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IMPACT OF THE ISOFLAVONE FORMONONETIN ON THE FORMATION OF TRIFOLIUM-GLOMUS MYCORRHIZAL SYMBIOSIS

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Aysegul Ozan

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Ronald J. Patterson
Major professor

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IMPACT OF THE ISOFLAVONE FORMONONETIN ON THE FORMATION OF *TRIFOLIUM-GLOMUS* MYCORRHIZAL SYMBIOSIS

By

Aysegul Ozan

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ABSTRACT

IMPACT OF THE ISOFLAVONE FORMONONETIN ON THE FORMATION OF *TRIFOLIUM-GLOMUS* MYCORRHIZAL SYMBIOSIS

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Activity and isozyme patterns of NAD-malate dehydrogenase and peroxidase were investigated at early stages of *Trifolium-Glomus* mycorrhizal development. Fungal NAD-malate dehydrogenase isozymes were detected as early as one week of growth. Activity of these isozymes increased in the presence of formononetin, a mycorrhiza-stimulatory isoflavone. Root colonization did not differ in one week old mycorrhiza with or without exogenously applied formononetin. By the end of two weeks, root colonization increased 50% in the presence of this isoflavone. At this stage, soluble peroxidase activity was lower in mycorrhizal roots than non-mycorrhizal roots. Formononetin caused a decrease in soluble peroxidase activity both in mycorrhizal and non-mycorrhizal roots. The results suggested that exogenously applied formononetin has a dual effect on the *Trifolium-Glomus* mycorrhiza formation: triggering the plant-dependent growth mechanisms of the mycorrhizal fungus and lowering the soluble peroxidase activity in roots, which may facilitate the intracellular spreading of hyphae.

In vitro effects of several plant phenolic compounds on the total soluble peroxidase activity of cell-free white clover root extracts were determined. Soluble peroxidases were able to utilize many plant phenolic compounds, except formononetin and caffeic acid, as hydrogen donors. The results suggested that formononetin mediated reduction in the specific

activity of white clover root peroxidases did not occur via direct inhibition of the enzyme.

Persistence of the exogenously applied bioactive isoflavones (formononetin and biochanin A) in soil was investigated. The amounts recovered did not change in sterile soil, however, recovery of the isoflavone amendments declined in non-sterile soil. The decline was more rapid with biochanin A than with formononetin. In soil planted with corn seedlings, the amounts recovered dropped dramatically for both isoflavones. The results suggested that soil microbial populations were able to metabolize these isoflavones. Measurements of vertical migration in soil indicated that when applied as a solution, higher concentrations of formononetin were recovered at several distances below the application point than when applied as a solid. The responses of several microbial populations to isoflavone amendments were measured in soil samples obtained from a potato field. The results indicated that the isoflavones formononetin and biochanin A were able to stimulate the growth of soil microorganisms.

To my parents and my husband

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CHAPTER 1

INTRODUCTION

Vesicular-arbuscular mycorrhizal fungi

Mycorrhizas are symbiotic associations that occur between soil fungi and plant roots. These associations are so widespread in nature that the plants that do not form mycorrhizal symbioses can be considered exceptional (Gerdemann, 1968; Newman and Reddel, 1987). Vesicular-arbuscular mycorrhizas are the most common of the mycorrhizal forms (Bonfante-Fasolo, 1987). The microsymbionts that form vesicular-arbuscular mycorrhizas belong to the taxonomic class Zygomycetes (Gerdemann and Trappe, 1974). Five major genera of the family Endogonaceae form vesicular-arbuscular mycorrhizal (VAM) associations: *Glomus*, *Gigaspora*, *Acaulospora*, *Sclerocystis* and *Scutellospora* (Trappe and Schenck, 1982).

In soil, clamydospores of VAM fungi germinate and hyphae elongate in a short period of time. If the elongating hyphae come in contact with plant roots, they continue to grow. However, if there are no plant roots in the vicinity, the growth ceases and the dormant state is resumed (Paul And Clark, 1989; Gianinazzi, 1991). Germination usually does not require plant factors, but the presence of certain plant volatile compounds and metabolites in root exudates have been found to favor subsequent hyphal

growth (Becard and Piche, 1989b). The direction of hyphal growth is thought to occur randomly. Since chemotaxis has not been demonstrated, the encounter of elongating hyphae with plant roots is likely a chance event. The possible signalling between the roots and the hyphae is thought to occur only within a few millimeters distance between the hyphal tip and the root (Becard *et al.*, 1989; Gianinazzi-pearson *et al.*, 1990).

The adhesion and penetration of the VAM fungal hyphae can occur through the root hairs or between epidermal cells. The hyphae then grow inter- and intracellularly in epidermal and cortical cells (Bonfante-Fasolo, 1984). As with some pathogen infections, the hyphal growth does not proceed beyond the cortex (Gianinazzi, 1991). Intercellularly growing hyphal branches form vesicles, which are the site of storage for fungal lipids (Cooper, 1984). Arbuscules are differentiated hyphal structures that occur inside the plant cells. These highly branched intracellular structures look like a small trees under the microscope. The arbuscule, with its high surface-to-volume ratio, is a major site of nutrient exchange between the plant cells and the fungus. This view on arbuscules as being the site of nutrient exchange is supported by an investigation that reported an increase in H^+-ATPase activity in arbuscular membranes as well as in peri-arbuscular membranes (PAM, plant membrane surrounding the arbuscule) as an indication of high transport activity (Gianinazzi-Pearson *et al.*, 1991). Arbuscules persist 4 to 10 days, then they are degraded by the plant cells and new ones are formed (Alexander *et al.*, 1988; Jacquelinet-Jeanmougin *et al.*, 1988).

In their work on the early events of vesicular-arbuscular mycorrhiza formation on Ri T-DNA transformed carrot roots, Becard and Fortin (1988) recognized three sequential stages in the development of infection. The

first stage is the attachment of the fungus to the root surface without penetration. The second is penetration and intercellular spreading of the hyphae. The final stage is the spreading of the intracellular hyphae (arbuscule formation). Becard and Fortin have found that there is a two day interval between attachment and penetration. They also suggested that this period of contact without penetration is the most critical step, in that either the infection proceeds or the following steps are aborted. They suggested that this interval may be an adaptation period to activate the recognition mechanisms and the synthesis of enzymes. Their work provided valuable information for the timing of the early events, since the experiments used only one spore of a VAM fungus and one piece of transformed root.

Dense hyphal growth does not occur either on the surface or in the roots in VAM associations. This fungus does not support dense hyphal growth in vitro either. Attempts to grow the fungus axenically have been unsuccessful thus far. Due to its dependence on living plants for its growth and propagation, this fungus is regarded as an obligate symbiont. Since growth can only be studied in conjunction with a living plant, biochemical and molecular biology investigations of the VAM fungi have been greatly hindered (Paul and Clark, 1989).

Methods of quantification for colonization and fungal mass

VAM associations do not produce obvious visual clues in plant roots, except in a few plant species where colonization by the fungus causes a slight yellowing of the roots. The colonization can be assessed microscopically after clearing the roots with potassium hydroxide and staining (Phillips and Hayman, 1970). Acid fuchsin and trypan blue are

the most commonly used dyes. The destaining step removes most of the stain from the plant cells, while the fungal hyphae retain the dye. Fungal structures can thus be observed under a microscope or a dissecting scope. Colonization is usually measured with the line intersect method (Kormanik and Mcgraw, 1982) and reported as percent root length colonized. This refers to the number of root pieces bearing fungal structures divided by the total number of root pieces counted. In this procedure, roots are cut into approximately 1 cm pieces. Colonization can be assessed biochemically also, in which the glucosamine (a component of fungal cell wall which is absent in plant roots) content of the roots is measured with a colorimetric assay (Ride and Drysdale, 1972).

Functioning of the mycorrhizal symbiosis

Many studies have demonstrated improved growth of plants colonized by VAM fungi compared to non-mycorrhizal plants under variety of conditions (Crews *et al.*, 1978; Fairweather and Parbery, 1982; Safir, 1980). It is well known that VAM fungi lack host-specificity (Gianinazzi-Pearson, 1984; Bonfante-Fasolo, 1987), however, effectiveness of different isolates or species can vary (Sylvia *et al.*, 1993). As VAM fungi are adapted to different edaphic conditions and may have unique physiological properties (Lambert *et al.*, 1980), the growth responses of the host plants can also vary (Haas and Krikun, 1985).

The major contribution of the fungi in the mycorrhizal symbiosis is in nutrient uptake and translocation. In return, plants supply carbon compounds necessary for the growth of the fungus (Jakobsen and Rosendahl, 1990). The mechanism of this enhanced nutrient uptake by mycorrhizal plants is partly due to the greatly increased soil volume explored by the

extramatrical hyphae of the VAM fungus (Hayman, 1983). The nutrients phosphorus, nitrogen (Haystead *et al.*, 1988), zinc (Gilmore, 1971), copper (Timmer and Leyden, 1980), sulphur (Gray and Gerdeman, 1973)and iron (Treeby, 1992) have been found to be absorbed and translocated (Rhodes and Gerdeman, 1975) to the host by mycorrhizal fungi. Among these mineral nutrients, phosphorus is considered by far the most important for functioning of the association (Gerdemann, 1968; Mosse, 1973). Mycorrhizal fungi are able to absorb phosphorus at lower solution concentrations than the uncolonized plant roots (Hayman and Mosse, 1972). In addition, the fungi have the ability to solubilize forms of phosphate, such as phytate, which is not accessible to plant roots (Jayachandran *et al*, 1992). Generally, plant growth is improved by the fungi in soils with lower concentrations of phosphate in the solution phase, provided that the soil has adsorbed phosphorus or phosphorus reserves in some insoluble forms. Many mycorrhiza-forming plants have the ability to inhibit mycorrhizal infection at high concentrations of phosphorus (Hepper, 1983).

In addition to improved mineral nutrient status, mycorrhizal plants tolerate drought. Drought-tolerance increases in mycorrhizal plants by the addition of phosphorus. The effect of mycorrhizal fungi on the tolerance has been found to be mediated by the ability of the fungus to maintain adequate phosphorus nutrition under water stress (Nelsen and Safir, 1982). Mycorrhizal plants also exhibit tolerance to salt stress, but salt tolerance may not be related to the improved nutrient status by the fungus (Rosendahl and Rosendahl, 1991).

The fungi protect the roots from pathogens (Rosendahl and Rosendahl, 1990), nematodes (Kellam and Schenck, 1980), and heavy metal toxicity (Read, 1986). The protection from heavy metal toxicity is especially

important in the highly contaminated areas such as mine spoils for land reclamation studies. Protection against pathogens is thought to occur due to the mechanical protection and antibiotic production by the fungi (Paul and Clark, 1989).

Although, there are many benefits of the mycorrhizal association which give a competitive edge to host plants in nature, the fungi can become parasitic when the nutrient levels in the soil are so low that the fungus can no longer extract minerals for the plants but continues to share plants' carbon. Therefore, the successful mutualism depends on the balance between the plants ability to supply the fungi with carbon compounds while receiving mineral nutrients and benefits provided by the fungi. In a well balanced symbiosis, carbon supply may not be costly since the host plant will more fully use its photosynthetic capacity, which may otherwise be underutilized (Paul and Clark, 1989).

Importance of isozymes in mycorrhizal research

Identification of VAM fungi based on their morphological traits has been a common practice. At the generic level, the formation and morphology of the spores, mode of germination and the morphology of the sporocarp (if present) serve as reliable characteristics for identification. However, species-level identification based on visual characteristics of resting spores has been considered less reliable since there is a low degree of morphological divergence at the species level. VAM fungi (*Glomus*-like species) have been recognized in the rhizomes of fossil plants (Nicolson, 1975; Pirozynski and Mallock, 1975; Koske, 1985). This indicates that selection pressure on the morphological traits of the VAM fungi has not been significant. However, a low degree of similarity

in isozyme patterns has been found among species. Therefore, it is thought that divergence of physiological processes of fungi has been faster than that of spore morphology (Rosendahl, 1989).

Sen and Hepper (1986) characterized several *Glomus* species by selective enzyme staining following electrophoretic separation on polyacrylamide gels of fungal proteins extracted from resting spores. This was the first study in which the mobility of specific enzymes during electrophoresis was used to investigate the physiological variations among the species of the same genus for VAM fungi, although mobility of specific enzymes has been used to characterize many other organisms, including fungi, for some time (Garber, 1973; Hall, 1969). Furthermore, it has been found that different isolates of the same species also exhibit variations in their isozyme patterns (Hepper *et al.*, 1988). This is thought to be an indication of a high degree of physiological adaptation of the fungi.

Since VAM fungi can not be isolated from the host plants and axenically cultured, identification of species colonizing plant roots has always been difficult, especially in the root samples collected from fields. In addition to the establishment of the isozyme patterns for several enzymes for the resting spores of some *Glomus* species, protein extracts from colonized roots of leek and maize plants have also been analyzed (Hepper *et al.*, 1986). Comparison of the isozyme patterns of resting spores with that of colonized and uncolonized roots offered the possibility of using some fungal isozymes for diagnostic purposes. External and internal hyphae have been removed and analyzed also. The preparation of internal hyphae requires enzymatic digestion of the plant roots followed by mechanical manipulation under the microscope, thus the

investigators emphasized that isozyme patterns obtained from hyphae, especially internal hyphae, should not be regarded as highly reliable.

Plant roots can be colonized by more than one VAM fungal species. The isozyme analysis based on diagnostic fungal enzymes indicated that the competitive ability of species differs (Hepper *et al.*, 1988). In addition, among co-occurring plant species the presence of particular groups of VAM fungi in roots is dependent on the host plant and the harvest time. Isozyme analysis, coordinated with morphological inspections, indicated that some species of VAM fungi can exclude less competitive species (Rosendahl *et al.*, 1989). Therefore, the fate of field inoculations of VAM fungi is determined by the competitive ability of the introduced species over indigenous species (Sen *et al.*, 1989).

Use of diagnostic enzymes has also allowed the quantification of VAM fungi colonizing the roots. Rosendahl *et al.* (1989) found a positive correlation between fungal enzyme peak heights (measured from the densitometric tracings of the bands appearing on the activity stained gels) and glucosamine content. This finding indicates that band intensity of a diagnostic fungal isozyme can be used to estimate the fungal mass associated with the roots. Determining the contribution of a single species in a mixed population has been possible with this technique. By using this technique in combination with an experimental procedure that involves splitting of the roots, it has been found that only minor changes occurred in levels of the root enzymes of the host plant, *Cucumis sativus*, but fungal malate dehydrogenase activity reached up to 40% of the host's activity at six weeks of growth (Rosendahl, 1992).

The enzyme systems used for the identification of fungal activity in roots have included NAD-malate dehydrogenase, glucose-6-phosphate

dehydrogenase, glutamate oxaloacetate transaminase, glutamate dehydrogenase, esterase, and peptidase. The enzymes used for diagnostic purposes are usually involved in the primary metabolic processes, and serve as indicators of fungal activity both in qualitative and quantitative assays.

Plant responses to VAM fungi as mutualistic colonizer

The attack of pathogenic microorganisms and interactions with gall mites elicit defense responses in plants. Defense responses may vary depending on host plants and pathogens. Generally, increased peroxidase activity has been observed following pathogen attack (Bashan *et al*, 1987; Parent *et al.*, 1985; Ward, 1986). This common reaction of plants is thought to be due to the non-specific induction of plant resistance (Kuc, 1966; Bronner *et al.*, 1991). Peroxidases catalyze cross-linking and polymerization of cell wall associated phenolic polymers, under the conditions that require reinforcement of cell walls (Ride, 1980; Dean and Kuc, 1987). These enzymes have been found to oxidize a variety of plant phenolic compounds (Putter and Becker, 1983). As they are involved in polymerization of the aromatic monomers to lignin for cell wall fortification, they are also active in cellular interconversion and catabolism of phenolic compounds (Barz, 1977).

Penetration of VAM fungi into the roots has been found to cause a transient increase in the activity of cell-wall-bound peroxidases in leek (*Allium porrum*) roots (Spanu and Bonfante-Fasolo, 1988). Similarly, chitinase activity has been found to increase in the cell-free extracts of mycorrhizal leek roots (Spanu *et al.*, 1989; Dumas-Gaudot *et al.*, 1992). Chitin is a major component of most fungal cell walls. Chitinases can

degrade chitin and also exhibit lysozyme activity (Boller *et al.*, 1983). Therefore, it has been suggested that chitinases like peroxidases may also be involved in defense reactions of plants against invading pathogens. Chitinase activity in VAM roots also exhibits a transient increase then falls below the activity of non-mycorrhizal roots. These studies on leek roots indicate that both peroxidase and chitinase activities peak between the second and third week of growth. In addition, the peak of chitinase activity occurs during the same time period in mycorrhizal alfalfa roots, then falls below the level of non-mycorrhizal roots (Volpin *et al.*, 1994). These findings indicate that mycorrhizal penetration and early stages of colonization elicit weak non-specific plant defense reactions, which later are suppressed. The suppression of defense reactions may have a physiological significance in facilitating the colonization of the mutualistic microsymbiont.

Leguminous plants as hosts for VAM fungi and Rhizobium

In the mycorrhizal roots, additional proteins have been detected, which are absent in the resting spores and in the uncolonized plants (Dumas *et al.*, 1989; Pacovsky, 1989). These symbiosis-specific proteins have been called mycorrhizins. Some of these proteins are immunologically cross-reactive with nodulins in soybean plants (Wyss *et al.*, 1990). Since nodulins are expressed in the leguminous plant species at the onset of *Rhizobium* infection, this finding suggests that in legumes mycorrhiza formation and rhizobium nodulation share some of the symbiosis-specific proteins of the host plant roots. The functions of some nodulins and all of the mycorrhizins are still not known.

Legumes are tripartite symbiotic associations that occur between a leguminous host plant, Rhizobial bacteria and mycorrhizal fungi (Paul and Clark, 1989). Nodulated mycorrhizal plants exhibit some biochemical differences compared to dual symbioses either with *Rhizobium* or with mycorrhiza (Pacovsky *et al.*, 1991).

Leguminous plants produce a variety of phenolic compounds (Porter *et al.*, 1985; Porter *et al.*, 1986; Smith *et al.*, 1986b; Gildersleeve *et al.*, 1991). Most of the research involved in determining the composition and metabolism of phenolic compounds has been done on soybeans, alfalfa, clover, chick peas and beans (*Phaseolus* spp.). Among phenolic compounds, flavonoid metabolism of leguminous plants has been given special attention, since these secondary plant metabolites are involved in variety of physiological activities. Flavonoid concentrations respond to environmental stresses (Osman and Fett, 1983) and pathogen attacks (Olah and Sherwood, 1971). Some isoflavones have been demonstrated to be the precursors of phytoalexins and antifungal compounds (Dewick, 1975). In addition, some isoflavones are found to act as a preinfectional inhibitor (Willeke and Barz, 1982). Flavonoids naturally released from the roots of alfalfa (*Medicago sativa*) plants have been found to stimulate or inhibit germination as well as hyphal elongation of VAM fungi (Tsai and Phillips, 1991). Similarly, some of these flavonoid compounds stimulate the growth of *Rhizobium* species in rhizosphere soil (Hartwig *et al.*, 1991) and some are actually *nod* gene inducers (Recourt *et al.*, 1991; Lean-Barrios *et al.*, 1993; Maxwell and Phillips, 1990). Therefore, some of the flavonoids are thought to play an important role in signalling between the microorganisms that can form symbiotic associations and the host plants (Phillips, 1992).

Stimulation of vesicular-arbuscular mycorrhiza formation by isoflavones

Root exudates from white clover plants under phosphate stress have been found to stimulate hyphal elongation of VAM fungi in vitro (Elias and Safir, 1987). The compounds that are active on the growth of VAM fungi have been isolated from white clover roots and identified as the isoflavones formononetin and biochanin A (Nair *et al.*, 1991). When these isoflavones are added to soil containing spores of a *Glomus* species, the rate of root colonization increases in white clover (Siqueira *et al.*, 1991a). In another study, exogenous application of these compounds have been found to reduce herbicide injury (Siqueira *et al.*, 1991b). This effect is mediated through the stimulation of mycorrhiza formation. Mycorrhizal plants are usually less affected by the adversities of compounds accumulated in the soils, provided that the concentrations are sublethal, such as in the case of herbicide carry-over. Of the two isoflavones tested, formononetin has exhibited more activity than biochanin A. Other flavonoids can also stimulate mycorrhiza formation, but require much higher concentrations than formononetin and biochanin A. Since formononetin and biochanin A are active at very low concentrations, these two isoflavones are thought to be good candidates to be investigated as signal molecules for mycorrhiza formation.

Although the stimulatory effects of the exogenously applied formononetin on mycorrhiza formation have been observed, the effects of formononetin on the physiological functions of the VAM fungi and plant roots have not been investigated. Once applied, how long the formononetin persists, how mobile it is in the soil and its effect on soil microbial populations is also not known. It is the purpose of this dissertation to approach some aspects of these questions.

In Chapter 2, the effects of formononetin on the isozyme activities of NAD-malate dehydrogenase and peroxidase were studied during the establishment of a *Trifolium-Glomus* mycorrhizal association. In Chapter 3, effects of a variety of plant phenolics on the peroxidase activity of white clover roots were determined with *in vitro* assays. In Chapter 4, recovery of exogenously applied isoflavones, formononetin and biochanin A, from sterile and non sterile soil and from soil planted with corn seedlings was measured at several time points. Vertical migration of formononetin was determined in soil columns. The response of soil microbial populations to the isoflavone amendments were investigated by using soil samples obtained from a Michigan potato field.

Based on the results of these experiments, a mechanism of action is proposed for the formononetin-mediated stimulation of *Trifolium-Glomus* mycorrhiza formation. In addition, this study reports the first information on the soil relations of the exogenously applied bioactive isoflavones formononetin and biochanin A.

CHAPTER 2

ISOZYME ACTIVITY OF DEVELOPING *TRIFOLIUM-GLOMUS* MYCORRHIZA AND ASSOCIATED EFFECTS OF THE ISOFLAVONE FORMONONETIN

INTRODUCTION

Hyphal growth of vesicular-arbuscular mycorrhizal (VAM) fungi is stimulated by volatiles and exudated compounds from roots (Elias and Safir, 1987; Becard and Piche, 1989b, Nair *et al.*, 1991). Hyphal elongation of VAM fungi (*Glomus* species) has been shown to be stimulated by the isoflavone formononetin (Nair *et al.*, 1991). This isoflavone has been isolated from white clover roots grown under phosphorus stress and characterized as a mycorrhiza-stimulatory compound. Soil application of formononetin has been found to increase the rate of root colonization by *Glomus* species at early stages of white clover (*Trifolium repens*) growth (Siqueira *et al.*, 1991a). Although positive effects of this isoflavone on hyphal growth and colonization have been demonstrated, the mechanism for its action is still not understood.

In this study, the effects of the exogenously applied formononetin on the isozyme activities of fungi and roots were investigated during the early stages of white clover root colonization by the VAM fungus *Glomus intraradix*. NAD-malate dehydrogenase was chosen to monitor the fungal activity in the roots. It has previously been found that fungal NAD-

activity in the roots. It has previously been found that fungal NAD-malate dehydrogenase (MDH) isozymes are expressed at high levels in mycorrhizas (Rosendahl, 1992). Some *Glomus* species synthesize high molecular weight NAD-MDH isozymes which can be detected easily on activity stained native gels (Rosendahl, 1989). In roots with sufficiently low molecular weight NAD-MDH isozymes, these high molecular weight isozymes serve as a diagnostic fungal indicator. The band intensities of fungal NAD-MDH isozymes have been found to correlate positively with the glucosamine content of the roots (Rosendahl, 1992). Therefore, it has been suggested that the band intensities of the fungal NAD-MDH isozymes can be used as an indicator of fungal mass. Although the relationship between the fungal NAD-MDH isozyme activity and fungal mass in roots has been established, the physiological significance of the high levels of this enzyme activity is not known. Furthermore the fungal NAD-MDH activity has not been investigated during the early stages of mycorrhiza formation.

Soluble peroxidase activity was chosen to monitor the non-specific plant defense response to accelerated colonization that occurs in the presence of formononetin. Cell-wall-bound peroxidase activity exhibits a transient increase during the early stages of mycorrhiza formation (Spanu and Bonfante-Fasolo, 1988). This indicates a weak elicitation of non-specific plant defense reactions to the colonizing fungi. After the transient increase, the peroxidase activity in VAM roots falls below the level of the activity in non-mycorrhizal roots. Peroxidases catalyze the polymerization of phenolic compounds to lignin. Their activity increases during the pathogen attacks where reinforcement of the cell walls are necessary (Dean and Kuc, 1987). Although the wall-bound total peroxidase

activity has been measured during the colonization of leek roots by VAM fungi, the effect on soluble peroxidases is not known. Soluble peroxidases play a major role in polyphenol metabolism of plant cells (Barz, 1977). Leguminous plant species synthesize a variety of phenolic compounds and some of these are accumulated under certain conditions (Porter *et al.*, 1986; Morandi, 1989). In this study, the effect of the exogenously applied isoflavone formononetin on soluble root peroxidases of white clover, a leguminous plant species that can synthesize and accumulate this isoflavone, was investigated.

The results of this study indicate that fungal NAD-MDH and plant peroxidase activities are affected by the exogenous application of formononetin. The physiological significance of the results are discussed in terms of association of VAM fungi with leguminous plant species.

MATERIALS AND METHODS

Growth conditions

White clover (*Trifolium repens* L., cv. Ladino) plants received either formononetin alone (-VAM+FOR), *Glomus intraradix* Schenck & Smith inoculum alone (+VAM-FOR), both (+VAM+FOR) or neither (-VAM-FOR, control), with six replications per treatment. Plants were harvested at one, two and three weeks post-emergence.

Clover seedlings were grown in plastic pots containing 1.6 kg of soil (1:1 mixture of steemed top soil and silica sand). The soil contained 114 mg NO_3, 0.2 mg P, 20 mg K, 271 mg Ca, 81 mg Mg, 36 mg Na, and 99 mg Cl per kg, pH 7.4.

Spores of *G. intraradix* were isolated from a commercial clay matrix (Nutralink, Native Plants Inc., Salt Lake City, UT) by wet sieving

thorough 420 and 38 μm mesh sieves. The amount of inoculum was adjusted to give approximately 4 spores per gram of soil and the spores were incorporated into the soil thoroughly in a small (66.5 L capacity) cement mixer (Granger Co. Lansing, MI).

Formononetin solutions were prepared at a concentration of 5 $mg.L^{-1}$. Formononetin was first dissolved in a small volume of warm methanol. The final methanol concentration was 0.5%. Designated pots received 200 ml of this solution (0.63 mg per kg of soil) once before the seeds were dispersed. Treatments without formononetin received the same amount of methanol.

About 30 seeds were dispersed onto the soil and were covered with a 0.5 cm layer of soil. Clover plants were grown in a greenhouse at 23°C and 18°C (day and night). Plants were illuminated with sodium vapor lamps in addition to sunlight, the photoperiod was 16 hours. A week after emergence the plants received 100 ml of a modified nutrient solution (Pacovsky and Fuller, 1988) containing 1.5 mM $CaCl_2$, 0.5 mM K_2SO_4, 0.25 mM $MgSO_4$, 2.5 mM NH_4NO_3, 0.4 mM KH_2PO_4, 0.6 μM $CoCl_2$, 0.4 μM $CuSO_4$, 2 μM $ZnSO_4$, 25 μM H_3BO_3, 20 μM FeNaEDTA, 0.4 μM H_2MoO_4. This nutrient solution and water was provided every other day.

Growth and colonization measurements

At harvest, plants were removed from the soil and gently washed in distilled water. Roots and shoots were separated and shoots were oven dried at 57°C for 12 hours. Roots were weighed wrapped in aluminum foil, frozen between blocks of dry ice and kept in a freezer at -20°C. Subsamples of roots from each treatment were used for colonization and dry weight measurements.

VAM-root colonization was assessed using a modification of the line-intersect method (Kormanik and McGraw, 1982) following the clearing and staining of root samples according to Phillips and Hayman (1970).

Data for root fresh weight, shoot dry weight, protein per gram of root dry weight and percent colonization were subjected to statistical analysis (ANOVA) and 2-tailed *t*-test for comparisons between treatments.

Preparation of root extracts

Roots were ground in a tissue grinder (Tekmar, Cincinnati,OH) twice, approximately 8 seconds each, in an ice bath following the addition of root extraction buffer (0.75 ml/g root) containing 50 mM Tris-HCl, 3.0 mM EDTA, 2.5 mM DTT, 250 mM sucrose, 50 mM NaCl, 2mM phenylmethylsulfonyl floride, and 2 mM N-ethyl-maleimide, at pH 7 (Pacovsky, 1989). Root extracts were then centrifuged for 10 minutes at 12,000 g at 4°C. Supernates of each extract were used for analysis. Protein concentrations in extracts were determined using a Bradford assay (Bradford, 1976) with bovine gamma globulin (Bio-Rad Laboratories Inc., Hercules, CA) as a standard.

Polyacrylamide native gel electrophoresis

Samples were prepared for electrophoresis according to Davis (1964). Soluble proteins (20-40 μg depending on the enzyme system) were seperated on discontinuous native gels: 4% polyacrylamide in the stacking gel, 7.5% polyacrylamide in the separating gel. During electrophoresis, a constant current of 25 mA per gel was applied while the samples were in the stacking gel (1.5 cm), and 35 mA during migration through the resolving gel (16 cm). The isozymes were visualized using the protocols developed

for NAD-malate dehydrogenase (NAD-MDH, E.C.1.1.1.37) and peroxidase (POX, E.C.1.11.1.7) by Shaw and Prasad (1970).

Densitometric analysis of isozyme bands

Densitometric analyses of NAD-MDH activity-stained gels containing protein extracts from one-week-old white clover roots were performed on 35 mm black and white photographic negatives using a computing densitometer (Model 300A, Molecular Dynamics, Innovative Optical Systems for Molecular Biology, Sunnyvale, CA) and MD Image Quant (IQ) software version 3.15.

The intensities of POX and NAD-MDH isozyme bands were also measured by using an image analysing system from a black and white photograph of activity stained gels. The photographs were viewed with a camera (Burle CCD). The images were frozen on the screen of a Sony Trinitron superfine pitch monitor attached to a Gateway 2000 computer. Software developed for image analysis (JAVA) was used to scan the intensities along the lanes of the gels. The data collected were imported to a computer program PLOT-IT for numerical transformations and line graphics were obtained by using a graphics program (Harvard Graphics version 3).

Total peroxidase activity measurements

Total peroxidase activity was determined spectrophotometrically according to Ridge and Osborne (1970a) with the following modifications. Reaction mixtures contained 4 ml of 14.2 mM hydrogen peroxide (H_2O_2) in 10 mM phosphate buffer at pH 7 and 1 ml of 9.8 mM guaiacol in water and 10 μg protein. The tubes were inverted twice quickly to mix the protein in the reaction mixture. Absorbance values were recorded every 30 seconds for 10 minutes at 480 nm in a Perkin-Elmer 35 spectrophotometer. For each of the

four experimental treatments, the total peroxidase activities of three replicates were measured. A computer program PLOT-IT was used to fit a non-linear regression model to the data obtained for each treatment.

RESULTS

Growth and colonization

Effects of VAM fungal inoculation and formononetin application on colonization, growth and soluble protein concentration were assesed. The percent of root length colonized increased rapidly between one and two weeks of growth (Fig. 2.1). At two weeks, root colonization of +VAM+FOR was 50% higher than for +VAM-FOR. The only significant difference in root fresh weights was observed at two weeks between control (-VAM-FOR) roots and mycorrhizal roots grown with formononetin (+VAM+FOR) (Table 2.1). Mycorrhizal roots grown in formononetin-amended soil were about twice the weight of control roots. There were no differences between the treatments in terms of shoot dry weight at two and three weeks. However, shoot dry weights of mycorrhizal roots without formononetin (+VAM-FOR) and non-mycorrhizal roots grown with formononetin (-VAM+FOR) differed in one-week-old plants. The concentration of soluble proteins extracted from roots ($mg.g^{-1}$ of dry weight) differed in one and two-week old plants but not at three weeks. Experiments were repeated at least three times with similar results.

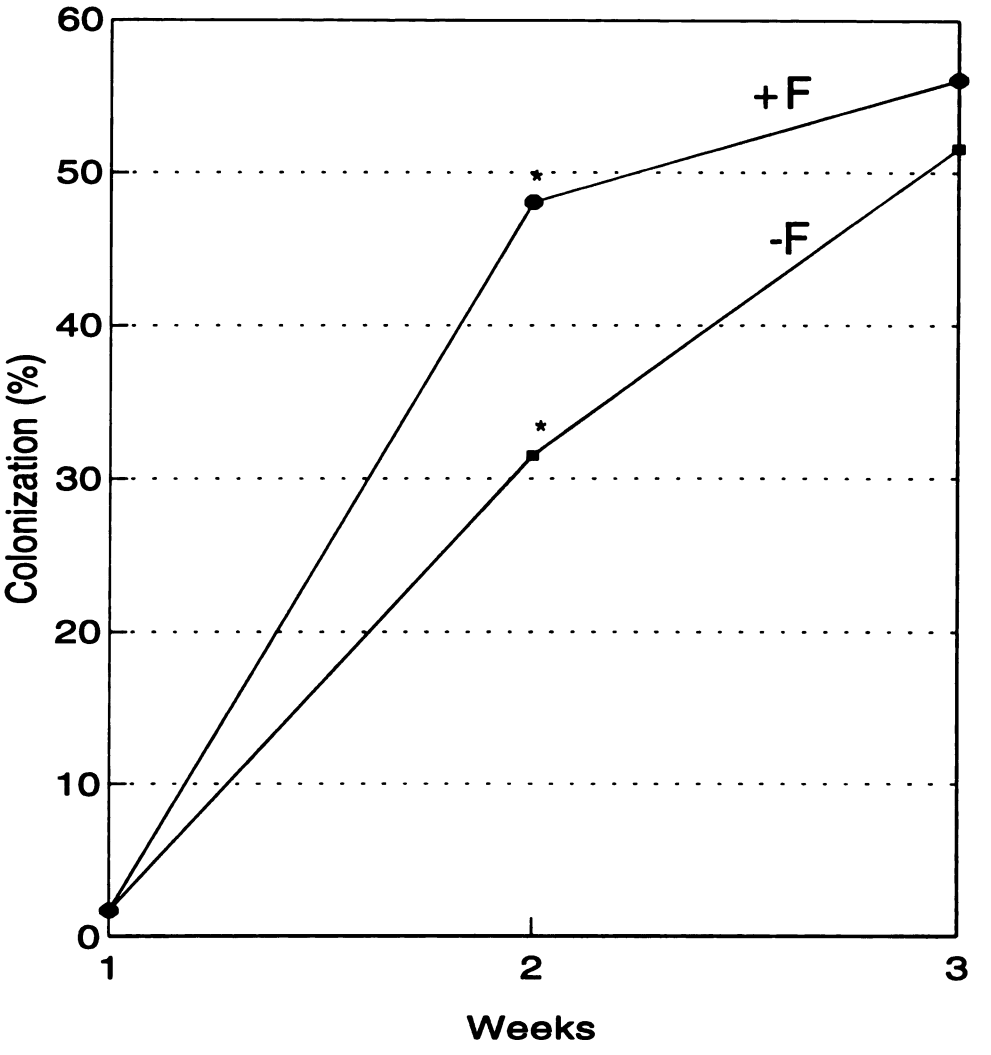


Figure 2.1. Effect of formononetin application on VAM colonization of white clover roots in a soil-sand mix inoculated with *Glomus intraradix* (4 spores.g^{-1} of soil) at one, two, and three weeks post emergence, with formononetin (+F) or without formononetin (-F). *Difference statistically significant at α=0.01. Symbols represent mean of six plants.

Table 2.1. Effects of VAM fungal inoculation and formononetin on growth and protein concentration of white clover plants.

Treatments	Root fresh weight (g)[b]	Shoot dry weight (mg)[b]	Protein ($mg \cdot g^{-1}$ rdw[c])[b]
Week 1			
-VAM -FOR[a]	0.26^{A}	36.6^{AB}	30.0^{A}
-VAM +FOR	0.31^{A}	45.9^{A}	15.1^{B}
+VAM -FOR	0.24^{A}	30.0^{B}	21.4^{AB}
+VAM +FOR	0.34^{A}	39.9^{AB}	25.4^{A}
Week 2			
-VAM -FOR	0.50^{N}	60.1^{M}	19.1^{O}
-VAM +FOR	0.63^{MN}	71.2^{M}	24.5^{NO}
+VAM -FOR	0.71^{MN}	74.7^{M}	35.8^{M}
+VAM +FOR	0.91^{M}	93.7^{M}	31.0^{MN}
Week 3			
-VAM -FOR	1.72^{X}	228.8^{X}	32.0^{X}
-VAM +FOR	1.60^{X}	238.1^{X}	38.3^{X}
+VAM -FOR	1.92^{X}	249.3^{X}	42.8^{X}
+VAM +FOR	2.17^{X}	274.3^{X}	41.1^{X}

a: Formononetin
b: Means followed by the same letter within a column do not differ significantly at $p \leq 0.05$
c: Root dry weight

NAD-Malate dehydrogenase isozymes

Six bands were seen in the malate dehydrogenase activity-stained gel for white clover roots grown in uninoculated soil (Fig. 2.2). In this first gel, lanes were loaded with equal volumes of the protein extract (40 μl) from three-week-old roots. In addition to these six activity bands VAM roots had three more bands with relative mobilities (R_m) of 0.21, 0.23 and 0.30 (MDH1a, MDH1b and MDH1c, respectively). Among these three isozyme bands, MDH1b appeared to have the strongest activity (Figure 2.2, lanes 3 and 4). Densitometric tracing also indicated the highest intensity peak at the region containing MDH1b band (Fig. 2.3 C and D).

These MDH1 activities were also observed in one-week-old VAM white clover roots (Fig. 2.4). In this gel, lanes were loaded with equal amounts of protein (40 μg). The optical densities of rectangular regions of the gel containing MDH1 activities were compared using a densitometer (792 data points were collected for each rectangle). The optical density of the MDH1 region in lane 3 (representing mycorrhizal roots) was 59.5 and that of lane 4 (representing mycorrhizal roots with formononetin) was 89.2. This indicated that MDH1 activities were stronger in mycorrhizal roots in the presence of formononetin. The MDH1 activities were not observed in roots from uninoculated soil, with or without formononetin, during the three weeks of growth (Fig. 2.3, lanes 1 and 2; Fig. 2.4, lanes 1 and 2).

Peroxidase isozymes

Ten bands were detected in the peroxidase activity-stained gel for both VAM and non-VAM roots (Fig. 2.5). The lanes were loaded with 20 μg of protein. Mycorrhizal colonization of roots caused a general reduction

Figure 2.2. NAD-Malate dehydrogenase isozymes visualized in a 7.5% polyacrylamide native gel. Protein extracts (40 μl / lane) from three-week-old white clover roots. Presence or absence of formononetin (FOR) or spores of *Glomus intraradix* (VAM) is denoted by (+), (-). Rm: Relative mobility.

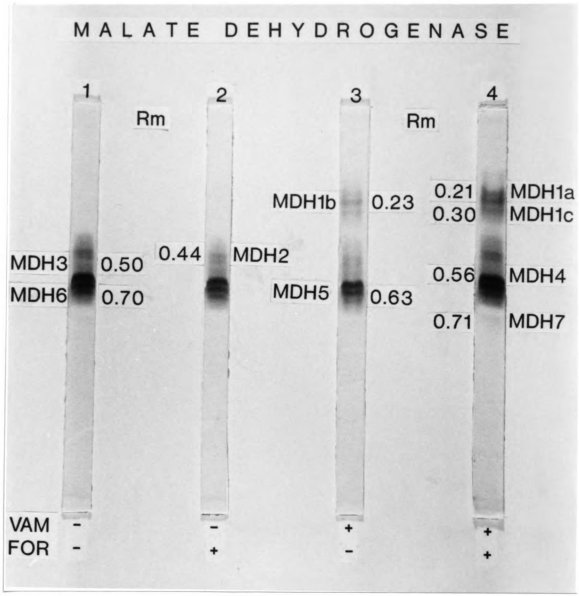

MALATE DEHYDROGENASE
1
2
3
4
Rm
Rm
MDH3
0.50
MDH6
0.70
0.44
MDH2
MDH1b
0.23
MDH5
0.63
0.21
MDH1a
0.30
MDH1c
0.56
MDH4
0.71
MDH7
VAM
FOR
- - + +
- + - +

Figure 2.3. Densitometric tracing of the NAD-malate dehydrogenase isozyme bands from a black and white photograph of the activity-stained polyacrylamide native gel in Figure 2.2. A. Lane 1 (-V-F); B. Lane 2 (-V+F); C. Lane 3 (+V-F); D. Lane 4 (+V+F). V (spores of VAM fungus *Glomus intraradix*), F (formononetin).

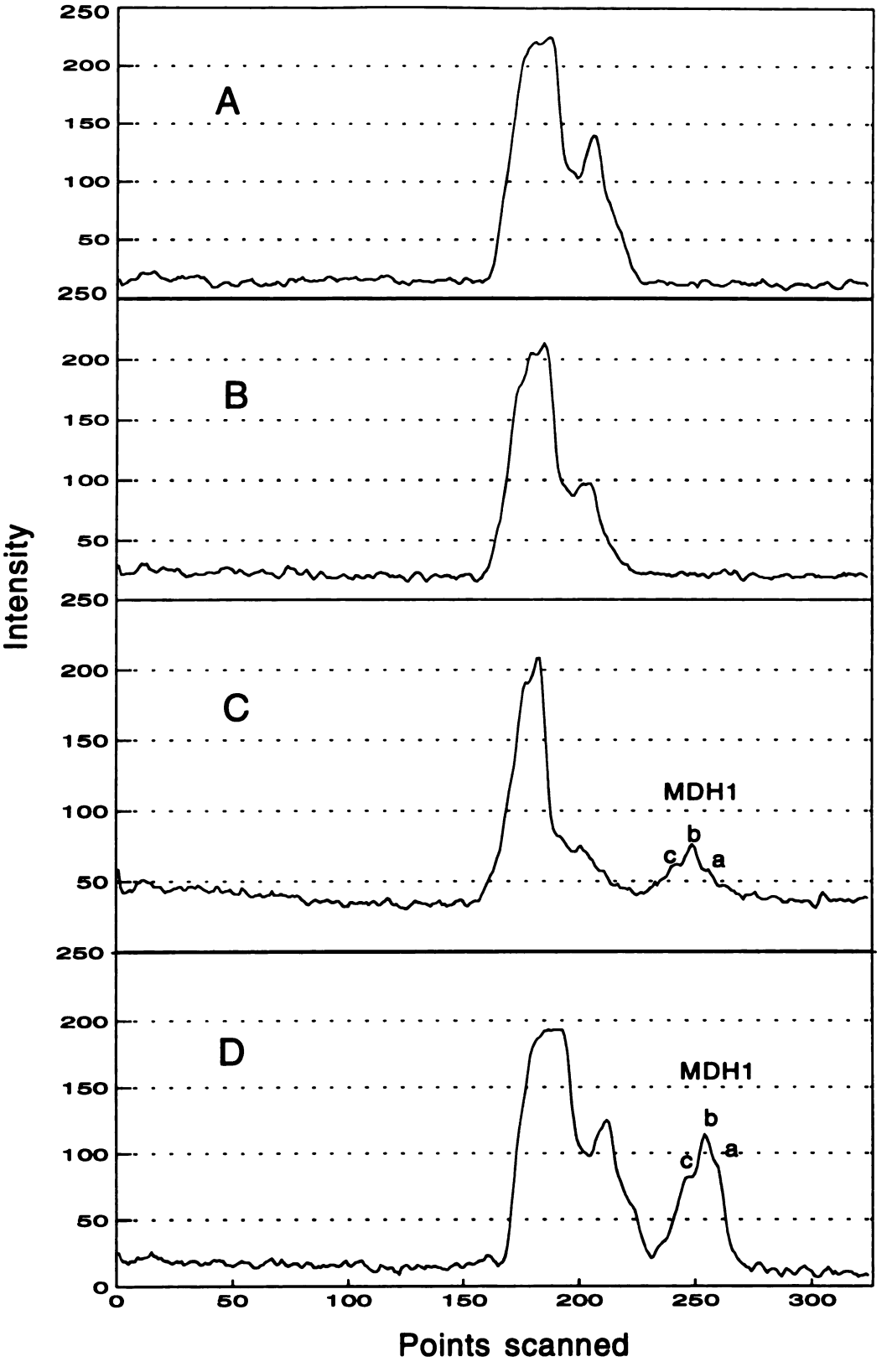

A
B
C
D
MDH1
MDH1
a
b
c
Intensity
Points scanned
0
50
100
150
200
250
300

Figure 2.4. NAD-malate dehydrogenase isozymes visualized in a 7.5% polyacrylamide native gel. Protein extracts (40 μg / lane) from one-week-old white clover roots. Presence or absence of formononetin (FOR) or spores of *Glomus intraradix* (VAM) is denoted by (+), (-). Rm: Relative mobility.

MALATE DEHYDROGENASE

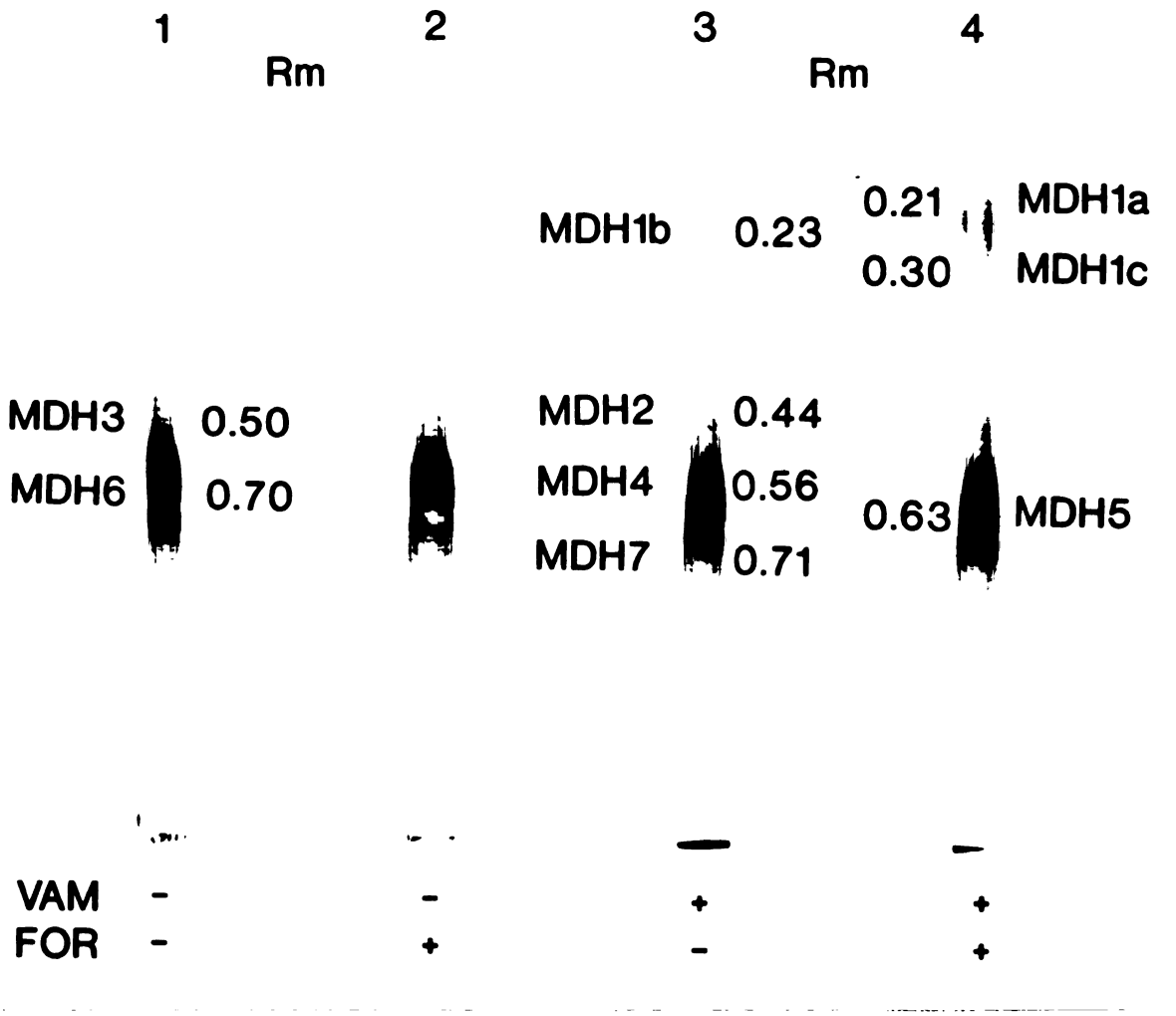


Figure 2.5. Peroxidase isozymes visualized in a 7.5% polyacrylamide native gel. Protein extracts (20 μg / lane) from one, two, and three-week-old white clover roots. Presence or absence of formononetin (FOR) or spores of *Glomus intraradix* (VAM) is denoted by (+), (-). Rm: Relative mobility.

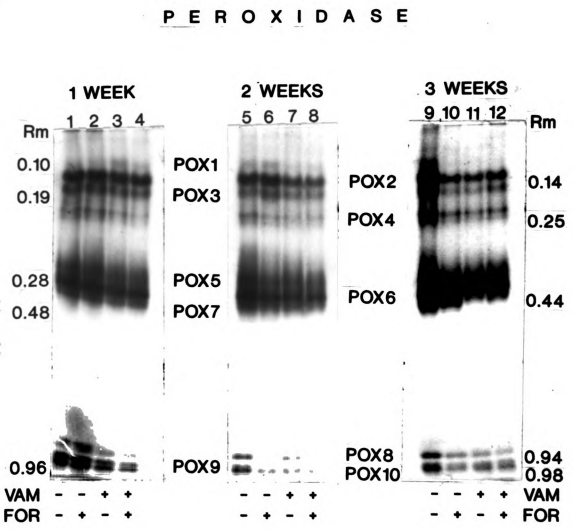

PEROXIDASE
1 WEEK
2 WEEKS
3 WEEKS
1 2 3 4
5 6 7 8
9 10 11 12
Rm
Rm
0.10
0.19
0.28
0.48
0.96
POX1
POX3
POX5
POX7
POX9
POX2
POX4
POX6
POX8
POX10
0.14
0.25
0.44
0.94
0.98
VAM - - + +
FOR - + - +
- - + +
- + - +
- - + + VAM
- + - + FOR

Figure 2.6. Densitometric tracing of the peroxidase isozyme bands from a black and white photograph of the activity-stained polyacrylamide native gel in Figure 2.5. A. Lane 1 (-V-F); B. Lane 2 (-V+F); C. Lane 3 (+V-F); D. Lane 4 (+V+F). V (Spores of VAM fungus *Glomus intraradix*), F (formononetin).

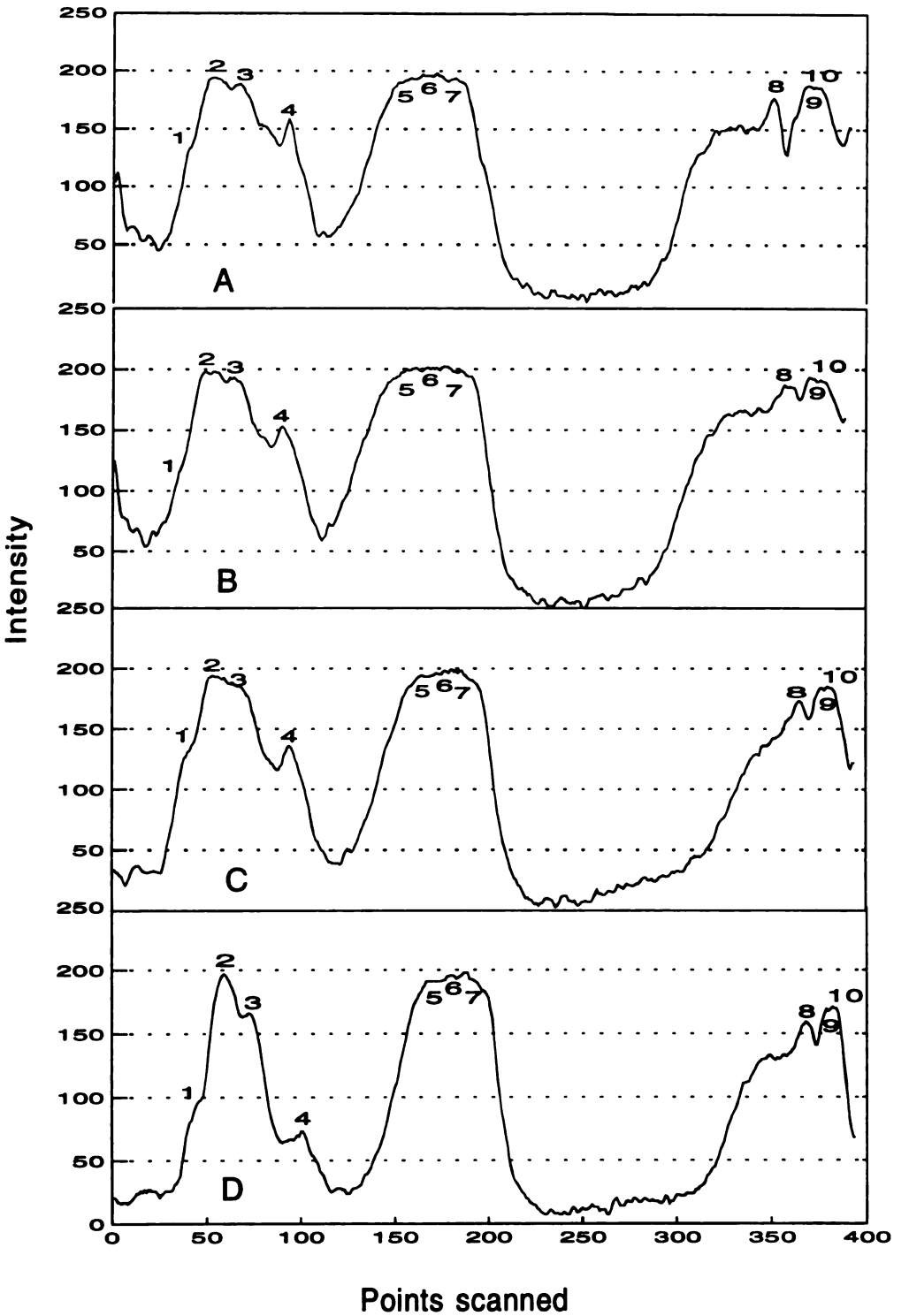

Intensity
Points scanned
A
B
C
D
0
50
100
150
200
250
300
350
400
1
2
3
4
5
6
7
8
9
10

Figure 2.7. Densitometric tracing of the peroxidase isozyme bands from a black and white photograph of the activity-stained polyacrylamide native gel in Figure 2.5. A. Lane 5 (-V-F); B. Lane 6 (-V+F); C. Lane 7 (+V-F); D. Lane 8 (+V+F). V (Spores of VAM fungus *Glomus intraradix*), F (formononetin).

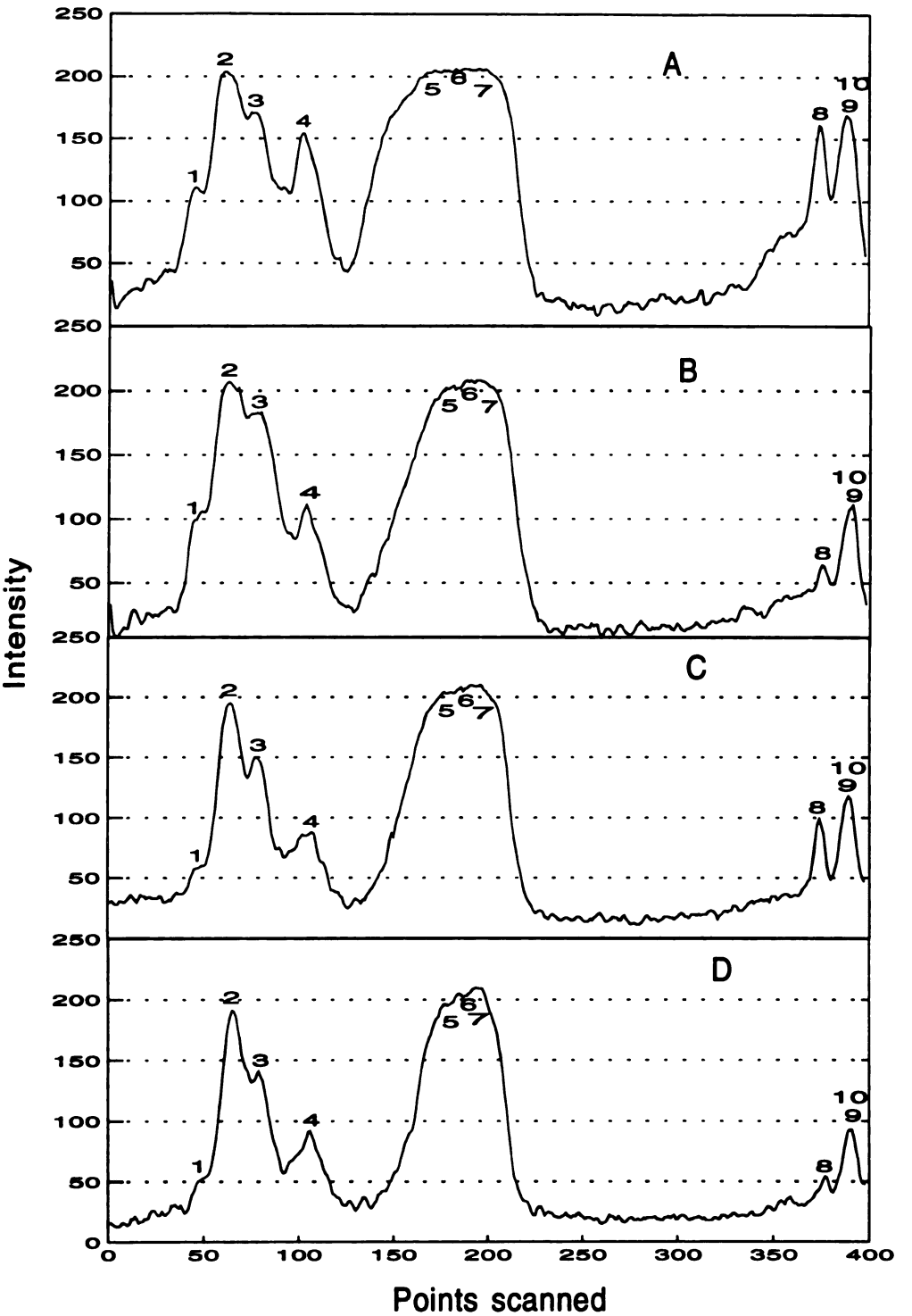
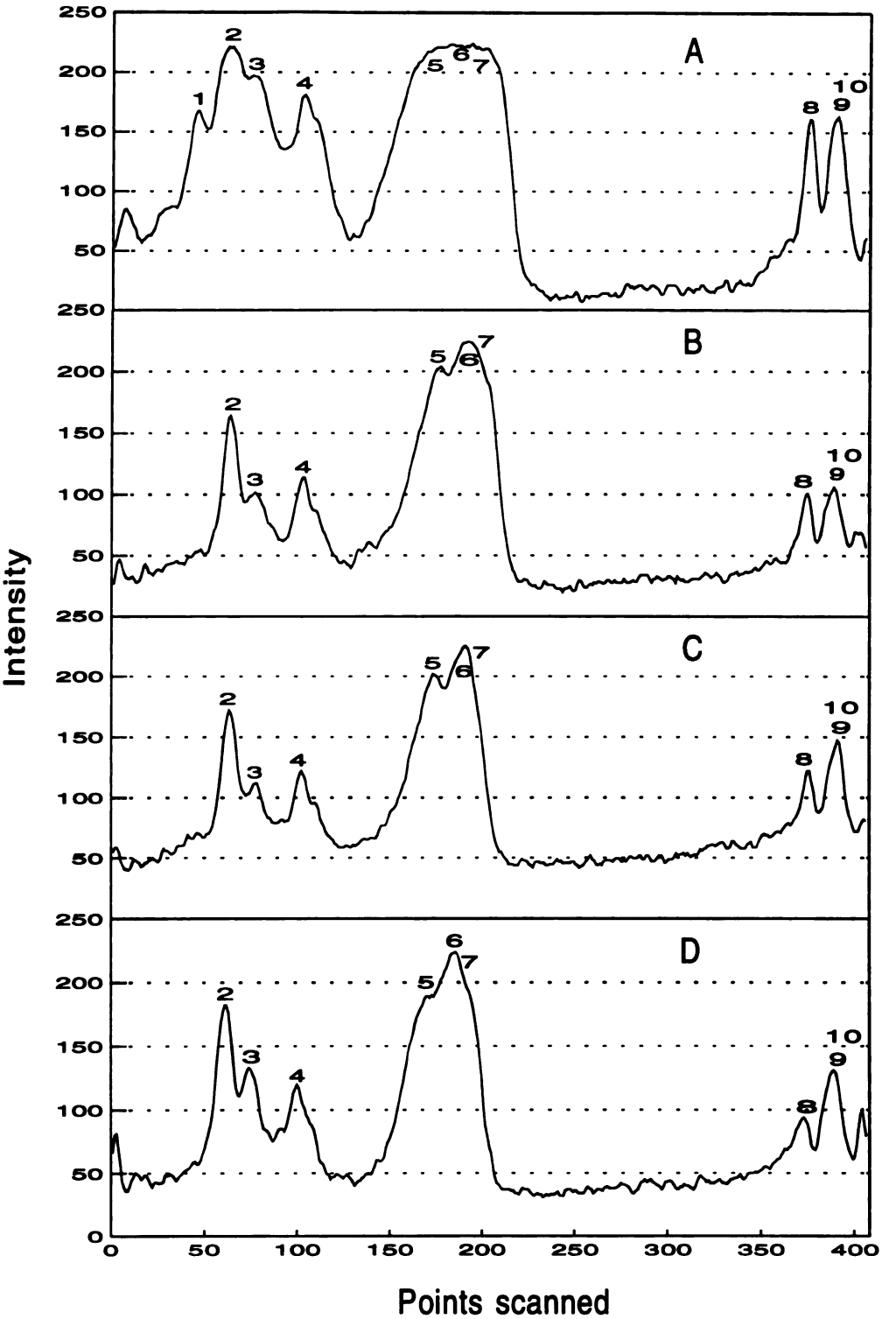

A
B
C
D
Intensity
Points scanned
0
50
100
150
200
250
300
350
400

Figure 2.8. Densitometric tracing of the peroxidase isozyme bands from a black and white photograph of the activity-stained polyacrylamide native gel in Figure 2.5. A. Lane 9 (-V-F); B. Lane 10 (-V+F); C. Lane 11 (+V-F); D. Lane 12 (+V+F). V (Spores of VAM fungus *Glomus intraradix*), F (formononetin).


A
B
C
D
Intensity
Points scanned
0
50
100
150
200
250
300
350
400

of peroxidase (POX) activity, as observed in polyacrylamide native gel (Fig. 2.5, lanes 7, 8, 11, and 12) at week 2 and 3. In addition, the presence of formononetin caused further reduction in peroxidase activity in VAM roots at two weeks (Fig. 2.5, lane 8) and a lesser reduction in non-VAM roots (Fig. 2.5, lane 6). Intensity tracing of the lanes indicated that POX4 activity was affected by formononetin application and mycorrhizal colonization as early as one week of growth. At this time, control roots had the strongest POX4 activity and +VAM+FOR roots had the weakest among the treatments (Fig. 2.6). In two-week-old roots, reduction in the activities of POX1, 2, 3, 8, 9, and 10, as well as POX4, was detectable from the areas and heights of intensity peaks (Fig. 2.7). The image analyzer detected POX5, 6, 7 bands as one large intensity peak. However, the variation in peak areas and heights of that region became more noticeable, especially at week three (Fig. 2.8).

Total peroxidase activity

Total peroxidase activity of two-week-old roots, in which colonization was significantly increased by formononetin, was quantified with a spectrophotometric assay (Fig. 2.9). *Glomus intraradix* colonization reduced the total POX activity by approximately 36% compared to control roots. Addition of formononetin reduced POX activity in mycorrhizal roots by 50% and non-mycorrhizal roots by 15% relative to -VAM-FOR roots. The non-linear regression equation parameters and correlation coefficients for total POX activity curves are summerized in Table 2.2.

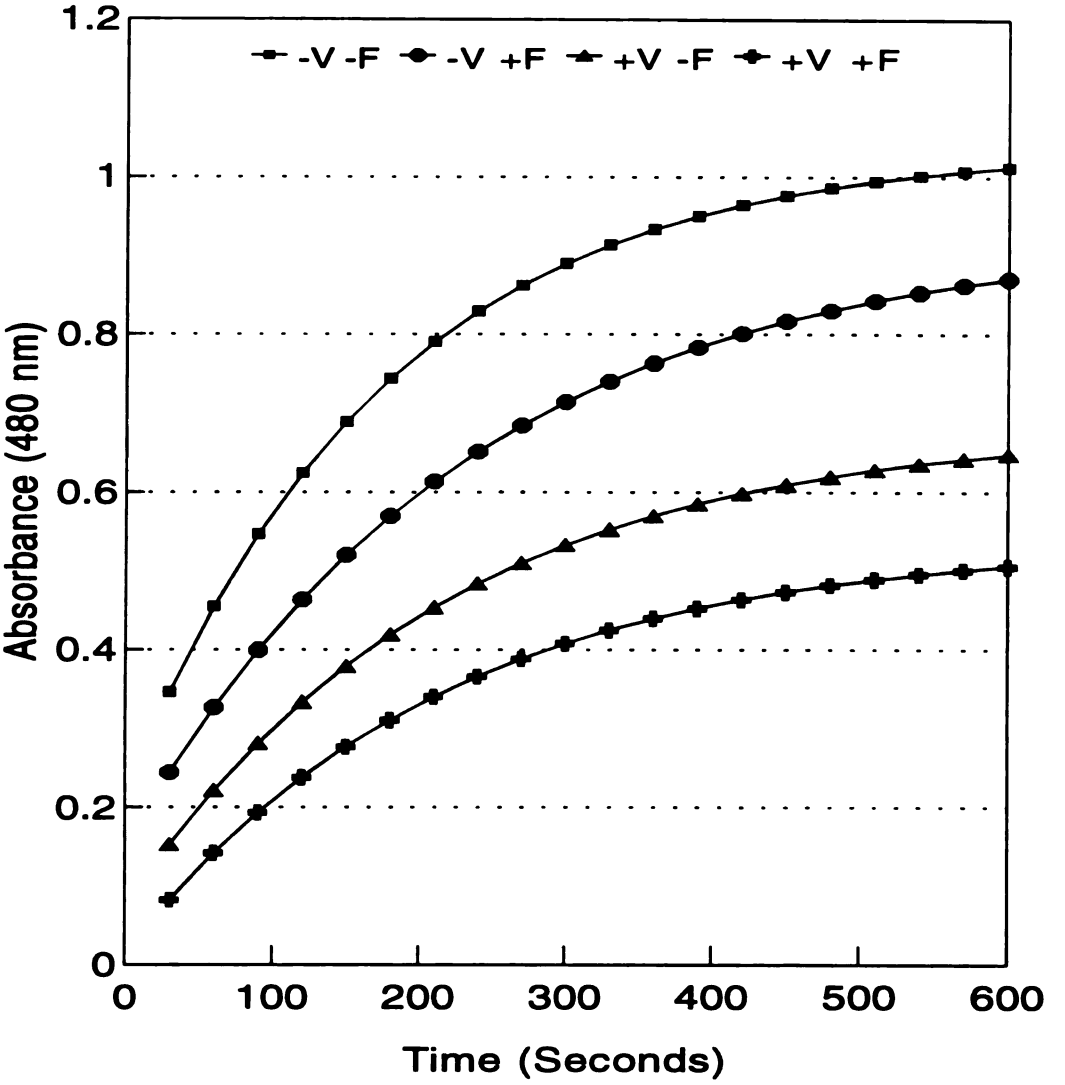


Figure 2.9. Total peroxidase activity in crude extracts of two-week-old white clover roots. Assay conditions: protein (10 μg), guaiacol (1.96 mM), H_2O_2 (11.36 mM), phosphate buffer (8 mM). Absorbance values represent three replicate treatments. Presence or absence of formononetin (F) or spores of *Glomus intraradix* (V) during the growth of white clover plants is denoted by (+), (-).

Table 2.2. Non-linear regression equation constants for progress curves of peroxidase reaction.

Equation : $Y = C_1 \exp^{C_2 X} + C_3$

Treatments	Constants C_1	C_2	C_3	R^2
+VAM +FOR[a]	-0.523	-0.00465	0.537	0.99
+VAM -FOR	-0.612	-0.00462	0.685	0.99
-VAM +FOR	-0.780	-0.00428	0.930	0.99
-VAM -FOR	-0.822	-0.00568	1.039	0.98

a: Formononetin

DISCUSSION

High molecular weight fungal NAD-MDH isozymes (MDH1) were detected in one-week-old *Trifolium-Glomus* mycorrhizas (Fig. 2.3). This was the earliest occurence of these NAD-MDH isozymes ever reported in mycorrhizal roots. Similar fungal isozymes have been observed in older *Cucumis sativus* roots. Six weeks after inoculation, these isozyme activities were found to be 40% of the host NAD-MDH activities (Rosendahl, 1992).

In this study, most of the colonization took place between the first and second week of growth (Fig. 2.1). The very early appearence of the MDH1 activities in easily detectable levels suggests that these functions may be important for the development of colonization. Similarly, in nodule crude extracts MDH appears to have the highest specific activity

among other enzymes involved in carbon metabolism in *Rhizobium*-legume association (Sheoran *et al.*, 1988). In addition, malate dehydrogenase activities of bacteroids have also been found to be higher than those of free-living *Rhizobium* (Suganuma and Yamomoto, 1987). The MDH activity of the plant component of the nodules is also lower than that of the bacterial component. These findings suggest that the malate produced during the CO_2 dark fixation in the host plant cells is supplied to bacteroids for nitrogen fixation in *Rhizobium*-legume associations. While carbon flow from plant cells to arbuscules and subsequent flow from the intraradical hyphae to extramatrical hyphae are documented, the form of the carbon skeletons still remains obscure. However, the high level of fungal MDH activity demonstrated here in mycorrhizal roots may indicate some similarities between *Rhizobium*-legume symbioses and VA mycorrhizal symbioses in terms of the form of carbon flowing from the host plant to the microsymbiont.

According to the results presented here, the activities of the MDH1 isozymes increased in the presence of formononetin. Although MDH1 activities were higher in +VAM+FOR roots, the percentage of root-length colonized was not different in one-week-old plants with or without formononetin (Fig. 2.1). The intensity of these fungal MDH bands has been positively correlated with glucosamine concentrations (Rosendahl, 1992). The higher MDH1 activities in +VAM+FOR roots may thus indicate more fungal mass per unit of colonized root.

Formononetin has been identified as a VA-mycorrhiza stimulating factor from white clover roots (Nair *et al.*, 1991). Since formononetin increases hyphal growth *in vitro*, it has been suggested that this isoflavone may be a host signal for the expression of fungal symbiotic and

growth genes (Nair *et al.*, 1991; Siqueira *et al.*, 1991a). Increased MDH1 activity, which was observed in this study, could possibly be a part of the sequence of biochemical events leading to a functional symbiosis. Therefore, NAD-MDH isozymes may be useful early and sensitive markers for monitoring fungal activity during the development of VAM association.

According to Siqueira *et al.* (1991a), soil application of formononetin reduces the lag phase and increases VAM colonization early in the growth period. Becard and Piche (1989a) have proposed a model for the formation of the VAM symbiosis. In their model they recognized two different mechanisms which are triggered by root factors during the growth of VAM fungi. The first mechanism (M1) is responsible for stimulation of hyphal growth from the germinated spore. During this stage, the hyphal growth is dependent on the spores and gradually slows down and ceases when there are no roots in proximity. The second mechanism (M2) is triggered during the formation of arbuscules. At this, stage the hyphal growth becomes dependent on the host and no longer requires the presence of the spores. Therefore, the two mechanisms represent switches in the nutritional source for the hyphae. Thus, according to the Becard-Piche Model the lag phase that is referred to by Siqueira *et al.* (1991) can be either at the M1 stage or M2 stage.

Becard and Fortin (1988) have found that there is a two-day period between contact-attachment and attachment-intracellular spread. They considered this two-day interval as a critical period for the formation of mycorrhizal associations. They hypothesize that this interval is an adaptation period for the development of recognition mechanisms or the synthesis of enzymes.

Based on the previous hypothesis, the results of this study suggest

that isoflavone formononetin may induce triggering of the M2 mechanism before the contact-attachment step of colonization. Thus, the two-day interval may be shortened and the colonization process is accelarated. Therefore, it is likely that the lag phase which is reduced by the formononetin, represents the beginning of the plant-dependent nutritional phase (M2).

Becard and Piche (1989b) presented conclusive evidence for the importance of CO_2, a root volatile, on hyphal elongation. In their work, the root exudates alone did not support hyphal elongation when CO_2 traps are present. However, in the presence of CO_2, root exudates highly enhanced the hyphal growth. While their work suggests that CO_2 may be the major factor in triggering the M1 mechanism, the synergistic effect of volatiles and exudated compounds is obvious. According to Elias and Safir (1987), hyphal elongation is stimulated by the exudates of plants grown under phosphorus-deficiency stress. This indicates that root factors may be altered under certain stress conditions. The concentrations of molecules involved in signalling VAM fungi (such as formononetin) may increase and may then be released in greater quantities to the rhizosphere soil.

In natural settings, exuded compounds occur within a few millimeters from the rhizoplane (Leon-Barrios *et al.*, 1993). Root volatiles can diffuse further in the soil than the exudates, it is likely that spore-dependent growth can be induced by CO_2. Since microbial metabolism also generates CO_2, roots are not the only CO_2 source in the soil. It is thus possible that the short-lived effect of root volatiles on spore-dependant growth can be enhanced by the gradient of the root exudates. When the hyphae reach the rhizosphere, the high concentrations of exuded compounds involved in the formation of mycorrhiza may trigger the plant dependent-

growth mechanisms in hyphae.

Aglycone and sugar-conjugates of formononetin are present in the normal (unstressed) intact roots of alfalfa and not found in the root exudates. The aglycone form is released under stress conditions (Maxwell and Phillips, 1990). Therefore, the leguminous plants growing in soils with suboptimal phosphorus levels (but not severely deficient levels) may not release formononetin in large quantities. This may explain the two-day lag phase between contact-attachment and attachment-intracellular spread of the hyphae. The formononetin concentrations necessary for switching the nutritional status will be sensed by the hyphae only after contact with the root. In this study, white clover plants were not grown under phosphorus deficient conditions. By three weeks of growth, root fresh weights and shoot dry weights of mycorrhizal and non-mycorrhizal plants were not different. The percent-root-length colonized did not differ in the presence or absence of formononetin at one week of growth. Thus, increased levels of fungal malate dehydrogenase at this stage in response to exogenously applied formononetin may present evidence for the involvement of this isoflavone in triggering the M2 mechanism of hyphal growth.

In this study, the soluble peroxidase (POX) activity was lower in two- and three-week-old VAM roots. Similarly, Spanu and Bonfante-Fasolo (1988) reported a net decrease in cell-wall-bound POX activity in three- to five-week-old roots of *Allium porrum* inoculated with *Glomus versiforme*. A peak POX activity was not detected in VAM white clover roots as was reported in their study. This may be due to the differences in the sampling times, or possibly any increases in the activities of cell-wall peroxidases may not occur in the soluble fraction used in my studies. In

some studies, however, the increase or decrease of POX activity has been found to correspond in soluble and wall peroxidase fractions (Ridge and Osborne, 1970a; Bronner *et al.*, 1991). Although they sometimes correspond, the changes in the soluble fraction have been observed to be less dramatic than those in the cell-wall-bound fraction.

Isozyme patterns did not differ between the mycorrhizal and non-mycorrhizal roots. Additionally, the intensity of peroxidase isozyme bands did not increase in mycorrhizal roots compared to non-mycorrhizal roots. Therefore, the presence of overlapping fungal and plant peroxidase isozymes was unlikely. Soluble extracts of *Rhizobium* and bacteroids in nodules of legumes do not show peroxidase activity, but nodule cell cytoplasm contains several peroxidase isozymes (Becana *et al.*, 1989). The results peresented herein indicated that the VAM fungus used did not exhibit peroxidase activity in clover roots. It is thus possible that VAM fungi do not have this function or the level of activity was undetectable.

The response of white clover to a colonizing mutualistic symbiont may be different from that of *A. porrum* (Leek). This may have an evolutionary significance, in that white clover, a leguminous plant species, may facilitate the colonization of a mutualistic symbiont by lowering its peroxidase activity. Leguminous plants make symbiotic associations with nitrogen fixing bacteria such as *Rhizobium*, as well as with VAM fungi. In natural settings, the roots of leguminous plants can host both VAM fungi and *Rhizobium* (Brown and Bethlenfalvay, 1987). This highly symbiotic nature of leguminous plants may exist due to the development of some means of mutual recognition with the symbionts which enable infection to occur and the association to develop (Dixon and Wheeler, 1986).

In this study, formononetin application further lowered the soluble

POX activity in VAM white clover roots. Interestingly, formononetin application also lowered the POX activity in non-VAM roots also. Exogenously applied formononetin mimicked the effect of VAM colonization on soluble peroxidase activity. POX activity has been shown to be affected by phytohormones. For example, ethylene increases the POX activity (Ridge and Osborne, 1970b) while gibberellin decreases the activity (Birecka and Galston, 1970). Phytohormones also influence isoflavone levels in another leguminous plant, *Cicer arietinum*. For example, TBA (2, 3, 6 trichlorobenzoic acid) has been shown to increase the rate of formononetin synthesis (Barz, 1977). In the same study, kinetin was shown to increase the rate of turnover without causing a change in the level of synthesis. Isoflavone concentration and peroxidase activities may thus be interrelated and regulated by some phytohormones.

Formononetin concentration increases in the roots and in root exudates of white clover plants grown under phosphorus stress (Nair *et al.*, 1991). Promotion of VAM colonization by phosphorus deficiency has been well documented (Sanders and Tinker, 1973; Mosse, 1973; Smith et al., 1986a). Increased formononetin concentration in root exudates is thought to be a signal for VAM fungi. This possibility has been strengthened by the observation that exogenously applied formononetin increases the rate of colonization (Siqueira *et al.*, 1991). Just as formononetin may act as a signal compound for VAM endophytes, its increased concentration in the roots may also lower the POX activities or expressions, which favors colonization. Electron microscopy studies indicate that a collar of wall material exists around the penetration point and the trunk of the arbuscule. However, this layer is absent in the branches of the arbuscule (Cox and Sanders, 1974). Peroxidases play a major role in secondary wall

formation and suberization, which contributes to the plant's resistance to invaders (Gianinazzi, 1991). The suppression of soluble peroxidase activity may provide a means of controlling VAM fungal intracellular growth and development by the host plant. Nevertheless, the molecular mechanisms underlying how exogenously applied formononetin causes a decrease in the peroxidase activities, observed in these experiments, is yet to be discovered.

The results suggest that exogenous application of formononetin has a dual effect on the early stages of *Trifolium-Glomus* mycorrhizal development. It triggers the plant-dependent growth mechanisms of the hyphae before contact-attachment, which reduces the adaptation period, as evidenced by increased MDH activity in one week old mycorrhiza. Additionally, it causes a decrease in the soluble peroxidase activity of white clover roots, which may facilitate the intracellular growth of the fungus.

CHAPTER 3

IN VITRO EFFECTS OF PLANT PHENOLIC COMPOUNDS ON ROOT PEROXIDASE ACTIVITY OF *TRIFOLIUM REPENS*

INTRODUCTION

Peroxidase utilizes hydrogen peroxide to oxidize a wide variety of hydrogen donors including phenolic compounds (Saunders *et al.*, 1964). Peroxidase plays an important role in plant polyphenol metabolism such as polyphenol degradation and polymerization (Barz, 1977). In cell-free extracts and purified preparations, many plant-derived phenolic compounds have been found to serve as substrates for peroxidase reactions (Pickering *et al.*, 1973; Badiani *et al.*, 1990; Lee and Kim, 1994). Typically, this enzyme system consists of a large number of isozymes in plants (Klapper and Hackett, 1965; Delincee and Radola, 1970; Liu and Lamport, 1973; Barz, 1977). Isoperoxidases differ in their affinity for polyphenolic substrates (Pickering *et al.*, 1973; Badiani *et al.*, 1990; Lee and Kim, 1994).

Peroxidase levels in plants are under the regulation of plant hormones and their level is inversely proportional with growth (Ridge and Osborne, 1970a,b; Birecka and Galston, 1970). Therefore, this enzyme system has been chosen to study hormonal control on plant growth processes (Galston and Davies, 1969). Steady-state concentrations of some

isoflavones such as formononetin, biochanin A and kaempherol have been shown to be regulated by phytohormones. This regulation occurs either at the level of biosynthesis or turnover (Zenk *et al.*, 1975; Barz, 1977). Peroxidase catalyzes the interconversion, degradation and polymerization of these isoflavones as well as other polyphenols (Barz, 1977).

Exogenously applied formononetin was shown to decrease the total soluble peroxidase activity in white clover roots (Chapter 2). However, the mechanism of this *in vivo* effect is not known. Although peroxidases from a number of plant species have been investigated, the catalytic properties of white clover peroxidases have not been studied. In this experiment, the *in vitro* effects of various bioactive plant phenolic compounds on total soluble peroxidase activity in white clover root extracts were investigated.

MATERIALS AND METHODS

Growth conditions

White clover (*Trifolium repens* L., cv. Ladino) plants were grown in 1 L capacity plastic cups containing 1.6 kg of soil. The soil was a mixture of one part steamed top soil and one part silica sand. The soil in each cup was moistened with 200 ml of water. White clover seeds (30 per cup) were dispersed into each cup and covered with approximately a 0.5 cm layer of soil. Plants were grown in a greenhouse, with the temperature regulated at 23°C and 18°C (day and night). Plants were illuminated with hanging sodium vapor lamps in addition to sun light (photoperiod of 16 hours). Plants received water every other day. A week after emergence, they received 100 ml of nutrient solution (1.5 mM $CaCl_2$, 0.5 mM K_2SO_4, 0.25 mM $MgSO_4$, 2.5 mM NH_4NO_3, 0.4 mM KH_2PO_4, 0.6 μM $CoCl_2$, 0.4

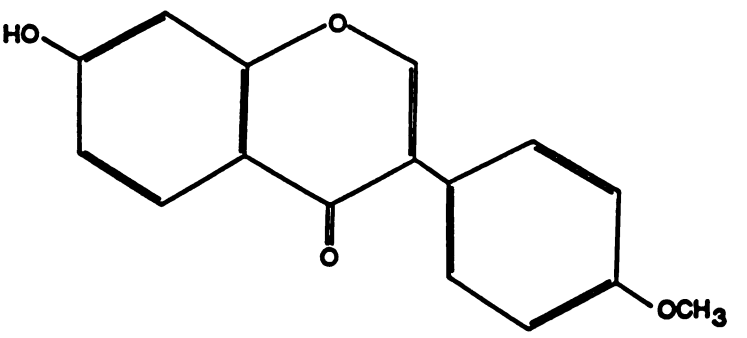
μM $CuSO_4$, 2 μM $ZnSO_4$, 25 μM H_3BO_3, 20 μM FeNaEDTA, 0.4 μM H_2MoO_4, Pacovsky and Fuller, 1988) every other day.

Protein extraction

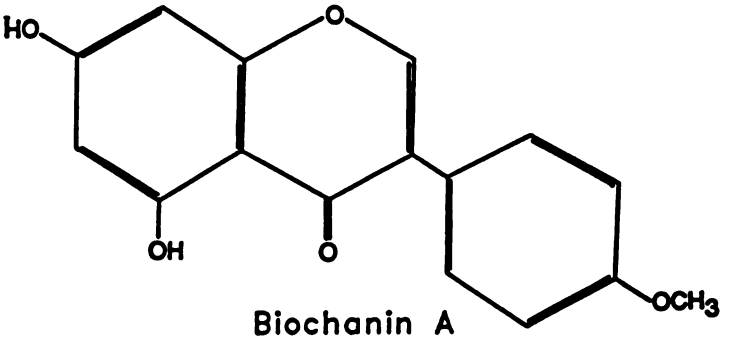
Plants were harvested two weeks after emergence. They were washed to remove the soil, and the roots and shoots were separated. The roots were weighed, wrapped in aluminum foil and frozen between dry ice blocks and stored -20°C. The roots were ground in tissue grinder twice, approximately 8 seconds each, in an ice bath following the addition of extraction buffer (0.75 $ml.g^{-1}$ root). This buffer contained 50 mM Tris-HCl, 3.0 mM EDTA, 2.5 mM DTT, 250 mM sucrose, 50 mM NaCl, 2 mM PMSF, and 2 mM N-ethyl-maleimide, at pH 7 (Pacovsky, 1989). The extracts were centrifuged at 12,000 g at 4°C. The supernates were transferred to microfuge tubes, dipped in liquid nitrogen and stored at -80°C. Protein concentrations were determined with a Bradford assay kit (Bio-Rad Laboratories Inc., Hercules, CA) by using bovine Γ globulin as a standard.

Peroxidase activity assay

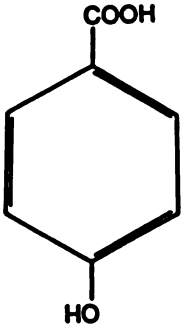
Total peroxidase activity was measured spectrophotometrically according to Ridge and Osborne (1970a) with the following modifications. The reaction mixture contained 3 ml 18.9 mM hydrogen peroxide (H_2O_2) in 10 mM phosphate buffer, pH 7, 1 ml of 9.8 mM guaiacol in water and 1 ml methanol. The final concentration of guaiacol and H_2O_2 were 1.96 and 11.36 mM respectively. One ml of methanol was used for controls and was substituted for the methanolic solutions of phenolic compounds. Ten μg protein was added to the reaction mixture for the assays. The tubes were inverted twice quickly and the absorbance was recorded at 30 second



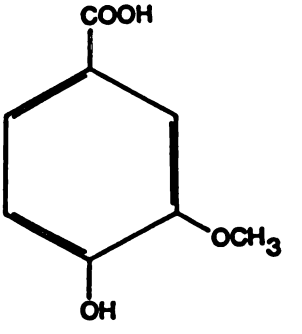
Formononetin



Biochanin A

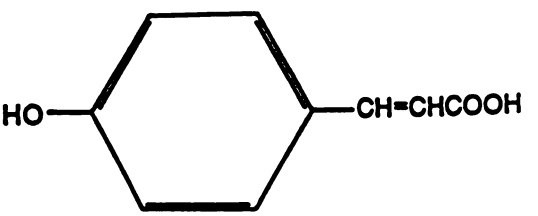


p-Hydroxybenzoic Acid

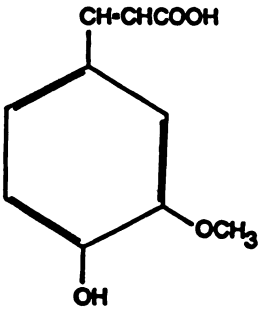


Vanillic Acid

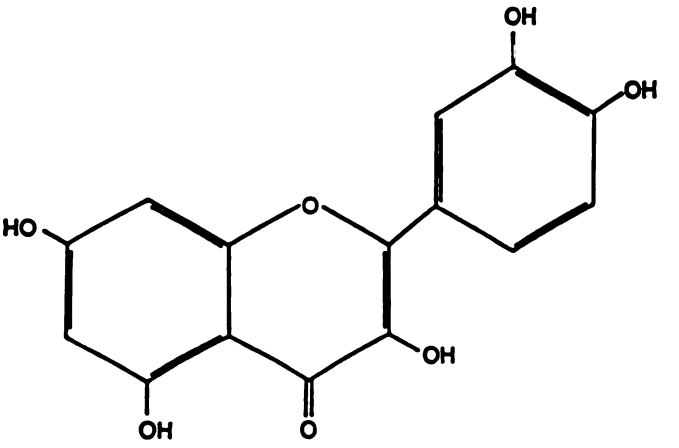
Figure 3.1. Structures of formononetin (7-hydroxy-4'-methoxyisoflavone), biochanin A (5, 7-Dihydroxy-4'-methoxyisoflavone), p-hydroxybenzoic acid, vanillic acid (4-Hydroxy-3-methoxybenzoic acid).



p-Coumaric Acid

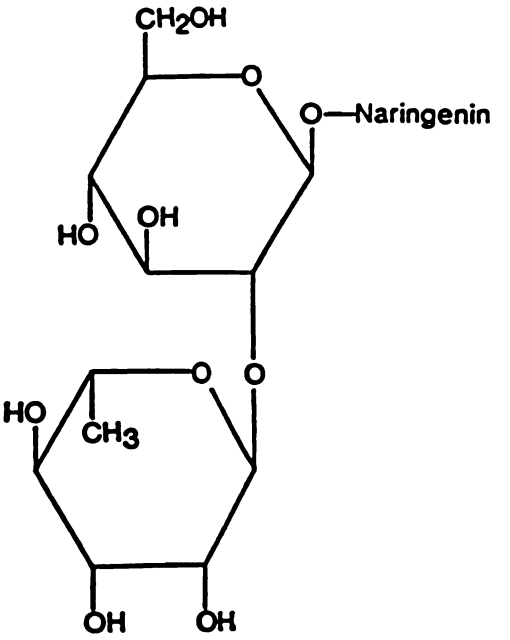


Ferulic Acid

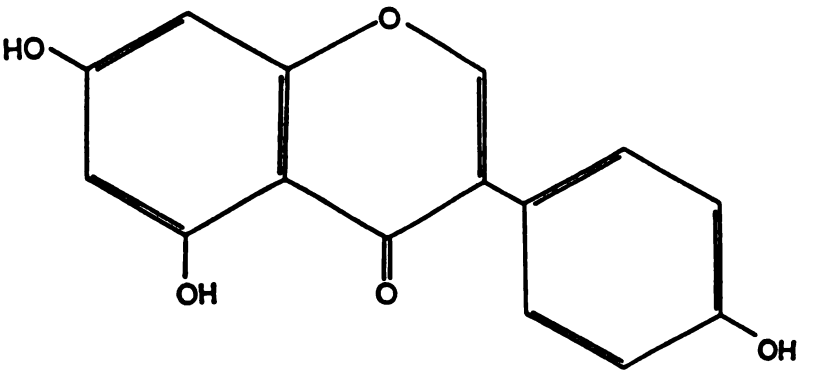


Quercetin

Figure 3.2. Structures of p-coumeric acid (3-(4-Hydroxyphenyl)-2-propenoic acid), ferulic acid (3-(4-Hydroxy-3-methoxyphenyl)-2-propenoic acid), quercetin (3, 3', 4', 5, 7-pentahydroxyflavone).

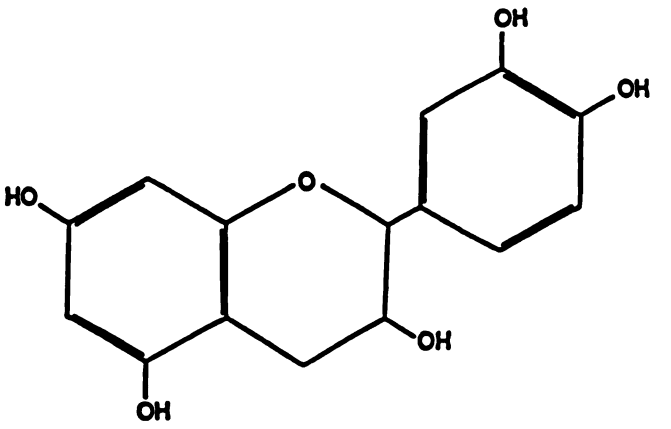


Naringin

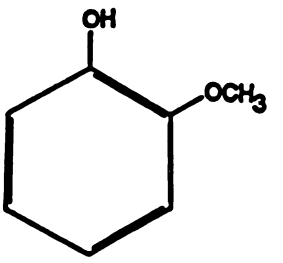


Genistein

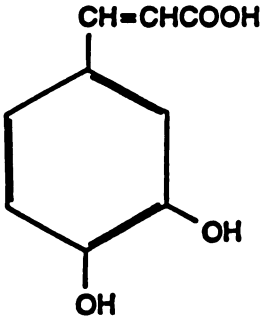
Figure 3.3. Structures of naringin (4', 5, 7-trihydroxyflavonone 7-rhamnoglucoside; Naringenin-7-rhamnoglucoside), genistein (4', 5 7-trihydroxyisoflavone).



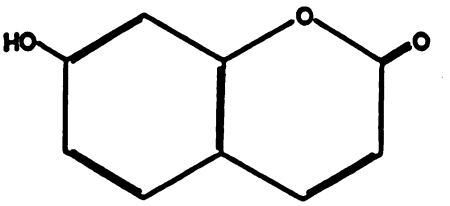
Catechin



Guaiacol

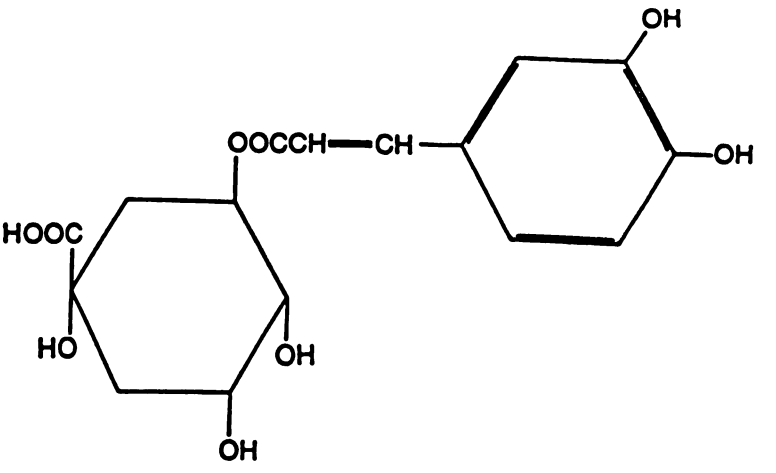


Caffeic Acid

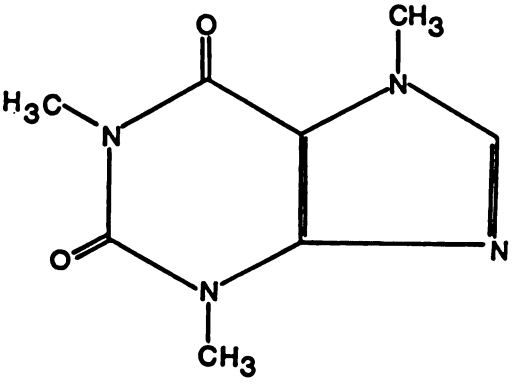


Umbelliferone

Figure 3.4. Structures of cathechin (3, 3′, 4′, 5, 7-flavan-pentol), guaiacol (o-methoxyphenol), caffeic acid (3-(3,4-dihydroxyphenyl)-2-propenoic acid), umbelliferone (7-Hydroxy-2H-1-benzopyran-2-one).



Chlorogenic Acid



Caffeine

Figure 3.5. Structures of chlorogenic acid (1, 3, 4, 5-tetrahydroxycyclohexane carboxylic acid 3-(3,4-dihydroxycinnamate)), caffeine (1,3,7-trimethylxanthine; 1,3,7-trimethyl-2,6-dioxopurine).

intervals for ten minutes at 480 nm in a Perkin-Elmer 35 spectrophotometer.

Effects of phenolic compounds on the rate of guaiacol oxidation

The phenolic compounds assayed for their effects on peroxidase activity were formononetin, biochanin A, p-Hydroxybenzoic acid, vanillic acid; (Fig. 3.1), p-coumeric acid, ferulic acid, quercetin; (Fig. 3.2), naringin, genistein; (Fig. 3.3), catechin, caffeic acid, umbelliferone, guaiacol; (Fig. 3.4), chlorogenic acid, and caffeine; (Fig. 3.5). The structures of the phenolic compounds were drawn according to Merck Index (Eleventh edition). The phenolic compounds were dissolved in methanol, 100 $mg.l^{-1}$. Methanolic solutions (1 ml) of these compounds were assayed for peroxidase activity. For ferulic acid and quercetin, in order to obtain smaller concentrations, original solutions were diluted further with methanol. Formononetin was prepared in two different concentration (0.1 $mg.\ ml^{-1}$ and 0.2 $mg.ml^{-1}$). Each assay was repeated at least three times. The absorbance values were analysed and non-linear regression equations were derived using a computer program PLOT-IT. Reaction-progress curves were obtained by using a graphics program (Harvard graphics version 3).

RESULTS

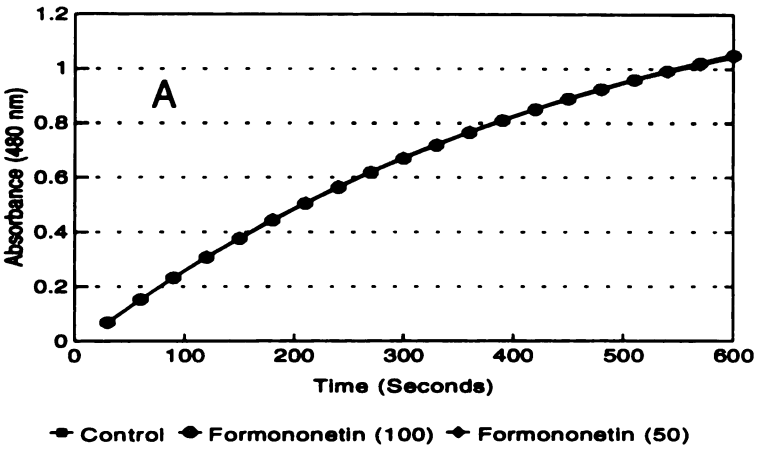
Cell-free extracts of white clover roots were assayed for total peroxidase activity. The results reported here refer to the activity of soluble peroxidases. Table 3.1 summarizes the effects of various naturally occuring plant phenolic compounds on the guaiacol oxidizing capabilities of white clover peroxidases.

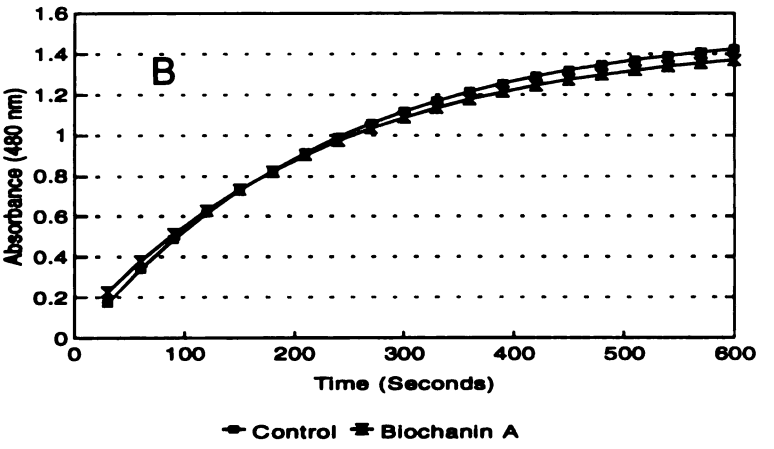
Table 3.1. Effect of various phenolic compounds on the initial velocity of peroxidase reaction.

Phenolic compounds added	Conc.(μM)	Initial velocity(%)
None	-	100
Formononetin	74	100
Formononetin	128	100
Biochanin A	70	108
Quercetin	66	25
Quercetin	33	47
Quercetin	17	70
Ferulic acid	102	324
Ferulic acid	51	298
Ferulic acid	26	245
Ferulic acid	10	168
p-Hydroxybenzoic acid	144	106
Vanillic acid	118	125
p-Coumeric acid	122	123
Naringin	34	102
Genistein	74	88
Catechin	68	56
Caffeic acid	112	100
Umbelliferone	124	108
Chlorogenic acid	56	19
Caffeine	102	100

Assay conditions were 1.96 mM guaiacol, 11.36 mM H_2O_2 in 8 mM phosphate buffer (pH 7.0), and 20% methanol (v/v).

Figure 3.6. Effects of formononetin (A), biochanin A (B) and quercetin (C) on the progress of peroxidase reaction. Assay conditions: 1.96 mM guaiacol, 11.36 mM H_2O_2 and 20% methanol methanol (v/v). Numbers in parantheses indicate the relative concentrations of the phenolic compound assayed.


A
Absorbance (480 nm)
1.2
1
0.8
0.6
0.4
0.2
0
0
100
200
300
400
500
600
Time (Seconds)
Control
Formononetin (100)
Formononetin (50)


B
Absorbance (480 nm)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
100
200
300
400
500
600
Time (Seconds)
Control
Biochanin A

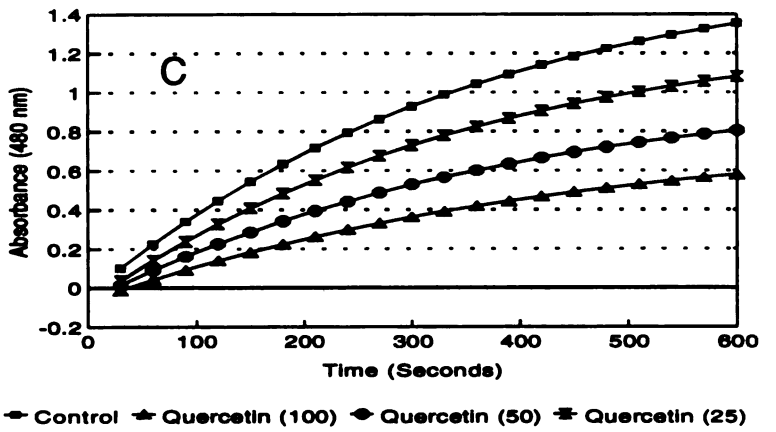
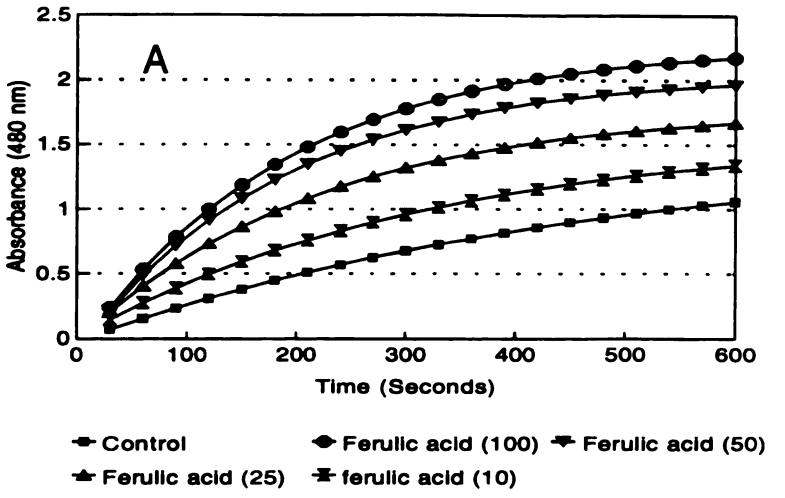
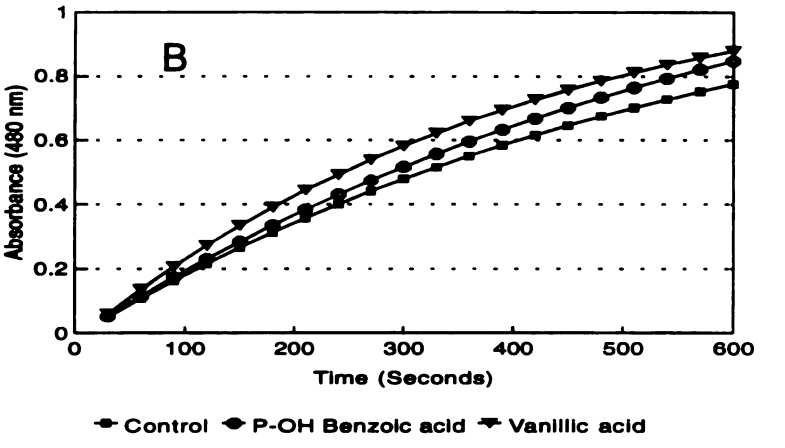

C
Absorbance (480 nm)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
0
100
200
300
400
500
600
Time (Seconds)
Control
Quercetin (100)
Quercetin (50)
Quercetin (25)

Figure 3.7. Effects of ferulic acid (A); p-hydroxybenzoic acid, vanillic acid (B); and p-coumeric acid (C) on the progress of peroxidase reaction. Assay conditions: 1.96 mM guaiacol, 11.36 mM H_2O_2 and 20% methanol methanol (v/v). Numbers in parantheses indicate the relative concentrations of the phenolic compound assayed.


A
Absorbance (480 nm)
Time (Seconds)
Control
Ferulic acid (100)
Ferulic acid (50)
Ferulic acid (25)
ferulic acid (10)


B
Absorbance (480 nm)
Time (Seconds)
Control
P-OH Benzoic acid
Vanillic acid

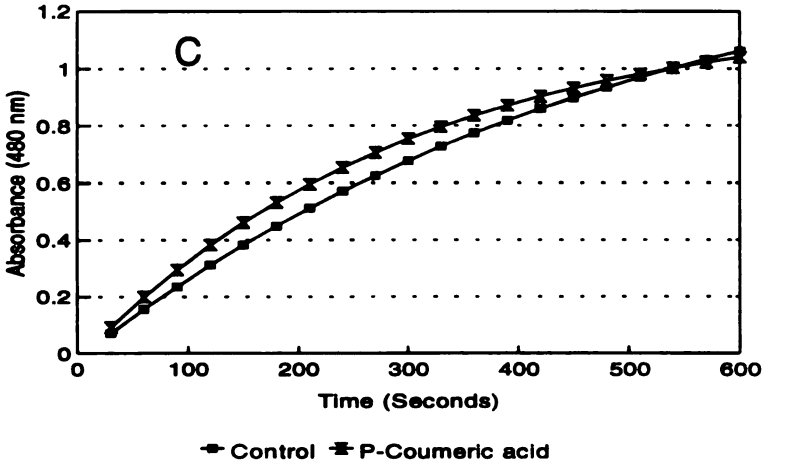
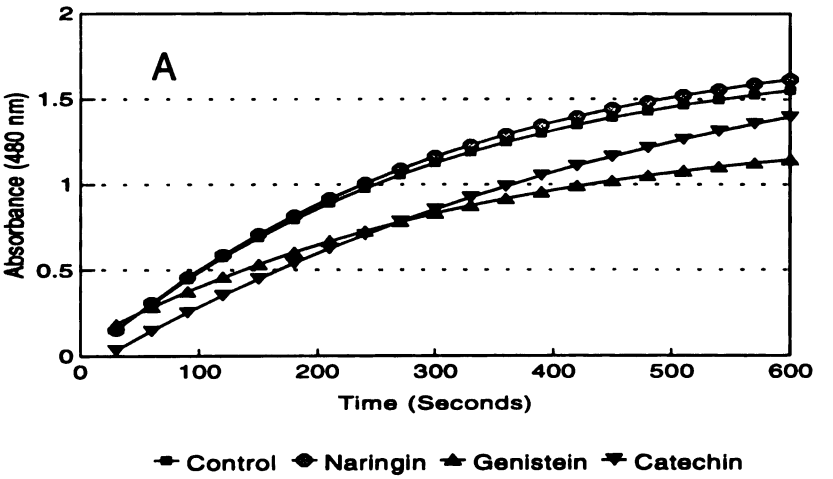
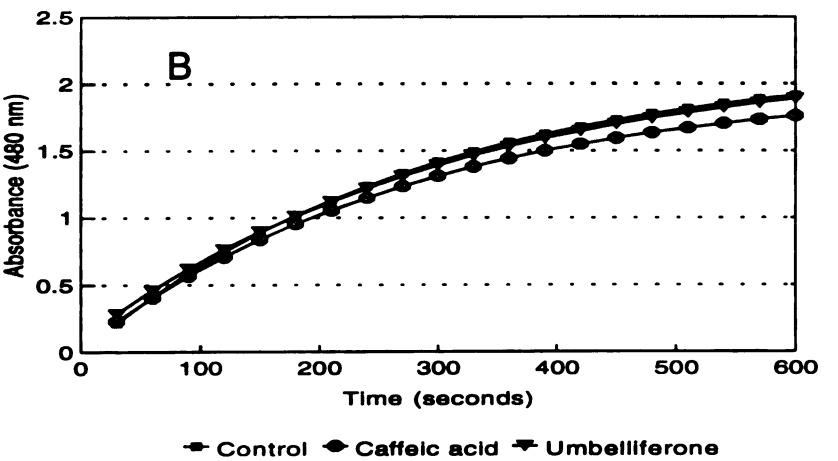

C
Absorbance (480 nm)
Time (Seconds)
Control
P-Coumeric acid

Figure 3.8. Effects of naringin, genistein,cathechin (A); caffeic acid, umbelliferone (B); chlorogenic acid and caffeine (C) on the progress of peroxidase reaction. Assay conditions: 1.96 mM guaiacol, 11.36 mM H_2O_2 and 20% methanol methanol (v/v).


A
Absorbance (480 nm)
2
1.5
1
0.5
0
0
100
200
300
400
500
600
Time (Seconds)
Control
Naringin
Genistein
Catechin


B
Absorbance (480 nm)
2.5
2
1.5
1
0.5
0
0
100
200
300
400
500
600
Time (seconds)
Control
Caffeic acid
Umbelliferone

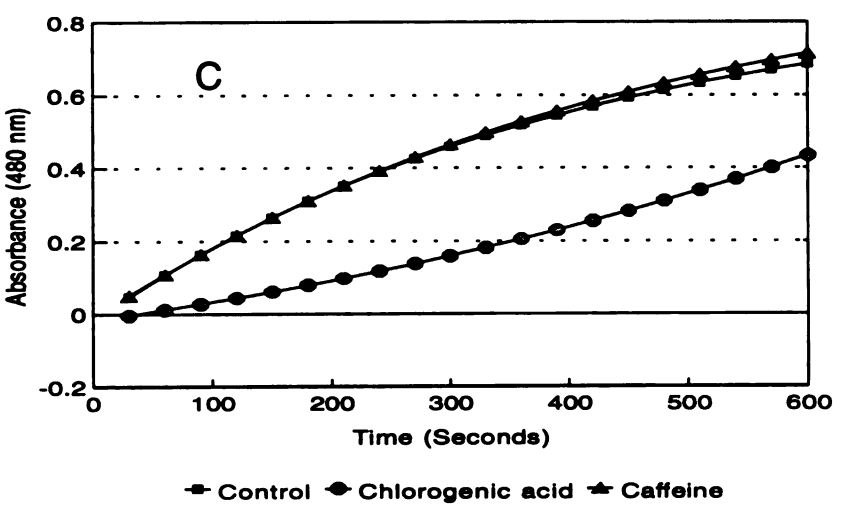

C
Absorbance (480 nm)
0.8
0.6
0.4
0.2
0
-0.2
0
100
200
300
400
500
600
Time (Seconds)
Control
Chlorogenic acid
Caffeine

Table 3.2. Non-linear regression model fitted to the data obtained from Peroxidase Assays and model constants.

		Non-linear regression equation: $Y = C_1 e^{C_2 X} + C_3$			
Phenolic Compounds	**Concentration (μM)**	**Constants** C_1	C_2	C_3	R^2
Formononetin	0	-1.523	-0.203	1.502	1.00
	74	-1.526	-0.201	1.505	1.00
	148	-1.483	-0.212	1.458	1.00
Biochanin A	0	-1.558	-0.004	1.539	1.00
	70	-1.422	-0.004	1.476	0.99
Quarcetin	0	-1.731	-0.002	1.696	1.00
	17	-1.431	-0.002	1.354	1.00
	33	-1.118	-0.002	1.051	1.00
	66	-0.870	-0.002	0.799	0.99
Ferulic Acid	0	-1.543	-0.002	1.523	1.00
	10	-1.584	-0.003	1.596	1.00
	26	-1.813	-0.004	1.788	1.00
	51	-2.150	-0.005	2.053	1.00
	102	-2.385	-0.005	2.280	0.99
P-Hydroxybenzoic Acid	0	-1.240	-0.001	1.228	1.00
	144	-1.405	-0.001	1.392	1.00
Vanillic Acid	0	-1.240	-0.001	1.228	1.00
	118	-1.187	-0.002	1.165	1.00
P-Coumeric Acid	0	-1.543	-0.002	1.523	1.00
	122	-1.229	-0.003	1.204	1.00
Naringin	0	-1.831	-0.003	1.808	1.00
	34	-1.924	-0.003	1.898	1.00
Genistein	0	-1.831	-0.003	1.808	1.00
	74	-1.293	-0.003	1.370	0.99
Catechin	0	-1.831	-0.003	1.808	1.00
	68	-2.215	-0.001	2.130	0.99
Caffeic Acid	0	-2.188	-0.003	2.183	0.99
	112	-1.974	-0.003	2.006	0.99
Umbelliferone	0	-2.188	-0.003	2.183	0.99
	124	-2.108	-0.003	2.199	0.99
Chlorogenic Acid	0	-0.909	-0.002	0.890	1.00
	56	-0.324	-0.001	-0.343	0.99
Caffeine	0	-0.909	-0.002	0.890	1.00
	102	-1.001	-0.002	0.987	1.00

Initial velocities were calculated from the linear increase phase of the reaction progress curves in Figures 3.6, 3.7, and 3.8. Velocity was defined as absorbance unit (AU) at 480 nm per minute. The constants for the non-linear equation, which was fitted to the reaction progress curves, are summerized in Table 3.2.

Some relationships between the structures of phenolic compounds and the total peroxidase activity can be inferred. When the phenolic group was in the para position to the unsaturated side chains (ferulic acid, p-coumeric acid) guaiacol oxidation was enhanced. In ferulic acid, the presence of an etherified second phenolic group in the ortho position to the free phenolic group enhanced the guaiacol oxidation capability of peroxidases greatest (Table 3.1, Fig. 3.7A). Since ferulic acid was highly stimulatory at the first concentration tested (102 μM), the assay was repeated with decreasing concentrations of ferulic acid. The results of the assays indicated that ferulic acid activation was concentration dependent.

A carboxyl group linked to the aromatic structure in para position to the free phenolic group (p-Hydroxybenzoic acid) caused a small activation in guaiacol oxidation. An additional etherified second phenolic group, in the ortho position to the free phenolic group (Vanillic acid) enhanced the guaiacol oxidation. This effect was similar to that seen in the case of p-coumeric acid and ferulic acid, but less dramatic. Therefore, an ortho-methoxy group and a carboxyl group, linked to the aromatic structure through an unsaturated side chain, resulted in the highest activation. Esterified phenolic group, in the para position to the free phenolic group (Umbelliferone) and two free phenolic groups in the meta position on the aromatic ring bonded to a 4H-1-pyran-4-one in the 4'-methoxyisoflavone

(Biochanin A) were not different in terms of their effect on total peroxidase activity. Both of these phenolic compounds caused a small activation, therefore, the presence of one phenolic group on the aromatic ring bonded to an alpha-pyran ring (Umbelliferone) was sufficient for this small activation. When the pyran ring is in gamma configuration, and the phenoxy group is in the fourth position, the presence of a side group in the third position of the pyran ring (such as the methoxylated B-ring of biochanin A) may be necessary for the observed effect on guaiacol oxidation.

Naringin increased the guaiacol oxidation slightly. This large molecule is a 7-rhamnoglucoside of the 4', 5, 7-trihydroxy- flavanone (Naringenin). It is possible that the flavonone itself (in aglycone form) may have a different effect on guaiacol oxidation. It is also possible that the presence of a large sugar moiety may cause a steric hinderance around the active site of the enzyme, thereby reducing the activation of guaiacol oxidation by the flavanone group.

Some of the phenolic compounds tested decreased the rate of guaiacol oxidation. When an intramolecularly esterified carboxyl group is attached to the 0-diphenol through an unsaturated side chain in the para position to one of the phenolic groups (Chlorogenic acid), the rate reduction of guaiacol oxidation was the greatest.

When there is an hydroxyl group in the third position of the flavone (quercetin) or flavan-pentol (catechin) structures, the rate of guaiacol oxidation decreased dramatically at comparable concentrations. Quercetin was more effective in the reduction of guaiacol oxidation than catechin, possibly due to the presence of 4H-1-4-one pyran to which the hydroxyl group was attached. In catechin however, this hydroxyl was attached to

the third position of dihydro-2H-1-benzopyran. The relative positions of the hydroxyl groups (ortho or meta) on the B-ring or on the benzene ring of these flavonoid structures may have less importance on the observed effects, since chlorogenic acid with o-diphenol configuration caused the greatest reduction in the rate of guaiacol oxidation. The effect of different quercetin concentrations on the rate of peroxidatic guaiacol oxidation was studied. The results indicated that the rate of decrease was concentration dependent.

The 5, 7, 4'-trihydroxyisoflavone (genistein) reduced the rate of guaiacol oxidation by peroxidases less than chlorogenic acid, quercetin and catechin. The results indicated that the presence of a 4'-methoxy group attached to the B-ring of the 5, 7-dihydroxy isoflavone (Biochanin A) activated the assay, whereas a 4'-hydroxyl group in a similar isoflavone (Genistein) decreased the rate of guaiacol reaction.

The 7-hydroxy 4'-methoxy isoflavone (Formononetin) did not change the rate of guaiacol oxidation by white clover root peroxidases in these in vitro assays at the two different concentrations tested. Biochanin A and genistein, the other isoflavones tested, did affect the rate of reaction. Both had two free phenolic groups in the meta position. The presence of another free phenolic group attached to the B-ring (genistein) reduced the rate of reaction. However, the presence of a methoxy group in B-ring caused a small amount of enhancement in the rate of guaiacol oxidation. These results indicate that more than one free phenolic group may be necessary for the isoflavones to be active in peroxidatic reactions.

Trimethylxanthine (caffeine) did not affect the rate of guaiacol oxidation. This molecule has none of the features (aromatic-ring, phenolic groups) which were thought to affect the rate of guaicol

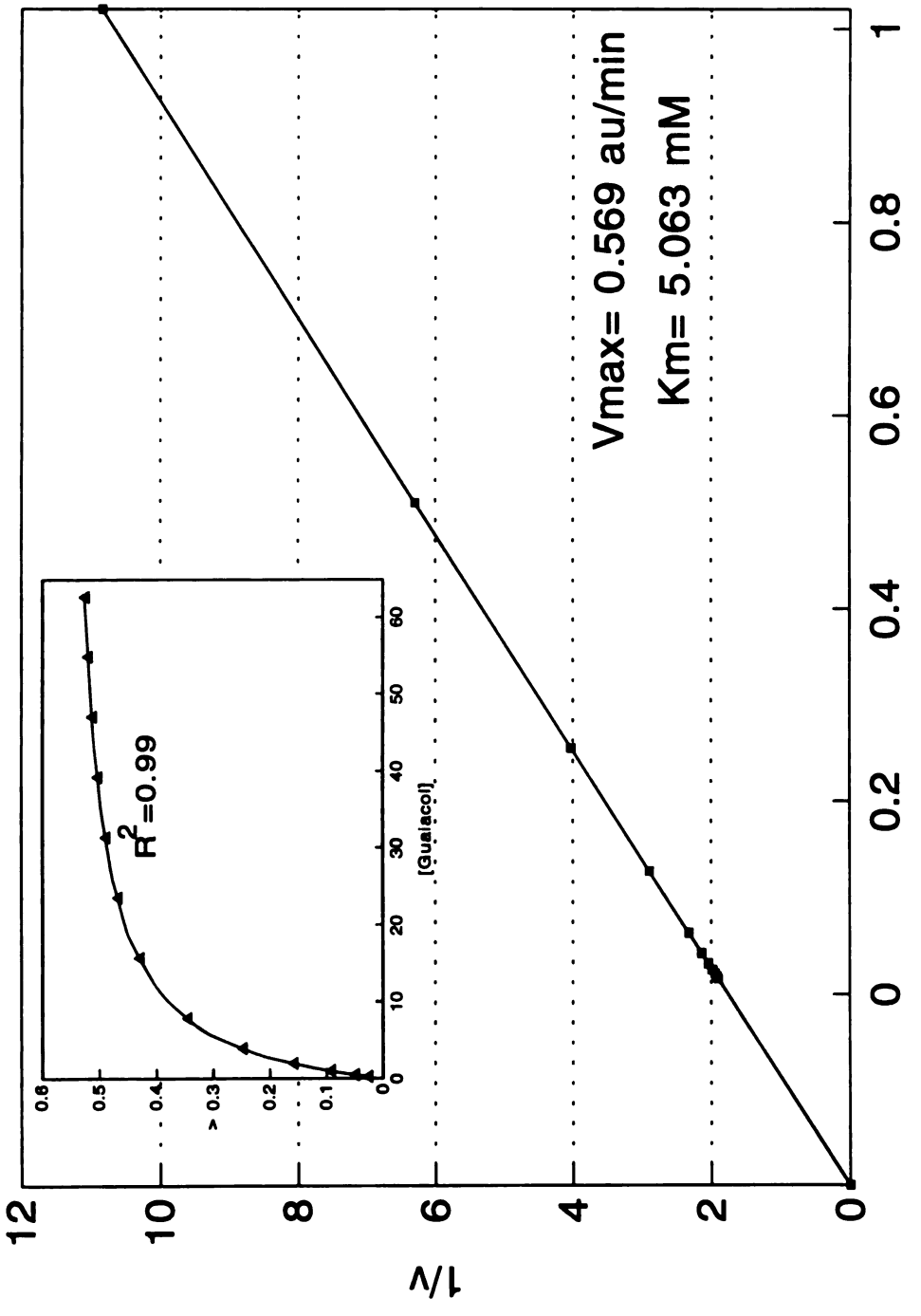
oxidation. Intrestingly, caffeic acid did not affect rate of the reaction either. Caffeic acid is similar to ferulic acid, but instead of an etherified phenolic group in the second position, it has another free hydroxyl group. Therefore, a free phenolic group in the para position to a carboxyl group linked to the aromatic ring via an unsaturated side chain activates the assay, as was seen in p-coumeric acid. An additional methoxy group in ortho position to the free hydroxyl (ferulic acid) greatly enhanced the guaiacol reaction. However, two free phenolic groups in such a structure (caffeic acid) was inactive.

When hydrogen peroxide was omitted from the assay mixture or when the soluble protein extracts were boiled for 10 minutes there was no color development. Additionally, no color developed in the absence of hydrogen peroxide when the assay mixture was thoroughly aerated five minutes after or prior to the additon of the soluble protein extract.

DISCUSSION

Soil application of formononetin reduced the specific activity of soluble white clover root peroxidases (Chapter 2). However, the mechanism of this effect is not known. Two possibilities were considered for the observed effect. Firstly, formononetin, which enters the roots via plant uptake, may inhibit the enzymes. Secondly, exogenously applied formononetin entering the roots via uptake may change the balanced concentration of endogenous formononetin and this change may be a signal for a shift in levels of peroxidase expression. This latter effect may be mediated by formononetin itself or one of its breakdown or interconversion products either in the soil or in the roots. The first possibility was tested, with an in vitro assay. Total soluble peroxidase activity of

Figure 3.9. Double reciprocal (1/v versus 1/[s]) Lineweaver-Burk plot for total peroxidase kinetics data. Velocity (v) refers to change in absorbance units (480 nm) per minute (au/min). Guaiacol was used as the substrate. H_2O_2 was 11.36 mM.


Vmax= 0.569 au/min
Km= 5.063 mM
1/V
1/[Guaiacol]
R2=0.99
[Guaiacol]
v

white clover root extracts was measured in the presence of formononetin. To better evaluate the in vitro activity of white clover root peroxidases, other potential hydrogen donors were also included in the study. The compounds assayed for their effects on total peroxidase activity were mainly isoflavones, flavones, phenolic acids, several other plant phenolics and a purine alkaloid.

Root peroxidases from white clover catalyzed the oxidation of guaiacol (2-methoxyphenol). The total peroxidase activity of white clover roots followed simple Michaelis-Menten kinetics in terms of guaiacol concentrations (Fig. 3.9). During the progression of the reaction a brown colored product forms. The product formation can be easily detected in the visible light range. Other compounds included in the guaiacol oxidation assay may change the reaction rate or may have no effect. The compounds that change the reaction rate may be substrates, inhibitors or effectors. When the effect is established, the mode of action can be determined with further investigations.

In several plant species, isozyme bands appear with common peroxidase stains when hydrogen peroxide is omitted. (Grison and Pilet, 1985; Sheen and Calvert, 1969; Srivastava and Van Huystee, 1977). Generally, these bands are a subset of the isoperoxidase bands (Badiani *et al.*, 1990; Ebermann and Stich, 1986). These findings demonstrated that peroxidases also possess phenoloxidase activity. In my study, when hydrogen peroxide was omitted, guaiacol was not oxidized by white clover root enzymes. If white clover were to have distinct phenoloxidases, guaiacol was not a substrate for these enzymes according to the results of this present study. Another possibility is that the phenoloxidase-like activity of white clover root peroxidases may require a pH different from that used

for the peroxidase assay in this study. Therefore, the results reflected hydrogen peroxide dependent oxidation.

The results indicated that some of the phenolic compounds tested were able to increase the rate of guaiacol oxidation. Phenolic acids with one free phenolic group (p-Hydroxy benzoic acid, vanillic acid, p-coumeric acid, and ferulic acid) stimulated the assay. Similarly, stimulation of guaiacol oxidation by ferulic acid occurs when activity of purified *Nicotiana tabacum* isoperoxidases are assayed (Pickering *et al.*, 1973). The rate of the reaction increased approximately to the same degree for both an anodic and a cathodic isoperoxidase assayed in the presence of ferulic acid. Futhermore, it has been demonstrated that ferulic acid is actually a substrate for the two *Nicotiana tabacum* isoperoxidases tested. Similar results have been obtained for the three anodic and two cathodic isoperoxidases which are purified from Korean radish roots (Lee and Kim, 1994). Some of these radish root isoperoxidases have ten times more affinity for ferulic acid than for guaiacol. Scopoletin also activated the guaiacol assay for one of the *Nicotiana* isoperoxidases and this phenolic compound has been shown to be a substrate for this isoperoxidase (Reigh *et al.*, 1973).

A similar increase in the rate of oxidation in the presence of another hydrogen donor has been demonstrated for the lignin peroxidase of the white rot fungi. The oxidation of 4-methoxy mandelic acid yields 180 times more of the product anisaldehyde in the presence of veratryl alcohol when both are in equimolar concentrations (Harvey *et al.*, 1992). Veratryl alcohol is a secondary metabolite of this fungus. The lignin peroxidase oxidizes veratryl alcohol to radical cations and it is proposed that the radical cations mediate the oxidation of lignin by charge transfer.

Veratrole (0-dimethoxybenzene) also increases the rate of product formation in the lignin peroxidase reactions. Therefore, the enhancement in guaiacol oxidation by ferulic acid implied that ferulic acid was a preferred substrate for soluble white clover root peroxidases and possibly participated in the non-enzymatic oxidation of guaiacol molecules. However, it is thought that phenoxy radicals do not engage in charge transfer reactions (Harvey *et al.*, 1992). If this is so, either ferulic acid oxidation by the enzyme does not produce phenoxy radicals in the usual manner or it is a different intermolecular exchange than charge transfer. The third possibility could be related to the transient enzyme intermediates formed during the oxidation. The oxidation of ferulic acid may produce a transient intermediate form of the enzyme with a higher oxidation potential which may favor the oxidation of guaiacol with greater ease than the native enzyme. Here, ferulic acid was given a greater emphasis since it activated guaiacol oxidation the most. The other phenolic acids which caused varying degrees of activation may share some of the possible mechanisms which were disscused for ferulic acid. The degree of activation appears to be related to the differential affinity of the enzymes for the hydrogen donors and the reductive capability of the phenolic molecule itself.

In addition to phenolic acids, the isoflavone biochanin A and the substituted benzopyran umbelliferone caused a small degree of activation. If these compounds were substrates for peroxidases, the affinity of the enzyme for them must be lower than guaiacol. Indeed, umbelliferone can be peroxidatically oxidized only by two of the twelve winterwheat leaf isoperoxidases (Badiani *et al.*, 1990).

The compounds that lowered the rate of guaiacol oxidation were most likely substrates for the white clover soluble root peroxidases rather than simple inhibitors. Chlorogenic acid decreases the rate of guaiacol oxidation by *Nicotiana* isoperoxidases drastically (Pickering *et al.*, 1973) and it is a substrate for all of the winter wheat soluble isoperoxidases (Badiani *et al.*, 1990). The caffeic acid moiety of chlorogenic acid undergoes a rapid turnover *in vivo*. The caffeic acid moiety is removed with oxidative reactions, followed by concomitant conversions, and is polymerized to lignin molecule (Taylor and Zucker, 1966; Berlin *et al.*, 1971; Aerts and Baumann, 1994). Interestingly, when the carboxyl group is intramolecularly esterified, as with chlorogenic acid, the resulting molecule is highly reactive. According to the results of this experiment, caffeic acid itself did not change the rate of guaiacol oxidation. This may indicate that it was not reactive with the clover peroxidases even when tested at two times higher concentration than chlorogenic acid. In *Nicotiana*, when tested in equimolar concentrations (0.4 mM), chlorogenic acid completely blocked guaiacol oxidation whereas caffeic acid highly activated the assay (Pickering et al., 1973).

Flavonols and flavonones are all degraded with their B-rings liberated as the equivalent benzoic acids (Barz and Hoesel, 1975). Flavanol degradation by peroxidases have been found to start with the formation of 2,3-dihydroxyflavonones. The formation of 2,3-dihydroxy flavonones is a sensitive peroxidase reaction for its requirement of hydrogen peroxide in stoichiometric amounts. The results of the present study suggested that quercetin and catechin could also be better substrates for white clover peroxidases than guaiacol. As it has been determined previously, quercetin is also a better substrate than IAA

(Indole acetic acid) in peroxidase and oxidase reactions (Barz, 1977). These two flavonoid compounds both have a hydroxyl group in the third position of the pyran ring. The flavonoids with a free hydroxyl group in the third position and with an hydroxyl or hydrogen substituent in position 3' are oxidized with relative ease (Barz, 1977). The lesser effect of genistein on lowering the rate of guaiacol oxidation confirmed this generalization. This isoflavone by nature carries the B-ring as attached to the third position. The results indicated that among the three isoflavones tested, genistein was the most active. This appears to be due to the presence of an hydroxyl group in 4' position.

Most of the phenolic compounds tested were able to exert an effect on guaiacol oxidation by white clover peroxidases in vitro. Caffeine was not expected to show any activity and the results verified this prediction. Catabolism of this purine alkaloid appears to be mediated through enzymes other than peroxidases.

Formononetin, the isoflavone that caused a reduction in the specific activity of soluble peroxidases in white clover roots, did not affect the rate of guaiacol oxidation in vitro. This indicated that direct inhibition of the enzymes by formononetin was unlikely. Accumulation of secondary plant metabolites is regulated by the induction or repression of the enzyme systems that are involved in the biosynthesis or turnover of such plant products (Barz, 1977). The peroxidase system has a significant contribution in the metabolism of secondary products (Putter and Becker, 1983). This family of enzymes differ in reactivities with a variety of substrates and they are expressed independently at different stages of development (Liu, 1975). It is known that formononetin accumulates in the green parts of clover and alfalfa, and the amounts accumulated appear to

be affected seasonally (McMurray *et al.*, 1986; Smith *et al*, 1986b). This accumulation may be achieved in these legumes such that the peroxidases or phenol oxidases that can oxidize formononetin may not be expressed constitutively or at certain times their rate of biosynthesis may be much higher than their rate of turnover.

Root feeding experiments with radiolabelled formononetin indicate that the isoflavone is metabolized by *Cicer arietinum* and the label can be recovered in several fractions of plants. However, in a similar experiment, formononetin is not metabolized by *Phaseolus aureus*, in which most of the isoflavone taken up by the roots is recovered intact, whereas the isoflavone daidzein is metabolized easily (Barz *et al.*, 1970). These previous findings suggest that there are differences among the leguminous plants in terms of isoflavone metabolism. Another possible explanation for the formononetin accumulation can be that the level of this isoflavone may be regulated via biosynthesis rather than turnover in clover or alfalfa roots. If this is so, the isoflavone metabolism in these two legumes is different from the *Cicer* system which has been studied in greater detail in this context.

These results suggested that formononetin mediated reduction in the specific activity of white clover root peroxidases did not occur via direct inhibition of the enzyme. The effect may be exerted through more complex interactions and the nature of which deserves further studies.

CHAPTER 4

RECOVERY OF EXOGENOUSLY APPLIED MYCORRHIZA-STIMULATORY ISOFLAVONES FROM SOIL AND THEIR EFFECTS ON SOIL MICROBIAL POPULATIONS

INTRODUCTION

The technique of adding and recovering biologically active, natural plant-derived phenolics from soil has been applied to determine their fate and behavior (Dalton, 1989). Such studies have almost exclusively explored phenolic acids, because of their association with allelopathic interactions in some ecosystems and in cultivated soils (Wang *et al.*, 1967; Patrick, 1971; Sparling *et al.*, 1981; Blum and Shafer, 1988). Phenolic acids enter the soil solution and are metabolised by soil microorganisms (Sparling *et al.*, 1981; Vaughan *et al.*, 1983). Alkaline solutions or water are the preferred extractants for the recovery of added phenolic acids (Wang *et al.*, 1967; Sparling *et al.*, 1981) to determine the active (bioavailable) concentrations remaining in soils (Dao *et al.*, 1987; Dalton, 1989). The rate of phenolic acid decomposition, the effects on soil biomass, and the response of soil microbial populations have been measured (Sparling *et al.*, 1981; Blum and Shafer, 1988). Studies on immediate response of soil biomass to added phenolic acids have indicated that some crops, such as potato, stimulate the growth of phenolic acid degrading microorganisms (Blum and Shafer, 1988). Isoflavones, another group of biologically active plant phenolics , have received less

group of biologically active plant phenolics , have received less attention in this context.

The isoflavones formononetin and biochanin A occur in shoots and roots of leguminous plants (Gildersleeve *et al.*, 1991; Volpin *et al.*, 1994) and in root exudates of some *Trifolium* and *Medicago* species (Nair *et al.*, 1991; Tsai and Phillips, 1991). These isoflavones are also found as sugar conjugates (Jaques *et al.*, 1985). Formononetin 7-O-glucoside has been detected in soils associated with a weed species, *Pluchea lanceolata* (Inderjit and Dakshini, 1992). Formononetin-7-O-glycoside has also been identified in methanolic extracts of Alfalfa rhizosphere soil (Leon-Barrios *et al.*, 1993). The occurence of formononetin glycoside in rhizosphere soil is thought to be due to either the large amounts of sugar conjugate in root exudates or its stability in soil.

Exogenous application of formononetin and biochanin A increases the rate of root colonization by vesicular-arbuscular mycorrhizal fungi during the early stages of symbiosis (Siqueira *et al.*, 1991a). However, the decomposition rates of these isoflavone aglycone additives and their effects on soil microbial populations are not known.

This study reports the recovery of added formononetin and biochanin A in methanolic extracts from sterile and non-sterile soil in the presence or absence of plants. The vertical migration of formononetin applied as solution or solid, and response of microbial populations to added isoflavones in soil from a potato field was also investigated.

MATERIALS AND METHODS

Extraction of added isoflavones from sterile and non-sterile soil

Soil material was prepared by mixing one part top soil with one part silica sand. Soil analysis of this mixture (Soil A) is summarized in Table 4.1. This soil A (10 g) was transferred into 2.5 cm x 15 cm test tubes. Half of the test tubes containing soil A were autoclaved, one hour, for three consecutive days. Formononetin (provided by American Cyanamid Co.) or biochanin A (purchased from Sigma Chemical Co. St. Louis, MO) was incorporated in solid form into the soil in the test tubes, 5 mg per tube, after thorough mixing each tube received 1 ml sterile distilled water to moisten the soil. The test tubes were capped with aluminum foil and kept in the dark at room temperature. The compounds were extracted from the soil with hot methanol (25 ml x 3) at 0, 5, 10 and 15 days. Extractions were performed by stirring the content of the test tubes over a heat gun (Master appliances Corp. HG 201). The combined total extracts (75 ml) were filtered through a No.1 filter paper (Whatman) and were evaporated to a final volume of 5 ml using a rotary evaporator (Rotavapor, Buchi). These concentrated solutions were filtered through 0.2 micron pore size filters (Acrodisc, milex FGS, Millipore) prior to HPLC analysis.

Extraction of added isoflavones from soil planted with corn

Corn seeds (Great Lakes hybrid) were germinated on moist filter paper in petri plates. Styrofoam cups (0.6 L capacity) were filled with 0.5 L of soil A. Each cup received 100 ml of formononetin or biochanin A solutions (5 mg L^{-1}) and 50 ml of one half strength Hoagland's nutrient solution (Hoagland and Arnon, 1950). The isoflavones were dissolved first

Table 4.1. Soil test results[a]

Parameters tested	Soil A	Soil B
Soil group	5 Sand	3 Sandy loam
Plow depth	9 inches	9 inches
Soil pH	7.5	5.7
Phosphorus	45 lb/A	140 lb/A
Potassium	71 lb/A	185 lb/A
Calcium	1448 lb/A	880 lb/A
Magnesium	176 lb/A	184 lb/A
Zinc	2.0 ppm	3.6 ppm
Manganese	24.2 ppm	19.0 ppm
Copper	1 ppm	1 ppm
Iron	5 ppm	51 ppm
CEC[b]	4.4 me/100g	9.2 me/100g

a: Soil samples were analyzed by Michigan State University, Soil Test laboratory
b: Cation exchange capacity

in a small volume of methanol (0.2%) and then added to water. The pre-germinated corn seedlings were planted into the soil, and grown in a greenhouse for three weeks (under conditions described in Chapter 2). The plants received 100 ml water every other day. At 7, 14, and 21 days after planting, the corn plants were removed from the soil, and the soil particles on the roots were loosened and recovered by shaking. Three 20 gram samples were taken from each treatment and were extracted in hot methanol (75 ml x 3) as described above. The extracts (225 ml) were evaporated to 5 ml volumes and filtered through 0.2 micron filters before HPLC analysis.

Measurement of vertical migration of formononetin in sterile soil

The migration of formononetin through soil was investigated with autoclaved soil A only. Plastic cups (1 L capacity, 13 cm high, with small holes punched in the bottom for drainage) were filled with the soil. The soil received 5 mg of formononetin in either solid or solution form. When applied as a solution, the formononetin (5 mg) was dissolved in 10 ml of methanol. The compound (solid or solution) was applied to the center of the soil surface and covered with a 0.5 cm layer of soil. The soil received 100 ml distilled water daily. Excess water which drained from the holes was collected and combined in flasks designated for each cup and stored at -4°C until further analyzed. This water was evaporated at 75°C for 24 hours. After evaporation the solids were recovered in 50 ml methanol and concentrated to 5 ml volume and prepared for HPLC analysis.

At six days the plastic cups containing the soil with solid or dissolved formononetin were cut horizontally into five disks. The disks were approximately 2.5 cm thick and the mid point of each slice

represented 1, 3.5, 6, 8, and 11 cm average distances from the top. The soil in each disk was placed in an aluminum plate and dried in a oven at 57°C for 24 hours, and the dry weights were recorded. After thorough mixing, two 10 g samples of the soil were taken from each disk. The samples were refluxed 15 minutes in 50 ml methanol. The refluxed soil was filtered and the methanolic extracts concentrated in a rotary evaporator to 5 ml volumes and prepared for HPLC analysis as mentioned above.

HPLC analysis

Aliquots of filtered concentrates (5-10 μl) were injected into a Waters high performance liquid chromatography (HPLC) system (Millipore Corp., Bedford, Mass) for recovery studies. The system was operated under isocratic conditions. The mobile phase solvent methanol:water, 5:1 (v/v), was used to elute the isoflavones from the HPLC column (C18, 10 mm x 250 mm, 10 micron). The isoflavones were detected at 254 nm with a Waters 490 programmable multi-wavelength detector. Peak integrations were made with a Waters 740 Data Module integrator. The analysis of methanol extracts for the migration study was performed with a Varian HPLC system (Varian associates Inc. Walnut Creek, Ca), a Waters model 481 detector, and a Shimadzu CR501 integrator, and a C8 (4.6mm x 125 mm) column (Chemcosorb, Dychrom, Sunnyvale, Ca.). Mobile phase and the aliquot volume injected were the same as above. The isoflavones were identified by comparing retention times with those of the standard solutions, pure formononetin and biochanin A dissolved in methanol (1 $mg.ml^{-1}$). For isoflavone quantitation, the peak areas of the recovered isoflavones were compared to the peak areas of the standard solutions. All percent recovery calculations were based on duplicate HPLC chromatogram readings for three

repetitions. For the migration study duplicate readings of two repetitions were averaged.

Enumeration of soil microorganisms on agar media

The effects of added formononetin and biochanin A on soil microbial populations were determined according to Blum and Shafer (1988). A soil subsample from a relatively low pesticide- and herbicide-input Michigan potato field was used for this study. The field had been rotated with alfalfa and potato several times. The soil analysis is summarized in Table 4.1 (Soil B). The soil was placed into 250 ml sterilized Erlenmeyer flasks (10 g soil per flask). Soil was amended with glucose, formononetin, or biochanin A in solid form (0.5 $mg.g^{-1}$ soil). The flasks were capped with aluminum foil and the amendments were mixed into the soil by shaking the flasks. To moisten the soil, 1 ml of sterile distilled water was added to the soil in each flask. Control soil received only water. Flasks were held at room temperature in the dark. At 3, 5, 10, and 15 days, 20 ml of sterile distilled water were added to each flask. The flasks were placed on an orbit shaker at 150 rpm for 15 min and diluted to 10^{-6} with 10-fold serial dilutions. Petri plates with agar media were spread with diluted soil and kept in an incubator for 6 days at 30°C in the dark. For enumeration of initial microbial populations in soil A and soil B, 20 ml of water was added to 10 g samples of air dried soil. Following the 10-fold serial dilutions, the soil suspensions were spread onto selective agar media described below. The LSD (least significant difference) was calculated among treatments at α=0.05 for each time point (3, 5, 10, 15 days) by using a computer program (MSTAT).

Medium ACT (1 g KH_2PO_4, 0.4 g $MgSO_4.7H_2O$, 0.13 g $CaCl_2.2H_2O$, 0.1 g

NaCl, 0.01 g $FeCl_3$, 0.50 KNO_3, 20 μl pimaricin as a fungal growth inhibitor, and 15 g agar per liter of distilled water) was used for enumeration of Actinomycetes. Medium BMS (ACT plus 1 g glucose) was used for enumeration of bacteria. For enumeration of fungi, the medium FGI (ACT without pimaricin plus 0.05 g rose bengal and 0.1 g chloramphenicol) was used. Medium BNA (23 g nutrient agar; Difco laboratories 23, 20 μl pimaricin, and 0.05 g tetrasodium phenolphtalein diphosphate) was also used for enumeration of bacteria including phosphatase positive colonies. Medium GNB for enumeration of gram-negative bacteria was similar to medium BNA but tetrasodium phenolphthalein was substituted with 0.004 g of crystal violet. The media were adjusted to pH 7.0 before autoclaving.

RESULTS

Recovery of added isoflavones from soil

The amounts of added isoflavones recovered from autoclaved and non-sterile soil differed over a 15 day period (Fig. 4.1). In sterile soil the amount of recovered isoflavones did not change, but in non-sterile soil a time dependent decline was observed. The rate of decline in the recovered amounts was faster with biochanin A than with formononetin. In the HPLC chromatograms, no peaks other than those of formononetin or biochanin A were detected at 254 nm.

The microbial counts were low in the non-sterile soil used for the recovery studies. The air-dried soil contained 30.10^4 c.f.u. for Actinomycetes and 23.10^4 c.f.u. for bacteria. The counts were lower than 10 c.f.u. in the smallest dilution (10^{-1}) for gram-negative, phosphatase-positive, bacteria (BNA) and fungi.

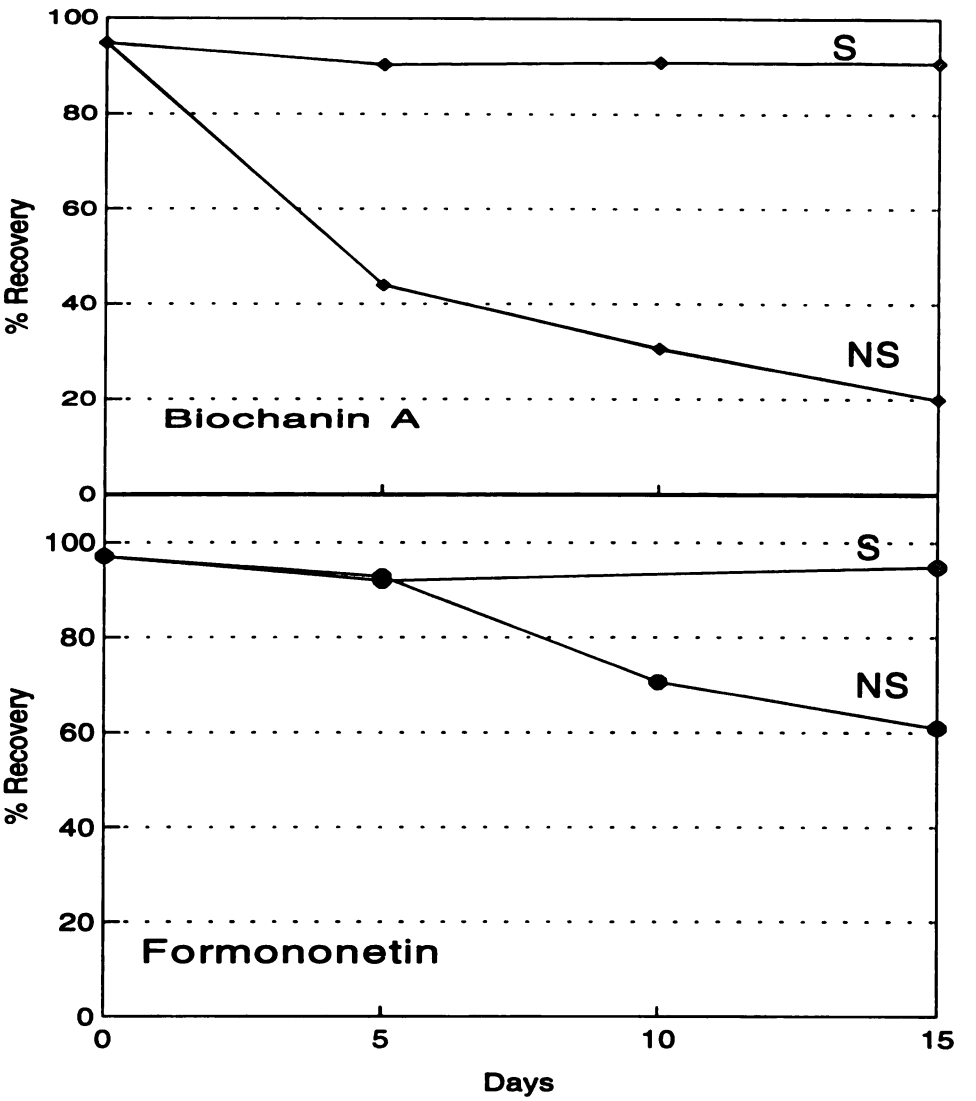


Figure 4.1. Recovery of formononetin and biochanin A amendments (0.5 mg. g^{-1} of soil) from soil-sand mixture. S: autoclaved soil; NS: non-sterile soil. Percent-recovery values represent the average of duplicate measurements of three replications. Differences between the treatments (S and NS) significant at $\alpha = 0.05$ at 5, 10, 15 days for biochanin A; at 15 days for formononetin.

In the presence of corn plants, much less exogenously added isoflavones was recovered (Fig. 4.2). One week after addition, more than 60% of formononetin was extracted back, however about 95% of biochanin A had disappeared. Although about 5% of the added formononetin was detectable by the end of three weeks, biochanin A was not detectable. HPLC chromatograms of the methanolic extracts of the soil planted with corn seedlings indicated several other peaks in addition to formononetin and biochanin A peaks. In these chromatograms, formononetin and biochanin A peaks were confirmed by mixing and co-injecting the methanolic soil extracts with the standard solutions of the isoflavones.

Vertical migration of formononetin in soil column

Migration of formononetin was monitored in autoclaved sandy soil. At several distances below the application point dissolved formononetin was recovered in higher amounts than solid formononetin (Fig. 4.3). These results indicate that when applied in solution form, more of the added formononetin migrated vertically downward into the soil column than when applied in solid form. Dissolved formononetin did not reach the bottom of the 13 cm cups in six days, as formononetin was not detected in either the drained excess water or deeper than 11 cm in the soil column.

Response of soil microbial populations to added isoflavones

Response of soil microbial populations to added isoflavones was measured in reference to glucose stimulation. For this study, soil from a potato field was used. The initial counts of microbial populations grown on selective media are summarized in Table 4.2.

The largest increase in actinomycete and the bacterial populations

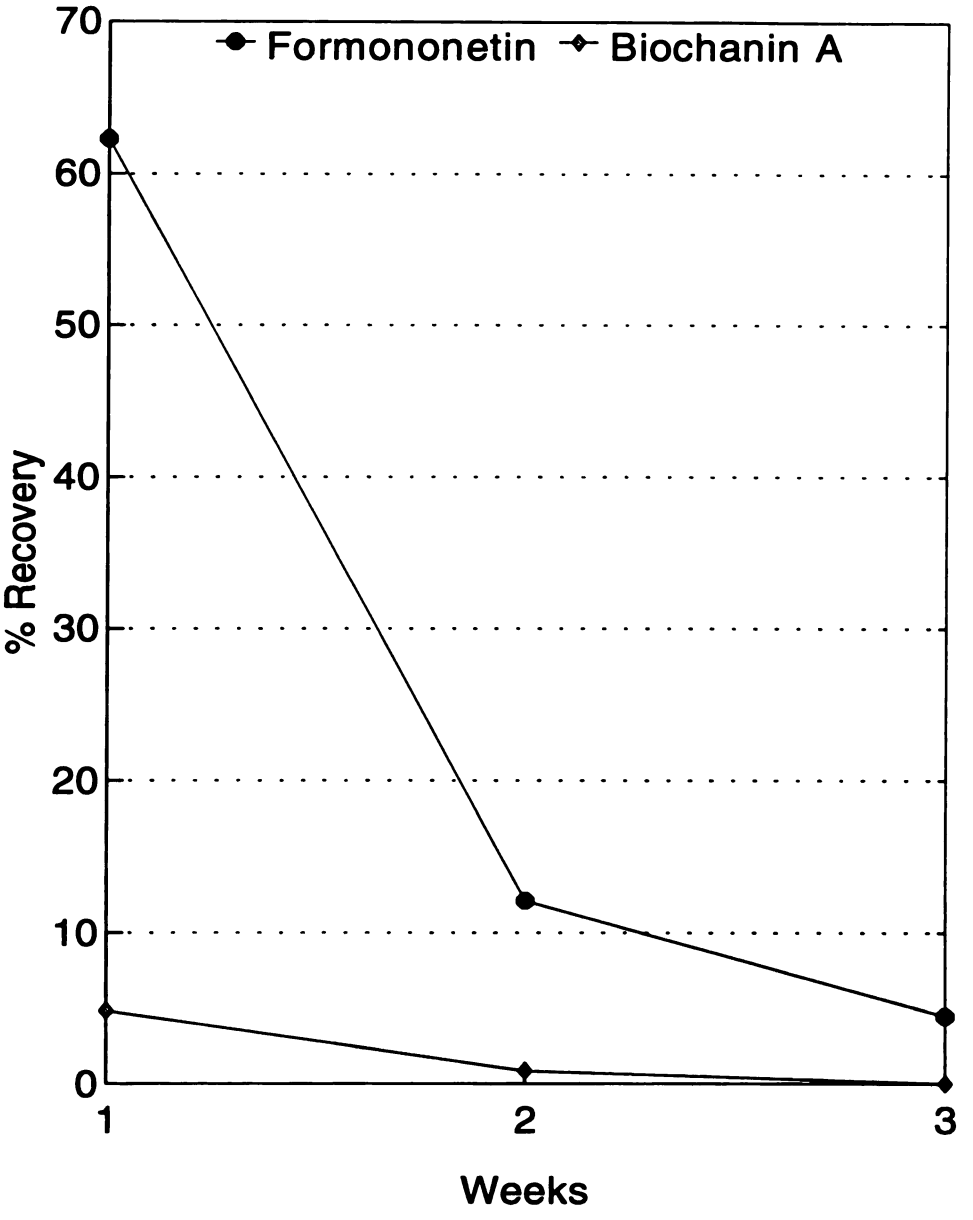


Figure 4.2. Recovery of formononetin and biochanin A amendments (0.625 $mg.kg^{-1}$ of soil) from soil planted with corn seedlings. Percent-recovery values represent averages of single measurements of three replicate samples.

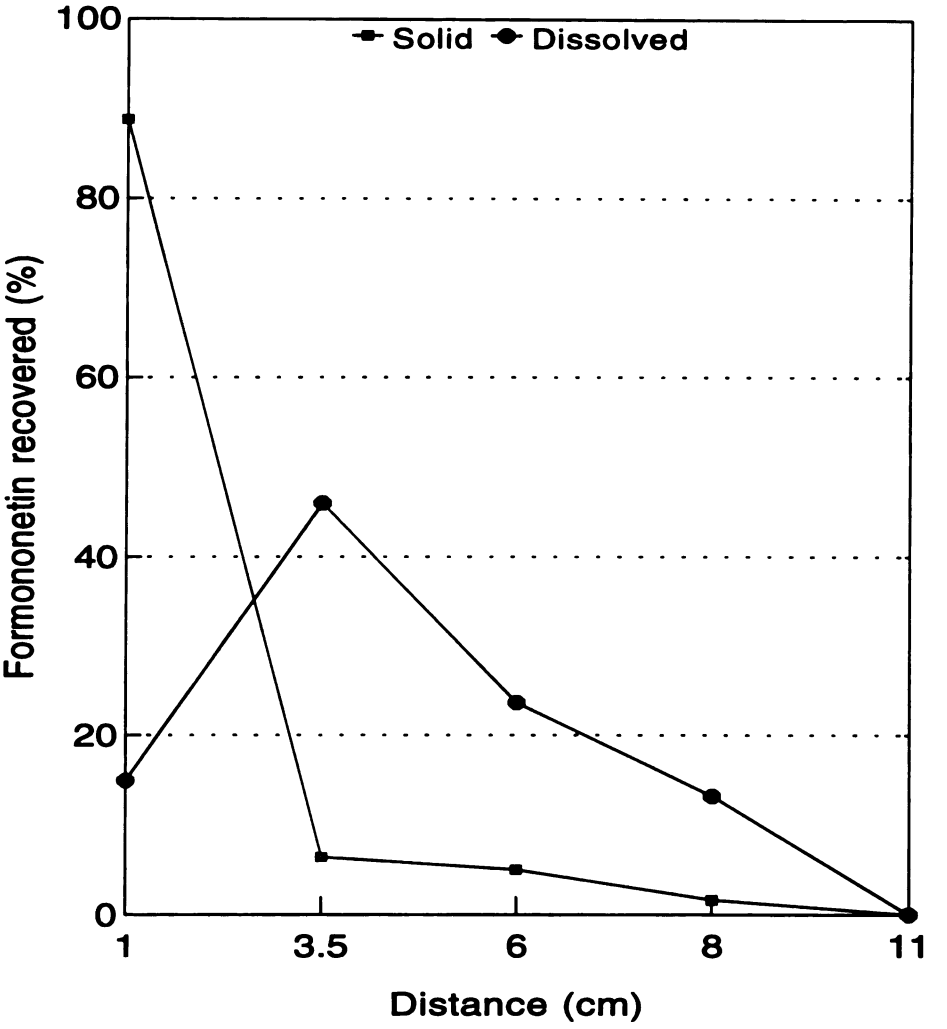


Figure 4.3. Vertical migration of formononetin in soil column. Formononetin applied as solution or as solid form (3.125 $mg.kg_{-1}$ of soil) to 1:1 mixture of top soil and silica sand (autoclaved). The soil received water (100 ml per day) for 6 days. Formononetin was recovered with hot methanol extraction. Percent-recovery values represent the averages of duplicate readings of two repetitions.

cultured on BMS and BNA media was observed with glucose in three days (Fig. 4.4 A, B, C). Similarly, both formononetin and biochanin A additions caused the largest increase in the actinomycete and bacterial population cultured on BMS at day three.

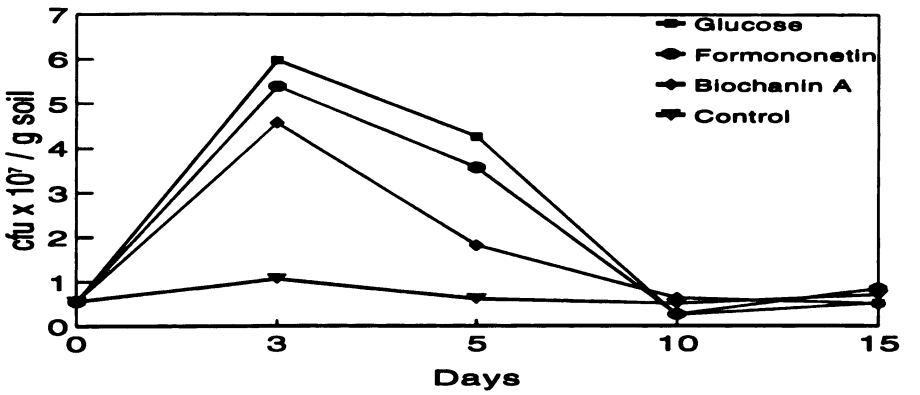
Table 4.2. Initial microbial population in soil B

Media	Microorganisms	10^4 c.f.u. g^{-1} dry soil
ACT	Actinomycetes	553.3 ± 42.4
BMS	Bacteria	693.3 ± 100.0
BNA	Bacteria	11.8 ± 1.2
BNA	Phosphatase$^+$ Bacteria	2.53 ± 0.1
GNB	Gram Negative Bacteria	6.26 ± 1.7
FGI	Fungi	3.73 ± 1.3

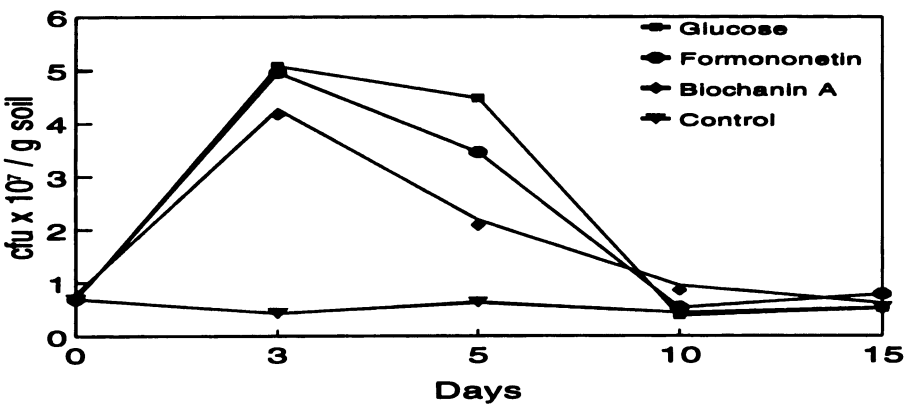
Formononetin addition increased the actinomycete population as much as glucose and biochanin A 76.5% of glucose stimulation (Table 4.3). Formonetin addition increased the number of bacteria cultured on BMS medium as much as glucose and biochanin A addition increased 82% of glucose stimulation (Table 4.3). The number of bacteria cultured on BNA medium was the highest in glucose amended soil (Table 4.3 and Fig. 4.4 C). On BNA medium, the number of bacteria increased with biochanin A addition only after 10 days while the number of bacteria in biochanin A amended

Figure 4.4. Response of soil microbial populations to isoflavone or glucose amedments (0.5 $mg.g^{-1}$ of dry soil). Soil samples were obtained from a potato field. Defined mineral salt media (ACT and BMS) were used for the enumeration of Actinomycetes and bacteria. For the enumeration of bacteria a complex medium (BNA) was also used.

A. ACTINOMYCETES



B. BACTERIA (BMS)



C. BACTERIA (BNA)

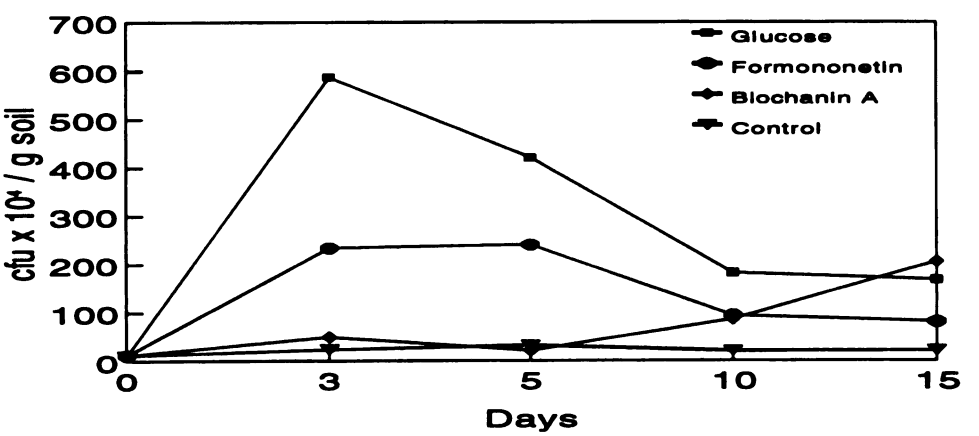
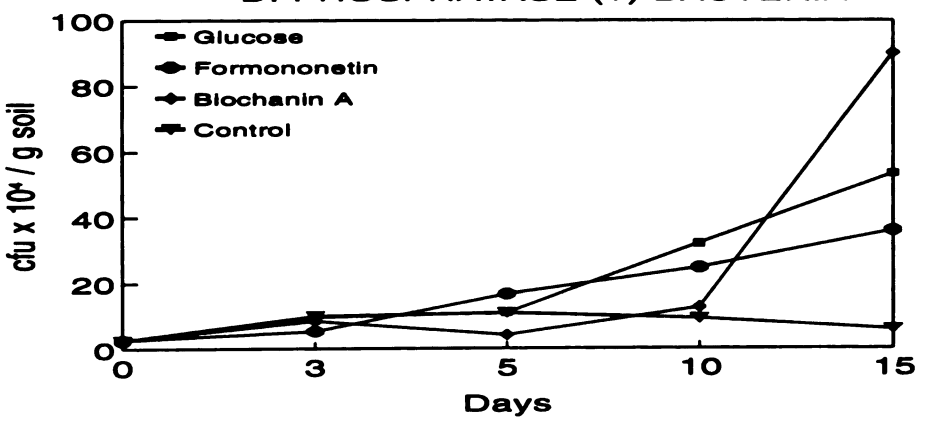
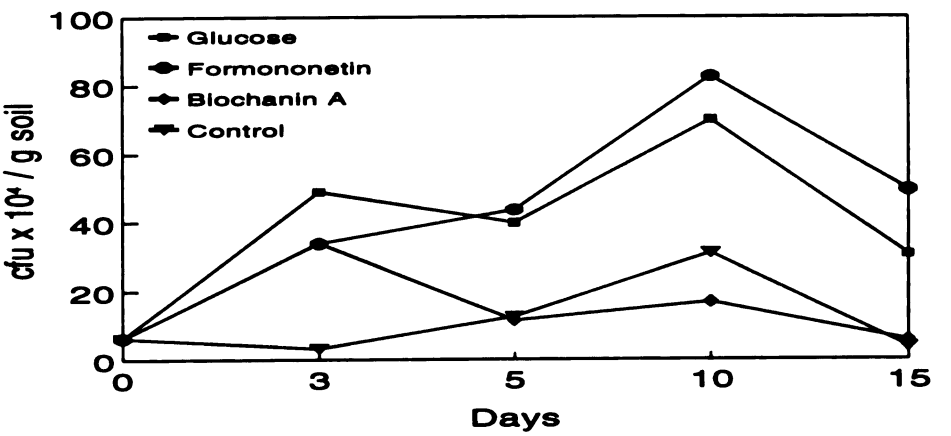


Figure 4.5. Response of soil microbial populations to isoflavone or glucose amedments (0.5 $mg.g^{-1}$ of dry soil). Soil samples were obtained from a potato field. Defined mineral salt medium (FGI) was used for the enumeration of fungi. For the enumeration of Gram negative and phosphatase-positive bacteria, complex media (GNB and BNA) were used.

D. PHOSPHATASE (+) BACTERIA



E. GRAM-NEGATIVE BACTERIA



F. FUNGI

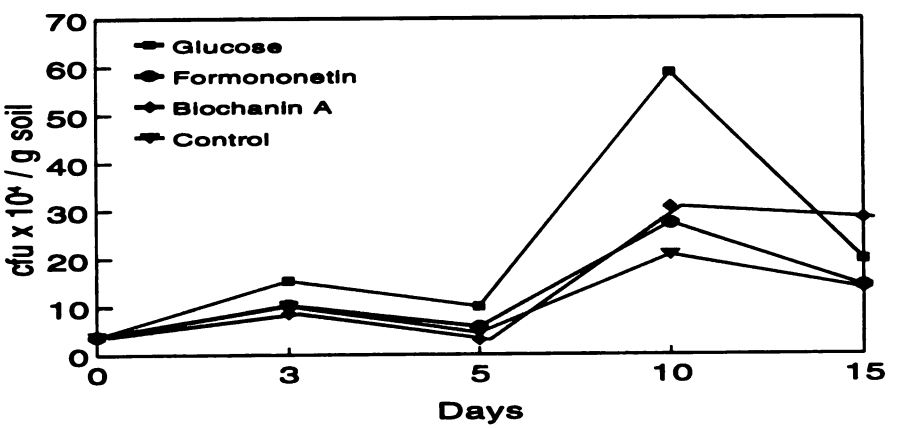


Table 4.3. Least significant difference (LSD) values determined for soil microorganisms (10^4x c.f.u. g^{-1} dry soil) growing several days in soil amended with glucose, formononetin and biochanin A.

	Days			
Microorganisms	3	5	10	15
Bacteria (BMS)				
Control	426.0	630.6	431.3	533.3
Glucose	5080.0	4473.3	380.0	514.0
Formononetin	4953.3	3460.0	533.3	782.0
Biochanin A	4166.6	2080.0	851.3	517.3
$LSD_{0.05}$	587.4	878.0	175.7	121.3
Gram Negative Bacteria (GNB)				
Control	3.2	12.6	31.0	3.7
Glucose	48.9	39.8	70.0	30.6
Formononetin	33.8	43.6	82.6	49.3
Biochanin A	34.0	11.4	16.8	5.6
$LSD_{0.05}$	6.8	5.7	18.2	9.5
Phosphatase (+) Bacteria (BNA)				
Control	10.0	10.9	9.2	6.0
Glucose	9.6	11.0	32.0	53.3
Formononetin	5.4	16.6	24.6	36.0
Biochanin A	8.5	4.2	12.6	90.0
$LSD_{0.05}$	2.6	10.1	8.5	66.0
Bacteria (BNA)				
Control	24.3	32.3	20.7	21.6
Glucose	586.7	420.0	182.0	168.0
Formononetin	233.3	240.0	94.0	80.6
Biochanin A	50.0	21.4	86.0	205.3
$LSD_{0.05}$	187.1	168.4	54.6	104.2
Actinomycetes (ACT)				
Control	1066.6	610.6	502.0	700.6
Glucose	5973.3	4260.0	515.3	515.3
Formononetin	5386.6	3566.6	849.3	849.3
Biochanin A	4566.6	1820.0	497.3	487.3
$LSD_{0.05}$	737.5	575.0	139.8	78.2
Fungi (FGI)				
Control	10.1	4.5	20.6	13.6
Glucose	15.4	9.9	58.6	19.8
Formononetin	10.3	5.7	27.3	14.2
Biochanin A	8.5	3.0	30.6	28.3
$LSD_{0.05}$	3.0	2.5	12.3	3.6

soil was similar to glucose amended soil at 15 days. The number of actinomycetes and bacteria culturable on BMS decreased in all cases at 5 days, and approached the initial counts at 10 days, and with no change between 10 and 15 days. However, on BNA medium the numbers of bacteria were still higher than the initial counts at 15 days with glucose and biochanin A.

The number of phosphatase positive bacteria increased gradually between 5 and 15 days (Fig. 4.5 A). At day fifteen, it was 143% of glucose stimulation in biochanin A amended soil (Table 4.3). The largest increase in numbers of gram negative bacteria was observed at 10 days with formononetin and glucose (Fig. 4.5 B and Table 4.3). At three days, biochanin A and formononetin stimulation was 69% of glucose stimulation. The number of gram negative bacteria decreased and reached the initial counts at 15 days in biochanin A amended soil, whereas the counts were still high with glucose and formononetin.

The fungal population responded to the amendments rather slowly (Fig. 4.5 C, Table 4.3). The highest stimulation was observed with glucose at 10 days. Although the number of fungi decreased in glucose and formononetin amended soil at day 15, it remained high in biochanin A added soil.

DISCUSSION

The mixture of steamed top soil and silica sand used for the recovery studies was similar to the soil material in which white clover plants were grown to test the effect of exogenously applied formononetin on root enzymes and colonization by mycorrhizal fungi (Chapter 2). The microbial counts were low in the soil, which may be to due steaming. Pasteurized

soil obtained by steaming is commonly used for greenhouse experiments to gain more controlled growth environments. The recovery experiments were designed to investigate the amount of exogenously applied isoflavones remaining in the soil at similar time points as the experiments conducted in Chapter 2.

In this study, methanol-extractable concentrations of added isoflavones did not vary over a fifteen day period in sterile soil (Fig. 4.1). However, decreasing amounts of isoflavones were recovered with methanol extraction in non-sterile soil. In all chromatograms, single sharp peaks of biochanin A and formononetin were detected. This indicated that either the isoflavones were decomposed completely or the breakdown products were not detectable at the wavelength used. Phenolic acids and many other compounds containing aromatic rings can be detected at 254 nm (Porter *et al.*, 1986; Smith *et al.*, 1986; Dalton, 1989; Ibrahim and Abul-Hajj, 1990), therefore it is likely that ring fissions occur during the decomposition of the isoflavones in soil (Barz,1977).

Since sterilization can change physical and chemical properties of soils (Lopes and Wollum, 1976; Williams-Linera and Ewel, 1984), Dalton (1989) suggests the comparison of immediate recovery of added compounds from sterilized and non-sterilized soils. In this study, the isoflavones were added on day 0 to non-sterile soil and extracted immediately. The amounts recovered at 5, 10, and 15 days from sterile soil did not differ from the amounts obtained on day 0. This indicated that the properties of the soil-sand mixture that was used in the recovery studies were not affected by sterilization with an autoclave. This stability may be due to the high sand content of the experimental soil material.

Water or sodium acetate has been used as extractants for the

recovery of bioavailable concentrations of phenolic acids (Dao, 1987; Dalton *et al.*, 1987). Bioavailable concentrations of phenolic acids include the molecules in the solution phase of soil and molecules attached to the particles via weak ionic bonds (Dalton, 1989). Water extraction recovers the phenolic acids from the solution phase of soil, while sodium acetate removes phenolic acids associated with soil particles via weak ionic bonds (Dalton, 1989). Methanolic extraction is highly effective in recovering water-insoluble phenolics. Leon-Barrios *et al.* (1993) have identified formononetin glycoside in methanolic extracts of alfalfa rhizosphere soil. However, in previous studies with water or acetate extractions, isoflavone aglycones were not detected. Hot methanol extraction, used in this study, recovers water-insoluble phenolic compounds (that are trapped in the soil via strong associations) as well as the molecules in the solution phase of soil. Therefore, this extraction method allows for the estimation of the amount of isoflavones decomposed in the soil based on the initial input, as opposed to water or aqueous extractants, which also accounts for the soil's trapping abilities. Different soils can trap added phenolic acids to varying degrees (Dalton, 1989). Tropical soils, for example, retain more added phenolic acids than temperate soils. Trapping ability is inversely related with the organic matter content of soils. Sterilization changes the physicochemical properties of tropical soils more than it does temperate soils. The trapping abilities and effects of sterilization have been studied by comparing the immediate recoveries with aqueous extractants (Dalton, 1989).

In this study, the decline of the methanol-extractable amounts in non-sterile soil suggested that the soil microbial population was able to

metabolize these isoflavones. Formononetin and biochanin A were added once at the beginning of the experiment and they were recovered at five day intervals for 15 days. Although some decreases in the amounts recovered were observed, the decrease was slow compared to the disappearance rates of added phenolic acids to soils (Sparling *et al.*, 1981; Blum and Shafer, 1988; Dalton, 1989). This variation in the rate of decline may be a result of the differences in quantity and composition of soil microbial populations. Phenolic acids in the solution phase of soil serve as a carbon source for many soil microorganisms. In fact, some bacterial species preferentially metabolize phenolic acids (Henderson and Farmer, 1955).

Unlike phenolic acids, isoflavone aglycones are not water soluble (Merck Index, eleventh edition). They may dissolve in the micropockets of soil with high pH and may then be utilized by soil microbes associated with these microenvironments (Patrick, 1971). In liquid cultures, formononetin is metabolized completely by rhizosphere bacteria (Barz *et al.*, 1970). However, the rate of utilization in soil environments has not been measured.

According to this study, added isoflavones were decomposed and likely metabolized by the soil microbial population. When soil microbial populations were eliminated with sterilization, the recovered amounts did not decrease. The decomposition was faster in the soil planted with corn seedlings. It is likely that more isoflavones were decomposed, since the mineral nutrient solution applied as fertilizer and organic metabolites from the root exudates enriched the soil and favored the growth of microorganisms. Similarly, studies on response of soil microbial populations to phenolic acid additions suggested that microbial

utilization of phenolic acids is dependent on nutrient availability (Blum and Shafer, 1988). Uptake of the added isoflavones by the roots may also have contributed to the faster rate of disappearance. However, ^{14}C-labelled feeding experiments in hydroponic cultures of *Cicer arietinum* indicated that rhizosphere microorganisms are mainly responsible for the degradation of formononetin (Barz *et al.*, 1970), thus the effect of root uptake may not be the major cause for the more rapid decrease. In this study, the observed difference between the rates of decline in the amounts of isoflavones recovered with or without plants was most likely due to the stimulation of soil microorganisms in the enriched soil.

Since leguminous plant species may also exude isoflavones, corn (*Zea mays*, a graminea) was chosen rather than clover (a leguminous plant) in this experiment to avoid the overestimation of the amounts recovered. Leguminous plants such as clover, alfalfa, or soybean synthesize various isoflavones, including formononetin and biochanin A, in milligram quantities per gram of dry weight (McMurray *et al.*, 1986; Smith *et al.*, 1986). These plants increase isoflavone synthesis under nutrient stress or in the presence of pathogenic organisms (Olah and Sherwood, 1971; Osman and Fett, 1983, Nair *et al.*, 1991). Corn, however, has not been cited for high production of isoflavones, and it is thought that corn may not exude large amounts of bioactive isoflavones (Simpson and Daft, 1990). The corn seedlings were provided with sufficient mineral nutrients and water to eliminate the possibility of stress conditions.

The disappearence rate of biochanin A was faster than that of formononetin in both the presence and absence of plants. This suggests that biochanin A may be a better substrate than formononetin for microbial degradation. The presence of an hydroxyl group at 5-C position may make

biochanin A a more reactive than formononetin. Catabolic degradation of biochanin A by *Fusarium* species has been monitored (Willeke and Barz, 1982; Willeke *et al.*, 1983). In most of the species tested, the degradation begins with the formation of 5, 7- dihydrobiochanin A . The hydroxyl group at 5-C position may facilitate the transport of the molecule into the cells since it has greater polarity than formononetin. In this study, formononetin persisted in the soil longer than biochanin A, possibly because its degradation is a slower process.

Formononetin migration was studied in sterile soil to avoid the interference of microbial breakdown of the isoflavone in the measurements (Fig. 4.3). Formononetin has been previously reported to stimulate vesicular-arbuscular mycorrhizal fungi (Nair *et al.*, 1991), and was found to be more effective in the growth of mycorrhizal plants than biochanin A (Siqueira *et al.*, 1991a). This may be related to its slow degradation rate. Application of formononetin to fields has been suggested to stimulate the indigenous VAM fungi (Siqueira *et al.*, 1991a). Stimulation of indigenous VAM fungi for low input agriculture may be advantageous since VA mycorrhizal symbiosis is known to benefit many crops for uptake of mineral nutrients such as phosphorus and nitrogen (Mosse, 1973; Sanders and Tinker, 1973; Bowen and Smith, 1981; Smith *et al.*, 1986a; Haystead *et al.*, 1988). The migration study indicated that when in solution, higher concentrations of formononetin were detected at various distances below the point of application than when in solid-form. Approximately 89% of formononetin applied in solid form was recovered near the site of application after a six day period. The action of formononetin has been found to be the most beneficial in the early days of VA mycorrhiza development, perhaps by reducing the lag phase of the infection process

(Siqueira *et al.*, 1991a). In the soil-sand mixture that was used in this experiment, most of the formononetin applied in solution was still in the upper horizon range (0-200 mm) even with plenty of irrigation. This suggested that when applied as a solution, formononetin would be in the vicinity of developing radicles for at least a week. This would be enough time for the stimulation of VAM fungi in the soil around the young roots.

The propagules of many VAM fungi have been found to be present primarily in the upper 15 cm of A horizon soil (An *et al.*, 1990). The results of the present study suggested that the previously observed success of field application of formononetin (Safir, unpublished) may be partly related to its rate of vertical migration.

However, in natural environments migration, breakdown and adsorption onto soil particles may likely occur simultaneously (Patrick, 1971). These factors would decrease the effective concentrations of the applied isoflavone in the soil. Migration studies of formononetin or other exogenously applied bioactive natural compounds in fields may become very complicated due to these multiple variables. Nevertheless, the results of such experiments would provide valuable information to determine the optimum amounts for soil application.

Response of soil microbial populations to added bioactive phenolic compounds have been investigated mostly with phenolic acids (Sparling *et al.*, 1981; Vaughan *et al.*, 1983; Blum and Shafer, 1988; Dalton, 1989). The relative amounts of phenolic acids recovered from the fields varies depending on the crop (Wang *et al.*, 1967). The studies also indicated that certain crops favor the growth of phenolic acid-degrading microorganisms (Sparling *et al.*, 1981). The response of soil microorganisms to added isoflavones was measured, after establishing the

possible contribution of soil microorganisms to the breakdown of added isoflavones in the high sand-content soil material that was used in recovery experiments (Fig. 4.1). For this part of the study, soil samples taken from a Michigan potato field were used. Blum and Shafer (1988) have previously demonstrated that potato cropping increases the number of phenolic acid degrading microorganisms.

The response of soil microorganisms to added isoflavones was measured in reference to glucose stimulation. The changes in the numbers of soil microorganisms were monitored at 3, 5, 10, and 15 days. Three-day measurements were included in the study because in soil respiration experiments, the highest stimulation to added phenolic acids and glucose occurres at this time (Sparling *et al.*, 1981). The results indicated that added isoflavones were able to stimulate the soil microorganisms. Formononetin caused a higher increase in numbers of Actinomycetes and bacteria than biochanin A. Glucose stimulation was the highest except in phosphatase positive bacteria. Formononetin stimulation was equal to glucose stimulation as observed in ACT and BMS media. Similarly, respiration studies indicated more biomass in soil amended with p-coumeric acid than glucose amended soil (Sparling *et al.*, 1981).

More bacteria grew on BMS medium than on BNA medium. BMS is a defined mineral salt medium. BNA, however, is a rich medium containing complex carbohyrates, proteins, vitamins and amino acids. This rich medium may favor the growth of r-strategist bacteria (Paul and Clark, 1989).

The culturable populations make up 1-5% of the total soil microorganisms (Paul and Clark, 1989). Therefore, these results represent the responses of a small group of soil microorganisms that are culturable

on laboratory media. It has also been reported that phenolic acids are metabolized in some soils without any changes in the numbers of culturable soil microorganisms (Blum and Shafer, 1988).

Bacterial populations from the potato field gave higher responses to formononetin, whereas fungal population responded more to biochanin A addition. However, in recovery studies biochanin A was depleted quicker than formononetin. This difference in the amount of recovery and response may be due to the initial composition of the microbial populations in the soils used in the experiments, or to the effect of microorganisms that can not be cultured on the selective media used.

The results of this study suggest that when applied as a solution, vertical migration of formononetin is limited to the upper A horizon range in the soil column for at least one week. In addition, the data indicate that soil microorganisms can metabolize the isoflavones formononetin and biochanin A and the growth of some soil microbial populations can be stimulated by these isoflavones.

CHAPTER 5

SUMMARY AND CONCLUSIONS

In this study, fungal malate dehydrogenase isozymes were observed as early as one week of *Trifolium-Glomus* mycorrhizal growth. Higher levels of malate dehydrogenase activity have been previously demonstrated in mature mycorrhizas compared to the activities of other fungal enzymes. Similarly, specific activity of malate dehydrogenase is the highest among the enzymes involved in carbon metabolism in nodules of *Rhizobium*-leguminous plant symbiosis. Although high malate dehydrogenase activity has been noted in mycorrhiza, the physiological significance of this phenomenon has not been discussed. This may be primarily because the fungal enzymes have been used primarily as diagnostic indicators for taxonomic purposes. The results of this study together with the previous observations of other investigators imply that there is a similarity between mycorrhizal and *Rhizobial* symbioses in terms of the form of carbon flowing into microsymbionts. Interestingly, malate is one form of carbon transported in and out through the mitochondrial membrane. According to the symbiont hypothesis, mitochondria may originate from intracellular prokaryotic parasites that had established a mutualistic symbiosis with the eukaryotic cells. At least in plant-microsymbiont relationships, the commonality of malate as a form of carbon transported between the symbiotic partners is noteworthy. However, although this argument may be

symbiotic partners is noteworthy. However, although this argument may be sensible for *Rhizobial* and mitochondrial symbioses, the mycorrhizal aspect remains speculative and needs further investigation.

The increased level of fungal malate dehydrogenase activity in the presence of formononetin suggested that this isoflavone may trigger the plant-dependant growth mechanisms in fungi. This mode of action may be likely since white clover synthesizes and accumulates this isoflavone. Under stress conditions the isoflavone is exuded into the rhizosphere. Once the hyphae are within a few millimeters of the root, an alteration in the nutritional dependance of the hyphae may be necessary for the establishment of the symbiosis. In fact, a low levels of phosphorus in the soil promotes colonization, perhaps due to the release of bioactive isoflavones under stress. In moderate levels of phosphorus, however, these isoflavones may only be encountered during the contact. Therefore, from an ecophysiological point, the stimulation of plant-dependant growth mechanisms by formononetin is more likely than stimulation of spore-dependant growth mechanisms. In natural settings, it may not be favorable for the fungus to support hyphal growth unless it is quite close to the rhizosphere.

The reduction in the soluble peroxidase activity of white clover roots, which occurred in the presence of formononetin, was not due to the direct inhibition of the enzyme by this isoflavone. During mycorrhizal colonization, the specific activity of soluble peroxidases decreased. In the presence of formononetin, mycorrhizal roots had lower peroxidase activity than the roots of plants grown without formononetin. In addition plants grown in the presence of formononetin had been colonized 50% more. There are two possible interpretations of these results. The suppression

of the soluble peroxidase activity may be enhanced due to the high levels of colonization. Alternatively, the suppression of the peroxidase activity by exogenously applied formononetin may have allowed fungi to spread intracellularly with greater ease and the resultant low peroxidase levels may be due to the positive feedback of these two interrelated events. The nature of this interaction requires further studies.

The mechanism proposed for the action of formononetin on *Trifolium-Glomus* symbiosis may be specific to the leguminous plant species in terms of soluble peroxidase suppression. The peroxidase levels of the leguminous plant roots may be more sensitive to the environmental concentrations of the isoflavones than non-leguminous plant species, since they themselves can synthesize and metabolize a variety of isoflavones.

Experiments involving the recovery of the added isoflavones formononetin and biochanin A indicated that both were stable in sterile soil. The time dependent decline in their concentrations in non-sterile soil suggested that soil microbial populations can metabolize these isoflavones. The disappearance rate of formononetin was slow compared to biochanin A. The difference in the rate of disappearance between these two isoflavones may be due to the presence of an additional hydroxyl group in biochanin A, which makes it more reactive. In soil planted with corn seedlings, both isoflavones declined more rapidly. This may have occurred due to the stimulation of microbial growth in the presence of root exudates and the nutrient solution applied to the soil as fertilizer. Plant uptake may have also contributed to this rapid decline. However, corn seedlings have fewer lateral roots and thus can not explore a large volume in the soil during the first week of growth. Therefore, microbial metabolism may have a greater contribution to the disappearance of these

isoflavones than plant uptake. Plant uptake may have a more pronounced effect on the decline of added isoflavones with a species that can make large number of lateral roots during the early stages of growth. The studies on vertical migration of formononetin suggested that this isoflavone is not likely to leach out from the A horizon of soil for at least a week. Exogenously applied formononetin is likely to be present in the upper A horizon range for a sufficiently long time where propagules of VAM fungi are frequently encountered. Vertical migration rate of formononetin may be an important factor for the success of field applications.

The response of soil microbial populations to the amendments of the isoflavones formononetin and biochanin A was measured. In this study, soil samples obtained from a potato field were used. It has previously been found that the potato cropping increases the number of phenolic acid-degrading microorganisms in soil. The purpose of these experiments was to compare the previously measured responses of soil microbial populations to added phenolic acids with the responses to added isoflavones. The results indicated that both isoflavones stimulated the growth of selected groups of soil microorganisms. Microorganisms that can be cultured on selective laboratory media represent a small percentage of the total soil microbial population. Therefore, the growth stimulation observed in this study may account only for this small subset of the soil microorganisms. However, the results indicated that the microbial populations of the soil under a potato crop were stimulated by the isoflavones.

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