roots and tops), alfalfa (roots and tops) and birdsfoot trefoil (tops) significantly reduced the germination of timothy. Extracts of roots and tops from birdsfoot trefoil, ladino clover tops and alfalfa roots significantly inhibited the germination of brome and orchardgrass seeds. All of these extracts significantly reduced the germination of reed canary grass seeds. Most studies with plant extracts have dealt with the establishment of grasses and legumes while work on growth subsequent to establishment has been neglected.

B. Effect of exudates on growth of grasses and legumes

Plant exudates are much more difficult to work with than extracts because they are present at very low concentrations in the

soil and soil solution. The effect of exudates generally are studied by passing a culture solution through the media in which the donor plant is growing and then using the solution to irrigate the acceptor plant.

The term exudate is used here to refer to any substance which gets into the medium directly from healthy, intact plant roots (Rovira, 1969). There is now ample evidence that plants exude sufficient quantities of allelopathic substances to influence the nutrient uptake and the productivity of crops.

Rice (1968) showed that root exudates of the grasses, Aristida oligantha, Bromus japonicus, and Digitaria sanguinalis significantly reduced the fresh weight of the legumes red kidney bean, korean lespedeza and white clover grown in sand culture with a low level of nitrogen; A. oligantha did not inhibit the growth of red kidney bean. Rice (1968) suggested that this reduction probably was due to the low supply of nitrogen available to the legumes which did not nodulate well. Where nitrogen is limiting as it is likely to be in many pasture situations, root exudates may directly affect the growth of legumes by inhibiting (interferring with) legume nodulation.

Sajise and Lales (1975) observed that the legume Stylosanthes guyanensis was severely inhibited by cogon (Imperata cylindrica), the dominant species. They found that the growth of S. guyanensis was reduced when grown in a mixture with cogon in pots and when receiving exudate from cogon in the field. Newman and Rovira (1975) showed that root exudates from the legume Trifolium repens significantly reduced the shoot weight of Anthoxanthum odoratum, but not of Cynosurus cristatus, Holcus lanatus, and Lolium perenne. Root exudates from

Trifolium repens, Anthoxanthum odoratum, and Lolium perenne, species which commonly grow together, also influenced phosphorus uptake of other grasses and legumes (Newman and Miller, 1977).

C. Effects of plant residues on germination and growth of grasses and legumes

Plant residues are materials left on or incorporated into the soil after harvesting. While the beneficial effects of crop residues are well known, their detrimental effects have been less well studied. Substances extracted or released from decomposing plant residues or produced as a result of residue-microorganism interactions have been shown to inhibit plant growth. This finding has significance when attempting to establish crops in fields where residues have been incorporated. For instance, some mulching materials have been observed to reduce the stand and growth of corn under certain conditions. Germination and seedling growth of corn was depressed more by sweet-clover (Melilotus sp.) residue than by alfalfa, wheat, or oat residues (McCalla and Duley, 1948). Guenzi and McCalla (1962) showed that 1:15 water extracts of the residues of soybean, sweetclover, oat, wheat, bromegrass, corn and sorghum inhibited the germination and growth of sorghum, corn, and wheat. McCalla (1968) showed that submerging crop residues in water prolonged the residual phytotoxic activity of wheat, oat, corn, and sorghum debris. Wheat and oats stubble lost more water soluble toxic substances within eight weeks of harvest than sorghum or corn.

Rice (1968) showed that residue of A. oligantha significantly inhibited the growth of white clover. Further study of how the residues of one crop affect the growth of the following crop and

further, which crop-residue associations lead to inhibition of growth or nitrogen fixation is badly needed. Increased understanding of residue-crop interactions could increase the available nutrient supply and the productivity of the cropping system.

2.3 Grass, Legume and Microorganism Interactions

Higher plants influence microbial activity in the soil which may then influence other higher plants, for example by the production of marasmins (Figure 1), but plant-microbe relationships have not been studied extensively. The implications for cropping systems are unknown. Starkey (1929) suggested that root exudates of plants influence the biological balance of organisms in the soil. Nickell (1959) showed that active antimicrobial substances were present in 157 vascular plant families. These results may implicate the plant as a factor in determining the distribution of microorganisms in soils.

A. Inhibition of bacteria by legumes and grasses

Some reports suggest that legumes produce chemicals which prevent infection of the roots of other legumes by desirable Rhizobium strains. Beggs (1964) reported the inhibition of nodulation of clover (Trifolium sp.) seeded into existing grasslands was a widespread practical problem in Marlborough, New Zealand. "The success of formalin treatment of the soil in overcoming this problem suggested that soil microflora are responsible" (Beggs, 1964). Thorne and Brown (1937) found that most legume-nodule bacteria were able to grow in freshly expressed juices of their host plants, but could not grow in the juices of other species. Elkan (1961) reported that a non-nodulating, near-isogenic soybean strain released a substance that inhibited nodulation of its normally nodulating sister strain.

Grasses also have been found to produce substances which inhibit nodulation of legume roots, free-living N_2 fixing bacteria and nitrifying bacteria. Rice (1964) showed that fresh extracts from Andropogon scoparius, Aristida oligantha, Bromus japonicus, B. tectorum, Chenchrus pauciflorus, and Setaria viridis inhibited one or more species of nitrogen-fixing (Azotobacter chroococcum ATC-9043, A. vinelandii ATC-9104, Rhizobium leguminosarum ATC-10314 and R. sp. ATC-10703) or nitrifying bacteria (Nitrobactor agilis ATC-14123, Nitrosomonas europaea Winogradsky, and Nitrosomonas sp.). Three of four species tested had more inhibitory activity in young than in old leaves. Rice (1964) found that extracts of A. oligantha significantly reduced the number of nodules on stringless greenpod bean and also inhibited nodulation of black valentine beans. Extracts of B. japonicus and D. sanguinalis reduced the number of nodules on two inoculated Phaseolus vulgaris cultivars. Also, Indigofera cordifolia grown in association with Aristida adscensionis had reduced nodulation in the field (Murthy and Ravinda, 1974). Murthy and Ravinda (1975) found that extracts of root, shoot and litter of A. adscensionis significantly inhibited the development of colonies of Azotobacter grown in culture. They also found that when soil samples were treated with the extracts of root and litter, the soil total nitrogen level was reduced significantly. The same effect was not found when extracts of the shoot were used. Extracts of A. adscensionis also inhibited the growth of Rhizobium in culture, and root extracts were generally more active than those from shoots and litter (Murthy and Nagodra, 1977). Festuca species were also found to produce compounds which were toxic to soil microbes. The rhizosphere of fescue

(Festuca spp.) had many fewer microorganisms associated with it and the densities of bacteria responsible for ammonification and nitrification were much lower in association with fescue roots than for many other plants (Zagallo and Bollen, 1962). Malone (1970) found that aqueous extracts of living roots and leaves of F. arundinacea were inhibitory to many bacteria (such as Azotobacter chroococcum, Rhizobium leguminosarum, Nitrobacter agilis, and Pseudomonas denitrifican). When roots and tops of fescue were killed the densities of soil bacteria increased regardless of whether or not the dead cover was removed and rates of organic matter decomposition were enhanced. Malone (1970) suggested that the proliferation of bacteria that occurred whenever living fescue was killed was due to the fact that toxic substances were no longer exuded from fescue.

B. Inhibition of bacteria by rhizoplane and rhizosphere microorganisms

The significance of rhizoplane (surface of roots) and rhizosphere (area around roots) bacteria in grass-legume associations probably has not been adequately established. However, there is some evidence that bacterial associations with plant roots do inhibit the growth of nitrogen-fixing bacteria. Van Der Merwe and Van Jaarsveld (1967) found that one strain of Pseudomonas isolated from the surface of Trifolium repens roots was more antagonistic to clover root nodule bacteria (Rhizobium trifolii) than any others tested. Hattingh and Louw (1969) found eighty-three different bacterial isolates from the rhizoplane of inoculated pasture clovers inhibited the growth of two Rhizobium trifolii strains. The antagonists belonged to the genera Pseudomonas, Xanthomonas, Achromobacter, Flavobacterium and

and Alcaligenes. Most of the antagonists , as well as the strongest ones, were confined to Pseudomonas bacteria. Leuck II and Rice (1976) showed that A. oligantha bacteria were antagonistic to the growth of Rhizobium and Azotobacter. While the evidence of bacterial inhibition of nitrogen fixing bacteria is not disputable, the ecological significance of bacteria-bacteria inhibition in the soil has not yet been adequately determined. Other microbe-microbe interactions may prevent Pseudomonas populations from building to levels where they are a significant factor in biological nitrogen fixation.

2.4 Allelopathic Chemicals in Grasses, Legumes and Soils

Most phytotoxic substances involved in allelopathy are secondary metabolites (Fraerkel, 1959). Whittaker and Feeny (1971) categorized these secondary compounds into five major groups--acetogenins, alkaloids, phenylpropanes, steroids, and terpenoids. Most substances from grasses and legumes which show allelopathic activity have been identified as phenolic compounds.

A. Phenolic inhibitors in grasses and legumes

Phenolic compounds which inhibited germination, growth, and nodulation of legumes have been found in a number of grasses and legumes. McCalla and Duley (1948) found that sweetclover residues contained large quantities of water-soluble substances, primarily coumarin, that depressed the germination and seedling growth of corn. They suggested that the effect of these substances in the soil when exposed to numerous microbial transformations may be entirely different than their effects in relatively pure culture. Fay and Duke (1977) found different amounts of the phytotoxin scopoletin (6-methoxy-7-hydroxy coumarin) exuded from different Avena species. Guenzi and

McCalla (1966a) reported that when the phenolic acids p-coumaric, ferulic, p-hydroxy-benzoic, syringic, and vanillic were quantitatively estimated in corn, oat, sorghum and wheat, p-courmaric acid was present in greater concentration than the others. Abdul-Wahab and Rice (1967) showed that seed germination and seedling growth of seven grasses were inhibited by rhizome extracts of johnsongrass. The inhibitors from johnsongrass were identified as p-coumaric acid, chlorogenic acid, p-hydroxybenzaldehyde, and the cyanogenic glucoside dhurrine. Chlorogenic, isochlorogenic, and sulfosalicylic acids were reported to be the chief phytotoxins produced by Digitaria sanguinalis (Parentic and Rice, 1969).

B. Phenolic inhibitors in soils and rhizoplane

Many phenolic acids have been isolated from the soil solution (Guenzi and McCalla, 1966b; Whithead, 1964). Glass (1976) analyzed several soil types for the presence of phenolic acids and reported that the soil solution concentration of free cinnamic and benzoic acids was commonly ofthe order of 10^{-5} M. Most plants contain phenols, lignin, tannins, and flavenoids, and other compounds which yield phenols upon degradation, and phenols also are produced by microbes so it is not surprising to find relatively large amounts of these compounds in the soil (McCalla and Norstadt, 1974). Van Der Merwe and Van Jaarsvelt (1967) found that 2,4-diacetyl-phloro-glucinol produced by Pseudomonas W78 collected from the rhizoplane of clover roots inhibited the growth of Rhizobium trifolii.

Phenolic acids have been found in many agricultural soils of Taiwan at concentrations that could inhibit the growth of corn, rice, soybean, sugarcane and wheat in culture solution (Wang et al., 1967b).

Purashothaman and Balaraman (1973) reported that the phenolics--anthranilic, p-aminobenzoic, cinnamic, ferulic, gallic, p-hydroxybenzoic acids and hydroquinone--extracted from black, lignite, peat, and red soils inhibited in vitro growth of Rhizobium spp., even at concentrations as low as 5×10^{-4} M. Blum and Rice (1969) found that gallic and tannic acids were highly effective at low concentrations in reducing nodulation and the amount of leghemoglobim produced in bean plants grown in sand culture or soil. Phytotoxic phenolic inhibitors and two unknown ninhydrin-positive compounds were found in twelve subtropical grasses and the soils in which they were growing (Chou and Young, 1975). The phytotoxins in the soils were identified as ferulic, syringic, trans-p-coumaric, cis-p-coumaric, o-coumaric, vanillic, p-hydroxybenzoic and o-hydroxy-phenylacetic acids.

The production of phytotoxins by plants and microorganisms and the toxicity of the compounds are influenced by the environment. Ohman and Kommedahl (1964) found phytotoxic substances from decomposing residues of quackgrass roots and rhizomes were produced only when decomposition was anaerobic. Lehman and Rice (1972) have shown that nutrient deficiency could increase the amount of toxic substances in a plant. In general, the production of phenolic compounds by plants increases under stress. However, the extent of exudation from plants under stress is not clear. No doubt plant residues from plants grown under stress would release greater amounts of phenolics into the soil than would nonstressed plants.

CHAPTER 3

EFFECTS OF ROOT RESIDUES ON THE GROWTH OF *DESMODIUM INTORTUM* AND *HEMARTHRIA ALTISSIMA* IN SOIL

3.1 Introduction

It is a commonly observed phenomenon that the growth of some crop species is reduced if the crop is planted in soil following certain types of crops. The observed growth reduction is often attributed to toxic residues which remain in the soil after the crop has been harvested (McCalla and Norstadt, 1974; Rice, 1974). The residues of plants remaining in soil after harvest could have no effect on the growth of the next crop or they could stimulate or inhibit growth. The stimulation of plant growth by crop residues may be due to an increase in soil nutrients upon residue decomposition. Stimulation could also be caused by compounds with growth regulator activity released from living roots or leached directly from residue or produced from residue by decomposition. The retardation of plant growth by root residue may be due to reduced nutrient availability as a result of removal by the previous crop(s), or by immobilization by soil micro-organisms. Inhibition could also be due to inhibitors which are released from residues or microorganisms.

The toxicity attributed to root residues could result from exudation of material from living roots prior to harvesting the crop and this broader definition of root residues is adopted here. It is generally assumed that the toxic substances are liberated from root residues upon decomposition although toxic substances could also be produced as result of residue-microorganism interactions (McCalla and Norstadt, 1974; Rice, 1974). The significance of these toxic or

allelopathic materials in cropping systems is not well known and has not been the subject of much intensive study.

The experiments described here were established to attempt to resolve the question of whether the difficulty of establishing Desmodium intortum (Mill.) Urb. (hereafter referred to as intortum) in an established sward of the tetraploid cultivar of Hemarthria altissima (Poir.) Stapf and Hubb cv. bigalta limpograss was due to allelopathy or competition. Establishment of intortum was not a serious problem with the diploid cultivar of H. altissima cv. greenalta limpograss. The first experiments were designed to test the effects of root residues remaining in soils in which bigalta and greenalta limpograsses, and intortum had been grown on the establishment and growth of intortum seedlings in those soils.

3.2 Materials and Methods

A. Plant and soil

The plant materials used in the studies were two clones of Hemarthria altissima, bigalta (a tetraploid cultivar) and greenalta (a diploid cultivar) limpograsses, and the legume Desmodium intortum cv. greenleaf. Seeds of intortum and cuttings of bigalta and greenalta limpograsses were collected from the Hawaii Agricultural Experiment Station farm at Kula on the island of Maui, Hawaii. Intortum seeds were surface sterilized with 5% H_2O_2, and cuttings of the two grasses were washed with water and then treated with a Benlate fungicide solution (0.63 g fungicide per liter of water) to control fungal pathogens. The forages were planted in the greenhouse in Paaloa soil (Humoxic tropohumult, clayey, oxidic, isothermic) collected from an

uncropped site in the Helemano area, Oahu, Hawaii. The soil was collected from an undisturbed area to reduce the possibility that the soil contained large quantities of root residues, particularly grasses. The topsoil was collected and passed through a 5 mm-sieve. The soil contained 0.18% nitrogen, 0.012 ppm available phosphorus in the soil solution and in me/100 g 0.06 potassium, 0.69 calcium, 0.16 sodium, and 0.03 magnesium. The soil pH in a 1:1 (weight basis) water-soil slurry was 5.3.

B. Design of Experiment I and II

The experimental design used for the two experiments was a randomized complete block with three replications. There were six treatments used in Experiment I and 22 in Experiment II (Table I). In Experiment I, bigalta and greenalta limpograsses and intortum were grown alone at two rates of phosphorus fertilization to provide base-line growth data on the three species and to provide soil in which each species had been grown as a substrate for Experiment II. In Experiment II, soil from Experiment I was utilized to observe the effects of root residues of the three forages on the growth of intortum.

C. Soil analysis, fertilization and planting in Experiment I and II

The method used in both Experiment I and II to determine the phosphorus required to bring the concentration in the soil solution to 0.3 ppm and 0.04 ppm was that of Fox and Kamprath (1970). Soil samples weighing 3 g were equilibrated at room temperature for six days in 30 ml of 0.01 M $CaCl_2$ containing graded amounts of $Ca(H_2PO_4)_2$. Toluene was added to prevent microbial proliferation. Equilibration was carried out in 50 ml plastic centrifuge tubes which were shaken in a shaker for a 30-minute period twice daily. After centrifugation

Table I. Treatments established to study the effects of phosphorus and root residues on the growth of three forage species

Experiment	Soil Treatment		Species	Number of Replicates
	P, ppm	Residue*		
I	0.3	None	D	15
		None	B	9
		None	G	9
	0.04	None	D	15
		None	B	9
		None	G	9
II	0.3	D	D	3
			B	3
			G	3
			D+B	3
			D+G	3
		B	D	3
			B	3
			D+B	3
		G	D	3
			G	3
			D+G	3
	0.04	D	D	3
			B	3
			G	3
			D+B	3
			D+G	3
		B	D	3
			B	3
			D+B	3
		G	D	3
			G	3
			D+G	3

* D = Desmodium intortum; B = Bigalta limpograss; G = Greenalta limpograss

(3000 rpm), the concentration of phosphorus in the supernatant was determined by the ascobic acid method of Watanabe and Olsen (1965). Soil pH was measured as described previously prior to the establishment of Experiment I, at the end of Experiment I, and at 35 days after planting Experiment II. Lime was added to adjust the soil pH to 5.5. A pH of 5.5 was used because Munns and Fox (1976) showed that the growth of intortum was depressed at soil pH values much greater than 6.

Fertilizers applied to the soil in Experiment I in ppm of the element were N (urea), 100; K (K_2SO_4), 100; Zn ($ZnSO_4 \cdot 7H_2O$), 10; Mo ($N_{a2}MoO_4 \cdot 2H_2O$), 1. Lime ($CaCO_3$) was added to adjust the soil pH to 5.5. Intortum was established from seed which had been inoculated with the appropriate Rhizobium strain in Experiment II, but not in Experiment I. The seeds of intortum and the grass cuttings were planted in 30 cm diameter black plastic pots (9.5 liters) containing 8 kg of air-dry soil. The three forages were over planted and then thinned to 16 intortum or eight grass plants in each pot. The plants were grown for 140 days. After Experiment I was harvested, the soil was screened through a 0.5 cm sieve to remove as many roots as possible from the soil in the pots. For Experiment II, the soil was replaced in the pots and N, P, K, Zn, Mo and $CaCO_3$ were added to the soil at the same rate as in Experiment I. Seeds of intortum and cuttings of bigalta and greenalta limpograsses were planted in various combinations in the soil from Experiment I (Table I). As in Experiment I, the three forages were over planted and then eight plants were selected in each pot.

D. Management and data collection

Water was supplied during the whole period to maintain growth

but at a rate designed to avoid leaching. In Experiment I, the forages were harvested after being grown for 30, 90 and 140 days after planting. Plant canopy height (from soil level to the top layer of leaves of the three crops) was measured at 81 and 140 days after planting and the tiller number of the grasses was counted prior to taking the second and third cuttings. In Experiment II, plant height (from the soil level to the stem apex) of the three crops was measured at 27, 31, 40 and 77 days after planting to provide a measure of the growth rates. The pots were harvested and dry matter yields determined at 77 and 152 days after planting. The dried tops of the three replications of intortum were combined and analyzed for K, P, Ca, Mg, Na, Zn, Fe, Mn and Cu by Atomic Absorption Spectrophotometry. Total nitrogen content was measured using the Kjeldahl procedure.

3.3 Results and Discussion

A. Productivity and growth characteristics of *H. altissima* and *D. intortum* in Experiment I

The purpose of Experiment I was to produce soil which contained plant residues of intortum and bigalta and greenalta limpograsses to determine whether the inhibition of intortum growth observed in the field could be reproduced in pot studies. The results of Experiment I also provided data on the growth of the three forages at two levels of soil phosphorus. Total shoot fresh weights of bigalta and greenalta limpograsses and intortum for the three harvests at the 0.04 level of soil phosphorus were 69.8, 84.9 and 55.7 percent respectively of the yields at the high level of soil phosphorus (Table II). The effects of phosphorus on root yields were variable. Fresh plant weights of

Table II. The average fresh weights of tops and roots (mean of three replications) for Desmodium intortum and bigalta and greenalta limpograsses grown at two levels of phosphorus

Soil P, ppm	Species	Top Production (g/pot) 1st Cutting	2nd Cutting	3rd Cutting	Root Production (g/pot)
0.3	Bigalta	115.2 a+	324.3 a	40.0 d	186.9 d
	Greenalta	49.5 c	270.0 b	183.3 b	449.6 a
	Intortum	--	138.6 cd	233.8 a	299.1 b
0.04	Bigalta	70.6 b	177.6 c	86.5 c	194.7 cd
	Greenalta	27.1 d	123.4 d	138.3 b	286.9 b
	Intortum	--	58.8 e	148.6 b	268.3 bc

+ Means within columns followed by the same letter are not significantly different ($p = 0.05$) by Duncan's multiple-range test

bigalta limpograss were greater than greenalta limpograss at the first and second cuttings at both levels of soil phosphorus. However, at the third cutting the yield of bigalta limpograss was lower than that of greenalta limpograss (Table II), probably because bigalta removed more nutrients than greenalta limpograss at the first two harvests. The total yields of tops of bigalta and greenalta limpograss in each pot for three harvests were not significantly different for the 0.3 ppm phosphorus treatment, but bigalta yielded significantly more than greenalta at the low level of phosphorus. Root production was highest in greenalta, second in desmodium, and lowest in bigalta limpograss at both levels of phosphorus (Table II).

Tiller number and canopy height are important characteristics in competition and could account in part for the reduced growth of intortum observed in the field. Canopy height of bigalta was significantly greater than that of greenalta limpograss and intortum at 81 days after planting in both levels of phosphorus but heights of the two grasses were similar at the third harvest. However, bigalta limpograss had fewer tillers per pot than did greenalta limpograss (Table III).

The pH values of the soil at the end of Experiment I were about 1 unit below the pH established at the outset (Table IV). The pH variation among all treatments was small.

B. Productivity and growth characteristics of intortum grown alone and in mixtures in Experiment II

1. Effect of root residue treatments on the height of intortum

In general, the height of intortum in Experiment II was greatest in the presence of greenalta residue, next in the intortum

Table III. The average canopy height and tiller number (mean of three replications) for bigalta and greenalta limpograsses and Desmodium intortum at two levels of phosphorus

Soil P, ppm	Species	Canopy Height (cm) at Days After Planting		Tiller Number at Days After Planting	
		81 Days	140 Days	60 Days	140 Days
0.3	Bigalta	91.3 a[+]	50.2 ab	26.8 c	24.2 c
	Greenalta	29.6 cd	48.9 ab	71.3 a	112.5 a
	Intortum	36.3 c	27.3 c	--	--
0.04	Bigalta	75.5 b	63.5 a	24.8 c	34.0 c
	Greenalta	40.9 c	48.0 b	37.5 b	75.2 b
	Intortum	22.3 d	20.4 c	--	--

[+] Means in columns followed by the same letter are not significantly different (p = 0.05) by Duncan's multiple-range test

Table IV. Mean soil pH after 140 days of growth of bigalta and greenalta limpograss and Desmodium intortum

Soil P, ppm	pH Value*		
	Bigalta	Greenalta	Intortum
0.3	4.8 ± 0.1**	4.7 ± 0.0	4.6 ± 0.0
0.04	4.7 ± 0.1	4.5 ± 0.1	4.5 ± 0.1

* Average of three replications

** s = standard deviation

treatment and lowest in the bigalta treatment (Table III). The height of intortum was reduced significantly when grown alone or in mixtures in soil in which bigalta limpograss had been grown previously (Figures 2, 3, 4 and 5). The average canopy height of intortum in Experiment I where no other crop residue was present was about 36 cm and 23 cm in 0.3 and 0.04 ppm P, respectively, after 81 days of growth. Plant height in Experiment II was measured after 77 days so the time periods are approximately comparable. Canopy height was measured from the soil level to the top layer of leaves and thus does not correspond exactly to the plant height measurement which was made from the soil level to the stem apex. However, large deviations between canopy height and plant height would suggest an effect due to treatment. A comparison of the data of Experiment I with that of Experiment II strongly suggests that the growth of intortum was not inhibited by residues of greenalta and intortum, and may have been slightly stimulated by them. Growth in height of intortum in the presence of bigalta residue was inhibited in all cases. The nutrients added in Experiment II were the same as in Experiment I. Therefore, the nutrients removed by the previous crop or immobilized by microorganisms would not likely cause nutrient deficiency. The results indicate that the soil in which bigalta had been grown contained growth inhibitors.

The increase in plant height, like other growth parameters such as dry weight and leaf area, tends to be exponential with time (Radford, 1967 and Hughes and Freeman, 1967) and thus can be described by an equation of the form $Y = ax^b$ or $\log Y = \log a + b \log x$. From the results of calculated coefficients of regression (Table V), it is concluded the growth rates of intortum in all treatments over the

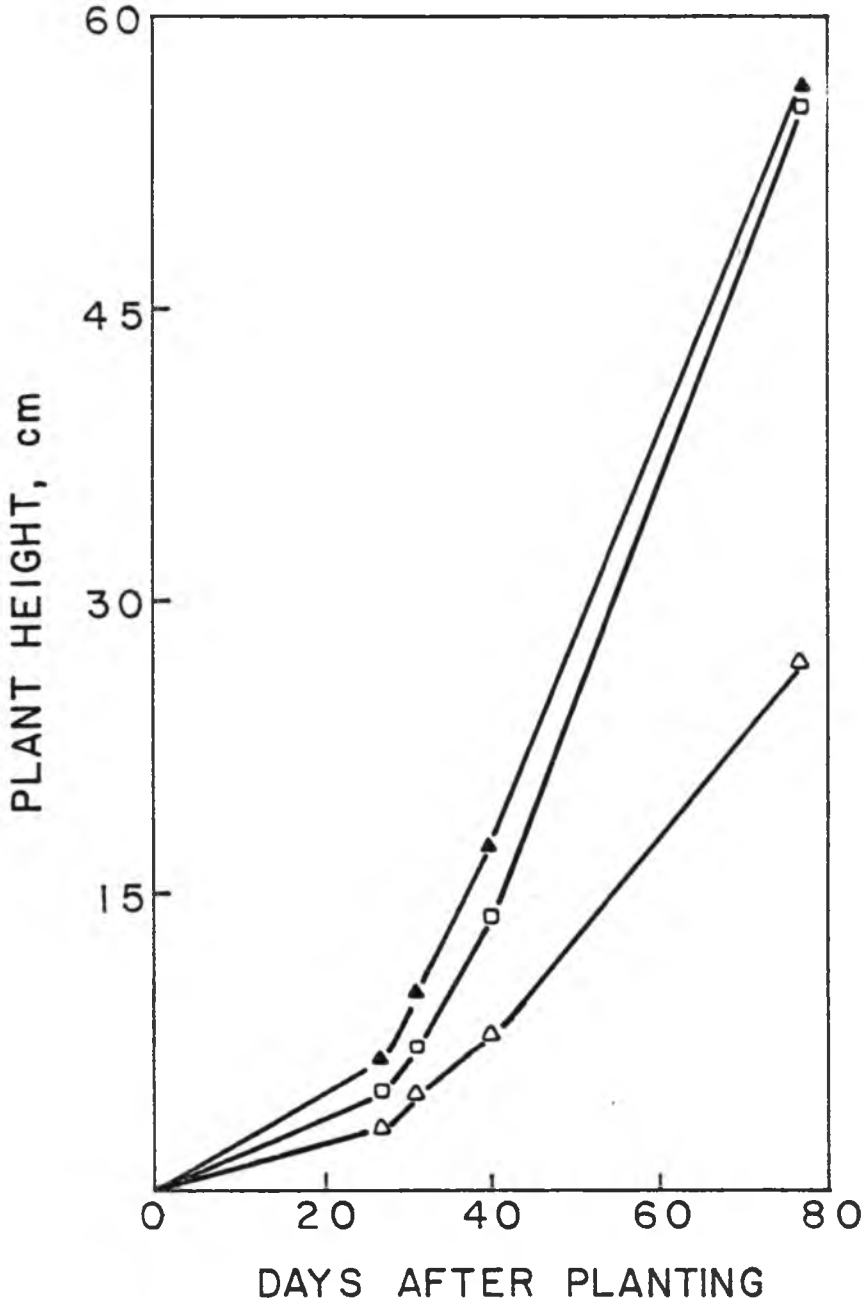

Figure 2. The height of D. intortum alone in soil containing 0.3 ppm phosphorus and residue from bigalta limpograss (△) greenalta limpograss (▲) or intortum (□).

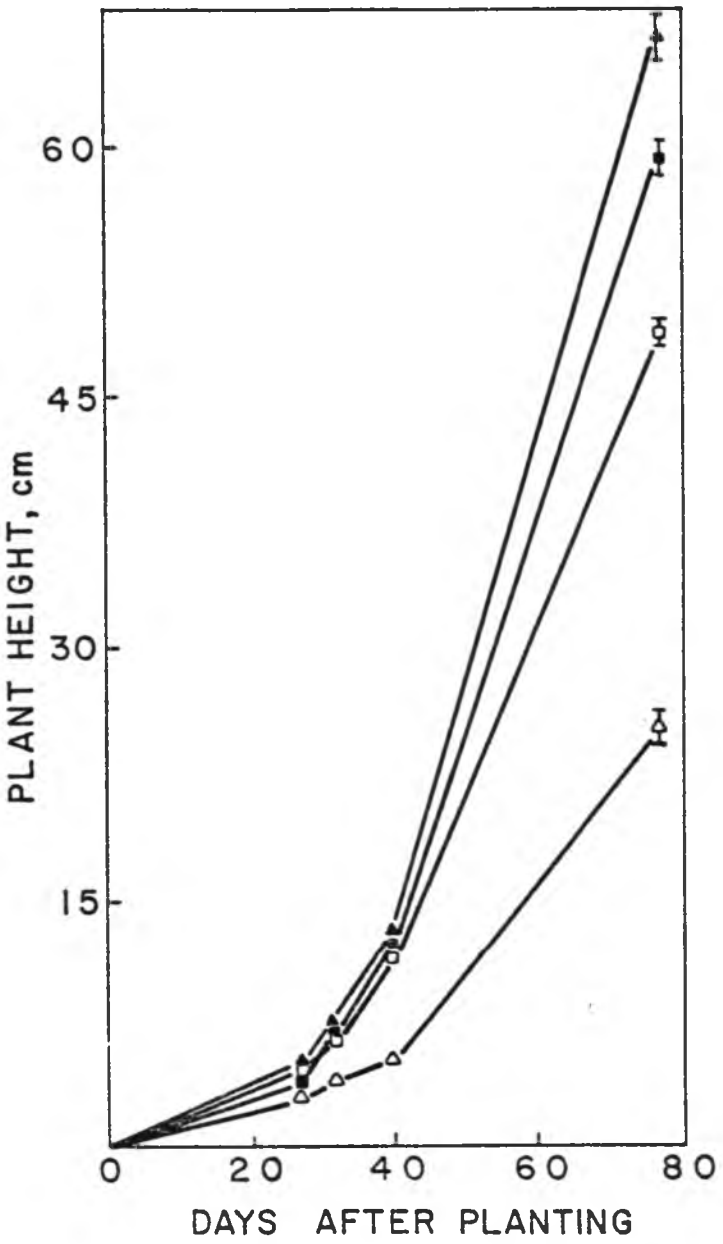

Figure 3. The height of Desmodium intortum when grown in a mixture with greenalta limpograss in soils containing residues of intortum (■) and greenalta limpograss (▲), and in a mixture with bigalta limpograss in soils containing residues of intortum (□) and bigalta limpograss (△). All soils contained 0.3 ppm phosphorus.

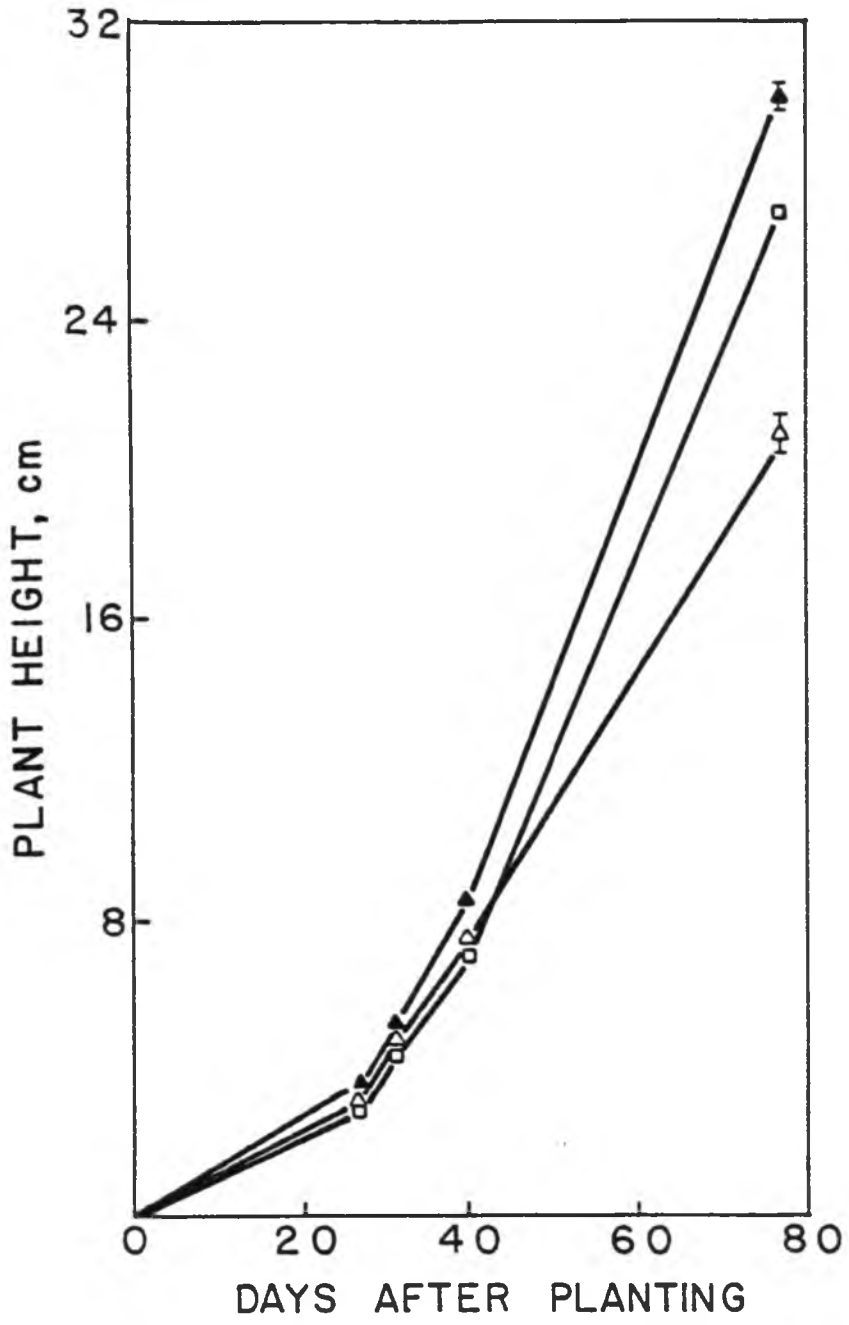

Figure 4. The height of D. intortum grown in soil containing 0.04 ppm phosphorus and residue from bigalta limpograss (△), greenalta limpograss (▲) or intortum (□).

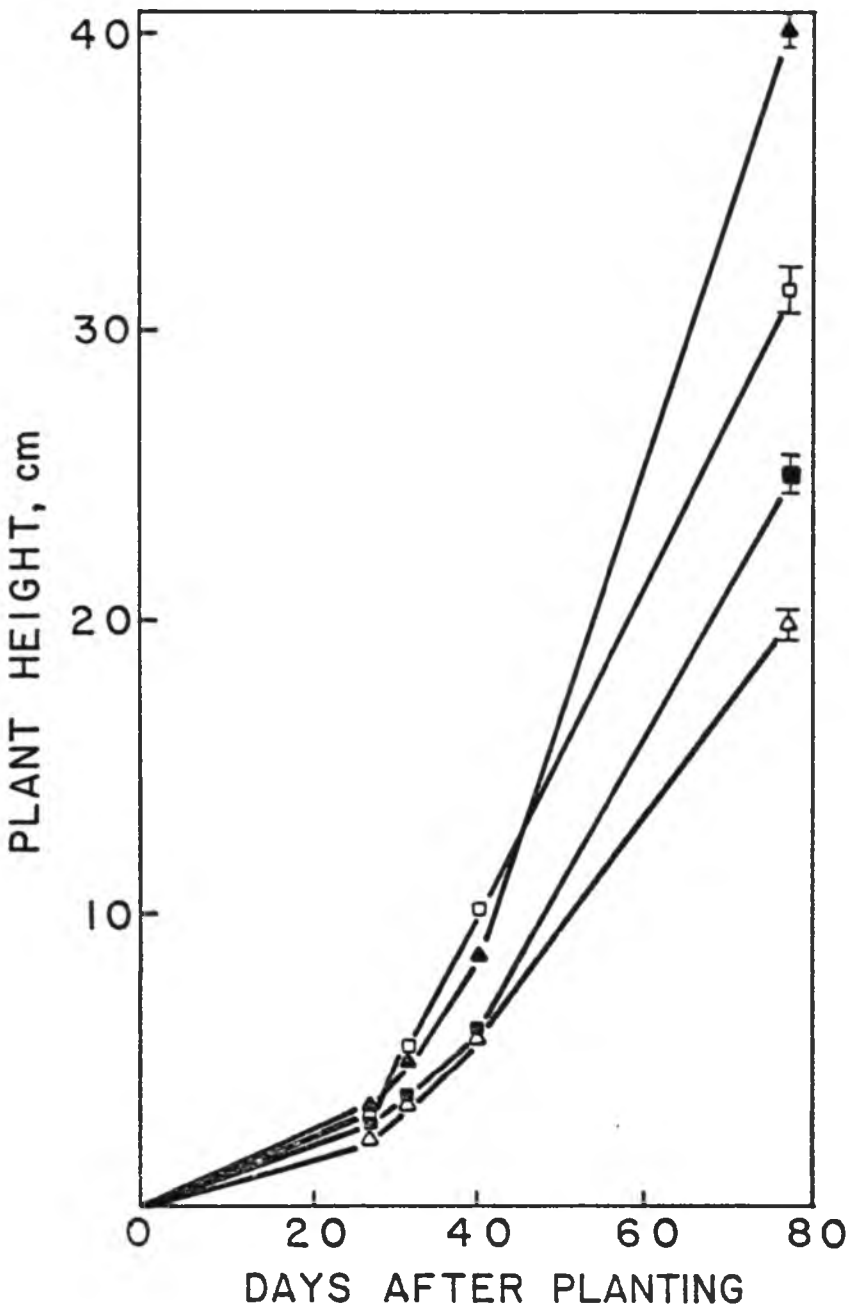

Figure 5. The height of Desmodium intortum when grown in a mixture with greenalta limpograss in soils containing residues of intortum (■) and greenalta limpograss (▲), and in a mixture with bigalta limpograss in soils containing residues of intortum (□) and bigalta limpograss (△). All soils contained 0.04 ppm phosphorus.

Table V. Regression coefficients ($Y = ax^b$) for plant height (Y) of D. intortum versus days (x) from 27 to 77 days after planting for several soil treatments

Crop+	Soil Treatment		Intercept	Coefficient of Regression	Coefficent of Correlation
	P, ppm	Residue++			
D	0.3	D	0.0025	2.310	0.97**
D		B	0.0058	1.913	0.85**
D		G	0.0110	1.975	0.99**
D+G		D	0.0046	2.158	0.91**
D+B		D	0.0115	1.905	0.88**
D+G		G	0.0012	2.516	0.96**
D+B		B	0.0053	1.889	0.85**
D	0.04	D	0.0032	2.055	0.87**
D		B	0.0082	1.811	0.91**
D		G	0.0033	2.121	0.94**
D+G		D	0.0035	2.027	0.91**
D+B		D	0.0030	2.135	0.86**
D+G		G	0.0037	2.129	0.96**
D+B		B	0.0038	1.934	0.86**

** Significant at 1% levels of probability

+ Desmodium (D) plants were planted alone and in mixtures with bigalta (D+B) or greenalta (D+G) limpograss

++ Soil was used which desmodium (D), bigalta (B) or greenalta (G) had been grown previously

50 day period from day 27 to day 77 were not significantly different, i.e. the slopes of height vs. time were the same. Since the slopes were not different, the data suggests that the retardation of intortum growth by bigalta limpograss residue occurs during germination or in the early growth stage (the first 27 days after planting in this experiment). However, in all cases the regression coefficients for growth of intortum in intortum residue were larger than coefficients for growth of intortum in bigalta residue. Thus it is possible that bigalta residue affected growth for more than the first 27 days after planting. Common b values (slope of the line) for the different treatments after 27 days would suggest that intortum became resistant to the allelopathic chemicals or that the chemicals were gradually degraded with time so that after 27 days, levels were no longer inhibitory to the growth of intortum.

2. Effect of root residue treatment on dry matter yield

Dry matter yields of intortum were significantly greater at the 0.3 ppm phosphorus level than at 0.04 ppm P for the first cutting, but not for the second one, and residue (R) effects were also highly significant (Table VI). However, because of a significant P x R interaction the various treatment effects will be discussed separately. Dry matter yields of intortum at the first cutting grown alone in soils containing plant residues decreased in the order, greenalta, intortum, and bigalta (Table VII). No differences between the treatments were present at 0.04 ppm P, thus the significant P x R interaction. The dry matter yield of intortum grown with greenalta in the greenalta residue treatment was highest at the first cutting. The yields of intortum whether grown alone or in a mixture with the two grasses in

Table VI. Analysis of variance of mean dry weights of the tops of Desmodium intortum grown in soil in which bigalta, greenalta and intortum had been grown previously and which contained 0.3 and 0.04 ppm phosphorus

Source of Variation	Degrees of Freedom	Mean Squares	
		1st Cutting	2nd Cutting
Replications	2	0.045 N.S.	0.002 N.S.
Treatments			
Phosphorus (P)	1	31.130**	4.774 N.S.
Residue (R)	6	4.757**	36.677**
PxR	6	2.080*	12.438**
Error	26	0.739	1.917

N.S. Not significant

*, ** Significant at 5% and 1% levels of probability, respectively

Table VII. Average dry weight of tops (mean of three replications) for Desmodium intortum grown in soil in which intortum and bigalta and greenalta limpograsses had been grown previously and which contained 0.3 and 0.04 ppm phosphorus

Crop[+]	Residue[++]	Average Dry Weight Per Plant (g) 1st Cutting	2nd Cutting
0.3 ppm P			
D	D	3.0 ab[§]	8.5 abc[§§]
D	G	3.9 ab	7.1 bcd
D	B	0.9 c	0.2 g
D+G	D	3.7 ab	8.6 abc
D+G	G	4.4 a	9.7 a
D+B	D	2.4 b	5.0 def
D+B	B	0.8 c	3.0 f
0.04 ppm P			
D	D	1.0 c	4.7 ef
D	G	1.2 c	9.3 ab
D	B	0.6 c	3.5 f
D+G	D	0.9 c	4.8 ef
D+G	G	1.5 c	6.8 cde
D+B	D	1.4 c	5.2 de
D+B	B	0.5 c	3.2 f

[+] Intortum (D) plants were planted alone and in mixtures with bigalta (D+B) or greenalta (D+T)

[++] Soil was used in which intortum (D), bigalta (B), or greenalta (G) had been grown previously

[§],[§§] Data within columns followed by the same letter are not significantly different (P = 0.05) by Duncan's multiple-range test

the intortum residue were not significantly different. Thus, there was no evidence for biochemical suppression or stimulation of growth of intortum in the presence of either greenalta or bigalta limpograsses. The intortum yield when grown in a mixture with bigalta in the presence of bigalta residue was significantly lower than all other treatments. The data show that the growth inhibition of intortum grown together with bigalta was due to the presence of the bigalta limpograss residue and not to the bigalta plants themselves.

The pattern of growth inhibition at the second cutting was similar to that obtained at the first cutting, but yields were somewhat greater, probably due to the fact that the plants were well established. However, the yield of intortum grown alone in the presence of bigalta residue was much lower than at the first cutting because only three of 24 intortum plants in the three replicates at 0.3 ppm P survived. Sixteen of the 24 plants in the 0.04 ppm P treatment survived. Where intortum was grown in a mixture with bigalta in the presence of bigalta residue, 13 of 24 intortum plants survived at the 0.3 ppm P level, but 21 of 24 plants survived at 0.04 ppm P. The higher survival rate of intortum when grown with bigalta than when grown alone in the presence of bigalta residue may be due to absorption of some of the bigalta residue toxins by the grass. The higher survival of intortum at 0.04 ppm P than at 0.3 ppm P may be due to the fact that root + top yields of bigalta grown at 0.04 ppm P were lower than when grown at 0.3 ppm P. The lower yield could result in the production of a smaller quantity of toxins. The growth and vigor of all intortum plants was low in the presence of bigalta residue and the low vigor could have been responsible for their failure to

survive after the first harvest. Plants of low vigor would also be more sensitive to residue toxins in the soil. Intortum growth in intortum residue was not inhibited by bigalta at the first cutting but growth was inhibited significantly at the second cutting; intortum grown with bigalta yielded significantly less than intortum grown with greenalta (Table VII). The toxicity of the bigalta treatment observed at the second harvest could be due to the release of inhibitors from roots which would die off after the first harvest and from exudates from the living roots which might accumulate in the soil.

After the first cutting in Experiment II, some intortum plants growing in soil in which bigalta limpograss had been previously grown started to die, while other seedlings survived and appeared to grow normally. Rotar (1970), in a study of the morphological characteristics of desmodium lines, reported that a large pool of genetic variability was present in D. intortum. The differences in growth made by intortum seedlings in the presence of bigalta limpograss residue suggests that it might be possible to develop a bigalta limpograss tolerant line of intortum through breeding and selection.

Various conditions, such as water stress (Vancura, 1964), wilting (Katznelson et al., 1954), high light intensity and high temperature (Rovira, 1959) have been found to increase the production of root exudates by many plants. Lehman and Rice (1972) showed that nutrient deficiency (N, K and S) increased the amount of toxic substances (chlorogenic acid and scopolin) in sunflower plants. However, the amount of inhibitory substances released to the soil from plant roots by exudation when the plants were stressed for nutrients has not yet been established.

In general, the dry matter production of bigalta limpograss was greater than that of greenalta limpograss (Table VIII). There was no effect of residue treatment on the dry matter yields of tops of bigalta or greenalta limpograss at 0.3 ppm soil P. However, in soil containing 0.04 ppm P and intortum residue, dry matter production by bigalta in a mixture with intortum was significantly higher than all other treatments (Table VIII). It is possible that the legume contributed nitrogen to the adjacent grass which increased its growth (Whitney and Kanehiro, 1967).

3. Effect of treatments on soil pH and nutrient contents of plants in Experiment II

Soil pH was readjusted to 5.5 by liming ($CaCO_3$) at the beginning of Experiment II and soil pH values were measured 35 days after planting to assess the effect of soil treatments on soil pH. Soil pH values were about one unit below the pH established at the beginning of the experiment in all treatments. Variation in the pH was small ranging from 4.5 to 4.9 (Table IX). The observed retardation in growth of intortum in which bigalta had been grown previously apparently was not due to differences in soil pH.

Since the variation in growth in the different treatments might have been caused by differences in amounts of available mineral nutrients, the tops of all plants were analyzed for nitrogen, phosphorus, potassium, calcium, magnesium, sodium, manganese, iron, copper and zinc. The range of concentration of each mineral in tops of intortum and grasses in all treatments was very small (Table X). Significant differences in the P, K, Mg, Na and Mn contents in the tops of intortum were obtained between the high and low phosphorus treatments;

Table VIII. Average dry weight of tops (mean of three replications) for bigalta (B) and greenalta (G) limpograsses grown alone and in mixtures with Desmodium intortum (D) in soil containing two levels of phosphorus and residues of bigalta, greenalta or intortum

Treatment		Dry Weight (gram)			
		0.3 ppm P Soil		0.04 ppm P Soil	
Crop	Residue	1st Cutting	2nd Cutting	1st Cutting	2nd Cutting
		Bigalta			
B	B	4.0a+	10.6a	1.1b	3.5b
B	D	5.6a	10.8a	1.5b	4.8b
B+D	B	2.7a	11.3a	1.2b	4.7b
B+D	D	4.9a	13.0a	3.3a	10.6a
		Greenalta			
G	G	2.1a+	6.0a	0.5a	1.9a
G	D	1.4a	6.2a	1.4a	6.3a
G+D	G	1.7a	8.5a	0.8a	5.3a
G+D	D	2.5a	11.4a	1.3a	6.8a

+ Data within columns for the same grass cultivar followed by the same letter are not significantly different (P = 0.05) by Duncan's multiple-range test

Table IX. Soil pH (mean of three replications) of soil containing crop residue after 35 days of growth of Desmodium intortum or mixtures of intortum with bigalta or greenalta limpograss

Soil P, ppm	Average Soil pH						
	Intortum			Intortum + Bigalta		Intortum + Greenalta	
	D+	B	G	D	B	D	G
0.3	4.7 ± 0.1++	4.7 ± 0.2	4.9 ± 0.1	4.8 ± 0.1	4.6 ± 0.2	4.8 ± 0.2	4.9 ± 0.3
0.04	4.6 ± 0.1	4.5 ± 0.1	4.9 ± 0.0	4.9 ± 0.1	4.6 ± 0.1	4.8 ± 0.1	4.8 ± 0.1

+ Soil in which intortum (D) and bigalta (B), or greenalta (G) limpograss had been grown previously

++ Values are ± one standard deviation

Table X. Mean of mineral nutrient contents of tops of D. intortum, and bigalta and greenalta limpograss grown at two levels of soil phosphorus

Soil P Level ppm	N	P	K	Ca	Mg	Na	Mn	Fe	Cu	Zn
			%				ppm			
					D. intortum					
0.3	3.25 ± 0.17+	0.21 ± 0.03	1.42 ± 0.17	2.21 ± 0.21	0.46 ± 0.05	0.04 ± 0.00	150.29 ± 29.84	195.86 ± 25.80	17.43 ± 5.88	58.45 ± 10.00
0.04	3.47 ± 0.24	0.16 ± 0.02	1.78 ± 0.24	2.23 ± 0.15	0.40 ± 0.02	0.03 ± 0.01	175.5 ± 7.91	174.43 ± 37.41	18.0 ± 6.19	52.45 ± 3.51
0.3/0.04	N.S.	**	**	N.S.	**	*	*	N.S.	N.S	N.S.
					Bigalta limpograss					
0.3	1.89 ± 0.18	0.16 ± 0.01	1.42 ± 0.31	0.92 ± 0.21	0.44 ± 0.1	0.34 ± 0.08	144.5 ± 5.97	233.5 ± 36.78	12.00 ± 1.63	61.75 ± 12.28
0.04	1.91 ± 0.13	0.13 ± 0.01	1.98 ± 0.24	0.82 ± 0.11	0.34 ± 0.04	0.34 ± 0.04	164.75 ± 18.28	216.75 ± 43.4	22.00 ± 1.63	57.50 ± 3.42
0.3/0.04	N.S.	**	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	**	N.S.
					Greenalta limpograss					
0.3	1.59 ± 0.05	0.15 ± 0.01	1.51 ± 0.12	0.51 ± 0.08	0.22 ± 0.03	0.29 ± 0.05	104.25 ± 2.06	203.00 ± 46.35	11.75 ± 0.50	63.75 ± 10.14
0.04	1.57 ± 0.08	0.13 ± 0.01	1.78 ± 0.15	0.47 ± 0.07	0.21 ± 0.01	0.21 ± 0.01	120.50 ± 7.55	152.5 ± 25.01	15.50 ± 1.91	49.25 ± 4.57
0.3/0.04	N.S.	*	N.S.	N.S.	N.S.	*	**	N.S.	**	*

+ Values are ± one standard deviation

*,** Significance at P = 0.05 and 0.01, respectively as determined by Student's t test

N.S. Not significant

P, K and Cu contents in the tops of bigalta and P, K, Na, Mn, Cu and Zn contents in the tops of greenalta limpograss were also significantly influenced by the phosphorus treatment. The potassium contents in intortum, and bigalta and greenalta limpograss generally were lower at the high level of P, perhaps due to the higher Ca from the application of monocalcium phosphate. However, no difference in calcium contents was obtained and differences in Mg were small or nonexistent suggesting that any Ca differential due to the application of $Ca(H_2PO_4)_2$ was very small.

The critical values of K and P in D. intortum were reported to be 0.8% and 0.22%, respectively (Andrew and Robbins, 1969). The data of Table X indicate no deficiency of K in any treatment. The P levels in the tops of intortum were below normal or marginal for normal intortum growth. The low phosphorus concentration in intortum tops in the high level of P may have been due to inhibition of P uptake by the inhibitory substances in the crop residue which remained in the soil. Newman and Miller (1977) found that among the species Trifolium repens, Anthoxanthum odoratum and Lolium perenne, which commonly are grown together, root exudates from one plant can influence phosphorus uptake by another. McClure et al. (1978) also found that ferulic acid in low concentration (0.5 and 1.0 mM) inhibited the absorption of phosphate by three soybean varieties.

3.4 Summary and Conclusions

Desmodium intortum seeds were planted alone and in mixtures with Hemarthria altissima, bigalta (a tetraploid cultivar) or greenalta (a diploid cultivar) limpograsses in fertilized soils in which intortum

and bigalta and greenalta limpograsses had been grown previously. The effects of crop residue in soil on the growth and mineral nutrient contents of plants and on soil pH were investigated.

1. The height of intortum grown alone and in a mixture with bigalta limpograss was reduced significantly relative to all other treatments in both 0.3 ppm and 0.04 ppm phoshporus when grown in soil in which bigalta limpograss had been grown previously. The data suggests that much of the retardation of intortum growth by bigalta limpograss residues occurs during germination or in the early growth stage.

2. Dry matter yields of the first and second cuttings of intortum were significantly reduced when grown in soil containing bigalta residue and 0.3 ppm phosphorus. Intortum growth was not reduced in the 0.04 ppm phosphorus treatment. In general, the yields of intortum in soil containing greenalta limpograss residue were higher than in soil containing intortum residue. The yields of intortum grown alone and in mixtures with bigalta limpograss in the bigalta residue treatments were not significantly different.

3. Dry matter yields of tops of bigalta and greenalta limpograsses generally were not significantly different in all treatments.

4. Variation of the soil pH values and mineral nutrient contents of tops of intortum in all treatments was small. It is concluded that the retardation of the growth of intortum grown in the soil in which bigalta limpograss had been grown previously was due to allelopathic substances in the root residues rather than to competition for environmental factors.

CHAPTER 4

EFFECTS OF ROOT EXUDATES OF *HEMARTHRIA ALTISSIMA* AND *DESMODIUM INTORTUM* ON THE GROWTH, NODULATION AND NITROGEN FIXATION OF *D. INTORTUM*

4.1 Introduction

The exudation of chemicals from roots is a major source of growth inhibiting substance. By definition, root exudates are substances released into the surrounding medium by healthy, intact plant roots (Rovira, 1969). The term root exudate is used in a broad sense here to refer to organic substances which are exuded from the root by any mechanism. Among the many factors which can influence the quantity and quality of root exudates are species, microorganisms, temperature, light intensity, nutrient and water stress, etc. (Rovira, 1969; Hale *et al.*, 1971).

The results reported previously showed that root residues of bigalta limpograss in soil retarded the growth of intortum. However, a soil system is a very complex mixture of nonliving inorganic and organic materials which interact with the soil microflora. In addition, plant-plant and plant-microbial interactions exist in the soil (Figure 1). It was not possible to separate the direct (plant-plant interactions) and indirect (plant-microbial-plant interactions) effects of root exudates and/or residues in the soil experiments described previously. A simplified system must be used in order to demonstrate chemical interactions between plants.

Most root exudate studies have been conducted in solution culture for convenience in sampling and analysis, but to investigate allelopathy due to root exudation it is necessary to do experiments with plants

grown in soil under natural conditions. However, it is known that soil components can adsorb some allelopathic substances, such as phenolic acids (Huang, et al., 1977). It is therefore difficult to establish an efficient system to observe the effects of root exudates from a donor plant on the growth of an acceptor plant in a soil experiment. Vermiculite is a clay mineral found in soil and the internal surfaces of vermiculite do not retain significant amounts of phenolic acids. A comparison of phenolic acid retention by vermiculite and other clay minerals showed the retention by vermiculite to be far lower than kaolinite, illite and noncrystalline hydroxy-Al and Fe components (Huang, et al., 1977). Therefore, hydroponic-vermiculite culture is an ideal system to study the role of root exudates in allelopathy because organic substances exuded from a donor plant can be carried in solution and easily transferred to the acceptor. The purpose of the experiment described in the following sections was to observe the effects of root exudates from bigalta and greenalta limpograsses and intortum on the growth, nodulation and nodule acetylene reduction rate of intortum in different nutrient solutions.

4.2 Materials and Methods

A. Effect of root exudates from three donor plants on intortum seedling growth, nodulation and acetylene reduction

The nutrient-vermiculite culture experiments were designed to eliminate competition between plants for nutrients, light and water. The experiments were set up so that the exudate produced by a previously established donor plant could be received by an acceptor plant. Because the total production of plant material is frequently

a function of the supply of mineral nutrients (Milthorpe and Moorby, 1974) and the amount of root exudates in a pot depends on the quantity and quality of roots and tops produced, three different nutrient solutions were used in the experiments described here (Table XI). Hoagland's solution contains more nutrients (e.g. N, P, K, Ca and Mg) than nutrient solution A (Table XI) so the production of plants in Hoagland's solution would be expected to be higher than with nutrient solution A. The N-free nutrient solution was included to observe the effect of root exudates on nodulation of intortum since Hoagland's solution, which contains 200 ppm N, would likely inhibit nodule formation. The production of grass would be lower than the production of legume in N-free nutrient solution because of the limited N supply in the solution.

Two kinds of pot designs were used in the experiments, one with a divider and the other without, to compare growth with and without direct contact between the root systems of intortum and the two grasses (Figure 6). A layer of 1.5 cm diameter crushed basalt rock was placed on the bottom of the pot for drainage and then the pot was filled with vermiculite. A randomized complete block design was used with three replications. Four cuttings, each of bigalta, greenalta and intortum were treated with 5% chlorox and established in one half of each glazed pot (30 x 30 cm, 8 liter volume) and grown for 44 days. Eight germinated seeds of intortum were inoculated with the appropriate Rhizobium bacteria (Nitragin Co., Inc., EL inoculant) and planted on the side of the pot opposite the donor plants. It was later thought that autoxicity might influence the growth of intortum so the control set in the experiments using Hoagland's nutrient solution and the

Table XI. Composition of the three nutrient solutions used to culture plants hydroponically for the study of allelopathy

Salt	Milligrams Nutrient Per Liter of Solution**		
	Hoagland's	Nitrogen-free	Nutrient solution A
Macronutrients:			
KH_2PO_4	136.1	136.1	68.0
KNO_3	505.5	--	--
$Ca(NO_3)_2$	820.5	--	--
$MgSO_4$	240.8	240.8	202.8
K_2SO_4	--	440.0	87.1
$CaSO_4 \cdot 2H_2O$	--	870.0	--
NH_4NO_3	--	--	200.1
$Ca\ Cl_2$	--	--	55.4
Micronutrients:			
H_3BO_3	2.86	2.86	0.72
$MnCl \cdot 4H_2O$	1.81	1.81	0.45
$ZnSO_4 \cdot 7H_2O$	0.22	0.22	0.055
$CuSO_4 \cdot 5H_2O$	0.08	0.08	0.02
$H_2MoO_4 \cdot H_2O$	0.02	0.02	0.005
Fe*	5.025	5.025	5.025

* Geigy-Sequestrene Fe 330 containing 10% iron as metal

** pH = 5.5

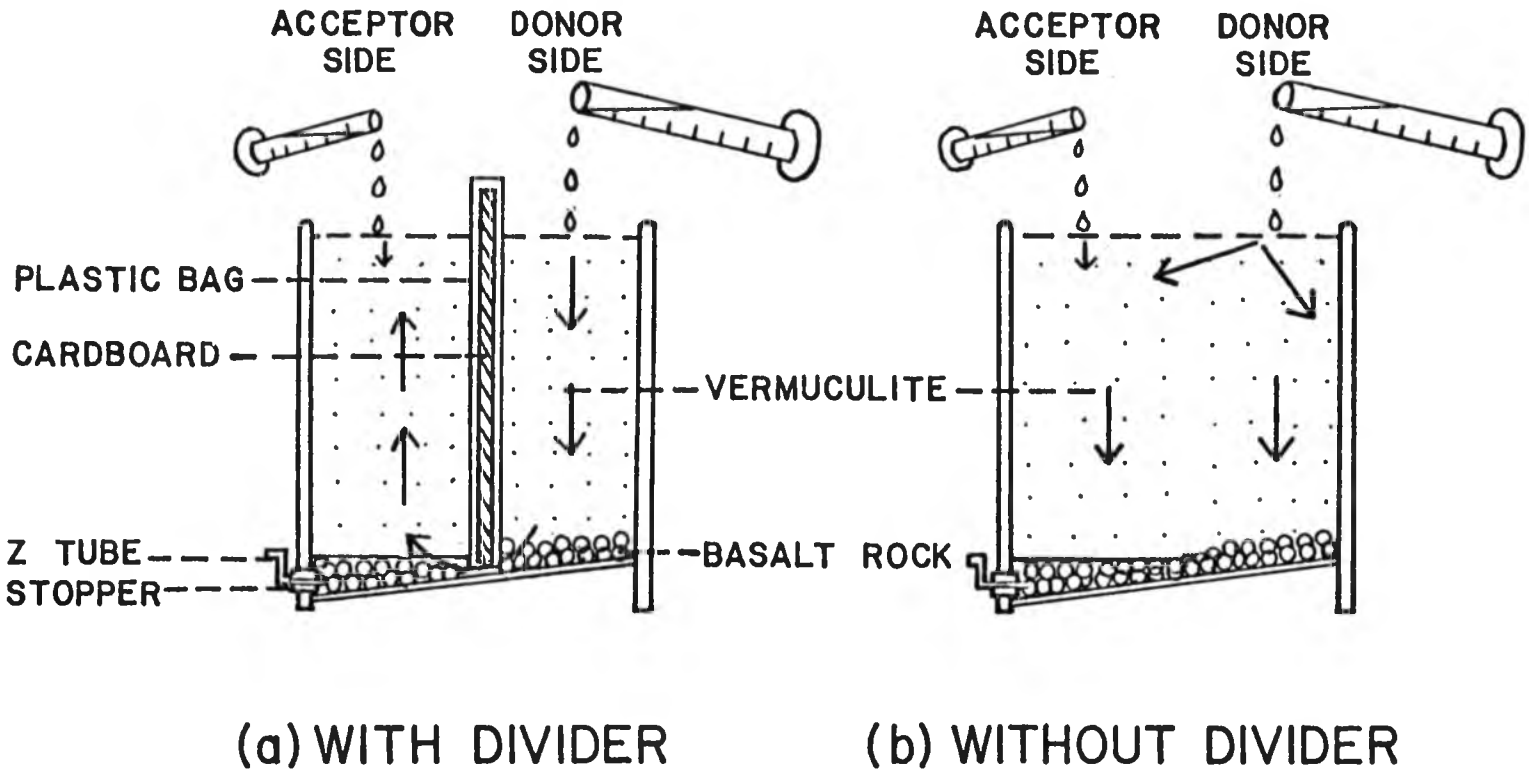

Figure 6. Pot designs and watering procedure used in hydroponic studies of allelopathy.

nitrogen-free nutrient solution contained only intortum seedlings. Pre-germinated intortum seeds were planted on the donor side of the control pot in the experiment using nutrient solution A. The plants were irrigated by applying nutrient solution and water to the donor side of the pot whether the divider was present or absent. Thus, the flow of nutrient solution was through the root of donor into the acceptor side in the pot. Each pot was watered with 300 ml of nutrient solution every morning. Distilled water was also added about 4 p.m. in the afternoon as needed to keep the vermiculite moist. An additional 200 ml of nutrient solution was also applied every two days at about 4 p.m. to the acceptor plants to assure that growth of intortum would not be retarded by a deficiency or imbalance of nutrients. The donor grasses in N-free nutrient-vermiculite culture were sprayed with 2% urea once every three days to supply N for their growth. Samples were collected from the bottom of each pot every four days to check the pH of nutrient solution. Dilute HCl was added to maintain the pH near 5.5. Plant height of intortum was measured at 30, 37 and 55 days after planting for Hoagland's nutrient-vermiculite culture; at 37, 55 and 61 days after planting for the nitrogen-free nutrient solution culture; and at 44, 52, 66 and 75 days for the nutrient solution culture; and at 44, 52, 66 and 75 days for the nutrient solution A-vermiculite culture. The fresh weight of tops and roots of the acceptor and donor plants in each pot was measured at harvest time which was on the last day that plant height was measured. Mineral contents of the tops of the acceptor were analyzed by the X-ray Quantometer.

Acetylene reduction by the intortum root system in each pot from Hoagland's and N-free nutrient-vermiculite cultures was measured by the procedure of Koch and Evans (1966) and Stewart et al. (1967). Roots were harvested between 11:00 a.m. and 1:00 p.m. and placed in 520 ml bottles stoppered with serum caps. Fifty ml of acetylene was added and the bottle was incubated at room temperature (about 25°C) for one hour. Ethylene production was measured with a gas chromatograph (Varian Aerograph Model 600-D) equipped with a hydrogen flame detector using a 0.5 ml gas sample. The column contained a stationary phase of 100-120 mesh Porapak N (Waters Association, Incorporated). The column temperature was 60°C and the nitrogen carrier gas flow rate was 20 ml per minute. Gases were identified by their retention times. Acetylene reduction, an indicator of nitrogenase activity, was calculated as μ mole ethylene per hour per pot and per gram of nodule fresh weight. Nodule number, fresh nodule weight and total root weight were determined after the acetylene reduction measurement.

B. Effects of the root exudates from three donor plants on the growth of intortum cuttings

Cuttings of intortum are sometimes used as a source of planting material in the field, especially where uniform material is desired. However, the effect of root exudates on different types of planting materials is not known. In this experiment, bigalta and greenalta limpograsses and intortum were used as the donor plants with intortum cuttings as the acceptor plant. A randomized complete block design was used with three replications. The experiment was carried out in a greenhouse. Two healthy cuttings of each donor plant were

treated with 5% chlorox and established first on the donor side of the pots (three liter capacity) which had a one liter plastic bag filled with perlite in the center of the pot (Figure 7). Thirty-five days after planting the donor plant tops were cut off to prevent the donor from competing with the acceptor for light. The central plastic bag was replaced with a new plastic bag which was perforated and filled with perlite. One healthy unrooted cutting of intortum was planted in the central plastic bag as an acceptor. No donor plant was present in the control pot. Each pot was watered with 100 ml nutrient solution A every four days from the donor plant side and with 10 ml from the acceptor plant side. The flow of nutrient solution was predominantly through the roots of the donor to the roots of the acceptor. Distilled water was also added as necessary to keep the perlite moist. The acceptor plants were grown for a period of 46 days. For the final two weeks of the experiment, the frequency of nutrient application was increased to once every two days. The final harvest was done at 46 days after planting and the fresh weight, height and number of branches of the tops of the acceptor plants were recorded.

4.3 Results and Discussion

A. Effects of root exudates from three donor plants on intortum seedling growth

In order to further evaluate the observed inhibition of intortum growth in soil containing bigalta limpograss residue, a simplified system was used to determine whether the inhibitory effect of bigalta limpograss root residues was due to plant-plant or plant-

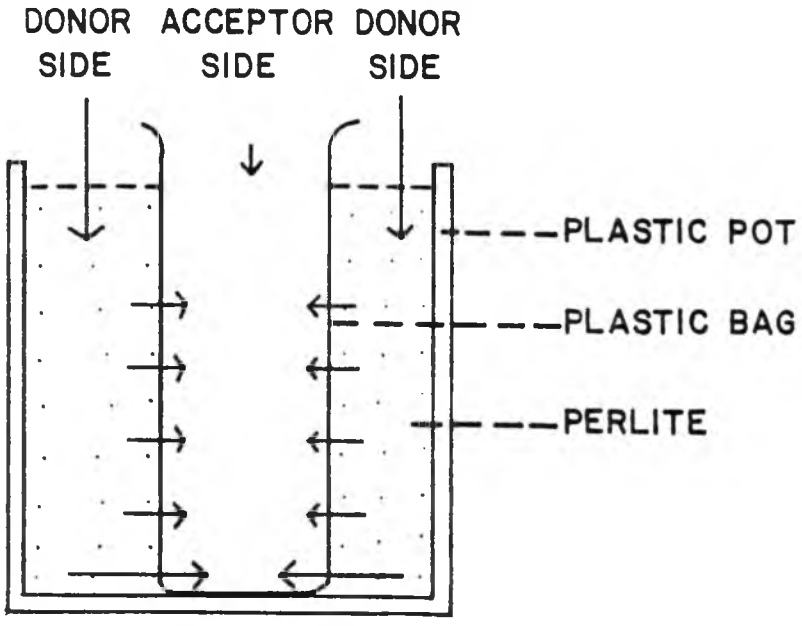

Figure 7. Pot design and the nutrient flow in the nutrient solution-perlite culture.

microbial interactions or to competition for nutrients. Nutrient-vermiculite cultures were used to minimize competition for nutrients, water and light. The divided pot design shown in Figure 6 (a) is better than growing the donor and acceptor in separate pots because root exudates from the readily and continuously diffuse from the donor side to the acceptor side. In the individual pot system, exudates are washed from the donor pot to the acceptor pot with a large volume of soultion, thus diluting the exudates to the point where effects tend to be very small.

1. Effect of donor treatments on the height of acceptor plant

The rate of growth in height of intortum seedlings receiving root exudates from bigalta and greenalta limpograsses and intortum was significantly slower than the control during the early growth stage in Hoagland's solution-vermiculite culture (Table XII). After 30 and 37 days, intortum was not significantly taller when grown with greenalta limpograss than when grown with bigalta limpograss, but intortum growth was significantly inhibited by bigalta at 55 days after planting. The presence or absence of a divider in the pots had no significant effect on the growth of intortum seedlings up to 55 days after planting.

Because the concentration of nutrients could affect the production of plant material and root exudates, two other nutrient solutions were used to examine the effects of nutrient concentration on intortum growth. The nutrient element contents in Hoagland's nutrient solution and two other solutions used are shown in Table XIII. N-free nutrient was a modified Hoagland's solution and nutrient solution A was somewhat less than half the strength of Hoagland's solution with the

Table XII. Growth in height of Desmodium intortum seedlings in the presence of root exudates from three previously established donor plants while growing in vermiculite cultures irrigated with Hoagland's nutrient solution

Pot Treatment	Donor Treatment	Days After Planting		
		30	37	55
		Plant Height+, cm		
With divider	Control	7.0 a+	16.6 a	37.7 a
	Bigalta	2.5 bcd	6.6 c	15.5 cd
	Greenalta	2.9 bc	7.9 c	22.6 b
	Intortum	2.6 bc	4.9 cd	12.6 cd
Without divider	Control	3.4 b	12.6 b	42.6 a
	Bigalta	1.3 d	3.0 d	10.9 d
	Greenalta	1.8 cd	5.0 cd	17.8 bc
	Intortum	1.8 cd	3.0 d	8.5 d

+ Values followed by a common letter are not significantly different (P = 0.05, Duncan's multiple-range test)

Table XIII. Concentration of each nutrient in three different nutrient solutions

Element	Hoagland's	N-free	Nutrient A
	ppm		
N	200	0	70.0
P	31	31	15.5
K	234	234	78.0
Mg	48	48	40.6
Ca	200	200	20.0
S	64	112	62.0
B	0.5	0.5	0.13
Cl	0.6	0.6	35.6
Cu	0.02	0.02	0.005
Fe	2.01	2.01	0.53
Mo	0.009	0.009	0.036
Mn	0.50	0.50	0.126
Zn	0.05	0.05	0.013

exception of Mg, Ca and S. Growth in height of intortum was much greater at 55 days in Hoagland's solution (Table XII) than in N-free solution (Table XV). The slowest growth rate occurred in solution A. While the plants in the different solutions weren't grown at the same time and thus were exposed to somewhat different environments, the data do indicate that the low level of nutrients in solution A were not adequate for optimum growth of intortum.

In nutrient solution A, intortum height was significantly reduced in pots with dividers by exudates from bigalta limpograss after 44 days of growth and by both grasses after 75 days (Table XIV). The inhibition due to greenalta exudates in pots with dividers was less than that due to bigalta exudates. The inhibition of intortum by intortum observed in the Hoagland's solution culture was not evident with the more dilute solution A. The lack of inhibition of intortum by intortum in the solution A experiment may be due to the fact that production of donor intortum which was much lower than in the Hoagland's nutrient solution.

The inhibitory effect of high levels of nitrogen fertilization on the nodulation of legumes has been known for many years (Vines, 1888; Orcutt and Wilson, 1935). Little is known, however, about the interaction between allelopathic substances and the growth, nodulation, and nodule activity of legumes. An N-free nutrient solution was used to study the effects of allelopathy on growth and nodulation of intortum which had been inoculated. The grasses were supplied with N by spraying 2% urea on the leaves at three day intervals. Early growth of intortum was slower in N-free solution than it was in the

Table XIV. Growth in height of Desmodium intortum seedlings in the presence of root exudates from three previously established donor plants while growing in vermiculite cultures irrigated with nutrient solution A

Pot Treatment	Donor Treatment	Days After Planting			
		44	52	66	75
		Plant height+, cm			
With divider	Control*	3.6 b++	6.1 b	13.5 b	23.6 ab
	Bigalta	1.8 c	2.4 d	6.3 d	9.7 e
	Greenalta	2.2 bc	3.5 cd	9.4 cd	15.4 cd
	Intortum	3.1 bc	5.5 bc	13.0 bc	20.4 bc
Without divider	Control	3.0 bc	3.6 cd	8.4 cd	19.9 bc
	Bigalta	1.8 c	2.5 d	6.2 d	10.6 de
	Greenalta	3.1 bc	5.0 bc	10.6 bc	19.0 bc
	Intortum	6.1 a	11.9 a	21.0 a	28.9 a

+ Means of three replications

++ Values followed by a common letter are not significantly different (P = 0.05, Duncan's multiple-range test)

* Desmodium intortum seedlings were used as the control

Table XV. Growth in height of Desmodium intortum seedlings in the presence of root exudates from three established donor plants while growing in vermiculite cultures irrigated with nitrogen-free nutrient solution

Pot Treatment	Donor Treatment	Days After Planting		
		37	55	61
		Plant height+, cm		
With divider	Control	2.5 ab++	15.7 b	20.4 a
	Bigalta	2.5 ab	17.0 ab	23.2 a
	Greenalta	3.1 a	20.3 a	25.9 a
	Intortum	2.4 ab	10.8 c	12.9 b
Without divider	Control	1.7 bc	9.0 c	15.6 b
	Bigalta	0.7 c*	3.3 d**	4.1 c
	Greenalta	1.1 bc	3.8 d	14.9 b
	Intortum	1.4 bc	1.5 d	4.9 c

+ Means of three replications

++ Values follow by a common letter are not significantly different (P = 0.05, Duncan's multiple-range test)

* Ten, 15 and 14 of 24 plants survived in the three replications for bigalta, greenalta, and intortum, respectively

** Five, 14 and 4 of 24 plants survived in the three replications for bigalta, greenalta, and intortum, respectively

solutions containing some N. The slow growth is probably due to the fact that nodulation and the initiation of N fixation by nodules does not contribute significantly to the nitrogen nutrition of legumes until a few weeks after planting (Kang, 1975). There were few significant differences in growth between treatments after 37 days of growth (Table XV). The height of intortum seedlings in the big treatment in the pots without a divider was significantly less than in the greenalta and control treatments at 61 days after planting (Table XV). This result is similar to the results in Hoagland's solution and solution A-vermiculite cultures at 55 days and 52 days after planting, respectively. Intortum survival was influenced by all three donor species but survival was lowest in the bigalta treatment. The reduced survival in the pots without dividers could be due to the intermingling of roots of the acceptor and donor plants. Also, because the intortum seedlings were small and weak during the early growth stages, they were probably more susceptible to allelopathic substance(s) which were present in the solution.

2. Effect of donor treatments on acceptor plant yield

The top and root yields of intortum grown in Hoagland's solution were significantly reduced in all donor treatments (Table XVI). The total yield of intortum in the bigalta treatment was only 25% of the control in the pot with the divider and only about 12% of the control in pots without a divider. The growth of intortum was inhibited significantly by intortum exudates in Hoagland's solution but not in solution A (Table XVII), perhaps because intortum growth was much less in the solution.

Table XVI. Yields of fresh tops and roots of Desmodium intortum seedlings after 55 days of growth as affected by root exudates from donor plants and the yield of the donor species grown in Hoagland's solution-vermiculite culture

Pot Treatment	Donor Treatment	Acceptor Production Per Pot, g[+]			Donor Production, g	
		Top	Root	Total Yield % of Control	Top	Root
With divider	Control	148.8[++]	78.3 a	--		
	Bigalta	28.0 a	26.6 b	24.0	375.3 a	120.0 a
	Greenalta	48.3 bc	37.9 b	38.0	258.7 b	91.0 b
	Intortum	19.7 c	28.4 b	21.2	375.0 a	95.0 b
Without divider	Control	154.4 a	39.9 b	--		
	Bigalta	20.0 bc	3.3 c	12.0	344.0 a	93.0 b
	Greenalta	42.9 bc	7.4 c	25.9	253.0 b	103.7 ab
	Intortum	17.9 c	4.1 c	11.3	365.7 a	75.3 b

[+] Means are derived from three replications

[++] Values within columns followed by the same letter are not significantly different (P = 0.05, Duncan's multiple-range test)

Table XVII. Effects of root exudates of donor plants on the fresh top and root production after 75 days of growth of Desmodium intortum and the yields of the donor plants grown in solution A-vermiculite culture

Pot Treatment	Donor Treatment	Acceptor Production Per Pot, g[+] Top	Root	Total Yield % of Control	Donor Production, g Top	Root
With divider	Control	42.2 b[++]	11.1 ab	--	42.7 d	52.0 c
	Bigalta	10.0 d	3.1 d	24.6	181.3 a	276.3 a
	Greenalta	22.5 c	5.8 cd	53.1	140.0 b	181.3 b
	Intortum	32.2 bc	10.9 ab	80.9	62.0 cd	58.3 c
Without divider	Control	38.0 b	7.6 bc	--	39.3 d	48.7 c
	Bigalta	11.2 c	2.3 d	29.6	156.7 ab	271.7 a
	Greenalta	37.7 b	5.9 cd	95.6	87.0 c	150.7 b
	Intortum	58.7 a	14.6 a	160.7	31.0 d	69.0 c

[+] Means are derived from three replications

[++] Values within columns followed by the same letter are not significantly different (P = 0.05, Duncan's multiple-range test)

The yield of intortum tops in the control treatment was significantly lower than in the bigalta and greenalta treatments in the pot with a divider in N-free solution (Table XVIII). However, root yields of intortum grown with the two grasses were only about 60 percent of the control (Table XVIII) while root yields of intortum were about 30% of the control in the other two nutrient solutions (Tables XVI and XVII). The yields of intortum tops and roots in the intortum treatment were significantly lower than in the control (Table XVIII). Intortum growth was inhibited less by the two grasses in the N-free solution because of the low production of both grasses. The top and root yields of intortum grown in pots without dividers were poor in all treatments. The result is due to the fact that only a few seedlings survived in these treatments.

It is recognized that nutrient requirements and efficiencies of nutrient uptake for the legume and the two grasses differ. Therefore, the relative growth of the donor grasses and the legume in a given nutrient solution would be different. As previously described, the amount of root exudates produced by a plant in a pot depends on the production of roots and tops of the plant. The results of the three experiments with nutrient culture have shown that the growth of intortum was reduced by root exudates from bigalta and greenalta limpograsses. Since the results of three experiments were similar but could not be compared directly because the methodology differed, the results were expressed as a percentage of the control for ease in comparison. The effects of donor production on the acceptor response expressed as a percentage of the control were linearized by plotting the logarithem of percent control against acceptor production.

Table XVIII. Effects of root exudates of donor plants on the fresh top and root production after 61 days of growth of Desmodium intortum and the yields of donor plants in pots grown in nitrogen-free nutrient solution-vermiculite culture

Pot Treatment	Donor Treatment	Acceptor Production Per Pot, g[+] Top	Root	Total Yield % of Control	Donor Production, g Top	Root
With divider	Control	40.3 b[++]	10.5 a	--		
	Bigalta	52.9 a	6.1 c	116.2	66.7 c	68.0 cd
	Greenalta	56.3 a	6.7 c	124.0	47.3 c	82.3 c
	Intortum	20.2 c	8.3 b	56.2	434.7 a	110.0 b
Without divider	Control	53.5 a	3.5 d	--		
	Bigalta [§]	4.1 d	0.4 f	7.9	90.3 c	86.0 c
	Greenalta	14.9 cd	1.3 f	28.3	152.0 c	58.7 d
	Intortum [§§]	3.2	1.0	7.2	535.6 b	133.0 a

[+] Means are derived from three replications

[++] Values with columns followed by the same letter are not significantly different (P = 0.05, Duncan's multiple-range test)

[§] Five and 14 of 24 plants survived in the three replications for bigalta and greenalta limpograsses

[§§] Only one pot data

Regression analysis showed that the production of intortum was significantly and negatively correlated with the total biomass produced by bigalta and greenalta limpograss (Figure 8). It is assumed that the amount of inhibitory substances produced by the donor cultivars was proportional to the biomass produced in the pot.

Since the variation in growth of intortum might have been caused by differences in amounts of available mineral nutrients, the mineral nutrient content of the tops was analyzed. The concentration of N, Mg, S, Na, Al, Fe and Cu in the tops of intortum receiving root exudates from bigalta in pots with dividers in the three nutrient-vermiculite cultures were not significantly different from the controls and similar results were obtained for Hoagland's and A solution in pots without dividers. The concentration of some of the nutrients in intortum tops exposed to bigalta exudates was significantly different than the control and those data are summarized in Table XIX (the actual data are in Appendix A Tables XXXVII, XXXVIII and XXXIX). The P contents of intortum tops in all cultures were significantly lower than the control. It is possible that root exudates from bigalta interferred with the absorption of P by the acceptor. However, all P concentrations in intortum tops were still higher than the critical level (0.22%) required for normal intortum growth (Andrew and Robbins, 1969). Therefore, reduced yield of intortum in the bigalta treatment in Hoagland's and the A solution probably were not due to the lower P contents. Where nutrient concentrations were greater than the controls, the levels obtained were not likely to be high enough to cause toxicity because the differences were small. Although the growth of intortum receiving root exudates from greenalta was significantly lower than the

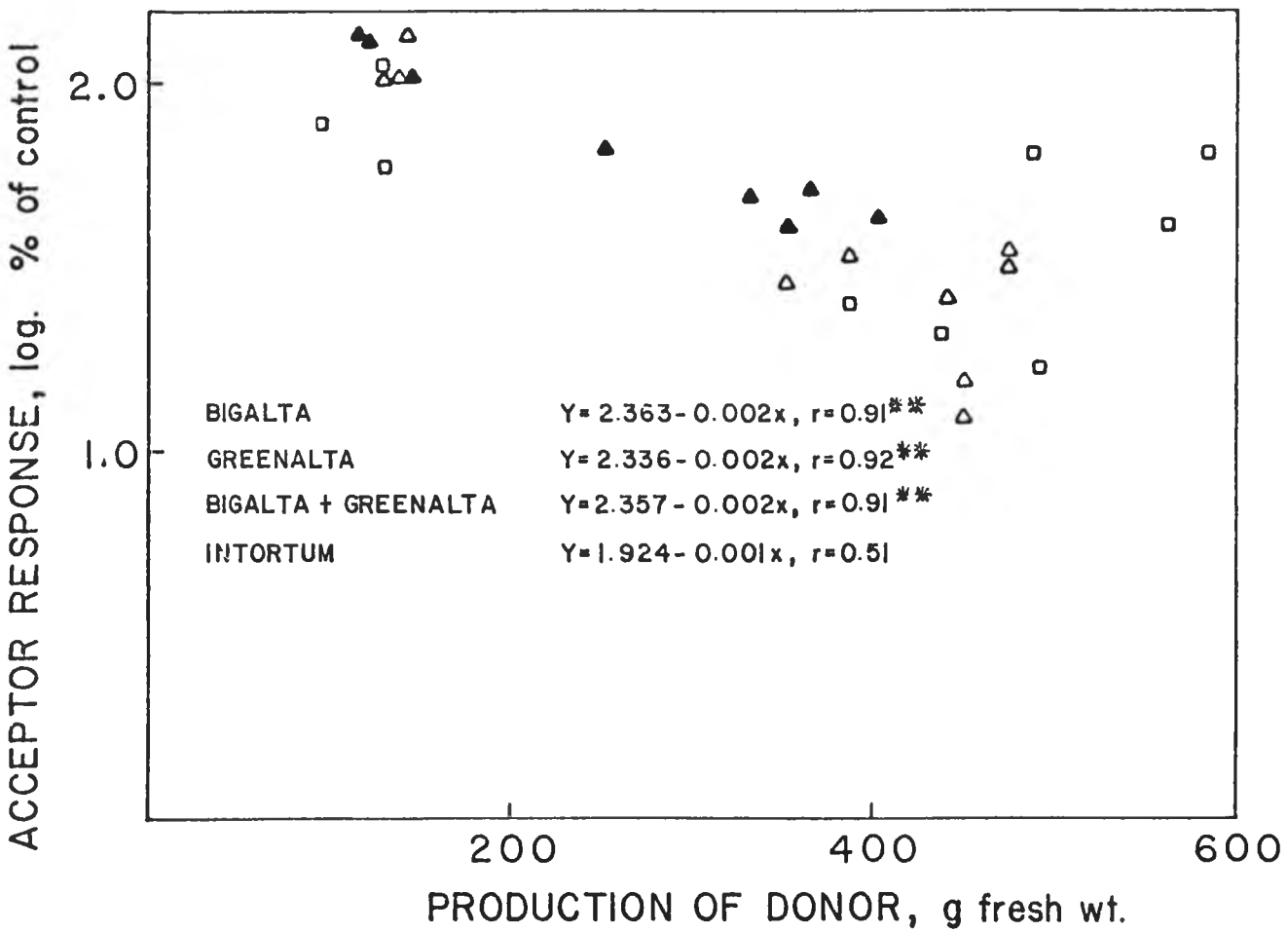

Figure 8. The relationship between donor production and the yield of intortum expressed as a percentage of the control receiving root exudates from the donor cultivars, bigalta limpograss, △; greenalta limpograss, ▲; and intortum, □.

Table XIX. Summary data on significant differences in mineral nutrient content of Desmodium intortum exposed to root exudates of bigalta limpograss and the control

Pot Treatment	Comparison	N	P	K	Ca	S	Si	Mn	Zn
With divider	Lower than control	-	HAF*	-	-	-	F	-	F
	Higher than control	-	-	A	H	-	-	H	-
Without divider	Lower than control	-	HA	-	-	-	HA	-	-
	Higher than control	H	-	-	H	A	-	-	H

* Data from Hoagland's solution-vermiculite culture (H), nutrient solution A (A) and N-free nutrient solution (F)

control in Hoagland's and the A solution in pots with dividers (Tables XVI and XVII), only the P content of the tops of intortum grown in Hoagland's solution and Si contents of intortum tops in all three cultures in pots with dividers were significantly lower than the control treatment (Table XX). The reduced N content of intortum tops in the greenalta treatment in pots without dividers (Table XX) is not easily explained because data obtained for the bigalta and greenalta limpograss treatments were not consistent.

The N content of intortum tops in the intortum treatment in N-free nutrient culture and the P contents in all three cultures were significantly lower than the controls in pots with dividers (Table XXI). The inhibition of intortum growth by the intortum treatment in pots with dividers might have been due to the direct inhibition of plant metabolism or to the inhibition of nodulation and nodule activity by intortum exudates. The lower N content of intortum tops in the intortum treatment than in the control in N-free nutrient culture suggests that nitrogen may have been a limiting factor for growth. However, differences in the levels of N in the tops were small. Levels of N, P and K in intortum tops in pots without dividers were significantly lower than the control (Table XXI) but did not cause a reduction in the yield of intortum in solution A cultures (Table XVII), probably because the differences were small. The mineral contents of intortum tops indicate that the reduction in the growth of intortum by the intortum treatment in Hoagland's solution was probably not due to nutrient deficiency but to autotoxicity.

3. Autotoxicity

Autotoxicity, the inhibition of a cultivar or species by that cultivar or species (Muller, 1966), is a well recognized

Table XX. Summary data on significant differences in mineral nutrient content of Desmodium intortum exposed to root exudates of greenalta limpograss and the control

Pot Treatment	Comparison	N	P	Ca	Si	Zn
With divider	Lower than control	-	H*	-	HAF	-
	Higher than control	-	-	-	-	-
Without divider	Lower than control	A	A	-	HAF	-
	Higher than control	-	F	F	-	-

* Data from Hoagland's solution-vermiculite culture (H), nutrient solution A (A) and N-free nutrient solution (F)

Table XXI. Summary data of significant differences in mineral nutrient content of Desmodium intortum exposed to root exudates of intortum and the control

Pot Treatment	Comparison	N	P	K	Ca	Mg	S	Si	Mn	Zn
With divider	Lower than control	F*	HAF	-	-	-	-	-	-	-
	Higher than control	-		-	HF	F	HAF	HAF	H	-
Without divider	Lower than control	A	A	A	-	-	-	-	-	-
	Higher than control	H	-	-	H	-	-	H	-	H

* Data from Hoagland's nutrient solution-vermiculite culture (H), nutrient solution A (A) and N-free nutrient solution (F)

phenomenon in many tree crops but little is known of the extent of autotoxicity in pasture vegetation. Since the extent of autotoxicity in forage plants is not known, strategies to deal with autotoxicity in forage species are not well developed. Particularly, autotoxicity could be important in some continuous cropping systems and in pure stands of pasture species, but there are no substantiating studies to confirm the existence of such a phenomenon.

The top and root yields of intortum as an acceptor in the presence of intortum as a donor in both Hoagland's and N-free nutrient-vermiculite cultures were significantly lower than in the control (Tables XVI and XVIII). The most striking inhibition was observed when intortum seedlings were grown in association with older plants in pots without dividers (Tables XVI and XVIII). It is possible that both allelopathic and competitive effects were responsible for the observed growth inhibition in pots without dividers. No autotoxicity was apparent in the solution A cultures (Table XVII) where the concentration of nutrients in solution A was much lower than in Hoagland's solution. The observed autotoxicity of intortum in Hoagland's and N-free solutions and its absence in solution A can be explained by the fact that growth of the donor intortum in the former two solutions was 4 times that in solution A. Thus, a greater quantity of root exudate likely would be present in cultures grown in Hoagland's and the N-free nutrient solutions. The existence of allelopathy in species such as intortum could be a natural mechanism of population control which prevents overcrowding. However, because autotoxicity may reduce the persistence of intortum in pastures under some climate conditions, the recognition of autotoxicity and the development of management

practices to overcome it may significantly increase pasture productivity.

4. Nodulation and acetylene reduction of intortum in the presence of donor plants

The supply of plant nutrients in soil affects legume nodulation and the rate of nitrogen fixation as indicated by acetylene reduction (ethylene production). The experimental design and management of the grass-legume association in the cultures used in this study made it possible to observe the effects of root exudates from donor plants on nodulation and ethylene production by intortum. The results show that nodule fresh weight, numbers, and ethylene production per pot in all treatments were significantly lower than the control (Table XXII). The only exception was that nodule number in the pots with dividers were not significantly different from the control. However, the specific activity of the nodules in μ moles C_2H_4 produced per g fresh weight per hour was not reduced significantly by the treatments (Table XXII). The nodule weight-root weight ratios of intortum which received root exudates from the three donor plants also were not significantly different from the control suggesting that the inhibition of growth by exudates was a general phenomenon rather than inhibition of a specific process such as nodulation or nitrogen fixation. The rate of nitrogen fixation by a legume depends on the supply of products from photosynthesis to the nodules (Allison, 1935; Hardy and Havelka, 1976; Bethlenfalvay and Phillips, 1977; Bethlenfalvay *et al.*, 1978 a,b) and photosynthesis is influenced by many factors, including allelopathic substances (Einhellig *et al.*, 1970). Results presented previously showed that root exudates from donor plants inhibited the growth of intortum. However, the mechanism of inhibition by root

Table XXII. Effects of root exudates of donor plants on nodulation and acetylene reduction rate of nodules of Desmodium intortum grown in Hoagland's solution-vermiculite culture

Pot Treatment	Treatment	Nodules Per Pot		Ethylene Production	
		Fresh weight, g	Number	$\mu M\ hr^{-1}\ pot^{-1}$	$\mu M\ hr^{-1}\ g^{-1}$
With divider	Control	0.174 a+	110 b	0.093 b	0.536 a
	Bigalta	0.027 cd	87 bc	0.016 d	0.819 a
	Greenalta	0.059 c	96 b	0.037 c	0.714 a
	Intortum	0.040 cd	63 c	0.016 d	0.411 a
Without divider	Control	0.112 b	153 a	0.134 a	0.971 a
	Bigalta	0.017 d	20 d	0.009 d	0.555 a
	Greenalta	0.027 cd	29 d	0.016 d	0.658 a
	Intortum	0.026 cd	17 d	0.010 d	0.375 a

+ Results within columns followed by the same letter are not significantly different (P = 0.05) by Duncan's multiple-range test

exudates is not yet known. The results from this experiment show that nodule weight, nodule number and ethylene production per pot were reduced by allelopathic substances and the nodule/root ratios suggest that the reduction in growth was due to direct effects of root exudates from the donor plant on the growth of the acceptor.

Kitamura and Nishimura (1977) showed that the average fresh weight of one nodule from intortum grown in full sunlight at temperatures of 20°, 25° and 30°C and N fertilizer rates of 0, 1, 2 and 4 kg N/acre ranged from 9.9 to 33.1 mg. Average weights of nodules obtained from intortum grown in Hoagland's solution were 1.0 mg or less in all treatments. The high nitrogen content (200 ppm) in Hoagland's solution was probably partly responsible for the low nodule weights and numbers obtained in this experiment. However the interaction of allelopathic substances and nitrogen levels on nodule size and nodule number of legumes also is not clear.

Several workers have reported that extracts of higher plants inhibited the growth of Rhizobium bacteria in in vitro culture (Rice, 1964; Malone, 1970; Murthy and Nagonra, 1977). Purashothaman and Balaraman (1973) reported that the phenolics -- anthranlic, p-amino-benzoic, cinnamic, ferulic, gallic, and p-hydroxybenzoic acids and hydroquinone -- extracted from black, lignite, peat and red soils inhibited in vitro growth of Rhizobium spp. Blum and Rice (1969) found that gallic and tannic acids at concentrations ranging from 33 to 300 ppm were highly effective in reducing nodulation of bean plants in sand and soil cultures. However, Husseir et al. (1976) has found that bacteriods from soybean nodules could be adapted to ultilize catechol, salicylic, p-hydroxybenzoic, gentisic, protocatechuic,

vanillic, syringic and ferulic acids at concentration of 1 μM/ml (10^{-9} M). Gaur and Pareek (1976) showed that 10^{-7} M concentrations of vanillic, salicylic, p-hydroxybenzoic, p-coumaric and protecatechuic acids stimulated the growth of Rhizobium leguminosarum; 10^{-5} M concentrations had no appreciable effect while levels of 10^{-3} M were injurious. Elkan (1961) reported that a non-nodulating, near isogenic soybean strain released an unknown substance that inhibited nodulation of its normally nodulating sister strain. While phenolic acids have been shown to inhibit the growth of Rhizobium, the mechanism(s) of inhibition of nodulation by these substances has not been established.

Intortum autotoxicity reduced fresh nodule weight, nodule number and acetylene reduction rate per pot (Tables XXII and XXIII). The specific activity of nodules in the intortum treatment was significantly less than the control in N-free solution, but not in Hoagland's solution. The inhibition of nodulation by seedlings receiving root exudates from older donor plants has not been reported previously. The inhibitory mechanisms may involve the direct or indirect inhibition of nodulation nodule growth and nitrogenase activity.

The production of the donor species bigalta and greenalta in N-free solution was very low (Table XVIII) although a 2% urea solution was regularly sprayed on the leaves of grasses. Average weights of nodules obtained from intortum grown in the N-free nutrient solution ranged from 5.4 mg to 3.9 mg and were much larger than those on plants grown in Hoagland's solution and nodule activity (ethylene production) was also higher. Thus the nitrogen content of the nutrient solution was an important determinant of the nitrogen fixing capacity

Table XXIII. Effects of root exudates from three donor species on nodulation and acetylene reduction rate of nodules of Desmodium intortum in N-free nutrient solution-vermiculite culture

Pot Treatment	Treatment	Nodules Per Pot		Etylene Production	
		Fresh weight, g	Number	$\mu M\ hr^{-1}\ pot^{-1}$	$\mu M\ hr^{-1}\ g^{-1}$
With divider	Control	4.196 b[+]	1090 a	7.27 a	1.72 a
	Bigalta	5.572 a	1036 a	7.66 a	1.44 ab
	Greenalta	5.640 a	1090 a	9.12 a	1.62 a
	Intortum	2.500 c	590 b	2.12 b	0.85 c
Without divider	Control	5.244 ab	1056 a	8.35 a	1.56 ab
	Bigalta[§]	0.437 c	73 c	0.46 b	1.01 bc
	Greenalta	1.576 c	256 c	1.37 b	0.86 c
	Intortum[§§]	0.517	120	0.28	0.55

[+] Results within columns followed by the same letter are not significantly different (P - 0.05) by Duncan's multiple-range test

[§] Five and 14 of 24 plants survived in the three replications of the bigalta and greenalta limpograsses treatments, respectively

[§§] Data from only one pot

of intortum, regardless of any effects of root exudates. Probably because of the low grass production, the limpograss treatments had no significant effect on nodule numbers, fresh nodule weight and the rate of acetylene reduction by nodules of intortum in the pots with dividers (Table XXIII). In contrast, nodule weight, numbers, and acetylene reduction rate per pot by nodules on the roots of intortum in both grass treatments in the pots without dividers was significantly lower than the control. It is possible that inhibition resulted from the intermingling of donor and acceptor roots in the treatments in pots without dividers which may have permitted toxic exudates to be rapidly taken up by the acceptor plants. It is also possible that competition for nutrient, light and space could reduce the growth of intortum, resulting in less photosynthate becoming available for nodulation and nodule development.

5. Effect of root exudates from three donor plants on the growth of intortum cuttings

The pot design for this experiment was similar to vermiculite culture system except that pearlite was used as the growing media (Figure 7). Cuttings of intortum were used as planting material because their growth is usually faster than that of seedlings. The yield of intortum tops in the bigalta treatment were significantly less than the yield from the greenalta treatment and the control (Table XXIV). The number of branches per plant in the bigalta treatment was significantly lower than in the control. However, the greatest depression in the growth of the acceptor was not associated with the greatest growth of the donor (Table XXIV). The results obtained with cuttings of intortum confirm those obtained with seedlings, i.e. that

Table XXIV. Effects of root exudates of three donor plants on the growth of Desmodium intortum cuttings in solution A-perlite culture

Donor Treatment	Intortum Growth[+]			Donor Fresh wt., g
	Top Fresh Weight, g	Branches Per Plant	Plant Height, cm	
Control	34.3 a[++]	3.0 a	122.3 a	--
Bigalta	9.0 c	1.0 b	66.3 a	95.3 a
Greenalta	18.3 b	2.0 ab	91.7 a	91.7 a
Intortum	16.3 bc	2.3 a	98.0 a	75.7 a

[+] Mean of three replications

[++] Results within columns followed by the same letter are not significantly different (P = 0.05) by Duncan's multiple-range test

exudates of bigalta were more toxic than those from greenalta or intortum. Some donor roots grew into the acceptor side of the container after starting the acceptor plants so it is also possible that competition for nutrients was involved in this experiment. The inhibition of intortum in the intortum treatment showed that autotoxicity was also present when mature stem cuttings of intortum were used as the acceptor plant. Thus older intortum plant material is also sensitive to toxins produced by intortum.

4.4 Summary and Conclusions

Hydroponic cultures in vermiculite and perlite and using three different nutrient solutions were used to study the effect of root exudates from the donor species bigalta and greenalta limpograss and intortum on the growth, nodulation and nitrogen fixation of intortum. The vermiculite and perlite cultures were designed so that competition for nutrients and light were minimized. The results are summarized as follows:

1. Exudates from bigalta and greenalta inhibited the growth of intortum seedlings and vegetative cuttings grown in Hoagland's solution and solution A, but not in nitrogen free nutrient solution. In all studies, the inhibition in the top production of intortum receiving root exudate from bigalta was significantly less than from greenalta in nutrient solutions containing nitrogen.

2. The data from the various nutrient showed that the degree of retardation of growth of intortum receiving root exudates from bigalta and greenalta was significantly correlated with the amount of biomass production by the donor plants.

3. The yields of intortum tops in the treatment where the donor and acceptor were both intortum showed that intortum growth was significantly inhibited by exudates from intortum.

4. Root nodule weight, nodule number and acetylene reduction per pot in all inhibited by exudates from the two grasses and intortum. However, the specific activity of the nodules were not significantly different.

5. The P content of the tops of intortum receiving root exudate from bigalta was significantly below the control. However, the P concentration in all nutrient-vermiculite cultures in the plant tissue was not below the level considered to be critical for growth of the plants.

The results of these experiments show that bigalta and greenalta limpograsses produced exudates which inhibited the growth of intortum and directly or indirectly inhibited nodulation and nodule growth. The data also show that intortum is an auto-inhibited species, producing exudates which inhibit its own growth.

CHAPTER 5

COLLECTION, ISOLATION AND CHARACTERIZATION OF ALLELOPATHIC SUBSTANCES FROM ROOT EXUDATES

5.1 Introduction

Hermarthria altissima was shown to inhibit the growth and nodulation of Desmodium intortum and this inhibition was assumed to be allelopathic and due to root exudates. However, it was recognized that growth inhibition could also have been due to substances produced or transformed by microorganisms present as contaminants in the culture system. Earlier approaches taken in characterizing the chemicals responsible for allelopathic responses was to collect root exudates directly from living plant roots grown hydroponically so that alteration of compounds by microorganisms or contamination by leachates from leaves and decomposing plant material was minimized (Rovira, 1969). The isolation of root exudates from collected solutions was accomplished by concentrating solutions with root exudates and passing them through columns with cation and anion exchange resins (Smith, 1976) or by lyophilization (Currier and Strobel, 1976; Vancura and Stanek, 1975). Organic compounds such as carbohydrates, amino acids, organic acids, nucleotides, flavanones, enzymes, and vitamins have all been found in the root exudates of numerous plants (Rovira, 1969; Hale et al., 1971). However, most allelopathic substances have been identified as phenolic or lipophylic compounds which are secondary metabolites (Rice, 1974; Whittaker and Feeny, 1971) and little work appears to have been done on such materials in root exudates.

The purposes of this study were to collect lipophylic substances in root extracts and exudates and to isolate and characterize the allelopathic substances in them. Since vermiculite and sand culture reproduce the root environment of natural soil more closely than does hydroponic culture without the chemical and physical complexity of soil, these methods were chosen for growing plants from which roots for extraction and root exudates were collected for further characterization.

5.2 Materials and Methods

A. Collection of allelopathic substances

Since the chemical nature of the allelopathic substances in roots and root exudates of H. altissima needs to be determined in order to chracterize their physiological effects, methods of collecting and concentrating these compounds must be developed. Methanol has commonly been used as a solvent for the extraction of inhibitory compounds from plant material because lipophilic substances are generally soluble in it. For the extraction of lipophilic substances, roots of bigalta and greenalta limpograsses were collected from 50-day old plants grown in nutrient solution A-vermiculite culture and washed in distilled water. The extraction procedure (Figure 9) involved homogenizing 7.2 g of fresh roots with 300 ml of methanol in a blender for 10 minutes after which the sample was centrifuged at 3000 rpm for 30 minutes. The supernatant was decanted and dried in vacuo in a flash evaporator at 50°C and then taken up in about 15 ml of distilled water. The water was extracted three times with 100 ml of methylene chloride. The methylene chloride fraction was concentrated

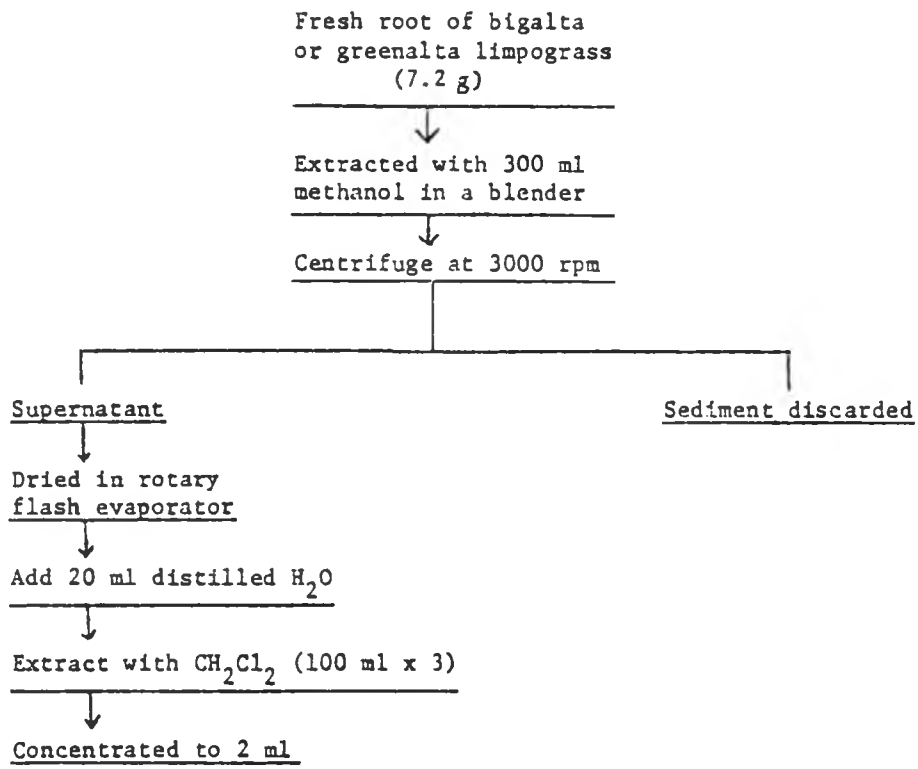

Figure 9. Procedure for the isolation of lipophilic substance(s) by direct methanol extraction of fresh roots of bigalta and greenalta limpograsses.

to about 10 ml in a rotary flash evaporator and to 2 ml by flushing with nitrogen gas. Activity of extracts was assayed by bioassay.

B. Collection of root exudates from limpograsses

Extraction of homogenized root tissue with methanol was a satisfactory method for the concentration of root-bound inhibitory substances. However, root exudates were assumed to be the actual source of allelopathic chemicals, and inhibitors in the exudates may or may not be the same as the root-bound inhibitors. Root exudates are difficult to isolate because they are present in the root environment in extremely low concentrations. Thus, a method must be developed to concentrate the exudates suspected of being active in allelopathy. Since most allelochemicals were assumed to be lipophilic (hydrophobic), it seemed possible that a hydrophobic resin might serve as a satisfactory extractant.

Amberlite XAD ploymeric adsorbents are hard, insoluble spheres of high surface area which have selective affinity for various chemicals depending on their polarity. Amberlite XAD-4 is a nonplolar resin which is used for the adsorption of organic substances of relatively low molecular weight from aqueous systems. If an organic molecule has a hydrophobic end, that end will attach to a hydrophobic adsorbent such as XAD-4. This is particularly true when the adsorption takes place from aqueous solution. The trapping efficiency of XAD-4 for allelopathic substance(s) in H. altissima root extracts was studied using the procedure shown in Figure 10. Prior to using the resin, it was cleaned in a Soxhlet extractor with acetone for a week. The activity trapped by the column was compared with direct methanol extraction using a standardized bioassay method to be described later.

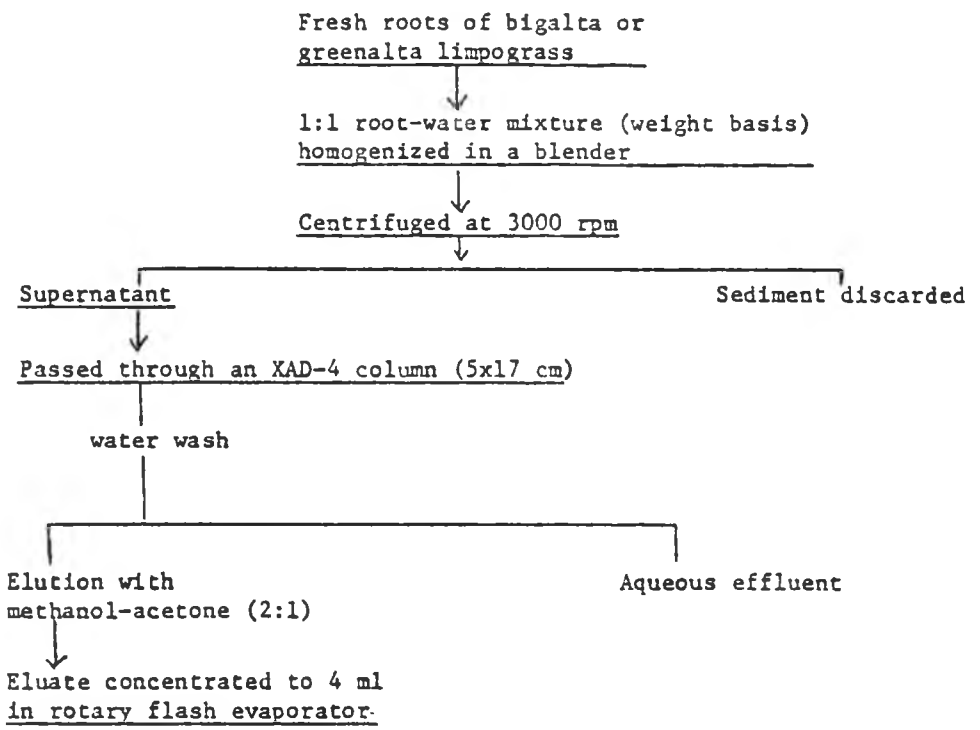

Figure 10. Procedure for the collection of lipophilic (hydrophobic) substances from root homogenates of bigalta and greenalta limpograsses using an XAD-4 resin column.

The bioassay results showed XAD-4 extraction to be as effective as methanol extraction. It was concluded that the XAD-4 column could be used to isolate allelopathic substance(s) from grass roots.

Since the amount of chemicals released from roots is likely to be minute, an effective method for the continuous collection of root exudates was needed. A trapping system designed for the continuous collection of root exudates from plants is shown in Figure 11. The top of the unit consisted of a brown bottle (2.5 liter capacity) with its bottom removed. The bottle was filled with a 2:1 (v/v) sand-crushed basaltic rock (<2 cm size) mixture with a 3 cm layer of the rock in the bottom. The top of the container was covered with aluminum foil and heat-sterilized for 2 days at 100°C. An XAD-4 resin column (12 g XAD-4 in a 1.6 x 12 cm glass column) was inserted into a teflon sleeve and connected to the neck of the bottle by a rubber stopper covered with thin teflon film. Air was bubbled into the side tube to circulate the nutrient solution. For the collection of root exudates, eight cuttings of bigalta and greenalta limpograss were surface sterilized with 5% chlorox and planted in the sand-rock mixture and grown for 30 days. Nutrients were provided with solution A (contents in Table XI) at the rate of 100 ml day. Additional water was supplied as needed to replace that lost by transpiration and evaporation. Each grass cultivar was grown in three jars. Control pots consisting of sterilized sand and nutrient solution were set up at the same time. After trapping the root exudates with the resin columns by recirculating the solution through the system (Figure 11) for 48 hours, the loaded column was detached and stored at about 4°C

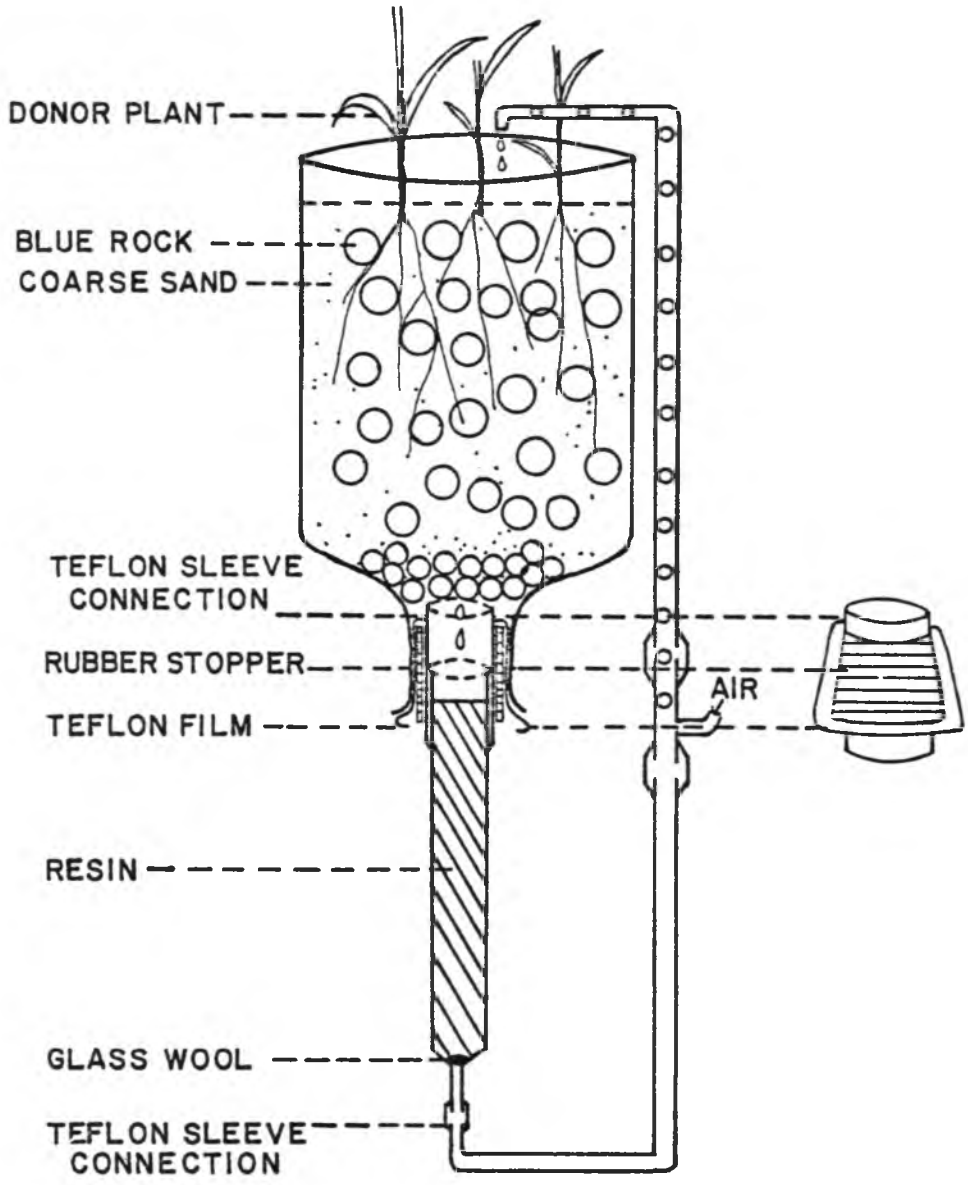

Figure 11. Apparatus for continuous trapping of root exudates.

to prevent microbial proliferation. Fifteen loaded columns for each cultivar were collected and eluted with methanol and acetone (1:1 v/v, 200 ml per column). The eluate was concentrated to an aqueous residue. A neutral fraction of the root exudate was obtained from the aqueous residue by CH_2Cl_2 extraction. After the neutral fraction had been removed, the aqueous fraction was acidified to pH 2.0 with 1N HCl and again extracted with CH_2Cl_2. A basic fraction was then obtained by adjusting the aqueous fraction to pH 11.0 followed by an additional CH_2Cl_2 extraction. The activities of the neutral, acidic and basic fractions were examined by the standard bioassay procedure.

C. Bioassays

A simple and fast bioassay is essential for the successful detection and isolation of inhibitory compounds.

Ideally, to confirm that the root exudates from bigalta were inhibitory to intortum growth they should be tested on intortum seedlings. However, the variation in intortum seed germination rate was found to be large making it difficult to develop an accurate bioassay based on intortum seedlings without using prohibitively large numbers of seeds. To determine the phytotoxicity of root extracts and exudates, a "lettuce seed bioassay" was used. The advantages of this bioassay were a high and rapid rate of germination and faster radicle growth. Prior to adopting the lettuce seed bioassay, the sensitivity of lettuce and intortum seed bioassays were compared using the neutral fraction of root exudates from bigalta limpograss.

1. Paper disc bioassay

Paper (Whatman No. 3 MM) was washed with 0.2% oxalic acid and then with distilled water to remove any toxic substances.

It was air-dried at room temperature. Aliquots of the root extracts and exudates were carefully applied on 3.5 cm^2 discs of the washed and dried paper. The solvent carrier was evaporated at room temperature by flushing with N_2 gas. The loaded paper discs were placed in 5.5 cm diameter petri dishes and 200 µl of distilled water were added to the paper discs. Ten lettuce seeds (Lactuca sativa var. Anuenue) were selected from seeds which would not float after soaking with tap water for two hours and were placed on the discs. The dishes were covered and placed in a plastic bag with sufficient water in the bag to maintain a saturated atmosphere. All treatments were replicated three times. After 48 hours at room temperature, the results were taken by measuring the length of the seedling radicle. The effects of root extracts on radicle growth were expressed as percent inhibition relative to the distilled water control. The data were analyzed statistically by Student's t test.

2. Chromatographic bioassay

A modification of the chromatographic bioassay described by McPherson et al. (1971) was used to determine the phytotoxicity of neutral and acidic fractions of root exudates from bigalta and greenalta limpograsses. Four 2 x 56 cm strips of Whatman No. 3 MM paper which had been washed and dried as described previously were cut to form a wedge shaped tip. The strips were spotted with 100 µl aliquots from the original 3 ml of root exudates. Four unspotted strips were used as a paper control and the strips were developed simultaneously in 2% glacial acetic acid by descending chromatography in a chromatography chamber (39 x 60 x 70 cm). After developing, the paper strips were air-dried at room temperature. All paper strips

were viewed under shortwave (254 nm) and longwave (336 nm) ultraviolet light to locate fluorescent and light quenching spots which were likely to contain allelochemical activity. One treated and one control strip was sprayed with diazotided p-nitroaniline (DPNA) followed by 10% sodium carbonate to detect phenolic compounds. The spots located by UV and the color reagent were used as a guide for cutting the strip into segments. The lettuce seed bioassay described previously was used to determine the phytotoxicity of the spots. Since spot size varied, 5 lettuce seeds and 120 µl of water per cm^2 were added to a segment. The total number of seeds and water added depended on the size of the segment. After 48 hours incubation, radicle lengths were measured and the data were treated as described in the bioassay procedure.

D. Characterization of allelopathic substances

Thin layer chromatography (TLC) was used to isolate individual allelopathic substances for further characterization. Since paper chromatography was used in the bioassay, the separation of compounds from extracts with both paper and thin layer chromatography were compared. Glacial acetic acid (2%) was used as the developing solvent for paper chromatography. For TLC, several solvent systems were used, including acetic acid (2%), tolulene-methyl formate-formic acid (TMF, 5:4:1), chloroform-acetic acid-water (CAW, 4:1:1), butanol-acetic acid-water (BAW, 5:4:1) and tolulene-chloroform-acetone (TCA, 8:5:7). The effectiveness of separation of compounds by conventional and wedged-tip thin layer strips were compared. Spots were detected by UV and the DPNA color reagent described previously.

5.3 Results and Discussion

A. Collection of growth inhibiting substances from root homogenates and exudates

The conventional approach to the study of compounds presumed to be responsible for allelopathy has been to selectively extract materials from root segements or homogenates (Rice, 1974). Plant extracts were chosen for study primarily for their convenience in spite of the fact that toxins in the extracts may or may not have been responsible for the observed allelopathy. Water of methanol extracts were commonly used to isolate substances from dead and broken cells (Rice and Parenti, 1967; Rice, 1965; Numata et al., 1973). Under those conditions, compounds may have been changed either enzymatically or chemically. Thus there is need to establish a new method for the collection of biologically active extracellular substances. The new method using an XAD-4 resin column described in the Materials and Methods was compared with conventional methanol extracts from root homogenates. The results showed that the efficiency of extraction of inhibitory substances from root homogenates by the column was no less than that of the methanol preparation (Figure 12). A solvent extract from resin alone did not exhibit any inhibitory activity. Inhibition of radicle growth by extracts from the bigalta limpograss root homogenate was higher than from greenalta limpograss root homogenate.

Various techniques have been used to grow plants for studies of root exudates including culture of seedlings on filter paper (Schroth and Snyder, 1961), in culture tubes (Rovira, 1956), or in sand or soil (Miller and Schmidt, 1965; Ayers and Thornton, 1968). Collection techniques are also varied; one commonly used method was to

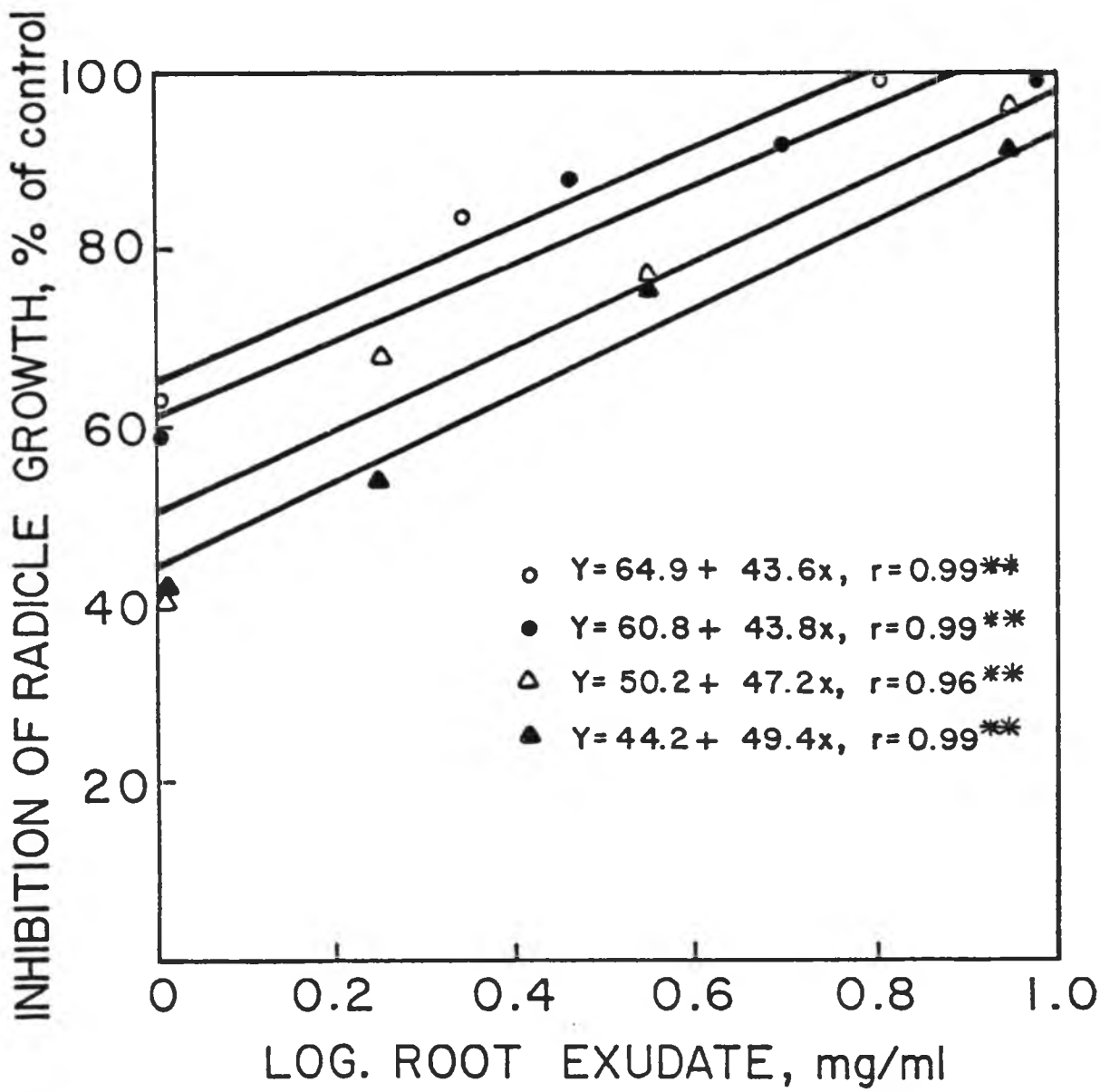

Figure 12, Relative efficiency of CH_3OH (triangles) and XAD-4 resin (circles) for the extraction of allelopathic substances from root homogenates as indicated by radicle growth of lettuce seedlings, Open and closed symbols indicate results for bigalta and greenalta root homogenates, respectively.

collect the nutrient solution or distilled water rinses from the root system and concentrate them to a smaller volume for solvent extraction or lyophilization. Procedures such as this were tedious and often ineffective because root exudates had to be concentrated from a large volume of aqueous solution. Thus the quantity of root exudates collected was limited. For example, Calum et al. (1949) grew 150,000 plants of tomato in sand culture and leached the sand on alternate days for one month to produce 12 g of crude exudate. The use of seedlings is questionable because they might produce exudates which differed in quantity and quality from those of older plants. The lyophilization method would be impractical for the collection of relatively volatile substances. In the methodology section, the unique continuous trapping apparatus designed for the collection of root exudates from growing donor plants was described (Figure 11). The advantage of this technique was that the inhibitors, despite their presence in extremely low concentrations, were adsorbed and accumulated on the resin column, while the mineral nutrients were recycled to the root system by an air-lift device. Amberlite XAD-4 is different from ion exchange resins because it has no ionic functional group incorporated into its resin structure. The inorganic nutrient elements in the circulating system are therefore not adsorbed by the resin.

B. Phytotoxicity of root exudates from bigalta and greenalta limpograsses

Intortum seeds should be used to determine the effect of allelopathic substance from root exudates on the growth of intortum seedlings in order to be able to draw conclusions about the potential phytotoxicity of bigalta to intortum. However, the variability in

intortum seed germination rate was a serious obstacle to its use in bioassays. Lettuce seeds with a high and rapid germination rate were therefore used for the bioassay. To examine if responses were comparable, intortum and lettuce were tested in one experiment. Lettuce seed was found to be more sensitive to the inhibitory substances than desmodium seed (Table XXV). The results also suggest that the inhibitory substances in root exudates from bigalta limpograss are not species-specific. The observed inhibition of radicle growth could be due to inhibition of cell division or cell elongation.

The root exudates from bigalta and greenalta limpograsses were collected from XAD-4 columns attached to the continuous trapping system and separated into neutral, acidic and basic fractions. The phytotoxicity of these three fractions was tested by the lettuce seed bioassay. The effects of the fractions are shown in Table XXVI Lettuce radicle growth was not inhibited by extracts from the resin control (eluate from XAD-4 column alone). However, there was some inhibition at the higher concentrations of the neutral and acidic fractions from the pot control (recirculating nutrient solution without plants). Apparently small amounts of hydrophobic organic compounds were formed in the pot even without the presence of any plants. Conceivably, microbial activities nursed by the nutrient solution could have produced toxins inhibitory to seedling growth. The unexpected result further suggests that the XAD-4 resin was a highly efficient trapping medium for lipophilic substances. The neutral and acidic fractions of root exudates from the grasses were much more potent inhibitors of radicle growth of lettuce seeds than comparable fractions from the pot control.

Table XXV. The length of the radicle of Lactuca sativa (Anuenue lettuce) and Desmodium intortum seeds in the neutral fraction of root exudate from bigalta limpograss

Treatment	Radicle Length, mm	
	Lettuce	Intortum
Distilled H_2O	12.9 ± 2.4[+]	11.8 ± 3.5
Neutral fraction	0	4.6 ± 2.0
% of control	--	39.0

[+] Average of 10 seeds ± 1 standard deviation

Table XXVI. Effects of neutral, acidic and basic fractions from a control pot and root exudates from bigalta and greenalta limpograsses on the radicle growth of lettuce seeds

Exudate, µl Per Replicate	Radicle Growth+, mm				
		Bigalta		Greenalta	
	Control	Test	Percent of Control	Test	Percent of Control
	Neutral Fraction				
50	11.5 ± 10.2** §	0** §§	0	6.7 ± 4.7* §§	58.3
30	12.0 ± 12.0**	0**	0	8.8 ± 8.9	73.3
10	24.7 ± 6.2	4.3 ± 7.1**	17.4	18.6 ± 6.9*	75.3
5	21.6 ± 4.5	15.9 ± 9.5**	73.6	20.3 ± 6.9	94.0
	Acidic Fraction				
50	16.9 ± 3.0*	0.1 ± 0.1**	0.6	0.5 ± 0.7**	3.0
30	19.8 ± 2.3	0.7 ± 0.5**	3.5	2.1 ± 2.1**	10.6
10	18.4 ± 3.5	16.0 ± 6.4	87.0	22.6 ± 6.0**	122.8
5	18.6 ± 4.9	20.8 ± 6.7**	111.8	22.2 ± 4.5**	119.4
	Basic Fraction				
50	19.8 ± 4.5	20.8 ± 3.8	105.1	19.5 ± 4.1	98.5
30	22.2 ± 4.9	22.5 ± 5.6	101.4	20.6 ± 3.6	92.8
10	22.0 ± 3.1	19.5 ± 4.7	88.6	20.3 ± 4.7	92.3
5	23.6 ± 4.0	25.1 ± 4.9	111.6	19.9 ± 3.8	84.3

Mean of distilled H_2O control = 20.6 ± 4.5
Mean of resin control = 21.1 ± 4.4

+ Three replications of 10 plants or an average of 30 seedlings, ± one standard deviation

*,** Significant at P = 0.05 and 0.01, respectively by Student's t test

§ Significantly less than resin control (data not shown) by Student's t test

§§ Significance compared to pot control by Student's t test

There were two types of inhibition observed. One type was complete inhibition of seed germination in the neutral fraction from bigalta limpograss. However, the seeds were shown to be able to germinate after washing and re-incubation in distilled water. Another type of inhibition was observed in the acidic fraction in which seed germination was not inhibited but radicle growth was suppressed, accompanied by browning of the root tips. Inhibition was invariably greater in exudates collected from bigalta limpograss than from greenalta limpograss (Table XXVI). These results indicated that bigalta limpograss exuded a greater quantity of inhibitors or that the inhibitory substances from bigalta limpograss were qualitatively different than those from greenalta limpograss. The results obtained with exudates corresponded to previous results (Chapters 3 and 4) which showed that root residues and exudates from bigalta limpograss caused a more severe reduction in the growth of intortum than those from greenalta limpograss. The neutral fraction from bigalta limpograss was a more potent inhibitor than the acidic fraction; however, the acidic fraction from greenalta limpograss inhibited radicle growth more than the neutral fraction (Table XXVI). Radicle growth was significantly stimulated at the lower concentrations of acidic fractions from bigalta and greenalta limpograss. It is possible that the acidic fraction contained plant hormones or chemicals which can stimulate radicle growth at low concentrations. The basic fraction did not exhibit any inhibitor activity, thus excluding the possible existence of alkaloid inhibitors.

While organic toxins are most likely responsible for the observed inhibition of radicle growth, pH, osmotic pressure, and

specific inorganic ions should also be considered as possible inhibitors of germination and growth. In the present study, the pH in all three fractions of the root exudates at a concentration of 50 µl of extract in 200 µl distilled water was about 6.0 (determined with pHast paper from EM Lab. Inc.). Osmotic pressures, measured with a Wescor vapor pressure osmometer, of all three root exudate fractions at the above rate were not significantly different from distilled water. Specific ion effects were assumed to be negligible because only organic chemicals are trapped by the XAD-4 resin column. Thus, it is concluded that the observed inhibition of radicle growth was due to organic phytotoxins. Although under the present experimental conditions, root exudation was the most plausible routes of release of allelochemicals from the plants, the phenomenon of exudation from the whole root system has not been thoroughly investigated. Considering the probable rapid turnover of root hairs or root epidermal cells after death, the contribution to allelopathy by residue decomposition cannot be easily determined at the present time.

Further studies of phytotoxicity in the neutral and acidic fractions of root exudates from bigalta and greenalta limpograsses are needed to identify the individual chemicals responsible for the inhibition observed in the bioassay. Paper chromatography (Whatman No. 3 MM) using 2% glacial acetic acid as the developing solvent was found to be a useful technique to resolve inhibitory substances in the extracts. The neutral fractions contained compounds having the greatest toxicity and the compounds causing the greatest inhibition of growth occurred in segment 7 and above segment 9 and had Rf values of 0.59 to 0.63 and 0.72 to 1.00 for bigalta limpograss (Table XXVII). Results

Table XXVII. Lettuce seed bioassays of the inhibitor activity of neutral fractions of root exudates from bigalta and greenalta limpograsses separated by paper chromatography

Segment	Radicle[+] Length, mm Pot Control	Rf Value	Bigalta Limpograss UV[§]	Bigalta Limpograss DPNA[§§]	Bigalta Limpograss Radicle Length, mm Exudate	Bigalta Limpograss % of Pot Control	Greenalta Limpograss UV[§]	Greenalta Limpograss DPNA[§§]	Greenalta Limpograss Radicle Length, mm Exudate	Greenalta Limpograss % of Pot Control
1	13.2 ± 2.9[†]	0.00-0.14	--	--	12.4 ± 5.6[†‡]	94	--	--	12.5 ± 5.7[†‡]	95
2	11.8 ± 3.0	0.14-0.28	--	--	13.9 ± 1.6	118	--	--	13.7 ± 2.7*	116
3	11.7 ± 2.6	0.28-0.41	f[++]	--	11.5 ± 3.7	98	--	--	12.9 ± 3.5	110
4	12.6 ± 3.1	0.41-0.48	wf	--	13.4 ± 4.0	106	f[++]	--	15.3 ± 3.4*	121
5	14.3 ± 3.1	0.48-0.53	b	--	14.5 ± 3.6	101	sf	--	17.1 ± 3.2*	120
6	14.6 ± 5.2	0.53-0.59	a	--	14.2 ± 2.7	97	a	--	13.2 ± 3.1	90
7	14.8 ± 3.0	0.59-0.63	f	--	11.8 ± 3.9*	80	f	--	13.2 ± 3.6	89
8	13.8 ± 3.4	0.63-0.72	--	--	13.6 ± 2.5	99	--	--	11.4 ± 4.2	83
9	12.0 ± 2.8	0.72-0.83	lf	Blue	8.3 ± 2.4**	69	--	--	10.3 ± 2.4	86
10	13.5 ± 3.4	0.83-0.86	sf[++]	Pink	8.5 ± 4.0**	63	lf[++]	Pink	11.1 ± 3.1*	82
11	11.3 ± 4.7	0.93-1.00	--	Yellow	0.1 ± 0.5**	1	--	--	0.5 ± 1.0**	4
12	10.1 ± 2.3**	0.86-0.93	a	Pink	8.0 ± 1.4*	79	--	--	8.9 ± 3.6	88

Mean of paper control = 12.4 ± 3.0
Mean of resin control = 13.0 ± 3.1

[+] Mean length of three replications ± standard deviation

[++] Visible only in shortwave ultraviolet light

[§] f = fluorescence; wf = white-fluorescence; b = blue; a = absorption; lf = light-fluorescence; sf = strong fluorescence

[§§] Color reagent= diazotized p-nitroaniline (DPNA) followed by 10% sodium carbonate

[†] Significantly less than resin control (data not shown) by Student's t test

[‡] Significance compared to pot control by Student's t test

*,** Significance at P = 0.05 and 0.01, respectively by Student's t test

obtained with the DPNA color reagent showed phenolic compounds to be present in these regions. The toxic spots in greenalta limpograss exudates were located in segments 8, 10 and 12 and had Rf values 0.6 to 0.72, 0.83 to 0.86 and 0.93 to 1.00 (Table XXVII). Radical growth was significantly stimulated by compounds in segment 2, 4 and 5 of the neutral fraction from greenalta limpograss, but not from bigalta limpograss. The results also show that the inhibition of radicle growth by the neutral fraction from bigalta limpograss were greater than from greenalta limpograss. Segment 9 from the acidic fractions of both limpograsses with an Rf of 0.69 to 0.76 significantly inhibited radicle growth and resulted in brown root tips similar to those observed when lettuce seed was germinated on the unchromatographed acid fraction (Table XXVIII). The results of the lettuce seed "chromatographic bioassay" of activity in the various fractions of root exudates separated with paper chromatography clearly indicated that allelopathic substances were present in the root exudates of bigalta and greenalta limpograsses. The isolation procedure which involved eluting compounds from the XAD-4 column with methanol and acetone ruled out the presence of large molecules such as pectins, polysaccharides, peptides or proteins. The segments which showed considerable activity may contain more than one inhibitory substance and the possibility of synergism among the compounds exists (Rasmussen and Einhellig, 1977).

C. Characterization of allelopathic substances

Isolation and purification of allelopathic compounds is essential for their characterization. Paper chromatography was useful for making crude separations for the chromatographic bioassay, but the quantity of sample required is quite large for UV or color detection.

Table XXVIII. Lettuce seed bioassays of the inhibitor activity of the acidic fractions of root exudates from bigalta and greenalta limpograsses separated by paper chromatography

Segment	Radicle[+] Length, mm Pot Control	Rf Value	Bigalta Limpograss UV[§]	Bigalta Limpograss DPNA[§§]	Bigalta Limpograss Radicle Length, mm Test	Bigalta Limpograss % of Pot Control	Greenalta Limpograss UV[§]	Greenalta Limpograss DPNA[§§]	Greenalta Limpograss Radicle Length, mm Test	Greenalta Limpograss % of Pot Control
1	12.2 ± 2.4*[†]	0.00-0.09	f	--	17.9 ± 3.6**[‡]	147	--	--	18.5 ± 3.6**[‡]	152
2	12.9 ± 2.6	0.09-0.22	f[++]	--	15.0 ± 2.9**	116	b[++]	--	16.0 ± 2.7**	124
3	13.2 ± 2.2	0.22-0.30	f	--	15.9 ± 3.8**	120	a	--	14.6 ± 3.5	111
4	12.7 ± 2.3	0.30-0.35	b	Blue	16.3 ± 3.5**	128	f	--	16.3 ± 1.9**	128
5	14.7 ± 2.5	0.35-0.43	f	--	15.8 ± 3.0	107	sf	--	18.1 ± 3.6*	123
6	12.9 ± 3.7	0.43-0.49	--	--	18.9 ± 4.0*	147	a	--	17.2 ± 4.9**	133
7	14.0 ± 3.1	0.49-0.64	--	--	18.3 ± 3.8**	131	--	--	20.0 ± 4.5**	143
8	11.3 ± 2.3	0.64-0.69	--	--	17.4 ± 2.1**	154	--	--	12.8 ± 2.4	113
9	15.3 ± 3.2	0.69-0.76	lf	--	7.8 ± 2.1**	51	a[++]	--	11.0 ± 3.2**	72
10	14.7 ± 2.6**	0.76-0.87	--	--	14.2 ± 3.3	97	--	--	15.5 ± 3.9	105
11	12.9 ± 2.5	0.87-1.00	--	--	12.8 ± 4.2	99	--	--	13.0 ± 3.7	101

Mean of paper control = 13.76 ± 3.29
Mean of resin control = 13.22 ± 2.73

[+] Mean length of three replications, each containing at least 5 seedlings ± 1 standard deviation

[++] Visible only in shortwave ultraviolet light

[§] f = fluorescence; lf = light fluorescence; sf = strong fluorescence; b = blue; a = absorption

[§§] Color reagents = diazotized p-nitroaniline (DPNA) followed by 10% sodium carbonate

[†] Significantly less than resin control (data not shown) by Student's t test

[‡] Significance compared to pot control by Student's t test

*,** Significance at P = 0.05 and 0.01, respectively by Student's t test

Thin layer chromatography is more sensitive to the color reagent and less time consuming. In a comparative experiment, the resolution of compounds in the neutral fraction of root exudates from bigalta limpograss was found to be much improved if strips on the TLC plates had a wedged-tip configuration (Figure 13). This technique was therefore used for part of the preparative chromatography in this study. Separation of compounds on paper and TLC produced similar results although separation was much more rapid and the bands were more distinct with the TLC procedure (Figure 13). Both the paper and the plate were cellulose so there is no reason to expect great differences in the patterns resulting from the two media.

Several solvent systems were used in attempts to improve the separation and purification of the compounds in the exudates. Other solvent systems tried were TMF, BAW, TCM and CAW. Two percent glacial acetic acid was found to be an effective solvent but the best separation was achieved with the TMF solvent system on silica gel plates. The results obtained with this system are shown in Table XXIX. Generally, the compounds in the neutral fraction of root exudates would include chemicals of the type R-H or R-OH and would include weak acids and phenolics. The acidic fraction would include organic acids and the basic fraction would include chemicals such as alkaloids. On the basis of results obtained with DPNA, there were more than 7 phenolic compounds present in the neutral fraction of exudates from bigalta limpograss, but only one in the neutral fraction of greenalta limpograss (Table XXIX). The acidic fractions from both grasses (Table XXIX) also contained only one DPNA-positive region. The results of both paper and TLC showed that the number of compounds visible in UV or

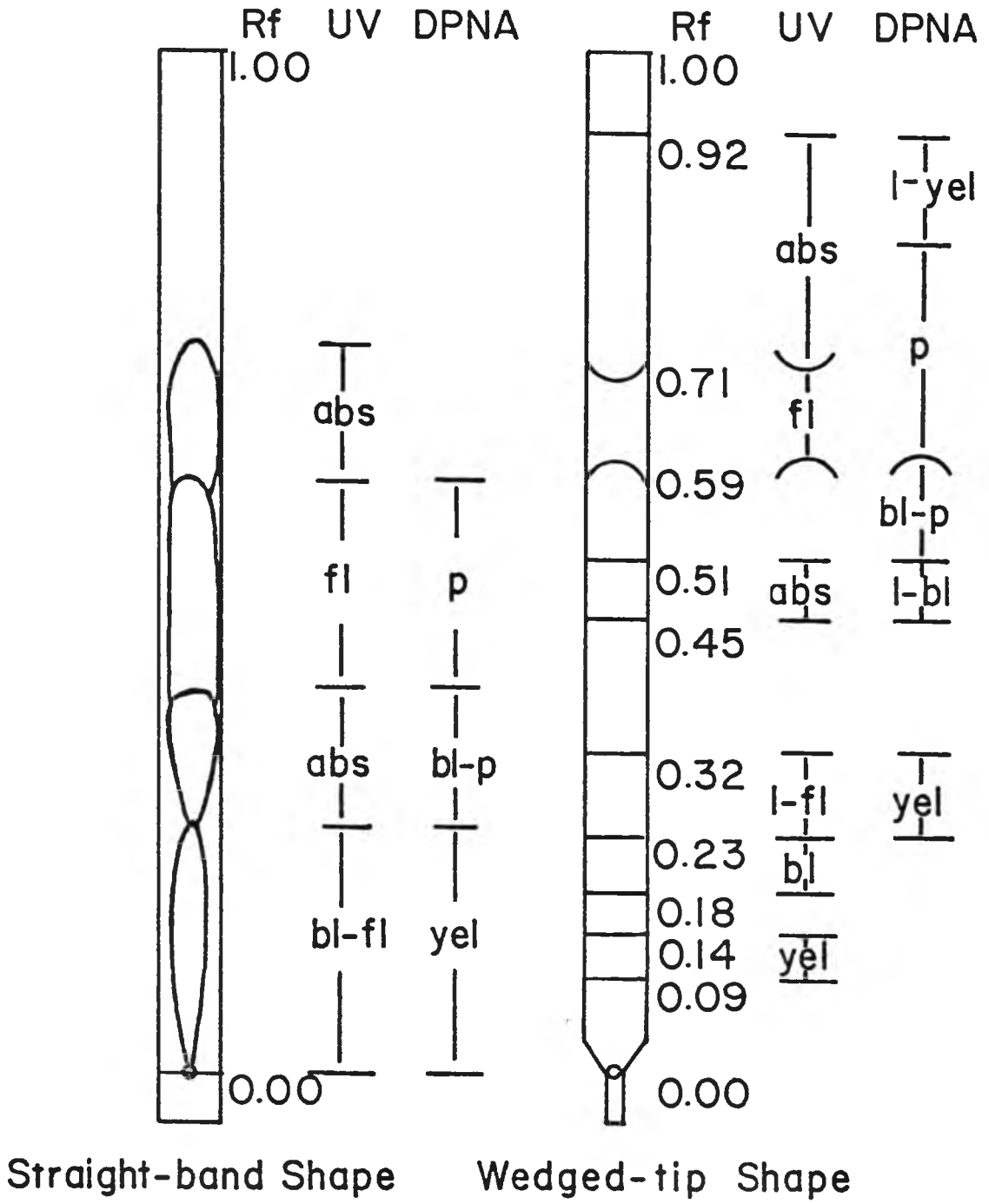

Figure 13. A comparison of the resolution of substances in the neutral fraction of root exudates from bigalta limpograss using conventional and wedged-tip thin layer chromatography. Chromatograms were developed with 2% glacial acetic at room temperature.

Table XXIX. Chromatographic separation of compounds in the neutral and acidic fractions of root exudates from bigalta and greenalta limpograss using tolulene-methyl formate-formic acid, 5:4:1, on silica gel thin layer plates

	Neutral Fraction							Acidic Fraction						
		Bigalta			Greenalta				Bigalta			Greenalta		
No.	Rf	UV	UV[§]	DPNA[§§]	UV	UV[§]	DPNA[§§]	Rf	UV[§]		DPNA[§§]	UV	UV[§]	DPNA[§§]
1	0.00-0.06	--	--	--	--	--	--	0.00-0.21	--	--	--	--	--	--
2	0.06-0.13	lf[†]	--	--	--	--	--	0.21-0.26	--	--	--	a	--	--
3	0.13-0.19	b	--	--	b	--	--	0.26-0.32	f	--	p	f	--	p
4	0.19-0.26	--	--	--	--	--	--	0.32-0.39	--	--	--	--	--	--
5	0.26-0.31	a	--	p	a	--	p	0.39-0.45	--	--	--	a	--	--
6	0.31-0.35	y	--	p	--	--	--	0.45-0.53	b	wf	--	b	wf	--
7	0.35-0.42	--	o	p	--	--	--	0.53-0.55	--	--	--	f	--	--
8	0.42-0.45	b	wf	p	b	wf	--	0.55-0.61	--	--	--	--	--	--
9	0.45-0.55	--	--	p	--	--	--	0.61-0.65	b	--	--	a	--	--
10	0.55-0.59	y	b	y	--	--	--	0.65-0.73	--	--	--	b	--	--
11	0.59-0.68	a	--	y	f	b	--	0.73-0.81	--	--	--	--	--	--
12	0.68-0.90	--	y	--	--	--	--	0.81-0.92	--	--	--	b	--	--
13	0.90-0.94	b	--	--	b	--	--	0.92-0.97	b	--	--	b	--	--
14	0.94-1.00	--	--	--	--	--	--	0.97-1.00	--	--	--	--	--	--

[§] Sprayed with 2N NaOH before viewing under UV

[§§] Color reagent = diazotized p-nitroaniline (DPNA) followed by 10% sodium carbonate

[†] lf = light fluorescence, f = fluorescence, b = blue, a = absorption, y = yellow, wf = white fluorescence, o = orange, p = pink

reacting with DPNA in the neutral fraction from tetraploid bigalta limpograss was quantitatively more than from diploid greenalta limpograss (Tables XXVII and XXIX). However, the opposite results were observed in the acidic fraction (Tables XXVII and XXIX). Since the neutral fraction from both grasses was more inhibitory to lettuce seed germination than the acidic fraction, these results suggest that the roots of tetraploid bigalta limpograss released more inhibitory substances than diploid greenalta limpograss. Although chemical characterization of the inhibitors is incomplete at this time, it is possible to speculate that the tetraploid cultivar released or accumulated more inhibitory compounds in both a quantitative and qualitative sense. This speculation is supported by the study of Levy and Levin (1971) who found two Phlox allotetraploids accumulated five flavonoids not observed in the parental species. They propose that hybridity and polyploidy have repressed or suppressed the activity of certain genes responsible for the production of enzymes involved in flavonoid synthesis. From the results obtained in this study it is also clear that the degree of inhibition in bioassays of root exudates from both tetraploid and diploid limpograss is in agreement with the degree of suppression of desmodium growth in soil, vermiculite and perlite cultures. Work towards characterization and identification is needed. Effects of the allelochemics on the physiology of the legume and its rhizobium symbiont also need to be examined.

5.4 Summary and Conclusions

A comparison of the relative efficiency of XAD-4 and methanol as extractants of allelochemicals from root homogenates of bigalta and

greenalta limpograsses showed that the efficiency of XAD-4 resin was greater than that of direct methanol extraction. In general, the inhibition of seed germination by extracts from bigalta root homogenates was greater than from greenalta root homogenates. A unique trapping apparatus was designed with the XAD-4 resin column for the continuous collection of root exudates from growing plants. The bioassay results showed that lettuce seed is more sensitive to the toxins from root exudates than desmodium seed. Both the neutral and acidic fractions of root exudates from bigalta and greenalta limpograss contained potent inhibitors of radicle growth of lettuce seeds. The basic fraction did not exhibit any inhibitory activity. The inhibition of radicle growth of lettuce seed was not due to pH, osmotic pressure or specific inorganic ion effects, thus demonstrating the presence of allelopathic substances. The results of chromatographic bioassays showed that there were five and two toxic spots in the neutral fraction of root exudates from bigalta and greenalta, respectively, and most inhibitory substances in bigalta root exudates appeared to be phenolic compounds. There was only one toxic spot in the acidic fraction from both grasses. The results of paper disc and paper chromatographic bioassays indicated that tetraploid limpograss cultivar bigalta released inhibitory compounds that were quantitatively and qualitatively different from those produced by greenalta, the diploid cultivar.

CHAPTER 6

CHANGES IN SOIL MICROORGANISMS AND SOIL ENZYME ACTIVITIES DURING ROOT DECOMPOSITION OF *HEMARTHRIA ALTISSIMA* AND *DESMODIUM INTORTUM*

6.1 Introduction

The effects of crops on soil biochemistry and microbiology has been little studied. The degree to which crop residues determine the population of a specific microorganism is not known and few data are available on rates of decomposition in soil of specific plant species. However, the chemical inhibition of higher plants by microorganisms has been reported (Hattingh and Louw, 1969). Microorganisms can play a role in allelopathy by influencing the rate of release and degradation of compounds in plant residues as well as by the direct production and release of allelo-chemicals. Organic matter decomposition in soil results from soil borne enzymes produced primarily by microorganisms. Enzyme activity in soil results from the accumulated extracellular enzymes of organisms and from enzyme activity associated with proliferating microorganisms (Kiss, *et al.*, 1972), although the enzymes can originate from organic residues. Among the enzymes which are released into soil by microorganisms and which are partially responsible for the extracellular degradation of soil organic matter are amylase, cellulase and invertase.

Pancholy and Rice (1973a) determined the activities of amylase, cellulase, invertase, dehydrogenase and urease in soil from two old-field successional stages and a climax stand in Oklahoma. They found that the activities of amylase, cellulase, and invertase were generally highest in the first stage of an old-field succession, intermediate in the second stage, and lowest in three types of climax

vegetation (tallgrass prairie, post oak-blackjack oak forest and oak-pine forest). They also found that no correlation existed between the level of activity of soil enzymes and soil organic matter content or pH. The addition of plant material from the first successional stage of an old-field succession to soil from the climax tallgrass prairie stand reslted in a continuous increase in the microbial population and the activities of amylase, cellulase, and invertase for a period of 45 days (Pancholy and Rice, 1973b). They also showed that the levels of amylase, cellulase and invertase activities in soil were influenced by the source of organic matter added to the soil.

Certain types of organic matter, tannins, and substances containing phenolics reduced the rate of organic matter decomposition in soil and have also been shown to inhibit seed germination and seedling growth (Rice, 1974). Basaraba and Starkey (1966) reported that a crude preparation of chestnut and wattle tannins combined with the proteins gelatin and gliadin resulted in material which was resistant to aerobic decomposition by microorganisms. The complexes were more resistant at pH 4.0 than at pH 7.0. Benoit *et al*. (1968) also showed that purified wattle tannin, a condensed tannin, reduced the rate of organic matter decomposition in soil. The purified wattle tannin also reduced the *in vitro* activities of purified polygalacturonase, cellulase and urease (Benoit and Starkey, 1968). They concluded that inactivation by tannins of exoenzymes from microorganisms which decompose compounds of high molecular weight (e.g. hemicellulose and cellulose) is an important part of the inhibitory effect of tannins on the microbiology and decomposition of plant residues.

The results presented in the previous chapters showed that the growth of intortum was inhibited by bigalta limpograss. The mechanism of inhibition could have been due to indirect effects on soil microbiology and biochemistry (Figure 1) as well as to direct chemical inhibition of intortum by the grass. If the inhibition was indirect, bigalta limpograss residue might detectably alter the levels of certain groups of microorganisms or of certain assayable enzymes presumed to be produced by them. The objectives of this work were to observe the changes in the microbial population, the activities of amylase, cellulase, invertase, and dehydrogenase, and CO_2 evolution from soil after adding roots of bigalta and greenalta limpograsses, and Desmodium intortum.

6.2 Materials and Methods

A. Roots and soil descriptions and decomposition treatments

Samples of ground roots of intortum and bigalta and greenalta limpograsses were incorporated into air dried soil at the rate of 0,15 g of dry root per 45.5 g soil (approximately 6,600 kg ha^{-1}). Soil without added roots was used as a control. All treatments were replicated three times. The elemental composition of the roots of intortum and bigalta and greenalta limpograsses are shown in Table XXX. The soil pH was 5.5. Twenty ml of water were added to the soils and they were incubated at 30°C for approximately 0, 3, 9 and 22 days. Since all measurements could not be made on the same day, data presented in the results show the number of days of incubation prior to determining enzyme activities or microbial counts.

B. Media for counts of microorganism

The number of bacteria and fungi were counted using a

Table XXX. Elemental analysis of the roots of bigalta and greenalta limpograsses and Desmodium intortum

Root	N	P	K	Ca	Mg	S
	%					
Bigalta limpo	0.52	0.11	0.30	0.17	0.19	0.05
Greenalta limpo	0.61	0.14	0.23	0.28	0.20	0.06
Intortum	1.65	0.26	1.15	0.33	0.31	0.30

dilution technique. Bacteria were counted by spreading appropriate dilutions of soil on plates of tryptic soy agar medium followed by incubation at 30°C for 24 hours. Fungi were counted on plates of potato-dextrose (PDA) and rose bengal-streptomycin (RBA) agar media after 4 days of incubation at 30°C.

C. Enzyme assay

The activities of amylase, cellulase, invertase and dehydrogenase were determined and the change in soil pH (1:1 water-soil by weight) was followed. The procedure of Ross (1966) was used to determine the activities of amylase and invertase, while cellulase activity was determined by the method of Pancholy and Rice (1973a). For the determination of amylase, cellulase and invertase activities, 5 grams of soil were placed in a 50 ml centrifuge tube along with one ml of toluene which was used to prevent microbial proliferation. The soil was vigorously mixed and placed at room temperature for 15 minutes. Ten ml of nitrate buffer (pH 5.9) was added followed by 10 ml of a solution containing either 1% starch, 1% carboxymethyl cellulose, or 5% sucrose to serve as the substrates of amylase, cellulase and invertase, respectively. The samples were then incubated for 24 hours at 30°C. Following the incubation period, the samples were centrifuged for 30 minutes at 3000 rpm and filtered through Whatman No. 5 filter paper. The volume of the filtrate was made up to 100 ml with distilled water and the reducing sugar content of the filtrate was determined by the method of Nelson (Clark, 1969). Dehydrogenase activity was determined by the method of Casida *et al.* (1964), by placing five grams of soil in a test tube and saturating it with 4 ml of a 1% solution of 2, 3, 5-triphenyltetrazolium chloride. The soil and

solution were mixed thoroughly, and the test tubes were tightly sealed and incubated at 30°C for 48 hours. After incubation, 10 ml of methanol was added to each test tube, and the contents stirred thoroughly. The suspension was then filtered through Whatman No. 30 paper and the concentration of the formazan read spectrophotometrically at 485 nm. A standard curve was made using various amounts of formazan formed from 2,3,5-triphenyl tetrazolium chloride. The results were expressed as mg formanzan per gram soil.

D. CO_2 evolution

Carbon dioxide evolution was measured by the method of Pramer and Schmidt (1964) with some modification. Four grams of soil were weighed into a 50 ml erlenmeyer flask which contained a small beaker. One ml of a solution containing 0.5% glucose and 0.006% NH_4NO_3 was mixed thoroughly into the soil. Two ml of 0.3N NaOH were added to the small beaker inside the flask. The flask was stoppered immediately and incubated at 30°C for 24 hours. The determination of CO_2 evolution from the soil was made by titrating the remaining NaOH, to which 2 drops of 0.1% phenolphthalein indicator and 1.0 ml of 50% $BaCl_2$ had been added, with standardized HCl. The phenolphthalein end point was determined by the disappearance of the pink color. The blank consisted of a similarly equipped flask without the soil sample.

6.3 Results

A. Dynamic changes in soil microorganisms

Bacterial and fungal populations in the Paaloa soil increased dramatically after moistening and incubating the soil for four days

(Figures 14, 15 and 16). In general, bacterial populations were highest in soil to which intortum roots had been added while smaller increases were measured in the soil containing the grass roots and in the control (Figure 14). After four days incubation, the fungal populations were highest in soil to which greenalta limpograss roots had been added (Figures 15 and 16). The dynamic changes in the fungal populations in soil to which bigalta and greenalta limpograss roots had been added were similar in both RBA and PDA. With intortum-treated soil, fungal counts peaked on day 4 on RBA and then declined but on PDA, the counts increased for 10 days before reaching a maximum (Figures 15 and 16).

B. Soil enzyme activities

Amylase and cellulase activities increased continuously with incubation time for a period of 9 days, except for the soil to which intortum roots had been added. In the intortum treatment, amylase activity decreased after 3 days of incubation (Figures 17 and 18). The activity of amylase in the soil to which intortum roots had been added was significantly higher than the other treatments. Differences in cellulase activity between treatments were small. The patterns of invertase and dehydrogenase activities were similar in all treatments (Figures 19 and 20). Invertase and dehydrogenase activities of the intortum treatment were greater than activities for other treatments at all measurement times. Based on quantity of product formed, the activity of invertase was highest in the experiment, amylase was second, and cellulase was lowest. Although fungal populations were high in the bigalta limpograss treatment (Figures 15 and 16), the activities of amylase, cellulase and invertase in the

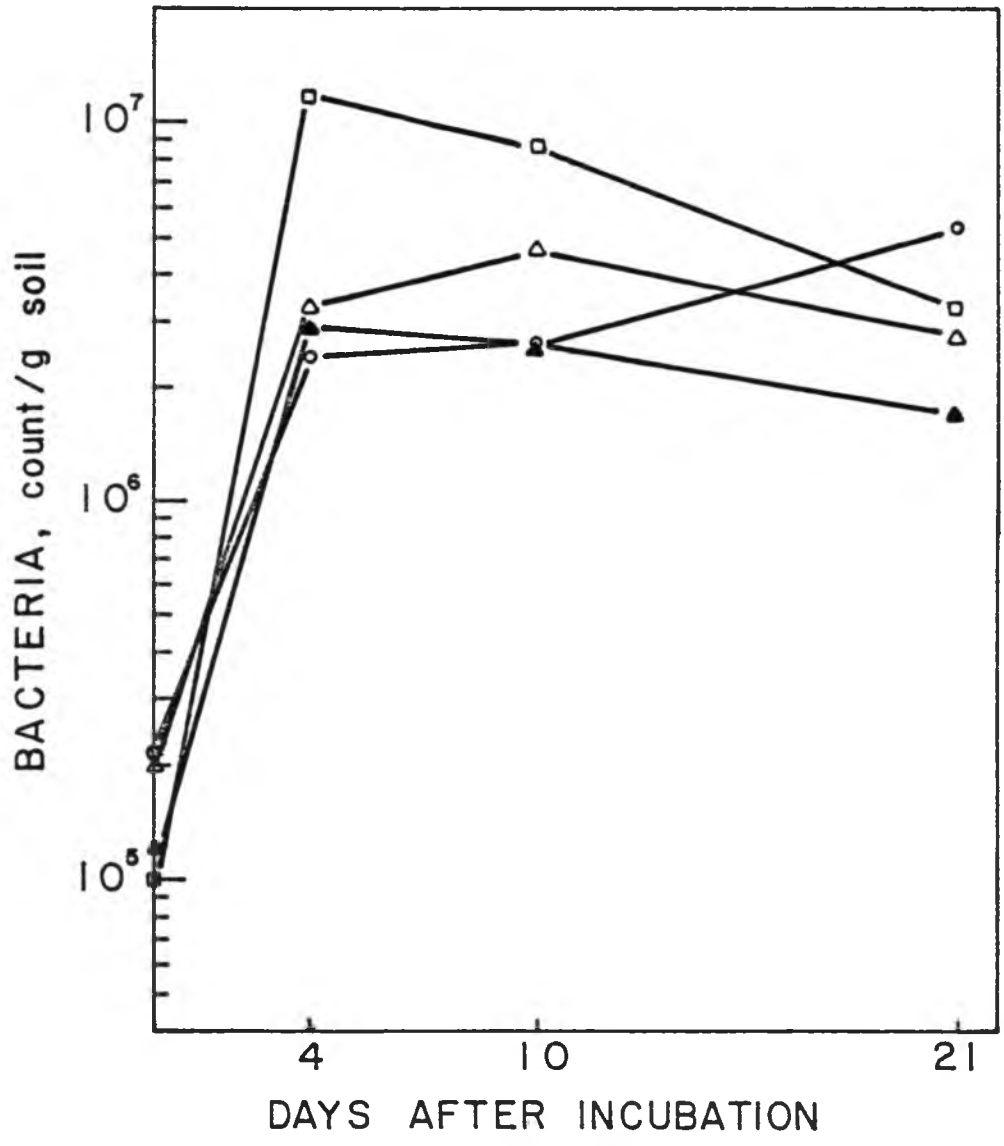

Figure 14. Effects of bigalta (△) and greenalta (▲) limpograsses and Desmodium intortum (□) roots on the bacterial counts in Paaloa soil using tryptic soy agar medium. Soil without added roots was the control (○).

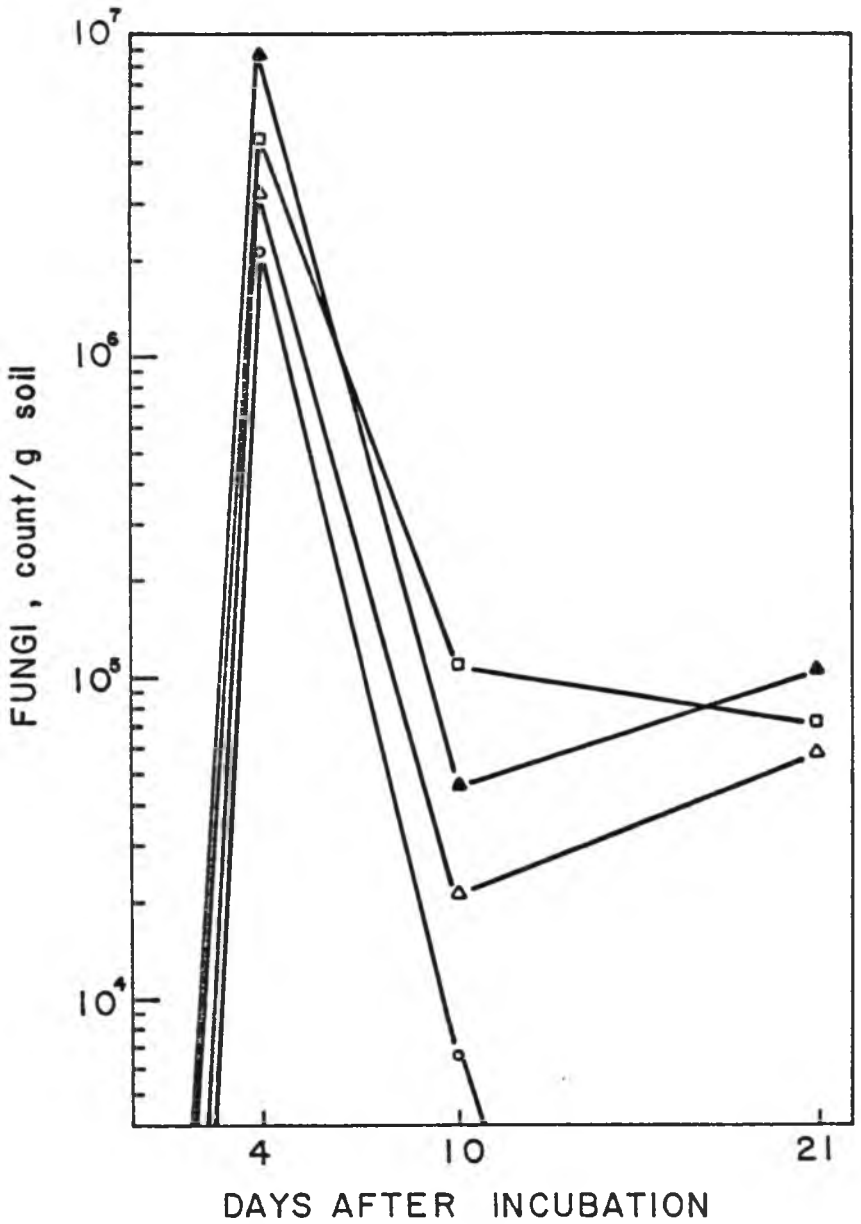

Figure 15. Effects of roots of bigalta (△) and greenalta (▲) limpograsses and Desmodium intortum (□) added to Paaloa soil on the fungal counts made using plates of rose bengal-streptomycin agar. Soil without added roots was the control (○).

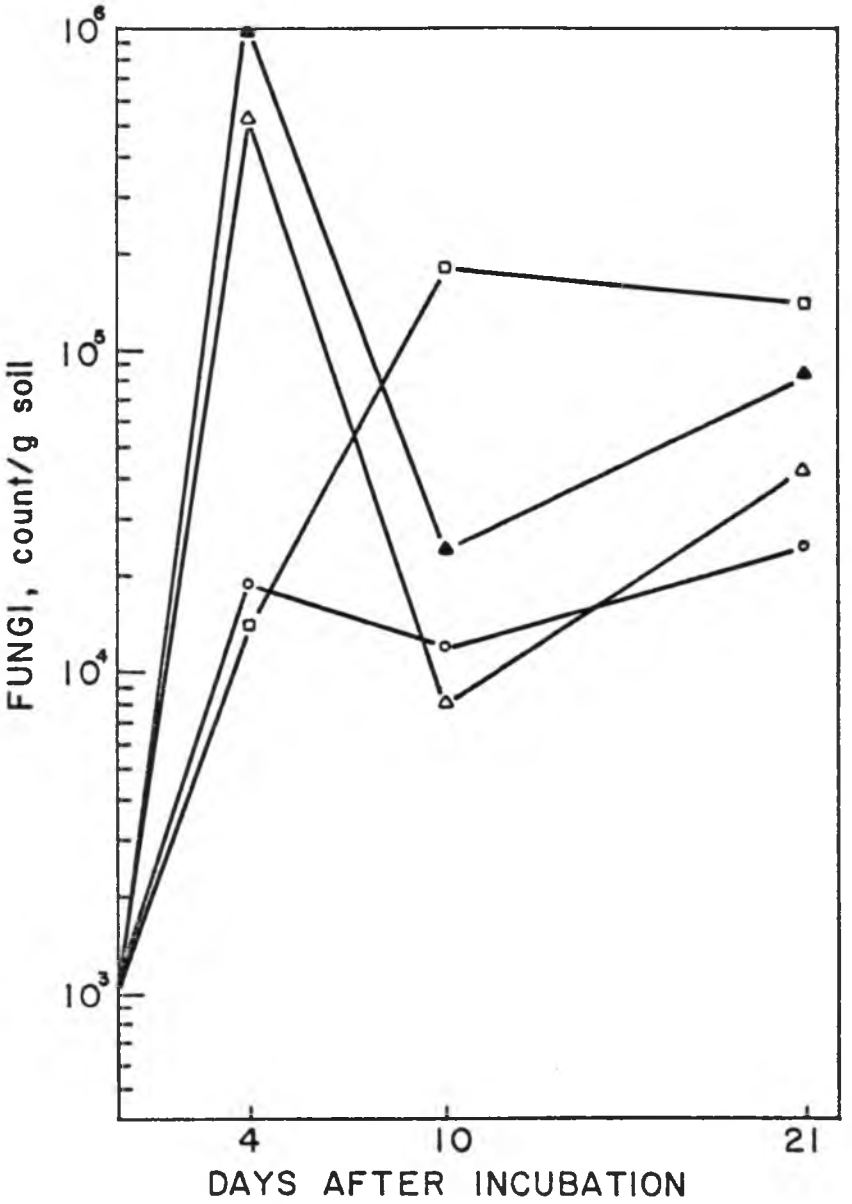

Figure 16. Effects of roots of bigalta (△) and greenalta (▲) limpograsses and Desmodium intortum (□) added to Paaloa soil on the fungal counts made using plates of potato-dextrose agar. Soil without added roots was the control (○).

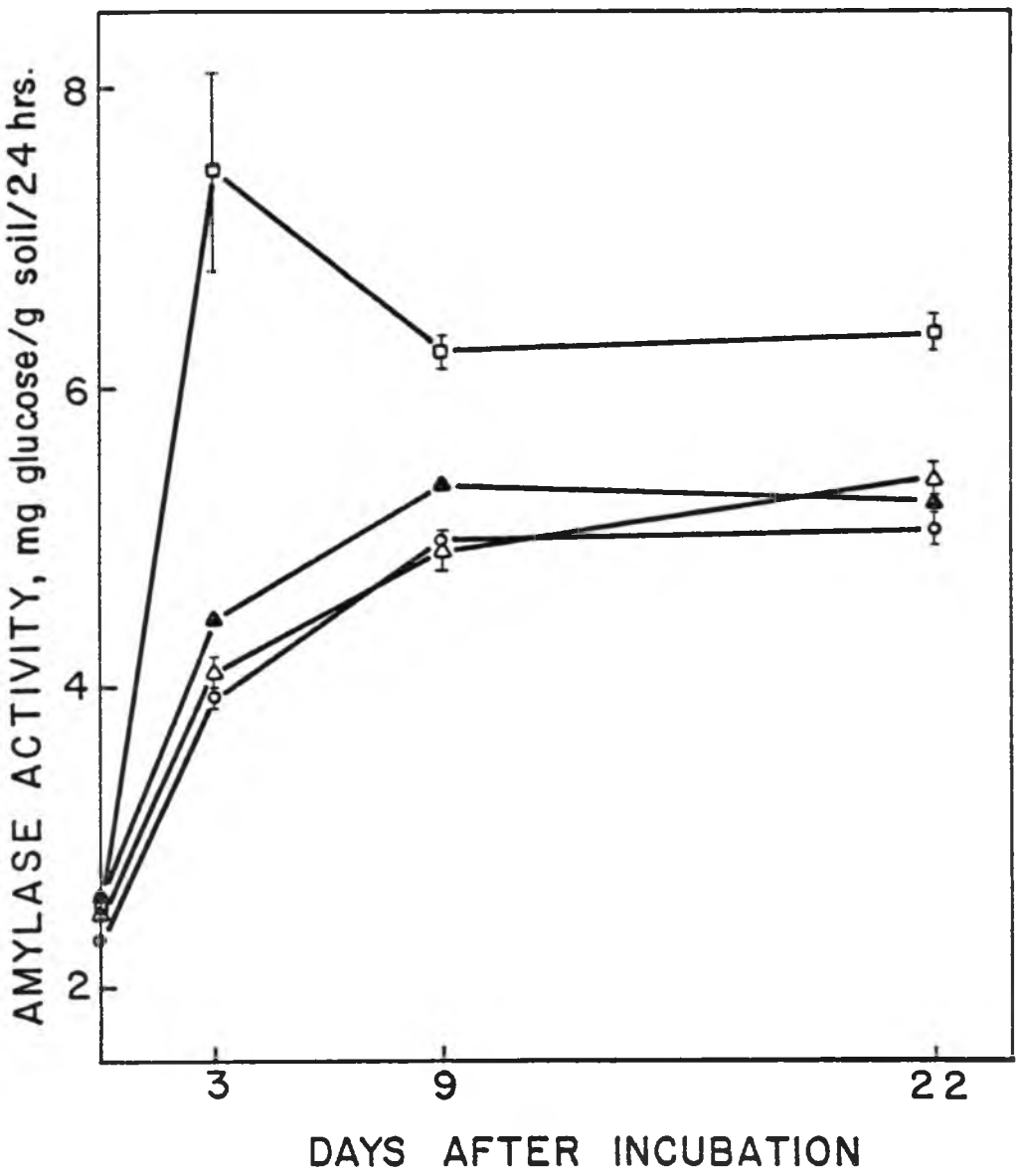

Figure 17. Effect of roots of bigalta (△) and greenalta (▲) limpograsses and Desmodium intortum (□) on the activity of soil amylase. Soil without added roots was used as a control (○).

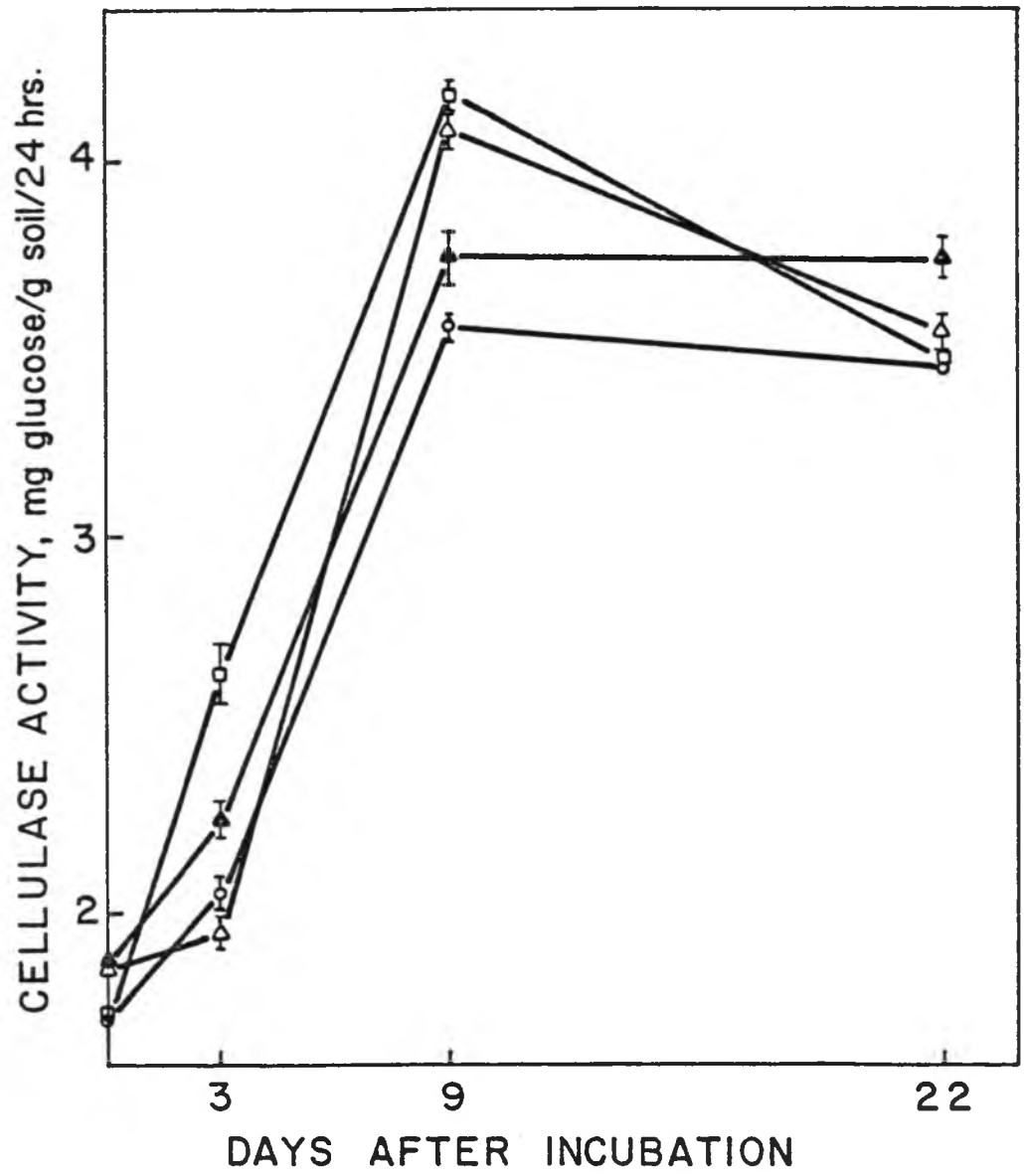

Figure 18. Effect of roots of bigalta (△) and greenalta (▲) limpograsses and Desmodium intortum (□) on the activity of soil cellulase. Soil without added roots was used as a control (○).

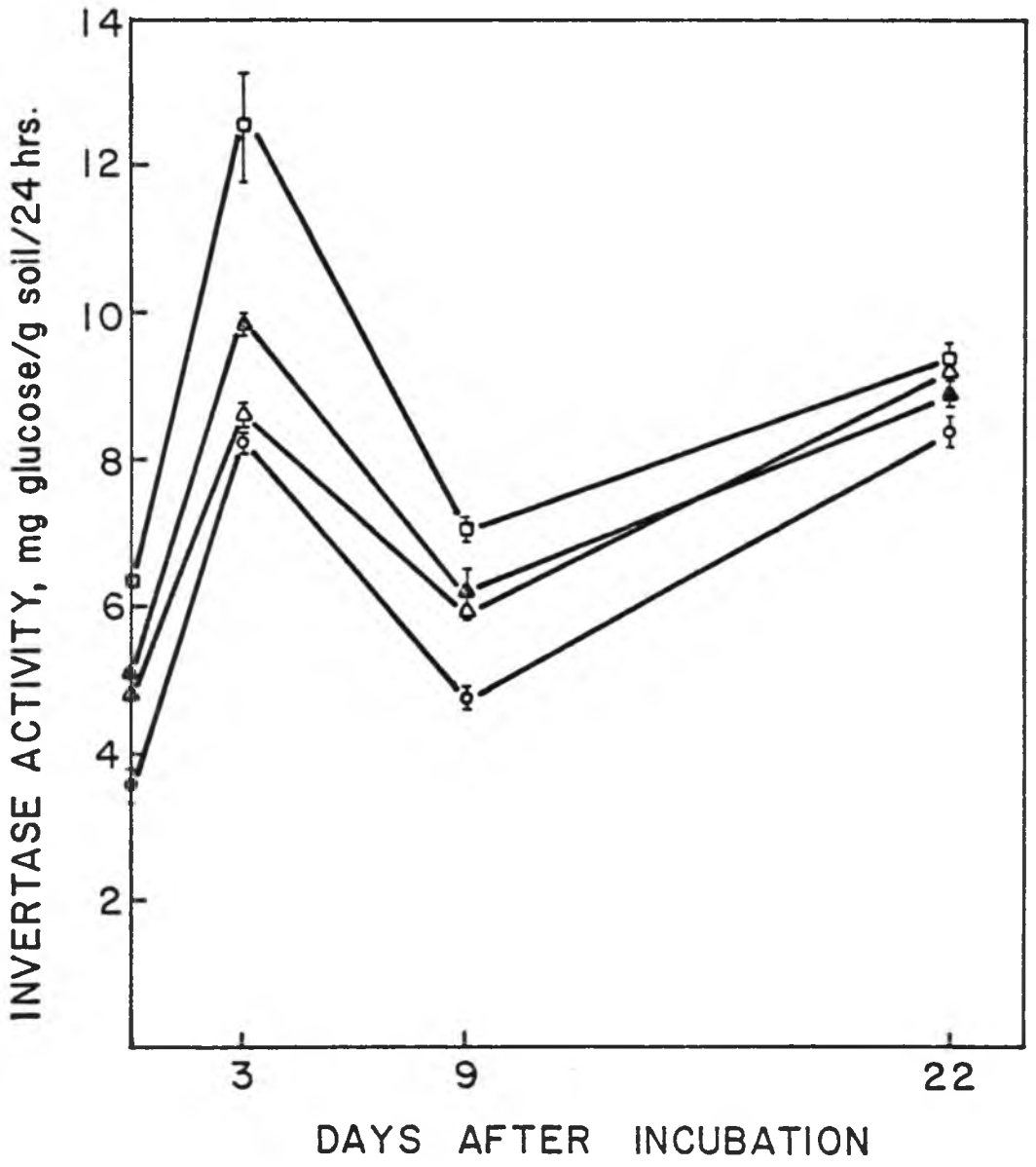

Figure 19. Effect of roots of bigalta (△) and greenalta (▲) limpograsses and Desmodium intortum (□) on the activity of soil invertase. Soil without added roots was used as a control (○).

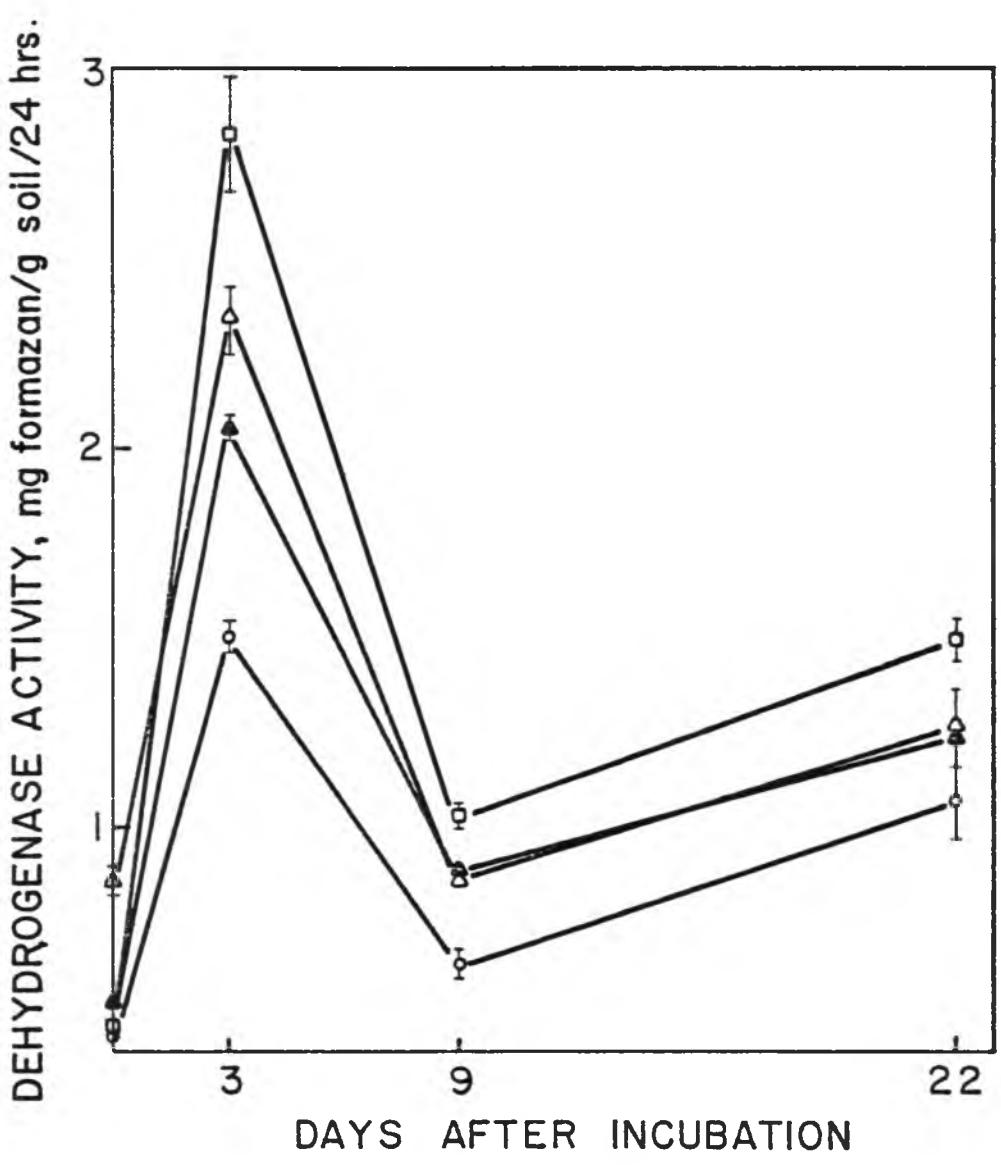

Figure 20. Effect of roots of bigalta (△) and greenalta (▲) limpograsses and Desmodium intortum (□) on the activity of soil dehydrogenase. Soil without added roots was used as a control (○).

treatment were not significantly different from the control soil 3 days after incubation.

C. CO_2 evolution

The CO_2 evolution rate from soil to which roots were added was highest after 3 days of incubation (Figure 21). The rate of CO_2 evolution decreased with time thereafter. All treatments, except the intortum treatment after 9 days incubation had significantly greater rates of CO_2 evolution than the control soil at all measurement times. Carbon dioxide evolution from the intortum treatment was much higher than that from other treatments after 3 days of incubation.

D. Soil pH

There was no obvious effect of treatment on soil pH. Soil pH generally increased with incubation time though the change was only about 0.3 units (Table XXXI).

6.4 Discussion

Greenhouse studies conducted since 1976 showed that well fertilized soil which previously had been planted to bigalta limpograss was inhibitory to intortum grown subsequently in the same pot. Leachate from roots of bigalta and greenalta limpograsses also reduced the growth of intortum to about one fourth that of the control in nutrient-vermiculite cultures.

The results of this study also show that the soil microbial population and some soil enzymes were differentially affected by the addition of the roots of intortum and the two grasses to the soil. In general, microbial populations, enzyme activities and CO_2 evolution from the soil to which desmodium roots had been added were higher than

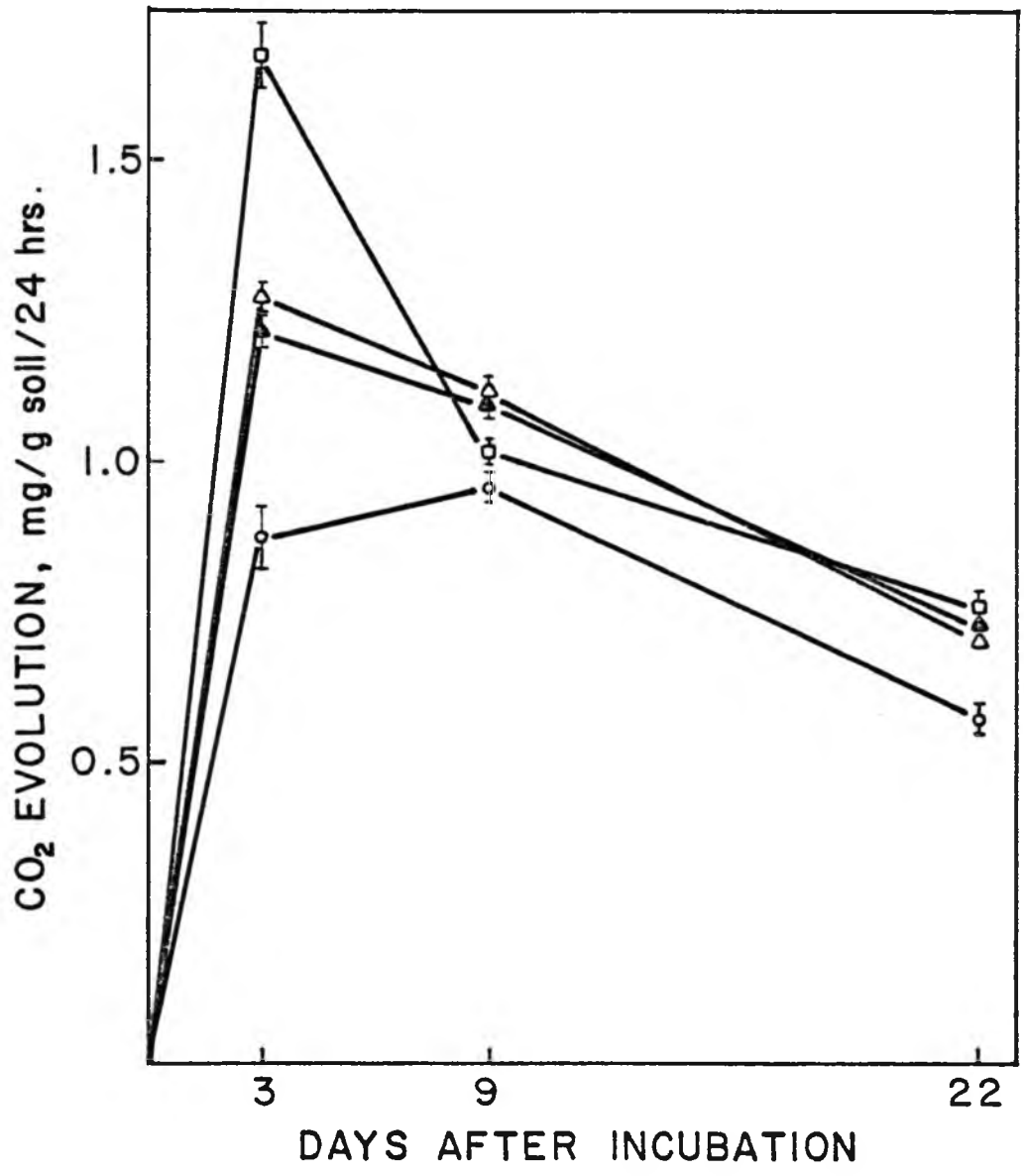

Figure 21. Effect of roots of bigalta (△) and greenalta (▲) limpograsses and Desmodium intortum (□) on the rate of CO_2 evolution from Paaloa soil. Soil without added roots was used as a control (○).

Table XXXI. Soil pH* at different times after incubating dry ground roots with Paaloa soil

Treatment	Days After Incubation		
	3	9	21
Bigalta limpograss	5.33 ± 0.02	5.35 ± 0.03	5.63 ± 0.03
Greenalta limpograss	5.27 ± 0.02	5.37 ± 0.02	5.40 ± 0.00
Intortum	5.23 ± 0.02	5.40 ± 0.03	5.50 ± 0.00
Control	5.20 ± 0.03	5.36 ± 0.02	5.48 ± 0.02

* Three replications ± 1 standard deviation

in soil to which bigalta and greenalta limpograss roots had been added. The higher bacterial and fungal counts in the intortum treatment may have been due to the higher nutrient contents in the desmodium roots than in the limpograsses (Table XXX). The nitrogen level of the tissue may have had the greatest influence because the soil nitrogen content was only 0.18%, a very low level. Ross and Roberts (1968) reported that the activities of amylase and invertase were highly correlated with the amount of organic carbon in four grassland soils in New Zealand. However, Pancholy and Rice (1973a) reported that the levels of amylase, cellulase and invertase in soil were not correlated with the amount of organic matter, but were affected by the type of organic matter (some herb and tree species) added to the soil. No measure of organic carbon content of the soil was made here. However, our results do show that the source of organic matter added to the soil did influence the total activity of the four enzymes assayed in this study. It is possible that chemicals leached from the ground limpograss roots or products produced during decomposition directly inhibited the activities of some soil enzymes, inhibited the growth of certain of the soil microorganisms or inhibited the release of enzymes from soil microorganisms.

Other studies of the associations between soil borne microorganisms and soil enzymes present results which are somewhat conflicting. Several workers have shown that dehydrogenase activity was correlated with bacterial activity (Stevenson, 1959; Hirte, 1963). However, Casida et al. (1964) found a correlation only between gram-positive bacteria and dehydrogenase activity but no relationship between enzyme activity and gram-negative bacteria, fungi or actinomycetes. More

recently Viswanath, et al. (1977) showed that fungi contributed to soil dehydrogenase activity as well.

The evolution of CO_2 from soil provides a measure of the total activity of soil microorganisms whereas plate counts of bacteria and fungi made from selective media indicate the levels of only a fraction of the soil microflora. Our results show that dehydrogenase activity for the combined treatments was quite highly and significantly correlated with CO_2 evolution (the data generally were best fitted by an equation of the type $y = a+bx$), and with bacteria and fungi measured in the two media (the data were best fitted by an equation of the type $y = ax^b$) suggesting that much of the soil microflora in Paaloa soil contributed to soil dehydrogenase activity (Table XXXII). Correlation analyses of the relationships between amylase, invertase and cellulase activity and soil borne organisms detected by plate counts on selective media resulted in coefficients which varied considerably. The source of root material apparently influenced the degree of correlation between enzyme level and selected microbial activity.

The bacteria population was significantly correlated with the activities of all enzymes (amylase, cellulase, invertase and dehydrogenase) when all treatments were combined, but was significantly correlated only with amylase when the treatments were examined separately. Fungi on RBA were significantly correlated with the activities of invertase (negative r), cellulase and dehydrogenase (positive r), for the combined treatments but it was positively and significantly correlated with the activities of amylase in intortum roots treatment and dehydrogenase in the bigalta + greenalta combined data, and was negatively correlated with the activities of amylase and cellulase

Table XXXII. Simple correlation coefficients calculated for relationships between soil enzymes activity and soil microorganisms

	Bacteria	Fungi[+]	Fungi[++]	Evolution
Amylase (T)[§]	0.93**	-0.28	0.67**	0.33*
Cellulase (T)	0.69**	0.89**	0.41	0.33*
Invertase (T)	0.66**	-0.68**	0.69**	0.36*
Dehydrogenase (T)	0.71**	0.83**	0.75**	0.62**
Amylase (B+G)	0.91**	-0.83*	0.62	0.63**
Amylase (D)	0.98**	0.99**	0.79	0.59
Cellulase (B+G)	0.63	-0.95**	0.10	0.54
Cellulase (D)	0.75	-0.88	0.99**	0.61
Invertase (B+G)	0.69	0.66	0.92**	0.17
Invertase (D)	0.67	0.84	0.22	0.76*
Dehydrogenase (B+G)	0.63	0.94**	0.92**	0.39
Dehydrogenase (D)	0.85	0.89	0.47	0.85**
Bacteria	--	0.14	0.73**	
Fungi (1)	--	--	0.37	
Fungi (2)	--	--	--	

[+]Fungal counts made on Rose bengal-streptomycin agar plates

[++]Fungal counts made on potato-dextrose agar plates

[§]Correlation coefficients were calculated using all data (T, n=12; but n = 35 for CO_2 evolution) or the separate sets of data obtained for bigalta (B and greenalta (G) limpograsses (B+G, n = 8, but n = 17 for CO_2 evolution) or for desmodium (D, n = 4; but n = 9 for CO_2 evolution)

*,**Significant at 5 and 1% levels of probability, respectively

in the B+G combined data.

It is known that the mineralization and decomposition of organic matter are inseparably related to the enzyme activities in soil and that extracellular enzymes are produced by many soil borne microorganisms. The ground root material of the three species did have a differential effect on the soil microflora and on the activity of some soil enzymes. The results of studies in hydroponic systems presented in Chapter 4 suggest that the growth inhibition of intortum by bigalta limpograss was due to the direct effect of allelochemicals in root exudates on the growth of intortum. The data obtained in the study reported here also indicates that the allelochemicals in bigalta limpograss are generally toxic to biological organisms rather than selectively inhibiting the growth of one or a few species of microorganisms.

The results of the study raised but do not provide answers to the following questions: 1. What effect does the allelochemical(s) have on the growth of specific microorganisms in soil? 2. What proportion of the total activity of a particular enzyme is released by a particular microorganism in soil? 3. Do chemicals released from organic residues change the proportion of extracellular enzymes produced by a microorganisms? 4. How long is the active life of an enzyme in a given soil environment? 5. How do these enzymes contribute to the recycling of soil nutrients and to the mineral nutrition of higher plants? The resolution of these questions will lead to a better understanding of the relationships between soil microflora and soil organic matter and their subsequent effects on mineral nutrition and plant growth.

6.5 Summary and Conclusions

The addition of finely ground roots of bigalta and greenalta limpograss to Paaloa soil followed by incubation resulted in differential increases in populations of fungi and bacteria. There were also increases in the activities of amylase, cellulase, invertase and dehydrogenase in the soil.

In general, the activities of the microorganisms and of the four enzymes were higher in soil containing desmodium roots. This may have been due to the higher nutrient levels, and particularly nitrogen in the desmodium root. In the presence of bigalta limpograss roots, after three days of incubation, the activities of amylase, cellulase and invertase were not significantly different from soil to which no organic matter had been added. It is possible that chemicals leached from the ground limpograss roots or products produced during decomposition directly inhibited the activities of some soil enzymes, inhibited the growth of certain of the soil microorganisms or inhibited the release of enzymes from soil microorganisms.

Appendix A Table XXXIII. Mineral nutrient contents of tops of Desmodium intortum (D) planted alone and in mixtures with bigalta (D+B) and greenalta (D+G) limpograss in soil containing two levels of phosphorus and plant residues of intortum (D), bigalta (B) and greenalta (G) limpograsses

Soil Treatment			N	P	K	Ca	Mg	Na	Mn	Fe	Cu	Zn
Crop	P, ppm	Residue	%						ppm			
D	0.3	D	3.15	0.21	1.42	2.11	0.46	0.04	152	172	17	52
D		B	3.60	0.15	1.44	2.67	0.40	0.04	206	165	14	78
D		G	3.10	0.21	1.31	2.11	0.46	0.04	118	236	28	58
D+G		D	3.16	0.23	1.37	2.13	0.52	0.04	126	188	14	50
D+B		D	3.37	0.24	1.19	2.12	0.53	0.04	146	184	23	57
D+G		G	3.20	0.21	1.46	2.23	0.46	0.04	135	207	14	50
D+B		B	3.20	0.24	1.75	2.13	0.41	0.05	169	219	12	64
		Mean	3.25	0.21	1.42	2.21	0.46	0.04	150	196	17	58
D	0.04	D	3.25	0.17	2.00	1.97	0.38	0.04	182	164	12	54
D		B	3.86	0.13	1.39	2.42	0.42	0.03	182	135	11	56
D		G	3.35	0.18	1.90	2.20	0.39	0.03	171	166	23	50
D+G		D	3.46	0.16	1.85	2.33	0.42	0.04	176	142	26	52
D+B		D	3.17	0.15	1.51	2.23	0.41	0.04	158	247	22	49
D+G		G	3.50	0.19	2.00	2.12	0.39	0.03	179	193	20	47
D+B		B	3.70	0.17	1.81	2.33	0.41	0.03	177	173	12	56
		Mean	3.47	0.16	1.78	2.23	0.40	0.03	176	174	18	52
	0.3/0.04		N.S.	**	**	N.S.	**	*	*	N.S.	N.S.	N.S.

*, ** Significant at P = 0.05 and 0.01, respectively as determined by Student's t test

N.S. Not significant

Appendix A Table XXXIV. Mineral nutrient contents of tops of bigalta (B) or greenalta (G) limpograsses planted alone and in mixtures with Desmodium intortum (B+D and G+D) in soil containing two levels of phosphorus and plant residues of intortum (D), bigalta (B) or greenalta (G) limpograss

Crop	Soil Treatment P, ppm	Residue	N	P	K	Ca	Mg	Na	Mn	Fe	Cu	Zn
			%						ppm			
B	0.3	D	1.70	0.15	1.50	0.75	0.56	0.36	152	230	10	52
B		B	1.97	0.15	0.97	1.13	0.44	0.25	138	224	12	62
B+D		D	1.77	0.17	1.53	0.72	0.44	0.44	146	284	12	54
B+D		B	2.10	0.15	1.67	1.07	0.31	0.31	142	196	14	79
		Mean	1.89	0.16	1.42	0.92	0.44	0.34	145	233	12	61
B	0.04	D	1.85	0.13	2.08	0.79	0.35	0.35	186	194	24	62
B		B	1.98	0.11	1.68	0.93	0.32	0.32	150	209	20	54
B+D		D	1.76	0.13	2.25	0.71	0.38	0.38	174	280	22	56
B+D		B	2.06	0.13	1.90	0.93	0.30	0.30	149	184	22	58
		Mean	1.91	0.13	1.98	0.82	0.34	0.34	165	217	22	57
	0.3/0.04		N.S.	**	*	N.S.	N.S.	N.S.	N.S.	N.S.	**	N.S.
G	0.3	D	1.55	0.14	1.60	0.47	0.20	0.32	107	214	11	54
G		G	1.60	0.15	1.34	0.57	0.27	0.32	104	254	12	78
G+D		D	1.55	0.15	1.59	0.42	0.20	0.28	104	202	12	62
G+D		G	1.65	0.15	1.51	0.58	0.22	0.22	102	142	12	61
		Mean	1.59	0.15	1.51	0.51	0.22	0.29	104	203	12	64
G	0.04	D	1.50	0.14	1.93	0.42	0.20	0.20	128	166	18	55
G		G	1.66	0.12	1.57	0.57	0.21	0.21	114	165	14	50
G+D		D	1.50	0.13	1.85	0.43	0.19	0.19	126	115	14	48
G+D		G	1.60	0.13	1.78	0.45	0.22	0.22	114	165	16	44
		Mean	1.57	0.13	1.78	0.47	0.21	0.21	120	152	15	49
	0.3/0.04		N.S.	*	*	N.S.	N.S.	*	**	N.S.	**	*

*,** Significant at P = 0.05 and 0.01, respectively as determined by Student's t test

N.S. Not significant

Appendix A Table XXXV. Effects of soil treatments on the average increase in plant height with time after planting (mean of three replications) of Desmodium intortum in soil containing 0.3 ppm phosphorus

Crop*	Residue**	Plant Height (cm) Days 27	31	40	77
D	D	4.9 b+	7.2 b	14.0 b	55.1 ab
D	B	3.1 d	4.7 d	8.0 d	27.3 c
D	G	6.6 a	10.3 a	17.8 a	56.5 ab
D+G	D	3.8 c	6.8 c	12.2 bc	59.3 ab
D+B	D	4.5 b	6.7 c	11.5 c	48.9 b
D+G	G	4.9 b	7.3 b	12.9 bc	67.8 a
D+B	B	2.9 d	3.9 d	5.5 e	25.1 c

* Intortum (D) plants were grown alone and in mixtures with bigalta (D+B) or greenalta (D+G) limpograss

** Soil was used in which intortum (D), bigalta (B), or greenalta (G) had been grown previously

+ Data within columns followed by the same letter are not significantly different (P = 0.05) by Duncan's multiple-range test

Appendix A Table XXXVI. Effects of soil treatments on the average plant height (mean of three replications) of Desmodium intortum in soil containing 0.04 ppm phosphorus at different times after planting

Crop*	Residue**	Plant Height (cm) Days 27	31	40	77
D	D	2.9 ab+	4.3 bc	7.0 bc	26.9 bc
D	B	3.0 ab	4.7 ab	7.5 bc	21.0 c
D	G	3.6 a	5.2 ab	8.5 ab	30.3 ab
D+G	D	3.0 ab	3.8 bc	6.3 c	25.0 bc
D+B	D	3.5 a	5.7 a	10.2 a	31.4 ab
D+G	G	3.7 a	5.5 a	8.6 ab	40.0 a
D+B	B	2.5 b	3.6 c	6.0 c	19.5 c

* Intortum (D) plants were planted alone and in mixtures with bigalta (D+B) or greenalta (D+G) limpograss

** Soil was used in which intortum (D), bigalta (B) or greenalta (G) limpograss had been grown previously

\+ Data within columns followed by the same letter are not significantly different (P = 0.05) by Duncan's multiple-range test

Appendix A Table XXXVII. Mineral nutrient contents of tops of acceptor, Desmodium intortum planted with various donor plants in pots with and without dividers in Hoagland's solution-vermiculite culture

For Treatment	Donor Treatment	N	P	K	Ca	Mg	S	Si	Na	Cl	Al	Mn	Fe	Cu	Zn
		%									ppm				
With divider	Control	2.93 ab+	0.31 a	2.67 a	1.56 b	0.47 a	0.17 c	0.47 bc	0.08 a	0.52 d	0 a	80.3 b	143 a	5.6 a	27.3 b
	Bigalta	3.22 a	0.25 b	2.58 a	1.96 a	0.58 a	0.18 bc	0.41 c	0.08 a	0.67 b	0 a	116.0 a	177 a	6.7 a	32.0 b
	Greenalta	3.02 a	0.24 b	2.59 a	1.70 b	0.51 a	0.17 c	0.37 d	0.08 a	0.55 cd	0 a	86.3 b	149 a	6.0 a	25.7 b
	Intortum	3.00 a	0.19 c	2.47 a	2.08 a	0.66 a	0.23 a	0.50 b	0.09 a	0.83 a	0 a	113.0 a	170 a	5.7 a	37.3 a
Without divider	Control	2.60 b	0.21 bc	2.56 a	1.66 b	0.53 a	0.19 bc	0.53 b	0.08 a	0.62 c	0 a	96.7 ab	225 a	2.7 a	29.7 b
	Bigalta	3.17 a	0.17 c	2.51 a	2.08 a	0.69 a	0.17 c	0.36 d	0.08 a	0.57 cd	0 a	89.7 b	330 a	3.3 a	40.3 a
	Greenalta	2.94 ab	0.16 c	2.51 a	1.87 ab	0.42 a	0.16 c	0.32 d	0.08 a	0.52 d	0 a	80.7 b	158 a	4.3 a	30.7 b
	Intortum	3.05 a	0.20 bc	2.44 a	2.06 a	0.72 a	0.21 ab	0.68 a	0.08 a	0.70 b	0 a	113.3 a	225 a	2.7 a	41.3 a

+ Means in columns followed by the same letter are not significantly different (P = 0.05) by Duncan's multiple-range test

Appendix A Table XXXVIII. Mineral nutrient contents of tops of acceptor, Desmodium intortum planted with variance donor plants in pots with and without dividers in the nutrient A-vermiculite culture

Pot Treatment	Donor Treatment	N	P	K	Ca	Mg	S	Si	Na	Cl	Al	Mn	Fe	Cu	Zn
		%									ppm				
With divider	Control*	3.07 b+	0.45 ab	2.68 b	1.64 a	0.67 a	0.29 b	0.71 a	0.08 a	0.87 b	0.0 a	131.0 a	550.7 a	5.33 a	36.33 a
	Bigalta	2.94 b	0.34 c	3.01 a	1.79 a	0.80 a	0.34 b	0.63 a	0.08 a	0.91 ab	0.0 a	179.7 a	756.0 a	7.00 a	38.67 a
	Greenalta	2.63 b	0.39 bc	2.85 ab	1.71 a	0.67 a	0.32 b	0.56 b	0.08 a	0.83 b	0.0 a	156.0 a	584.7 a	6.33 a	34.00 a
	Intortum	3.01 b	0.33 c	2.67 b	1.81 a	0.80 a	0.43 a	0.70 a	0.08 a	1.16 a	0.0 a	143.7 a	635.3 a	7.00 a	49.67 a
Without divider	Control	3.48 a	0.49 a	2.81 ab	1.63 a	0.73 a	0.23 c	0.66 a	0.08 a	0.69 b	0.0 a	130.0 a	394.00	4.67 a	31.00 a
	Bigalta	3.13 ab	0.33 c	2.98 a	1.61 a	0.67 a	0.33 b	0.44 c	0.08 a	0.84 b	0.0 a	145.7 a	693.7 a	8.00 a	32.67 a
	Greenalta	2.80 b	0.39 bc	2.66 b	1.77 a	0.75 a	0.30 bc	0.60 b	0.08 a	0.90 ab	0.0 a	154.3 a	117.3 a	5.33 a	34.00 a
	Intortum	2.69 b	0.34 c	2.42 c	1.55 a	0.64 a	0.35 ab	0.65 a	0.08 a	1.04 ab	0.0 a	129.7 a	380.3 a	7.67 a	39.67 a

+ Data in columns followed by the same letter are not significantly different (P = 0.05) by Duncan's multiple-range test

* Desmodium intortum seedlings were used as the control

Appendix A Table XXXIX. Mineral nutrient contents of tops of acceptor, D. Intortum planted with various donor plants in pots with and without dividers in N-free nutrient-vermiculite culture

Pot Treatment	Donor Treatment	N	P	K	Ca	Mg	S	Si	Na	Cl	Al	Mn	Fe	Cu	Zn
		%									ppm				
With divider	Control	3.63 a+	0.53 a	3.10 a	1.18 b	0.31 bc	0.40 b	0.62 b	0.08 a	1.02 bc	0 a	91 ab	260 ab	9 a	62 a
	Bigalta	3.57 a	0.45 b	3.09 a	1.27 ab	0.28 c	0.36 b	0.41 c	0.08 a	0.88 c	0 a	71 b	213 b	10 a	50 b
	Greenalta	3.42 a	0.49 ab	3.08 a	1.21 b	0.28 c	0.33 b	0.38 c	0.08 a	0.89 c	0 a	73 b	218 b	12 a	47 b
	Intortum	3.07 b	0.22 c	2.94 a	1.37 a	0.47 a	0.63 a	0.84 a	0.07 a	1.49 a	0 a	104 a	242 b	10 a	62 a
Without divider	Control	3.72 a	0.41 b	3.17 a	1.22 b	0.30 bc	0.37 b	0.66 b	0.08 a	1.12 b	0 a	101 a	223 b	9 a	61 a
	Greenalta	4.24 a	0.51 a	3.32 a	1.37 a	0.34 b	0.35 b	0.35 c	0.08 a	0.84 c	0 a	90 ab	310 a	13 a	64 a

+ Means in columns followed by the same letter are not significantly different (P = 0.05) by Duncan's multiple-range test

Appendix A Table XXXX. Effect of dried root material on CO_2 evolution from soil at different incubation times

Root Material Added	Incubation Time, Days		
	3	9	22
	mg CO_2/g soil/24 hrs		
Control	0.85 c	0.95 b	0.58 b
Bigalta	1.26 b	1.12 a	0.71 a
Greenalta	1.22 b	1.11 a	0.74 a
Intortum	1.68 a	1.03 ab	0.76 a

+ Data within columns followed by the same letter are not significantly different (P = 0.05) by Duncan's multiple range test

Appendix A Table XXXXI. Effects of dried root material on the activities of soil enzymes after different incubation times

Root Material Added	Amylase+ Incubation Times, Days				Invertase Incubation Times, Days				Cellulase Incubation Times, Days				Dehydrogenase Incubation Times, Days			
	0	3	9	22	0	3	9	22	0	3	9	22	0	3	9	22
Control	2.27 b++	3.95 b	4.95 c	5.07 b	3.57 c	8.29 b	4.78 c	8.43 a	1.71 b	2.05 b	3.57 b	3.48 a	0.46 b	1.50 c	0.64 c	1.07 a
Bigalta	2.50 a	4.10 b	4.93 c	5.39 b	4.81 b	8.63 b	5.99 b	9.24 a	1.86 a	1.95 b	4.09 a	3.56 a	0.85 a	2.35 ab	0.85 b	1.26 a
Greenalta	2.59 a	4.46 b	5.35 b	5.21 b	5.08 b	9.87 b	6.16 ab	8.94 a	1.87 a	2.24 b	3.76 b	3.75 a	0.54 b	2.05 b	0.87 b	1.24 a
Intortum	2.57 a	7.45 a	6.25 a	6.32 a	6.37 a	12.57 a	7.09 a	9.29 a	1.91 a	2.63 a	4.19 a	3.47 a	0.48 b	2.82 a	1.02 a	1.50 a

+ Activities of amylase, invertase and cellulase are mg glucose per g soil per 24 hrs and dehydrogenase activity is mg formazan per g soil per hr

++ Data within columns followed by the same letter are not significantly different (P = 0.05) by Duncan's multiple-range test

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