PHYSIOLOGICAL RESPONSES OF MAIZE *(ZEA MAYS* L.) TO EXOGENOUS FERULIC ACID

THESIS SUBMITTED FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
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By
S. Rama Devi
(Enrolement No.: PL 5944)

DEPARTMENT OF PLANT SCIENCES
SCHOOL OF LIFE SCIENCES
UNIVERSITY OF HYDERABAD
HYDERABAD - 500 134
INDIA

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To my parents

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UNIVERSITY OF HYDERABAD

SCHOOL OF LIFE SCIENCES
HYDERABAD-500 134. INDIA

Phones : 253901
FAX: 91-040-253145
Telex : 04292-205 UHYO IN

DECLARATION

I hereby declare that the work embodied in the presented thesis "Physiological responses of maize (Zea *mays* L.) to exogenous ferulic acid" has been carried out by me under the supervision of Dr. M.N.V. Prasad and that this work has not been submitted for a degree or diploma of any other university.

S. Rama Devi

S. Rama Devi
Candidate

M. Prasad

UNIVERSITY OF HYDERABAD

SCHOOL OF LIFE SCIENCES
HYDERABAD-500 134. INDIA

Phones : 253901
FAX: 91-040-253145
Telex : 04292-205 UHYO IN

CERTIFICATE

This is **to** certify **that Miss. S. Rama Devi** has carried out the research work embodied in the present thesis **"Physiological responses of maize *(Zea mays L.*) to exogenous ferulic acid" for** the **full** period prescribed under the Ph.D ordinance of this university. The thesis is recommended for submission to the University of Hyderabad for the **Doctor of Philosophy in Plant Sciences.**

Dr.M.N.V. Prasad
Supervisor

Prof.A.R.Reddy
Head, Dept. of Plant Sciences

7.9.94
Prof. N.C.Subrahmanyam *FNA*
Dean, School of Life Sciences

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S. Rama Devi

LIST OF ABBREVIATIONS

ABA	Abscisic acid
ADP	Adenosine dinucleotide phosphate
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CAD	Cinnamylalcohol dehydrogenase
DCMU	3-(3,4-Dichlorophenyl), 1 -Dimethyl urea
DCPIP	2,6, Dichlorophenol indophenol
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
FA	Ferulic acid
GA	Gibberellic acid
HEPES	4-(-2-(Hydroxyethyl)piperazine-l -ethane sulfonic acid)
1AA	Indole-3-acetic acid
MV	Methyl viologen
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
PAGE	Polyacrylamide gel electrophoresis
PAL	Phenylalanine ammonialyase
PMS	Phenazine metho sulphate
POD	Peroxidase
POPOP	1,4 Bis (2,5,-Phenyloxazyl benzene)
PPO	Polyphenol oxidase
PPO	Diphenyloxazole
PS 1	Photosystem I
PS II	Photosystem II
SDS	Sodium dodecyl sulphate
STN buffer	Sucrose Tricine Sodium chloride buffer
TBA	Thiobarbituric acid

TCA	Trichloro acetic acid
TEA	Tetra ethyl ammonium
TEMED	N,N,N,N, tetramethyl ethylene diamine
TRIC1NE	N-Tris-(Hydroxymethyl) methyl glycine
Tris	Tris (hydroxymethyl) aminomethane

PART I

INTRODUCTION

ALLELOCHEMICALS - AN OVERVIEW

In the process of evolution, higher plants have evolved a complex array of biochemical pathways through which a variety of secondary metabolites are synthesized and accumulated which enable them to recognize and respond to signals from the environment. Most of these compounds are released into the environment in appreciable quantities via root exudation and as leachates during litter decomposition and play a significant role in allelopathy as allelochemicals.

The term allelopathy coined by Molisch (1937) generally refers to the direct or indirect detrimental effect of one plant (including micro organisms) on the germination, growth and development of other plants mediated by the chemicals that are released into the soil. Allelopathic interactions among plants have been implicated in the patterning of vegetation (Smith and Martin 1994), weed growth in agricultural systems (Aldrich 1987, Inderjit and Dakshini 1994, Putnam 1985, Rice 1987) and yield of agricultural crop plants (Bansal ***et al*** 1992, Chou 1990, Liu and **Lovette** 1993, Ramamoorthy and Paliwal 1993). In addition to its role in terrestrial ecosystems, allelopathic mechanisms are often well observed in aquatic ecosystems (Gopal and Goel 1993).

In the recent years, allelopathy in agriculture is receiving considerable attention due to the potential of allelochemicals in reducing the agricultural yields (Chou 1982, Chou *et al* 1981, 1990, Moitra *et al* 1994) and for **their** potential as natural herbicides and pesticides (Bernard *et al* 1990).

1.1.1. Chemical nature of allelochemicals:

The chemistry of various allelochemicals in terrestrial ecosystems shows a wide range of chemical compounds identified from soil (Whitehead 1964) and plants (Glass 1974, Hoagland and williams 1985, Miles *et al* 1993) which are implicated in allelopathy. These include compounds ranging from simple

gases and aliphatic compounds to complex multiranged aromatic acids including acetic and butyric acids, long chain fatty acids, quinones, simple phenols, phenolic acids derived from cinnamic and benzoic acids, coumarins, flavonoids, several hydrolyzable and condensed tannins, terpenoids, alkaloids and various nitrogenous compounds (Chou 1982, Mandava 1985, Rice 1987).

1.1.2. Release of allelochemicals into the environment:

Most of the allelochemicals synthesized in the plants are released into the environment by various mechanisms. Mandava (1985) critically reviewed the mechanisms of release of these compounds which include 1. Exudation of volatile compounds from plant parts, 2. Leaching of water soluble compounds from plants due to rain, fog or dew, 3. Exudation of water soluble compounds from the roots (Yu and Matsui 1994), 4. The release of toxic chemicals from the non living plant parts through leaching of toxins from litter.

Based on the route from which allelochemicals are released into the environment, these compounds have been identified as four groups 1. Root exudatcs, 2. Leaf leachates and decomposition products, 3. Volatile toxicants, 4. Sick soil toxicants includes the accumulation of toxic compounds from the previous crops which inhibit the growth of successive crop (Mandava 1985).

1.1.3. Factors affecting the allelochemical production:

The production or release of allelochemicals vary with plants type, age (Wardle *et al* 1993) and its microenvironment, which include a number of soil physico-chemical factors like texture, nutrient status, pH, temperature, and rhizospheric microbes (Barz and Koster 1981, Blum *et al* 1987, 1992, Einhellig 1987, 1989, Hoagland and Williams 1985, Putnam 1985, Rice 1987, Whitehead *et al* 1981). In addition to these factors, compounds like glucose, methionine and nitrate are also known to modify allelopathic effects of allelochemicals (Blum *et al* 1993). Moreover the final active concentration of allelochemicals in the soil depends on the relative rates of addition and inactivation (Barz and Koster 1981, Hoagland and Williams 1985) as these chemicals occur in soils as

released products during litter decomposition by the action of soil bacteria (Einhellig 1987, Huang *et al* 1993) and in aquatic ecosystems the degradation of phenolic compounds by fresh water algae has been observed (Ellis 1977).

In most of the cases, the allelopathic effects usually are the result of the combined actions of several allelochemicals, often with each below the threshold concentrations for its effect (Barz and Koster 1981). In most of the allelopathic situations, the concentrations of the chemicals ranged from 10-lOOOppm for each compound and often additive and synergistic effects have been demonstrated (Einhellig and Rasmussen 1979, Einhellig *et al* 1982). Such combined interactions are very important in the field conditions as they determine the final allelopathic effect.

1.1.4. Detoxification of allelochemicals:

Plants have acquired a number of detoxification mechanisms to minimize the levels and toxic effects of allelochemicals (Shimabukuro 1985, Shimabukuro *et al* 1982). Mandava (1985) recognized three types of detoxification mechanisms which include phase I reactions like oxidation, reduction and / hydrolysis that include the peroxidases, monoxygenases and other oxygenase enzymes which occur commonly in the plants (Shimabukuro 1985).

Another type of detoxification reaction that is most commonly observed in plants is the conjugation reactions which include the conjugation of allelochemicals with an endogenous substrate to form a new compound. The importance of conjugation reactions lies in the greater water solubility of the conjugates (Balke *et al* 1987). The structural modification of the allelochemical during the conjugation reduces the toxicity and the higher molecular weights of the conjugates impose a limitation for its free movement. The most important conjugation mechanism in plants include glucosylation, which involves the conjugation of allelochemicals with glucose mediated by an enzyme glucosyl trasferase. A large number of glucosyl transferases based on the type of

allelochemical being conjugated have been identified and purified (Balke *et al* 1987, Sun and Hrazdina 1991, Yalpani *et al* 1992a,b).

In addition to these above discussed mechanisms, an another additional mechanism has been observed in plants which include further conjugation of primary conjugates to other endogenous substrates that results in tertiary conjugates. This mechanism is found to be the most important process which ultimately reduces the toxicity of the allelochemicals to considerable extent. In general these conjugation reactions are thought to be the ultimate processes involved in the detoxification of the allelochemicals to a greater extent from sites of continuous metabolic activity in the organism (Mandava 1985). The final toxicity of the compounds, however is determined as to what extent an organism is able to reduce the accumulation of allelochemicals from site of action.

1.2.1. Phenolic compounds as allelochemicals:

Among the allelochemicals identified todate, phenolic compounds constitute an important group which are of great significance in plant soil interactions (Kuiters 1990, Rice 1987, Siqueira *et al* 1991). Recently Zaprometov (1992) reviewed the functional role of phenolic compounds in plants. Some of the commonly identified phenolic compounds that are found in plants (Harborne 1980, Putnam and Tang 1986, Rice 1984, Waller 1987) are listed in table 1.

Most of them are found as an integral part of the structural matrix (Lam *et al* 1992), serve as flower pigments, confer resistance against invading pathogens (Abrahamson *et al* 1991, Leon *et al* 1994), function as signal molecules during infection (Raskin 1992) and as allelopathic compounds (Rice 1987) thus regulate the cell and plant growth (Abrahamson *et al* 1991, Gerig and Blum 1991, Harborne 1980, Liu and Lovette 1993, Lynn and Chang 1990, Muller-Harvey and Reed 1992, Pellissier 1993a,b, Putnam and Tang 1986, Rice 1984, Richter and Wild 1992, Vidyasekaran 1988, Waller 1987). In addition to

Table 1. Some of the phenolic compounds commonly found in plants

Category	Name of the phenolic compound
Simple phenols	Phenols, catechol, hydroquinone, phloroglucinol, pyrogallol
Phenolic and benzoic acids	p-hydroxy benzoic, catechuic acids: vanillic, gallic, syringic, salicylic protocatechuic and gentisic acids
Cinnamic acids	p-coumaric, cinnamic, caffeic, ferulic and sinapic acids
Flavonols	Kaempeferol, quercetin and myrcetin

these varied roles, Palm and Sanchez (1991) observed interference of phenolic compounds with nitrogen release from leaves of tropical legumes during decomposition and Hartley (1992) observed the regulation of cell wall biodegradation by phenolic compounds.

Furthermore, these compounds which are released into the soil during decomposition of litter are known to influence variety of metabolic processes in plants. The most important role of phenolic compounds in argicultural ecosystems is their involvement in the autointoxication mechanism which is well observed in Taiwan rice fields where in a considerable reduction in the yield of second crop has been observed when compared to first crop due to allelochemicals (mostly phenolic compounds) released from the leftover residues of the first crop (Chou *et al* 1981).

1.2.2. Biosynthesis of phenolic compounds:

Phenolic compounds which accumulate in plants are synthesized in a multibranched metabolism represented primarily by a phenylpropanoid pathway and flavonoid/chalcone pathway and most of them are 6 to 10 carbon skeleton compounds (Gross 1981, Hahlbrock and Scheel 1989). Some of the key enzymes involved in the synthesis of these compounds include phenylalanine ammonialyase, chalcone synthase and enzymes of cinnamic acid pathway which are partially self regulated and known to be influenced by both biotic and abiotic stresses (Bolwell *et al* 1986, 1988, Liang *et al* 1989). The general biosysnthetic pathway of phenolic compounds is shown in figure 1. The concentrations of these compounds in the tissue vary according to the rate of their biosynthesis, storage, degradation and are affected by internal balance of plant growth regulators (Bell and Charlwood 1980, Harborne 1980, Stumpf and Conn 1981, Tokhver and Palm 1991).

1.2.3. Accumulation of phenolic compounds **in plants and soil:**

Plants contribute to a large extent in the accumulation of phenolic compounds in the soil. The major route of entry for these compounds into the

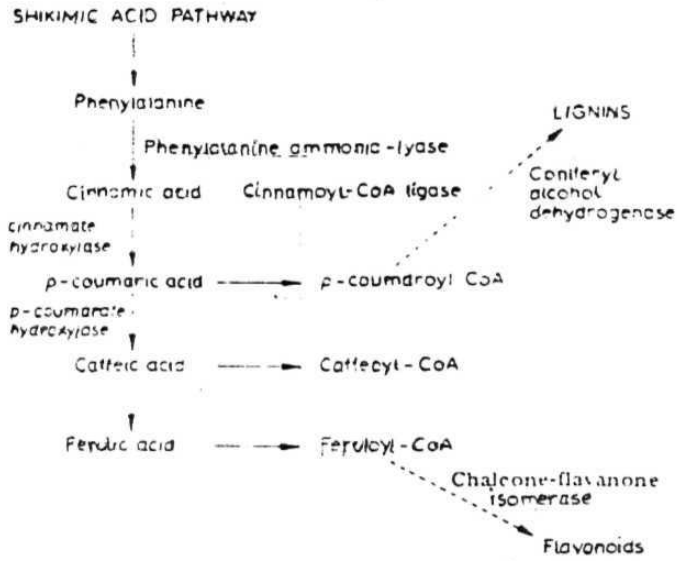


Fig. 1. The biosynthetic pathways of phenolics.

environment include root exudates and litter decomposition (Yu and Matsui 1994). In addition to their release into the soil, phenolic compounds undergo continuous cycles of deposition, decomposition, plant uptake, leaching and chemical immobilization as shown in figure 2 (Siqueira *et al* 1991). Whether the available concentration of inhibitors in the soil is adequate to inhibit seedling growth, finally depends on the above said factors (Fig. 2).

1.3.1. Allelopathic potential of ferulic acid:

Ferulic acid (FA), a derivative of cinnamic acid is commonly found in plants (Akin *et al* 1992, Blade *et al* 1991, Colton and Einhellig 1980, Tan *et al* 1991, 1992), soil (Chou and Lin 1976, Dalton *et al* 1987, Katase 1981, Lodhi 1975, Whitehead *et al* 1981), as a product of lignin degradation in the soil (Turner and Rice 1975) and is identified as a potential allelochemical (Mc Pherson 1971, Rasmussen and Einhellig 1977). The concentration of FA in the soil usually range from $4x10^{-4}$ to $5x10^{-5}$ which however found to depend on various biotic and abiotic factors (Whitehead 1964, Wang *et al* 1967).

FA has been identified as a structural component in the cell walls of both monocot and dicot plants (Kamisaka *et al* 1990, Locher *et al* 1994, Tan *et al* 1991, 1992). Cyclodimers of FA as substitutes in the cell wall polysaccharides in graminaceous plants has been observed (Hartley and Jones 1976, Smith and Hartley 1983). Ohashi *et al* (1987) observed 5- hydroxy ferulic acid in maize and barley and Locher *et al* (1994) noticed isomers of FA in growing maize roots. Esters of FA have been observed in the stem bark of *Pavetta* (Blade *et al* 1991). FA linked to arabinoxylan in the cell walls has been identified in cell cultures of *Festuca arundinaceae* (Myton and Fry 1994). Weidner *et al* (1992) reported the occurrence of FA in barley seeds as a dormancy factor. In addition to its association mostly with cell walls, FA is also identified in its bound form in the aleurone layers of barley (Gubler and Ashford 1985, Gubler *et al* 1985) and as N-acyl terminal group in a protein of barley seeds (Vansumere *et al* 1973) linked directly to glycine and phenylalanine and has been detected as a

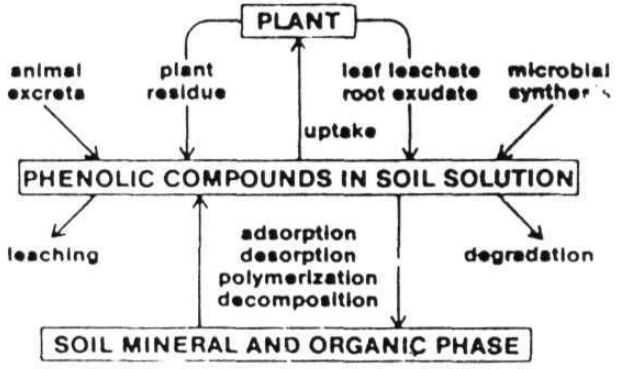


FIGURE 2. Major processes regulating phenolics in plant-soil systems

lipid conjugant (Chatterjee *et al* 1977). Ehmann (1974) reported N-ferulyl tryptamine in kernals of *Zea mays* and as germination inhibitor (Vansumere *et al* 1972). In addition to its existance mostly in bound form, often free forms of FA are observed in some plants such as sugar beet and cereals (Hartley and Jones 1976). Apart from FA, several of the metabolic products of FA released during microbial metabolism, such as vanillic, caffeic, and protocatechuic acids are also known to be toxic either equally and in some cases even more toxic than FA (Turner and Rice 1975)

1.3.2. Biosynthesis of ferulic acid:

It is well documented that most of the phenolic compounds including FA are derived from the shikimic acid path way and the precursor being the phenylalanine derived from the shikimic acid (Gross 1981, Hahlbrock and Scheel 1989). Earlier it has been demonstrated that the parent compound cinnamic acid undergoes ring substitutions in a series of hydroxylation and methylation steps yielding various p-hydroxylated cinnamic acids (Fig. 2).(Gross 1981).

Deamination of phenylalanine is catalyzed by phenylalanine ammonialyase which results in cinnamic acid or p-coumaric acid (Jones 1984, Jorrin *et al* 1990). Among the hydroxylase enzymes involved in the cinnamic pathway i.e. cinnamic 4-hydroxylase and p-coumarate 3-hydroxylase catalyzing the sequence cinnamic acid > p-coumaric acid > caffeic acid, have been isolated and characterized as membrane associated mixed function oxygenases (Billett and Smith 1980, Vaughan *et al* 1975). The methyl group of FA is formed by methylation of the metahydroxyls of its precursor 5-hydroxy FA by the action of o-methyltransferase (Pellegrini *et al* 1993)

1.3.3. Effects of exogenous ferulic acid:

Vast information is available on the toxicity of FA since its identification as an allelochemical. A number of physiological and biochemical

processes are known to be altered by FA either alone or in combination with other phenolic acids acting synergistically or antagonistically.

a. Germination:

FA has been reported to inhibit the germination of sorghum, cucumber, wheat and many other crop plants either alone or incombination with other phenolic acids in the soil (Blum and Dalton 1985, Blum and Rebbeck 1989, Leather and Einhellig 1985, Rasmussen and Einhellig 1979, Williams and Hoagland 1982). Khan and Ungar (1986) observed an inhibition upto 80% in the germination of *Atriplex triangularis* seeds with FA. It is also known to retard the germination of isolated barley embryos (Weidner *et al* 1992). In addition to crop plants, FA is also known to inhibit the germination of fungal spores and conidia (Alfenas *et al* 1982, Kasenberg and Traquair 1988). Similar to FA, some of its derivatives are known to impose self inhibitory effect on spore germination of rust fungi (Allen 1972, Faudin and Macko 1974).

b. Growth:

In addition to germination, FA is reported to alter the growth of the seedlings by reducing the shoot and root growth. However, the primary effect being observed to be on roots. Earlier studies observed a reduction in the growth of primary root and decrease in the number of secondary roots which tan to brown frequently with deformed root tips (Blum and Dalton 1985, Blum and Rebbeck 1989, Blum *et al* 1984). Reduction in growth (Blum and **Dalton** 1985, Blum and Rebbeck 1989, Holappa and Blum 1991), fresh weight and dry weight of shoots and roots have been observed in cucumber, tomato, maize and soybean (Blum and Dalton 1985, Blum and Rebbeck 1989, Devi and Prasad 1992, Holappa and Blum 1991, Patterson 1981). Furthermore, inhibition in leaf expansion and wilting of plants is observed in variety of crop and vegetable plants even after **a** short period of application (Blum and Dalton 1985, Blum *et al* 1984, Einhellig *et al* 1985, Holappa and Blum 1991, Klein and Blum 1990, Waters and Blum 1987). Interference in the vegetative and reproductive growth

of *Phaseolus* has been observed by Waters and Blum (1987). Reduction in the growth of *Oryza sativa* coleoptiles due to a decrease in the cell wall extensibility by FA is well observed (Kamisaka *et al* 1990, Tan *et al* 1991, 1992).

c. Stomatal functions:

FA, together with many other phenolic acids are known to interfere with stomatal function. Several studies observed a decrease in stomatal conductance of the leaves (Balke 1985, Blum and Dalton 1985, Einhellig and Kuan 1971, Einhellig and Stille 1979, Einhellig *et al* 1985, Patterson 1981). In addition to stomatal conductance and stomatal closure, reduction in transpiration rates of leaves has been observed for a number of plants (Einhellig and Kuan 1971, Einhellig and Stille 1979).

d. Water potentials:

The uptake of water which is essential to maintain turgor in the cell is adversely affected by FA. A significant reduction in leaf water potentials are observed in soybean, cucumber, sorghum and tomato (Patterson 1981) and water utilization (Blum and Dalton 1985, Blum *et al* 1985a,b) with exogenous FA. Further, Einhellig *et al* (1985) observed interference of FA with water metabolism of grain sorghum. However, Blum *et al* (1985b), and Klein and Blum (1990) did not observe any change in the water potential of bean plants, indicating differential sensitivity of the plants to FA. The decrease in the stomatal conductance, transpiration and leaf expansion have been ascribed to FA induced reduction in water potentials (Einhellig *et al* 1985) and ascribed this reduction in water potentials due to reduction in osmotic potential and turgor pressure (Einhellig *et al* 1985).

e. **Ion uptake**

The uptake of water and ions by the roots is usually facilitated by the transport of these across the cell membranes (Tyerman 1992). FA like many

other phenolic compounds has been reported to interfere with ion uptake. Bergmark *et al* (1992) observed an inhibition in the uptake of nitrate, ammonium and potassium by maize roots and inhibition in the uptake of potassium has been noticed by Booker *et al* (1992). Inhibition in the uptake of phosphate by barley and *Glycine max* roots (Glass 1975a, Mc Clure *et al* 1978) and potassium absorption by roots of *Hordeum vulgare* (Glass 1974) and *Avena sativa* (Harper and Balke 1981) with exogenous FA is observed. The inhibition in the uptake is found to be either non-competitive (Mc Clure *et al* 1978) or uncompetitive (Glass 1973). Even low concentrations of FA (100µM) are reported to inhibit the rubidium absorption in Pauls scarlet rose within lOmin (Danks *et al* 1975). Lyu and Blum (1990) noted an inhibition in the capacity of roots to absorb phosphorus and potassium due to FA.

f. **Photosynthesis:**

A significant reduction in the photosynthetic rates with allelochemicals have been documented earlier. FA is known to inhibit the photosynthetic functions in a number of crop plants. Patterson (1981) observed significant reduction in net photosynthetic rates and chlorophyll content in soybean with FA along with many other phenolic compounds. Lodhi and Nickell (1973) observed similar reduction in the net CO_2 exchange rates in three grasses. Reduction in the chlorophyll content in soybean has been observed with FA (Einhellig and Rasmussen 1979). Blum and Rebbeck (1989) observed significant reduction in the proportion of carbon allocated to roots in addition to reduction in the photosynthetic rates and chlorophyll content. In addition to its effect at whole plant level, FA is found to inhibit photosynthetic functions at the cellular level. Moreland and Novitzky (1987) reported an inhibition in the electron transport rates and ATPase activities of chloroplasts incubated with FA. Mersie and Singh (1993) observed an inhibition in the photosynthetic rates of isolated leaf cells of velvet leaf by FA and other phenolic compounds.

g. **Interaction with plant growth regulators:**

Many of the researchers are of the opinion that phenolic acids exert their effect by altering the levels of endogenous growth regulators or by blocking their sites of action. Tayal and Sharma (1981, 1985) observed changes in IAA levels on FA treatment. Tomaszewski and Thimann (1966) observed a synergistic interaction of IAA and phenols in rooting of oat and pea seedlings. FA, when applied alone at concentrations below 0.1mM increased root length in *Phaseolus vulgaris* hypocotyl cuttings (Waters and Blum 1987). However, a decrease in root length was observed in the presence of FA+IAA indicating the antagonistic nature of FA towards IAA (Tayal and Sharma 1985). Recently Kathiresan *et al* (1990) observed an interactive effect of auxin-phenol determining the rooting of mangrove and Ray and Laloraya (1983) observed interaction of phenolic compounds with GA and ABA as examined earlier (Nutbeam and Briggs 1982). Rasmussen and Einhellig (1979) observed an inhibition of GA3 stimulated germination in sorghum by FA together with p-coumaric acid and vanillic acid. In addition to its action on the functions mediated by these growth regulators, FA also alters the levels of growth regulators in the plants. FA induced increase in the levels of ABA is observed in cucumber seedlings (Holappa and Blum 1991). Li *et al* (1993) observed a similar increase in the endogenous ABA levels of lettuce with FA and other phenolic compounds. Kamisaka *et al* (1990) observed an inhibition in the auxin stimulated elongation of cells in rice with FA application. Ishi and Saka (1992) also observed a similar effect of FA in rice lamina joints and reported the involvement of feruloylated arabinoxylans in regulation of the IAA induced growth.

h. Interaction with enzymes:

Many of the phenolic compounds are known to modify the levels and activity of enzymes in both *in vitro* and *in vivo.* Interference of FA with activities of a variety of enzymes that are involved in various metabolic processes has been observed in a number of situations. Hoover *et al* (1977)

observed an inhibition in glucose 6-phosphate dehydrogenase activity and isozymes with FA and many other phenolic compounds. Several nitrogen fixing enzymes are inhibited by FA (Dhir *et al* 1992). In majority of the cases, FA is known to modify the IAA levels by regulating the activities of IAA oxidase and peroxidase which are involved in the IAA oxidation and the FA effect found to be concentration dependent (Lee *et al* 1982). An increase in the peroxidase activity has been observed with FA treatment (Shann and Blum 1987b, Van Huystee and Zheng 1993).

1.4.1. Problem and prospects of allelopathic research:

As the impact of allelochemicals on the plant growth are increasing, the need to understand the mechanism of action of these chemicals has become inevitable for the proper management of weeds and crops in agroecosystems, and to develope natural herbicides and pesticides for better yields (Aldrich 1987)

Recently Lovette (1991) critically reviewed the utilization of these allelopathic mechanisms in biological control of weeds. For proper management of weeds and crops, these compounds are to be tested in various combinations to find out the threshold concentrations for their activity (Rippin *et al* 1994). Crop varieties are to be screened or new varieties need to be developed to exploit the potential of allelochemicals for controlling weeds. Similarly, if crop varieties allelopathic to pests are developed, their residues can be used for pest control (Wiseman *et al* 1992).

In agroforestry, agricultural crops are grown together with forest plants to increase the yields (Chou *et al* 1987). However, the major limitation of this system is, the allelochemicals released during the litter decomposition of forest plants that are toxic to crop plants. Therefore, the suitable management systems to select the complimentary tree species and crop plants for better yields in agroforestry is possible only with thorough understanding of allelopathy and mechanism of allelochemical's action.

Synthesis of the bio-pesticides and growth promoters is emerging as an important area in crop improvement. The understanding of the mechanism of action of these allelochemicals is useful which can be exploited in developing natural pesticides that can replace the synthetics (Bernard *et al* 1990, Miles *et al* 1993)

Further the beneficial effects of allelochemicals can be extended to use them as growth promoters as many of them are known to promote growth at very low concentration. Developments in crop management based on allelopathic studies would not only increase production but also reduce the expenditure on farm labour, and reduction in use of synthetic agrochemicals. It is even possible that allelochemical production can be induced by genetic manipulation into cultivars, to provide an inexpensive safe and permanent means of biological pest control.

Objectives of the present work:

Though earlier studies reported the inhibitory nature of FA on various metabolic processes, the possible sites and mechanism of action particularly, the physiological and biochemical aspects through which it exerts deleterious effects on the plants are not critically investigated. Further more, most of these studies were aimed in observing the FA effects on a particular process in a plant. Information is rather scanty on the FA effect on various physiological processes in a single experimental system. It is thus important to understand how FA affects these interactive physiological processes in a sigle experimental system starting from seed germination to seedling development which would provide better understanding of FA effects and physiological basis of allelopathy. Hence, the present work has been carried out to study the FA action on growth of maize seedlings with respect to the following:

1. Percentage of seed germination; length, fresh weight and dry weight of shoots and roots.

2. Activities of hydrolytic enzymes viz., amylase, maltase, invertase, acid phosphatase protease and interaction with gibberellic acid.
3. Activities of oxidative enzymes viz., peroxidase, catalase, 1AA oxidase, and polyphenol oxidase.
4. The activities of key phenylpropanoid enzyme *viz.* phenylalanine ammonialyase and cinnamylalcohol dehydrogenase.
5. Accumulation of hydroxyphenolic compounds and lignin.
6. Photosynthetic functions viz., net CO_2 assimilation rate, stomatal conductance electron transport rates, fluorescence emission, cyclic and non-cyclic photophosphorylation, Mg^{2+} and Ca^{2+} ATPase activities.
7. The uptake of phosphate.
8. Lipid peroxidation and ferricyanide reduction in roots.

PART II

EXPERIMENTAL

EXPERIMENTAL

2.1. Plant material and growth conditions:

a. Plant material:

Maize seeds *{Zea mays* cv.Ganga-5) purchased from the local market were used for the experiments. Seeds were surface sterilized with 5% sodium hypochlorite solution for 15min and washed three times with distilled water. The sterile seeds were soaked in water for 12h and placed on filter paper disks in polypropylene germination boxes (9x9 cm, inner dimensions, 25 seeds per box). The seedlings raised under different growth conditions were used for experiments.

b. Growth conditions

Growth measurement:

For growth analysis experiments seeds germinated in germination boxes were treated with 30ml of 1mM to 5mM (intervals of 1mM) FA (Sigma Chemical Co., St.Louis, MI, USA) for eight days starting from germination. FA was dissolved in a small volume of ethanol (1% v/v) and was made upto the required volume with Hoagland's solution and pH was adjusted to 5.5. Hoagland's solution grown plants were considered as control. The seedlings were grown under continuous light provided by four 60 Watts cool white fluorescent and four 60 Watts incandescent bulbs which yielded 80 $\mu E\ m^{-2}\ s^{-1}$ light. The temperature ranged from 25 to 30° C. Length, fresh and dry weight of shoots and roots of eight days old seedlings was measured.

Hydrolytic enzymes:

Seedlings grown under similar conditions except that grown in dark (to exclude any light and photosynthetic effects on enzymes) were used to measure the activities of hydrolytic enzymes. Growth and hydrolytic enzyme activities

of seeds was also measured in six days old seedlings raised in dark treated with 30ml of 1mM FA, 3mM FA, 0.1mM GA and 1mM and 3mM FA together with 0.1mM GA for six days.

Oxidative and phenylpropanoid enzymes, hydroxy phenolic compounds and lignin:

Seedlings grown for eight days under continuous light (80 $\mu Em^{-2}s^{-1}$) treated with 30ml of 0.5mM to 3mM FA (intervals of 0.5mM) for eight days were used to measure the activities of oxidative and phenylpropanoid enzymes, phenolic compounds and lignin.

Photosynthetic functions:

Seedlings raised under continuous light ($80\mu Em^{-2}s^{-1}$) treated with 30ml of 0.25mM, 0.5mM, 1mM and 3mM FA for 24h, 48h and 72h were used to study photosynthetic functions.

Lipid peroxidation and ferricyanide reduction:

Seedlings of eight days old, grown under uniform light and temperature conditions treated with 30ml of 0.5mM to 3mM FA for 24h, 48h and 72h starting from fifth day were used to study lipid peroxidation and ferricyanide reduction.

Phosphate uptake:

Seedlings raised under continuous light for 4 days in the presence of 0.5mM $CaCl_2$ were used for uptake studies.

2.2. Growth analysis:

Length, fresh and dry weights of shoot and root portions of the seedlings were measured. Dry weight was measured after drying the seedlings in oven at 70° C for 48h.

2.3. Enzymes:

Hydrolytic enzymes:

On the specified days (2, 4, 6 and 8 days and for zero time 12h soaked seeds were used) the shoot and root portions of the seedlings were seperated and seed portion (1 g) was washed with water and used for the preparation of crude enzymes.

Amylase (E.C.3.2.1.1.):

Seed material was homogenized in 5ml of 50mM sodium acetate buffer pH 4.8 containing 20mM calcium chloride at 4° C. The extract was centrifuged at 10,000g for 20min at 4° C and the supernatant was used to determine the enzyme activity. Amylase activity was determined by the modified method of Anonymous (1979). Reaction mixture (1ml) containing 2mg of amylopectin, 25µg of enzyme protein and 50mM acetate buffer with 20mM calcium chloride pH 4.8 was incubated at 37° C for 10min. Reaction was terminated by adding 1ml of Dinitro salicylic acid reagent (Miller 1959) and placed the tubes in boiling water bath for 5min. After cooling the tubes to room temperature the samples were diluted by adding 2ml of double distilled water and absorbance was measured at 540nm using maltose (50-500µg) as standard reducing sugar. One unit of amylase activity was equivalent to the quantity of protein that liberates one µmole of reducing sugar.

Preparation of Dinitrosalicylic acid reagent: 3,5 Dinitro salicylic acid lg was dissolved in 25ml of 4% sodium hydroxide and 30g of sodium potassium tartarate was added. The volume was finally made upto 100ml. The solution was filtered and stored in a brown bottle at 4° C.

Maltase (E.C.3.2.1.20):

Seeds were homogenized in 5ml of 50mM sodium acetate buffer pH 5 and the extract was centrifuged at 10,000g for 20min at 4° C. The supernatant was used to determine the activity of maltase and invertase. Maltase activity was measured by the method of Yamasaki and Suzuki (1980). Reaction mixture

containing 0.1ml of 0.1% maltose, 0.35ml of 50mM acetate buffer pH 5 and enzyme extract containing 50ug of protein in a final volume of 0.5 ml, was incubated at 37° C for 1h and the reaction was stopped by keeping the tubes in a boiling water bath for 5min. The amount of glucose formed was estimated using arsenomolybdate reagent (Nelson 1944). Maltase activity was represented as umoles of glucose equivalents released per mg of protein per hour.

Invertase **(E.C.3.2.1.26):**

Invertase was assayed following the method of Billett *et al* (1977). Reaction mixture (1ml) containing 100mM sucrose, enzyme extract containing 50ug of protein in 100mM sodium acetate buffer pH 5 was incubated at 35° C for lh. Reaction was terminated by keeping the tubes in boiling water bath for 5min and the released glucose was measured using arsenomolybdate reagent (Nelson 1944). Invertase activity was expressed as umoles of glucose equivalents released per mg of protein per hour.

Acid phosphatase **(E.C.3.1.3.2):**

Acid phosphatase was extracted by homogenizing the seeds in 5ml of lOOmM sodium acetate buffer pH 5 containing 20mM magnesium chloride. Extract was centrifuged at 10,000g for 20min at 4° C and the supernatant was used to determine the enzyme activity. Acid phosphatase activity was measured according to Shinshi and Kato (1979). The reaction mixture containing 0.1M acetate buffer pH 5 containing 20mM magnesium chloride, 50ug of protein and 0.5mM p-nitrophenyl phosphate in total volume of lml was incubated for 10 minutes at 30° C. The reaction was terminated by adding 2ml of 1N sodium hydroxide and the absorbance was measured at 400nm. One unit of enzyme activity was defined as the amount which liberates 1umole of p-nitrophenol per minute. The molar extinction coefficient of 18,000 was used in the calculation.

Protease (E.C.3.4.11.2):

Protease was extracted by homogenizing the seeds in 5ml of 100mM sodium acetate buffer containing 10mM mercaptoethanol. The extract was centrifuged at 10,000g for 20min at 4° C and the supernatant was used to determine the enzyme activity. Protease activity was measured according to Mathur *et al* (1988). Reaction mixture containing 0.5ml of 1% casein, 0.1M acetate buffer containing 10mM mercaptoethanol pH 5 and 100µg of enzyme protein in a total volume of 2ml was incubated for 1h at 40° C and the reaction was stopped by adding 2ml of 10% TCA and the precipitated protein was removed by centrifugation. The supernatant was used to estimate the amino acids released during the reaction by the method of Rosen (1957) using ninhydrin. Protease activity was expressed in µmoles of amino acids released per hour per gm fresh weight of tissue using a standard curve for L-leucine.

Assay of **oxidative** enzymes: Activities of oxidative enzymes were measured in leaves and roots of eight day old seedlings.

Peroxidase (E.C.1.11.1.7):

Leaf and root material (1g) was extracted with 5ml of lOOmM sodium phosphate buffer pH 7 and the extract was centrifuged at 10,000g for 20min at 4° C and 400ul of the supernatant was desalted on sephadex G-25 (2cm X 0.5cm). The desalted extract was considered as soluble peroxidase. The pellets were resuspended in Triton X-100 and centrifuged at l,000g for 5min and then washed with water until no activity was detected in the supernatants. Pellets were then washed (x3) with 1ml of 1M NaCl. The washes were pooled and assayed for ionic peroxidase. The NaCl washed pellets were resuspended in 2ml of 0.05M phosphate buffer (pH 7) containing 10mg cellulase and incubated at 25° C overnight. After a 10min spin at l,000g, supernatant was assayed for covalent peroxidases.

Activity of peroxidase was measured according to Shinshi and Noguchi (1975). Reaction mixture contained 50mM sodium phoshate buffer pH 7,

0.2mM guaiacol, and 2mM hydrogen peroxide and required protein in a total volume of 3ml. The absorbance was read at 470nm in Shimadzu UV-160A spectro-photometer, Japan. Enzyme activity was calculated using an extinction coefficient of 25.5 cm^{-2} x $umol^{-1}$ for tetraguaiacol.

Catalase **(E.C.1.11.1.6):**

Leaf and root tissue (1g) was homogenized in 5ml of 100mM sodium phosphate buffer pH 7 and centrifuged at 10,000g for 20min at 4° C. Supernatant was used to measure the activity of catalase according to Abei (1975) in 3ml of reaction mixture containing 10mM H_2O_2, lOOmM phosphate buffer and 25 µg protein. The change in absorbance was measured spectrophotometrically at 240nm. The change of 1 O.D. represents 25 umoles of hydrogen peroxide decomposed per ml.

IAA oxidase:

Leaf and root tissue (lg) was extracted with lOOmM potassium phosphate buffer pH 6 and centrifuged at 10,000g for 20min at 4° C. The supernatant was used to determine the enzyme activity. IAA oxidase activity was measured according to Talwar *et al* (1985). Reaction mixture contained 0.2mM DCP, 0.2mM $MnCl_2$, 20ug of IAA and 50µg of protein in a total volume of 1ml. The mixture was incubated for 15min in dark and the reaction was stopped by addition of 2ml of solkowski's reagent and incubated in dark for 30 min. The colour developed was measured at 530nm. One unit of activity represents 1µg of IAA oxidized/ml in 15 min.

Polyphenol Oxidase **(E.C.1.14.18.1):**

Leaf and root material (1g) was homogenized in 5ml of lOOmM sodium phosphate buffer pH 7 and centrifuged at 10,000g for 20min at 4° C. The supernatant was used to measure the enzyme activity.

Polyphenol oxidase was assayed according to Tremolieres and Bieth (1984). Reaction mixture contained lOOmM sodium phosphate buffer pH 7, 10mM catechol and 10µg of protein in a total volume of 3ml. The change in

absorbance was read spectrophotometrically at 420nm. One unit of activity is the change in absorbance of 0.001 /min. Polyphenol oxidase activity was also measured in the presence of different concentrations of FA (25μM-3.0mM) *in vitro.*

Assay of phenylpropanoid enzymes:

Phenylalanine ammonialyase (E.C.4.3.1.5):

Leaf and root material (1g) was homogenized in 5ml of 50mM borate buffer pH 8.8, and centriftiged at 10,000g for 20min at 4° C. The supernatant was used as the enzyme.

PAL activity was determined according to Southerton and Deverall (1990). The reaction mixture containing 200ug protein of leaf or root enzyme extract was incubated at 4° C with 0.6ml of borate buffer containing 6umoles of L-phenylalanine. A blank with no phenylalanine was also prepared. After 2h, reaction was stopped by the addition of 100ul of 6N HC1. The product cinnamic acid, was extracted by adding 1ml of chloroform and mixed thoroughly using a vortex mixer. After centrifuging at 7,000 rpm for 5min, 0.2ml aliquot was carefully taken from the lower chloroform phase, and the chloroform was allowed to evaporate. The residue was dissolved in lml of borate buffer and the absorbance was read at 270nm. Cinnamic acid 1-10μg/ml was used for the standard curve. Enzyme activity was expressed as ug of cinnamic acid/gm freshweight/h.

Cinnamylalcohol dehydrogenase (E.C. 1.1.1.195):

Leaf and root material (1g) was ground in 5ml of 100mM potassium phosphate buffer pH 7.6 containing 10mM mercaptoethanol. The extract was centriftiged at 10,000g for 20min at 4° C. The supernatant (400ul) was desalted on sephadex G-25 (2cm X 0.5cm) with 500ul extraction buffer. The desalted enzyme extract was used to measure the enzyme activity.

Cinnamylalcohol dehydrogenase activity was measured according to (Wyrambik and Grisebach 1975). Reaction mixture (2.7ml) contained lOOmM

potassium phosphate buffer pH 7.6, 300nmoles of cinnamaldehyde, 300nmoles of NADPH and 50ug of either leaf or root protein. The decrease in absorbance was measured spectrophotometrically at 340nm for 2min. Reaction mixture without cinnamaldehyde was used as blank.

Assay of ATPase:

For the assay of chloroplastic ATPase, chloroplasts were isolated according to Biswal and Mohanty (1978). The homogenation buffer contained 0.4M sucrose, 10mM NaCl and 20mM Tris-HCl buffer pH 8 and filtered through four layers of muslin cloth. Slurry was centrifuged at 100g for 5min to remove debris. The supernatant was centrifuged again at 3,000g for 10min. The pellet was washed thoroughly and resuspended in a small volume of isolation buffer.

Light activated Mg^{2+} ATPase:

Light activated Mg^{2+} ATPase was measured according to Aslop and Moreland (1975). The enzyme activity was determined in 5ml of the reaction mixture which contained in (μmol): Tris-HCl buffer (pH 8) 50; NaCl 50; MgC12 10; sucrose 1800; DTT 30; PMS 0.1 and chloroplasts equivalent to 75ug chlorophyll. The tubes were irradiated as described for photophosphorylation experiments. After 5min of irradiation, 7umol of ATP was added to the reaction tube and incubated in dark for 15min at 25° C. The reaction was arrested by addition of 1ml of 10% TCA. The release of inorganic PO_4 by the action of enzyme was determined spectrophotometrically (Fiske and Subbarow 1925).

Trypsin activated Ca^{2+} ATPase of coupling factor I (CF_1):

The isolation of CF_1 and assay of Ca^{2+} ATPase activity were carried out according to Biswasl and Mohanty (1978). Chloroplats were isolated in STN buffer (pH 7.8). The pellets were washed thoroughly and resuspended in 10mM NaCl and slowly stirred at 4° C for 10min in vortex mixer. The chloroplast suspension was centrifuged for 20min at 20,000g and resuspended in a small

volume of 10mM NaCl. The suspension was diluted with 0.75mM EDTA (pH 7.8) to approximately 100µg chlorophyll per ml, shaken for 5min at room temperature and centrifuged at 20,000g for 30min at 20° C. The supernatant containing CF_I was used for estimation of EDTA solubilized protein (Lowry *et al* 1951).

Trypsin activation:

The supernatant (0.5ml) containing CF_I was mixed with 0.5ml of trypsin solution containing 50ug of trypsin, 25umol of tricine-NaOH buffer (pH 8), 2.5umol EDTA and 0.5umol ATP. After 5min of incubation the digestion was stopped by the addition of 0.5ml of trypsin inhibitor solution containing 300ug of trypsin inhibitor. Activated enzyme extract was used to determine Ca^{2+} ATPase activity following the method of Biswal and Mohanty (1978). Reaction mixture (4ml) contained 20mM Tris-HCl buffer (pH 8), chloroplasts equivalent to 50ug chlorophyll and 350uM ATP and 3mM Ca^{2+}. The whole suspension was incubated in dark for 15min, at 37° C in a water bath. The reaction was terminated by addition of 1ml of 30% TCA and the amount of inorganic phosphate released was measured according to Fiske and Subbarow (1925).

2.4. Non-denaturing polyacrylamide gel electrophoresis:

Non-denaturing polyacrylamide gel electrophoresis was performed in 1mm thick slab gels using the electrophoretic buffer system of Davis (1964). The running gel was prepared with 7.5% (w/v) acrylamide, 0.37M Tris-HCl, pH 8.9, 0.07% (w/v) ammonium persulphate and 0.05% (v/v) TEMED. The running gel was overlaid with stacking gel consisting 2.5% (w/v) acrylamide, 61.25mM Tris-HCl pH 6.7, 0.07% (w/v) ammonium per sulphate and 0.05% (v/v) TEMED. The electrode buffer consists of 25mM Tris and 192mM glycine, pH 8.3.

Isozymes of amylase:

Amylase isozymes were seperated in 7.5% non-denaturing polyacrylamide gel electrophoresis in alkaline buffer system at 4° C for 12h.

After electro-phoresis, the gel was stained for amylase according to Segundo *et al* (1990) by incubating the gel in 2% starch in 50mM sodium acetate buffer pH 4.8 for 1h and washed thoroughly for 2 times with double distilled water to remove excess starch. Later the gel was developed with staining solution containing 6mM I_2, 50mM KI and 0.2M HC1 until white bands appear on a dark blue back ground. The gel was washed with water and photographed immediately.

Isozymes of acid phosphatase:

Acid phosphatase isozymes were seperated on non-denaturing 5% polyacrylamide gel and the electrophoresis was carried out at 4° C for 12h. Acid phosphatase isozymes were detected following the method of Wetter and Dyck (1983). Staining was carried out by incubating the gel in 0.2M sodium acetate buffer containing 20mM $MgCl_2$ for 30min and then transferred to staining solution containing 4mg/ml α-naphthyl phosphate disodium salt, 4mg/ml Fast blue RR salt with constant shaking until brown bands appear in the gel. The gel was washed thoroughly with water and stored in 7% acetic acid.

Isozymes of protease:

Acid protease isozymes were seperated on 7.5% native polyacrylamide gel containing 0.1% gelatin and the electrophoresis was carried out at 4° C for 12h. In each lane 200µg of protein was loaded. After electrophoresis, the gel was incubated in 50mM sodium acetate buffer containing 10mM mercaptoethanol for 14h. Following incubation, the gel was stained with 0.1% w/v solution of amido black in methanol: acetic:water (30:10:60) for 30min and then destained in ethanol:acetic acid:water (10:10:80) according to Hay *et al* (1991). The active proteases appear as clear white bands against uniformly stained blue background.

Isozymes of **IAA** oxidase:

The isozymes of IAA oxidase were seperated by 5% native polyacrylamide gel in alkaline buffer system at 4° C for 12h. In each lane 200µg protein was loaded. The IAA oxidase isozymes were developed following the method of Talwar *et al* (1985). After electrophoresis the gel was immersed in a staining solution containing 1 part of solution A (4mg Fast Blue BB/ml ethanol), 2 parts of solution B (16.4mg p-coumaric acid+17.6mg IAA dissolved in alcohol to a total volume of 50ml+2.4ml 0.1% hydrogen peroxide) and 1 part of solution C (2M acetate buffer pH 4.2) until brown bands of IAA oxidase appear. The gel was washed with water and stored in 7% acetic acid.

2.5. Measurement of photosynthetic rates:

Net carbon dioxide assimilation rates of primary leaves of eight day old seedlings were measured using an open system Infrared gas analyser LCA 2 interfaced to data logger (DL-2), Air supply unit (Mass flow) (ASMU) and plant leaf chamber (PLC-N) (Analytical Developmental Co., Hoddesdon, UK). Fully expanded leaf was placed in the leaf chamber and the net CO_2 assimilation rate and stomatal conductance were recorded.

Isolation of thylakoids:

Thylakoids were isolated from fresh primary leaves of 8 day old seedlings according to Atal *et al* (1991). Leaves were homogenized in prechilled mortar and pestle in ice cold isolation medium containing 0.4M sucrose, 20mM tricine (pH 7.8), 5mM $MgCl_2$, 10mM NaCl, 1mM sodium ascorbate and 0.1% BSA. The slurry was filtered through 4 layers of muslin cloth and the thylakoids were sedimented at 5,000g for 10min at 4° C in Beckman J2-21 M/E centrifuge (USA). The pellets were washed and resuspended in a small volume of isolation buffer. Chlorophyll was estimated according to Arnon (1949).

2.6. Photosynthetic electron transport:

Whole chain electron transport (water —> MV):

The reaction mixture contained 20mM tricine (pH 7.8), 0.4M sucrose, 10mM NaCl, 5mM $MgCl_2$, 1mM sodium azide and 0.5mM MV and thylakoids equivalent to 20ug chlorophyll was used in the assay and the samples were illuminated as described for PS I activity measurement.

PS I **activity:**

Elelctron transport activities were performed according to Atal *et al* (1991). Light mediated uptake of oxygen (PS I) was measured polarographically with Gilson 5/6 Oxygraph (USA). The assay mixture contained 20mM tricine, 10mM NaCl, 5mM $MgCl_2$, lmM sodium ascorbate, lmM sodium azide, 0.5mM MV and 5uM DCMU and chlorophyll equivalent to 20ug was used in a final volume of 1.8ml and illuminated at 1600 uE m^{-2} s^{-1} light provided by 4x150 V comptalex bulbs and temperature was maintained at 25° C using circulatory waterbath.

PS II activity:

PS **II** activity was measured spectrophotometrically by measuring the photoreduction of DCPIP. The reaction mixture (3ml) for the assay of hill reaction consists of 50mM HEPES (pH 7.8), 5mM $MgCl_2$, 10mM NaCl, 5mM NH_4Cl and 25uM DCPIP. Thylakoids equivalent to 5ug chlorophyll were used in the assay. Saturating actinic light was filtered through a red glass filter (Corning 2-58) and the detector was protected with a blue filter (Corning 4-96). PS II activity was measured as DCPIP reduction at 600nm (E = 18.9 mM^{-1} cm^{-1}). PS II activity was also measured in the presence of 10mM NH2OH.

***In vitro* effect of FA on PS I, PS II and whole chain electron transport rates:**

The activities of PS I, PS II and whole chain were also measured using thylakoids isolated from control plants and incubated with various

concentrations of FA for different time periods (10min, 20min, 30min, 40min, 50min and 60min).

2.7. **Fluoscence measurements (Atal *et al* 1991):**

Fluorescence emission spectra for chloroplasts were recorded at room temperature and low temperature (77° K) using Hitachi fluorescence spectrophotometer (model 4010), Japan. The spectra were recorded at 490nm excitation wave length, using 5nm as extinction bandpass, 5nm emission bandpass and instrument response 2. Fluorescence emission at room temperature was recorded by suspending chloroplasts in assay buffer containing 20mM HEPES (pH 7.5), 100mM sucrose, 2mM $MgCl_2$, 10mM NaCl and 10 µg of chlorophyll. Fluorescence at 77° K was recorded using liquid nitrogen attachment and circulating nitrogen gas through the inlet and out let of the low temperature attachment of the Hitachi fluorescence spectrophotometer. The instrument parameters were similar to the parameters used for recording room temperature fluorescence emission spectra. The suspension buffer used to measure emisssion at low temperature was similar to that of room temperature emission recording except that the suspension buffer contained 50% glycerol.

2.8. Photophosphorylation

Cyclic photophosphorylation catalyzed by PMS was monitored according to Heber *et al* (1973). Assay mixture consisted of 0.1ml of 50mM $MgCl_2$, 0.1ml of lOmM sodium phosphate, 0.1ml of 20mM ADP (adjusted to pH 7) and 0.1ml of 0.5mM PMS and 30ug of chlorophyll. Samples were illuminated for 10min at an irradiance of 1800 $\mu E\ m^{-1}\ s^{-1}$ light and at 20° C temperature. Reaction was stopped with 0.1ml of 50% TCA.

Non-cyclic photophosphorylation was also determined according to Heber *et al* (1973) in a similar assay buffer as that of cyclic phosphorylation except that PMS was replaced by 0.1ml of 5mM potassium ferricyanide. Inorganic phosphate was estimated by the modified method of Fiske and Subbarow(1925).

2.9. Protein estimation (Lowry *et al* 1951):

To each 0.2ml of protein sample, 1ml of alkaline copper sulfate solution (23ml of reagent A [4% sodium carbonate in 0.2M NaOH] mixed with lml of reagent B [1% $CuSO_4.5H_2O$ and lml of 2% sodium-potassium tartarate]), was added and the mixture was allowed to stand for 10min. Then 0.2ml of Folin's reagent (commercial preparation diluted with water in a ratio of 1:1) was added and mixed immediately. After 30min of incubation at 25° C, the absorbance was read at 750nm. A standard curve was prepared with BSA ($5\text{-}50 \mu g\ ml^{-1}$)

2.10. Inorganic phosphate estimation:

Inorganic phosphate was measured by the modified method of Fiske and Subbarow (1925). The reaction mixture (0.1ml) was made upto lml with deionized water. To the above solution, lml of 5N H2SO4, lml 2.5% ammonium molybdate and 0.1ml of freshly prepared 25mg/ml reducing reagent (1.28g sodium sulfite anhydrous, 1.28g sodium metasulfite, 0.2g ANSA) were added. The contents were mixed and allowed to stand at room temperature for 20min and the absorbance was read at 660nm.

2.11. Chlorophyll estimation:

Chlorophyll of the leaves was estimated according to Arnon (1949) by extracting the leaf discs with 10ml of 80% acetone and centrifuged at 5,000rpm for 5min. The supernatant was read at 664nm and 647nm. The total chlorophyll,

chlorophyll a and chlorophyll b were calculated as follows

Total chlorophyll = 7.93 X A664 + 19.53 X A647

Chlorophyll a = 13.1 XA664 + 2.57 XA647

Chlorophyll b = 22.1 X A647 + 5.26 **X A664**

2.12. Hydroxy phenolic compounds estimation:

Total hydroxy phenolics were extracted from leaves and roots of eight days old FA treated maize seedlings with 75% alcohol at 4° C. **The**

homogenate was centrifuged at 20,000g for 20min. An aliquot of the supernatant was used to estimate the phenolic compounds.

Total hydroxy phenolics was estimated colorimetrically following the method of Seigler *et al* (1986). An aliquot of the extract (0.2ml) was made upto 2ml with water, 2ml of Folin-Denis reagent (prepared by mixing sodium tungstate [41.24g], phosphomolybdic acid [8.25g] 85% phosphoric acid [20ml] and water [300ml]. The mixture was refluxed for 2h and diluted to 1l) was added and allowed to stand for 3min. Then 2ml of sodium carbonate solution (prepared by dissolving 106g of sodium carbonate in 1000ml of water) was added. After colour development (2h), the absorbance was measured at 725nm. Phenolic content was determined using tannic acid standard curve. Phenolic concentration was expressed as mg of phenolics per gm fresh weight.

2.13. Lignin estimation:

Leaves and roots of eight day old seedlings were used to extract lignin according to (Kevers and Gaspan 1985, Shann and Blum 1987b). Samples were freeze dried and ground to pass through a 60um mesh sieve. Samples were pre-extracted with water 100° C for 30min, 3% SDS (120° C) 30min (x2), 85% ethanol (75° C) for 30min, and acetone (56° C) for 30min. After the final extraction the material was filtered and the residue was dried for overnight in an oven at 30° C. The residue (10mg) was digested with 2.5ml acetyl bromide for 30min at 70° C dry heat in 50ml volumetric flask. After cooling on ice, 15ml of 2N NaOH and glacial acetic acid mixture (1:5) was added to the digest. Before bringing the mixture to 50ml with acetic acid, 0.25ml of 7.5M hydroxylamine hydrochloride was added to each flask and the absorbance at 280nm was measured after 2h. The amoumt of lignin was expressed as percentage of final dry weight following the formula with a coefficient of lignin standard =24

OD at 280nm/20 x 100

e x final dry weight (g)

2.14. Lipid peroxidation:

Lipid peroxidation was measured according to Heath and Packer (1968). Root samples of the seedlings were washed in distilled water (x3) and cut into 0.5mm segments. Root material (0.1g) in 4ml of 0.25% TBA in 10% TCA was incubated at 95° C for 30min. The samples were cooled to room temperature and the solution was decanted and centrifuged at 3,000rpm for 15min. The absorbance of the clear supernatant was measured at 532nm and also at 600nm. The non specific absorbance at 600nm was subtracted from O.D at 532nm. The mM extinction coefficient of TBA 155 was used in the calculation to measure the concentration of malanoldehyde formed during the reaction.

2.15. Ferricyanide reduction:

Ferricyanide reduction was measured following the method of Federico and Giartosio (1983). Roots of the seedlings were cut into 1cm segments and 100mg of root material was incubated in 1ml of the incubation mixture containing 1mM Tris-HCl pH 7.2, 0.5mM $CaCl_2$, 50mM KCl and 0.8mM potassium ferricyanide for 30min. The solution was decanted and the absorbance of the solution was measured at 420nm. The mM extinction coefficient of ferricyanide ($e = 1.0mM^{-1}\ cm^{-1}$) was used to measure the amount of ferricyanide reduced. For zero time measurements the absorbance of the solution was measured immediately after placing the roots in incubation medium which was subtracted from the values after 30min incubation.

2.16. Phosphate uptake:

The terminal 4cm of the primary and two seminal roots of 4days old seedlings were excised and cut into lcm segments. Segments were stored on moist filter paper over ice until used. Root material (300mg) was considered as one experimental unit. Uptake of phosphate was measured using radioisotope

$KH_2 32PO_4$ (BARC, India, specific activity 10mCi/mmole) according to Mc Clure *et al* (1978) with some modifications.

Measurement of radio activity:

Each unit root material was ground in 1ml of double distilled water and the whole slurry was carefully added to 10ml of Bray's scintillation fluid and the radioactivity was measured in Beckman liquid scintillation spectrometer (USA).

Composition of Bray's Scintillation fluid:

Naphthalene	60g
PPO	4g
POPOP	200mg
Methanol	100ml
Ethylene Glycol	20ml

The solution was made upto 1L with l-4,dioxane.

Time course of phosphate uptake:

Each experimental unit of root material (300mg) was preincubated in 0.5mM $CaCl_2$ pH 5.5 for 4h. Each unit was blotted, weighed and placed in 50ml of incubation solution containing 100umoles of KH_2PO_4 containing 1uCi of phosphate, 0.5mM $CaCl_2$ and pH adjusted to 5.5. The samples were incubated for different time periods with continuous oxygen bubbling. The samples were incubated up to 3h and the phosphate uptake was measured at different intervals of time. Following incubation, roots were removed and placed in desorption solution containing 200uM KH_2PO_4 and 0.5mM $CaCl_2$ (pH 5.5) for 15min on ice. The segments were again filtered and the radioactivty was measured immediately.

Uptake of phosphate at different phosphate concentrations:

Each experimental unit after preincubation in 0.5mM $CaCl_2$ pH 5.5 for 4h, was placed in 50ml of absorption solution containing different concentrations of phosphate (2µM, 5uM, 10µM, 20uM, 50uM, 100µM,

200µM, 400µM, 600uM, 1000µM, 1500µM, and 2000µM), 0.5mM $CaCl_2$ pH 5.5 and 1uCi of labelled phosphate. The samples were incubated for 2h under continuous oxygen bubbling.

After absorption, the samples were transferred to desorption solution containing unlabelled phosphate and 0.5mM $CaCl_2$ and incubated on ice for 15min. The samples were filtered and the radioactivity was measured in the roots as described earlier.

Influence of pH on Phosphate uptake:

Phosphate absorption was measured similar to earlier experiments containing 300mg of experimental unit each placed in 50ml of absorption solution containing 200uM KH_2PO_4 and 1uCi of radioactive phosphate, 0.5mM $CaCl_2$, at four different pH's i.e. 4.5, 5.5, 6.5 and 7.5. The uptake was also measured in the presence of 0.25mM, 0.5mM, 1.0mM and 3mM FA at these four pH values. The roots were incubated for 2h and then transferred to non labelled desorption solution containing 200uM KH_2PO_4, 0.5mM $CaCl_2$ and were kept on ice for 15min. The solution was filtered and the radioactivity in the roots was measured as described earlier.

Uptake of phosphate by excised roots at different **phosphate concentrations:**

Root material (300mg) after preincubation in 0.5mM $CaCl_2$ for 4h was transferred to 50ml of absorption solution containing different concentrations of KH_2PO_4 (5uM, 20uM, 200uM, 800uM and 2mM), different concentrations of FA (0.25mM, 0.5mM, 1mM and 3mM) and 1uCi of radioactive phosphate. The samples were incubated for 2h under continuous oxygen bubbling through out the uptake period. After 2h, the roots were transferred to desorption solution containing non labelled 200uM KH_2PO_4, 0.5mM $CaCl_2$ pH 5.5 and incubated on ice for 15min. After desorption, the radioactivity in the roots was measured as described earlier.

Uptake **of** phosphate by **intact** roots **at different phosphate concentrations:**

Single seedling was preincubated in 0.5mM $CaCl_2$ for 4h was transferred to 20ml of absorption solution in such a way that only root portion was immersed in solution. The absorption solution contained 5uM, 20µM, 200µM, 800µM and 2mM KH_2PO_4 and 1µCi of radioactive phosphate, 0.5mM $CaCl_2$ and different concentrations of FA (0.25mM, 0.5mM, 1mM and 3mM). The samples were incubated for 2h under continuous oxygen bubbling. After 2h, entire root portion was cut and transferred to desorption solution containing 200µM KH_2PO_4, 0.5mM $CaCl_2$ pH 5.5 and allowed to stand for 15min on ice. The roots were blotted and weighed and the radioactivity was measured as described earlier.

Recovery of absorption capacity:

The tissue was tested for its capacity to recover from the effects of 1mM FA on phosphate absorption. Root material 300mg was incubated for 1h in absorption solution containing 200µM KH_2PO_4, 0.5mM $CaCl_2$ pH 5.5 with 1uCi of radioactive phosphate and incubated for different time periods upto 60min The roots were then transferred to absorption solution containing 200µM KH_2PO_4, 0.5mM $CaCl_2$, 1mM FA, and 1uCi of radioactive phosphate pH 5.5. The samples were incubated for 1h. Following treatment, the solution was filtered and the roots were washed with desorption solution containing no radiotracer for 10min and transferred to absorption solution containing no FA for lh at pH 5.5. The radioactivity was measured at appropriate time intervals as described earlier in absorption measurements.

Effect of FA and TEA^{2+} on phosphate uptake:

Root material (300mg) was incubated in absorption solution containing 200uM KH_2PO_4, 0.5mM $CaCl_2$, and 1uCi of $KH_2{}^{32}PO_4$, 10mM TEA^{2+} alone or in the presence of FA and tested for phosphate uptake at different intervals of time by measuring the radioactivity of the samples as described earlier.

2.17. Chemicals:

Amylopectin, maltose, ferulic acid, casein, gibberellic acid, gelatin, guaiacol, cellulase, benzidine, indole acetic acid, p-coumaric acid, catechol, L-phenylalanine, cinnamic acid, sodium azide, dichlorodimethyl urea (DCMU), Tricine, HEPES, dithiothreitol, trypsin, trypsin inhibitor, leucine and thiobarbituric acid were purchased from Sigma Chemical Co., St. Louis, U.S.A.

Ninhydrin, a-naphthyl phosphate disodium salt, fast blue BB and RR, fast garnet GBC, amido black, Triton X-100, cinnamaldehyde, sodium ascorbate and methyl viologen were purchased from Fluka Chemie AG, (Switzerland).

NADPH, PMS, ATP, ADP, PPO, POPOP were obtained from Spectro-chem chemical Co, India. All other chemicals were of analytical grade purchased from local commercial establishments.

PART III

RESULTS AND DISCUSSION

CHAPTER I

Influence of ferulic acid on growth and hydrolytic enzymes of maize

Introduction

In nature a number of biotic and abiotic factors are known to influence the germination and growth of the seedlings. In addition to these, a variety of chemical compounds which occur either endogenously within the seed (Chow and Lin 1991, Sathiyamoorthy 1990) or present in the soil are known to interfere with the growth of the seedlings.

Phenolic compounds constitute a larger portion of germination inhibitors and are known to modify growth and development of plants (Bansal *et al* 1992, Chow and Lin 1991, Glass 1975b, Kefeli and Kutacek 1977, Khan and Ungar 1986, Kumar and Tayal 1982, Rasmussen and Einhellig 1979, Tayal and Sharma 1985, Vansumere *et al* 1972, Williams and Hoagland 1982). However, phenolic compounds like caffeic and chlorogenic acid at low concentrations (< 0.1 mM) are reported to increase germination (Tomaszewski 1964).

During the course of their action, many of the phenolic compounds are known to interact with endogenous GA, IAA and ABA either synergistically and / or antagonistically thus accordingly modify the growth (Corcoran *et al* 1972, Kakkar and Rai 1986, Kathiresan *et al* 1990, Khan and Ungar 1986, Nanda *et al* 1977, Nutbeam and Briggs 1982, Pawar and Laloraya 1988, Perez and Nepomuceno 1991, Ray 1986, Tayal and Sharma 1981, 1985, Tomaszewski and Thimann 1966).

Seed germination represents an important phase in life cycle of the plants. Early phase of the seedling growth requires the mobilization of reserve food material (Bewely and Black 1985). Hydrolytic enzymes perform a crucial role in the mobilization of reserve food material which supply the nutrients to the developing embryo and other metabolic processes associated with early seedling development (Bewely and Black 1985, Karuppaiah *et al* 1989, Mayer

and Poljaklof-Mayber 1982). In mature seeds, the degradation of starch to glucose via amylolytic pathway invoves amylase, maltase and invertasc (Nomura *et al* 1969). Phosphatases regulate the breakdown of reserve phosphates and protein reserves are acted by proteases (Mayer and **Poljakolf**-Mayber 1982). It is well established earlier that GA is the key regulator in the synthesis of these enzymes. GA produced in the embryo trigger the *de novo* synthesis of hydrolytic enzymes (Bailey *et al* 1976, Callis and Ho 1983, Jacobsen and Varner 1967, Panbieres *et al* 1989, Segundo *et al* 1990).

The wide spread occurrence of phenolic compounds and their antagonistic nature towards GA (Jacobsen and Corcoran 1977) further suggest the role **of** phenolic compounds in the regulation of GA induced processes.

In the present work, FA effect on growth and hydrolytic enzymes has been studied in detail and examined the possibility of FA interaction with GA in regulating the growth and hydrolytic enzyme activities.

Results

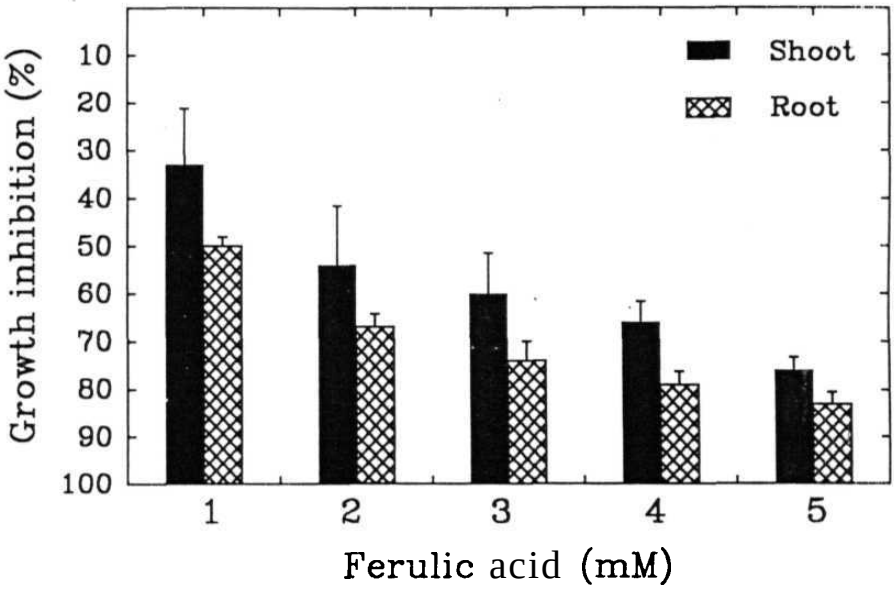
Germination:

The germination bioassay indicated an inability of FA (1mM and 2mM) to alter the seed germination. However, 3mM-5mM FA reduced the germination by 30%, 35% and 60% respectively.

Analysis of growth:

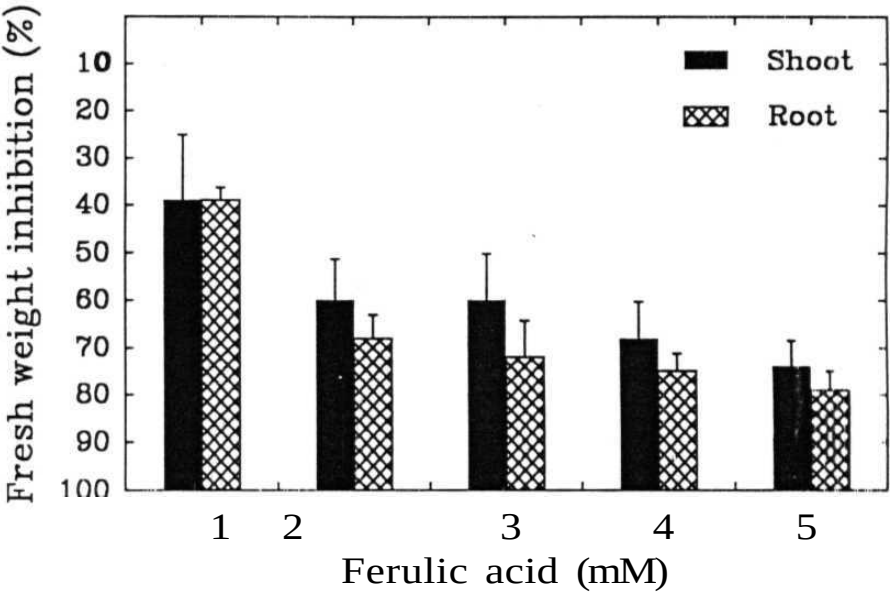
Application of FA (lmM to 5mM) to germinating seedlings showed a concentration dependent decrease in the shoot length by 33% to 76% and 50% to 83% reduction in root length (Fig. 1.1). Similar decrease in shoot (39%-74%) and root (39%-79%) fresh weight was observed with lmM-5mM FA (Fig. 1.2). Correspondingly, a decrease in the dry weight of shoots (30%-61%) and roots (32%- 81%) (Fig. 1.3) was observed with lmM-5mM FA. The three growth parameters studied (length, fresh and dry weights) exhibited a **maximum** decrease upto 3mM FA and with further rise in FA concentration (4mM and 5mM) showed a small increase in the percentage of decrease.

Fig. 1.1



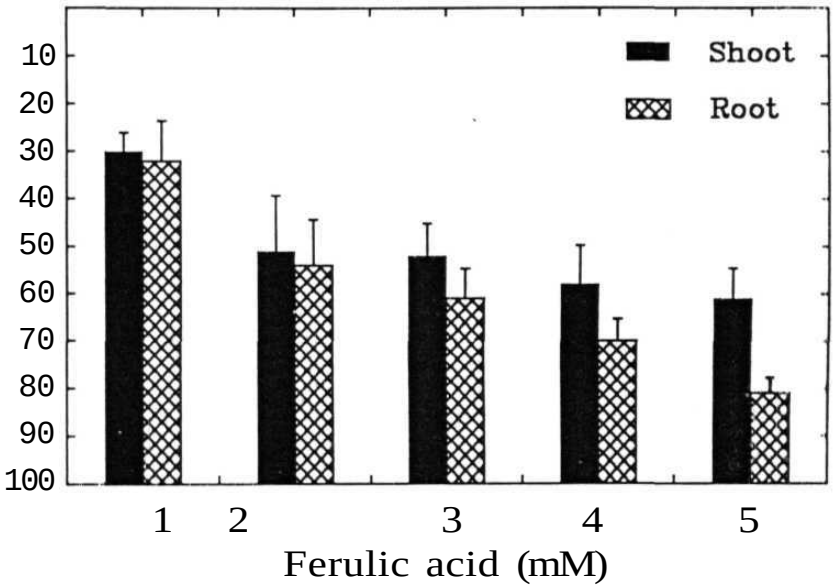
Effect of ferulic acid on the shoot and root **length of eight** days old maize seedlings (n- 25).

Fig. 1.2



Effect of ferulic acid on the fresh weight of shoots and roots of eight days old maize seedlings (n= 25).

Fig. 1.3



Effect of ferulic acid on the dry weight of shoots and roots of eight days **old maize** seedlings (n= 25).

Hydrolytic enzymes:

Amylase: In control seeds amylase activity increased gradually and reached maximum on sixth day of germination. Initially (2 days), application of FA has resulted in an increase of 7%-20% and as the germination progressed, significant reduction in the amylase activity was observed. Reduction upto 9%-21%, 29%-49% and 18%-44% was noticed in 4, 6, and 8 days old germinating seeds respectively (Table 1.1).

Maltase: No remarkable change in maltase activity was observed with FA during early growth (2 days) except with a small rise i.e. 6%-7% with 2mM and 3mM FA while rise in FA concentration (3mM and 4mM) increased activity by 16%-33% on fourth day of germination. Contrary, 5mM FA inhibited the activity significantly (58%) in four days old seedlings. As the germination progressed i.e. 6 and 8 days, a considerable decrease in the activity i.e. 15%-52% and 35%-54% was observed with 1mM to 3mM FA (Table 1.2).

Invertase: Invertase activity exhibited a differential response to FA concentration. An increase in the activity (18%) was noticed with lmM-2mM FA in 2 day old germinating seeds while 5mM FA decreased the activity by 33%. A rise in the activity upto 28% and 15% was observed in four day old germinating seeds with 2mM and 3mM FA. However, 4mM and 5mM FA treated seeds didnot show any significant change in activity compared to control. With progress of germination (6 days) 15%-41% decrease in activity was observed with 1mM-5mM FA compared to control. Further reduction in the activity upto 18%-46% was observed with FA treatment by eigth day (Table **1.3).**

Acid phosphatase: Application of FA to germinating seeds resulted in an increase (4%-15%) in the activity of acid phosphatase intially (2 days) with 1mM and 2mM FA, while a decrease in activity (10%-27%) was observed with 3mM-5mM. In four days old seedlings, except lmM FA, all the other concentrations of FA (2mM-5mM) inhibited **the** activity by 13%-30% depending on concentration. As the germination progressed (6 and 8 days) a

Table 1.1

Effect of ferulic acid on the amylase activity in maize seeds

Ferulic acid (mM)	μ moles of maltose/mg protein/min			
	2 days	4 days	6 days	8 days
Control	1.54+0.03	2.02+0.04	2.68±0.01	1.65+0.06
1.0	1.86+0.05	1.72+0.02	1.89+0.01	1.23+0.03
	(+20.77)	(-14.85)	(-29.47)**	(-25.45)
2.0	1.47+0.04	1.80+0.02	1.57+0.01	1.23+0.01
	(-4.57)	(-10.89)	**(-41.41)***	(-25.45)
3.0	1.97+0.06	1.58+0.01	1.43+0.03	1.35+0.03
	(+27.92)	(-21.78)	**(-46.64)***	(-18.18)
4.0	1.73+0.04	1.82+0.03	1.45+0.05	0.92+0.02
	(+12.33)	(-9.90)	(-45.89)*	(-44.24)*
5.0	1.66+0.04	1.71+0.02	1.36+0.02	1.05+0.01
	(+7.79)	(-15.34)	(-49.25)*	**(-36.36)***

Figures in parentheses are % of increase (+) or decrease (-) over control.
•P<0.001 **P<0.005

Table 1.2

Effect of ferulic acid on the maltase activity in maize seeds

Ferulic acid (mM)	μ moles of glucose/mg protein/h			
	2 days	4 days	6 days	8 days
Control	9.72+0.35	5.49+0.39	7.53+0.23	5.56+0.30
1.0	9.67+0.70	6.77+0.18	4.96+0.48	3.53+0.38
	(-0.51)	(+23.31)	(-34.13)**	(-36.15)*
2.0	10.46+0.74	5.42+0.16	3.75+0.36	3.61+0.17
	(+7.61)	(-1.27)	(-50.19)*	(-35.07)*
3.0	10.34+0.29	7.32+0.16	6.37+0.25	3.24+0.28
	(+6.37)	(+33.33)**	(-15.40)	(-41.72)*
4.0	8.70+0.20	6.39+0.33	3.60+0.31	**3.24+0.11**
	(-10.49)	(+16.39)	(-52.19)*	**(-41.72)***
5.0	8.53+0.25	**8.71+0.11**	3.57±0.20	2.52+0.22
	(-12.24)	(-58.65)*	(-52.58)*	(-54.67)*

Figures in parentheses are % of increase (+) or decrease (-) over control.

* $P < 0.001$ ** $P < 0.005$

Table 1.3

Effect of ferulic acid on the invertase activity in maize seeds

Ferulic acid (mM)	μ moles of glucose/mg protein/h			
	2 days	4 days	6 days	8 days
Control	5.34+0.30	5.67+0.31	5.53+0.16	4.56+0.19
1.0	6.32+0.19	5.59+0.15	4.65±0.26	2.83+0.13
	(+18.35)	(-1.41)	(-15.91)	(-37.93)*
2.0	6.33+0.29	7.28+0.32	4.66+0.23	2.43±0.17
	(+18.53)	**(+28.39)****	(-15.73)	**(-46.71)***
3.0	5.33±0.31	6.55+0.27	4.29+0.28	3.70±0.22
	(-0.18)	(+15.52)	(-22.42)	(-18.85)
4.0	5.41+0.27	5.33+0.30	4.62+0.39	3.23+0.19
	(+1.31)	(-5.99)	(-16.45)	**(-29.16)****
5.0	3.56+0.40	5.74+0.16	3.22+0.15	2.55+0.30
	(-33.33)**	(-1.23)	**(-41.77)***	(-44.07)*

Figures in parentheses are % of increase (+) or decrease (-) over control.
*P<0.001 **P<0.005

Table 1.4

Effect of ferulic acid on the acid phosphatase activity in maize seeds.

Ferulic acid (mM)	μ moles of p-nitrophenol/mg protein/min			
	2 days	4 days	6 days	8 days
Control	57.78+2.4	67.99+4.5	85.21+3.0	74.84+11.8
1.0	60.30+5.9	68.89+7.6	70.85+4.8	57.81±5.6
	(+4.36)	(+1.32)	(-16.85)	(-22.75)
2.0	66.46+8.7	58.86+8.3	65.71+5.9	58.35+2.5
	(+15.02)	(-13.42)	(-22.88)	(-22.03)
3.0	51.59+6.7	54.77+2.0	55.59+7.4	46.60+5.8
	(-10.71)	(-19.44)	(-34.76)*	(-37.73)*
4.0	42.08+5.1	52.81+3.5	55.84+1.0	39.91+1.8
	(-27.17)**	(-22.32)	(-34.46)*	(-46.67)*
5.0	57.10+4.3	46.92+4.9	47.62+4.7	38.97+2.4
	(-1.17)	(-30.97)*	(-44.11)*	(-47.92)*

Figures in parentheses are % of increase (+) or decrease (-) over control.
* $P < 0.001$ **$P < 0.005$

significant decrease in the activity (16%-44%) in six days old and 22%-47% in eight days old seedlings (Table 1.4) was observed.

Protease: Similar to the other enzymes studied, the activity of protease exhibited 9%-24% stimulation with lmM-3mM FA while 5mM FA increased the activity by 35% in 2day old seedlings. However, the activity was reduced by 7% with 4mM FA. FA significantly inhibited the protease activity as germination progressed. Reduction upto 18%-57%, 40%-70% and 38%-63% was observed with lmM-5mM FA after 4, 6, and 8 days respectively (Table 1.5).

Having observed the FA influence on the growth and hydrolytic enzymes, further experiments were carried out to see whether FA had exerted its action by interacting with endogenous GA. Additional analysis of growth and hydrolytic enzyme activities were measured in maize treated with FA and / GA.

Application of GA to maize seedlings (0.1mM, maximum effect was noticed at this concentration) increased the shoot length by 29% while, 1mM and 3mM FA inhibited the length by 26% and 59% respectively compared to control. However, the application of GA (O.lmM) along with 1mM FA increased the shoot length by 11% compared to control (completely restored the 1mM FA induced inhibition) while GA could not alleviate the 3mM FA induced inhibition though a small decrease in the percentage of inhibition due to 3mM FA was observed (59% inhibition with 3mM FA alone and only 44% inhibition with GA + 3mM FA) (Fig. 1.4).

Unlike shoot length, application of GA had reduced the root growth by 11% and 1mM and 3mM FA further reduced the root growth to 33% and 90% respectively compared to control. Application of GA (O.lmM) to seedlings simultaneously with FA, did not alleviated the FA caused reduction **in root** growth except that a small reduction (10%) in the percentage of inhibition of 3mM FA by GA was observed (Fig. 1.4).

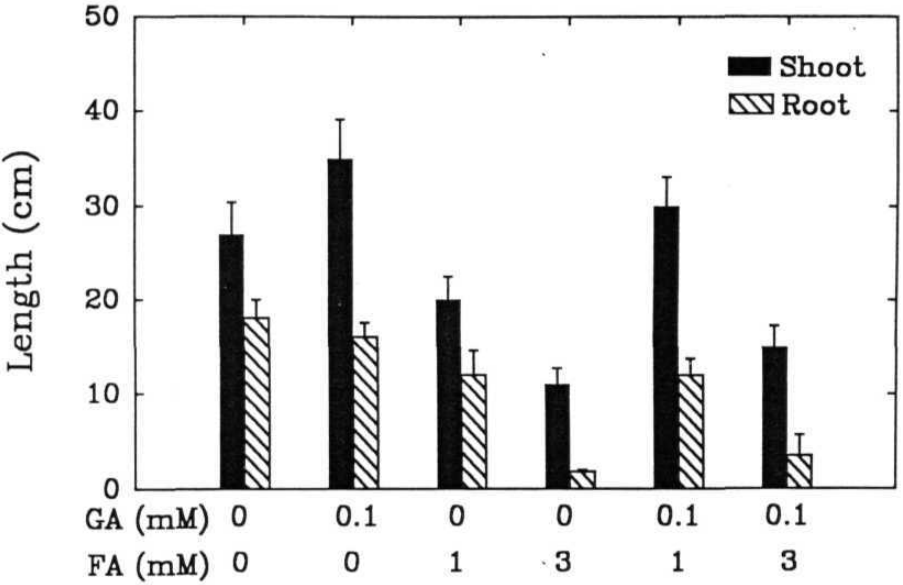
Table 1.5

Effect of ferulic acid on the protease activity in maize seeds

Ferulic acid (mM)	μ moles of amino acids/g f.wt./h			
	2 days	4 days	6 days	8 days
Control	7.19+0.11	11.96+0.25	15.58±0.21	9.74+0.88
1.0	7.22+0.29	7.74+0.18	9.31+0.12	6.01+0.45
	(+0.38)	(-35.28)*	(-40.24)*	**(-38.29)***
2.0	8.96+0.13	7.70+0.29	8.69+0.77	5.77+0.13
	(+24.58)	(-35.61)*	**(-45.22)***	**(-40.75)***
3.0	7.89±0.27	5.60+0.13	5.49+0.1	4.72±0.16
	(+9.70)	(-53.17)*	(-64.76)*	(-51.54)*
4.0	6.68+0.10	9.69+0.91	4.64+0.10	3.54+0.70
	(-7.11)	(-18.97)	(-70.21)*	**(-63.65)***
5.0	9.72+0.38	5.12+0.15	4.64+0.81	3.75+0.59
	(+35.55)*	(-57.19)*	(-70.21)*	(-61.49)*

Figures in parentheses are % of increase (+) or decrease (-) over control.
* $P < 0.001$

Fig. 1.4



Effect of ferulic and gibberellic acids (singly or together in various concentrations) on shoot and rootlength of six days days old maize seedlings.

The analysis of shoot fresh weight revealed an increase upto 7% with GA treatment. In contrast, treatment with FA (1mM and 3mM) decreased the fresh weight by 11% and 57% respectively. However, application of GA together with FA (1mM) completely alleviated the inhibition while failed to influence the inhibition due to 3mM FA (Percentge of reduction with 3mM FA and 3mM FA along with 0.1mM GA was 57% and 54% respectively) (Fig.1.5).

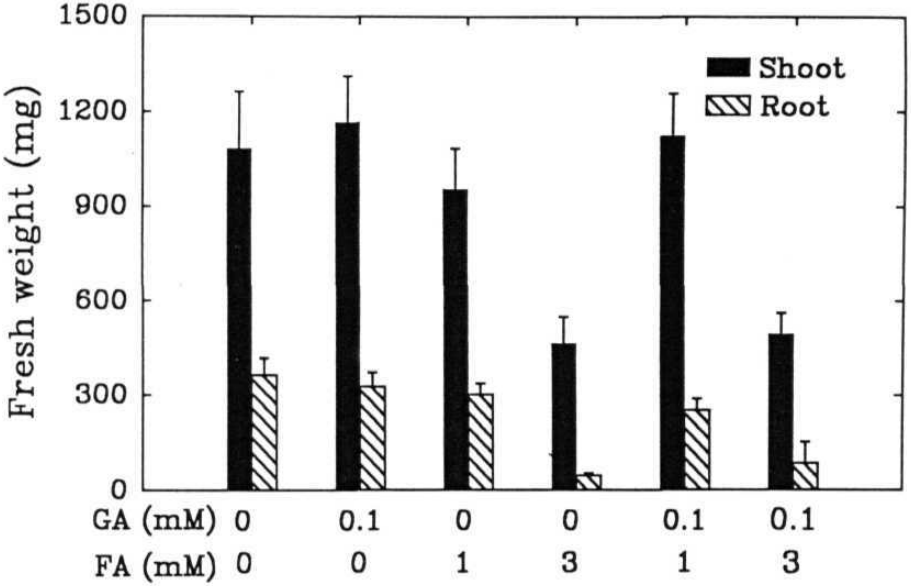
A non-significant decrease (10%) in root fresh weight was observed in GA treated seedlings and further reduction upto 17% and 87% was noted with 1mM and 3mM FA respectively compared to control. Supply of GA simultaneously with 1mM and 3mM FA exhibited reduction in the root fresh weight to 30% and 76% (Fig. 1.5).

The dry weight analysis showed an increase in dry weight of shoots by 9% with application of GA (O.lmM) and 7% with 1mM FA. 3mM FA decreased the dry weight by 24% compared to control. Seedlings treated with GA and 1mM FA simultaneously showed an increase in dry weight by 14% and GA along with 3mM FA had no effect on the 3mM FA caused inhibition (25% inhibition for both
3mM FA and 3mM FA plus O.lmM GA) (Fig. 1.6).

Besides, GA application decreased the root dry weight by 11% and 1mM and 3mM FA further decreased the dry weight by 15% and 80% respectively. Seedlings treated with GA along with 1mM and 3mM FA exhibited 7% and 73% decrease as against 15% and 80% reduction with 1mM and 3mM FA alone (Fig. 1.6).

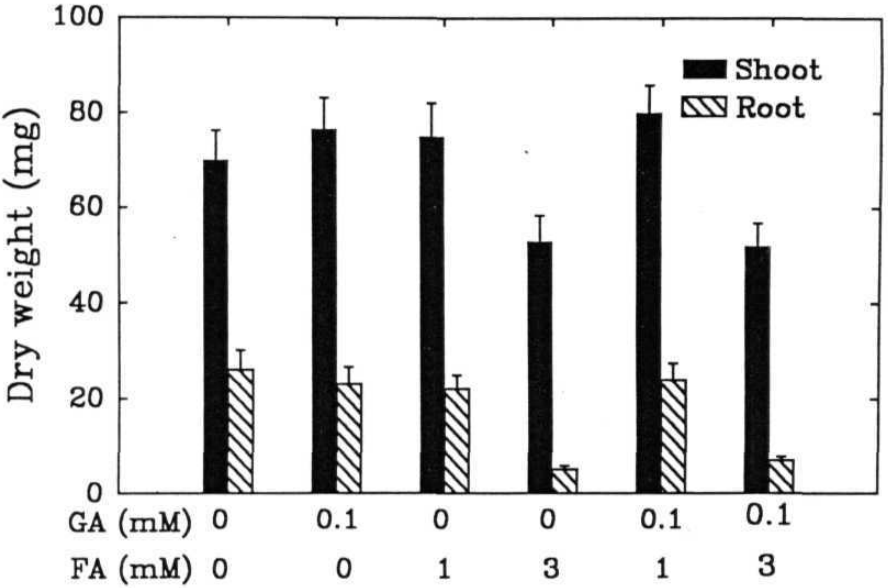
Corresponding with growth analysis of maize seedlings, the activities of hydrolytic enzymes were also examined in 6 days old germinating seeds treated with GA and FA either alone or in combination. A marked increase in the amylase activity (43%) was observed with application of GA while 1mM and 3mM FA decreased the activity by 21% and 30% respectively (Fig. 1.7). However, the application of GA simultaneously with 1mM and 3mM FA

Fig. 1.5



Effect of ferulic and gibberellic acids (singly or together in various concentrations) on fresh weight of shoots and roots of six days old maize seedlings.

Fig. 1.6



Effect of ferulic and gibberellic acids (singly or together in various concentrations) on dry weight of shoots and roots of six days old maize seedlings.

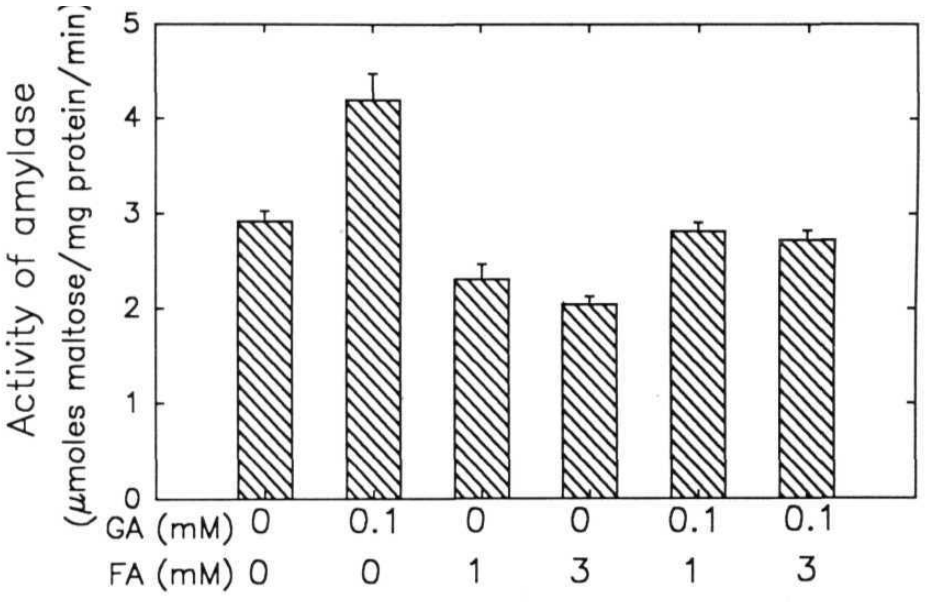
during germination resulted in only 3.7% and 6.8% reduction in the activity as against 21% and 30% with lmM and 3mM FA alone indicating the restoration of FA inhibited activity with GA (Fig. 1.7).

Reduction in the amylase activity caused by FA was further confirmed by the additional analysis of the isozyme pattern. Amylase of germinating seeds seperated on non-denaturing polyacrylamide gel was resolved into five isozymes (A-E) in control seeds(Fig. 1.8, lane 1). GA treatment has resulted in considerable increase in the activities of all the isozymes (lane 2) while lmM and 3mM FA decreased the activities of all the isozymes (lane 5,6) depending on the concentration. A complete restoration in the activity of isoforms was observed with application of GA simultaneously with lmM (lane 3) and 3mM FA (lane 4). These results are in agreement with those obtained from the measurement of activity (Fig. 1.7).

Similar to that of amylase, GA increased the activity of acid phosphatase by 54% while lmM and 3mM FA inhibited the activity by 17% and 33% respectively. However, application of GA simultaneously with FA (lmM and 3mM) increased the activity by 23% and 12% compared to control (Fig. 1.9) indicating the complete restoration of FA caused inhibition by GA and even greater activity compared to control (Fig. 1.9).

The analysis of acid phosphatase isozyme pattern revealed three isoforms (A,B and D) in control seeds (Fig. 1.10, lane 1). Induction of new isozyme C was observed with GA treatment (lane 2) in addition to an increase in the activity of other three isozymes (A,B and D). FA, (lmM) did not alter the isozyme pattern except that the relative activities of isoforms decreased (Fig. 1.10, lane 6). while 3mM FA treatment resulted in complete disappearance of isozyme B (Fig. 1.10, lane 5) in addition to decrease in the activity of other isozymes. Complete reversal of FA effect i.e. restoration of the isozyme expression of "B" and increase in the activity of the other isozymes of

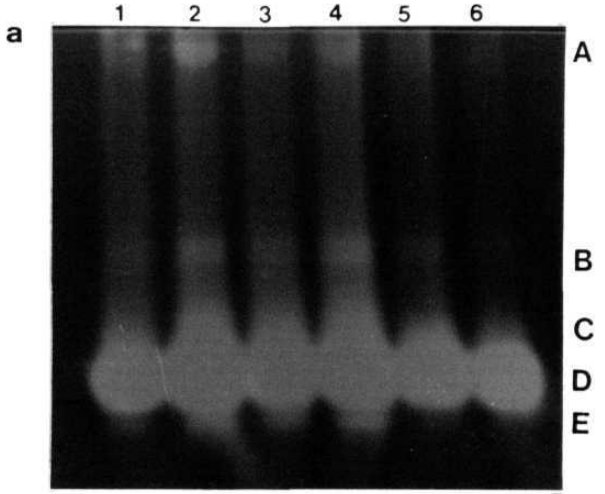
Fig. 1.7



Influence of ferulic and gibberellic acids (singly or together in various concentrations) on the activity of amylase in seeds of six days old maize seedlings.

Fig. 1.8a: Non-denaturing PAGE of amylase isozymes of 6days old germinating maize seeds treated with different concentrations of ferulic acid and gibberellic acid either alone or incombination.Electrophoresis was carried out in 7.5% gel at 4° C for 12h. lOunits of amylase activity was loaded in each lane. Gel was incubated in starch and stained with iodine solution until white bands appear on blue background. Amylase showed five isoforms (A-E) in control (lane 1) as well as treated seeds (lanes 2-6). GA (0. lmM) treatment resulted in significant increase in all the isozymes (lane 2) and FA significantly decreased the all the five isozymes (lanes 5 and 6) and GA application together with FA completely restored the FA induced inhibition (lanes 3 and 4).

b. Zymogram of the PAGE of amylase.


a
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2
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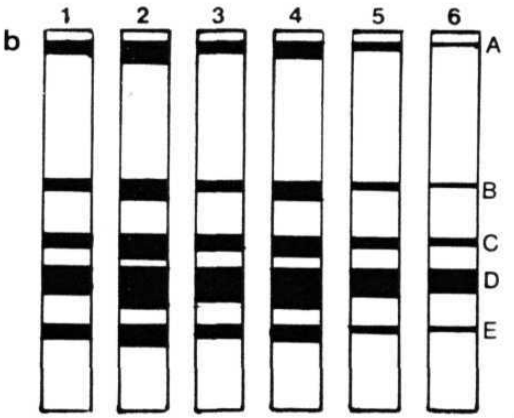
6
A
B
C
D
E


b
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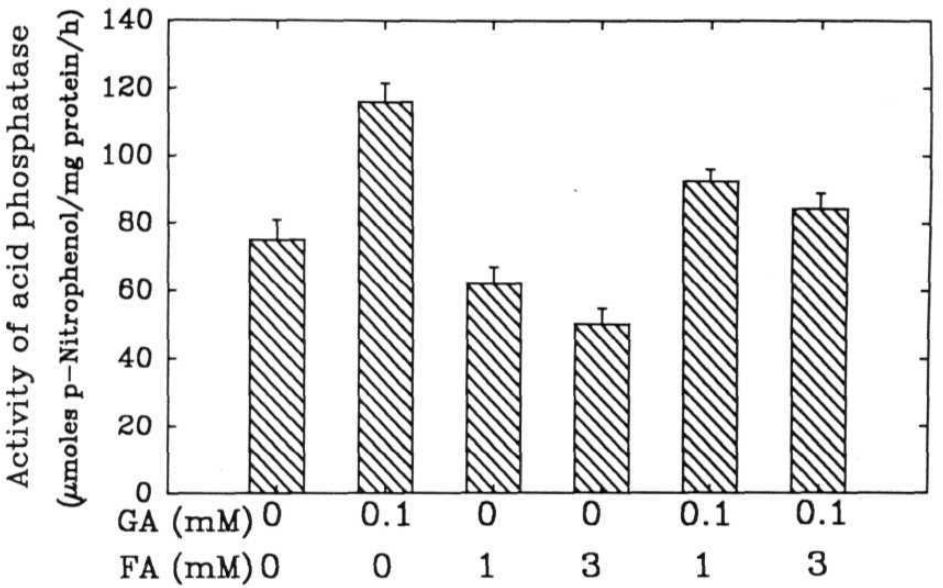
E

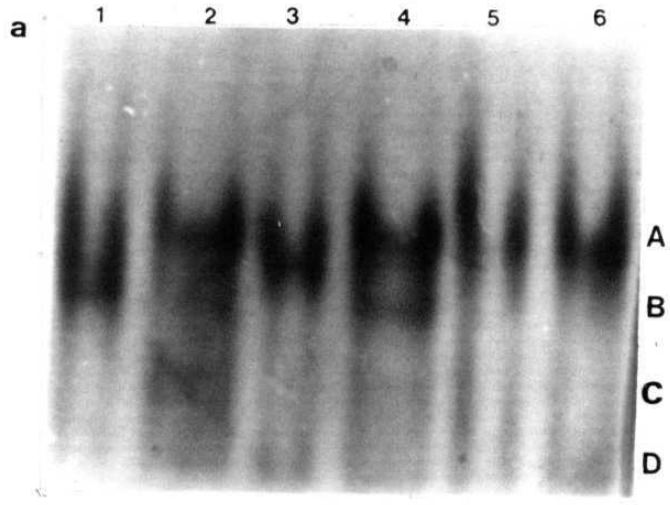
Fig. 1.9

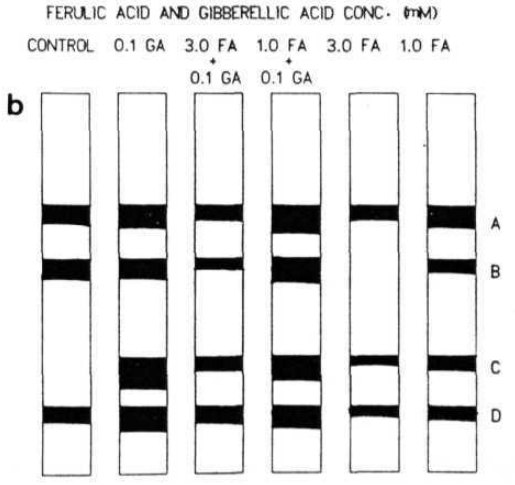


Influence of ferulic and gibberellic acids (singly or together in various concentrations) on the activity of acid phosphatase in seeds of six days old maize seedlings.

Fig. 1.10a: Non-denaturing PAGE showing acid phosphatase of 6days old germinating maize seeds treated with FA and GA either alone or incombination. Electrophoresis was carried out in 5% gel at 4° C for 12h. The gel was stained for acid phosphatase as given in experimental. In control seeds acid phosphatase resolved into three isoforms (A,B and D, lane 1). GA induced an isozyme C (lane 2). FA (1mM) showed reduction in the activities of all the three isozymes (A,B and D, lane 6) and 3mM FA resulted in disappearance of isozyme B (lane 5) in addition to reduction in the activity of isozymes A and D. GA application along with lmM (lane 4) and 3mM FA (lane 3) antagonzied the FA effect (restored the isozyme expression of B).

b. Zymogram of the PAGE of acid phosphatase.


a
1
2
3
4
5
6
A
B
C
D


FERULIC ACID AND GIBBERELLIC ACID CONC. (mM)
CONTROL
0.1 GA
3.0 FA + 0.1 GA
1.0 FA + 0.1 GA
3.0 FA
1.0 FA
b
A
B
C
D

acid phosphatase was observed in seeds treated with FA and GA simultaneously (Fig. 1.10, lanes 3,4)

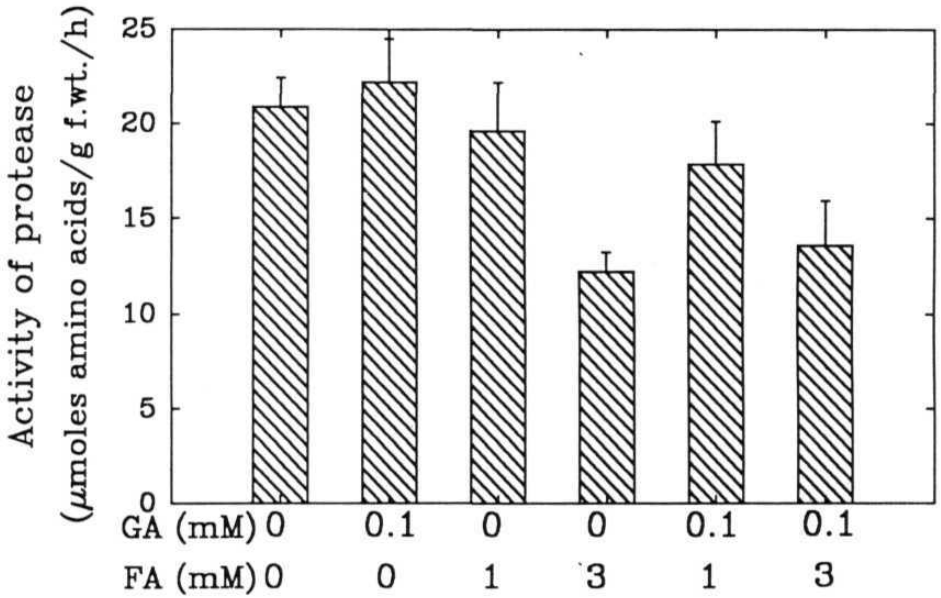
Unlike amylase and acid phosphatase, GA had no marked influence on the activity of protease wherein only 6% increase in protease activity was observed. Treatment with lmM and 3mM FA decreased the activity by 6% and 41% respectively compared to control. lmM and 3mM FA together with GA resulted in \4% and 34% inhibition in protease activity indicating the inability of GA to alleviate the FA mediated inhibition completely (Fig. 1.11).

The isozyme pattern of protease revealed eight isozymes (A-H) in control (Fig. 1.12, lane 1) as well as GA treated seeds (Fig. 1.12, lane 2). Though the relative activities of the isozymes increased with GA, but it did not altered the isozyme pattern. FA treatment (1mM) decreased the activity of D, E and F isozymes (Fig 1.12, lane 5) and 3mM FA resulted in decrease in the activity of B,D,E,F,G and H (Fig. 1.12, lane 6) isozymes. The exogenous supply of GA along with FA (lmM and 3mM) increased the activities of isozymes E,F and H similar to control (Fig. 1.12, lane 3 and 4) but could not restored the activity of isozyme D (Fig. 1.12, lane 3 and 4).

Discussion

The present study clearly established the toxic nature of FA to maize growth. Seed germination is less affected compared to growth. Inhibition of germination by FA has been observed earlier for soybean, wheat and sorghum (Leather and Einhellig 1985, Patterson 1981, Rasmussen and Einhellig 1977, 1979). Considerable inhibition in growth has been noticed i.e. shoot and root length (Fig. 1.1), fresh weight (Fig. 1.2) and dry weight (Fig. 1.3) in FA treated maize seedlings. This observation supplements the earlier reports of FA action on growth of a number of crop plants (Blum and Dalton 1982, 1985, Blum and Rebbeck 1989, Blum *et al* 1984, 1985a,b, Einhellig *et al* 1982, 1985, Glass 1975b, Klein and Blum 1990, Leather and Einhellig 1985, Patterson 1981).

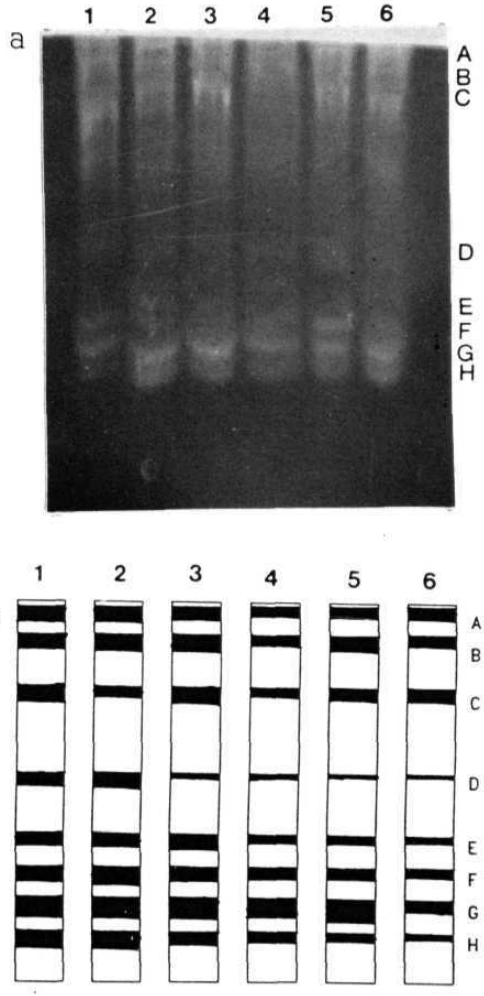
Fig. 1.11



Influence of ferulic and gibberellic acids (singly or together in various concentrations on the activity of protease in seeds of six days old maize seedlings.

Fig. 1.12a: Non-denaturing PAGE of the isozymes pf protease of 6days old germinating maize seeds treated with different concentrations of FA and GA either alone or in combination. Electrophoresis was carried out in 7.5% gel containing 0.1% gelatin at 4° C for 12h. The gel was stained with amidoblack as given in experimental. Protease izozymes appeared as white bands. Protease showed eight isozymes in control seeds, A-H (lane 1) as well as GA treated seeds (lane 2). FA (lmM) decreased the activity of isozymes E and F (lane 5), and 3mM FA resulted in decrease in the activity of B,D,E,F and H (lane 6). Application of GA along with FA resulted in increase in activity of isozymes E,F, and H (lanes 3 and 4).

b. Zymogram of the PAGE of protease.


a
1 2 3 4 5 6
A
B
C
D
E
F
G
H
b
1 2 3 4 5 6
A
B
C
D
E
F
G
H

Root growth is inhibited to a greater extent compared to shoot growth, probably due to immediate and constant exposure of roots to FA.

In earlier studies FA mediated inhibition in the growth has been attributed to several factors such as decrease in the cell wall extensibility due to the accumulation of phenolic compounds and rigidification due to diferulate cross links in the cell walls (Tan *et al* 1991, 1992) and lignin accumulation (Shann and Blum 1985b). Apart from its action on cell walls, FA is also known to decrease the water potential in treated plants resulting in the loss of cell turgor pressure that is essential for extensibility of cells (Blum *et al* 1985a, Holappa and Blum 1991, Lyu and Blum 1990). These might have acted either independently or synergistically to bring about reduction in growth of maize.

The role of hydrolytic enzymes during growth is well documented (Bewely and Black 1985, Morris and Arthur 1984). Among several of these, amylase, maltase and invertase are the key enzymes involved in the breakdown of starch to glucose via amylolytic pathway (Nomura *et al* 1969). Invertase is also responsible for the hydrolysis of sucrose to glucose and fructose (Morris and Arthur 1984), acid phosphatase acts on the endogenous reserve phosphates and protease acts on the reserve proteins (Bewely and Black 1985). Any alterations in the levels/activity of these enzymes would certainly alter a number of metabolic processes associated with growth.

A considerable decrease in the activity of amylase (Table 1.1), maltase (Table 1.2) and invertase (Table 1.3) has been observed in the germinating seeds with FA. The interference in the activity of these enzymes by FA would certainly limit the availability of glucose which is an important substrate for several metabolic reactions including respiration (Bewely and Black 1985, Mayer and Poljaklof-Mayber 1982).

Furthermore, a decrease in the activity of acid phosphatase has been observed with FA (Table 1.4). As phosphates are involved in various energy

dependent and energy mediated processes such as reduced activity of acid phosphatase at high FA concentrations would curtail the phosphate turnover and the metabolism of compounds containing phosphorus such synthesis of ATP, cell membrane componets such as phospholipids, nucleotide synthesis, and respiration (Bewely and Black 1985) would be further disturbed due to the deleterious effect of FA on acid phosphatase.

The decrease in protease activity by FA (Table 1.5) would limit the supply of smaller peptides and aminoacids that are essential for a number of metabolic processes including the synthesis of hydrolytic enzymes themselves (Mayer and Poljaklof- Mayber 1982).

In the light of the above results it is proposed that the reduction in growth could be due to the cut down in the supply of nutrients leading to slowing down of cell metabolism of growing seedlings that might have contributed for the FA caused growth reduction in the maize seedlings.

A number of biotic and abiotic factors viz. drought, water stress, herbicides, pathogens and heavy metals ABA, GA and various phenolic compounds (Akiyama and Suzuki 1981, Bhargava and Sachar 1983, Billett *et al* 1977, Bishnoi *et al* 1993, Chander *et al* 1987, Corradi *et al* 1993, Kabi *et al* 1981, Mathur *et al* 1988, Miyamoto *et al* 1993, Nolan *et al* 1987, Sharma *et al* 1991) are known to alter the hydrolytic enzymes which in turn have reflected on growth further suggesting a correlation between growth and hydrolytic enzymes as observed earlier (Morris and Arthur 1984).

Earlier studies revealed that a number of phenolic compounds are known to exert their action by interfering with GA, IAA and ABA either antagonistically or synergistically (Datta *et al* 1979, Kakkar and Rai 1986) which would alter a variety of metabolic processes mediated by these growth regulators. This is further evident in the present study as application of GA simultaneously with FA alleviated the FA induced inhibition in shoot growth (Fig. 1.4), fresh weight (Fig. 1.5) and dry weight (Fig. 1.6) either fully or

partially. In addition to growth, GA application has restored the FA induced inhibition in the activities of amylase (Fig. 1.7, 1.8), acid phosphatase (Fig. 1.9, 1.10) and protease (Fig. 1.11, 1.12) either partially or fully suggesting that FA might be acting as GA antagonist probably interfering with some process mediated by GA, or some proteins that recognizes GA or rendering the molecule incapable of performing its normal function.

GA is the key regulator in the synthesis and activation of these hydrolytic enzymes which trigger the *de novo* synthesis of the enzymes in the aleurone layer which later released into endosperm to act on the food reserves (Akiyama and Suzuki 1984, Bailey *et al* 1976, Callis and Ho 1983, Deikman and Jones 1985, Jacobsen and Varner 1967, Panabieres *et al* 1989, Segundo *et al* 1990). GA is also known to regulate the shoot extensibility (Keyers *et al* 1990, Zack and Loy 1984) by increasing the cell wall extensibility (Hohl and Schopfer 1992, Zack and Loy 1984) and / or have acted on the osmotic pressure of the cells (Cleland *et al* 1968) or by preventig the cell wall rigidification by inhibiting the peroxidase secretion (Fry 1979).

In contrast to GA, FA is known to decrease water potential, increase peroxidase activity and decrease the cell wall extensibility suggesting that FA probably is acting as antagonist of GA by inhibiting the GA mediated processes. The additional supply of GA to reverse the FA action further suggests this.

Studies of Gubler and Ashford (1985) and Gubler *et al* (1985) showed that GA helps in the release of FA from isolated aleurone layer walls with increased release of hydrolytic enzymes into the endosperm to act on the food reserves. Thus, the exogenous supply of GA might have helped in the removal of FA accumulated in the aleurone layers.

Jacobsen and Corcoran (1977) reported tannin inhibited activities of amylase and acid phosphatase and proposed its action by blocking the native function mediated by GA and by acting as regulators of GA or by

inhibiting transport of GA into the endosperm acting as GA antagonists. Similar antagonistic action of tannins has been observed by Green and Corcoran (1975). The restoration of FA mediated inhibition in the activities of amylase (Figs. 1.7, 1.8) and acid phosphatase (Figs 1.9 and 1.10) further suggests the possibility of FA acting as GA antagonist.

To sumup, the study clearly suggests that FA inhibited growth of maize could be probably due to reduction in the hydrolytic enzyme activities limiting the supply of nutrients to the growing seedlings. It also appears that FA is exerting its action probably acting as GA antagonist interfering with GA or GA mediating processes.

CHAPTER II

Ferulic acid mediated changes in oxidative enzymes of maize seedlings

Introduction

Phenolic allelochemicals are gaining considerable importance in crop improvement due to their potential to control weeds and pests (Liu and Lovette 1993, Miles *et al* 1993). Several of them are known to inhibit germination, seedling growth and influence many physiological processes (refer to part I, section 1.2.1). FA, a derivative of cinnamic acid is well known for its potential to modify growth of a number of crop plants. However, the physiological and biochemical mechanism of action of FA has not been clearly established. Apart from its role in allelopathy, FA also exists as a structural component in the cell walls of many angiosperms and serves as a precursor in lignin synthesis (refer to part III, chapter 3).

The role of oxidative enzymes viz. peroxidase, catalase, IAA oxidase, polyphenol oxidase in growth regulation has been well documented (Gasper *et al* 1990, Goldberg *et al* 1986, Omran 1980, Ros Barcelo and Munoz 1992). Peroxidases have been implicated in cross linking of cell wall polysaccharides (Fry 1986), resistance to infection by pathogens (Hammerschmidt *et al* 1982, Hrubcova *et al* 1992, 1994), catalyze the oxidation of phenolic compounds (Pedreno *et al* 1987, Spiker *et al* 1992, Srivastava and Van Huystee 1973, 1977, Zapata *et al* 1992), oxidation of H_2O_2 (Mader and Fisher 1982), IAA oxidation (Grambow and Langenback-Schwich 1983, Ferrer *et al* 1992b) and the most important being its role in lignin synthesis by catalyzing the oxidative polymerization of lignin precursors i.e. cinnamyl alcohols (Grisebach 1981, Grison and Pilet 1984, Lewis and Yamamoto 1990, Mader and Fussel 1982, Mc Dougall 1992, Mc Dougall *et al* 1982, Tadeo and Primo-Millo 1990, Yoshizawa *et al* 1991).

In addition to peroxidase, catalase also participates in the oxidation of H_2O_2 (Havir and Mc Hale 1987, 1989, Monk *et al* 1987, Nir *et al* 1986) thus

protecting the cellular systems from the toxic H_2O_2 and other free radicals. Furthermore, catalases are also observed to oxidize phenolic compounds similar to peroxidases and known to possess peroxidative functions (Halliwell 1974, 1978, Havir and Mc Hale 1987, Leek *et al* 1972).

The endogenous IAA levels in the plants are regulated by IAA oxidase. A direct evidence for correlation between growth, IAA and IAA oxidase has been well documented in the literature (Mukherjee and Choudhuri 1981, Omran 1980). Polyphenol oxidase catalyzes the oxidation of monophenols to diphenols and inturn to quinones and regulate the turnover of endogenous phenolic compounds (Butt 1980, Vaughn and Duke 1984). The relation of growth to phenolic compounds is well documented in the literature (Spiker *et al* 1992, Tomaszewski and Thimman 1966).

A variety of biotic and abiotic factors are often known to modify these oxidative enzymes synthesis and /activities (Binova 1992, Gasper *et al* 1990, Ferrer *et al* 1992a). In addition to these, a number of phenolic compounds are also known to alter the activities *in vitro* (Grambow and Lagenbeck-Schwich 1983, Imbert and Wilson 1972, Lee *et al* 1980, Ros Barcelo *et al* 1990). However, the *in vivo* action of these compounds has not been understood.

Apart from their independent action and regulation, these enzymes are found to act coordinately in growth regulation (Beffa *et al* 1990, Canal 1988, Machackova *et al* 1975, Shinshi and Noguchi 1975, Srivastava and Van Huystee 1977).

Therefore in the present study, FA effect on the activities of peroxidase, catalase, IAA oxidase and polyphenol oxidase enzymes have been examined and the significance of these changes in relation to FA altered maize growth are discussed.

Results

The study clearly showed significant alterations in the activities of oxidative enzymes with FA. Considerable increase in the activity of peroxidase

(soluble, ionic and covalent) was observed though the relative activities varied among the three forms. In leaves, 0.5mM-1.5mM FA increased the activity of soluble peroxidase by 13%-21% and 2mM-3mM FA increased the activity by 30%-41% (Table 2.1). The increase in ionic peroxidase was 7%-25% with 0.5mM-1.5mM FA compared to control and the activity was further increased to 38%-78% with 2mM-3mM FA (Table 2.1). Furthermore, covalent peroxidase activity was increased by 51%-121% with 0.5mM-3mM FA. The order of increase in the activity of different forms of peroxidase was covalent > ionic > soluble.

Roots exhibited an increase of 16%-33% in the activity of soluble peroxidase with 0.5mM-1.5mM and with rise of FA concentration (2mM-3mM), activity was increased to 38%-46%. Except with 0.5mM FA, wherein a negligible increase (2%) in activity was observed and the other FA concentrations (lmM 3mM) significantly increased the ionic peroxidase by 34%-86%. Covalent peroxidase activity was increased in the order of 11%-28% with 0.5mM-1.5mM and 32%-44% increase with 2mM-3mM FA (Table 2.2). Unlike leaves, the order of increase in peroxidase activity was ionic > soluble > covalent.

The relationship between peroxidase activity and phenolic content exhibited a positive correlation in roots (r=0.96, Fig. 2.1) suggesting the increased phenolic compounds might have been utilized by peroxidase for the peroxidation reactions (refer part III, chap 3, table 3.3)

Catalase activity of leaves and roots responded differently FA treatment. In leaves, 0.5mM-1.5mM FA caused 4%-19% increase, while 2mM-3mM FA caused 10%-31% decrease in activity (Table 2.3). In contrast, roots exhibited a concentration dependent increase in activity of catalase with FA treatment. FA (0.5mM-lmM) increased the catalase by 12%-13% and 1.5mM-3mM FA increased the activity by 54%-296% (Table 2.3).

Table 2.1

Effect of ferulic acid on peroxidase activity in leaves of maize seedlings

Ferulic acid (mM)	Peroxidase activity (μmoles/g fresh weight/h)		
	Soluble	Ionic	Covalent
Control	8.03+0.67	1.30+0.05	1.33±0.07
0.5	9.10+1.2 (+13.3)	1.40+0.60 (+7.69)	2.02+0.09 (+51.8)*
1.0	9.79+2.0 (+21.9)	1.55+0.30 (+19.2)	2.07+0.10 (+55.6)*
1.5	9.75+1.5 (+21.4)	1.63+0.50(25.3)	2.14+0.98 (+60.9)*
2.0	10.50+1.4 (+30.7)**	1.80+0.70 (+38.4)**	2.31+0.60 (+73.6)*
2.5	10.80+1.2 (+34.6)**	1.80+0.80 (+38.4)**	2.93+0.70 (+120.3)*
3.0	11.40+1.4 (+41.9)*	2.32+0.90 (+78.4)*	2.95+1.20 (+121.8)*

Figures in parentheses are % of increase (+)/ decrease (-) over control
* $P < 0.001$, ** $P < 0.005$

Table 2.2

Effect of ferulic acid on peroxidase activity in roots of maize seedlings

Ferulic acid (mM)	Peroxidase activity (μmoles/g fresh weight/h)		
	Soluble	Ionic	Covalent
Control	26.7+2.2	19.7+1.6	23.4+1.9
0.5	34.1±2.7(+27.7)	20.1 + 1.4 (+2.03)	28.3±2.6 (+20.7)
1.0	31.0+1.9(16.4)	26.4±2.5 (+34.09)**	26.0+1.7 (+11.1)
1.5	35.6+2.8 (+33.6)	28.8+3.4 (+46.3)*	30.0+1.6 (+28.2)
2.0	36.8+2.4 (+38.0)*	32.3+2.0 (+63.6)*	31.0+2.7 (+32.1)**
2.5	38.6+3.1 (+44.6)*	37.2+2.1 (+88.7)*	33.1+1.8 (+41.2)*
3.0	39.1±2.9 (+46.6)*	36.8+3.2 (+86.5)*	33.8±2.6 (+44.4)*

Figures in parentheses are % of increase (+)/ decrease (-) over control

* $P < 0.001$, ** $P < 0.005$

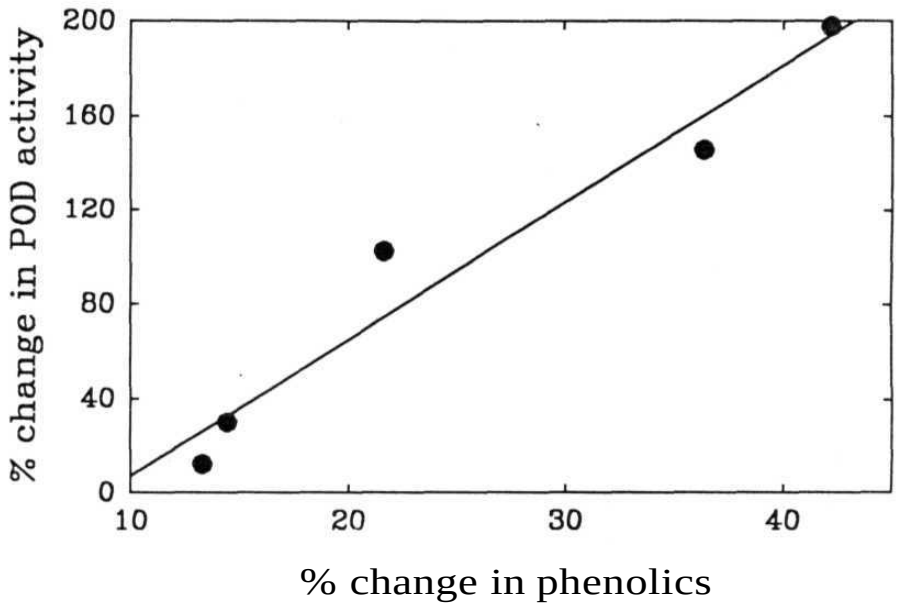
Table 2.3

Effect of ferulic acid on the activity of catalase in leaves and roots of maize seedlings

Ferulic acid (mM)	Units of catalase/mg protein/h	
	Leaves	Roots
Control	12.00+2.30	2.76+0.45
0.5	10.75+1.87 (-10.4)	3.10±0.39(+12.3)
1.0	12.48+2.51 (+4.00)	3.12±0.60 (+13.0)
1.5	14.28+2.74 (+19.0)	4.26+0.99 (+54.3)*
2.0	10.77+1.66 (-10.2)	4.86+0.53 (+76.8)*
2.5	8.20+1.08 (-31.0)**	5.94+1.12 (+115.2)*
3.0	8.28±1.01 (-31.3)**	10.94+2.10 (+296.3)*

Figures in parentheses are % of increase (+)/ decrease (-) over control
* $P < 0.001$, •• $P < 0.005$

Fig. 2.1



Relationship between peroxidase activity and phenolic content in roots of eight days old maize seedlings treated with difTe- rent concentrations of ferulic acid. A good correlation was observed (r= 0.96)

The endogenous level of IAA is important for the growth of plant. Any alterations in the IAA levels would certainly reflect on the growth. In plants IAA levels were normally regulated by IAA oxidase and to some extent by peroxidase. FA treatment has significantly increased the activity of IAA oxidase in both leaves as well as roots (Table 2.4). In leaves, 0.5mM FA increased the activity by 20% and lmM-3mM FA increased the activity by 55%-141%. In roots, 0.5mM and 1mM FA increased the activity by 16%-25% and 1.5mM-3mM FA caused 662%-686% increase (Table 2.4).

The increase in IAA oxidase due to FA was further confirmed by the additional analysis of isozymes of IAA oxidase. Non-denaturing electrophoresis revealed three isozymes (A, B and C) of IAA oxidase in control (Fig. 2.2, lane 1) as well as treated leaves (Fig. 2.2, lanes 2-5). A significant increase in the activity of all the three isozymes was observed with FA treatment depending on the concentration (Fig. 2.2, lanes 2-5) compared to control (Fig. 2.2, lane 1).

IAA oxidase activity was resolved into two isozymes (A and C) (Fig. 2.3, lane 1) in control roots. Treatment with FA (0.5mM and lmM) increased the activity of both isozymes (Fig. 2.3, lanes 2 and 3) while induction of new isozyme "B" was observed in seeds treated with lmM and 3mM FA in addition to an increase observed in the activity of the isozymes A and C (Fig. 2.3, lanes 4 and 5).

Polyphenol oxidase regulate the turnover of phenolic compounds in plants. FA treatment did not alter the activity of polyphenol oxidase significantly in leaves as well as in roots except with 3mM FA. In leaves, 1mM-2.5mM FA decreased the activity by 3%-13% and 3mM FA inhibited the activity by 28%. In contrast, roots exhibited an increase in PPO activity by 13% and 4% with 0.5mM and 1.5mM while lmM-2.5mM FA decreased the activity by 1%-16% and 31% decline in activity was observed with 3mM FA (Table 2.5).

Table 2.4

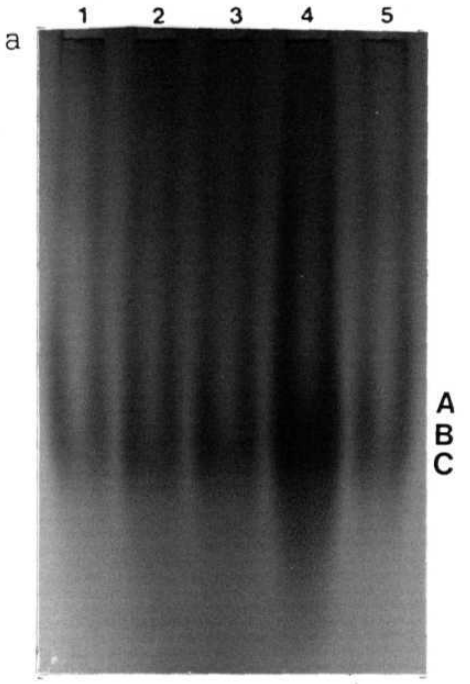
Effect of ferulic acid on the activity of IAA oxidase in leaves and roots of maize seedlings

Ferulic acid (mM)	μg of IAA oxidized/mg protein/h	
	Leaves	Roots
Control	61.24+9.90	87.37+12.3
0.5	73.51 + 12.2 (+20.0)	101.67+5.98 (+16.3)
1.0	94.97+8.2 (+55.0)*	109.27+14.2 (+25.0)
1.5	110.16+9.4 (+79.8)*	666.36+16.0 (+662.6)*
2.0	120.21+ 11.7(+96.2)*	678.65+15.4 (+676.4)*
2.5	129.82+8.0 (+111.9)*	686.92+14.2 (+686.2)*
3.0	147.92+4.1 (+141.5)*	687.59+14.9 (+686.9)*

Figures in parentheses are % of increase (+)/ decrease (-) over control
* $P < 0.001$.

Fig. 2.2a: Non-denaturig PAGE of IAA oxidase in leaves of 8days old seedlings treated with different concentrations of FA. Electrophoresis was carried out in 5% gel at 4° C for 12h. The gel was stained for IAA oxidase isozymes with Fast Blue BB as given in experimental until brown appeared. In control leaves IAA oxidase was seperated into three isozymes A-C (lane 1). Treatment with 0.5mM to 3mM FA resulted in concentration dependent increase in all the three isozymes. 0.5mM (lane 5), ImM (lane 2), 2mM (lane 3), 3mM (lane 4).

b. Zymogram of the PAGE of IAA oxidase


a
1
2
3
4
5
A
B
C

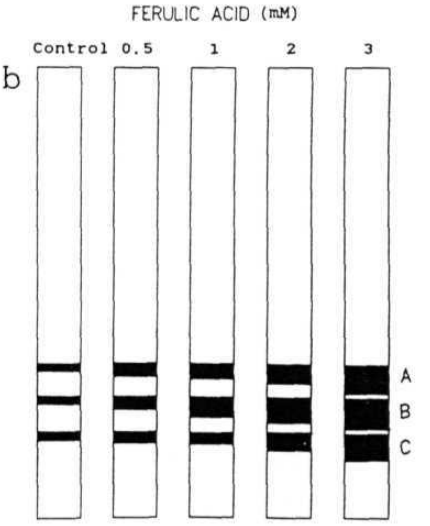
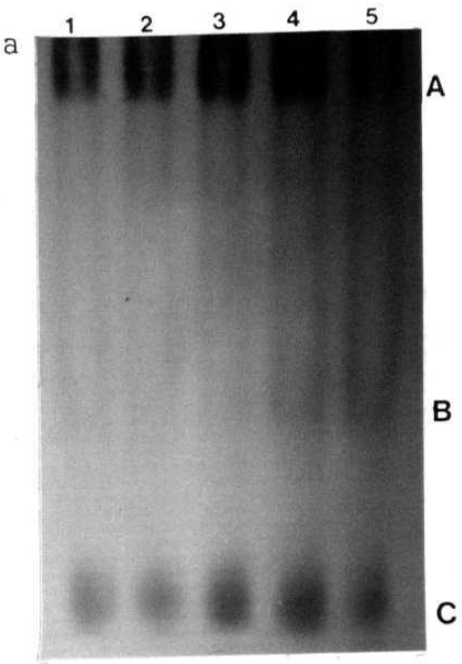

FERULIC ACID (mM)
Control
0.5
1
2
3
b
A
B
C

Fig. 2.3a: Non-denaturing PAGE showing IAA oxidase isozymes in roots of 8days old maize seedlings treated with different concentrations of FA. Electrophoresis and staining for IAA oxidase was carried out as given in experimental using Fast Blue BB for staining. In control roots IAA oxidase was seperated into two isozymes A and C (lane 1).). Treatment with 0.5mM FA (lane 2) and 1mM (lane 3) showed an increase in the activity of both isozymes i.e A and C. Treatment with 2mM FA (lane 4) and 3mM FA (lane 5) showed an induction of new isozyme B in addition to an increase in the activity of isozymes A and C.

b. Zymogram of PAGE of root IAA oxidase


a
1
2
3
4
5
A
B
C

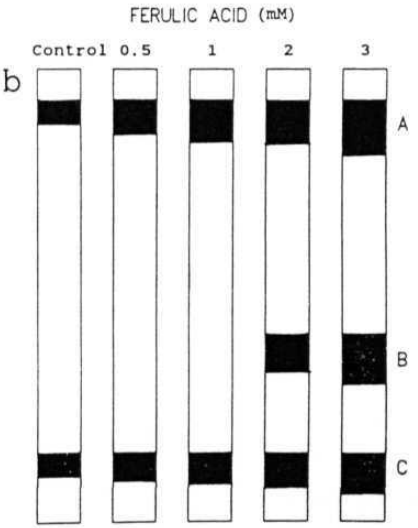

FERULIC ACID (mM)
Control
0.5
1
2
3
b
A
B
C

Table 2.5

Effect of ferulic acid on the activity of polyphenol oxidase in leaves and roots of maize seedlings

Ferulic acid (mM)	PPO activity(units/mg protein /min)	
	Leaves	Roots
Control	1837.1 + 184.68	4280+719.2
0.5	1828.5±170.4 (+0.4)	4850+683.4 (+13.3)
1.0	1720.0+277.4 (-6.3)	4200+792.8 (-1.86)
1.5	1766.6+103.2 (-3.8)	4474+575.0 (+4.50)
2.0	1587.5+203.1 (-13.6)	4000+479.0 (-6.54)
2.5	1625.0+183.2 (-11.5)	3577+603.4 (-16.4)
3.0	1316.6+276.2 (-28.3)**	2942+754.2 (-31.2)**

Figures in parentheses are % of increase (+)/ decrease (-) over control

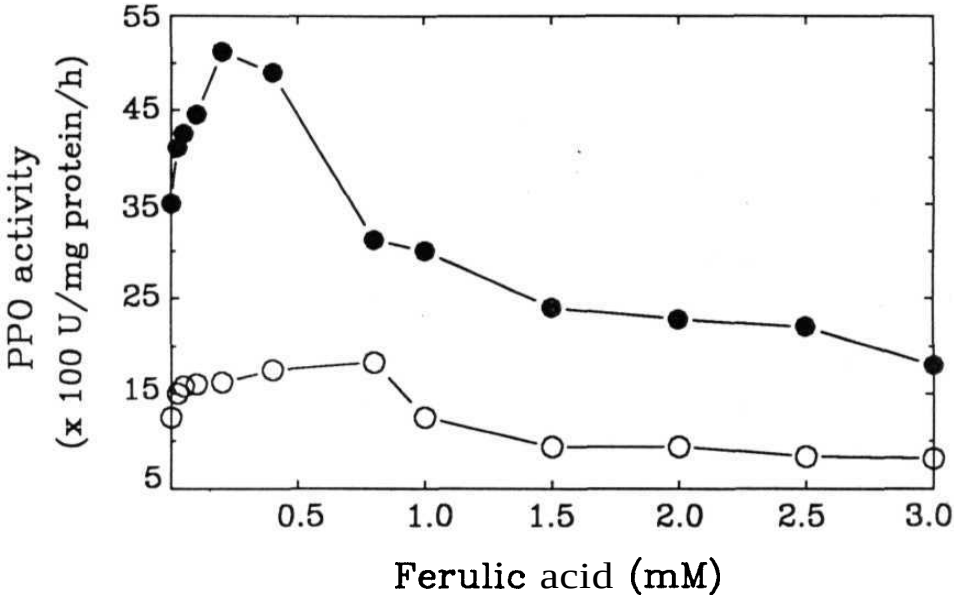
•* $P < 0.005$

In addition to *in vivo* effect of FA on polyphenol oxidase activity, the *in vitro* effect was tested with different concentrations of FA. In leaves the activity was significantly increased upto 0.8mM and higher FA concentrations significantly decreased the activity. Similarly, in roots the activity was increased upto 0.4mM and further increase in FA concentration resulted in considerable decrease in activity (Fig. 2.4).

Discussion

This study clearly shows considerable changes in the activities of the oxidative enzymes of maize on FA treatment. FA treatment has resulted in significant increase in the activity of soluble, ionic and covalent peroxidases in leaves as well as roots though the extent of increase varied among the three forms (Table 2.1, 2.2). Compared to soluble peroxidases, activity of wall bound (Ionic and Covalent) peroxidases has been increased to a greater extent. In plants the localization of peroxidases depends on their function (Grison and Pilet 1984). Soluble peroxidases are mostly associated with cytoplasm and catalyze most of the peroxidative reactions in the cell (Ridge and Osborne 1970). Among the wall bound, ionic peroxidases are associated with middle lamellae and covalent peroxidases with pectins and hemicellulose of cell walls (Grison and Pilet 1984). The role of wall bound peroxidases is to catalyze the oxidative polymerization of lignin precursors leading to synthesis of lignin (Bruce and West 1988, Mc Dougall *et al* 1994, Van Huystee and Zheng 1993). The greater increase in wall bound peroxidases (Tables 2.1 and 2.2) is presumably due to more availability of lignin precursors (FA and its derivatives) and to utilize them in lignin synthesis. Similar increase in peroxidase activity associated with binding of FA to cell walls has been observed earlier (Imberty *et al* 1985, Whitemore 1976, Van Huystee and Zheng 1993). The role of peroxidase in binding of FA to cell walls was demonstrated by Whitemore (1976) and Shann and Blum (1987b) observed a substantial increase in lignin content and peroxidase activity with exogenous supply of FA

Fig. 2.4



Effect of ferulic acid *(in vitro)* on the polyphenol oxidase activity of leaves (0) and roots (I) of maize seedlings. Note an increase in the activity upto **0.8mM** in leaves and 0.4mM in roots. Deviations for the values were less **than 10%.**

in barley. The present results further suggests the role of peroxidase in lignin synthesis as a significant increase in peroxidase activity (Tables 2.1, 2.2) and lignin content (refer to part III, chapter 3, table 3.4) has been observed with FA application.

In addition to its role in lignin synthesis, peroxidases are (soluble) also known to participate in the H_2O_2 dependent oxidation of phenolics (Nakajima *et al* 1991, Takahama 1988a,b Takahama and Oniki 1992, Spiker *et al* 1992). Many Phenolic compounds are natural substrates for peroxidases (Fry 1986) which provide OH" groups for the peroxidation reactions both *in vitro* and *in vivo* (Spiker *et al* 1992, Takahama and Oniki 1992). In addition to lignin, a substantial increase in the phenolic compounds has been observed with FA application (refer to part III, chapter 3, Table 3.3) which suggests that the increase in the soluble peroxidase activity with FA might be due to more availability of phenolic compounds which provide OH" groups for their oxidation reactions. This inter-relationship between peroxidase and phenolic compounds is evident from figure 2.1, which showed a postitive correlation between peroxidase activity and phenolic compounds. Similar relation between phenolic compounds and peroxidase has been observed earlier (Binova 1992, Hrubckova *et al* 1992, 1994).

Peroxidase mediated lignification results in decline in cell wall elongation and growth (Fry 1983, Ishi and Saka 1992, Mac Adam *et al* 1992a,b, Mc Dougall 1992, Rama *et al* 1982, Tan *et al* 1991, 1992). Thus in light of the present results, it is clear that the increase in the activity of wall bound peroxidases with FA application might have contributed to growth reduction via increased rigidification of the cell walls.

Catalases play a significant role in breakdown of H_2O_2 to water and oxygen thus protecting the cells from toxic H_2O_2. In addition to its role in breakdown of H_2O_2, plant catalases are also known to possess peroxidase function (Halliwell 1974, Havir and McHale 1987, Leek *et al* 1972).

Significant increase in the catalase activity observed in roots (Table 2.3) with FA treatment suggest efficient removal of H_2O_2 produced during various metabolic processes. In contrast, high concentrations of FA decreased the activity of catalase in leaves which would result in the accumulation of H_2O_2 and may aid in the peroxidation of chloroplast membrane lipids and reduce the effeciency of photosynthetic processes in FA treated plants. Similar involvement of singlet oxygen radicals in lipid peroxidation has been observed (Chakraborthy and Tripathy 1992). The relation between lipid peroxidation and antioxidant enzymes (Hurng and Kao 1994, Sung and Jeng 1994) suggest that these enzymes are involved in the detoxification of free radicals. Many of the phenolic compounds and flavonoids are known to act as hydroxyl radical scavengers (Husain *et al* 1987).

The endogenous IAA levels in the plants are regulated by IAA oxidase and to some extent by peroxidases (Gebhardt 1982, Zheng and Van Huystee 1992). In few plant systems, IAA oxidase and peroxidase activities are observed to associate with same protein (Talwar *et al* 1985). Stimulation in IAA oxidase (Table 2.4) and peroxidase (Tables 2.1 and 2.2) with FA, further support the existing notion of the role of these two in IAA oxidation as reported earlier (Beffa *et al* 1990). Recently, Krylov *et al* (1993, 1994) observed the regulation of peroxidase catalyzed oxidation of IAA by caffeic acid. The greater increase in IAA oxidase with FA would lower the endogenous IAA levels and would result in growth reduction as is observed by Ferrer *et al* (1992a,b). The observed reduction in maize growth, particularly root growth with FA might be probably due to reduced increased IAA oxidase activity which would decrease the endogenous IAA levels. A number of phenolic compounds including FA are known to affect IAA oxidation under *in vitro* conditions (Krylov *et al* 1993, Lee *et al* 1982) wherein phenolic compounds act as mediators for the transfer of electrons for IAA oxidation. Similar increase in IAA oxidation with FA was reported earlier (Gelinas 1973).

Increase in IAA oxidase activity due to FA is further confirmed by the additional analysis of isozymes of IAA oxidase. FA treatment enhanced IAA oxidase activity of all the 3 isozymes in leaves (Fig. 2.2) in addition to an induction of a new isozyme "B" in roots (Figs. 2.3) suggesting that FA has increased the activity at the isozyme level.

Polyphenol oxidase regulates the turnover of phenolic compounds and a variety of phenolic compounds are known to act as substrates, inhibitors or activators of polyphenol oxidase *in vitro* (Tremolieres and Bieth 1984). The observed inability of FA (upto 2.5mM) to bring about any significant change in polyphenol oxidase suggests the existance of another mechanism for the regulation of phenolic levels *in vivo.* Accordingly, Canal *et al* (1988) suggested that peroxidase is probably the enzyme normally involved in phenolic oxidation and hydroxylation *in vivo* in these tissues. However, at high concentrations of FA (3mM) a decrease in polyphenol oxidase activity (Table 2.5) has been observed which could be probably due to inactivation of enzyme by these inhibitory phenolic compounds. However, *in vitro* studies revealed a signifcant stimulation of polyphenol oxidase upto 1mM FA (Fig. 2.4) might be the result of an increase in the formation of an enzyme substrate activator complex and a decrease with high concentration of FA (greater than 1mM) could be due to its action as a competitive inhibitors. Similar concentration dependent regulation of polyphenol oxidase by phenolic compounds has been reported earlier (Tremolieres and Bieth 1984).

It is concluded that FA application to maize seedlings had altered the activities of the oxidative enzymes. Since the proposed role of peroxidases and IAA oxidases in plant growth and differentiation is based mainly in their involvement in lignin biosynthesis and IAA oxidation, the increase in the activities of these enzymes would result in the accumulation of more lignin thus rigidifying the cell walls, decreased endogenous IAA levels and accumulation of growth inhibitory phenolic compounds. Further these enzymes might also

have acted in a co-ordinated manner in controlling the lignin synthesis, accumulation of phenolic compounds and regulating IAA levels.

CHAPTER III

Influence of ferulic acid on the activities of phenylpropanoid enzymes and accumulation of phenylpropanoid intermediates in maize seedlings

Introduction

During the growth and development of plants, a number of secondary metabolites are synthesized and accumulated in various parts of the plants. Ferulic acid in addition to its potential to inhibit growth of plants occurs as a structural component in the cell walls (refer to part I, section 1.3.1 to 1.3.3) and is an important precursor of lignin synthesis (Akin *et al* 1992, Blade *et al* 1991, Bookern *et al* 1991, Bolwell *et al* 1986, 1988, Borg-Olivier and Monties 1993, Myton and Fry 1994, Ralph *et al* 1992a,b, Shann and Blum 1987b, Vansumere *et al* 1972).

A number of secondary metabolites such as phenolic compounds, flavonoids, coumarins and lignin are synthesized from L-phenylalanine via a series of biosynthetic pathways involving phenylpropanoid metabolism which play an important role in growth and development of plants (Davin and Lewis 1992, Douglas *et al* 1992, Hahlbrock and Scheel 1989). The chemistry, enzymology, and molecular biology of phenylpropanoid metabolism has been gaining considerable attention due to their varied roles in growth regulation (Hahlbrock and Scheel 1989). A number of phenylpropanoid intermediates are the precursors for the lignin synthesis (Bolwell *et al* 1988, Borg-Olivier and Monties 1993, Ralph *et al* 1992a,b). The accumulation of lignin has been observed under various stress conditions such as pathogen attack to confer resistance (Kumar *et al* 1991, Nagarathna *et al* 1993, Siqueira *et al* 1991, Southerton and Deverall 1990) and known to reduce growth of the plants by reducing the cell wall extensibility (Dellagreca *et al* 1991, Ishi and Saka 1992, Hartley and Jones 1976, Kefeli and Kutacek 1977, Stafford and Brown 1976, Tan et al 1991, 1992, Yamamaoto and Towers 1985).

Phenylalanine ammonialyase (PAL) and cinnamylalcohol dehydrogenase (CAD) are the key phenylpropanoid enzymes regulate the early stages of the propanoid metabolism leading to synthesis of a number of phenylpropanoid intermediates and lignin synthesis (Hahlbrock and Scheel 1989). A variety of factors are known to modulate phenylpropanoid metabolism (Dixon and Bolwell 1988).

PAL catalyzes the conversion of L-phenylalanine to t- cinnamic acid, a first step for biosynthesis of phenylpropanoid skeleton in higher plants (Dixon 1986, Jones 1984, Hanson and Havir 1988) and the levels of the enzyme is known to be influenced by a number of physical, chemical and biotic factors (Dixon *et al* 1992, Jorrin *et al* 1990, Orr *et al* 1993, Sato *et al* 1982) and has been reviwed by Jones (1984).

CAD catalyzes the reduction of cinnamaldehydes to corresponding alcohols in the presence of NADPH which is the last step in the formation of lignin precursors (Grand *et al* 1985, Greisebach 1981, Hibino *et al* 1993, Mitchell *et al* 1994, Wyrambik and Greisebach 1975). This enzyme has been purified to homogenity from both gymnosperms and angiosperms (Grand *et al* 1985, Hibino *et al* 1993, Wyrambik and Griesebach 1975).

FA, being precursor for lignin synthesis and is a substrate for peroxidases, the exogenous supply of FA to seedlings would alter synthesis and / activity of these enzymes. In the present study FA effect on the activities of PAL and CAD has been examined to understand the mechanism of action of FA induced growth reduction as these enzymes are involved in the synthesis of lignin and phenolic compounds in the cells.

In the present study, the correlation among the enzyme levels and accumulation of phenolic compounds and lignin are investigated and the significance of these changes in relation to maize growth reduction is discussed.

Results

The results presented here clearly shows the FA induced alterations in the phenylpropanoid metabolism. FA treatment significantly increased the activity of PAL in leaves and roots (Table 3.1). Though an increase in PAL activity was observed with FA, but didnot exhibit a linear increase with rise in FA concentration. Except with 0.5mM, lmM 3mM FA increased the activity by 8%-99% while 0.5mM FA caused 41% inhibition in PAL activity in leaves (Table 3.1). In contrast to leaves, FA treatment has resulted in a significant concentration dependent increase (8%-495%) in the activity of PAL in roots.

Treatment with FA has resulted in significant increase in the activity of CAD in leaves as well as roots (Table3.2). Unlike PAL, CAD activity exhibited a concentration dependent increase in leaves. FA (0.5mM-2.5mM), increased the activity of CAD by 7%- 24% and with increase in FA concentration (3mM), the activity was increased to 39% (Table 3.2). In roots, FA treatment has resulted in a considerable increase in CAD activity except with 0.5mM FA wherein a nonsignificant increase (3% increase) was observed. FA (1mM-3mM), increased the activity of CAD by 25-65% compared to control (Table 3.2).

The role of PAL in phenolic synthesis is well studied. Any alterations to the enzyme activity reflects on the accumulation of phenolic compounds. Accordingly, a signinficant increase in the phenolic content was observed with FA treatment (Table 3.3) in accordance with PAL activity. In leaves, 0.5mM and lmM FA increased the phenolic content by 14% and 25% respectively and further increase in the phenolic content by 47%-107% was observed with increase in FA (2mM-3mM) compared to control (Table 3.3). Roots also exhibited a similar response to FA treatment. An increase of 12% and 42% in the phenolic content was observed with 0.5mM and lmM FA respectively

Table 3.1

Effect of ferulic acid on phenylalanine ammonialyase in leaves and roots of maize seedlings

Ferulic acid (mM)	µg cinnamic acid/g f.wt./h	
	Leaves	Roots
Control	6.1+0.4	20.5+1.0
0.5	3.5+1.2 (-41.5)*	18.7+2.3 (-8.8)
1.0	7.8+0.5 (+27.5)**	22.3+2.6 (+8.8)
1.5	6.6+0.5 (+8.1)	49.9+4.7 (+143.4)*
2.0	7.7+2.4 (+26.4)	55.6+6.7 (+171.2)*
2.5	12.2+2.7 (+99.9)*	89.0+2.3 (+334.1)*
3.0	6.9+1.9 (+13.3)	122.0+2.0 (+495.1)*

Figures in parentheses are % of increase (+)/ decrease (-) over control
* $P < 0.001$, ** $P < 0.005$

Table 3.2

Effect of ferulic acid on cinnamylalcohol dehydrogenase activity in leaves and roots of maize seedlings

Ferulic acid (mM)	µmoles cinnamaldehyde/mg protein/h	
	Leaves	Roots
Control	1.51+0.55	1.54+0.43
0.5	1.71+0.70 **(+13.2)**	1.60+0.24 (+3.8)
1.0	1.62+0.51 (+7.28)	1.94+0.34 (+25.9)**
1.5	1.88+0.58 (+24.5)	1.97+0.70 (+27.9)**
2.0	1.75+0.62 (+15.8)	2.13+0.61 (+38.3)*
2.5	1.79+0.66 (+18.5)	2.55+0.48 (+65.5)*
3.0	2.10+0.70 (+39.0)*	•2.55+0.59 **(+65.5)***

Figures in parentheses are % of increase (+)/ decrease (-) over control
* $P < 0.001$, •* $P < 0.005$

Table 3.3

Effect of ferulic acid on the phenolic content in leaves and roots of maize seedlings

Ferulic acid (mM)	mg phenolics/gm fresh weight	
	Leaves	Roots
Control	2.43+0.17	1.33+0.11
0.5	2.79+0.09 (+14.8)	1.49+0.09 (+12.0)
1.0	3.04+0.11 (+25.1)	1.89+0.23 (+42.0)
2.0	3.58+0.27 (+47.3)*	2.69+0.18 (+102.2)*
2.5	4.24+0.28 (+74.4)*	3.27+0.22 (+145.8)*
3.0	5.04+0.11 (+107.4)*	3.96+0.35 (+197.7)*

Figures in parentheses are % of increase (+)/ decrease (-) over control
* $P < 0.001$. •* $P < 0.005$

while 2mM-3mM FA treatment resulted in 102%-197% increase over control (Table 3.3).

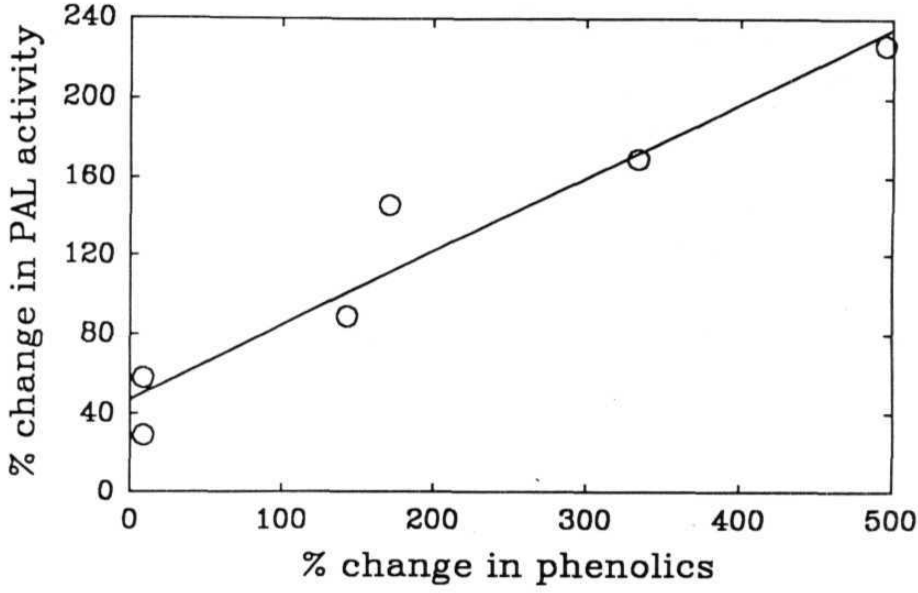
The relation between PAL activity and phenolic content exhibited a positive correlation (r= 1.06) in roots suggesting the increase in phenolic content may be due to increase in PAL activity with FA treatment (Fig. 3.1).

FA being the precursor of lignin and has stimulated the enzymes which metabolize FA a quantitative change in the lignin content would be expected with FA treatment. Accordingly, significant increase in the lignin content was observed in roots treated with FA (Table 3.4). An increase upto 4% and 16% was observed with 0.5mM and 1mM FA respectively while increase in FA concentration (1.5mM-3mM) increased the activity to 27%-56% increase in lignin content in roots suggesting the FA utilization in lignin synthesis. In contrast to roots, leaves exhibited a decrease lignin content. FA, (0.5mM-3mM) resulted in 7% to 23% reduction in lignin levels (Table 3.4).

Discussion

The results presented in this study clearly showed FA induced changes in the phenylpropanoid metabolism of maize seedlings. Though a number of biotic and abiotic factors are known to regulate the activity of PAL, the important regulaion in plants is observed with some of the intermediate phenylpropanoid metabolites. Among these t-cinnamic acid the immediate product of PAL action known to regulate the endogenous levels of PAL via feed back inhibition ((Jones 1984, Jorrin *et al* 1990, Sato *et al* 1982, Shields *et al* 1982). Jorrin *et al* (1990) reported the inhibition of PAL activity with p-coumaric acid, caffeic acid, ferulic acid and sinapic acid at concentrations above 10mM. However, the effectiveness of these compounds as inhibitors of PAL has been lower than cinnamic acid and further more, among the cinnamic acid derivatives FA has been found to be least sensitive for PAL inhibition. In contrast, Shields *et al* (1982) observed no significant decrease in PAL activity even with concentrations higher than 5mM FA. Further more these regulations

Fig. 3.



Relationship between phenylalanine ammonialyase activity and phenolic content in roots of eight days old maize seedlings treated with different concentrations of ferulic acid. A good correlation was observed (r= 1.06).

Table 3.4

Effect of ferulic acid on lignin content in leaves and roots of maize seedlings

Ferulic acid (mM)	Lignin % / dry wt.	
	Leaves	**Roots**
Control	37.63+4.65	42.52+5.26
0.5	37.69+3.29 (+0.1)	44.49+3.77 (+4.6)
1.0	34.77+3.96 (-7.6)	49.55+4.67 (+16.5)
1.5	33.13+4.42 (-11.9)	54.25+4.00 (+27.5)**
2.0	33.94+5.36 (-9.8)	57.60+6.97 (+35.4)*
2.5	31.05+4.13 (-17.4)	60.33+5.84 (+41.8)*
3.0	28.66+3.46 (-23.8)	66.55±6.65 (+56.5)*

Figures in parentheses are % of increase (+)/ decrease (-) over control
* $P < 0.001$. ** $P < 0.005$

were mostly observed under *in vitro* conditions. However, with in the plants the levels of cinnamic acid will be simultaneously altered due to diversion to other phenylpropanoid metabolic processes. Correspondingly, present study also showed a significant increase in activity of PAL activity with FA which is a derivative of cinnamic acid (Table 3.1). These results clearly suggests that though the activity of PAL is known to be regulated by its product to some extent but under *in vivo* conditions there will be continuous and rapid diversion of these phenylpropanoid intermediates into the lignin synthesis as most of these acts as precursors for lignin. This possibility is further evident from an increase in the activity of CAD (Table 3.2) which catalyzes the alcohol to aldehydes of lignin precursors. The increase in CAD might have utilized these intermediate metabolites leading to more accumulation of lignin as observed in roots of treated seedlings (Table 3.4). Such induction of CAD has been observed earlier with a number of biotic and abiotic factors along with an increase in the phenylpropanoid metabolites (Grand *et al* 1985, Mitchell *et al* 1994). These results suggest that application of additional FA might have activated the enzymes of PAL and CAD to utilize these lignin precursors leading to synthesis of lignin and phenolic compounds. In contrast to roots, a decrease in lignin content has been observed in leaves of FA treated seedlings (Table 3.4) probably due to the transport of lignin precursors to the shoots thus accumulation of lignin in shoots rather than leaves as was observed earlier (Shann and Blum 1987b). Induction of PAL along with an increase in phenolic compounds and lignin has been observed under various stress conditions (Berlin and Widholm 1977, Cahill and Mc Comb 1992, Claudot *et al* 1992, Cvikrova *et al* 1991, Duke and Hoagland 1978, Hoagland *et al* 1978, Notsu *et al* 1994). In addition, many of the phenylpropanoid intermediates especially a number of phenolic compounds are known to act as substrates for various peroxidase (Spiker *et al* 1992) which catalyzes the oxidative polymerization of lignin precursors to lignin (Spiker *et al* 1992, Zieslin and Ben-Zaken 1991).

This is further evident from the present study where in an increase in the peroxidase activity has been observed with FA treatment in both leaves and roots (refer to part III, chapeter 2, Table 2.1, 2.2). Inhibition in plant growth with increased phenolic compounds is well documented earlier (Duke and Hoagland 1978, Kefeli and Kutacek 1977). In addition to phenolic compounds, the increase in lignin also known to reduce the growth of plants by rigidifying the cell walls (Kamisaka *etal* 1990, Tan *et al* 1991, 1992).

These results lead to conclusion, that FA inhibited growth of the maize seedlings could be due to an increase in the PAL and CAD activity resulting in an increase in lignin and phenolic compounds which rigidy the cells and modify a number of metabiloc processes associated with growth.

CHAPTER IV

Influence of ferulic acid on CO_2 assimilation, photosynthetic electron transport, fluorescence emission and photophosphorylation of maize thylakoids.

Introduction

Allelochemicals released into the soil are known to cause considerable reduction in crop yield. Earlier, significant decrease in the rice yield in Taiwan has been attributed to phenolic compounds released from the previous crop residue during decomposition (Chou and Lin 1976, Chou *et al* 1984). The eco-physiological significance of these phenolic compounds have been discussed earlier (refer to part I).

A number of phenolic compounds, coumarin and flavonoids are reported to inhibit CO_2 dependent evolution of O_2 and photophosphorylation of isolated thylakoids (Arntzen *et al* 1974, Einhellig *et al* 1970, Moreland and Novitzky, 1987, Tissut *et al* 1980). Kaemferol is known to inhibit photophosphorylation capacity of pea thylakoids by acting as energy transfer inhibitor (Arntzen *et al* 1974). Interference of phenolic compounds with chloroplast thylakoid membranes is reported by Muzafarov *et al* (1988). Phenolic compounds, viz., salicylic acid, tannic acid, benzoic acid and their derivatives are known to enhance at lower concentrations and subsequently curtail hill reaction in rice with rise in inhibitor concentration (Sharma and Singh 1987). However, the exact site (s) or mechanism of action of these chemicals is presently unknown. Many of the classical phenolic herbicides and substituted phenols are known to uncouple the electron transport from phosphorylation (Miyoshi *et al* 1990, Pfister and Schreiber 1983). However, whether the action of phenolic compounds mimic the action of herbicides on the processes of photosynthesis or not is unknown. Most of the earlier studies dealing with action of phenolic

compounds on photosynthesis were short term *in vitro* experiments using isolated thylakoids.

Information dealing with effects of FA on the function of chloroplasts *in vitro* and *in vivo* are rather scanty. Hence, the aim of the present study is to investigate the influence of single and multiple doses of FA on net CO_2 assimilation rates, stomatal conductance, electron transport rates, fluorescence emission of chloroplasts and photophosphorylation ability of the thylakoids to determine the site (s) and mechanism of FA action under *in vitro* and *in vivo* conditions.

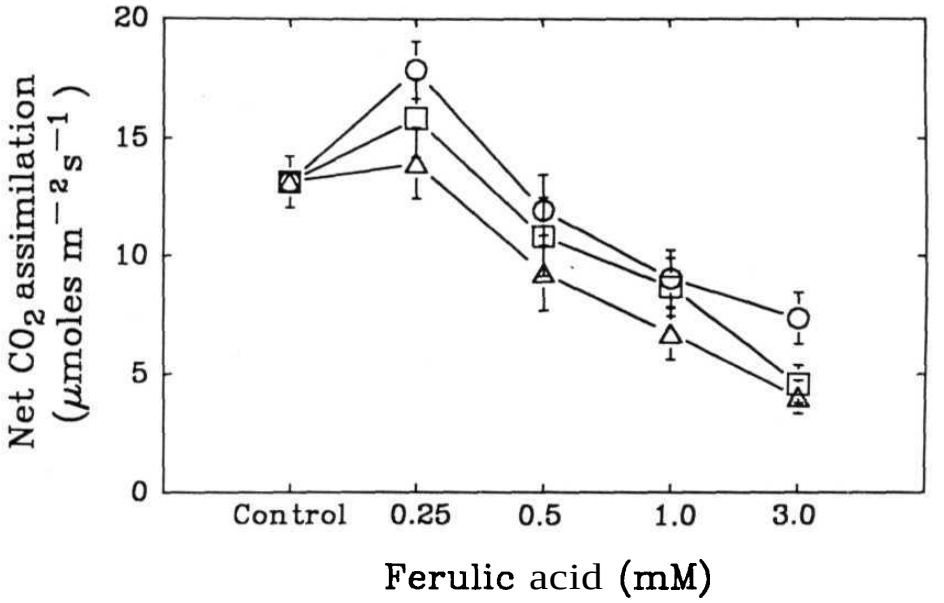
Results

Gas exchange measurements:

The results clearly indicate the interference of FA with photosynthetic processes. Treatment with 0.25mM FA increased the rate of CO_2 assimilation by 35% after 24h while, further increase in the duration of treatment reduced the percentage of increase to 20% and 5% after 48h and 72h respectively (Fig. 4.1). In contrast, 0.5mM, 1mM and 3mM FA caused significant reduction in the CO_2 assimilation rates depending on the duration of treatment. FA (0.5mM) reduced the rate by 9% (24h) and increase in the treatment time to 48h and 72h further reduced the rate by 17% and 29% respectively (Fig. 4.1). Similarly the increase in the concentration of FA (1mM) reduced the assimilation rate by 31%, 34% and 49%. High concentration of FA (3mM) drastically reduced assimilation rate by 44%, 65% and 69% after 24h, 48h and 72h respectively (Fig. 4.1).

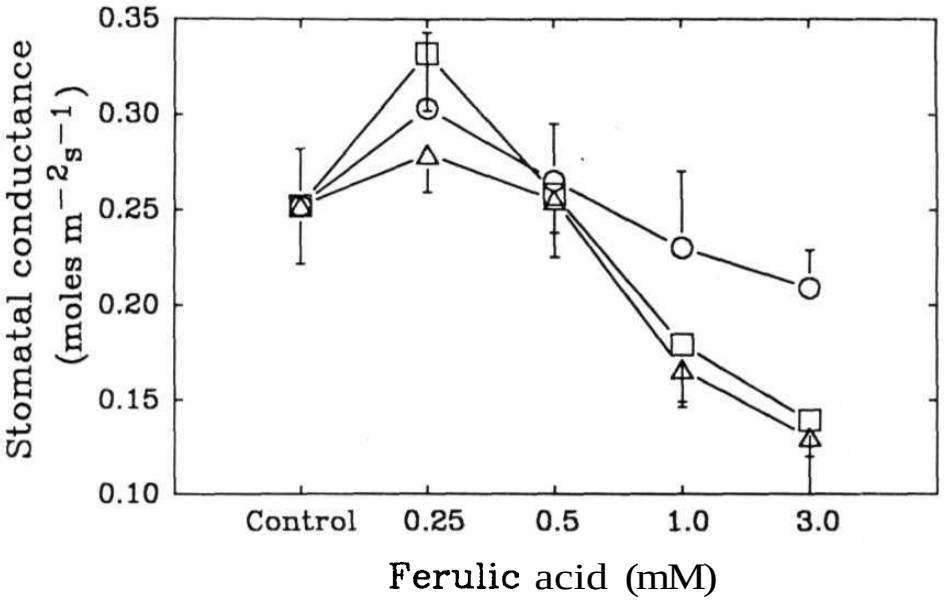
The observed reduction in assimilation rates correlated with stomatal conductance. Treatment with 0.25mM FA increased the stomatal conductance of leaves by 20%, 31% and 10% after 24h, 48h and 72h respectively (Fig. 4.2). However, 0.5mM FA treatment resulted 5% and 23% decrease in stomatal conductance after 24h and 48h respectively. Considerable inhibition in stomatal conductance was observed with 1mM and 3mM FA depending on

Fig. 4.1



Effect of ferulic acid on net CO_2 assimilation rate by leaves of maize seedlings treated with different concentrations for 24h (0), 48h (□), 72h (A).

Fig. 4.2



Effect of ferulic acid on stomatal conductance of leaves of maize seedlings treated with different concentrations for 24h (0), 48h (a), 72h (A).

concentration and duration of application of the inhibitor. 1mM FA inhibited the conductance rates by 8%, 28% and 34% respectively while 3mM FA further enhanced the attenuation of the rates by 17% 44% and 48% after 24h, 48h, and 72h of treatment respectively (Fig. 4.2).

FA treatment had no siginificant effect (increase or decrease) on the chlorophyll content after 24h, 48h and 72h of treatment (Table 4.1).

Electron transport rates:

Having observed a significant reduction in CO_2 assimilation, FA effect on electron transport rates of PS I and PS II and whole chain were examined. Treatment with 0.25mM showed a negligible increase in whole chain activity (5%, 10% and 13% after 24h, 48h and 72h respectively). However, increase in the FA concentration showed a significant inhibition in whole chain activity (Fig. 4.3). Treatment with 0.5mM FA inhibited the activity by 33%, 35% and 24% after 24h, 48h and 72h respectively. The elevation in FA concentration to lmM caused 33%, 35% and 37% reduction and 3mM FA further decreased the activity by 36%, 37% and 50% after 24h, 48h, and 72h respectively (Fig. 4.3).

PS I supported electron transport (DCPIP --> MV) activity increased with 0.25mM FA treatment by 13%, 8% and 6% respectively, while 0.5mM treatment increased the activity by 11%, 7% and 8% at the end of 24h, 48h and 72h respectively. In contrast, lmM and 3mM FA inhibited the activity. Though 1mM FA showed reduction in activity (9%, 9% and 5% after 24h, 48h and 72h respectively) the inhibition was not significant compared to control. Further rise in the FA concentration (3mM) lowered the PS I electron transport by 11%, 17% and 25% after 24h, 48h and 72h treatment respectively (Fig. 4.4) indicating that PS I electron transport was not as sensitive to FA treatment when compared to that of whole chain.

Compared to PS I activity, 0.25mM and 0.5mM FA significantly increased the PS II activity. Application of 0.25mM FA increased the PS II electron transport by 41%, 44% and 32% respectively while 0.5mM enabled

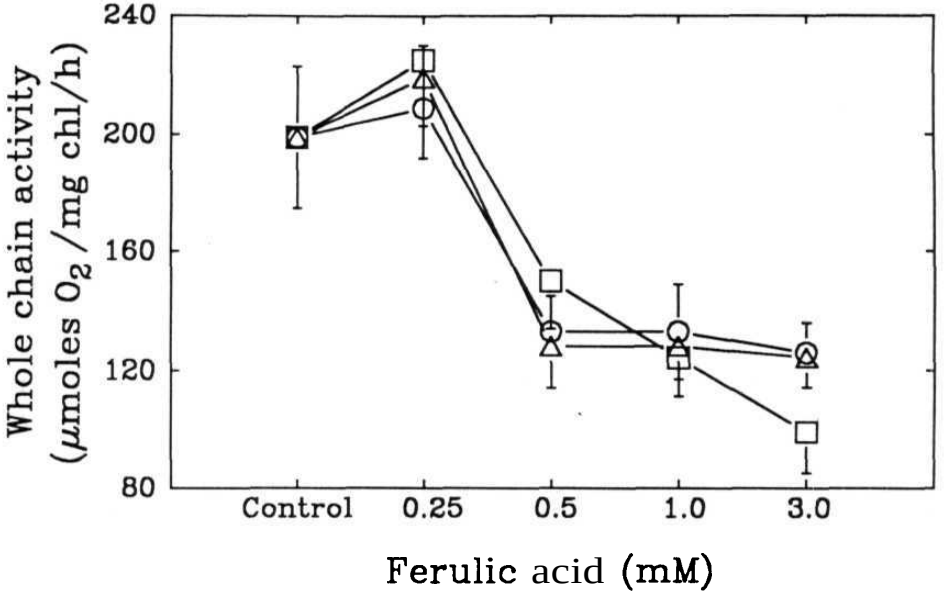
fable 4.1

Effect of ferulic acid on chlorophyll content in leaves of maize seedlings

Ferulic acid (mM)		Chl cm^{-2}			
		Chl a	Chl b	Total chl	Chl a/b
24h	Control	15.1 + 1.1	4.4+0.6	19.5+1.7	4.0+0.2
	0.25	16.7+1.3	4.1+0.6	20.9+1.6	3.7+0.1
	0.5	16.6+1.6	3.7+0.3	20.4+1.9	4.3+0.4
	1.0	16.2+1.6	4.2+0.7	20.4+1.7	3.9+0.2
	3.0	15.8+1.1	3.9+0.9	19.8+1.3	3.7+0.3
48h	Control	15.1 + 1.1	4.4+0.6	19.5+1.7	4.0+0.4
	0.25	18.3+1.6	5.1+0.5	23.5+1.6	4.1+0.4
	0.5	15.9+1.4	4.0+0.2	20.0+1.7	3.9+0.2
	1.0	16.6+1.7	4.3+0.2	20.9+1.9	4.2+0.5
	3.0	15.4+1.6	4.3+0.2	19.7+1.6	3.8+0.1
72h	Control	15.1+1.1	4.4±0.6	19.5+1.7	4.0±0.2
	0.25	15.9+1.0	4.2±0.2	20.1 + 1.2	3.8+0.2
	0.5	14.9+1.1	3.7+0.3	18.7+1.7	3.8+0.1
	1.0	17.1 + 1.8	3.4+0.3	20.5+1.1	3.8+0.1
	3.0	17.8+1.2	5.0+0.5	20.4+1.2	3.6+0.1

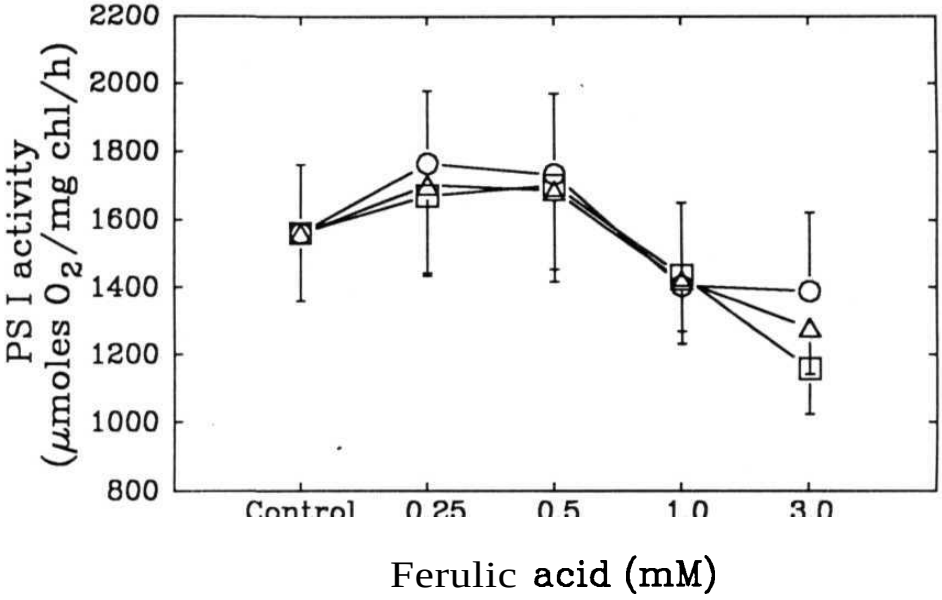
The changes are not significant compared to control

Fig. 4.3



Ferulic acid effect on whole chain electron transport activity of thylakoids isolated from leaves of eight days old maize seedlings treated with different concentrations for 24h (0), 48h (a), 72h (A).

Fig. 4.4



Ferulic acid effect on PS I electron transport activity of thylakoids isolated from leaves of eight days old maize seedlings treated with difTerent concentrations for 24h (0), 48h (A), 72h (a).

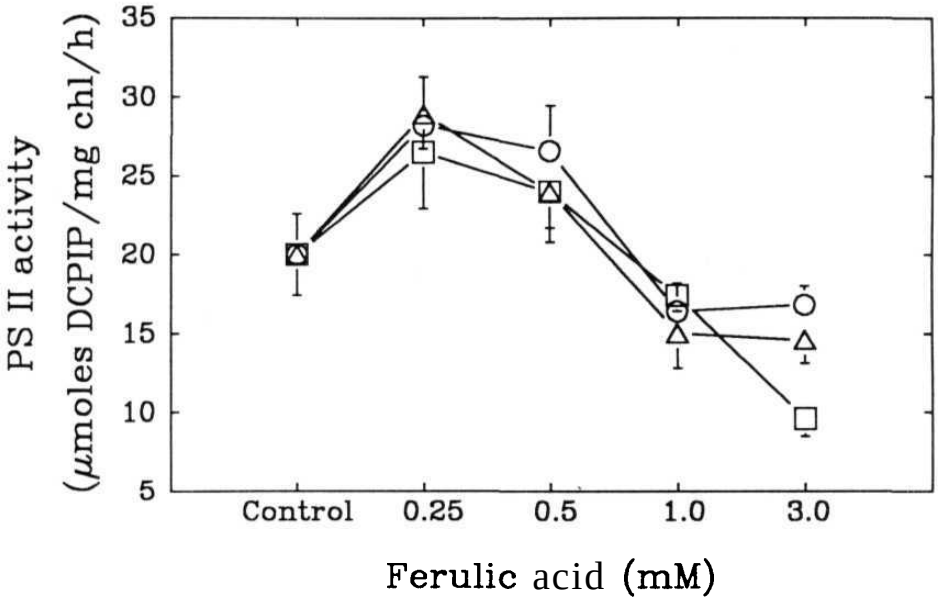
33%, 20% and 20% rise at the end of 24h, 48h and 72h respectively (Fig. 4.5). In contrast, 1mM FA decreased the activity of PS II by 18%, 25% and 13% respectively whereas, 3mM FA caused 16%, 27% and 52% inhibition at the end of 24h, 48h and 72h respectively (Fig. 4.5).

In order to locate the possible site of inhibition of the transport of electrons through PS II, DCPIP photoreduction was measured using exogenous electron donor (10mM NH_2OH) (Table 4.2). Thylakoids isolated from 0.25mM FA treated plants exhibited an increase (14%) in PS II activity. Whereas, 0.5mM, lmM and 3mM FA treated plants exhibited reduction in the activity (16%, 25% and 41% respectively) (Table 4.2). In contrast, the thylakoids of FA treated plants when incubated with 10mM NH_2OH showed negligible decrease in activity suggesting the restoration of PS II activity by NH_2OH. (Table 4.2).

PS I, PS II and whole chain activities were also measured in the presence of different concentrations of FA under *in vitro* conditions by incubating the thylakoids with FA for different durations. The measurement of degree of inhibition would be useful in characterizing the binding site of the inhibitor. Whole chain electron transport decreased by 12%, 25% and 28% with 0.25mM FA at the end of 10, 20, and 30 min of incubation respectively. Similarly 0.5mM FA inhibited whole chain electron transport by 18%, 29% and 29% at the end of 10min, 20min and 30min of incubation. lmM and 3mM FA inhibited electron transport by 39% and 47% respectively at the end of lOmin of incubation. while lmM FA decreased the activity by 50% and 51% at the end of 20min and 30min respectively. Inhibition upto 66% was observed with 3mM FA at the end of 20min and 30min indicating that the maximum inhibition occurred after 20min of treatment. Further rise in incubation time had no effect on the extent of inhibition (Fig. 4.6).

The decrease in PS I electron transport with 0.25mM (13%) and 0.5mM FA (17%), after 30min of incubation, was non-significant. FA (lmM) reduced the PS I activity by 35%, 40% and 45% at the end of lOmin, 20min and 30min

Fig. 4.5



Ferulic acid effect on PS II electron transport (DCPIP photoreduction) activity of thylakoids isolated from leaves of eight days old maize seedlings treated with different concentrations for 24h (0), 48h (A), 72h (a).

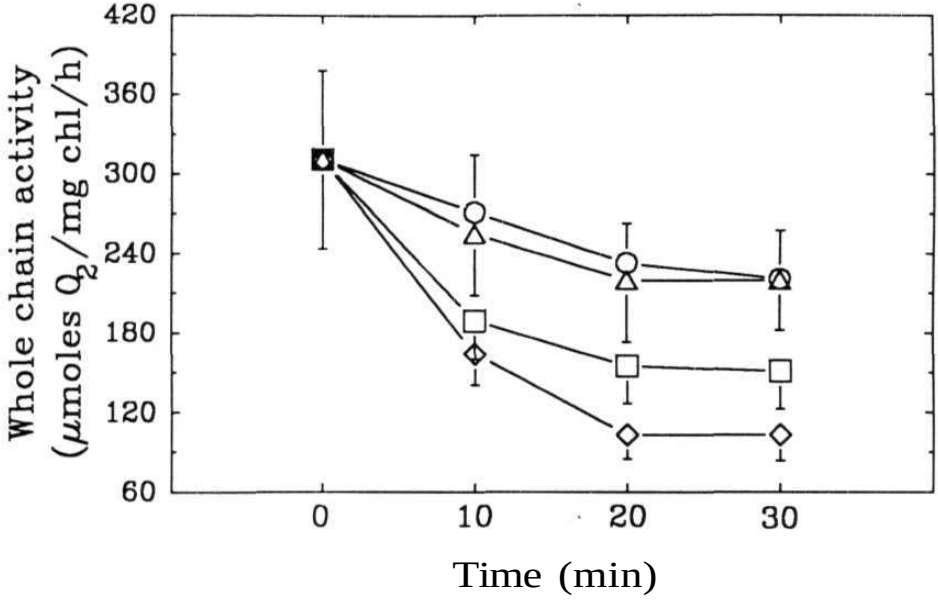
Table 4.2

Effect of ferulic acid on PS II elctron transport in the presence and absence of 10mM NH_2OH.

Ferulic acid (mM)	µmoles DCPIP reduced/mg chl/h	
	$-NH_2OH$	$+NH_2OH$
control	23.2+1.2	31.6+6.2
0.25	26.6+2.8 (+14.4)	31.1+5.6 (-1.5)
0.5	19.4+2.2 (-16.4)	31.3+3.6 (-0.9)
1.0	17.4+2.2 (-25.0)**	29.6+5.7 (-6.2)
3.0	13.5+2.9 (-41.9)*	28.7+5.0 (-9.0)

Figures in parentheses are % of increase (+)/ decrease (-) over control
* $P < 0.001$. ** $P < 0.005$

Fig. 4.6



Time course of inhibition of whole chain electron transport of maize thylakoids incubated for 10min, 20min and 30min at 4° C with different concentrations of ferulic acid.
0.25mM (0), 0.5mM (A), 1mM (a), 3mM (0).

respectively. Similarly, 3mM FA showed a time dependent decrease in the PS 1 activity i.e. 77%, 82% and 84% at the end of 10min, 20min and 30min respectively suggesting that 1mM and 3mM FA had inhibited the activity of PS I with in 10min of incubation and increase in time of incubation (20 and 30min) did not show much increase in inhibition (Fig. 4.7).

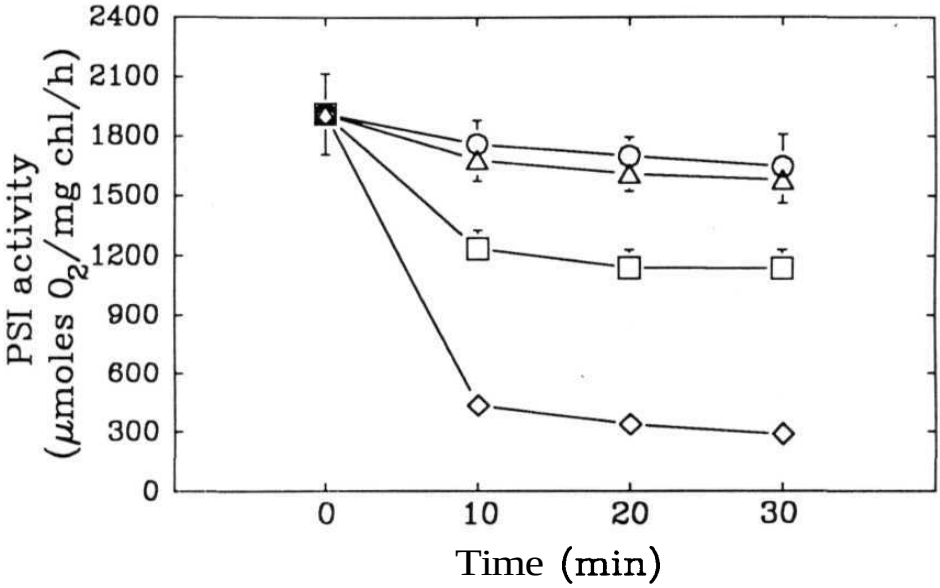
In contrast to PS I elctron transport, PS II was found more sensitive even at low concentrations of FA (0.25mM and 0.5mM). A significant reduction was observed within lOmin of treatment. Thylakoids incubated with 0.25mM FA exhibited 31%, 33% and 41% reduction while 0.5mM FA caused 35%, 40% and 45% decrease in PS II activity at the end of 10, 20 and 30min of incubation respectively (Fig. 4.8). The inhibition was further increased on the application of lmM FA (37%, 47% and 56%). FA (3mM) caused 41%, 49% and 69% reduction at the end of lOmin, 20min and 30min of incubation respectively. Thus FA had exerted a maximum effect on PS II activity which is a concentration and time dependent (Fig. 4.8).

Unlike the *in vivo* effect, FA *in vitro* did not increase the activity of either PS I, PS II and whole chain rates with low FA concentrations. Under *in vitro* conditions, all the tested concentrations of FA showed a time dependent decrease in the activity. PS II was reduced considerably than PS I *(in vitro* and *in vivo)* though the extent of inhibition varied.

Chlorophyll fluorescence emission:

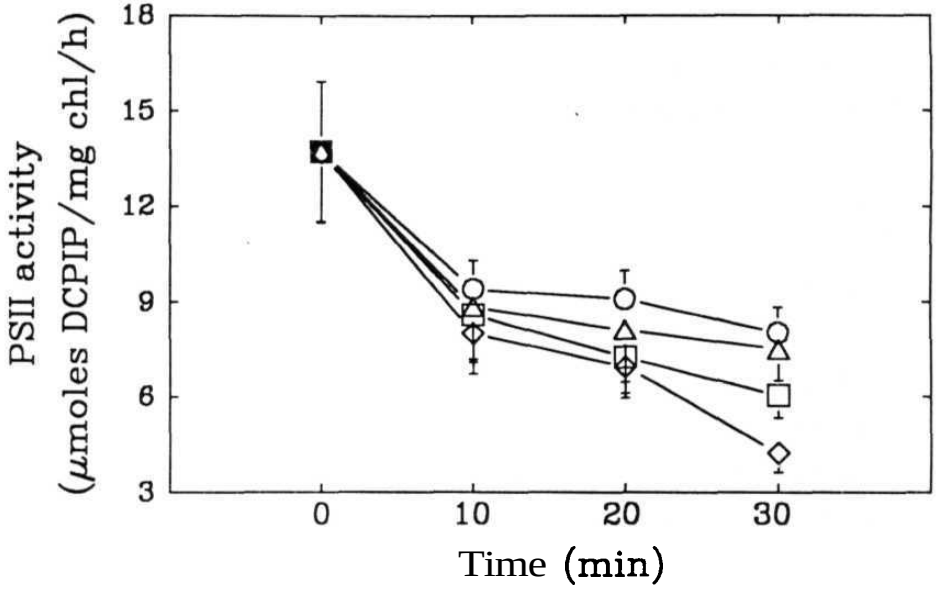
Chrolorophyll fluorescence emission at room temperature is known to provide information regarding the intrinsic heterogenity in both the structure and function of the various chlorophyll protein complexes which constitute the building blocks of photosynthetic membranes, as well as the very rapid exchange of energy among these complexes. At room temperature, the emission mostly consists of a single band centered around 685nm and at 77° K, a characteristic pattern of 2 bands located at 685nm and 735nm are observed. These have been named as F_{685} and F_{735} respectively.

Fig. 4.7



Time course of inhibition of PS I electron transport of maize thylakoids incubated for 10min, 20min and 30min at 4° C with different concentrations of ferulic acid. 0.25mM (0), 0.5mM (A), 1mM (a), 3mM (◇).

Fig. 4.8



Time course of inhibition of PS II electron transport of maize thylakoids incubated for 10min, 20min and 30min at 4° C with different concentrations of ferulic acid. 0.25mM (0), 0.5mM (A), 1mM (•), 3mM (◇).

The room temperature fluorescence emission spectra of chloroplasts isolated from control and FA treated plants for 24h, 48h and 72h excited at 490nm are shown in Figs 4.9-4.11. Chloroplasts isolated from FA treated plants (24h) showed quenching in the fluorescence emission of PS II, however the inhibition in the intensity is not significant on treatment with FA (Fig. 4.9).

An increase in the duration of treatment (48h) decreased the fluorescence emission of PS II, however the inhibition was significant only with 3mM FA treatment (Fig. 4.10). Further increase in the duration of FA treatment (72h) resulted in a similar effect as that of 48h treated plants (Fig. 4.11).

Seedlings treated for 24h showed a significant decrease in the fluorescence intensity of PS I (F735) at 77° K (Fig. 12). In addition to F735, the F_{685} peak which originates from PS II also reduced on treatment with FA (Fig. 4.12).

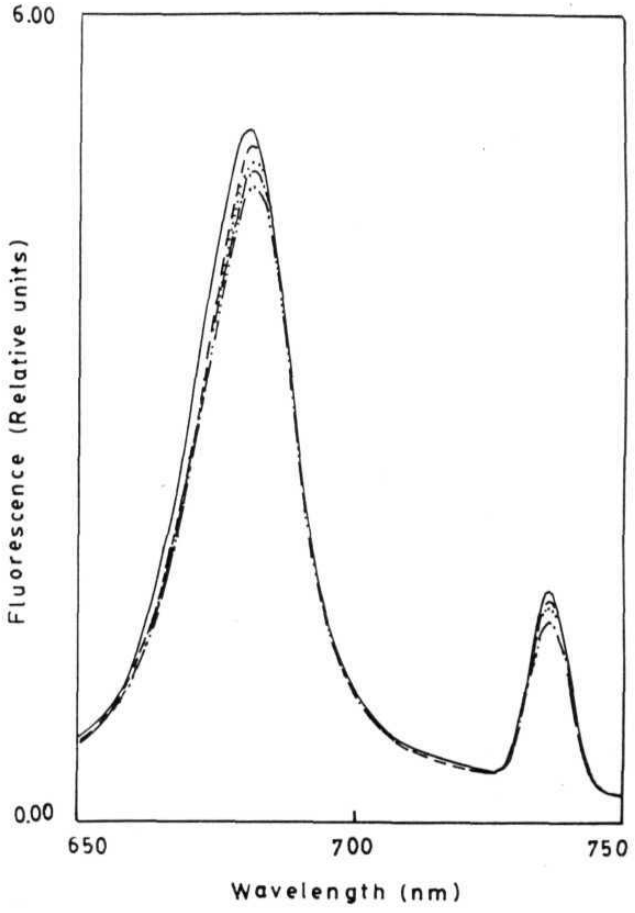
An increase in the duration of treatment (48), FA significantly inhibited the fluorescence intensity from PS I and PS II in a concentration dependent manner (Fig. 4.13). Similarly seedlings treated for 72h exhibited a significant reduction (upto 75%) in the fluorescence emission from both PS I and PS II even with low concentrations of FA (0.25mM and 0.5mM) tested (Fig. 4.14).

The F_{685}/F_{735} ratio was lowered at the completion of 24h, 48h and 72h of FA application (Table 4.3). It was observed that the F_{685} at room temperature also decreased under *in vitro* conditions in a time dependent manner on incubation of chloroplasts with 1mM FA (Fig. 4.15). FA induced inhibition was completely restored when the chloroplasts were incubated with 1mM FA plus 10mM NH2OH, an artificial electron donor to PS II (Fig. 4.16).

Photophosphorylation:

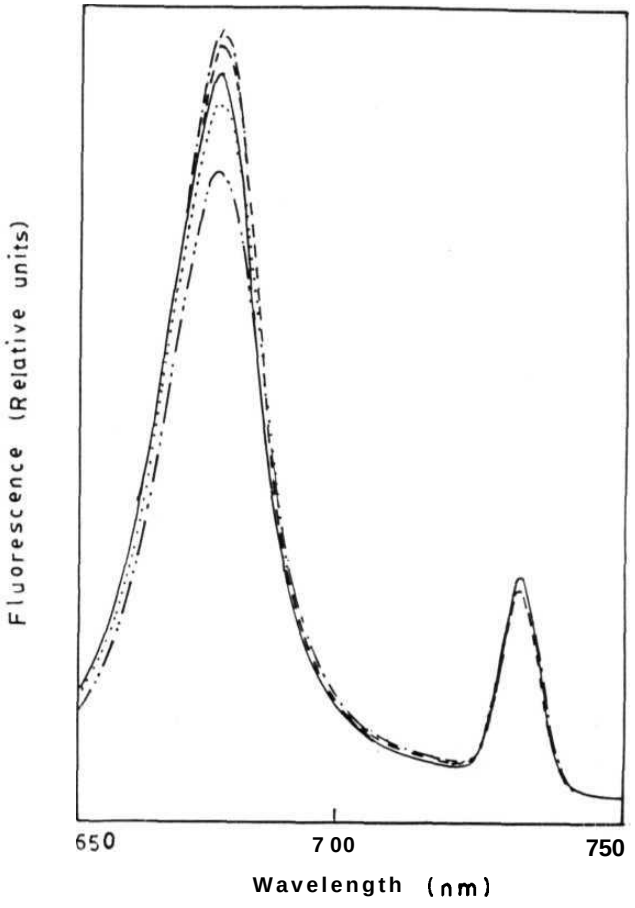
During photosynthetic electron transport, protons are pumped into the thylakoid lumen and a pH difference (A pH) builds up across the thylakoid membrane which is the driving force for the synthesis of ATP. Photosynthetic

Fig. 4.9



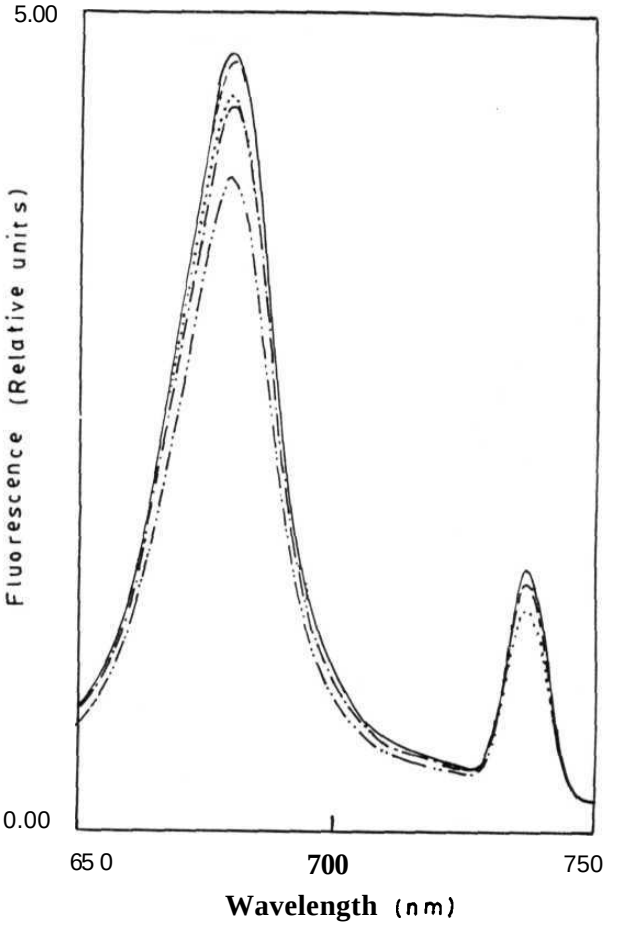
Room temperature fluorescence emission spectra of chloroplasts isolated from control and ferulic acid (24h) treated maize seedlings. Other details are given in materials and methods. Control (——), 0.25mM (——), 0.5mM (-——), 1mM (–·–·–), 3mM (——).

Fig. 4.10



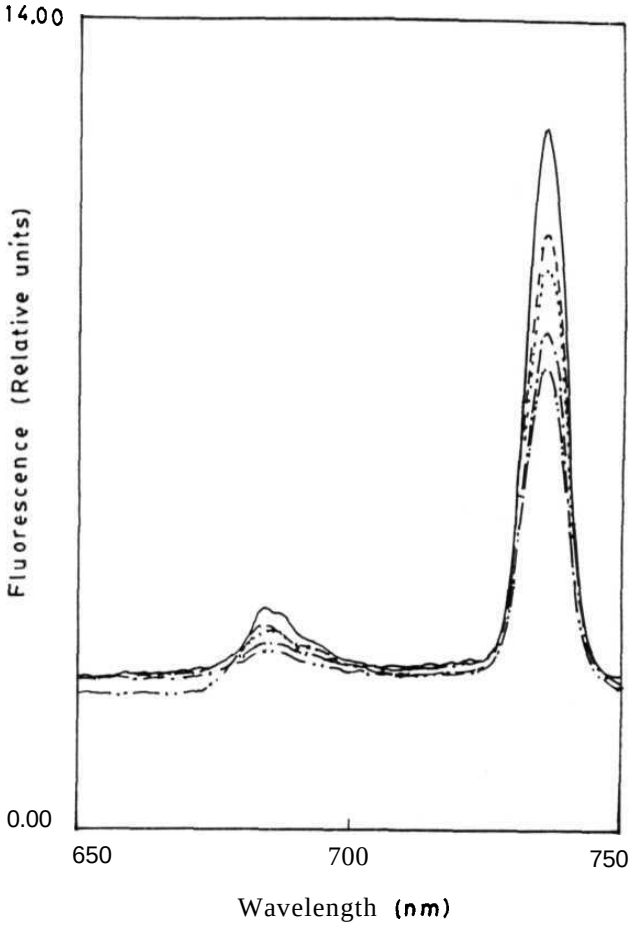
Room temperature fluorescence emission spectra pf chloroplasts isolated from control and ferulic acid (48h) treated maize seedlings. Other details are given in materials and methods. Control (—), 0.25mM (---), 0.5mM (. . . .), 1mM (——), 3mM (——).

Fig. 4.11



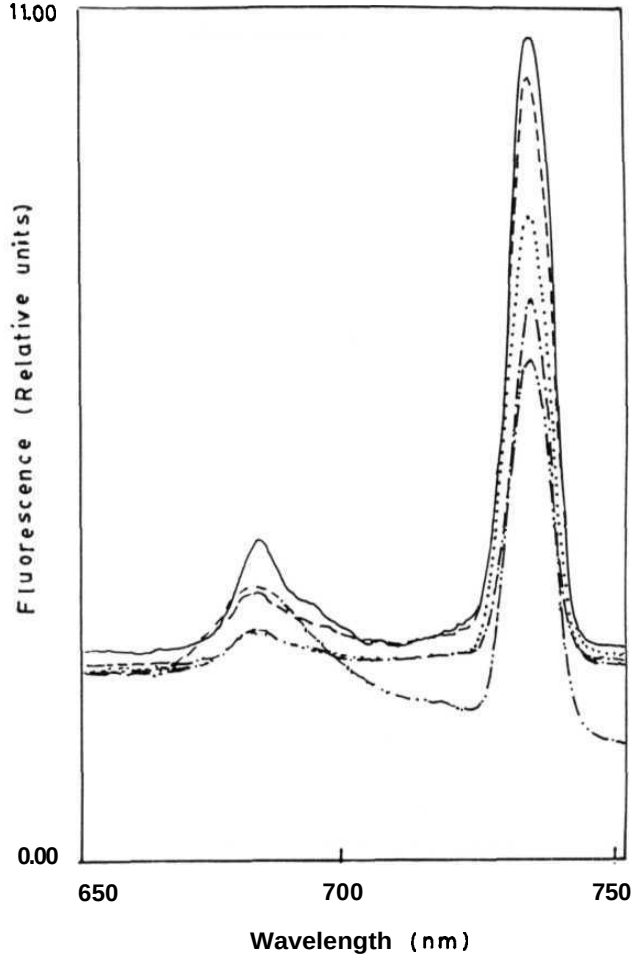
Room temperature fluorescence emission spectra of chloroplasts isolated from control and ferulic acid (72h) treated maize seedlings. Other details are given in materials and methods.
Control (—), **0.25mM (——)**, 0.5mM **(·——)**, **1mM (——)**, **3mM (——).**

Fig. 4.12



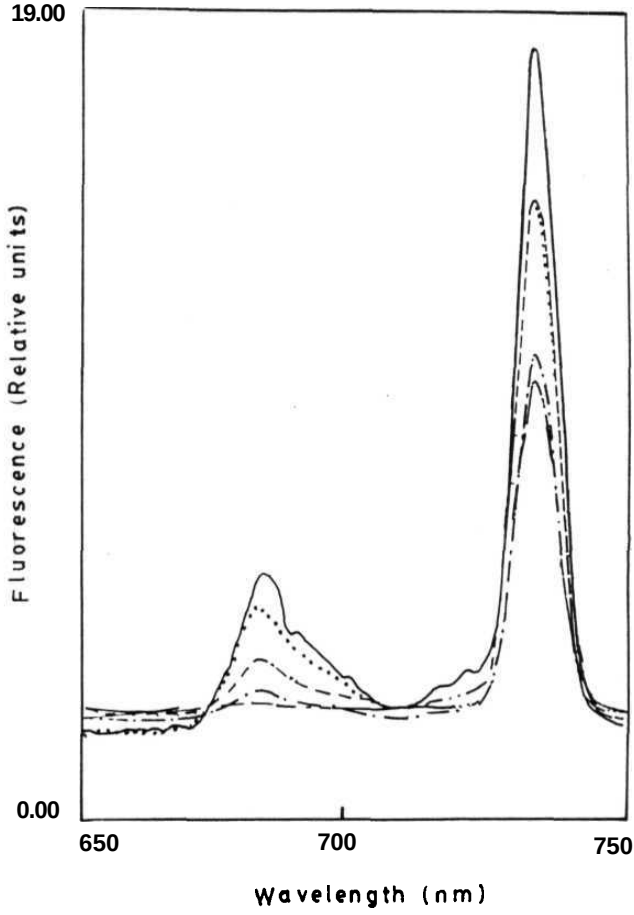
Low temperature (77° K) emission spectra of chloroplasts iso-lated from control and ferulic acid **treated** (24h) maize seed- lings. Details are given in materials and method.
Control (—), **0.25mM** (——), **0.5mM** (——), **1mM** (——), 3mM (-·—·-).

Fig. 4.13



Low temperature (77° K) emission spectra of chloroplasts isolated from control and ferulic acid treated (48h) maize seed- lings. Details are given in materials and methods.
Control (—), 0.25mM (---), 0.5mM (- - - -), 1mM (-——-), 3mM (-——).

Fig. 4.14



Low temperature (77° K) emission spectra of chloroplasts isolated from control and **ferulic** acid treated (72h) maize seed- lings. Details are given in materials and methods.

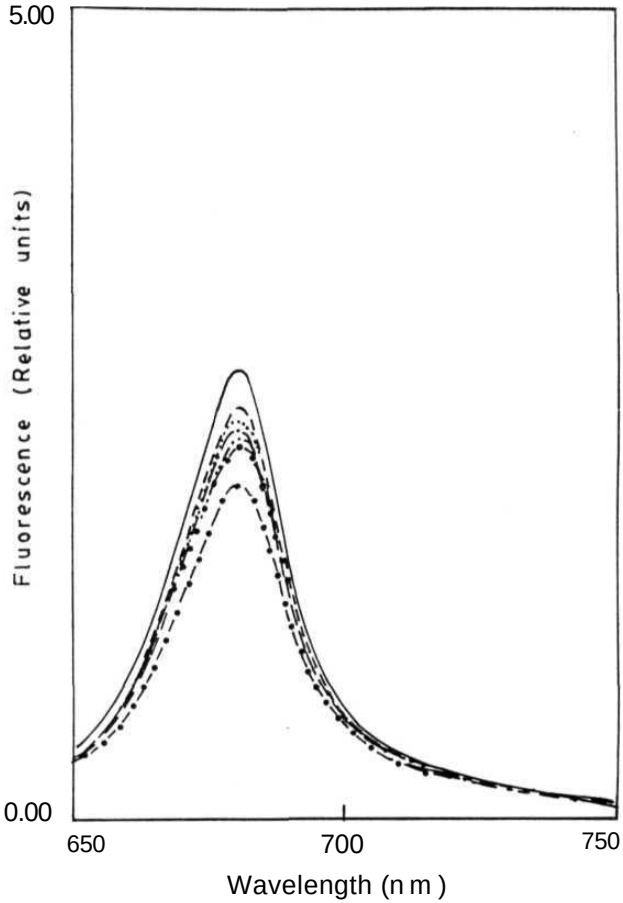
Control (——), **0.25mM** (---), **0.5mM** (——), **1mM** (—.-), 3mM (·····).

Table 4.3

Effect of ferulic acid on the F_{685}/F_{735} ratio of chlorophyll fluorescence emission at room temperature

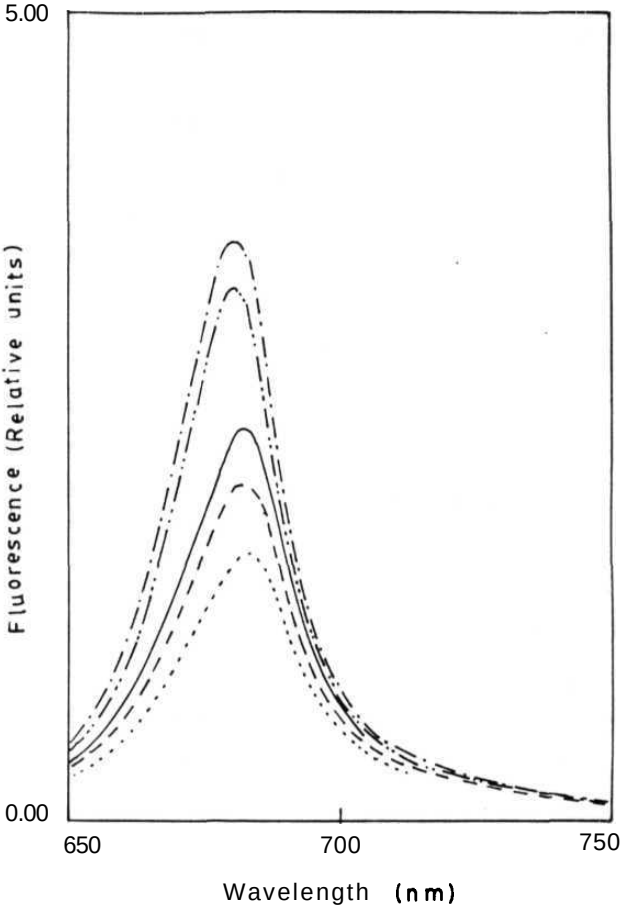
Ferulic acid (mM)	F_{685}/F_{735} ratio		
	24h	48h	72h
Control	0.39	0.48	0.31
0.25	0.37	0.41	0.34
0.5	0.36	0.41	0.27
1.0	0.35	0.41	0.26
3.0	0.32	0.35	0.25

Fig. 4.15



Room temperature fluorescence emission spectra of chloroplasts incubated with 1mM ferulic acid for different time periods. Control (—), 10min (——), 20min (·——), 30min (——), 40min (·——), 50min (·-·-·), 60min (·-·-·).

Fig. 4.16



Room temperature fluorescence emission spectra of chioroplasts incubated with 1mM ferulic acid for 30min (--), 60min (---) and 1mM ferulic acid plus 10mM NH_2OH for 10min (——) and 30min (——). Control (——)

control of electron transport is regulated through the generation of pH gradient. Since FA had significantly decreased the rates of electron transport of both photosystems, photophosphorylation ability of the chloroplasts treated with FA was examined.

PMS catalyzed cyclic photophosphorylation was significantly altered by FA treatment (Table 4.4). FA, 0.25mM increased the rate of cyclic photophosphorylation by 23%, 37% and 21% on treatment for 24h, 48h and 72h respectively. FA treatment (0.5mM) increased the rates by 8%, 44% at the end of 24h and 48h. However, increase in the duration of treatment (72h), 0.5mM FA reduced the rate by 15%. In contrast to 0.25mM and 0.5mM FA, lmM and 3mM FA caused significant inhibition in the rates of cyclic photophosphorylation which showed a time dependent decrease. FA (1mM) caused 13%, 28% and 40% reduction and 3mM FA caused 28%, 43% and 48% reduction at the end of 24h, 48h and 72h treatment (Table 4.4).

FA treatment decreased the rate of non-cyclic photophosphorylation. FA (0.25mM) initially increased the rate by 19% after 24h when the duration of treatment increased to 48h and 72h, the rate of non-cylic phosphorylation was decreased by 16% and 11% respectively where as, 0.5mM FA decreased the rate by 8%, 24% and 34% at the end of 24h, 48h and 72h respectively. Further rise in FA concentration (lmM and 3mM) showed a decrease in non-cyclic photophosphorylation. Reduction upto 19%, 31% and 45% with lmM and 28%, 47% and 57% with 3mM after 24h, 48h and 72h treatment was observed (Table 4.5). Non-cyclic phosphorylation was more inhibited compared to that of cyclic phosphorylation.

Light activated Mg^{2+} ATPase of thylakoids decreased with FA treatment. However, the inhibition was significant only with 3mM FA. The lower concen-trations of FA application (0.25mM and 0.5mM) caused 5-11% and 3-16% reduction in activity. Reduction to the extent of 11%-24% was

Table 4.4

Effect of ferulic acid on cyclic photophosphorylation of maize thylakoids

Ferulic acid (mM)	μ moles of phosphate used/mg chl/h		
	24h	48h	72h
Control	25.8+4.5	25.3+5.1	23.1+2.2
0.25	31.9+7.1	34.9+3.2	28.0+2.0
	(+23.7)	(+37.8)*	(+21.0)
0.5	28.0+3.0	36.5+5.3	19.6+2.8
	(+8.68)	(+44.1)*	(-15.3)
1.0	22.3+1.3	18.3+2.4	13.8+2.7
	(-13.2)	(-28.0)**	(-40.2)*
3.0	18.5+2.1	14.2+1.6	11.8+1.7
	(-28.1)**	(-43.3)*	(-48.7)*

Figures in parentheses are % of increase (+) or decrease (-) over control
* $P < 0.001$ ** $P < 0.005$

Table 4.5

Effect of ferulic acid on non-cyclic pliotophosphorylation of maize thylakoids

Ferulic acid (mM)	μ moles of phosphate used/mg chl/h		
	24h	48h	72h
Control	14.5+2.2	15.7+1.7	16.1 + 1.2
0.25	17.3+3.2	13.0+1.8	14.3+1.5
	(+19.4)	(-16.8)	(-11.2)
0.5	13.2+2.1	11.9+1.3	10.4+1.6
	(-8.8)	(-24.0)	(-34.9)*
1.0	11.7+1.6	10.7+1.4	8.8+1.7
	(-19.28)	(-31.52)*	(-45.0)*
3.0	10.4+1.1	8.2+1.3	6.8+1.0
	(-28.8)**	(-47.3)*	(-57.5)*

Figures in parentheses are % of increase (+) or decrease (-) over control
* $P < 0.001$ •• $P < 0.005$

observed with 1mM FA while 19%-24% reduction was noticed with 3rnM FA at the end of 24h, 48h and 72h treatment (Table 4.6).

Trypsin activated Ca^{2+} ATPase exhibited significant inhibition only at higher concentrations of FA (3mM FA treatment for 72h). Lower concentrations of FA, i.e. 0.25mM slightly increased the activity (1.7%) whereas 0.5mM FA caused 0.8%, 2.7% and 15.3% reduction after 24h, 48h and 72h treatment. Ca^{2+} ATPase was more resistant to FA treatment upto 48h treatment and even the high concentrations of FA (1mM and 3mM) could not induce any significant changes in Ca^{2+} ATPase (Table 4.7). It was observed that Ca^{2+} ATPase activity could withstand FA treatment compared to Mg^{2+} ATPase activity (Table 4.6 and 4.7).

Discussion

A differential regulation of photosynthetic assimilation rates and stomatal conductance at lower and higher concentrations of FA application observed in maize sedlings is in accordance with earlier studies by other workers (Einhellig *et al* 1970, Lodhi and Nickell 1973, Patterson 1981). The dissimilarity in the mode of action of FA at lower and higher concentrations of FA is presently unknown.

The co-relation observed between stomatal conductance and photosynthetic rates (Fig. 4.1 and 4.2) on FA treatment suggests that regulation of stomatal conductance might be responsible for observed changes in photosynthetic rates. A correlation between the CO_2 assimilation rates and stomatal conductance has beeen observed earlier (Bunce *et al* 1992). Previous studies with FA and other phenolic acids like p-coumaric acid, cinnamic acid and their derivatives demon-strated the ABA mediated closure of stomata (Apte and Laloraya 1982, Laloraya *et al* 1986, Manthe *et al* 1992, Purohit *et al* 1991, 1994, Rai *et al* 1986) which is reversed by these compounds at low concentrations by increasing the K^+ uptake by guard cells (Laloraya *et al* 1986). However, higher concentrations of these phenolics were shown to

Table 4.6

Effect of ferulic acid on light activated Mg^{2+} ATPase activity of maize thylakoids

Ferulic acid (mM)	μ moles of phosphate /mg chl/h		
	24h	48h	72h
Control	28.6+6.9	27.4+1.9	26.9+1.8
0.25	29.2+7.2	26.0+2.7	23.8+1.8
	(+2.00)	(-5.10)	(-11.5)
0.5	27.6+4.2	23.2+3.6	22.5+1.5
	(-3.50)	(-15.3)	(-16.3)
10	25.4+2.4	**21.4+1.2**	20.4+1.2
	(-11.1)	(-21.8)	(-24.1)
3.0	**23.0+1.2**	20.8+1.6	20.6+1.2
	(-19.5)	(-24.0)**	(-23.4)**

Figures in parentheses are % of increase (+) or decrease (-) over control

** $P < 0.005$

Table 4.7

Effect of ferulic acid on trypsin activated Ca^{2+} ATPase activity of maize thylakoids

Ferulic acid (mM)	μ moles of phosphate /mg chl/h		
	24h	48h	72h
Control	112+19	109+11	117+16
0.25	114+16	111+14	106+13
	(+1.7)	(+1.8)	(-9.40)
0.5	111+20	106+10	99+13
	(-0.3)	(-2.7)	(-15.3)
1.0	102+17	97+18	91+7
	(-8.9)	(-11.0)	(-22.2)
3.0	100+15	88+7	87+8
	(-10.7)	(-19.2)*	(-25.4)**

Figures in parentheses are % of increase (+) or decrease (-) over control

** $P < 0.005$

restricted the stomatal movement (Purohit *et al* 1991). Apart from interference of FA with stomatal functions, it is also known to decrease the water potential (Blum and Rebbeck 1989, Blum *et al* 1985a,b, Klein and Blum 1990) and increase the levels of ABA (Holappa and Blum 1991). These factors can also be attributed for the observed decrease in stomatal conductance in maize under FA treatment which correlated with photosynthetic rates.

In earlier studies the lowered photosynthetic assimilation due to the application of phenolic compounds has been attributed to the reduction in chlorophyll content (Buttery and Buzzell 1977, Chander *et al* 1988, Choudhary 1990, **Einhellig** and Rasmussen 1979, Patterson 1981, Mohnot and Choudhary 1990, Todorov *et al* 1992. In contrast to these previous studies in the present study maize seedlings treated with FA did not show any changes in the chlorophyll content (Table 4.1) which precludes the possibility of chlorophyll content as a limiting factor for the observed lower photosynthetic rates. Therefore the present work strengthens earlier notion that the rate of photosynthesis depends on manifold factors and not only on the chlorophyll content (Gabrielson 1984). The present study identifies that the regulation of photosynthesis by FA is at the photosystem level since the electron transport ability of PS I and PS II and whole chain were reduced to a considerable extent both under *in vivo* (Figs. 4.3- 4.5) and *in vitro* (Figs. 4.6-4.8) conditions. The order of inhibition under ***in*** *vivo* is PS II > whole chain > PS I, whereas under *in vitro* conditions PS II > PS I > whole chain. Therefore PS II is the major site of inhibitory action of FA even though it also affected the PS I function. This is in accordance with the earlier investigations which proposed that the principle target of allelochemicals is PS II (Moreland and Novitzky 1987). The negligible observed discrepancies of inhibi-tory effect of FA between ***in*** *vivo* and *in vitro* conditions could be due to the differences in the time of exposure to FA and concentration of available FA. The present study clearly suggest that the FA mediated curtailment in electron transport capacity of the photosystems

could be due to either lowered functional ability of component (s) of the electron transport pathway or alterations of thylakoid membrane structure.

Further, an increase in the electron transport ability of the photosystems with low concentrations of FA (0.25mM and 0.5mM) as observed in figures 4.3 4.5 could be due to an increase in the faster turnover of photosystems reaction centres or an alteration in energy distribution between PS II and PS I. Tiwari and Mohanty (1993) observed similar increase in the PS II activity with low concentrations of cobalt chloride.

A number of stress factors like application of herbicides, heavy metals, un optimal water, temperature and light are known to inhibit assimilation rates with concomitant lowering of electron transport ability of both photosystems (Brewer *et al* 1979, Critchley 1981, Curwiel and Van Rensen 1993, Draber *et al* 1993, Laasch and Weis 1988, Tietjen *et al* 1993, Tripathy and Mohanty 1980, Tripathy *et al* 1981, York *et al* 1981). However the relative intensity of modulation of both photosystems varied among the employment of different stress factors. Many of the phenolic herbicides are known to act primarily on the photosystem II by displacing the secondary electron acceptor Q_B from its binding site at the reducing side of PS II (Draber *et al* 1989, 1993, Tietjen *et al* 1993, Wildner *et al* 1990). More over nitro phenols are also known to act on the donor side of PS II (Ranger 1973) in addition to displacement of Q_B from its binding site. The secondary interactions with thylakoid membranes structure is found to contribute to a greater extent for observed inhibition in electron transport rates. The greater susceptibility of PS II to FA treatment than PS I under *in vitro* and *in vivo* conditions suggest that FA is exerting its action mainly on the PS II compared to PS I. Similarly the susceptibility of PS II than PS I was observed earlier with other kinds of stresses such as heavy metals and herbicides (Draber *et al* 1989, 1993, Mohanty *et al* 1989, Renganathan and Bose 1989, Tietjen *et al* 1993, Trebst *et al* 1985, 1993, Wildner *et al* 1990). The lack of inhibitory effect of FA on PS II function when NH_2OH was used as

artificial electron donor to PS II (Table 4.2) indicates that FA acts on the oxidizing side of PS II close to the reaction centre since it is observed earlier that NH_2OH donates electrons close to the reaction center of PS II (Izawa 1970, Izawa and Good 1972, Mie and Yocum 1993).

F_{685} at room temperature (Figs. 4.9-4.11) is associated with PS II activity (Breton 1982, Chou and Govindjee 1970, Goedheer 1972). FA exhibited a time dependent decrease in PS II activity at room temperature (Fig. 4.15). At Low temperature (77° K) 685nm and 735nm peaks are known to originate from PS II and PS I (Figs. 4.12-4.14) respectively (Murata and Satoh 1986), whereas at room temperature fluorescence is emitted only from PS II (F_{685}). Any inhibition of electron flow to the PS II reaction centre from donor side of PS II lowers the chl a fluorescence yeild, whereas a block on the acceptor side increases the fluore-scence yeild (Tripathy and Mohanty 1980). The lowering of F_{685} band by FA (Figs. 4.9-4.14) in maize is proposed to be due to lowering the light harvesting chl complexes of energy transfer to PS II. In addition to F_{685}, FA has also significantly reduced the fluorescence of F735 band suggesting that FA caused structural alterations in chloroplast membrane which resulted in inactivation of PS II and PS I centers. Similar functional alterations in the chloroplast thylakoid membranes with phenolic compounds is reported earlier (Muzafarov *et al* 1988) and Wullschleger *et al* (1992) observed the correlation between electron transport and fluorescence. The complete restoration of FA induced inhibition of fluorescence of PS II by NH_2OH (Fig. 4.16) substantiates the earlier proposition that FA acts on the oxidising side of PS II close to the reaction centre. The decrease in F_{685}/F_{735} ratio (Table 4.3) in the present study indicates that when FA is applied, it interferes with energy transfer between the photosystems. Similar quenching of fluorescence emission has been observed with heavy metals like Ni^{2+} (Tripathy *et al* 1981), Zn^{2+} Tripathy and Mohanty 1980) and Cd^{2+} (Atal *et al* 1991).

The photophosphorylation of thylakoid membranes is affected by FA treatment. Non-cyclic phosphorylation (Table 4.5) is more susceptible compared to cyclic phosphorylation (Table 4.4). In addition, lower concentrations of FA (0.25mM and 0.5mM) enabled a significant rise in the cyclic compared to that of non cyclic phosphorylation (Tables 4.4, 4.5). The greater reduction in non-cyclic photophosphorylation correlated with greater limitation on non-cyclic electron transport. On the other hand cyclic phosphorylation showed less alteration which is in accordance with less reduction in PS I electron transport. The role of electron transport rates and involvement of coupling factors in the process of photophosphorylation is well documented before (Anthon and Jagendorf 1984, Choudhury and Biswal 1984, Curwiel and Van Rensen 1993, Ohata *et al* 1993). The membrane bound H^+-ATPase from chloroplasts (CF_o-CF_1) catalyzes the ATP synthesis/hydrolysis coupled with a transmembrane proton flux (Curwiel and Van Rensen 1993, Shiying *et al* 1993). The changes in Ca^{2+} ATPases should give some information on structural modification of the coupling factor (Jagendorf 1975), While the changes in the activity of the light activated membrane bound Mg^{2+} ATPases reflects the alterations in the energization capacity of plastid membrane (Biswal and Mohanty 1978, Choudhury and Biswal 1984, Jagendorf 1975). Earlier decreased rates of photophosphorylation has been attributed to decreased activities of Ca^{2+} and Mg^{2+} ATPases via interacting with coupling factors (Choudhury and Biswal 1984, Shiying *et al* 1993, Qweirolo *et al* 1981a,b). The fact that FA did not alter the function and structure of chloroplast coupling factor even upto 2.5mM FA (Tables 4.6, 4.7) suggests that the observed lowering of photophosphorylation (both cyclic and non cyclic, Tables 4.4, 4.5) could be due to curtailment in electron transport rates. In contrast to the action of FA, the other flavonoids like naringenin, quercetin, coumarin and derivatives of cinnamic acid act more like energy transfer inhibitors while inhibiting the photophosphorylation (Arntzen *et al* 1974, Moreland and

Novitzky 1987). Surprisingly at higher concentrations (3mM) FA seems to act both as energy transfer and electron transport inhibitor in regulating the process of photophosphorylation. Mc Carthy (1977) observed similar relation between electron transport and photophosphorylation.

In conclusion the present study identified that FA acts on thylakoid membranc function by inhibiting PS II at the water splitting Mn^{2+} complex and interferes with energy transfer between photosystems acting as energy transfer inhibitior resulting in lower photophosphorylation. These factors either synergistically or independently might have contributed to decreased photosynthetic rate.

CHAPTER V

Influence of ferulic acid on the uptake of phosphate by maize roots

Introduction

A large number of benzoic and cinnamic acid derivatives commonly found in soil are known to interfere with a variety of physiological processes (Blum and Dalton 1985, Blum and Rebbeck 1989, Holappa and Blum 1991, Klein and Blum 1991). Roots being the first organ to come in contact with allelochemicals, their growth was particularly affected compared to shoots (Blum and Dalton 1985, Blum and Rebbeck 1989, Devi and Prasad 1992, Lyu and Blum 1990).

The uptake of water and nutrients are the major functions of roots and any damage to the roots would alter these functions (Sattelmacher *et al* 1993). Phenolic acids including FA decrease plant growth by lowering the absorption of mineral nutrients and water (Balke 1985, Blum and Dalton 1985, Blum and Rebbeck 1989, Blum *et al* 1985a,b, Einhellig 1986, Holappa and Blum 1991, Kobza and Einhellig 1987, Lyu and Blum 1990, Mersie and Singh 1988). Previous studies revealed that FA significantly decrease the uptake of phosphate, K^+ and NO^{3-} (Bergmark *et al* 1992, Booker *et al* 1992, Glass 1973, 1974, 1975a, Lyu and Blum 1990, Lyu *et al* 1990, Mc Clure *et al* 1978). In addition to these phenolic compounds, a number of biotic and abiotic factors are known to influence ion uptake viz. pH of the absorbing solution (Borst-Paumels and Peters 1981, Baon *et al* 1994, Sentenac and Grignon 1985), concentration of the substrate, temperature, ATP supply and electrical potential difference across the membranes and the other ion concentrations such as potassium, magnesium and manganese (Bowling and Dunlop 1978). Glass (1975a) and Matula (1992) suggested that the toxicity of phenolic compounds depends on their solubility which inturn depend on the partition coefficient (Glass 1973, 1974).

The uptake of phosphate commonly involves the OH-/ PO_4 an part (Lin 1979, Ullrich-Eberius *et al* 1981). At higher concentrations of external phosphate the uptake is also mediated by H^+ cotransport in addition to OH^-/PO_4^- antiport (Ullrich-Eberius et al 1981).

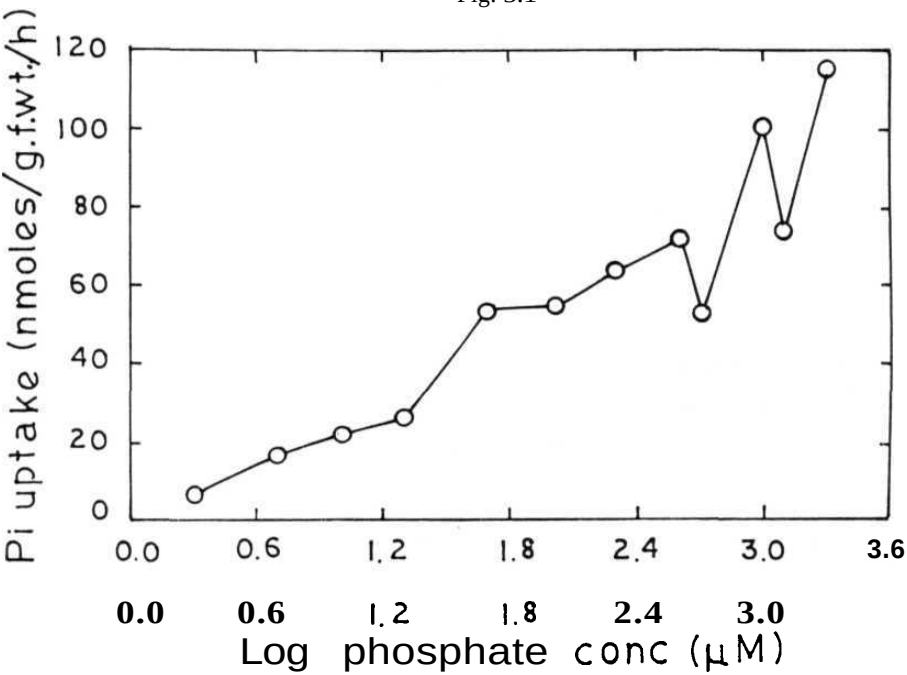
Though the toxic nature of phenolic compounds on ion uptake is evident from the earlier studies, the physiological and biochemical mechanisms involved are little known. Thus the present study is carried out to examine the FA influence on the uptake of phosphate by maize roots at various pH, substrate concentration and duration of treatment. The possible mechanism of action of FA to bring about these alterations with reference to lipid peroxidation, ferricyanide reduction are discussed.

Results

The results of the initial phosphate uptake under controlled conditions in the presence of different concentrations of phosphate (2,5,10,50,100,200,400, 600, 1000, 1500 and 2000|iM) showed a multiphasic uptake (Fig. 5.1). The uptake exhibited a biphasic nature upto 400uM external phosphate concentration. Increase of phosphate in the external medium exhibited an abrupt multiple increase and decrease in the phosphate uptake (Fig. 5.1). No physiological significance was claimed for this multiphasic uptake.

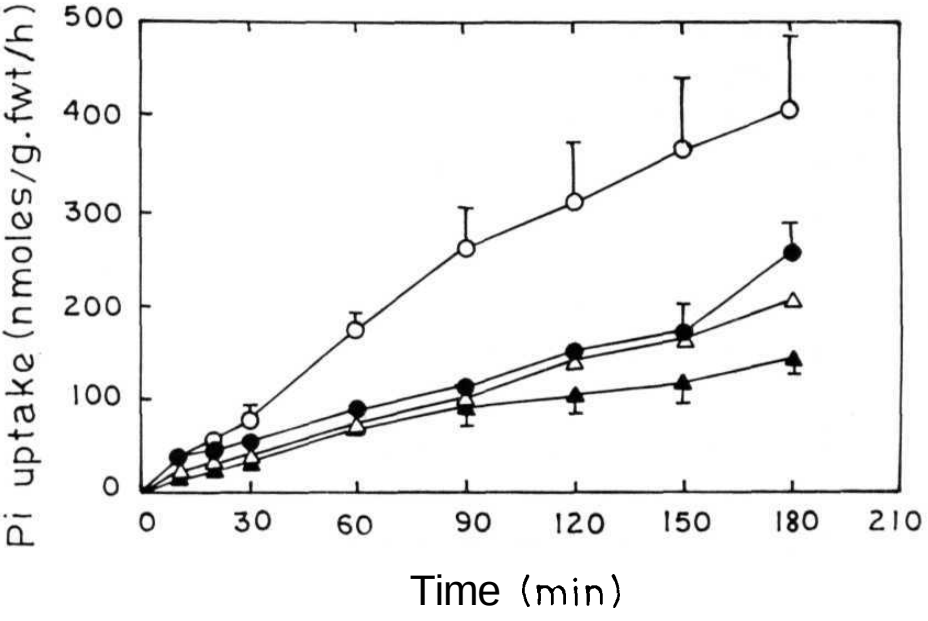
Preliminary experiments have established that the rate of active absorption of phosphate by control roots was linear upto 3h without any indication of reduction (Fig. 5.2). FA treated roots followed the same pattern of uptake as that of controls, but they exhibited significant decrease in the uptake by 53%, 60% and 67% with 0.25mM, 0.5mM and 1mM FA respectively (Fig. 5.2). The decrease in the uptake was linear upto 150min with 0.25rriM FA and further increase in the time of uptake to 180min decreased the inhibition to 37%. Similarly, 0.5mM FA decreased the uptake linearly upto 90min (60%) and the inhibition reduced to 49% at the end of 180 min of incubation. In

Fig. 5.1



Uptake of phosphate by excised roots of four days old maize seedlings incubated with different concentrations of ferulic acid for 2h.

Fig. 5.2



liptake of phosphate by excised roots of four day old maize seedlings incubated for different durations with different concentrations of ferulic acid.
Control (0), 0.25mM (●), 0.5mM (A), 1mM (A).

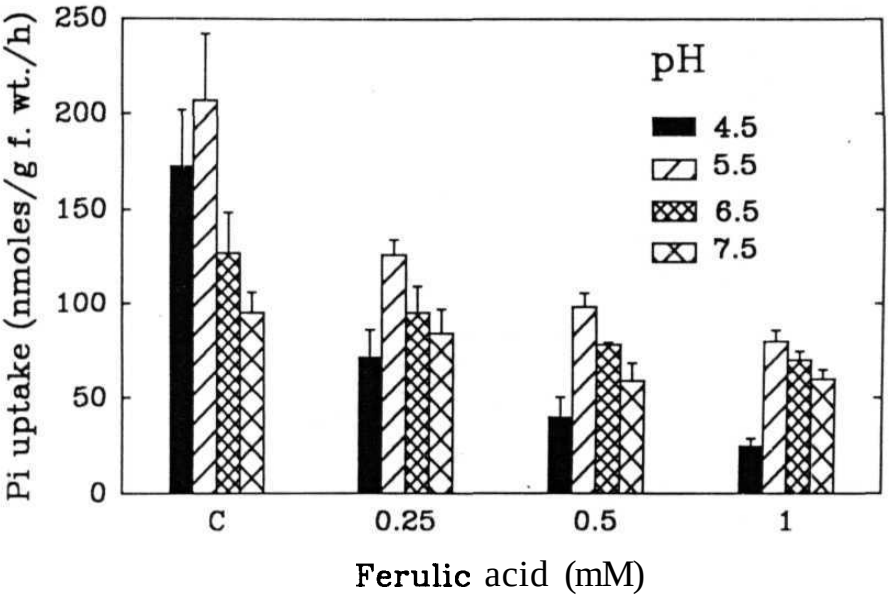
contrast, 1mM FA showed a continuous and linear decrease in uptake upto 180min(Fig. 5.2).

The uptake of phosphate as a function of pH (4.5, 5.5, 6.5 and 7.5) was measured in control as well as in FA treated roots (Fig. 5.3). The results indicated a maximum uptake of phosphate at pH 5.5 in control as well as in FA treated roots. However, FA treated roots exhibited significant decrease in uptake compared to control and the degree of inhibition varied with pH. At 4.5 pH, 0.25mM, 0.5mM and lmM FA decreased the phosphate uptake by 58%, 76% and 85% respectively. When pH was increased to 5.5, uptake was reduced by 39%, 52% and 61% with 0.25mM, 0.5mM and lmM FA respectively. Further increase of pH to 6.5 resulted in 25%, 38% and 44% reduction of uptake and at pH 7.5 the inhibition was 11%, 37% and 36% with the inclusion of 0.25mM, 0.5mM and lmM FA. These results clearly indicate that the FA inhibites phosphate uptake maximally at pH 4.5 and at higher pH FA has less influence in phosphate uptake.

The phosphate uptake by excised roots (in the presence of FA) was measured in the presence of different concentrations of phosphate (Fig. 5.4). Roots treated with 0.25mM FA did not inhibit the uptake to a significant level (9%) at all phosphate concentrations tested. FA (0.5mM) decreased the uptake linearly (25%) upto 0.02mM phosphate and the inhibition reduced to 15% with an increase in phosphate concentration to 2mM. Similarly roots treated with lmM FA reduced the uptake by 35% upto 0.2mM phosphate and as the external phosphate concentration in the medium increased to 2mM there was less inhibition (28%) due to FA. FA (3mM) caused 64% inhibition upto 0.02mM phosphate, and further increase in phosphate concentration to 2mM caused 44% reduction in the phosphate uptake (Fig. 5.4).

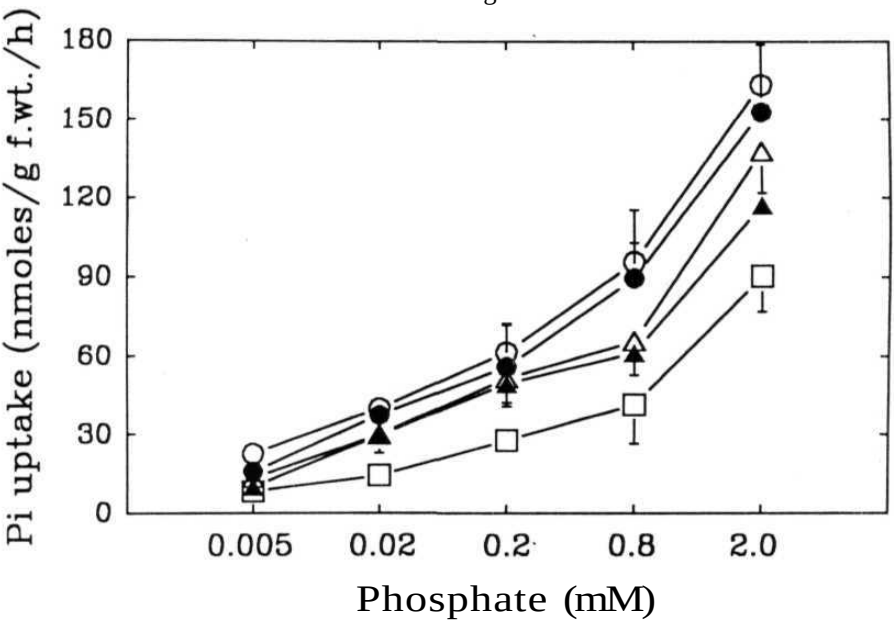
Intact roots also exhibited a significant decrease in phosphate uptake with FA treatment. 0.25mM and 0.5mM FA decreased the uptake (38% and 44% respectively) in a linear phase upto 0.2mM phosphate and as the

Fig. 5.3



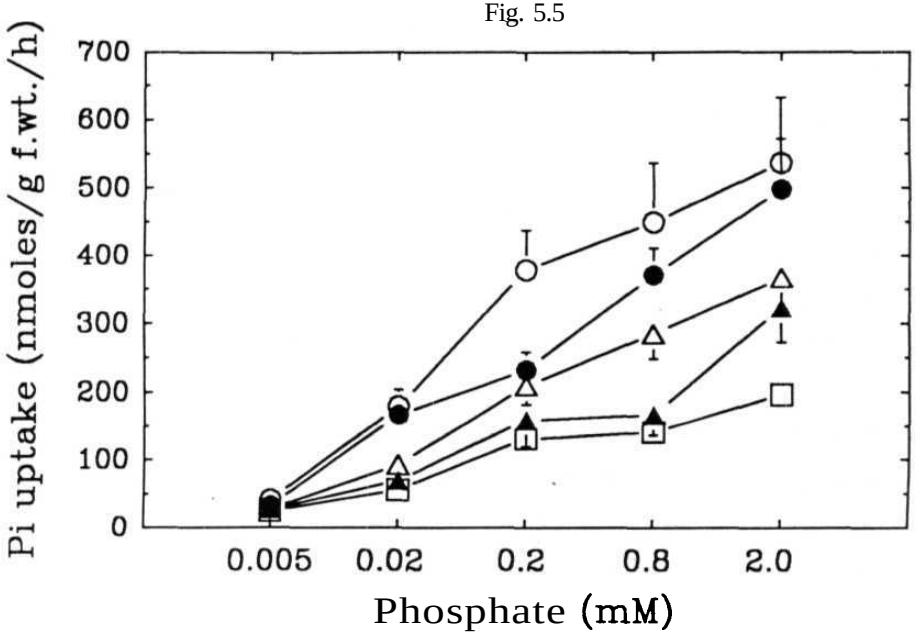
Effect of ferulic acid on the uptake of phosphate by excised roots of four day old maize seedlings at different pH (4.5, 5.5, 6.5 and 7.5) incubated for 2h.

Fig. 5.4



Uptake of phosphate by excised roots of four day old maize seedlings incubated for 2h with different concentrations of ferulic acid. Control (0), 0.25mM (•), 0.5mM (△), 1mM *(A)*, 3mM fla).

Fig. 5.5



Uptake of phosphate by intact roots of four day old maize seedlings incubated for 2h with different concentrations of ferulic acid.
Control (0), 0.25mM (•), 0.5mM (△), 1mM (▲), 3mM (a).

concentration of phosphate increased to 2mM the inhibition was reduced to 31%. However, roots treated with 3mM FA exhibited a continuous linear decrease in uptake at all phosphate concentrations studied (Fig. 5.5). There was a liner decrease in the V_{max} at all the tested concentrations of FA both in excised and intact roots (Table 5.1).

Studies on the reversibility of FA induced inhibition exhibited a significant decrease in the uptake with 1mM FA (Fig. 5.6, first arrow) compared to a linear increase in control roots. However, when the roots were transferred to solution devoid of FA (Fig.56, second arrow), the inhibition was reversed (uptake was recovered) after 50min of transfer. However, the earlier reports proposed that the uptake was restored immediately within 10min of transfer to FA free solution. The roots treated with 10mM TEA^+ decreased the uptake by 27% and lmM FA caused 69% reduction in uptake compared to control. FA along with TEA^+ inhibited the uptake by 76% (Fig. 5.7) compared to control.

Many of the phenolic compounds are known to exert their effect by partitioning between lipid layer of the membrane and external medium (partition coefficient). The membrane integrity application of FA is further studied which revealed significant increase in the lipid peroxidation. The increase in lipid peroxidation was significant with FA concentrations greater than 1.5mM though marginal rise in peroxidation was observed at lower concentration of FA. Roots treated with 2mM-3mM FA for 24h showed an increase in lipid peroxidation by 26%-36% while at the end of 48h, 37-62% increase was observed. The lipid peroxidation was increased by 38%-101% at 1.5mM to 3mM FA concentrations after 72h treatment (Table 5.2).

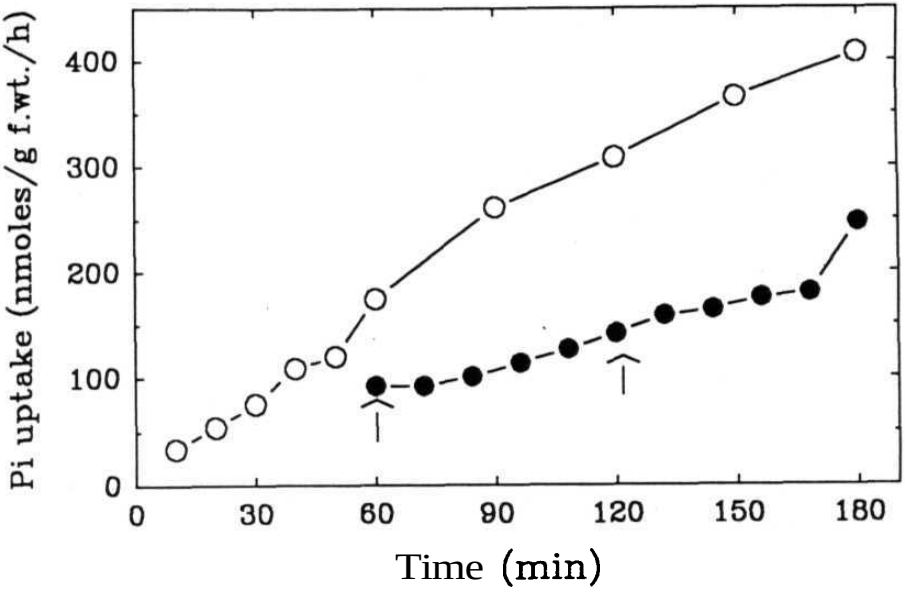
Ferricyanide reduction is a. measure of redox activity in roots which provide energy required for movement of solutes and ions across the membranes. The ferricyanide reduction was lowered at all FA concentrations

Table 5.1

Effect of ferulic acid on the V_{max} of the phosphate uptake; by excised and intact roots

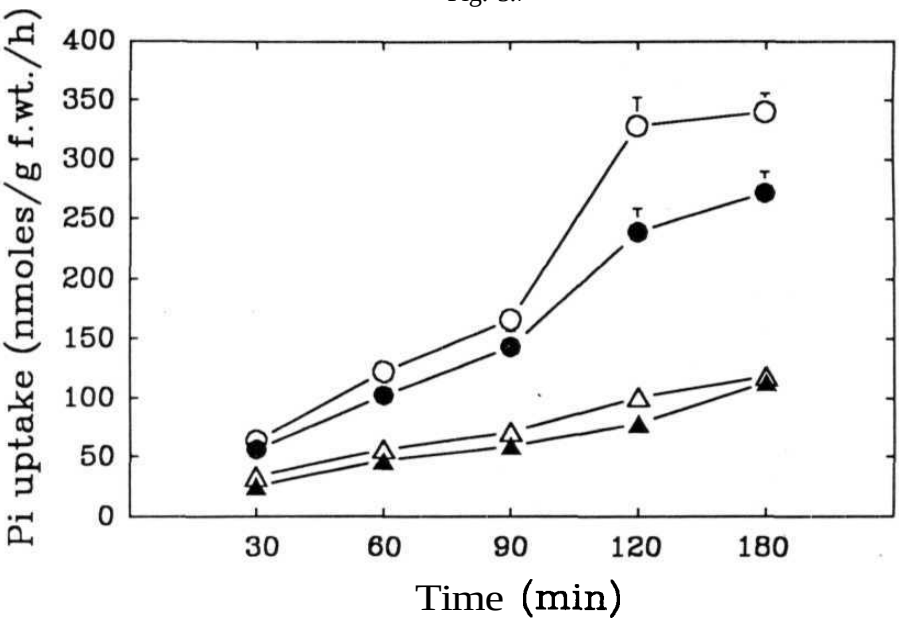
Ferulic acid (mM)	V_{max}	
	Excised	Intact
Control	85.23	624
0.25	85.43	510
0.5	73.55	**314**
1.0	70.05	**197**
3.0	36.98	**146**

Fig. 5.6



Reversibility of ferulic acid induced inhibition of phosphate uptake by roots. Control (O), 1mM (•). First arrow indicates the time when ferulic acid was added to the root samples and second arrow indicates the time when the roots were returned to the inhibitor free solution.

Fig. 5.7



Uptake of phosphate by excised roots of maize seedlings on treatment with combinations of 10mM tetraethyl ammonium (TEA*) and lmM ferulic acid. Control (○), control + lOmM TEA' (●), lmM ferulic acid (△), 1mM ferulic acid + lOmM TEA^{+} (▲).

Table 5.2

Effect of ferulic acid on the lipid peroxidation in maize roots

Ferulic acid (mM)	μ moles of malonaldehyde/g f.wt./h		
	24h	48h	72h
Control	9.4+1.24	8.6+1.14	8.4+1.06
0.5	10.8+1.34	9.3+0.98	9.3+0.52
	(+14.7)	(+8.13)	(+10.8)
1.0	11.1 + 1.43	9.6+0.77	9.5±0.77
	(+17.9)	(+11.6)	(+13.8)
1 5	11.6+1.20	10.7+1.57	**11.6+1.31**
	(+23 2)	**(+24.2)**	**(+38.9)***
2.0	11.9+1.18	11.8+1.89	12.1 + 1.21
	(+26.4)**	(+37.2)*	(+44.4)*
2 5	11.5+0.80	12.9+1.34	12.8+4.84
	(+22.3)	(+49.4)*	**(+52.4)***
3.0	12.8+1.46	14.0+1.46	**16.9+4.23**
	(+36.3)*	(+62.1)*	**(+101.2)***

Figures in parentheses are % of increase (+) or decrease (-) over control
*P< 0.001 ** P < 0.005

tested (Table 5.3). However, the inhibition was significant only with 2.5mM and 3mM FA treatment for 48 and 72h i.e. 26% and 27% respectively.

Discussion

It is clear from the data presented that FA is capable of exerting a strong inhibitory effect on the uptake of phosphate by maize roots. Preliminary studies on phosphate uptake by control roots exhibited a multiphasic character when tested over a wide range of phosphate concentrations (Fig. 5.1). In earlier studies, similar multiphasic uptake was observed for phosphate (Nandi *et al* 1987, Nissen 1973, Nissen 1980), sulphate (Holmern *et al* 1974, Nissen 1973) and potassium (Nissen 1980) and no physiological significance was claimed for this multiphasic uptake and was hypothesized that each solute or group of related solutes are taken up across the plasma membrane by a single proteinacious carrier/channel which changes confirmations as a result of all or none interactions with membrane lipids.

In the present study time dependent uptake of phosphate revealed FA induced decrease in phosphate uptake within 10min of treatment. However the decrease was significant only after 30min of incubation with FA (Fig. 5.2) incontrast to earlier reports that claimed significant reduction in phosphate uptake with in 10min of exposure to FA (Glass, 1973, 1974, 1975a).

Under normal conditions the uptake of phosphate mostly involves the $H_2PO_4^-$ rather than $HPO4^{2-}$ since $HPO4^{2-}$ exists only at pH greater than 6 (Sentenac and Grignon 1985) and is coupled to an OH^-/PO_4^- antiporter system operating against electrochemical gradient (Lin 1979, Ullrich-Eberius *et al* 1981). In addition to OH^-/PO_4^- antiport, uptake of phosphate is also known to depend on the H^+ co-transport (Ullrich- Eberius *et al* 1981). The later is substantiated by the influence of pH on phosphate uptake by maize roots in the present study. The observed maximum uptake of phosphate at pH 5.5 might be due to the existance of PO4-, as H2PO4" form which can enter easily into the roots (Fig. 5.3). The decrease in uptake at higher pH may be due to an increase

Table 5.3

Effect of ferulic acid on the rate of ferricyanide reduction in maize roots

Ferulic acid (mM)	μ moles of ferricyanide/ g f.wt./h		
	24h	48h	72h
Control	2.16+0.4	1.90+0.3	2.23+0.7
0 5	2.12+0.5	1.89+0.4	2.09+0.7
	(-1.85)	(-0. 05)	(-6.27)
1 0	2.04+0.5	1.64+0.4	1.96+0.6
	(-5.5)	(-13.6)	(-12.1)
1.5	1.98+0.4	1.56+0.3	1.95+0.5
	(-8.3)	(-17.8)	(-12.5)
2.0	1.89+0.5	1.63+0.4	1.90+0.5
	(-12.5)	(-14.3)	(-14.7)
2.5	1.90+0.3	1.42+0.4	1.66+0.5
	(-12.0)	(-25.2)**	(-25.5)**
3.0	1.86+0.4	1.39+0.3	1.61±0.4
	(-13.8)	(-26.8)**	(-27.8)**

Figures in parentheses are % of increase (+) or decrease (-) over control

*• P < 0.005

in OH^- ions in the medium increasing the back pressure of OH^- ions which ultimately leads to a decrease in the rate of phosphate influx. Similar pH effect on ion uptake was observed earlier (Harper and Balke 1981). Alternatively, the rise in pH might have resulted in a variety of phosphate species due to their higher degree of ionization thus lowering the levels of available $H_2PO_4^{2-}$. FA treatment didnot alter the pH optimum for absorption (pH 5.5) however, decreased the uptake to a significant level depending on the concentration. The decrease in uptake due to FA varied with pH (Fig. 5.3). The inhibition was pronounced at pH 4.5 ranging upto 82% with 3mM FA and as the pH increased to 7.5 the inhibition was decreased to 44% even at highest concentration of FA (3mM) tested (Fig. 5.3). presumably at lower pH i.e. 4.5 and 5.5, more of FA might have entered into roots compared to at pH 6.5 and 7.5. Similar uptake maxima for FA at lower pH has been observed earlier (Shann and Blum 1987a). Phenolic acids predominantly exists in undissociated (permeable) state at lower pH which is near to its pKa of the phenolic compounds (Putnam and Duke 1978). The pKa of FA is 4.8 at which FA present mostly in undissociated form and can easily penetrate into roots thus increasing the enormity of inhibition of phosphate uptake. Further rise in pH enhances the dissociated form of FA which cannot enter the roots easily. Similar pH effect on the uptake of phosphate and K^+ by salicylic acid was reported (Balke 1985, Harper and Balke 1981).

In excised roots, the inhibition with FA was maximum upto 50uM phosphate in the medium and as phosphate concentration increased the percentage of inhibition due to FA was reduced (Fig. 5.4) but could not restored the uptake completely.

Phosphate uptake by intact roots exhibited a reversal of inhibitory action of low concentrations of FA (0.25mM) with an increase in the phosphate concentration in the medium suggesting the competitive nature of FA inhibition. However, there was no significant change in the magnitude of

reduction in phosphate uptake by higher concentrations of FA (0.5mM, 1mM and 3mM) even with further rise in substrate concentration (Fig. 5.5). Therefore the possibility of competitive nature of the inhibition is ruled out. From the present study it can be concluded the non- competitive/uncompetitive role of FA on phosphate uptake.

The observed decrease in V_{max} (Table 5.1) further substantiates the non competitive inhibition of phosphate. Similar non-competitive/uncompetitive nature of uptake inhibition was observed with many other phenolic acids earlier (Glass 1973, 1975a, Mc Clure *et al* 1978).

Many of the phenolic compounds are known to depolarize the membranes (Galss and Dunlop 1976) and are known to mediate their action by altering the membrane permeability as evident from the restoration of uptake of ions immediately after transfer of roots to inhibitor free solution (Glass 1973, 1974, 1975a, McClure *et al* 1978). However, in the present study revival of FA induced inhibition of phosphate uptake was observed only after 50min of transfer to inhibitor (FA) free solution (Fig. 5.6) suggesting that the blockade of ion channels by FA or disrupting the ion gradient across the membrane. Thus it is likely that FA in addition to altering the membrane permeability might have damaged the carrier proteins. This notion is further strengthened by the uptake of phosphate in the presence of FA and TEA^+ (Fig. 5.7) where in a greater inhibition of uptake is observed with $FA+TEA^{2+}$ compared to TEA^{2+} alone. A similar inhibition to ion carriers and/or secondary gradient-coupled transport system by FA is reported earlier (Booker *et al* 1992). TEA^+ is a well known K^+ channel blocker which inhibits the potassium uptake by roots (Kochain *et al* 1985, Stanfield 1983, Tester 1988). The maximum inhibition observed in the present studty in the phosphate uptake in presence of FA and TEA^+ when compared to TEA^+ alone suggests that the effect of FA is an additive effect to TEA^{2+} action. The cation uptake by K^+ channels also contribute to some extent to phosphate uptake (Balke and Hodges 1979a,b, Lin 1979). Thus alterations in

confirmation of K^+ channels by FA might have contributed to the observed inhibition in phosphate uptake by the inhibitor.

In addition to ion uptake, many of the phenolic compounds are known to increase the efflux of ions (Bergmark *et al* 1992, Booker *et al* 1992, Murphy et al 1993) suggesting structural alterations to the membranes. The entry of phenolic acids into the roots depend on their lipid partition coefficient (Glass 1974,1975a). Therefore any change in membrane proteins and/or lipids will significantly alter the ion uptake. In the present study FA treatment resulted in significant increase in the lipid peroxidation (Table 5.2) which would cause changes in the confirmation of membrane lipids of cells consequently the damage to the membrane permeability barrier which results in the leakage of ions thus disrupts the ionic gradient across the membrane and affect the uptake of ions. Similar reduction in the membrane permeality due to altered membrane lipids has been observed earlier (Hayeshi *et al* 1992). Increased lipid peroxidation with a heavy metals like copper has been examined with concomitant decrease in the membrane permeability (Ric De Vos *et al* 1989).

The uptake of phosphate is an active process which involves energy for the transport of ions. The ferricyanide reduction (Table 5.3) is a measure of redox activity of membranes that provide energy for many important biological processes (Morre *et al* 1988, Rubinstein *et al* 1984). The involvement of redox energy in the proton efflux in various plant and animal cells (Lin 1979, Rubinstein *et al* 1984) is reported earlier. In addition, this redox activity linked to antiport has been observed for animal cells (Morre *et al* 1988) previously. It is believed that alterations in redox potentials will modify the proton efflux which regulates ionic gradient and concomitantly ion transport across membrane. As FA (2mM-3mM) has resulted in significant inhibition of redox activity (Table 5.3) of roots an alterations in proton efflux and energy for driving of the ions across membranes would be expected and might have further contributed to for the observed inhibition in the phosphate uptake. The

inhibition of phosphate uptake due to a collapse of proton gradient in the membranes by gamma radiation, carbonyl cyanide, phenylhydrazine and other phenolic compounds are reported earlier (Lin 1979).

Present work clearly suggests that FA inhibited the phosphate uptake by altering the membrane permeability, damage to carrier proteins, structural damage to membrane and inhibiiton in the redox activity of root membranes. The mode of interaction of these factors (either synergistically and or additively) might have that contributed for the observed inhibition in ion uptake. Though these experiments are of short term, it is conceivable that prolonged exposure to phenolic acids at concentrations in the tested range for soil solution could produce significant and permanent inhibition in phopshate uptake.

Summary

FA induced physiological responses in maize with respect to growth, hydrolytic enzymes, oxidative enzymes, phenylpropanoid enzymes, phenylpropanoid intermediates, photosynthesis, uptake of phosphate by roots were investi-gated to understand the physilogical basis of its (FA) action as allelochemical. The results of the study revealed a significant alterations in the above physiological processes. Considerable reduction in the growth of the seedlings (length, fresh weight and dry weight) and activities of hydrolytic enzymes viz. amylase, maltase, invertase, acid phosphatase and protease were observed with FA treatment. The reduction in the activity of hydrolytic enzymes was also observed at isozymes level where in marked decrease and / complete disappearance of isozymes was observed. Application of GA restored the growth and activities of hydrolytic enzyme\ activities and their isozymes of FA treated seedlings either fully or partially depending on the concentration of FA. GA being the key regulator of growth and *de novo* synthesis of hydrolytic enzymes it is assumed that FA is acting as GA antagonist by interfering with GA or GA mediated process. This is evident from the restoration of growth and hydrolytic enzymes with additional supply of GA.

FA treated seedlings showed a greater increase in activities of peroxidase, catalase and 1AA oxidase in leaves and roots which would result in wall rigidification and reduction in endogenous IAA levels.

Leaves and roots of FA treated seedlings exhibited a significant increase in PAL, CAD activities with concomitant increase in lignin and growth inhibitory phenolic compounds depending on concentration indicating the correlation between these enzymes and accumulation of these phenylpropanoid metabolites.

FA treated seedlings exhibited reduced net CO_2 assimilation rates and stomatal conductance of leaves. The chloroplasts isolated from FA treated seedlings showed considerable reduction in the electron transport effeciency of

photosystems and particularly PS II. Exogenous hydroxylamine, an artificial electron donor to PS II restored the PS II activity completely suggesting the FA action on the oxidising side of PS II close to reaction center. This is further evident from fluorescence emisssion at room temperature and at low temperature where in considerable decrease in fluorescence emisssion of F_{685} and F735 probably due to interference with energy transfer between photosystems and hydroxylamine has completely restored the FA inhibited fluorescence emission strongly suggesting the FA action on oxidising side of PS II.

FA decreased the cyclic and non-cyclic photophosphorylation of maize thyalkoids with non-cyclic being more affected compared to cyclic acting as energy transfer inhibitors in addition to electron transport inhibitors as FA has decreased the ATPases (Ca^{2+} and Mg^{2+}) at higher concentrations indicating that FA is acting more like electron transport inhibitor.

FA reduced the capacity to absorb phosphate from the medium by both cxicised and intact roots. The abosrtption of phosphate showed a continuous decrease even at the end of 3h. Phosphate uptake was maximum at pH 5.5. FA induced inhibition in phosphate uptake was more at pH 4.5 and 5.5 compared to 6.5 and 7.5 probably due to the entry of more FA into the roots at pH 4.5 and 5.5 at which most of the FA exists in its undissociated form. FA induced inhibition in the uptake was either non-competitive or uncompetitive as increase in the external phosphate concentration in the medium did not alleviate the FA induced inhibition. A significant reduction in the V_{max} of uptake by FA treated roots. Treated roots did not show reversal of their inhibition (restoration of uptake) to absorb phosphate even upto 50min after transfer to FA free medium suggests the FA action on the ion carriers in addition to membrane damage. This is evident from the uptake in the presence of TEA2+ and FA, which showed a greater inhibition in phosphate uptake when compared to either of it alone. The membrane damage to roots was futher ft

evident from the FA induced increase in the root lipid peroxidation which would damage the permeability barrier. The decrease in ferricyanide reduction which is measure of redox activity with FA treatment suggesting the possibility of low supply of ATP necessary to drive ions and solutes across the membranes.

Thus to sumup, these results lead to the conclusion that FA has exerted its action:

1. By decreasing the hydrolytic enzymes which results in low availability of nutrients for growing seedlings acting as GA antagonist.
2. By increasing the peroxidase and IAA oxidase which results in wall rigidification and low levels of endogenous IAA.
3. By increasing the activities of PAL and CAD leading to accumulation of lignin and growth inhibitory phenolic compounds.
4. By inhibiting the photosynthesis of the plants
5. By reducing the capacity of the plants to obtain nutrients (phosphate)

These factors either independently and /synergistically are responsible for observed growth reduction. This study is the first report of a detailed investigation to examine the allelochemical action of FA on variety of physiological processes in a single experimental system i.e. maize (*Zea mays.* L). This knowledge would provide better understanding of the mechanism/s of action that is necessary to exploit these allelochemicals for increasing the crop yields by way of designing natural herbicides and their mode of action. Thus from the present study a hypothetical FA action is proposed as seen in the figure (S)

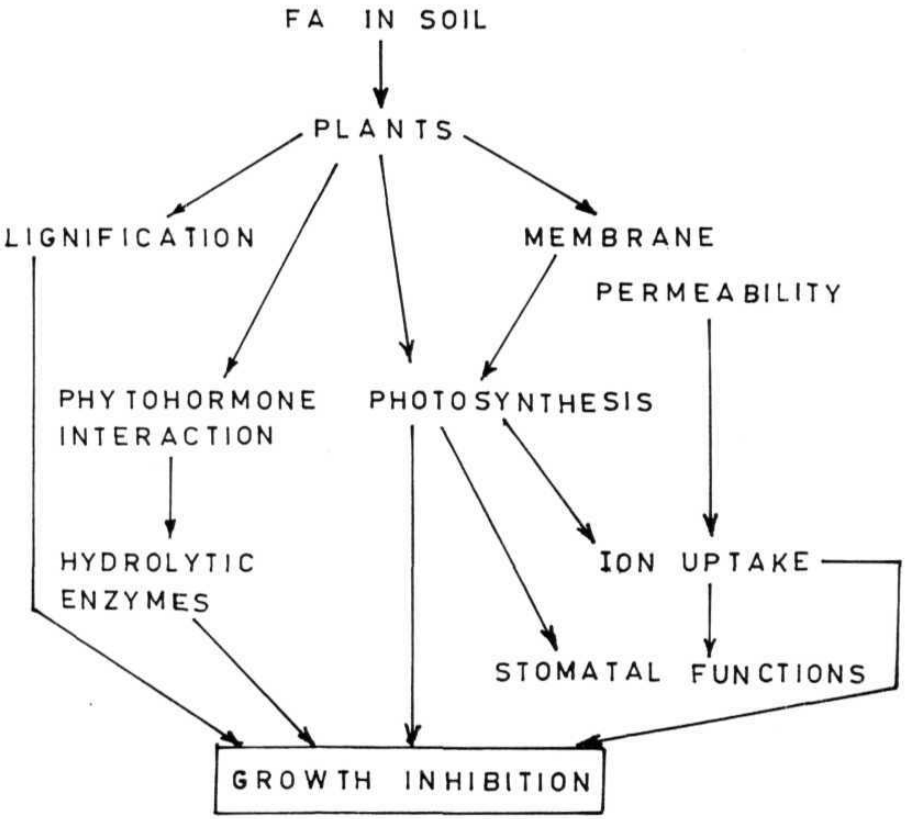


Fig.S: Suggested hypothetical ferulic ncid action

PART IV

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APPENDIX

LIST OF PUBLICATIONS

1. Devi, S.R. and Prasad, M.N.V. (1989). *Acacia* tree crops for coproduction of tannins, related polypehnols and other useful products. In: Proceedings Bio-Energy Society Sixth Convention and Symp`89. (ed.) R.N. Sharma, O.P. Vimal, H.L. Sharma and K.S. Rao. BESI, DNES, 136-146, New Delhi.

2. Prasad, M.N.V. and Devi, S.R. (1989). *Terminalia bellerica* Roxbo-Potential source of polyphenols for industrial use. In: Biomass for energy and industry. 5th European conference (eds) G. Grassi, G. Gosse and G. Dos Santos. PP 1.541-1-546, Lisbon, Portugal.

3. Devi, S.R. and Prasad, M.N.V. (1990). Phytotoxicity *of Acacia* nilotical pod phenolics: *In vitro* bioassays. *Biochem. Arch.* 6: 75-82.

4. Devi, S.R. and Prasad, M.N.V. (1991). Tannins and related polyphenols from ten common *Acacia* species of India. *Bioresouce. Technol.* 36: 189-192.

5. Devi, S.R. and Prasad, M.N.V. (1992). Effect of ferulic acid on growth and hydrolytic enzyme activities of germinating maize seeds. *J. Chem Ecol.* 18: 1891-1990.

6. Devi, S.R. and Prasad, M.N.V. (1993). Effect of ferulic acid on the photosynthetic electron transport activities of maize *(Zea mays* L.) thylakoids. In: Proceedings of DAE symposium on photosynthesis and plant molecular biology. BRNS, DAE, Bombay, 72-79.

7. Devi, S.R. and Prasad, M.N.V. (1994). Ferulic acid altered growth and metabolism of oxidative enzymes in maize *(Zea mays* L.) seedlings (communicated).

8. Devi, S.R. and Prasad, M.N.V. (1994). Interaction of ferulic acid and gibberellic acid on the isozyme expression of amylase, acid phosphatase and protese in germinating maize seeds (communicated).