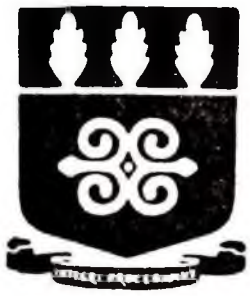

# PHENOLIC OXIDASES IN GALL DISEASE OF *MILICIA* SPECIES

A Thesis Presented to the

Board of Graduate Studies, University of Ghana

Legon, Accra, Ghana.

By

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Masters of Philosophy (M.Phil.) Degree in Biochemistry.

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Legon, Accra, Ghana.

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# DEDICATION

To Mr. & Mrs. Dorleku
and to Mr. & Mrs. Kportufe-Tamakloe
for their love and support.

# DECLARATION

I declare that the information contained in this thesis is the result of research done by me at the Department of Biochemistry, University of Ghana, Legon, under the supervision of Dr. W.S.K. Gbewonyo.

All references made have duly been acknowledged.

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To my family, I can only say; YOU have brought me this far. A simple thank you is definitely inadequate.

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# ABSTRACT

*Milicia regia* and *M. excelsa* are invaluable plant species in Ghana for their durable timber. Their numbers are however dwindling. Efforts by the forestry department to establish plantations have been unsuccessful due primarily to attack by *Phytolyma* species, resulting in gall formation.

Phenols and phenolic oxidases appear to be important in the defence mechanisms of some plants as well as in wound healing and regulation of growth in these plants. Thus, they may be natural resistance factors of the *Phytolyma*-induced gall disease of *Milicia* species.

The activities of phenolic oxidases, [polyphenol oxidase (PPO) and peroxidase (PRO)] and the phenolic content in galls and leaves of *Milicia* species differing in susceptibility to *Phytolyma* attack were determined. Polyphenol oxidase and peroxidase were also extracted and partially purified from acetone powders prepared from the galls and leaves.

The activity of polyphenol oxidase was found to be higher in the leaves of the resistant plants than in the

tolerant and susceptible plants. Galls of the seedlings also showed higher activity of the enzyme than in the normal leaves. Similar trends were also observed for the activity of peroxidase in the samples but the differences in activity were insignificant. Phenol and o-dihydroxyphenol contents of galls of seedlings were slightly lower than in the normal leaves. The resistant plants also showed slightly higher phenol content than the tolerant and susceptible plants. The susceptible plants on the other hand appeared to contain slightly higher o-dihydroxyphenol than the resistant plants.

Peroxidase and polyphenol oxidase were extracted and partially purified from acetone powders prepared from galls and leaves of the samples. The peroxidase and polyphenol oxidase from the galls were purified 10 and 14-fold, respectively, using gel filtration followed by ion-exchange chromatography. The peroxidase and polyphenol oxidase from the leaves were also purified 37 and 17-fold, respectively, using ion-exchange chromatography followed by gel filtration. The activities of the two enzymes could not be separated as they co-eluted in the same fractions. The purified enzymes from both sources did not oxidize monophenols and had an estimated molecular weight of 34 kDa.

# CHAPTER ONE

## INTRODUCTION

Much of the land area in Ghana supports natural vegetation that is either forest or savannah. These areas support numerous woody tree species. Roughly, fifty-five percent of Ghana's 238,500km$^2$ land area is savannah while one third (83,000km$^2$) is high forest. Because various tree species exist naturally in all of these areas, forest insect problems can be found throughout the country. The dry semi-deciduous type of forest, which occupies a range of environmental zones, constitutes the transition zone between the higher rainfall forest zone and the Guinea Savannah. It covers approximately 26 percent of the total forest area. Important commercial tree species that occur in this zone include *Antiaris africana, Milicia* spp. and *Triplochiton scleroxylon* among others (Hall and Swaine, 1981).

In recent years, the world's attention has been drawn to the loss of natural forest and the threat this poses to the global environment and national economies. Vast areas are being deforested by spreading agriculture and

human settlement, by commercial timbering, and by gathering of firewood, which has been spurred on to an unprecedented scale by the rising cost of domestic fuels such as liquefied petroleum gas and kerosene. Of particular concern to countries in West Africa, including Ghana, is the loss of valuable commercial timber species that contribute significant revenue to the economies of these countries. Timber exports earned Ghana an estimated one hundred and ninety-one million US dollars ($191,000,000) and one hundred and forty-seven million US dollars ($147,000,000) in 1995 and 1996, respectively i.e. 4.5 percent and 4.4 percent of Gross Domestic Product for the respective years (ISSER, 1997).

As part of efforts to regenerate the forest, the Forestry Department by 1991 had established roughly 75,000 hectares of forest plantations with ninety-four tree species having been introduced into the country mainly for plantation purposes (Wagner *et al.*, 1991). Common exotic species used in Ghana include *Azadirachta indica, Cassia siamea, Eucalyptus* spp., *Gmelina arborea, Pinus* spp., and *Tectona grandis* (Nkansa-Kyere, 1972). Probably the most successful are those of teak (*Tectona grandis*) plantations. This species grows well in all the regions of Ghana and produces excellent fire and

termite resistant electricity poles. The establishment of several major indigenous species of high economic value such as *Khaya* and *Milicia* spp. in plantations has however been a complete operational failure. Forest insects have been identified as a major factor that limits the regeneration effort (Wagner *et al.*, 1991; White, 1968). There are large numbers of plant-feeding insects, which cause pathological disturbances of tissues that cannot be ascribed merely to mechanical injury or microbial contamination. These disturbances may manifest as galls and other growth disorders. Approximately fifteen thousand gall inducing insects spanning a range of insect orders have been identified around the world (Rohfritsch and Shorthouse, 1982).

A major factor affecting the regeneration of *Milicia* species that is well established is the influence of a gall-forming insect, *Phytolyma lata*. This insect is commonly called the Iroko gallfly in West Africa. It attacks young seedlings (i.e. young trees of less than three years old) and saplings (i.e. young trees of over three years old) and induces the formation of plant galls (Wagner *et al.*, 1991). The *Phytolyma* spp. belongs to the insect family of *Psyllidae*. They are small insects about the size of aphids and are usually very

active, moving rapidly in a combination of leaping and flying motions, but are incapable of sustained flight. These insect species are found in tropical Africa throughout the range of *M. regia* and *M. excelsa*.

**Odum Plant *(Milicia* Spp)**

The *Milicia* spp. is considered the most important timber species in the family Moraceae. In addition to the two species, *M. regia* and *M. excelsa* known in Africa, another species, *M. tintorius* is found in South America. The African species are very similar and not distinguished in the timber trade. The two species are commonly referred to as Iroko plant in West Africa and Odum plant in Ghana (Hall and Swaine, 1981). Both plant species are invaluable in Ghana for their durable and strong timber.

The most recent inventory analysis has revealed that while these species are well represented in the mature age class, for some unknown reasons there are very few young trees. Growing stock in forests of Ghana as of 1987 was 834,000 cubic metres for immature stock (0.305 - 2.135m girth) and 5,269,000 cubic metres for mature stock (2.135m girth or more) (Alder, 1989). On the basis of estimated current extraction rate of these

species from the Ghanaian forests (172,983 cubic metres per year) and the annual growth rate of only 28,650 cubic metres per year, this timber will no longer be available for harvest after 2000.

Continued harvesting of mature trees without regeneration would therefore have a significant effect on future supplies and in reducing the biodiversity of the available genetic resources. Efforts have therefore been made to regenerate this species, particularly, in areas where the forest is degraded or poorly stocked but without any success. The menace of the gall-forming insect, *P. lata*, has however, remained the major setback in this effort.

**Host-Pest Relationship**

In Ghana where the two *Milicia* species overlap, three *Phytolyma* species, *P. lata, P. tuberculata, and P. fusca* have been identified. In areas dominated by *M. regia,* the most common of these insect species is *P. lata.* On *M. excelsa,* the predominant species is *P. fusca. Phytolyma lata* breeds successfully on *M. regia* in Sierra Leone and possibly eastward to Ghana. From eastern Ghana to Tanzania, on the east coast of Africa, the predominant species is *M. excelsa*, which is infested by

another *Phytolyma* species or possibly by a complex of species. The habits of the three *Phytolyma* species are however, very similar (Wagner *et al.*, 1991).

Various measures have been applied to control *Phytolyma* attack with little or no success primarily because of the cryptic habit of the insect (White, 1968; Cobbinah, 1983). Previous studies on *M. regia* have, however, shown that considerable variability exists in infestation intensity among wild naturally occurring seedlings and young trees raised from the same seed source (Cobbinah, 1988). Planting genetic strains of *Milicia* spp. inherently resistant to *Phytolyma* attack can therefore be one method of exerting long term control over the menace of the gall-inducing insects and thus sustaining the regeneration efforts.

The system of classifying the seedlings into resistant and susceptible varieties presently relies on the dieback scars for identification. The susceptible varieties are those on which the insects establish permanent infestations and stimulate the formation of galls. The resistant varieties are those on which the insects establish temporarily but are either unable to produce galls or produce small sized galls which harden

without opening and the trapped insect(s) dies. The limitation of this method of identification and classification of seedlings is that seedlings earmarked for transplanting must first be exposed to *Phytolyma* attack for a minimum period of two years before resistant varieties may be identified. In order to develop a quicker, reliable and reproducible biochemical method of identifying seedlings that are inherently resistant to *Phytolyma* attack, an understanding of the mechanism of gall development and resistance among these plant species may be required. A study of the underlying biochemical changes associated with the development of galls and resistance of the Odum plant to *Phytolyma*-induced gall formation is therefore necessary.

Previous work indicated a high incidence of polyphenol oxidases (PPO) and peroxidases (PRO) in *Phylloxera* galls and in various other galls when compared to the healthy tissues (Tandon and Arya, 1982; Tayal *et al.*, 1981; Schaefer, 1980; Purohit *et al.*, 1979). The cells adjacent to the wound cope with the parasite attack by the production of various kinds of phenols which may be oxidized to quinones by polyphenol oxidases and peroxidases (Kahl, 1982; Levin, 1971). Beckman *et al.* (1972), established that phenols are involved in defence

mechanisms as well as in wound healing and in the general synthesis and regulation of the important growth hormone, indole acetic acid, a feature which could be related to the excessive cellular hyperplasy in *Calycopteris*.

## Aim

The aim of this study therefore, is to investigate the role of polyphenol oxidase and peroxidase and some related secondary metabolites (phenolic compounds) in susceptibility or development of resistance to *Phytolyma*-induced gall formation on *Milicia*.

## Objective

The objectives of the study are to:

**i.** compare the levels of dihydroxyphenol and total phenolic compounds in leaves of the susceptible, tolerant and resistant saplings as well as in leaf and leaf galls of two species of *Milicia* seedlings (*M. regia* and *M. excelsa*),

**ii.** compare the total activity of polyphenol oxidase and peroxidase in crude extracts of the leaf and gall samples and

**iii.** partially purify the crude enzymes.

# LITERATURE REVIEW

Most plant diseases including insect-induced galls are generally associated with changes in the activity of oxidative enzymes (Shekhawat *et al*, 1978; Mayer and Harel, 1979). There is no uniformity as to the activation or inactivation of these enzymes in diseased plants with the apparent exception of polypenol oxidases, which seem to be activated whenever a tissue is attacked by parasites. The accumulation of phenolic compounds is also a widespread phenomenon characteristic of most diseases. This unique property of polypenol oxidases makes it tempting to speculate about the role played by these enzymes and their substrates (phenolic compounds) in the defence mechanism of host tissues. In most gall diseases, there is a widely held believe that the interaction between polyphenol oxidases, peroxidases and phenolic compounds results in the formation of the auxin, indole acetic acid, which subsequently stimulate tissue growth leading to gall formation.

## Plant Galls

Galls are manifestations of growth, positive or negative and of abnormal differentiation induced on a plant by animal or plant parasites (Kant and Ramani, 1990).

These can be divided into self-limiting overgrowths (e.g. galls) and non self-limiting overgrowths (e.g. tumours), which involve hyperplasia or hypertrophy or both. Each species of gall-forming insects induces a unique self-limiting overgrowth. These are more or less circular, fleshy and granular with patches of solid parenchyma (3-10mm in diameter). They may arise on shoots, roots, petioles, stipules, leaf-blades, vegetative and flower buds, inflorescence axis, bracts, flowers, fruits, etc. Leaf galls are less globular to ovate and most commonly occur on the midrib, while galls formed on stems or shoots are somewhat oblong. Several such galls on young leaves and shoots may coalesce and become a bunched mass of gall tissue. Other gall inducing agents include physical and chemical agents, genetic constitution, bacteria, fungi, viruses, nematodes, and mites (Kant and Ramani, 1990).

**Insect-Induced Plant Galls**

Insect-induced galls are a product of an interspecific association between a plant and an insect. All insect induced galls are initiated by a tissue-stimulating action supplied by the gall-forming insects (cecidozoans) (Norris, 1979). The development of these galls involves factors that release the cells of the

host from their normal morphogenetic co-ordinating influences and become cytologically active. The nucleic acid content of such cells increases and the cell nuclei enlarge. Chloroplasts degenerate and starch is converted to sugar; cell permeability is increased and cells enlarge. Young parenchymatous cells may become meristematic and the rate of divisions among such cells increases. New vascular tissues appear in the gall and these join the plant's vascular system. The host plant is thus provoked into surrounding the feeding site of the insect with several layers of highly nutritious cells. This encloses the insect in a sheltered chamber and becomes the insect's sole source of food. The polarity of the tissues of the gall is related to the insect and not to the rest of the plant (Rohfritsch and Shorthouse, 1982)

*Phytolyma-Induced Plant Galls*

The *Phytolyma* species lay their eggs (approximately 0.27mm x 0.08mm dimensions) in rows, or more rarely, scattered singly on buds, shoots or leaves of the host plant. After approximately eight days of incubation, the first instar nymphs emerge from the eggs. The young nymphs then burrow into the plant tissue, breaking down the epidermal cells, which cause fermentation of

parenchyma cells of the affected tissues. A gall is then formed within one or two days completely enclosing the insect nymph, which feeds on the gall tissue until the insect turns adult. The gall then becomes turgid and splits open, usually at the point of the original infestation, to release the adult approximately two to three weeks after the initiation of the gall (Wagner *et al.*, 1991).

The accompanying dieback, following eruption of the gall to release the adult insect, also causes growth reduction and seedling mortality in many cases. Occasionally, however, the gall may harden without opening and the trapped insect dies (White, 1968; Wagner *et al.*, 1991).

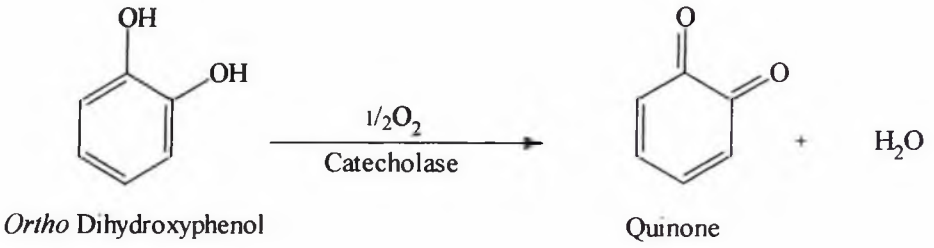
**Initiation of Insect-Induced Galls**

Although there is little doubt that the insect provides the stimulus for gall formation, the identification and mode of action of the stimulus is not clear. The insect acts through mechanical injury, but it may also apply cecidogenetic active substances, which could be present in the insect's saliva that baths the plant cells surrounding the feeding site. Several substances, including auxins (mainly indole acetic acid), and other

growth promoting substances, amino acids and amides, and numerous digestive enzymes have been detected in the saliva of the gall inducing insects. Actual biosynthesis of indole acetic acid from tryptophan has been demonstrated in the salivary glands of cecidogenic insects (Rohfritsch and Shorthouse, 1982; Hori and Miles, 1977).

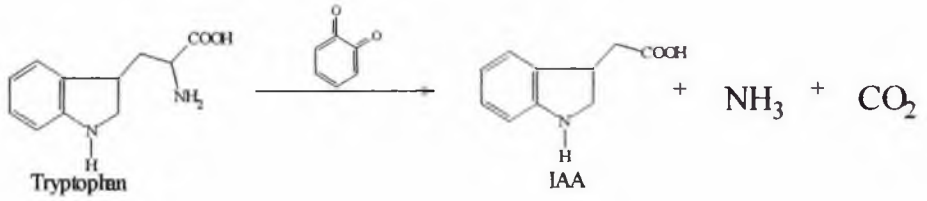
A widely held opinion is that cecidogenetic active substances applied by the gall-forming insect interfere with basic sub-cellular processes in the attacked plant tissues. This results in an imbalance in auxin and phenolic compounds in the meristematic tissues. Such auxin imbalances may also result directly from the withdrawal of such chemicals as auxin inhibitors from the tissues along with the ingested liquified contents of the disrupted cells or injection of auxin activators during feeding or oviposition. These imbalances in auxins may also result indirectly from altered levels of amino acids, oxidative enzymes, sugars, protein-carbohydrate ratio, phenols, auxin precursors or a combination of these factors during feeding or oviposition. Thus, tissue damage from insect feeding may initiate a series of wound-response reactions in the plant tissues, and in some circumstances (e.g. a

hypersensitive reaction), phenols in the damaged and surrounding cells are oxidized to highly toxic quinones in a reaction as shown below.




*Ortho* Dihydroxyphenol Quinone

These quinones may directly kill plant cells or they may transform tryptophan, by polyphenol oxidase catalyzed oxidative deamination, to toxicogenic amounts of indole acetic acid as shown in the reaction below.




Tryptophan IAA

The indole acetic acid so formed may then stimulate the growth of meristematic plant cells to initiate the gall formation. It has also been suggested that the

increased levels of chlorogenic acid (an inhibitor of auxin oxidation) measured after infestation of carrot by the root-knot nematode resulted in decreased oxidation of auxin and thus increased tissue growth and gall formation (Knypl *et al.*, 1975).

Tissue damage due to herbivory, in addition to increasing the incidence of auxin, causes significant increases in peroxidation and hydroxyl radical formation. The activity of several oxidative enzymes including peroxidases, polyphenol oxidase, ascorbic acid oxidase and NADH oxidase have been observed to increase after herbivory on foliage. Increased activity of polyphenol oxidase and peroxidase has been demonstrated cytologically in galled tissues at all stages of development when compared with healthy tissues in several plants. Vigorous activity of these enzymes was observed in the nutritive zone cells and cells further away, which suggests a triggering of the synthesis of these enzymes by the feeding stimulus of the gall-forming insect and subsequent wound initiation (Gopinathan and Ananthakrishnan, 1985).

The accumulation of phenolic compounds is also a widespread phenomenon characteristic of most diseases

(Bisth and Gupta, 1986). A series of reviews have dealt with the increased synthesis of phenolics, lignins, and isoflavonoid phytoalexins in diseased plants (Friend, 1981; 1976; Van Etten and Pueppke, 1976; Vance *et al.*, 1980). In some cases, the enhanced production of the various phenylpropanoid derivatives in response to mechanical injury or infection of tissues, results in the compounds accumulating faster in the resistant species than in the susceptible species. This increase follows increases in the activity of phenylalanine ammonia-lyase (PAL) and cinnamate 4-hydroxylase and hydroxycinnamoyl-CoA:quinate hydroxycinnamoyl transferase. Changes in the activity of these enzymes are usually induced by wounding or by pathogen attack and suggest a function in protecting the exposed surfaces. On the other hand, the levels of foliar anti-oxidants such as ascorbic acid, total carotenoids and non-protein thiols decreased significantly following herbivory (Beshir, 1994; Bi and Felton, 1995). The oxidative responses resulting from the increased levels of phenolic compounds and the activity of the phenolic oxidases (polyphenol oxidase and peroxidase) may cause a decrease in herbivory but an increase in oxidative damage to the plant (Dowd and Vega, 1996). However, several anti-oxidative processes seem to be activated in

susceptible plant cultivars, which diminish the damaging effects of oxidative stress (El-Zahaby *et al.*, 1995). The salient benefit to the plant, in terms of insect resistance, is the balance between these opposing effects. These findings implicated primary compounds (e.g. ascorbic acid and proteins); secondary metabolites (e.g. phenolics) and reactive oxygen species (e.g. hydroxyl radical, hydrogen peroxide) as multiple components of induced resistance to herbivory (Bi and Felton, 1995).

## Phenolic Compounds

Nieman and Baayen (1988), demonstrated that accumulation of certain phenolic compounds roughly correlated with the degree of host resistance to pathogenic attack. The resistance of potatoes to the scab fungus *Streptomyces scabies* and to *Verticillum* wilt has been related to their chlorogenic acid contents. Susceptible varieties have markedly lower quantities of this acid and resistant varieties have become susceptible when their chlorogenic acid contents fell below a certain minimum level.

Although the presence of phenolic compounds is in some cases related to resistance to certain diseases, in

others it is their accumulation after infection, or the further metabolism of phenolics already present which is more significant. Several investigators have shown that products formed by oxidation of these phenols inactivate enzymes including hydrolytic enzymes in the gut of herbivorous insects and hydrolytic enzymes produced by plant pathogenic fungi (Chamberlain and Baxton, 1968; Patil and Dimond, 1967).

Many of these phenolics arise from the shikimic acid pathway as shown in Figure 1.1. Among these are the acids cinnamic, p-coumaric, caffeic, ferulic, chlorogenic and protocatechuic (Figure 1.2). These simple phenolics are important to the plant not because they are abundant in uncombined (free) form, but because they are converted into several derivatives besides proteins. These derivatives include phytoalexins, coumarins, lignin and various flavonoids including anthocyanins. The content of these phenolics in plants is profoundly influenced by a variety of environmental factors including light, temperature and nutrient status. The content is also influenced by some abnormal treatments (e.g. injury or infection) of the plant that may cause either some quantitative interference in the normal metabolism of the plant or the appearance of some

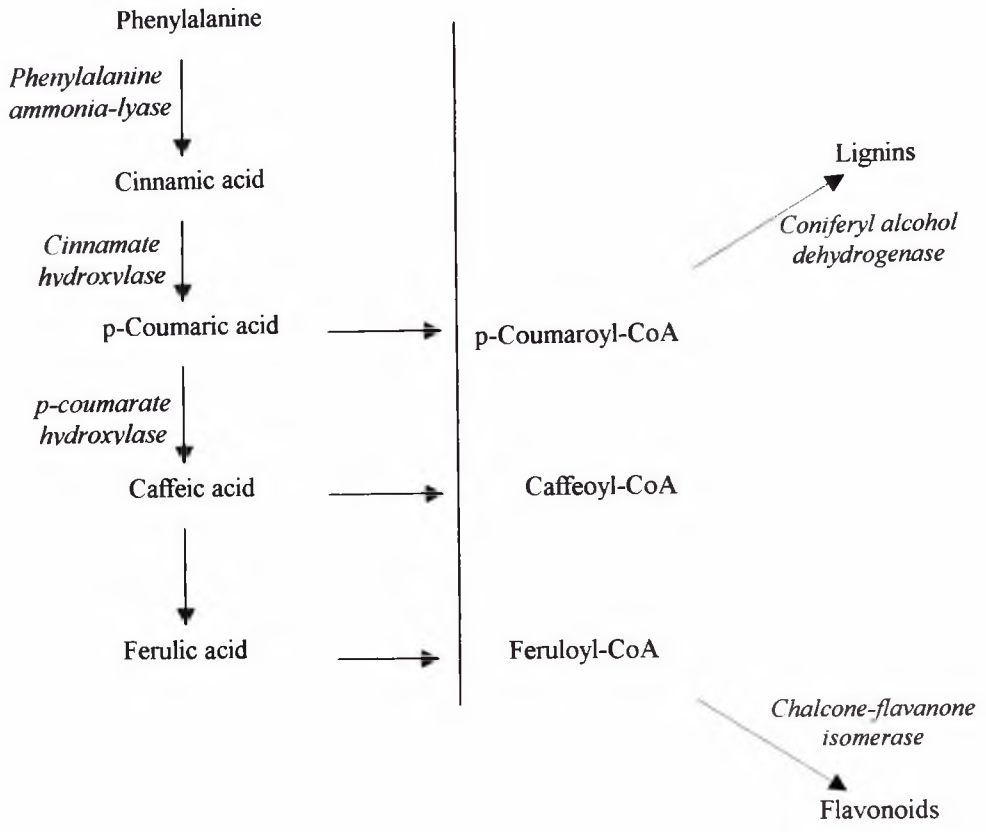



**Figure 1.1:** Shikimic Acid Pathway: the Biosynthetic Pathway of Phenolics in Plants.

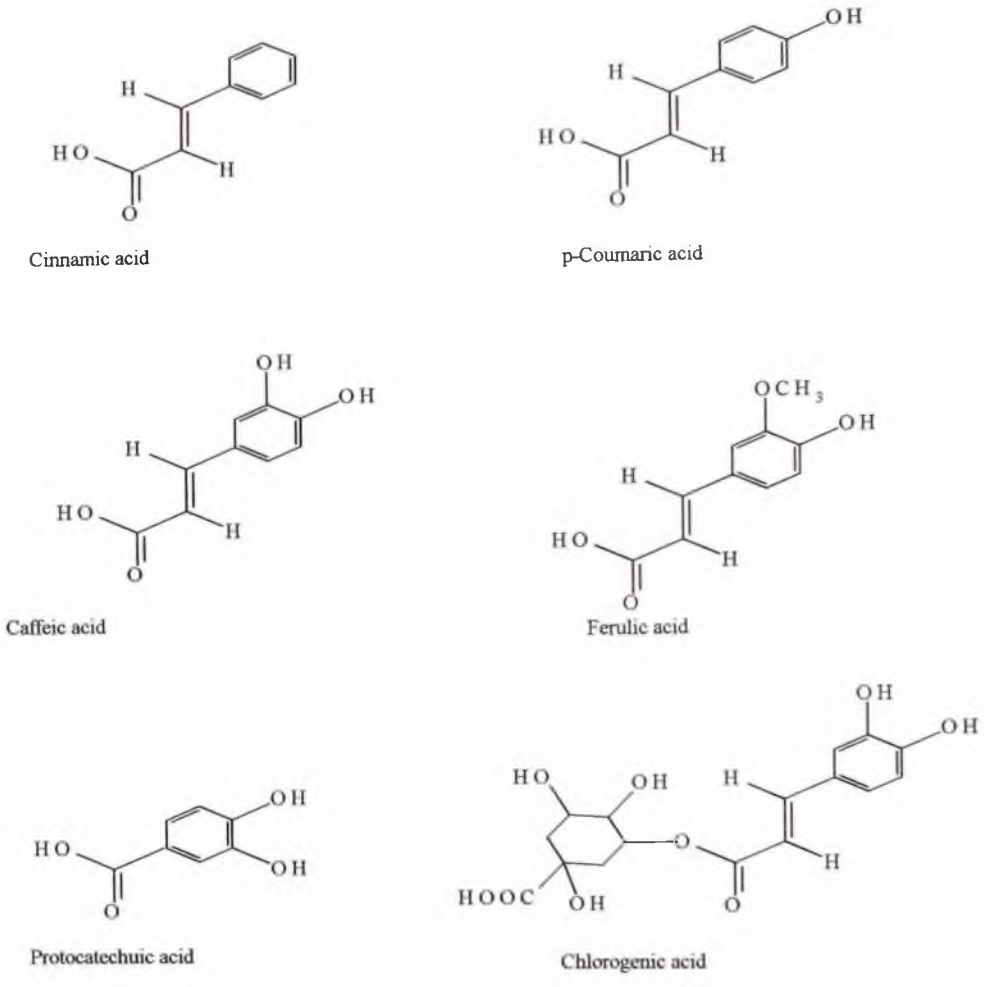



**Figure 1.2:** Structures of Phenolic Acids often found in Plants. (*All are shown in the trans-form*).

new phenolic metabolites (Shekhawat *et al.*, 1978; Koradge *et al.*, 1980; Candela *et al.*, 1995). These changes involve increases in the activity of enzymes at several points along the shikimic acid pathway.

**Isolation and Quantification of Phenols**

Phenols are very susceptible to enzymatic oxidation and phenolic materials may be lost during isolation procedures, due to the action of specific polyphenol oxidases present in all plants. Extraction of the phenols from plant tissues is therefore accomplished with boiling alcohol, which prevents enzymatic oxidation of the phenols from occurring during the extraction process. Additionally, the use of alcohol ensures high yield, due to its ability to completely penetrate the plant tissues to solubilise the phenols.

Methods used for the detection and estimation of most phenols are not highly specific and, are in principle, applicable to phenolic compounds in general but different phenols give different responses to the various reagents. Consequently, unless the nature of the phenols being estimated is known, the experimental values obtained by quantitative application of colour reactions cannot be correctly interpreted since it will

be uncertain whether a reliable standard for colorimetry has been selected. The Folin-Ciocalteu test is, however, a very sensitive test given by a large number of phenols and is extensively used in the quantitative determination of total phenols. It depends on the reduction of compounds containing sexivalent tungsten and molybdenum to coloured compounds in which the metals have a lower valence. It is however, liable to give colours with non-phenolic reducing substances, e.g. nitrophenols and thiophenols. O-dihydroxyphenols on the other hand react with nitrous acid to produce yellow coloured compounds whose intensity depends on the concentration of the o-dihydroxyphenols. This property of the o-dihydroxyphenols is exploited to determine the amount of this class of phenols in plant extracts.

While the functions of some classes of phenolic compounds are well established (e.g. the lignins as structural materials of the cell wall; the anthocyanins as flower pigments), the purpose of other classes have not yet been firmly established. Chlorogenic and protocatechuic acids (o-dihydroxyphenols) probably have special functions in disease resistance of certain plants. Protocatechuic acid is one of the compounds that prevented smudge in certain coloured varieties of

onions, a disease caused by the fungus *Colletotrichum circinans*. This acid occurs in the scales of the neck of coloured onions that are resistant to the pathogen, but it is absent from the susceptible white cultivars (Salisbury and Ross, 1992).

Large amounts of chlorogenic acid might similarly prevent certain diseases in resistant cultivars, but evidence for this is weak. Its oxidation followed by a free radical polymerization causes formation of large uncharacterised quinones responsible for the darkening of freshly cut tubers. Polyphenol oxidase, a copper containing enzyme catalyzes this and similar reactions using molecular oxygen as the electron acceptor (Butt and Lamb, 1981; Mayer, 1987).

The involvement of polyphenol oxidase in affecting the regulation of plant growth has also been implied in various ways. It has been demonstrated that quinones can interact with tryptophan to form indole acetic acid ultimately. The proposed reaction mechanism may, however, not be followed in normal tissues on the basis of the spatial separation of polyphenol oxidase and phenols *in vivo* and so prevented from interaction at significant rates (Gordon and Paleg, 1961). The

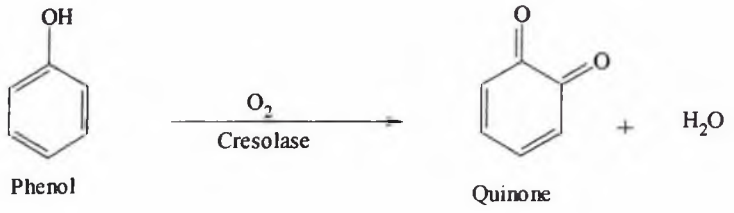
tryptophan-polyphenol oxidase-phenol reaction could thus be considered as a latent, potentially operative, mechanism for auxin formation in the plant. However, the lysis, which occurs after wounding of plant tissues, during which activation of the polyphenol oxidase complex takes place, may produce abnormal quantities of the growth hormone via the phenol-tryptophan reaction. This phenomenon of abnormal auxin and polyphenol metabolism was observed in various abnormal tissue growths (Beckman *et al.*, 1972; Shekhawat and Arya, 1979).

## Polyphenol Oxidases

Polyphenol oxidases (EC. 1.14.18.1) are a group of ubiquitous, membrane-associated plastid enzymes that oxidize phenols to quinones at the expense of oxygen. The role of these enzymes in plant diseases generally involves the formation of toxic quinones, which may subsequently react to form products that afford the plant some protection against further parasitic attack. These copper containing enzymes catalyze two distinct phenol oxidation reactions:

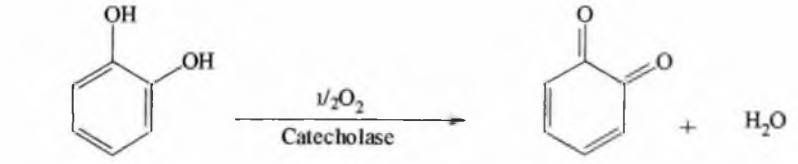
i. the insertion of oxygen in a position *ortho* to an existing hydroxyl group, usually followed by

oxidation of the dihydroxyphenol to the corresponding quinone, which is often referred to as the cresolase activity shown in the reaction below and




Phenol Quinone

ii. the oxidation with hydrogen abstraction from o-dihydroxyphenol which is often referred to as the catecholase activity.




*Ortho* Dihydroxyphenol Quinone

This enzyme is also referred to variously as catechol oxidase, phenolase, and tyrosinase depending on the substrate being used in the enzyme assay.

### Intracellular Location and Physiological Activity

In functional chloroplast, polyphenol oxidase may also be involved in some aspect of oxygen chemistry - perhaps mediation of pseudocyclic photophosphorylation (Mayer and Harel, 1979; Vaughn and Duke, 1984).

Recent evidence supports the view that polyphenol oxidase is a plastidic enzyme that is nuclear-coded, but is inactive until incorporated into the plastid. In healthy green tissues, polyphenol oxidase exists in a latent form on the thylakoid membrane and is not involved in synthesis of phenolic compounds. Polyphenol oxidase normally functions as a phenol oxidase *in vivo* only in senescence or damaged cells (Vaughn and Duke, 1984).

### Role of Polyphenol Oxidases in Disease Resistance

Cell damage brings together o-dihydroxyphenols and polyphenol oxidases following the release of vacuolar contents into the protoplasm resulting in the oxidation of o-dihydroxyphenols to o-quinones. The o-quinones produced then undergo rapid self-polymerization with further oxidation resulting in the formation of relatively insoluble brown polymers (Dey *et al.*, 1997; Wong and Wilson, 1976). Such polymers protect the

surface temporarily against invasion during the period before the newly exposed cells can protect themselves by suberization or lignification (Friend, 1976).

Apart from the rapid self-polymerization, (which involves C—O and C—C bond formation), o-quinones may also be readily attacked by other nucleophilic compounds and groups, such as the ϵ-amino groups of lysyl residues and the thiol groups of cysteinyl residues in proteins (Pierpoint, 1969). In consequence, proteins released by the destruction of cell organisation may be tanned, affording further physical protection, or during pathogen attack, the surface proteins (e.g. fungal mycelia or the hydrolytic enzymes in the gut of the herbivorous insect) may be incorporated into these polymers. This complexing of proteins by o-quinones has been found in some cases to affect the feeding habits of insects (Dowd and Vega, 1996). These responses to tissue damage may not depend on the presence of o-dihydroxyphenols but require at least substances that can be converted to them.

The sensitivity of this enzyme to dihydroxyphenols present in crude homogenates and the reactivity of proteins, including the enzyme proteins, to quinones

formed require their immediate removal usually by the preparation of acetone powder. The acetone powder can then be treated by the normal standard methods of enzyme purification. Procedures which have been employed in the purification of polyphenol oxidase include removal of inactive protein by precipitation with protamine sulphate or calcium acetate, fractionation with ammonium sulphate, ion exchange chromatography (with DEAE-cellulose) and gel filtration (Mayer and Harel, 1979).

The enzymes prepared from *Neurospora* and a number of higher plants show the existence of multiple forms corresponding to sub-unit aggregation-desegregation. Most reports on the molecular weight of the enzyme are based on estimates (e.g. by gel filtration or acrylamide gel electrophoresis) using partially purified preparations. On present evidence, it is not possible to decide to what extent different forms are artifacts of preparative methods used or whether the molecular weights given (30-40 kDa, 60-70 kDa and 85-180 kDa) are those of sub-units rather than of the intact enzyme (Mayer and Harel, 1979). Although many of the multiple forms described may be artifacts of the homogenization procedure, controlled changes in the isozyme pattern during development or infection suggests the possibility

that these different forms may have evolved in relation to the plant during its life time. Changes in the activity of polyphenol oxidase under the action of pathogens or during the development of tissues, especially under the influence of hormones such as indole acetic acid or ethylene, cannot therefore be taken to imply simple increase in the level of enzyme already present. With present level of evidence, it is not clear how far the increase in polyphenol oxidase activity in plants after infection is due to the acceleration of host senescence or to the pathogen invasion itself; the two effects are difficult to distinguish and the relationship between enzyme response and infection resistance is difficult to establish.

In spite of the numerous detailed studies on polyphenol oxidases, its reaction mechanism is still unclear. Polyphenol oxidase oxidizes phenolic substrates, utilising molecular oxygen. The enzyme undergoes monomer-dimer-tetramer transitions and it is not always certain which of the forms is being investigated from the point of view of mechanism. In spectrophotometric assays, catechol, chlorogenic acid and 4,5-dimethylcatechol have been used as substrate. The polyphenol oxidase activity of crude plant extracts and

of purified enzyme preparations can be measured by oxygen uptake or spectrophotometrically by o-quinone formation. Nevertheless, accurate assays with many preparations are difficult because of the high reactivity of the o-quinone product, which may self-condense, condense with other phenolic compounds in extracts, react with proteins present or even react with the enzyme itself. In consequence, spectrophotometric assays or oxygen uptake measurements with the oxygen electrode remain linear for short periods only. The most convenient, but in some way inaccurate, method is to follow the initial rate of formation of the quinone spectrophotometrically (Mayer and Harel, 1979).

## Peroxidases

Peroxidases (EC 1.11.1.7) are a heterogeneous group of heamoproteins, localised both intra- and extra-cellularly, that catalyze the oxidation and peroxidation of a variety of substrates (Salisbury and Ross, 1992). These iron-containing enzymes catalyze two separate reactions that lead to polymerization (Mäder and Amberg-Fischer, 1982). They are involved in the final stages of lignin synthesis in which coniferyl, 4-coumaryl, synapyl and related alcohols are oxidized with radical formation prior to polymerization to lignin (Salisbury

and Ross, 1992). They have also been implicated in the initial stages of the break down of flavonoids and benzoic acids in plant cells, in which these compounds appear to undergo turnover.

Peroxidase exists in several isozyme forms, a few of which exist in the cell walls. These isozymes apparently function first by forming hydrogen peroxide from NADH and oxygen. Next they remove a hydrogen atom from each of two aromatic alcohols and combine the two hydrogen atoms with hydrogen peroxide to release water as by-product. The remaining part of each aromatic alcohol is now a free radical and several kinds of electronic shifts allow migration of unpaired electrons to other parts of the molecule. Many such free radicals combine spontaneously in various ways to form bonds between the alcohols to form lignin.

**Reaction Mechanism**

The mechanism of chemical reactions underlying the functions of peroxidase are not known in detail but many properties of the intermediates and kinetics of the enzyme reaction are well understood. According to

Maehly and Chance (1954), the reactions are in principle as follows:

Formation of green, active enzyme (E) - substrate ($H_2O_2$) complex I:

$$E + H_2O_2 \xrightarrow{k_1} \text{complex I}$$

Transition of complex I to pale-red, active complex II:

$$\text{Complex I} + AH \xrightarrow{k_2} \text{complex II} + A$$

Recovery of enzyme by reduction of complex II:

$$\text{Complex II} + AH \xrightarrow{k_4} E + A + 2H_2O$$

To a certain degree, spontaneous decomposition of complex II and the formation of inactive complex III from complex II in an excess of $H_2O_2$ could also occur:

$$\text{Complex II} \xrightarrow{k_3} E + \text{products}$$

$$\text{Complex II} + H_2O_2 \longrightarrow \text{complex III}$$

Thus, two types of peroxidative activities can be measured, depending on the experimental conditions (Maehly, 1955):

i. the disappearance of the substrate ($H_2O_2$)

$$E + H_2O_2 \xrightarrow{k_1} \text{complex I}$$

ii. the oxidation of the donor substance - AH

$$\text{Complex II} + AH \xrightarrow{k_4} E + A + 2H_2O$$

**Lignification in Relation to Disease Resistance**

Lignification is a common response to infection or wounding in plants. A definite role for lignin

formation in relation to disease resistance is indicated by a number of experiments. When tuber discs of susceptible and resistant varieties of potato were inoculated with a sporangial suspension of *Phytophtera infestans*, there were increases in the level of phenylalanine ammonia-lyase, chlorogenic acid, and lignin (Friend *et al.*, 1973). Lignification in the resistant strain was faster and was concentrated in the centre of the infected area. Similar observations were made upon infection of radish roots (Asada *et al.*, 1975) and wheat leaves (Ride, 1975). In wheat leaves for example, lignin was rapidly synthesized around the areas infected by non-pathogenic fungi, and subsequent fungal growth was limited to the wounds. The rate of lignification was slower in response to pathogenic fungi, and these fungi were not restricted to the wounds. Ride (1975), suggested that virulent pathogens inhibit the development of lignin barriers by diverting lignin precursors into other metabolic pathway.

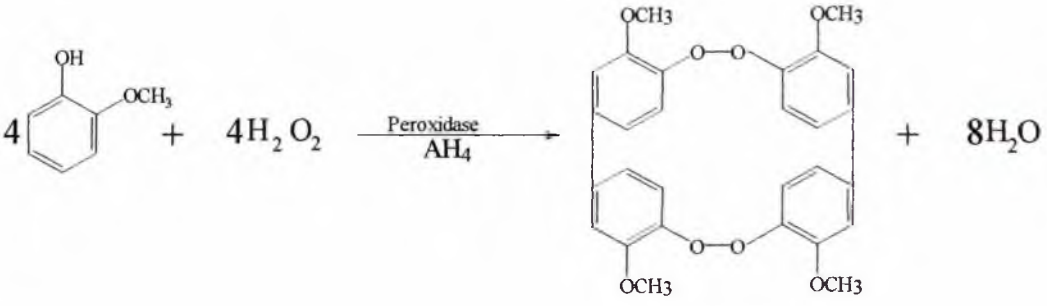
**Phenols and the Regulation of Plant Growth**

The potential role of peroxidase in development, besides that of lignification, is indicated by peroxidase activity in the oxidation of indole acetic acid, ethylene biosynthesis, and hydroxylation of proline

(Elstner, 1983). The oxidative destruction of indole acetic acid constitutes an important regulatory mechanism in the control of levels of indole acetic acid in plants. The enzyme that catalyzes this reaction is indole acetic acid oxidase. Several indole acetic acid oxidase isozymes exist and all or nearly all are identical to the peroxidase involved in the early stages of lignin formation. The peroxidase and indole acetic acid oxidase activities as well as polyphenol oxidase activity have been reported to reside on the same protein molecule (Srivastava and van Hustee, 1977). The oxidative reaction is inhibited by polyphenols (Schaeffer *et al.*, 1967; Runkova *et al.*, 1972). These naturally occurring indole acetic acid oxidase inhibitors of phenolic nature, range from free chlorogenic, ferulic and protocatechuic acids to high molecular weight auxin protectors. Some monophenols however, act as co-factors of the oxidative process in addition to requiring indole acetic acid as electron donor (Witham and Gentile, 1961; Novak and Galston, 1971; Atsumi and Hayashi, 1978). Phenols thus appear to be important in regulating growth in plants.

Most peroxidases are relatively unspecific for the hydrogen donor, which include phenolic substances,

cytochrome C, ascorbate, indole acetic acid, amines and certain inorganic ions (Butt, 1980). They however, have an absolute requirement for peroxide as an oxidant but their metabolic roles vary with the tissue in which they are found. An extensive list of donor substances, which gives coloured oxidation products, has been listed in numerous publications. One of the most favourable assay methods for peroxidases involves the oxidation of the hydrogen donor, guaiacol. This compound, catechol monomethyl ether, forms part of the guaiaconic acid molecule. On oxidation with peroxidase and hydrogen peroxide, it forms tetraguaiacol (complex II) in the presence of hydrogen donors ($AH_4$). The reaction is shown below.




Guaiacol Tetraguaiacol

Tetraguaiacol solutions have a red-brown colour, which begin to fade a few minutes after its formation. The concentration of such solutions can therefore be

determined spectrophotometrically only if the measurements are made within one or two minutes.

# CHAPTER TWO

# MATERIALS AND METHODS

## Reagents and Chemicals

The following reagents and chemicals were obtained from Sigma Company Limited, St. Louis M.O., USA: molecular weight markers, bovin serum albumin (BSA), sodium dodecyl sulphate (SDS), glycine and Tris·Cl (TRIZMA) used for polyacrylamide gel electrophoresis, guaiacol, phenol, catechol, Sephadex G-150 and DEAE-cellulose. Acrylamide, N,N'-methylenebisacrylamide, dithiothrietol, hydrogen peroxide and N,N,N',N'-tetramethylenediamine (TEMED) were all obtained from Fluka Chemical Company, Buschs, Switzerland. Ammonium sulphate, copper sulphate, silver nitrate, sodium molybdate, and sodium nitrite were obtained from Hopkins and Williams Laboratory Reagents, Essex, England.

The chromatographic columns were obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden.

## Plant Materials

Leaves of *Milicia* saplings (2-7 years old plants) classified as being "resistant", "tolerant" and

"susceptible" to *Phytolyma* attack were obtained separately from Forestry Research Institute of Ghana (FORIG) experimental plot at Fumesua, Kumasi.

Normal leaves, galls and gall bearing leaves of *M. regia* and *M. excelsa* seedlings (<2 years old plants) were obtained from an experimental plot established at the Dept. of Biochemistry, Legon.

This work involved three main aspects, namely:

i) the isolation and quantification of phenolic compounds,

ii) the extraction and determination of activities of polyphenol oxidase and peroxidase in the samples and

iii) the partial purification of these phenolic oxidases.

## Sampling

Fresh leaves (usually from about the third to sixth leaf from the apex) were plucked from randomly selected plants and grouped into individual species. Samples from saplings were kept in sealed transparent polythene bags on ice and transported to the Laboratory for storage at -20°C within six hours. Acetone powder was

prepared from samples for enzyme analysis within 24 hours while samples for phenolic analysis were used within two weeks. Samples obtained from seedlings for analysis of phenolics (total phenols and o-dihydroxyphenols) and acetone powder preparation were used within one hour of harvesting without storage.

## Determination of Phenolic Compounds

Fresh samples of selected plant tissues (400mg from each cultivar) were washed under tap water and blotted dry between a pile of tissue papers. The samples were chopped into small pieces and boiled in 10ml of 80% methanol for 5 minutes to prevent the oxidation of the phenols by phenolic oxidases. The boiled samples were ground up in the hot methanol. The volume of the homogenate was made up to 20ml with more methanol and spun at 6,000 X g for 45 minutes. The supernatant was used for the determination of the contents of the phenolic and o-dihydroxyphenolic compounds.

### The Folin-Ciocalteu Test for Phenols

The supernatant obtained was diluted 1/10 with distilled water and the concentration of phenols determined by adding 1.0ml of Folin-Ciocalteu reagent to 10ml of the diluted extract. The mixture was made alkaline by the

addition of 1.5ml of 20% sodium carbonate solution. This was heated in a boiling water bath for 2 minutes for blue-black colour to develop. The colour intensity (proportional to the phenol content of the extract/tissue) was measured spectrophotometrically at 750nm and the phenol concentration estimated from a standard curve prepared with pure phenol.

**The Arnow's O-dihydroxyphenol Test**

Methanol extract of the samples was diluted to twice its volume. A portion of the diluted extract (2.0ml) was used for the determination of o-dihydroxyphenols by the addition of 1.0ml of 0.5M hydrochloric acid and 1.0ml of Arnow's reagent (1.5M sodium nitrite and 0.5M sodium molybdate solution). Water (5ml) was then added to the reaction mixture. The resulting yellow solution was made alkaline by the addition of 1.0ml of 0.5M sodium hydroxide solution changing the yellow colour of the solution to a characteristic intense orange-red colour given by o-dihydroxyphenols (Arnow, 1937). The colour intensity was measured at 515nm, 5 minutes after addition of the sodium hydroxide. The o-dihydroxyphenol concentration was estimated from a standard curve prepared from a 1.0M catechol stock solution.

## Determination of Enzyme Activity

### Preparation of Acetone Powder

The tissues were first extracted with 80% acetone pre-chilled to -20°C to remove phenolic compounds, chlorophyll and other low molecular weight compounds which interfere with the enzyme assay.

Freshly collected leaf and gall samples were washed under tap water and finely ground in 3 volumes of the pre-chilled acetone. The homogenate obtained was centrifuged at 10,000 X g for 5 minutes at -5°C and the supernatant discarded. The residue was re-suspended in 3 volumes of the pre-chilled acetone with gentle shaking for three minutes and centrifuged. This was repeated. The final residue was spread on aluminium foil to dry under fan at -5°C in the cold room. The dried residue (referred to as acetone powder) was stored in airtight plastic containers at -20°C.

### Preparation of Crude Enzymes

The acetone powder (1.0g) was ground in 10ml of 0.1M sodium phosphate buffer (pH 6.8). The homogenate was left to stand on ice with constant stirring for one hour and centrifuged at 13,000 X g for 30 minutes at 4°C. The supernatant was retained as the crude enzyme.

**Enzyme Assays**

Polyphenol oxidase activity on catechol and the peroxidase activity on guaiacol were assayed by spectrophotometric determination of the initial rate of formation of the respective products according to the methods described by Srivastava and van Huystee (1973). Preliminary work had indicated that at room temperature, both polyphenol oxidase and the peroxidase enzymes had maximum activity at pH 6.8 in 0.1M sodium phosphate buffer. A unit of enzyme activity was defined as the amount of enzyme that caused a 0.001 change in absorbance per minute at the appropriate wavelength.

*Assay for Polyphenol Oxidase Activity*

A reaction medium containing 30mM catechol in 3.0ml of 0.1M sodium phosphate buffer (pH 6.8) was prepared to which was added 200µl of the enzyme preparation. The reaction mixture was incubated at room temperature for 20 minutes and the absorbance measured at 480nm. The blank sample contained 200µl of the enzyme pre-heated to 100°C for 45 minutes on boiling water bath to ensure complete enzyme inactivation.

temperature for 10 minutes and 250µl of Folin-Ciocalteu solution added. The reaction tubes were again incubated at 45°C in a water bath for 10 minutes. Absorbance measurements were taken at 750nm and the total protein content of the enzyme preparations estimated from a calibration curve of the bovin serum albumin solutions.

## Statistical Methods

A statistical test of significance (one-way analysis of variance), was applied to the data obtained. If the preliminary analysis of variance indicated that there was a significant difference between sample means, a Student t-test was applied to determine the level of significance of individual differences between sample means.

## Partial Purification of Enzymes

The acetone powders of the gall and susceptible leaf samples were further studied. Crude enzyme extracts were separately prepared from 20g acetone powders of the galls and the susceptible leaves in 10mM sodium phosphate buffer (pH 6.8) as previously described.

### Ammonium Sulphate Fractionation

Enough ammonium sulphate was added to the crude enzyme preparation to obtain 20% saturation on ice. The solution was stirred for 30 minutes and centrifuged at 13,000 X g and 0°C for 30 minutes to remove the precipitated proteins. More solid ammonium sulphate was added to the supernatant to raise the ammonium sulphate concentration to 30%. This was also stirred for 30 minutes and centrifuged at 13,000 X g for 30 minutes. Further additions of ammonium sulphate were made until 100% saturation was obtained. The precipitated proteins were re-dissolved in (just enough) sodium phosphate buffer (pH 6.8) and dialysed against four changes of 750ml of the phosphate buffer on ice. The fractions showing significant enzyme activities were pooled together and applied to a gel filtration column for further purification.

### Column Chromatography

*Gel Filtration*

Gel filtration with Sephadex G-150 was carried out in a 2.6 x 40cm column in the cold (5 - 10°C). The column, which had been equilibrated with 10mM sodium phosphate buffer (pH 6.8) was loaded with the partially purified enzymes or crude extract and eluted with the sodium

phosphate buffer. Flow rate was usually around 0.5ml/min and 2.5ml per fraction was collected. Fractions, which showed significant enzyme activity, were pooled together and assayed for polyphenol oxidase and peroxidase activities as well as total protein content.

*Ion Exchange Chromatography*

Ion exchange chromatography was performed on diethylaminoethyl (DEAE) cellulose packed in a 2.6 x 35cm column. The partially purified gall enzymes or crude enzyme of leaf extract was applied to the DEAE-cellulose column pre-equilibrated with 10mM sodium phosphate buffer (pH 6.8). The enzymes were eluted from the column with a 0.00 to 0.75M stepwise change in sodium chloride gradient prepared in 10mM sodium phosphate buffer (pH 6.8). Fractions (2.5ml) were collected at flow rates maintained at 0.75ml/min. Fractions, which showed significant enzyme activity, were pooled together and assayed for polyphenol oxidase and peroxidase activities. Total protein content was also determined.

**Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis (SDS-PAGE)**

The sodium dodecyl sulphate - polyacrylamide gel electrophoresis for the enzyme preparations was performed on 10% vertical slab minigel as described by Laemmli (1970) and modified by Sambrook *et al.*, (1989). Aliquots of the partially purified proteins and standard low molecular weight protein markers were added to equal volumes of sample buffer (made of 50mM Tris-HCl, pH 6.8, 100mM dithiothreitol, 2% SDS, 20% glycerol and 0.1% bromophenol blue) and heated to 100°C. The samples (15µl) were loaded into the bottom of the wells alongside the molecular weight markers. A voltage of 8V/cm was applied until the tracking dye had travelled to the interface between the 5% stacking and 10% resolving gels. The voltage was then increased to 15V/cm and run for 90 minutes.

The gel was fixed in 100ml of ethanol : glacial acetic acid : water (30:10:60) mixture overnight and silver stained (Sambrook *et al.*, 1989). The silver-stained gel was soaked in 30% ethanol solution containing 3% glycerol overnight to minimise the risk of cracking while drying. The gel was then overlaid at either side with porous cellophane and dried at 75°C for 5 hours.

# CHAPTER THREE

# RESULTS

## Phenolic Compounds

Biochemical analysis of normal and galled tissues of *Milicia* seedlings revealed an identical pattern for phenolic and *ortho*-dihydroxyphenolic content in the tissues of both *M. regia* and *M. excelsa* (Figs. 3.1 and 3.2). Decreased levels of phenol and *ortho*-dihydroxyphenol were found associated with gall tissues of both species relative to their normal counterparts. The gall tissue of *M. excelsa* and *M. regia* showed 14.8% and 10.7% decrease in phenols, and 22.2% and 8.6% decrease in *ortho*-dihydroxyphenol content, respectively. The levels of phenol and *ortho*-dihydroxyphenol in normal leaves as well as galls of *M. excelsa* appeared to be higher than in normal leaves of *M. regia* and its galls, respectively.

The differences recorded between the galls of the two species and their normal counterparts with respect to phenolic content (*M. regia*: $p = 0.513$; *M. excelsa*: $p = 0.318$) and *ortho*-dihydroxyphenolic content (*M. regia*: $p = 0.486$; *M. excelsa*: $p = 0.064$) were

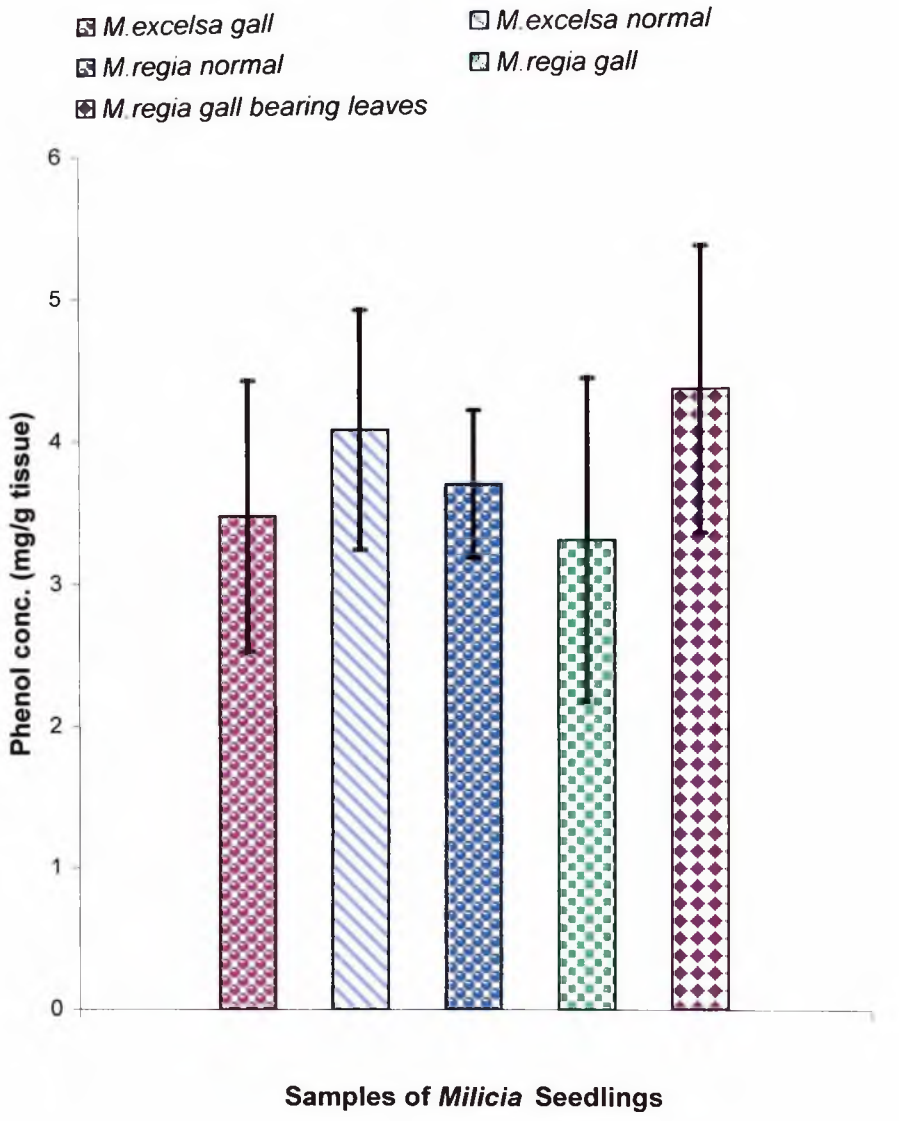



**Figure 3.1:** Phenolic Content of Alcoholic Extracts from Leaf and Gall Samples of *Milicia* Seedlings. ($^{\Psi}$n = 18).

$^{\Psi}$n is the number of duplicate analyses used for the calculation of the sample mean

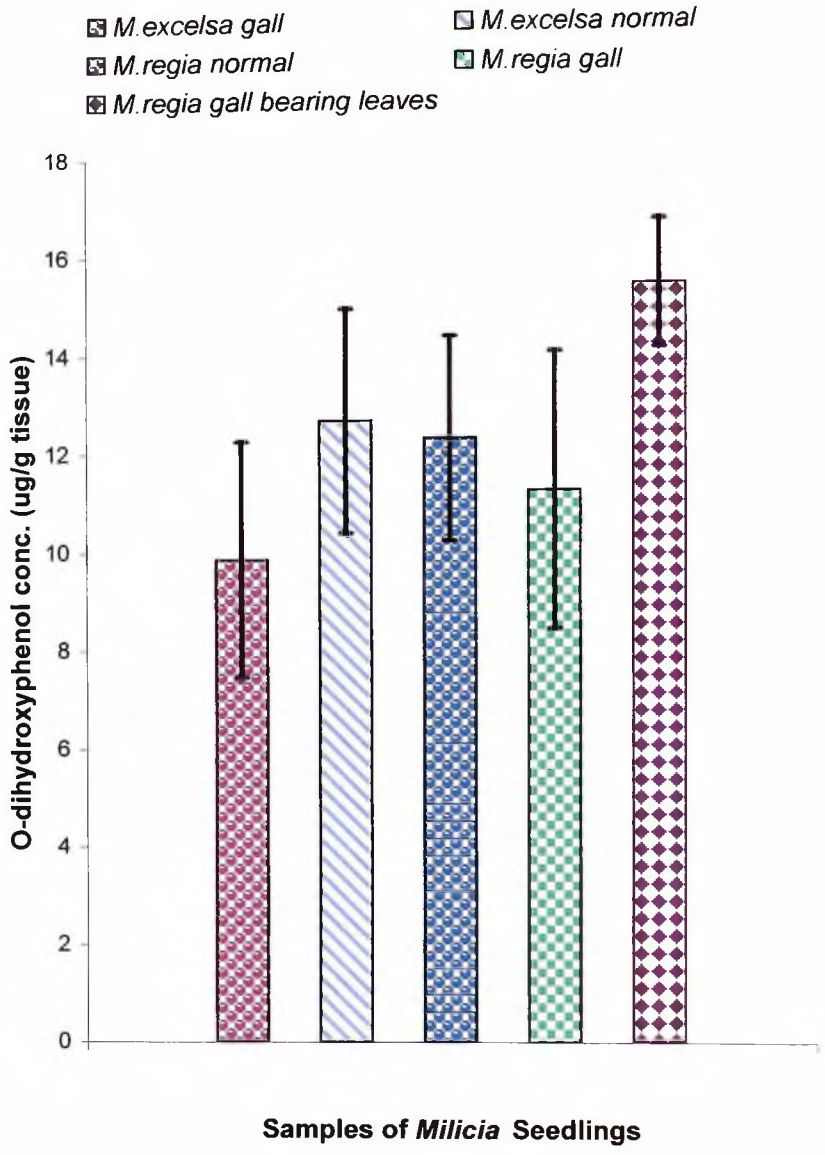



**Figure 3.2:** O-Dihydroxyphenol Content of Alcoholic Extracts from Leaf and Gall Samples of *Milicia* Seedlings. ($^{\Psi}n = 16$).

$^{\Psi}n$ is the number of duplicate analyses used for the calculation of the sample mean

however, not statistically significant at 95% confidence level (Figs. 3.1 and 3.2).

The difference between the two species, *M. regia* and *M. excelsa*, with respect to phenolic content (galls: $p = 0.800$; normal leaves: $p = 0.418$) and *ortho*-dihydroxyphenolic content (galls: $p = 0.384$; normal leaves: $p = 0.790$) were also not statistically significant at 95% confidence level.

Phenol concentration in saplings (Fig. 3.3) appears to be marginally higher in the resistant variety than in the tolerant and susceptible varieties. The resistant variety was only about 17% more in phenolic concentration than the susceptible variety, which recorded the lowest phenolic concentration. The difference recorded was however, not significant ($p = 0.735$) at 95% confidence level. With respect to *ortho*-dihydroxyphenol concentration however, the highest *ortho*-dihydroxyphenol concentration was recorded in the susceptible variety whilst the tolerant variety, turned out to be lower in *ortho*-dihydroxyphenol content than in both the resistant and susceptible varieties (Fig. 3.4). Statistically, there was no significant difference at 95% confidence level among the varieties ($p = 0.262$).

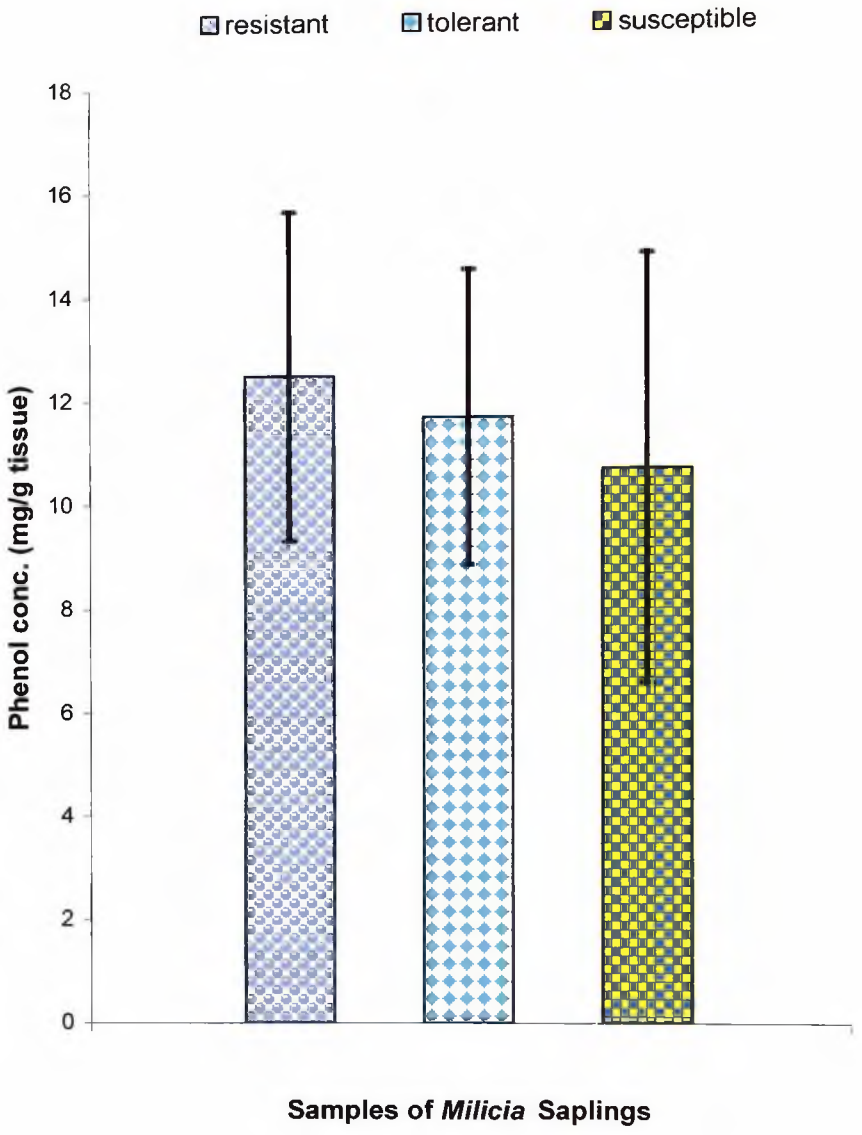



**Figure 3.3:** Phenolic Content of Alcoholic Extracts from Leaf Samples of *Milicia* Saplings.
($^{\Psi}$n = 18).

$^{\Psi}$n is the number of duplicate analyses used for the calculation of the sample mean

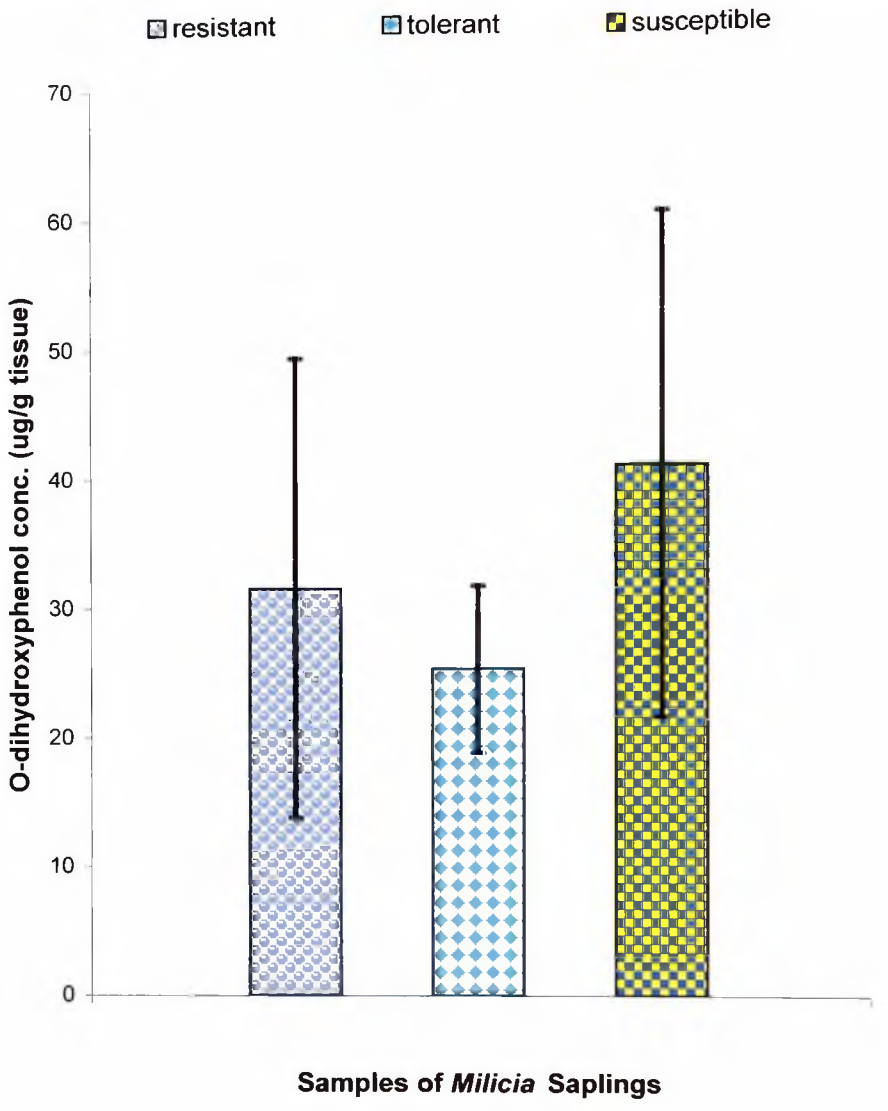



**Figure 3.4:** O-Dihydroxyphenol Content of Alcoholic Extracts from Leaf Samples of *Milicia* Saplings. ($^{\Psi}$n = 16).

$^{\Psi}$ n is the number of duplicate analyses used for the calculation of the sample mean

## Enzyme Assays

The gall tissues of seedlings of the individual species showed highly significant increase in polyphenol oxidase activity relative to the activity in their respective normal leaves (Fig. 3.5). In *M. excelsa* galls, the activity of the enzyme was increased by over 1½ times that of the normal leaves. *M. regia* galls also showed considerable increase in activity of the enzyme. The activity was about 3.4 times more in the galls than in the leaves.

Similarly, the activity of peroxidase enzyme in the gall tissues was higher in the two *Milicia* species than in their normal leaves (Fig. 3.6). However, the recorded increase in activity was significant only between *M. regia* galls and its normal leaves ($p = 0.045$).

The resistant variety of the saplings showed very highly significant difference ($p \approx 0.000$) between its polyphenol oxidase activity and the activity of the enzyme in the tolerant and susceptible varieties (Fig. 3.7). The activity was more than twice that in either the susceptible or resistant varieties. There was however, no difference in the polyphenol oxidase

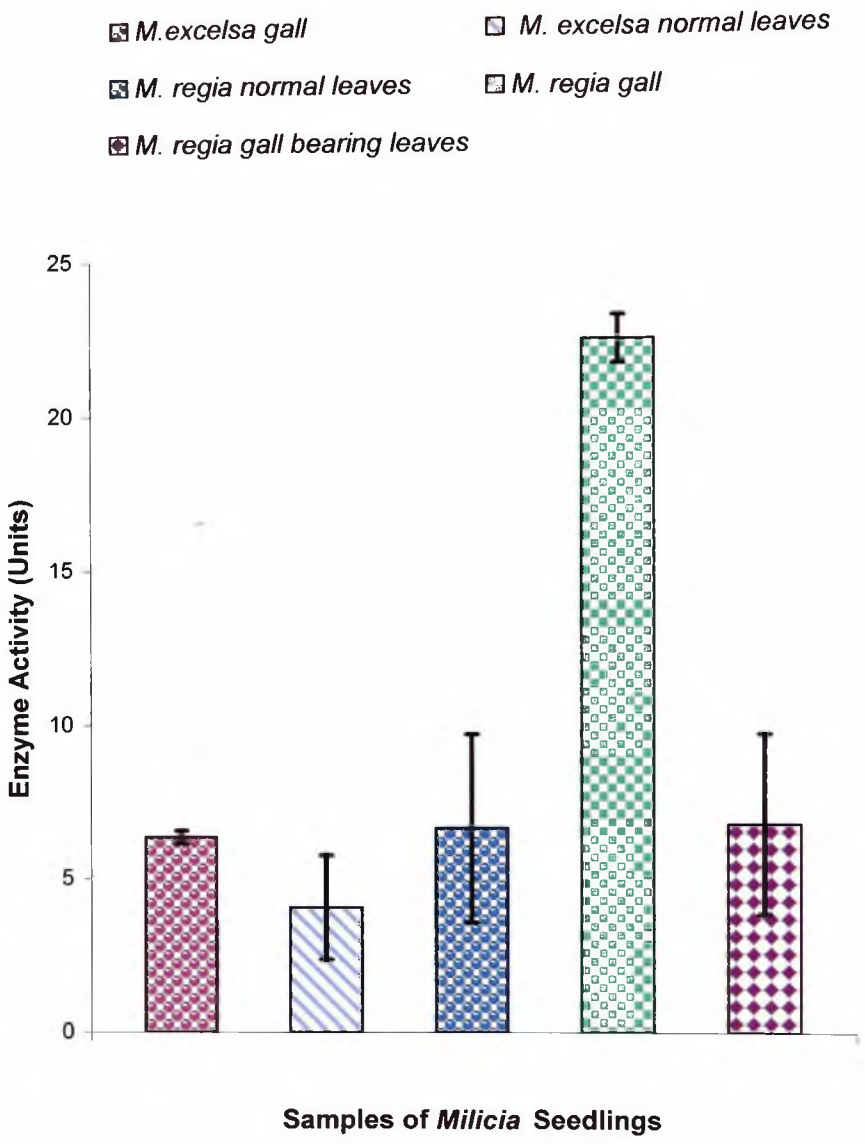



**Figure 3.5:** Polyphenol Oxidase Activity of Crude Extracts from Leaf and Gall Samples of *Milicia* Seedlings.
($^{\Psi}$n = 16)

$^{\Psi}$ n is the number of duplicate analyses used for the calculation of the sample mean

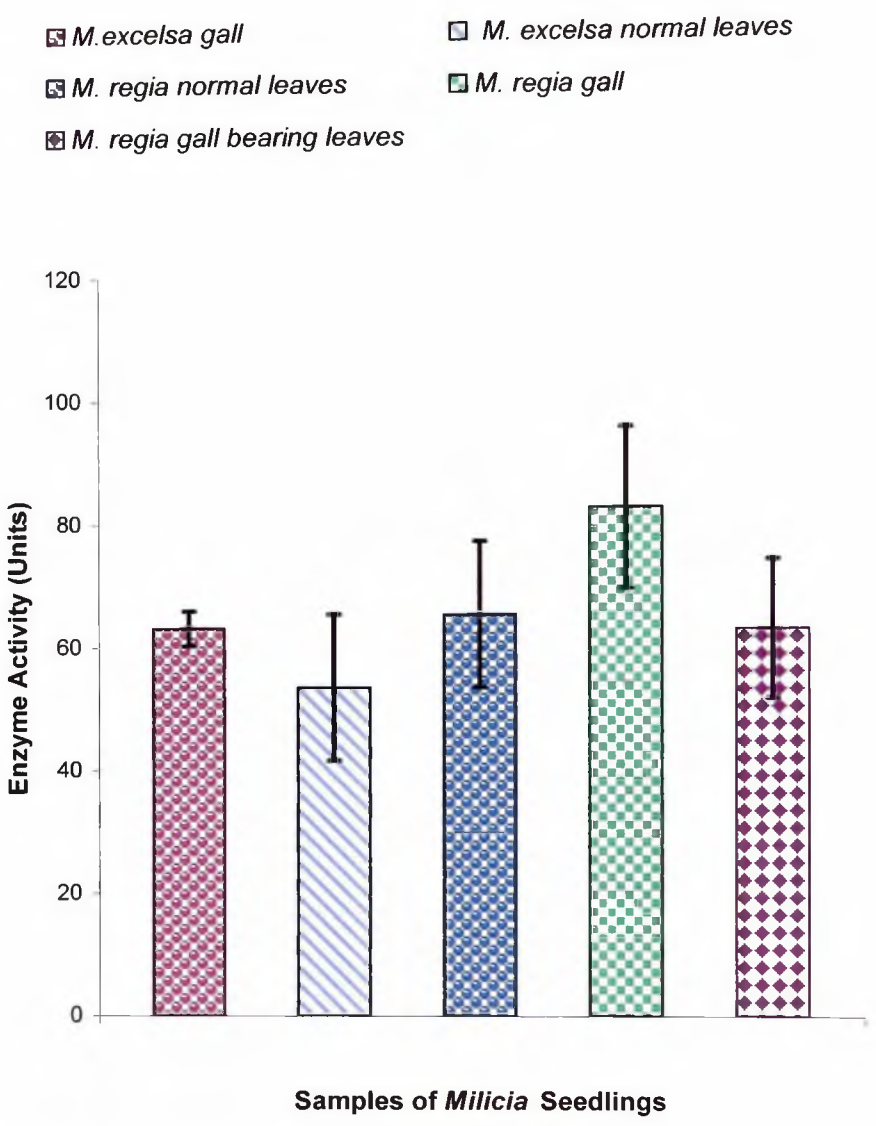



**Figure 3.6:** Peroxidase Activity of Crude Extracts from Leaf and Gall Samples of *Milicia* Seedlings. ($^{\Psi}$n = 16)

$^{\Psi}$ n is the number of duplicate analyses used for the calculation of the sample mean

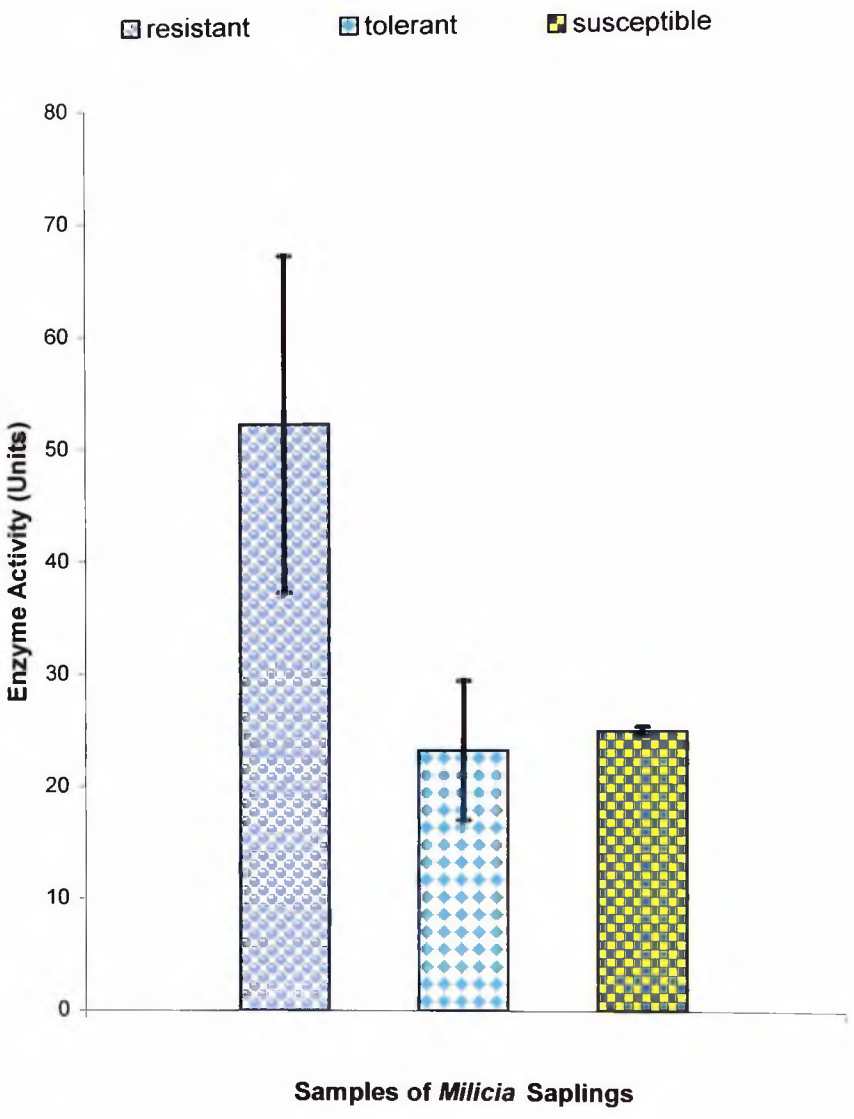



**Figure 3.7:** Polyphenol Oxidase Activity of Crude Extracts from Leaf Samples of Three Varieties of *Milicia* Saplings.
($^{\Psi}$n = 16)

$^{\Psi}$ n is the number of duplicate analyses used for the calculation of the sample mean

activity of the tolerant and susceptible varieties (Fig. 3.7). With respect to peroxidase activity, the resistant variety appeared to have higher activity than the tolerant and the susceptible varieties (Fig. 3.8). The difference was however, not significant at 95% confidence level ($p = 0.608$).

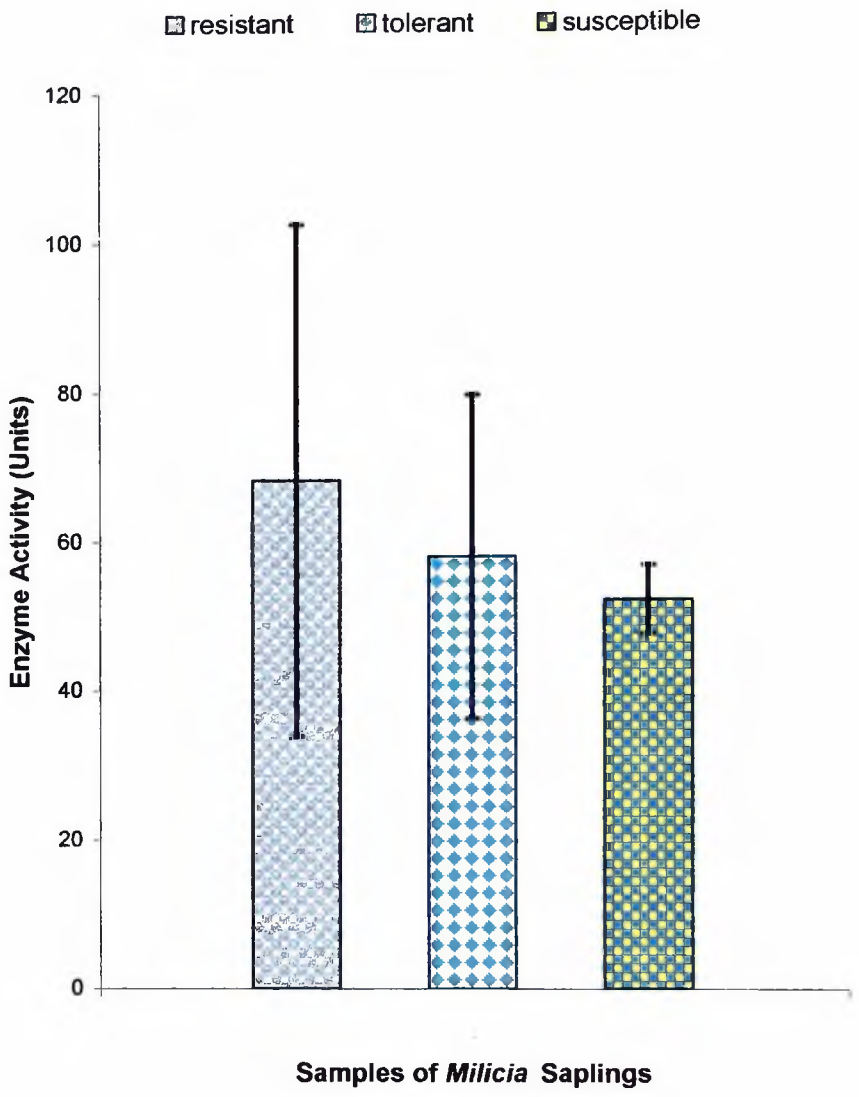



**Figure 3.8:** Peroxidase Activity of Crude Extracts from Leaf Samples of Three Varieties of *Milicia* Saplings. ($^{\Psi}$n = 16)

$^{\Psi}$ n is the number of duplicate analyses used for the calculation of the sample mean

## Partial Purification of Enzymes

### Ammonium Sulphate Fractionation of Crude Extracts

Proteins precipitating between 0 and 20% ammonium sulphate saturation at 0°C had some polyphenol oxidase activity while the fraction precipitating between 20% and 60% showed only minimal peroxidase but no polyphenol oxidase activity. However, the fraction precipitating between 60 and 100% saturation showed both polyphenol oxidase and peroxidase activities (Fig. 3.9).

However, further purification of the active fraction (60-100%) on Sephadex G-150 or DEAE-Cellulose ion-exchange columns resulted in a significant loss of the activities of the enzymes from the gall extracts.

Ammonium sulphate fractionation of the susceptible leaf extracts did not yield any precipitates even at 100% saturation.

### Purification of Polyphenol Oxidase and Peroxidase from Galls

*Gel filtration on Sephadex G-150:*

Crude enzyme extracts from galls run through Sephadex G-150 showed a single peak for both polyphenol oxidase and peroxidase activities (Fig. 3.10). The peroxidase enzyme activity appeared at an elution volume of

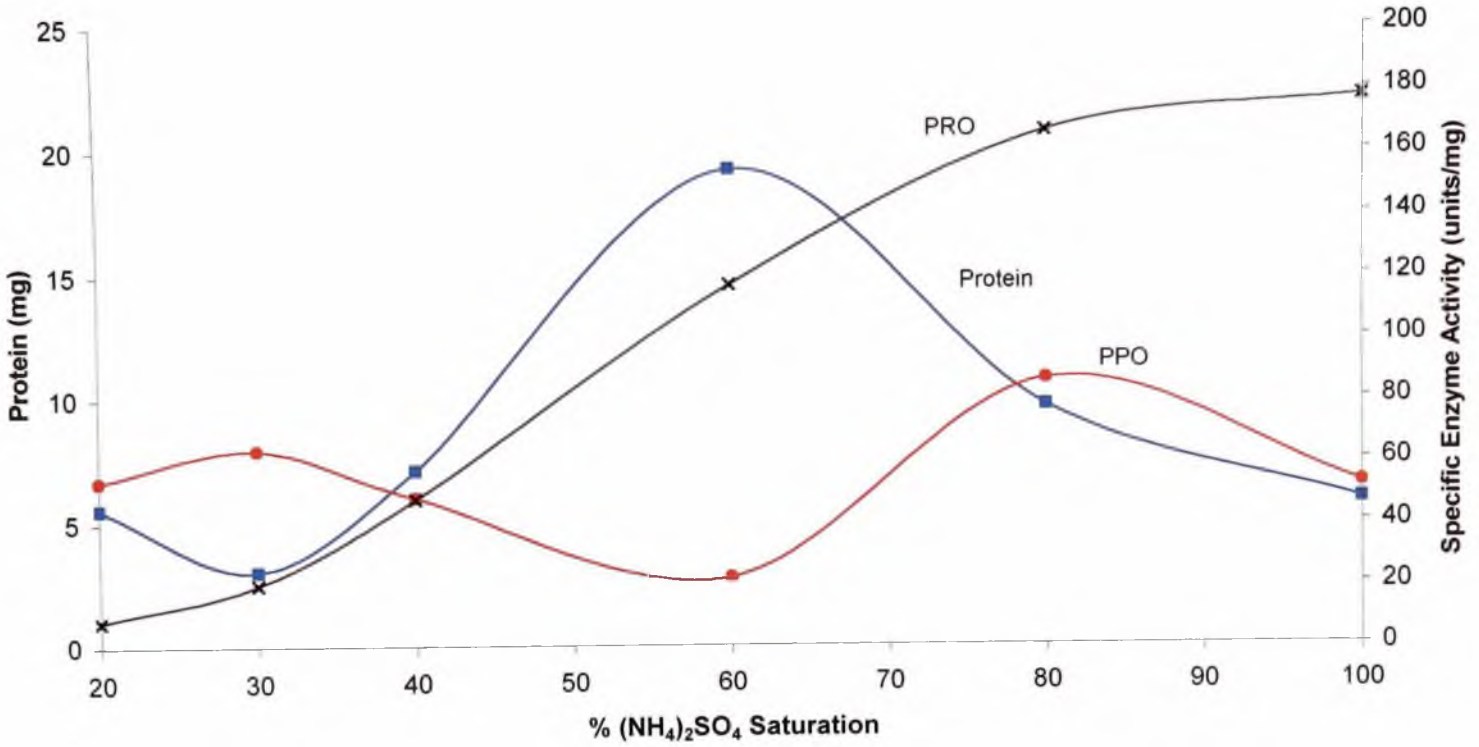



**Figure 3.9:** Ammonium Sulphate Fractionation of the Crude Enzyme Preparation from *Milicia* Galls

The proteins precipitated at the various ammonium sulphate saturations were collected by centrifugation. The precipitates were redissolved in 5 ml of 10mM sodium phosphate buffer (pH 6.8) and dialysed against four changes of 750 ml of the 10mM sodium phosphate buffer.

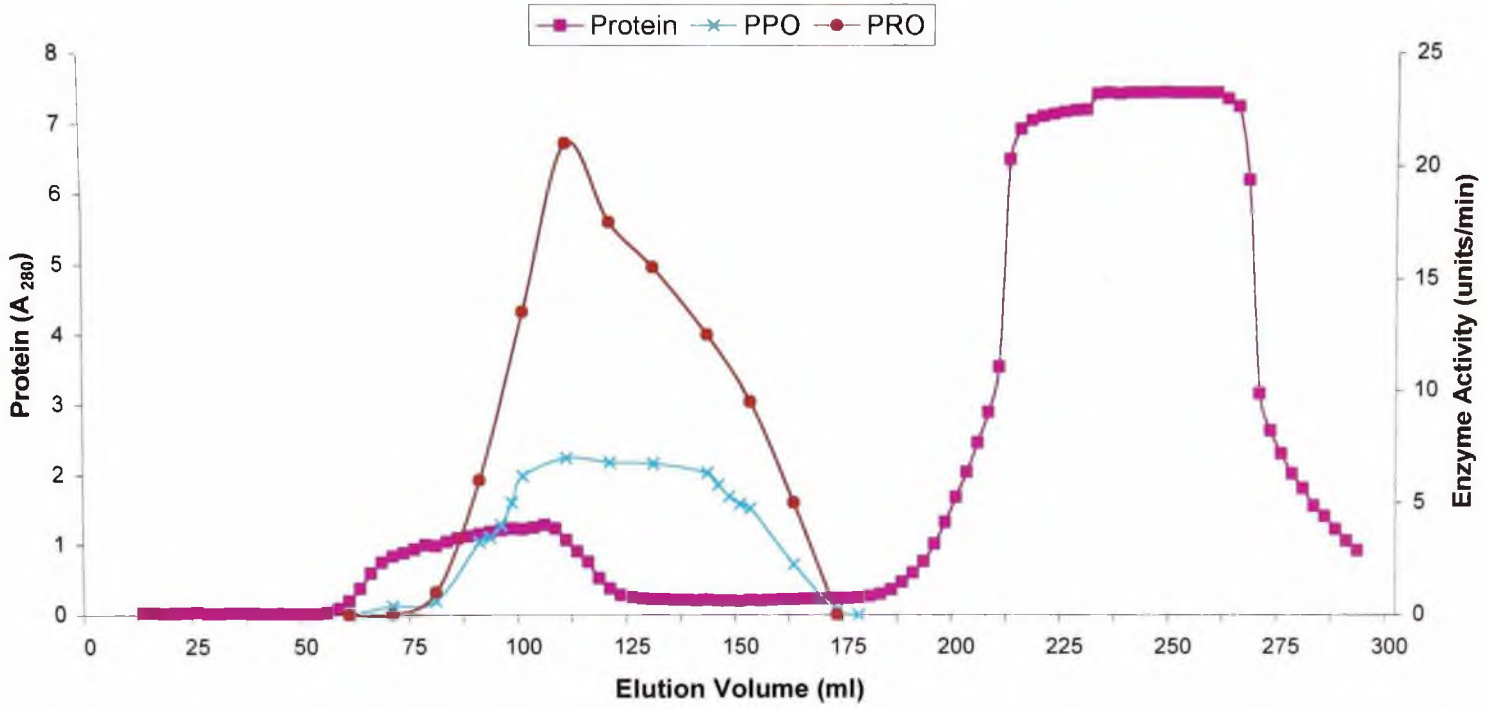



**Figure 3.10:** Gel Filtration of Crude Extract from Galls

The crude extract (50ml) applied to the Sephadex G-150 gel filtration column (2.6 X 40cm) was eluted with 10mM sodium phosphate buffer (pH 6.8) at 5°C. Fractions (2.5 ml) were collected at a flow rate of 0.5 ml/min.

approximately 110ml in a sharp peak while polyphenol oxidase appeared in a broader peak between 100ml and 160ml. A major protein peak appeared well after the enzyme peak with another eluting just before the enzyme peak. Fractions eluting from 110ml to 142.5ml elution volumes were pooled together and applied to ion-exchange column for further separation.

*Ion-exchange on DEAE-Cellulose:*

Fractions pooled together from the gel filtration column were run through a column of DEAE-Cellulose and eluted with 0.01M sodium sulphate buffer (pH 6.8) with a stepwise change in sodium chloride concentration (from 0.0 and 0.75M). The loaded sample was fractionated into five prominent protein peaks (Fig. 3.11). Both polyphenol oxidase and peroxidase activities were associated with the second protein peak at an elution volume of 115ml. No activity of either enzyme appeared in the four other protein peaks. Fractions eluting from 112.5ml to 125.0ml were pooled and assayed for the enzyme activities and total proteins.

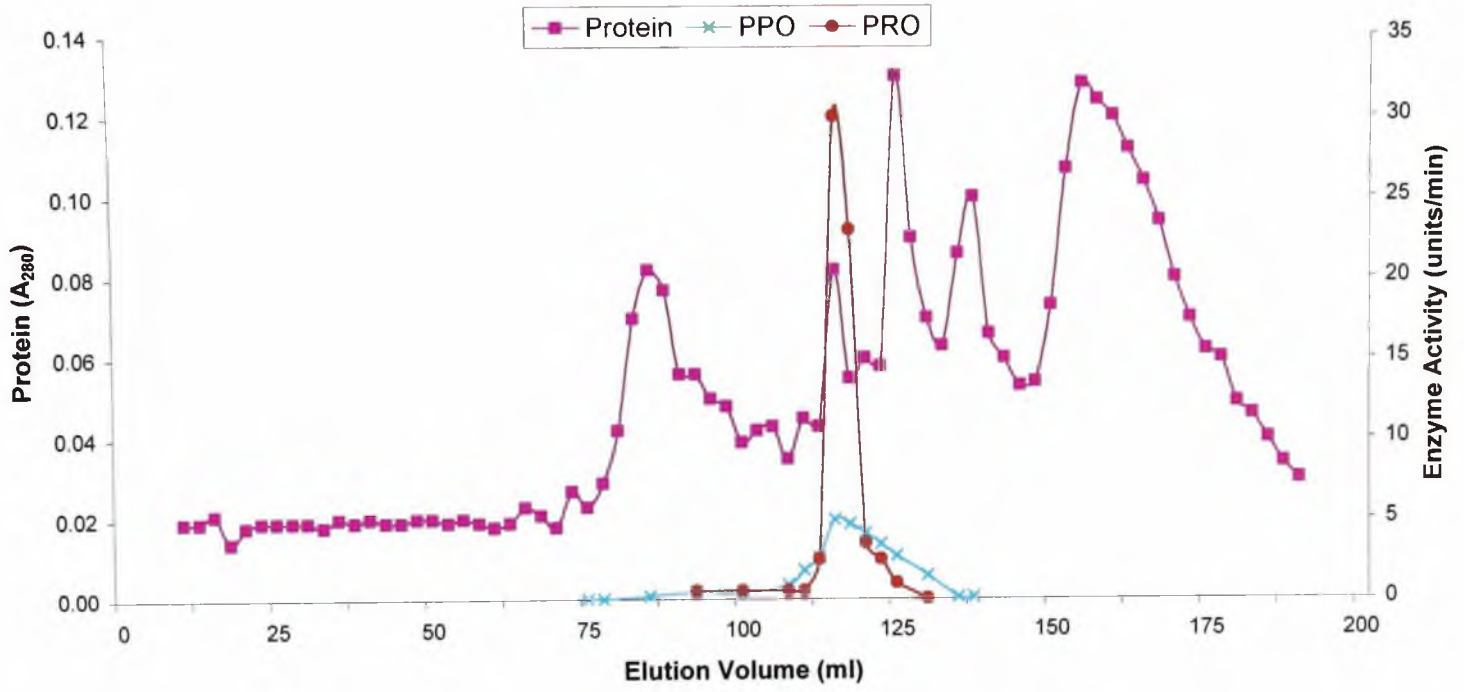



**Figure 3.11:** Ion-Exchange Chromatography of Partially Purified Gall Extract

Pooled fractions from gel filtration column showing enzyme activities were applied onto DEAE – Cellulose column (2.6 X 35cm). The applied sample (25 ml) was eluted with a stepwise change of 0.0 to 0.75M NaCl gradient prepared in 10mM sodium phosphate buffer (pH 6.8). Fractions (2.5 ml) were collected at a flow rate of 0.75ml/min.

**Purification of Polyphenol Oxidase and Peroxidase from the Susceptible Leaves**

In the case of crude extract from the susceptible leaves, the purification protocol followed was as described below

*Ion-exchange on DEAE-Cellulose:*

The crude enzyme extract from the susceptible leaves was applied first to the ion-exchange column and eluted with sodium phosphate buffer (pH 6.8) with a stepwise change in sodium chloride concentration from 0.0 to 0.75M. The polyphenol oxidase eluted in a broad peak between 150ml and 367.5ml whilst the peroxidase enzyme eluted in a sharp peak at 322.5ml (Fig. 3.12). Other prominent protein peaks appeared before and after the enzyme peak. Fractions eluting between 292.5ml and 342.5ml elution volumes were pooled together for further purification on gel filtration column.

*Gel filtration on Sephadex G-150:*

The partially purified fractions were passed through a column of Sephadex G-150. The elution patterns of the polyphenol oxidase and peroxidase enzyme (Fig. 3.13) were identical to the pattern obtained for the enzymes obtained from the galls (Fig. 3.10).

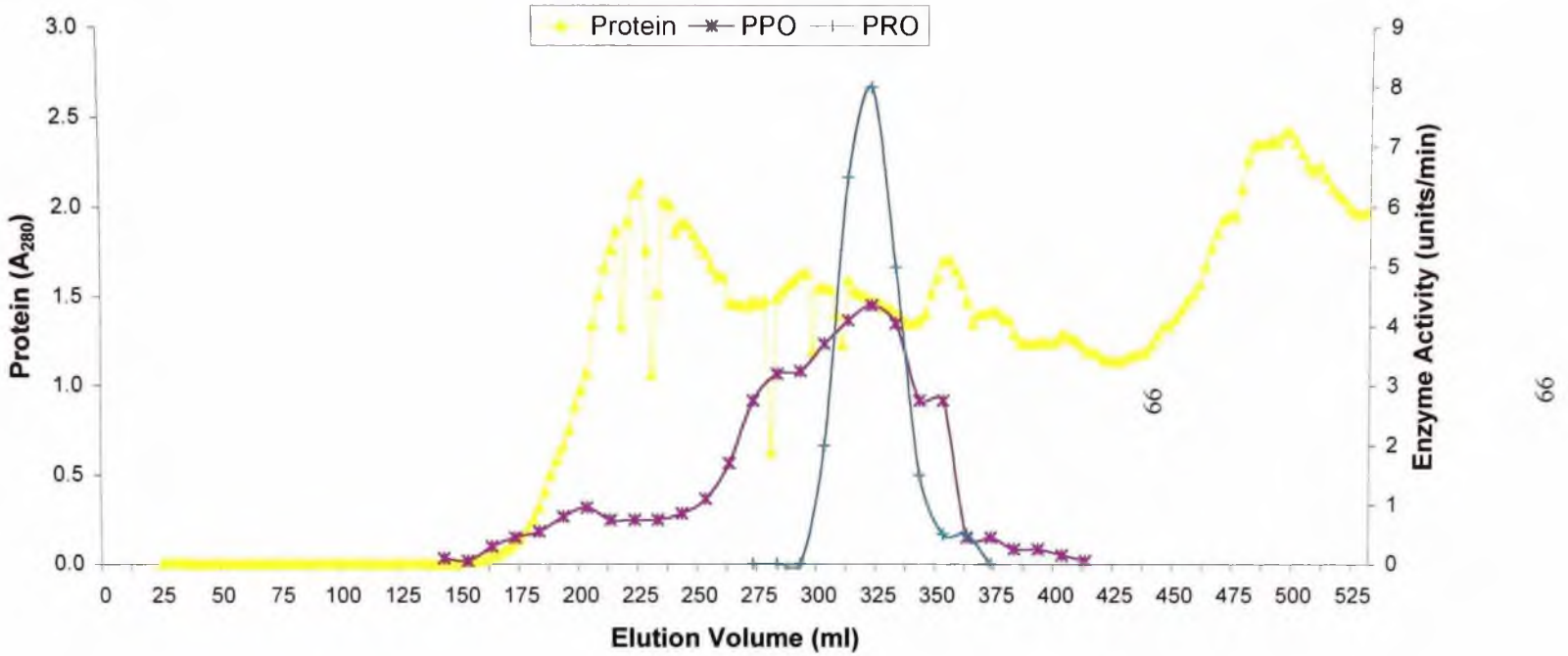



**Figure 3.12:** Ion-Exchange Chromatography of Crude Extract from Susceptible Leaves

Crude extract (60ml) from susceptible leaf samples was applied onto DEAE-Cellulose column (2.6 X 35cm) and eluted with a stepwise change of 0.0 to 0.75M NaCl gradient prepared in 10mM sodium phosphate buffer (pH 6.8). Fractions (2.5ml) were collected at a flow rate of 0.75ml/min.

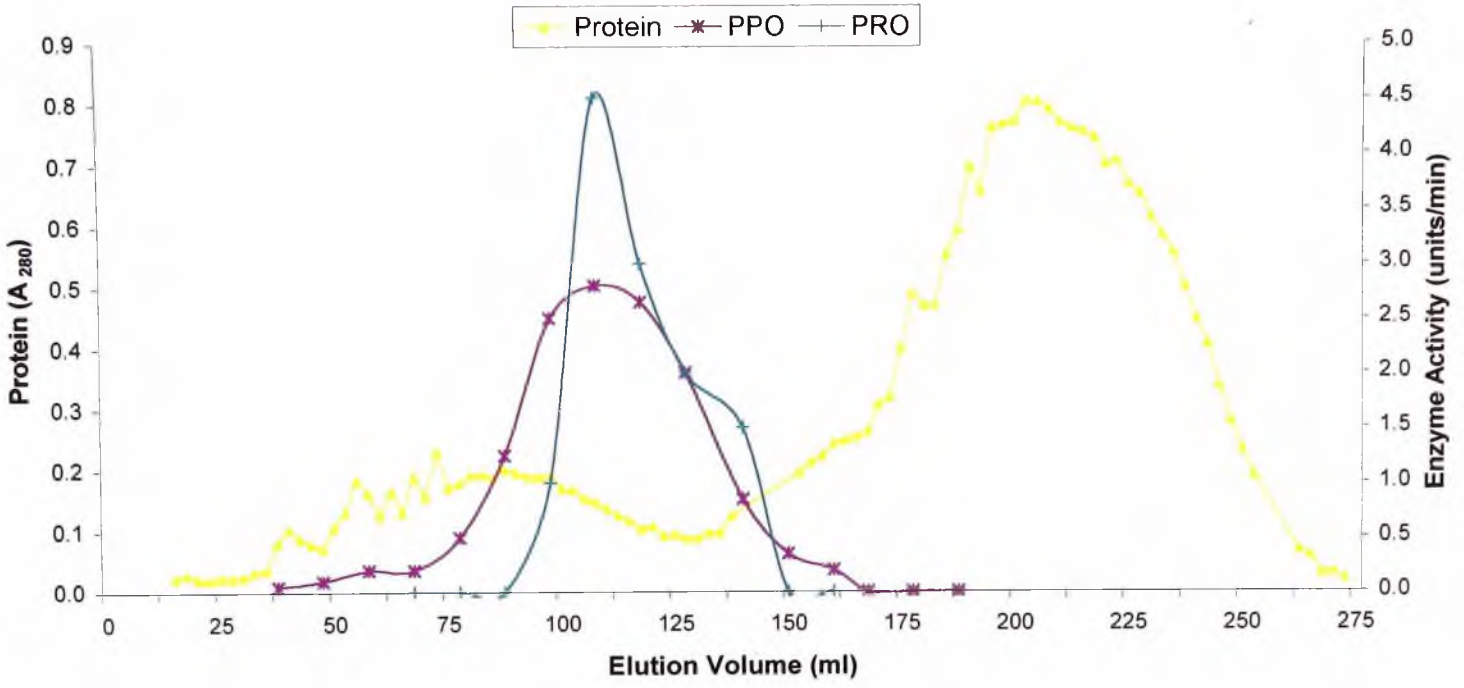



**Figure 3.13:** Gel Filtration of Partially Purified Susceptible Leaf Extract

Pooled fractions from ion-exchange column showing enzyme activities were applied onto Sephadex G-150 column (2.6 X 40cm) and eluted with 10mM sodium phosphate buffer (pH 6.8) at 5°C. Fractions (2.5 ml) were collected at a flow rate of 0.5 ml/min.

The enzymes appeared at approximately, an elution volume of 110ml with a protein peak just ahead and a clearly resolved protein peak after the enzyme peaks. Fractions eluting from 105.0ml to 117.5ml of elution volumes were pooled together and the activities of the enzymes and total protein determined as previously described.

**Purification Profile**

The purification of polyphenol oxidase from the galls and susceptible leaves using catechol to monitor enzyme activity through two purification steps resulted in nearly 14-fold and 17-fold purification of polyphenol oxidases from the galls and leaves, respectively (Tables 3.1 and 3.2). The 10-fold purification obtained for gall peroxidase (Table 3.3), using the peroxidation of guaiacol to monitor the enzyme activity, was considerably lower than the 37-fold increase in activity obtained for the leaf samples (Table 3.4).

Tyrosine, p-cresol and phenol were not oxidised by the polyphenol oxidase at any stage of its purification.

**Table 3.1: Activity of Polyphenol Oxidase from *Milicia* Galls During Purification**

| Step | Purification procedure | Total protein (mg) | Specific activity (units/mg) | $^{\Phi}$Yield (%) | $^{/}$Purification (fold) |
|---|---|---|---|---|---|
| 1 | Crude extract (non-purified) | 46.60 | 54.86 | 100 | 1.00 |
| 2 | Gel filtration on Sephadex G-150 | 1.40 | 620.54 | 44.86 | 11.31 |
| 3 | Ion-Exchange Chromatography on DEAE-Cellulose | 0.52 | 721.83 | 14.74 | 13.78 |

$^{\Phi}$Yield is calculated as: $\frac{\text{total activity of fraction x 100\%}}{\text{total activity of crude extract}}$

where total activity is calculated as total volume of fraction x activity/ml of fraction

$^{/}$ Purification factor is calculated as: $\frac{\text{specific activity of fraction}}{\text{specific activity of crude extract}}$

**Table 3.2: Activity of Polyphenol Oxidase from *Milicia* Leaves During Purification**

| Step | Purification procedure | Total protein (mg) | Specific activity (units/mg) | [Φ]Yield (%) | [/]Purification (fold) |
|---|---|---|---|---|---|
| 1 | Crude extract (non-purified) | 146.64 | 19.33 | 100 | 1.00 |
| 2 | Ion-Exchange Chromatography on DEAE-Cellulose | 32.08 | 20.15 | 18.45 | 1.04 |
| 3 | Gel filtration on Sephadex G-150 | 0.23 | 324.32 | 2.65 | 16.78 |

[Φ]Yield is calculated as: $\frac{\text{total activity of fraction} \times 100\%}{\text{total activity of crude extract}}$

where total activity is calculated as total volume of fraction x activity/ml of fraction

[/] Purification factor is calculated as: $\frac{\text{specific activity of fraction}}{\text{specific activity of crude extract}}$

**Table 3.3: Activity of Peroxidase from *Milicia* Galls During Purification**

| Step | Purification procedure | Total protein (mg) | Specific activity (units/mg) | [Φ]Yield (%) | [/]Purification (fold) |
|---|---|---|---|---|---|
| 1 | Crude extract (non-purified) | 46.60 | 115.34 | 100 | 1.00 |
| 2 | Gel filtration on Sephadex G-150 | 1.40 | 774.65 | 39.91 | 6.72 |
| 3 | Ion-Exchange Chromatography on DEAE-Cellulose | 0.52 | 1160.71 | 7.52 | 10.06 |

[Φ]Yield is calculated as: $\frac{\text{total activity of fraction} \times 100\%}{\text{total activity of crude extract}}$

where total activity is calculated as total volume of fraction x activity/ml of fraction

[/] Purification factor is calculated as: $\frac{\text{specific activity of fraction}}{\text{specific activity of crude extract}}$

**Table 3.4: Activity of Peroxidase from *Milicia* Leaves During Purification**

| Step | Purification procedure | Total protein (mg) | Specific activity (units/mg) | $^{\Phi}$Yield (%) | $^{/}$Purification (fold) |
|---|---|---|---|---|---|
| 1 | Crude extract (non-purified) | 146.64 | 18.41 | 100 | 1.00 |
| 2 | Ion-Exchange Chromatography on DEAE-Cellulose | 32.08 | 36.83 | 35.42 | 2.00 |
| 3 | Gel filtration on Sephadex G-150 | 0.23 | 675.68 | 5.79 | 36.70 |

$^{\Phi}$Yield is calculated as: $\frac{\text{total activity of fraction x 100\%}}{\text{total activity of crude extract}}$

where total activity is calculated as total volume of fraction x activity/ml of fraction

$^{/}$ Purification factor is calculated as: $\frac{\text{specific activity of fraction}}{\text{specific activity of crude extract}}$

**Polyacrylamide Gel Electrophoresis**

Analysis of the peaks from the ion exchange and gel filtration columns by SDS-PAGE indicated that the enzyme was fairly pure after the two purification steps (Fig. 3.14). Samples of the susceptible leaves showed two protein bands A and B, of 34 kDa and 32 kDa, respectively after gel filtration following an initial ion-exchange purification step. Pooled fractions from ion-exchange purification of susceptible leaf extracts also yielded two closely associated bands C and D of the same molecular weights as bands A and B, respectively. The gall sample also showed two prominent protein bands E and F of molecular weights 34 kDa and 50 kDa, respectively after the first purification step using gel filtration chromatography. Neither of these bands could however, be located in fractions pooled from the second purification step (ion-exchange chromatography).

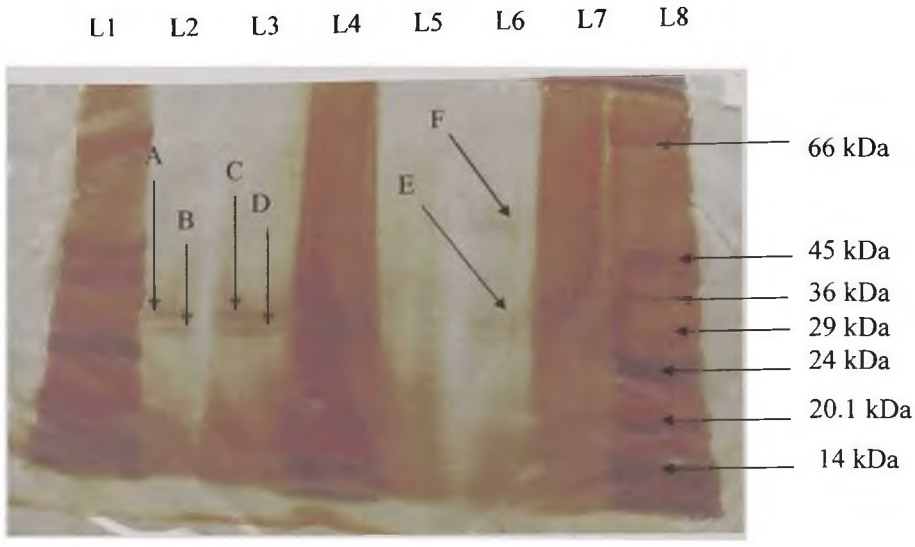



**Figure 3.14:** Silver Stained SDS-PAGE of Partially Purified Polyphenol Oxidase and Peroxidase from Galls and Susceptible Leaves.

| Lane | Sample |
|---|---|
| L1 & L8 | -- molecular weight protein markers. |
| L2 | -- susceptible leaf samples: active fractions pooled from gel filtration column. |
| L3 | -- susceptible leaf samples: active fractions pooled from ion-exchange column. |
| L4 | -- crude extracts from susceptible leaves. |
| L5 | -- gall samples: active fractions pooled from ion-exchange column. |
| L6 | -- gall samples: active fractions pooled from gel filtration column. |
| L7 | -- crude extracts from galls. |

| Protein Band | Estimated Molecular Weight |
|---|---|
| A, C & E | 34 kDa |
| B & D | 32 kDa |
| F | 50 kDa |

# CHAPTER FOUR

## DISCUSSION

This study principally investigated the relationship between levels of phenolic compounds and the phenolic oxidases, polyphenol oxidase and peroxidase, in relation to the degree of resistance of the plant to *Phytolyma*-induced gall disease. The objective was to identify one or more of the measured parameters as molecular biomarker(s) of resistance to the gall disease.

In a study of young apple trees, Picinelli *et al.* (1995) showed that it was possible to differentiate scab susceptible from resistant apple varieties on the basis of their phenolic content. Biochemical components could therefore serve as the basis of developing a quick and reliable biochemical technique for identifying and selecting resistant varieties of *Milicia* seedlings.

In the study, there were generally no statistical differences between the levels of phenolic compounds found in the normal leaves and galls as well as between the susceptible and resistant varieties of the *Milicia* samples. The seemingly higher amounts of

dihydroxyphenols in the susceptible varieties as compared to the resistant variety (Fig. 3.4) may however, suggest some level of involvement of this class of secondary metabolites in susceptibility of *Milicia* saplings to the gall disease. It has been suggested that increased levels of chlorogenic acid, a dihydroxyphenol, measured after infestation of carrot roots by nematodes resulted in decreased oxidation of indole acetic acid and thus increased tissue growth and increased gall formation (Knypl *et al.*, 1975).

In their study of plant phenols, Beckman *et al.* (1972) established that phenols are involved in defense mechanisms as well as in wound healing and in the general synthesis and regulation of the important growth hormone, indole acetic acid, which has been implicated in gall formation (Rohfritsch and Shorthouse, 1982; Hori and Miles, 1977). Though not statistically significant, trends observed in this study indicated a decreasing phenolic content with respect to increasing susceptibility to *Phytolyma* attack and gall formation (Fig. 3.3). This corresponded with the decreasing polyphenol oxidase and peroxidase activity with respect to increasing susceptibility to the attack (Fig. 3.7 and 3.8). Jiao *et al.* (1992) observed similar trends in

their study of phenolic oxidases (polyphenol oxidase and peroxidase) and total phenolic content of citrus leaves differing in susceptibility to infection by *Xanthomonas* sp. The oxidative responses resulting from the increased levels of phenolic compounds and the activity of the phenolic oxidases may have resulted in decreased herbivory (Dowd and Vega, 1996). There was not enough evidence in this study, however, to confirm the involvement of phenols or dihydroxyphenols in the resistance or development of *Phytolyma*-induced galls on *Milicia* since the differences between samples were not significant.

The diseased tissues (i.e. galls) of the two species of *Milica* seedlings showed significant increases in polyphenol oxidase activity with the increase being highest in the *M. regia* galls. The increase in polyphenol oxidase activity (3-fold) following infestation of the *M. regia* seedlings (Fig. 3.5) suggests that this species (*M. regia*) is more resistant to *Phytolyma* attack than *M. excelsa*. This is in line with the suggestion made by Dowd and Vega (1996), that increased synthesis of quinone and other phenolic oxidation products resulting from increased activity of

polyphenol oxidase results in decreased herbivory by insect predators.

The increased polyphenol oxidase activity would also result in increased oxidation of dihydroxyphenols, which are inhibitors of indole acetic acid oxidase. This would in turn lead to increased oxidation of indole acetic acid and therefore decreased tissue growth and gall formation. Bashen (1986) has shown that during disease development on cotton seedlings, polyphenol oxidase activity accumulated faster in the leaves of the resistant varieties than in the susceptible plants. Though not statistically significant, the *M. regia* species generally showed slightly higher polyphenol oxidase and peroxidase activities than the *M. excelsa* species (Fig. 3.5 and 3.6). Hegazi *et al.* (1993) made similar observations in studies of the brown leaf spot disease of rice. The slightly higher levels of polyphenol oxidase in *M. regia* and the significantly higher increase in the activity of the polyphenol oxidase in galls of this species as compared to *M. excelsa*, suggest that *M. regia* seedling may be more resistant to *Phytolyma*-induced gall formation than the *excelsa* species. The significantly higher polyphenol oxidase activity measured in the resistant varieties of

fractionation was performed on the gall extract prepared from the acetone powder. A peak for polyphenol oxidase activity occurred at 20% ammonium sulphate saturation. This may be due to membrane bound fractions of the enzyme. This fraction lacked peroxidase activity and also lost its polyphenol oxidase activity very rapidly. The major peak of activity, which occurred between 60 and 100% ammonium sulphate saturation, may be due to the cytosolic enzyme (Mayer and Harel, 1979). The loss of enzyme activity after dialysis of the 60-100% fraction may be due to destabilization of the enzyme protein due to the process of dialysis. Ponting and Joslyn (1948) discovered that polyphenol oxidase and peroxidase activity were reduced by over 50% subsequent to dialysis even at temperatures as low as 0°C.

Crude extracts from the susceptible leaf sample did not yield any protein precipitates during ammonium sulphate fractionation because of the very viscous nature of the extract.

Gel filtration of the crude extracts from galls resulted in the separation of the crude proteins into two main peaks (Fig. 3.10) with the enzyme peak appearing between these protein peaks. Further separation of the pooled

fractions from the gel filtration column on DEAE-Cellulose produced five protein peaks, only one of which was associated with the activity of the two enzymes. This two-step purification process resulted in approximately 14 and 10-fold purification, respectively, of the gall polyphenol oxidase and gall peroxidase. The extracts of susceptible leaf were, however, found to be viscous and slimy. As a result, there was difficulty in the use of gel filtration on Sephadex G-150 as the first purification step. Consequently, the chromatographic steps were carried out as shown in Tables 3.2 and 3.4, with ion-exchange chromatography of the viscous crude extracts on DEAE-Cellulose preceding the gel filtration on Sephadex G-150. Approximately 17-fold purification was obtained for polyphenol oxidase and approximately 37 fold for peroxidase. Thus, the direct application of the viscous and slimy enzyme extract to the ion-exchange column resulted in the successful separation of the enzyme proteins from the viscous and slimy materials in the extract. However, this procedure could not separate polyphenol oxidase activity from peroxidase enzyme activity, as both enzyme activities were present in the same fractions.

Unlike the reported experience of multiple enzyme peaks with the enzymes from grape, banana, mushroom etc. (Mayer and Harel, 1979) the two chromatographic steps used for each sample resulted in a single superimposing peak of activity for the two enzymes towards their respective substrates, catechol and guaiacol.

Contrary to what has been observed in green olives and white yam tubers (Ben-Shalom *et al.*, 1977; Ikediobi, 1982), tyrosine, p-cresol and phenol were not oxidized by *Milicia* polyphenol oxidase at any stage during the extraction and purification process. The lack of activity towards monophenols may suggest the absence of hydroxylase action by the enzyme in this plant species.

The results of the chromatographic separation, based on differences in size (gel filtration) and net charge (ion-exchange chromatography), thus suggest that polyphenol oxidase and peroxidase activity may be dual catalytic functions of a single enzyme. Dual catalytic enzymes are not uncommon in plants (Srivastava and van Huystee, 1973). Widely recognised among such enzymes are plant phenolic oxidases, which have dual capabilities of hydroxylation and oxidation of phenolic compounds as well as peroxidative action on a variety of

substances (Srivastava and van Huystee, 1973, Mayer and Harel, 1979).

The appearance of the polyphenol oxidase and peroxidase enzyme peaks at the same elution volumes (112.5ml) for both leaves and galls (Figs. 3.10 and 3.12) also suggests that these enzymes have the same molecular weight and native conformation in the two tissues investigated namely, gall and susceptible leaves.

Analysis of the purified enzyme fractions of susceptible leaves by sodium deodocyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) resulted in two closely associated protein bands C and D of molecular weights 34 KDa and 32 KDa. The 34 KDa band (band C) matched one of the protein bands (band E) obtained for the gall sample following gel filtration (Fig. 3.14). This band appears to be the polyphenol oxidase-peroxidase protein band.

The degree of purification of polyphenol oxidase achieved in this work, about 14-fold and 17-fold for the galls and leaves, respectively, is much lower than what was obtained for the enzyme from green olives (239-fold) (Ben-Shalom *et. al.*, 1977) and yam tubers (112- fold) (Ikediobi and Obasuyi, 1982). The estimated molecular

weight of 34 kDa appears to be in the lower 30-40 kDa range of the three molecular weight ranges (30-40 kDa, 60-70 kDa and 85-180 kDa) that has been observed for catechol oxidases from a variety of sources (Mayer and Harel, 1979).

# CONCLUSION

High polyphenol oxidase activity was found in the study to be associated with high resistance to the *Phytolyma*-induced gall disease while the least activity of the enzyme was associated with very high susceptibility to the disease. The level of activity of polyphenol oxidase in the leaves of *Milicia* plants may therefore be a useful biomarker of resistance to *Phytolyma*-induced gall disease on seedlings of the plant. The enzyme, which lacked cresolase activity, has an estimated molecular weight of 34 kDa. The enzyme protein appears to be one with catalytic sites for both polyphenol oxidase and peroxidase since the activity of these two enzymes could not be separated.

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