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EFFECT OF TANNIN EXPOSURE ON ASCORBATE PEROXIDASE ACTIVITY IN GERMINATING SEEDLINGS OF *Vigna radiata*

A PROJECT REPORT

Submitted by

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BONAFIDE CERTIFICATE

Certified that this project report **"Effect of Tannin Exposure on ascorbate peroxidase activity in germinating seedlings of *Vigna radiata*"** is the bonafide work of **Vivekanandan. S and Agasthiya Raj. S,** who carried out the project work under my supervision.

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ABSTRACT

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The appearance of photosynthetic organisms during the course of evolutions, led to the conversion of the earth's atmosphere from predominantly reducing environment to that of an aerobic environment.The advent of molecular oxygen led to the inevitable generation of reactive oxygen species(ROS),which constitute a group of partially reduced oxygen molecules.(The ROS are highly reactive, and cause potentially harmful macromolecular damage, including DNA mutations.).Organisms therefore have evolved a battery of non enzymatic and enzymatic defense mechanisms to counter the deleterious effects of ROS.Among the various anti oxidant enzymes of plants ascorbate peroxidase constitutes an important enzymatic defense against ROS. Ascorbate peroxidases are widely distributed in plants from algae to higher plants.Several iso enzymes are known to exist in different sub cellular organelles, underscoring the importance of this enzyme for anti oxidant defense.Previous studies from laboratories have addressed the role of various agents including herbicide, ozone and in modulating the response of ascorbate peroxidase.In the persent study seedlins of *Vigna radiata* were supplemented with commercial tannic acid and isolated tannic acid (from sorghum), during growth and the response of ascorbate peroxidase was determined.It has emerged from the results that ascorbate peroxidase activity is modulated differentially at lower and higher concentrations.

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1. AOX-	ascorbate oxidase
2. APX-	ascorbate peroxidase
3. AsA-	ascorbate
4. cAPX-	cytosol ascorbate peroxidase
5. Cat-	catalse
6. CCP-	cytochrome c peroxidase
7. chlAPX-	choloroplast ascorbate peroxidase
8. ETC-	extra celluar matrix
9. Fd-	ferredoxin
10.GPX-	glutathione peroxidase
11.GR-	glutathione reductase
12.GSH-	glutathione
13.GSSG-	disulphide form of glutathione
14.H_2O	water
15.mAPX-	microbody ascorbate peroxidase
16.MDAsA-	monodehydroascorbate
17.MDAsAR-	monodehydroascorbatereductase
18.mitAPX-	mitochondrail ascorbate peroxidase
19.NADH-	nictotinamide adenine dinucleotide
20.OX-	oxidase
21.POD-	peroxidase
22.PS-	photo system
23.ROS-	reactive oxygen species
24.sAPX-	stromal ascorbate peroxidase
25.SOD-	superoxide dismutase

INTRODUCTION

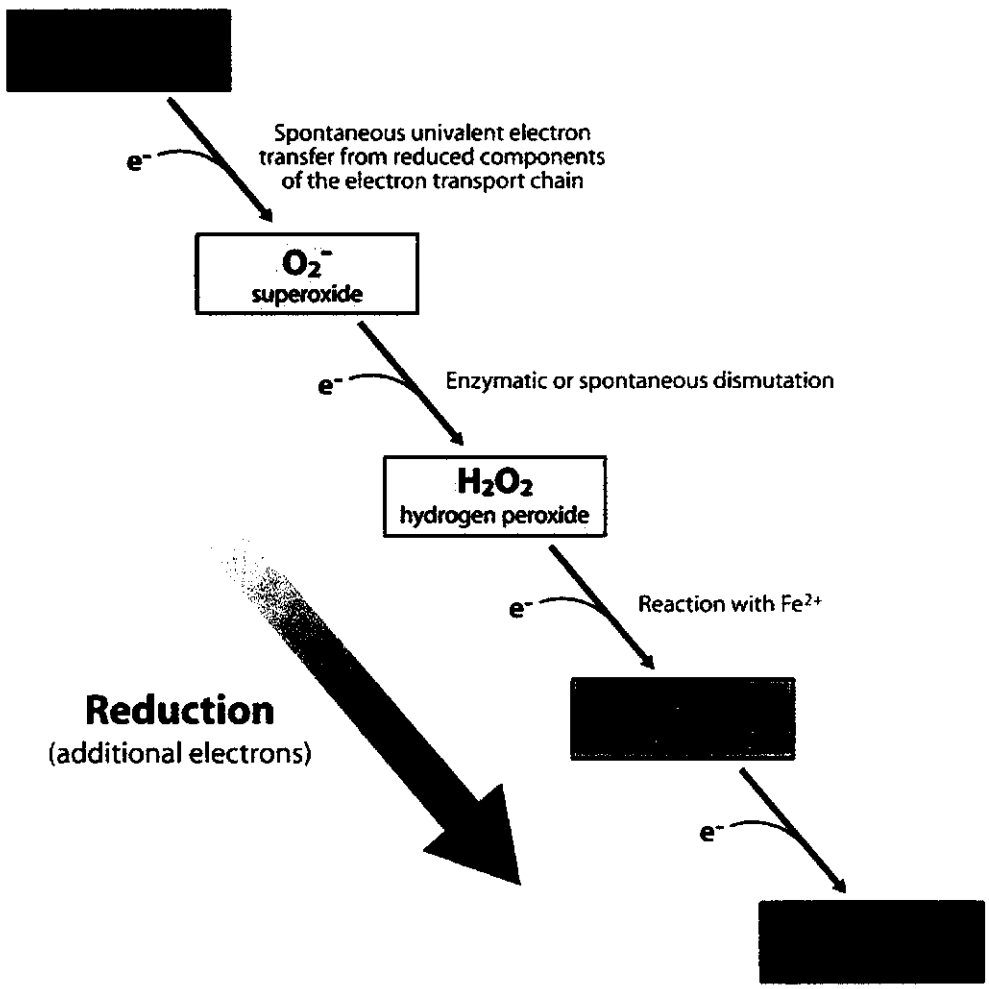
1. INTRODUCTION:

The advent of molecular oxygen (O_2) into the earth's atmosphere, by evolving photosynthetic organisms, about two million years ago, resulted in the production of reactive oxygen species (ROS). Since then, ROS have been the deleterious companions of aerobic metabolism. In contrast to oxygen, these partially reduced or activated derivatives of oxygen (O_2, H_2O_2, and $HO^{\cdot}$) are highly reactive and toxic, leading to the oxidative destruction of cellular macromolecules. Consequently, the evolution of all aerobic organisms has been dependent upon the development of efficient ROS scavenging mechanisms. In recent years a new role of ROS has been identified: the control and regulation of biological processes such as programmed cell death, hormonal signaling, stress responses and development. The current knowledge on the importance of ROS in plant biology suggest a dual role: (a) as toxic by-products of aerobic metabolism and (b) key regulators of metabolic and defense pathways.

1.1 Generation of ROS in biological systems

During aerobic respiration, the production of ROS is an unavoidable consequence. The terminal electron acceptor, cytochrome c oxidase, transfers four electrons to oxygen forming as water . However, occasionally oxygen can react with other electron transport components, as a consequence only one electron is transferred resulting in the production of superoxide anion (O_2^-). In mammalian cells it has been estimated that 1–2% of the oxygen consumed is converted to superoxide as a by-product. Through a variety of reactions, superoxide leads to the formation of hydrogen peroxide, hydroxyl radical, and other ROS (Fig 1.1).

Fig 1.1: Sequential reduction of molecular oxygen leads to formation of a group of reactive oxygen species:



In plants, hydrogen peroxide is formed by the dismutation of the superoxide radicals produced through the autooxidation of the photoreduced, primary electron acceptor in photosystem I and the photo reduced ferridoxin (Asada and Takahashi 1987). **Major sub cellular ROS gener**ating locations in plants are summarized in Table 1.1.

Table 1.1: Production of reactive oxygen species in plants

Cellular location of ROS production	Localization	ROS
Photosynthesis ET and PSI or II	Chloroplast	O_2^-
Respiration ET	Mitochondria	O_2^-
Glycolate oxidase	Peroxisomes	H_2O_2
Exicited cholorophyll	Chloroplast	O_2^-
NADPH oxidase	Plasma membrane	O_2^-
Fatty acid oxidation	Peroxisomes	H_2O_2
Oxalate oxidase	Apoplast	H_2O_2
Xanthine oxidase	Peroxisomes	O_2^-
Peroxidases and NADH	Vacoules	H_2O_2, O_2^-
Amine oxidase	Apoplast	H_2O_2

In mammalian mitochondria, complexes I and III are the main sites of ROS production and these two complexes certainly contribute to ROS production in plant mitochondria.

ROS production is highest in both mammalian and plant mitochondria under conditions in which the electron transport chain is relatively reduced. This means that any mechanism that can minimize this reduction will limit ROS production. Thus, activation of both the alternative oxidase and the uncoupling protein causes a marked reduction in ROS production probably through a signal transduction pathway like that shown in Figure 1.2

Fig 1.2: Stress related ROS generation in mitochondria

Stress

Constraint on ETC

Higher reduction level of
key ETC components

Increased ROS production

ROS accumulation

ROS mediated activation of
general stress genes(aox)

Increased concentration of AOX and
other stress proteins in mitochondria

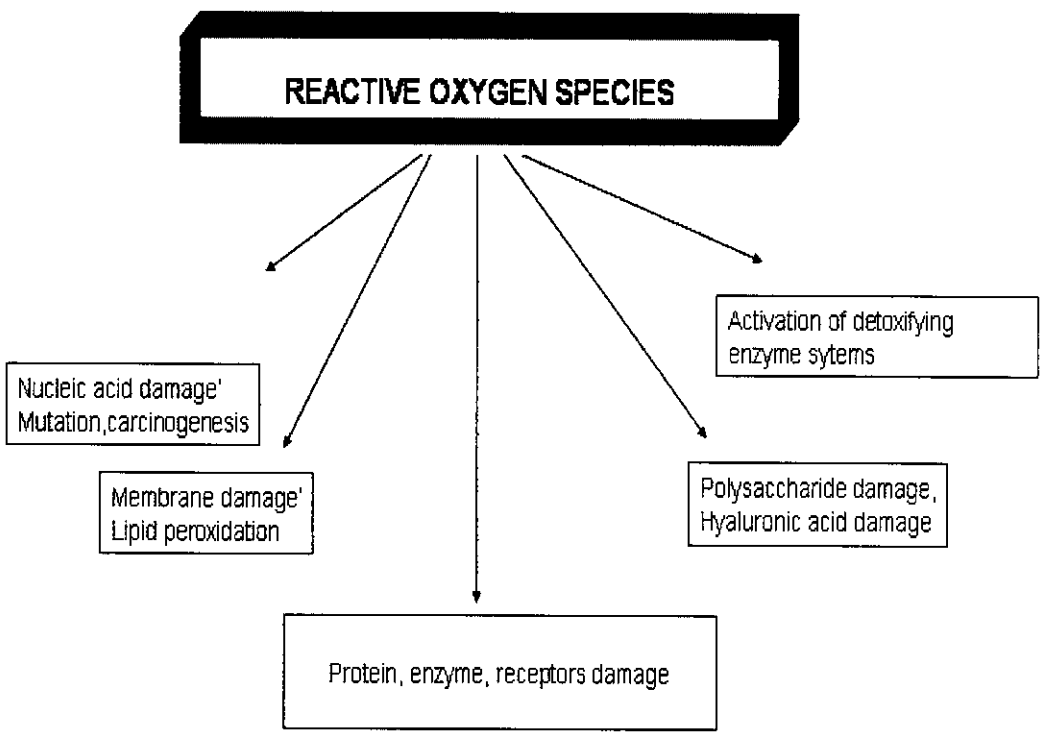
Lower reduction of level of
key ETC components

Lower ROS production
and concentration

1.2 ROS Can Damage Lipids, Proteins, and DNA

Reactive oxygen species particularly hydroxyl radical ($OH^{\cdot}$) are highly reactive and can react with variety of cellular macromolecules leading to modification and loss of function.

Fig. 1.3: Generalized scheme for oxidative injury to macromolecules



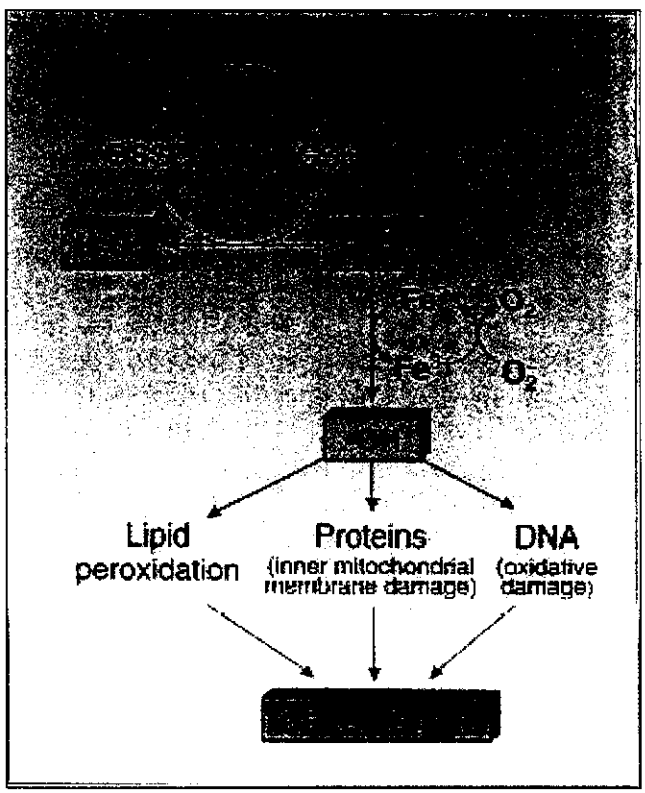
Peroxidation of polyunsaturated fatty acids by ROS attack can lead to chain breakage and shortening which will increase membrane fluidity and permeability. When isolated, mammalian mitochondria are exposed to oxidative stress, the membrane phospholipid diphosphatidylglycerol is damaged presumably, *via* peroxidation of the polyunsaturated fatty acids, 18:2 and 18:3, which are the main fatty acids in this lipid. The damage to diphosphatidylglycerol causes an inhibition of

cytochrome c oxidase, which appears to require this phospholipid for activity. Peroxidation of membrane lipids can have numerous effects, including:

- increased membrane rigidity
- decreased activity of membrane-bound enzymes (e.g. sodium pumps)
- altered activity of membrane receptors.
- altered permeability

In addition to effects on phospholipids, radicals can also directly attack membrane proteins and induce lipid-lipid, lipid-protein and protein-protein cross linking, all of which obviously have effects on membrane function.

Fig.1.4: Consequences of ROS induced macromolecular damage



Proteins can be damaged by ROS either through direct chemical interaction or indirectly involving end products of lipid peroxidation. A number of amino acids can be modified for example, cysteine can be oxidized to cysteine and both proline and

arginine are converted to glutamyl semialdehyde. Such modifications can affect the function of the proteins. In some cases the damaged amino acids are repaired *in situ*, whereas in other cases the entire protein is removed and degraded.

Finally, ROS can cause mutations in mtDNA. During aging in mammals, mutations in mtDNA accumulate faster than in nuclear DNA. This might be because mtDNA is closer to the site of ROS synthesis and/or because mtDNA is not protected by histones. Excision and replacement of the modified nucleotides can repair the DNA damage.

1.3 Cellular defense against ROS:

Life on earth evolved in the presence of oxygen, and necessarily adapted by evolution of a large battery of antioxidant systems. Some of these antioxidant molecules are present in all life forms examined, from bacteria to mammals, indicating their appearance early in the history of life.

Many antioxidants work by transiently becoming radicals themselves. These molecules are usually part of a larger network of cooperating antioxidants that end up regenerating the original antioxidant. For example, vitamin E becomes a radical, but is regenerated through the activity of the antioxidants vitamin C and glutathione.

Table 1.2: Various agents for scavenging of ROS

Antioxidant	Localization	Radical species
Super Oxide Dismutase	Chl,Cyt,Mit,Per,Apo	O_2^-
Ascorbate Peroxidase	Chl,Cyt,Mit,Per,Apo	H_2O_2
Catalase	Per	H_2O_2
Glutathione peroxidase	Cyt	H_2O_2, ROOH
Peroxidases	CW, Cyt, Vac	H_2O_2
Thioredoxin peroxidases	Chl, Cyt, Mit	H_2O_2
Ascorbic acid	Chl,Cyt,Mit,Per,Apo	H_2O_2, O_2^-
Glutathione	Chl,Cyt,Mit,Per,Apo	H_2O_2
α-Tocopherol	Membranes	ROOH, O_2^-
Caretenoids	Chl	O_2^-
Ascorbate Oxidase.	Chl, Mit	O_2^-

Chl: Chloroplast; Cyt: Cytosol; Mit: Mitochondria; Per: Peroxisomes; Apo: Apoplast; Vac:Vacuoles.

Enzymatic Antioxidants: Three groups of enzymes play significant roles in protecting cells from oxidant stress.

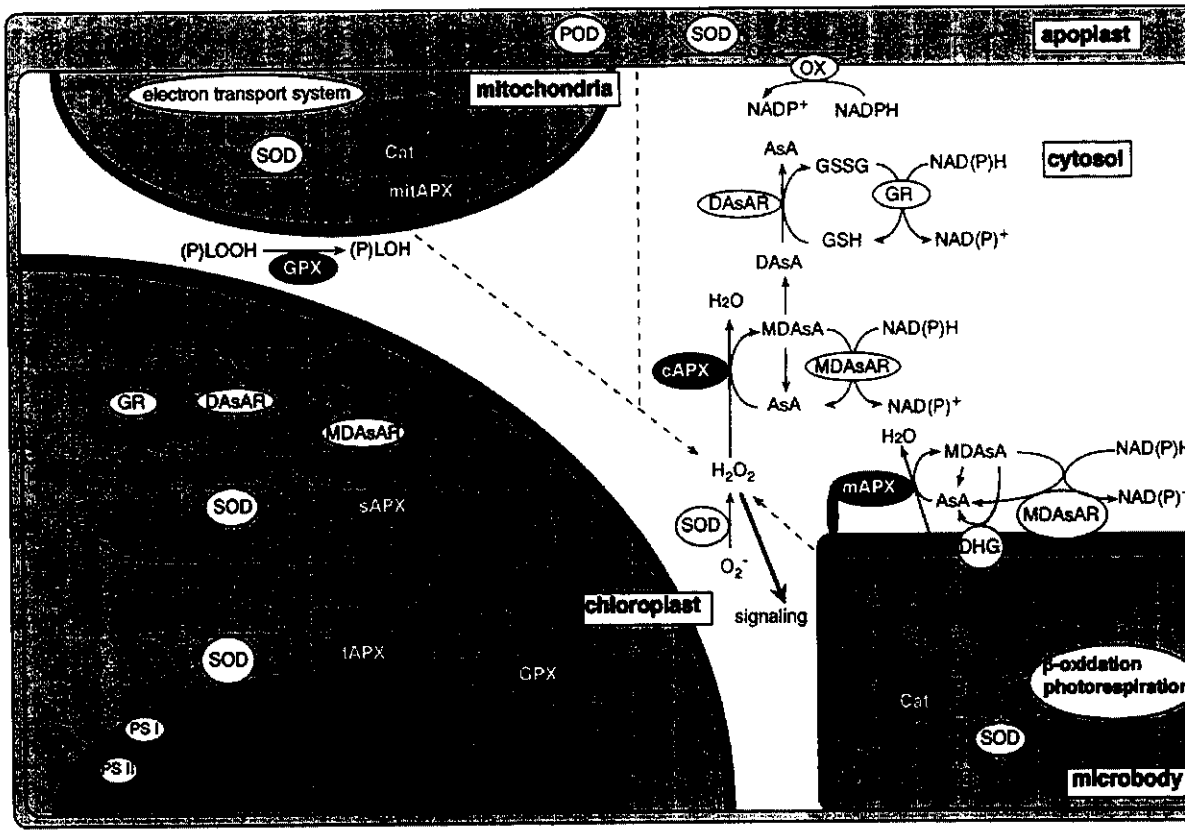
Superoxide dismutases (SOD) are enzymes that catalyze the conversion of two superoxides into hydrogen peroxide and oxygen. The benefit here is that hydrogen peroxide is substantially less toxic that superoxide. SOD accelerates this detoxifying reaction roughly 10,000-fold over the non-catalyzed reaction.

$$O_2^{\bullet -} + O_2^{\bullet -} \xrightarrow{SOD} O_2 + H_2O_2$$

SODs are metal-containing enzymes that depend on bound manganese, copper or zinc for their antioxidant activity. In mammals, the manganese-containing enzyme is most abundant in mitochondria, while the zinc or copper forms predominant in cytoplasm. Interestingly, SODs are inducible enzymes - exposure of bacteria or vertebrate cells to higher concentrations of oxygen results in rapid increases in the concentration of SOD.

The steady state level of ROS in the different cellular compartments is determined by the interplay between multiple ROS producing pathways, and ROS scavenging mechanisms. These are controlled by the ROS-signal transduction pathway and constitute the basic ROS cycle. During normal growth and development this pathway monitors the level of ROS, produced by aerobic metabolism, and controls the expression and activity of ROS-scavenging pathways. The basic ROS cycle may also perform fine metabolic tuning, e.g., suppression of photosynthesis, to reduce the production rate of ROS.There are many potential sources of ROS in plants. The enhanced production of ROS during stress can pose a threat to cells, and many stress conditions enhance the production of ROS-scavenging enzyme. However it is also thought that during stress ROS are actively produced by cells and act as signals for the induction of defense pathways. Thus, ROS may be viewed as cellular byproducts of stress metabolism, as well as secondary messengers involved in the stress-response signal transduction pathway.

Fig 1.5: Metabolic pathways involved in detoxification of ROS



Catalase is found in peroxisomes in eukaryotic cells. It degrades hydrogen peroxide to water and oxygen, and hence finishes the detoxification reaction initiated by SOD.

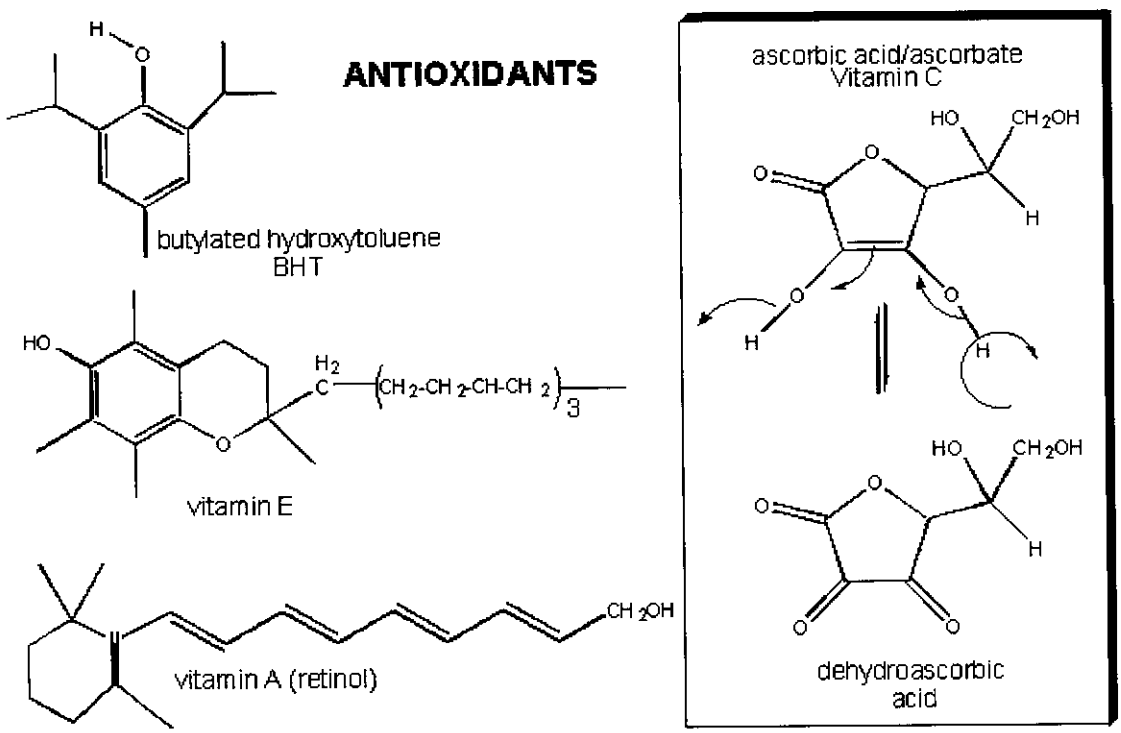
Glutathione peroxidase is a group of enzymes, the most abundant of which contain selenium. These enyzmes, like catalase, degrade hydrogen peroxide. They also reduce organic peroxides to alcohols, providing another route for eliminating toxic oxidants.

In addition to these enzymes, glutathione transferase, ceruloplasmin, hemoxygenase and possibly several other enzymes may participate in enzymatic control of oxygen radicals and their products.

Non-enzymatic Antioxidants: Three non-enzymatic antioxidants are of particular importance-vitamin E, vitamin C and Glutathione

Vitamin E is the major lipid-soluble antioxidant, and plays a vital role in protecting membranes from oxidative damage. Its primary activity is to trap peroxy radicals in cellular membranes.

Fig1.6: Chemical antioxidants found in biological systems



Vitamin C or ascorbic acid is a water-soluble antioxidant that can reduce radicals from a variety of sources. It also appears to participate in recycling vitamine E radicals. Interestingly, vitamin C also functions as a pro-oxidant under certain circumstances.

Glutathione may well be the most important intracellular defense against damage by reactive oxygen species. It is a tripeptide (γ-glutamyl-cysteinyl-glycine). The cysteine provides an exposed free sulphydryl group (SH) that is very reactive, providing an abundant target for radical attack. Reaction with radicals oxidizes glutathione, but the reduced form is regenerated in a redox cycle involving glutathione reductase and the electron acceptor NADPH.

LITERATURE REVIEW

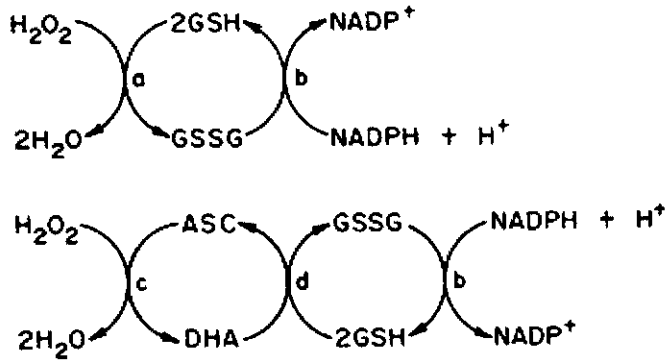
2. LITERATURE REVIEW:

Plant cells have various mechanisms of detoxifying ROS. One of the main defensive mechanisms against these reactive intermediates is superoxide dismutase (EC 1.15.1.1), an enzyme that is ubiquitous in aerobic organisms including both eukaryotes and prokaryotes . Superoxide dismutase catalyzes the conversion of 0_2^- to H_20_2. The H_20_2 may then be decomposed in the presence of catalase, which is widespread in plants and animals . In addition, many higher animals contain the selenoenzyme glutathione peroxidase (EC 1.11.1.9) which catalyzes the peroxidation of reduced glutathione (GSH), forming the oxidized disulfide form of glutathione (GSSG) as a product . The supply of GSH is regenerated in an NADPH-dependent reaction catalyzed by glutathione reductase (EC 1.6.4.2).

2.1 Ascorbate Peroxidase

Ascorbate peroxidase (APX) exists as isoenzymes and plays an important role in the metabolism of H_2O_2 in higher plants. APX is also found in eukaryotic algae. Ascorbic acid (ascorbate, ASC) is an essential molecule for higher plant cells. It is known that ASC is involved in several defence mechanisms against oxidative stress (Noctor and Foyer, 1998), as well as in the regulation of cell proliferation (Arrigoni and De Tullio, 2002 De Tullio *et al.*, 1999) and elongation (Hidalgo *et al.*, 1989 González-Reyes *et al.*, 1994 Córdoba-Pedregosa *et al.*, 1996). In addition, ASC has an important role in excess energy dissipation in thylakoids (Müller-Moulé *et al.*, 2003).

Another type of peroxidase system has been identified in plants . The initial peroxidase reaction of this system utilizes ascorbate as an antioxidant and produces dehydroascorbate. Ascorbate is then regenerated in a GSH-dependent reaction catalyzed by dehydroascorbate reductase. Finally, the GSSG is reduced back to GSH in a reaction, that involves glutathione reductase and NADPH. The enzymes involved are ascorbate peroxidase (EC 1.11.1.7), dehydroascorbate reductase (EC 1.8.5.1), and glutathione reductase. The initial product of the ascorbate peroxidation reaction is actually monodehydroascorbate, which spontaneously forms dehydroascorbate. The sequence of reactions is given below:



a. Glutathione peroxidase b.glutathione reductase

c. Ascorbate peroxidase d. Dehydro Ascorbate reductase

Ascorbate peroxidase is a homodimeric haeme containing enzyme that is used by plants to catalyze the reduction of hydrogen peroxide to water at the expense of ascorbate this function is important for the plant to regulate the levels of potentially damaging oxidative radicals that can accumulate in plant tissues.

Ascorbate peroxidase activity has been demonstrated in higher plants, eukaryotic algae and certain cyanobacteria. Plant ascorbate peroxidases are intracellular enzymes encoded in the nucleus. The code number (EC 1.11.1.11) for APX was first given on the basis of detailed information that was available about the enzymological properties of the Euglena enzyme (Shigeoka *et al.,* 1980).

In functional terms, ascorbate peroxidases differ from Class III peroxidases by exhibiting a higher affinity for ascorbate as reducing substrate. Furthermore ascorbate peroxidases are inhibited by thiol reagents and salicylic acid and chloroplast ascorbate peroxidase can lose activity in the absence of ascorbate.

APX isoenzymes are distributed in at least four distinct cell compartments, the stroma (sAPX) and thylakoid membraine (tAPX) in chloroplasts, the microbody (mAPX), and the cytosol (cAPX) (Asada, 1992; Miyake and Asada, 1992; Ishikawa *et al.,* 1998). A second family of cAPX has also reported in various plant species such as spinach, Arabidopsis, soybean, and rice (Ishikawa *et al.,* 1995; Santos *et al.,* 1996; Jespersen *et al.,* 1997; Caldwell *et al.,* 1998). More recently, Jiménez *et al.* (1997) reported the detection of APX activity in pea mitochondria, but the corresponding protein and cDNA have not yet been identified.

The involvement of APX in the scavenging of hydrogen peroxide has been confirmed by the detection of the H_2O_2 dependant photo evolution of O_2 (Asada and Badger 1984).APX has been purified from the leaves of several plants (Asada *et al.*,1989) from the root nodule of legumes(Dalton *et al.*,1986),and from euglena.

2.2 Ascorbate-glutathione cycle

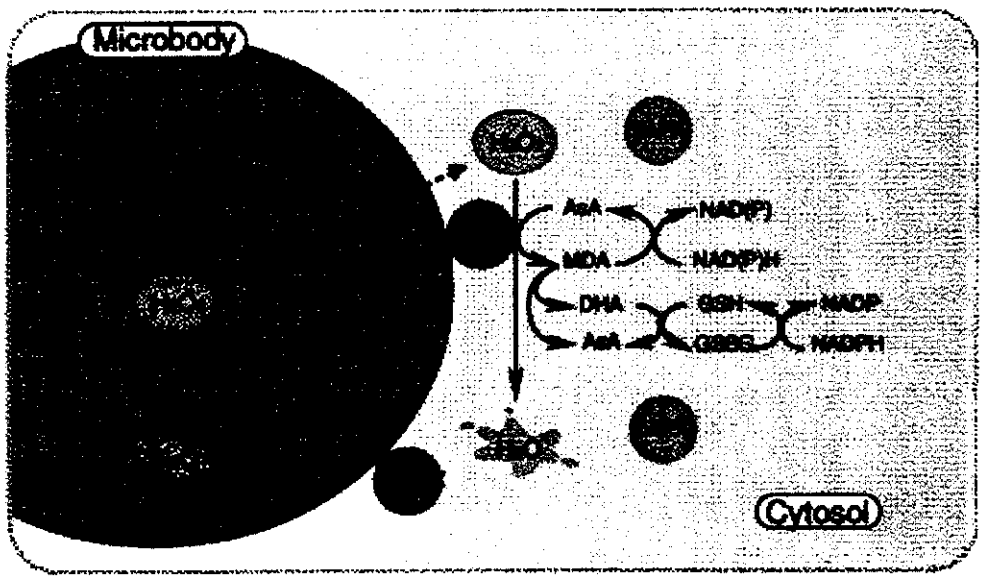
APX utilizes ascorbic acid as its specific electron donor to reduce H_2O_2 to water with the concomitant generation of monodehydroascorbate (MDAsA), a univalent oxidant of Ascorbic acid , monodehydroascorbate is spontaneously disproportionated to ascorbic acid and dehydroascorbate (DAsA). Monodehydroascorbate is also directly reduced to ascorbic acid by the action of NAD(P)H-dependent monodehydroascorbate reductase. Dehydroascorbate reductase utilizes glutathione (GSH) to reduce dehydroascorbate and thereby regenerate ascorbic acid. The oxidized GSH is then regenerated by GSH reductase, utilizing reducing equivalents from NAD(P)H. Thus, APX in combination with the effective ascorbic acid –GSH cycle functions to prevent the accumulation of toxic levels of H_2O_2 in photosynthetic organisms (Asada, 1992, 1997).

2.3 Isoenzymes of Ascorbate Peroxidase

APX has been identified in many higher plants and comprises a family of isoenzymes with different characteristics. Many isoenzymes of guaiacol peroxidase (GP) in plant tissues are localized in vacuoles, the cell wall, and the cytosol, but not in organelles (Asada, 1992). By contrast, APX isoenzymes are distributed in at least four distinct cellular compartments: stromal APX (sAPX) and thylakoid membrane-bound APX (tAPX) in chloroplasts, microbody (including glyoxysome and peroxisome) membrane-bound APX (mAPX), and cytosolic APX (cAPX) (Chen and Asada, 1989; Miyake *et al.*, 1993; Yamaguchi *et al.*, 1995; Bunkelmann and Trelease, 1996; Ishikawa *et al.*, 1996, 1998). A fifth APX isoenzyme (mitAPX) occurs in a mitochondrial membrane-bound form (Jiménez *et al.*, 1997; Leonardis *et al.*, 2000). Recently, the cDNA encoding a putative APX in the chloroplast thylakoid lumen of *Arabidopsis* was identified (Kieselbach *et al.*, 2000). APX has also been found in the protozoan *Trypanosoma cruzi* (Boveris *et al.*, 1980) and the bovine eye (Wada *et al.*, 1998). The

finding of APX in the bovine eye suggests that APX may also contribute, together with glutathione peroxidase (EC 1.11.1.9), to the detoxification of AOS in AsA-rich tissues of animals.

Fig 2.1: Role of ascorbate peroxidase in mitigating reactive oxygen species



APX isoenzymes have high specificity for AsA as the electron donor, which is especially the case for the chloroplastic APX (chlAPX) and mitAPX isoenzymes (Yoshimura *et al.,* 1998; Asada, 1999; Leonardis *et al.,* 2000). In addition to oxidizing AsA, cAPX and mAPX of higher plants and algal APXs can also oxidize artificial electron donors such as pyrogallol or guaiacol at appreciable rates (Chen and Asada, 1989; Ishikawa *et al.,* 1995, 1996; Yoshimura *et al.,* 1998; Asada, 1999)

APX is inhibited by cyanide and azide (Shigeoka *et al.,* 1980; Chen and Asada, 1989). APX is also inhibited by thiol-modifying reagents such as p-chloromercuribenzoate and suicide inhibitors such as hydroxylamine, p-aminophenol, and hydroxyurea (Chen and Asada, 1990). Since these suicide inhibitors do not affect GP, these reagents are useful for assays that differentiate between APX and GP (Amako *et al.,* 1994). One of the most characteristic properties of APX is its instability in the absence of AsA.

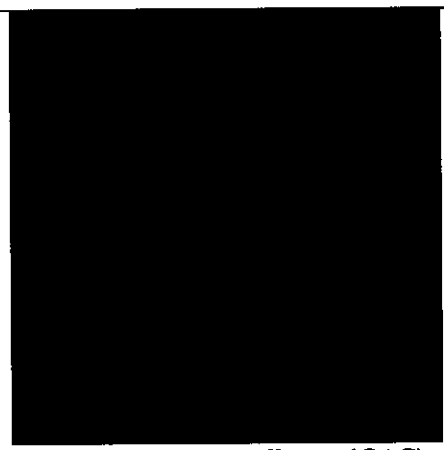
ChlAPX isoenzymes exist in a monomeric form, but cAPX is a dimer consisting of identical subunits with a molecular mass of 28 kDa (Mittler and Zilinskas, 1991; Miyake *et al.,* 1993).

As for chlAPX isoenzymes, the molecular mass of tAPX is about 4.5 kDa larger than that of sAPX (33.2 kDa); the difference in molecular mass between tAPX and sAPX is related to the requirement for membrane binding (Chen and Asada, 1989; Miyake *et al.*, 1993; Ishikawa *et al.*, 1996). The molecular masses (31 kDa) of mAPX and mitAPX are similar (Yamaguchi *et al.*, 1995; Ishikawa *et al.*, 1998; Leonardis *et al.*, 2000). The X-ray crystal structures of several ascorbate peroxidase enzymes have deduced (Fig 2.2, Fig. 2.3, Fig. 2.4).

On the basis of the amino acid sequences, plant peroxidases can be classified into three well-separated classes (Welinder, 1992). Class I includes the intracellular peroxidases of prokaryotic origin, Class II the fungal peroxidases, and Class III the classical plant peroxidases. APX, yeast cytochrome c peroxidase (CCP) and cyanobacterium CPX belong to the Class I family (Welinder, 1992; Mutsuda *et al.,* 1996; Zámock'y *et al.,* 2000). On the other hand, GP belongs to the Class III family, which constitutes a separate lineage from APX. A novel Class III peroxidase in tea leaves showed high specificity for AsA as an electron donor (Kvaratskhelia *et al.*, 1997).

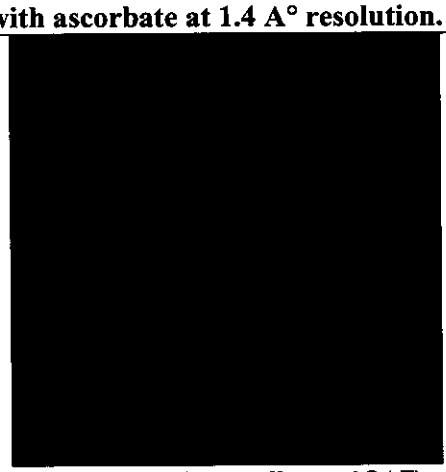
A phylogenetic tree constructed using the deduced amino acid sequences of the catalytic active domains of APX isoenzymes, not including the transit peptide and the membrane-spanning sequences, shows that APX isoenzymes in higher plants and algae can be divided into four groups (cAPX I, cAPX II, chlAPX, and mAPX) (Fig. 2.5.).

Fig 2.2: Crystal structure of ascorbate peroxidase from soybean cytosol at 1.75 A° resolution.



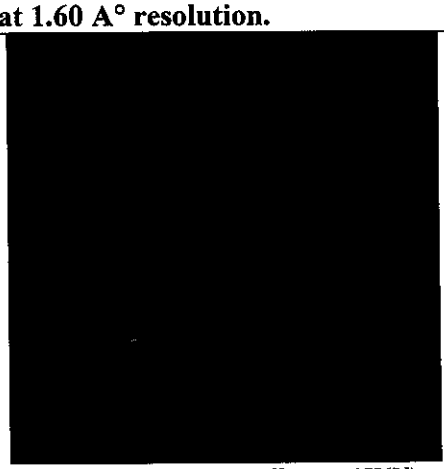
(source:http://www.pdb.org; 1OAG)

Fig 2.3: Crystal structure of ascorbate peroxidase from soybean cytosol in complexation with ascorbate at 1.4 A° resolution.



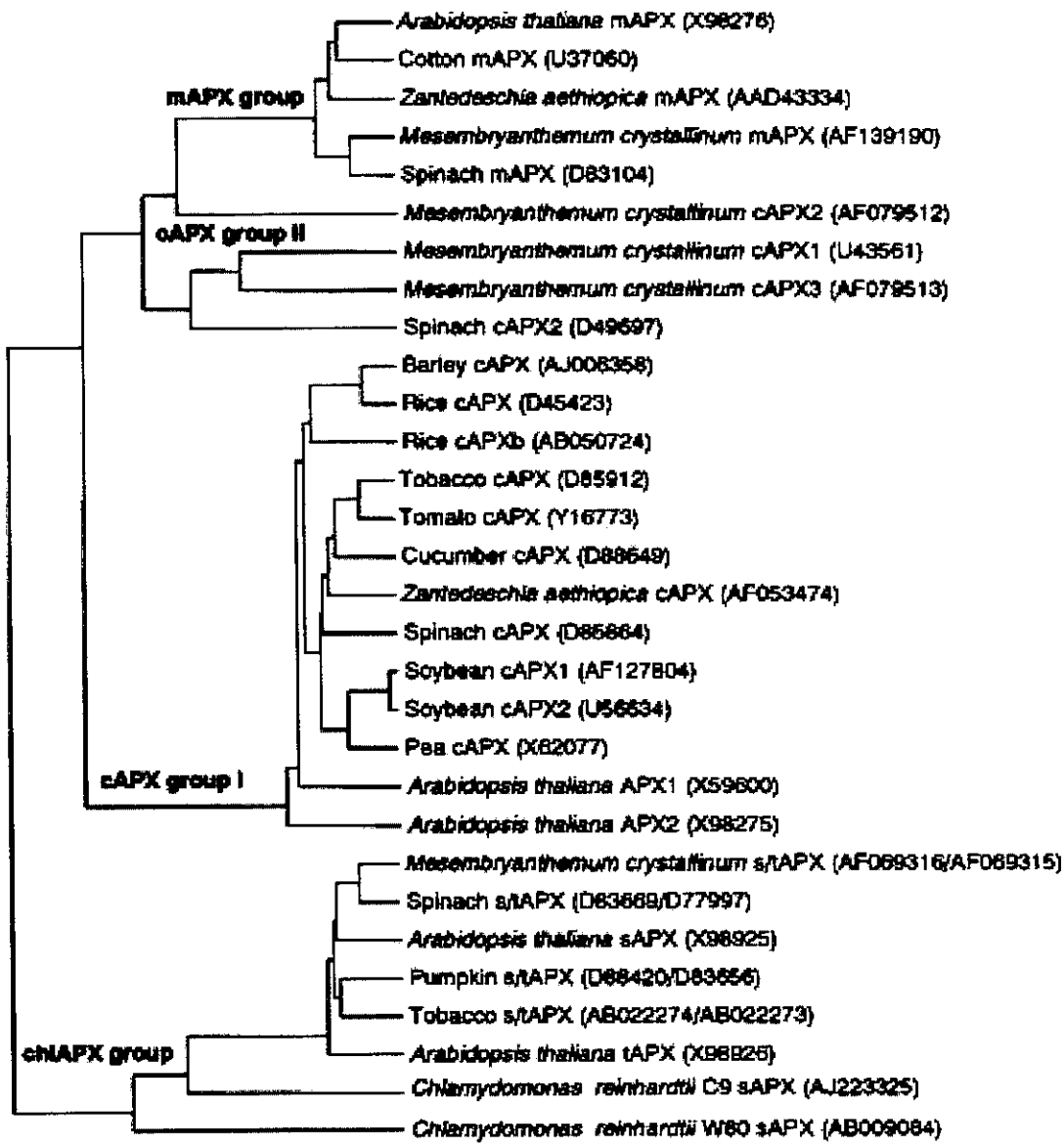
(source:http://www.pdb.org; 1OAF)

Fig 2.4: Crystal structure of chloroplast isoenzyme of ascorbate peroxidase from tobacco plants at 1.60 A° resolution.



(source:http://www.pdb.org; 1IYN)

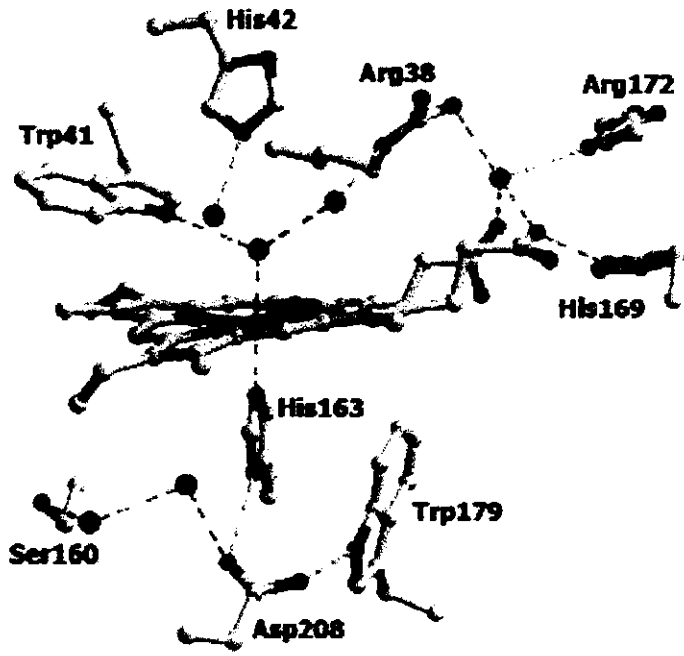
Fig 2.5: Classification of ascorbate peroxidase from various organisms based on sequence similarities



2.4 APX active site:

Some functional amino acid residues which are related to the binding of heame and AsA have been identified by mutation analysis using recombinant pea cAPX.

Fig 2.6: X-ray crystal structure of the active site residues of recombinant ascorbate peroxidase



Arg-172 of pea cAPX has an important role in AsA utilization to form Compound II (Bursey and Poulos, 2000). Glu-112 is located at the dimer interface, and is related to an alteration in solvent structure (Mandelman *et al.,* 1998). Arg-38 has a functional role in the control of substrate binding and orientation (Celik *et al.,* 2001). The active site structures, including the hydrogen-bonding interactions between the proximal His-163, buried Asp-208, and Trp-179, are nearly identical to those of CCP.

2.5 Tannins

Tannins are phenolic compounds that precipitate proteins. They are composed of a very diverse group of oligomers and polymers. One of the most satisfactory definition of tannins was given by Horvath (1981) "Any phenolic compound of sufficiently high molecular weight containing sufficient hydroxyls and other suitable groups (i.e. carboxyls) to form effectively strong complexes with protein and other macromolecules under the particular environmental conditions being studied"

Classification of Tannins

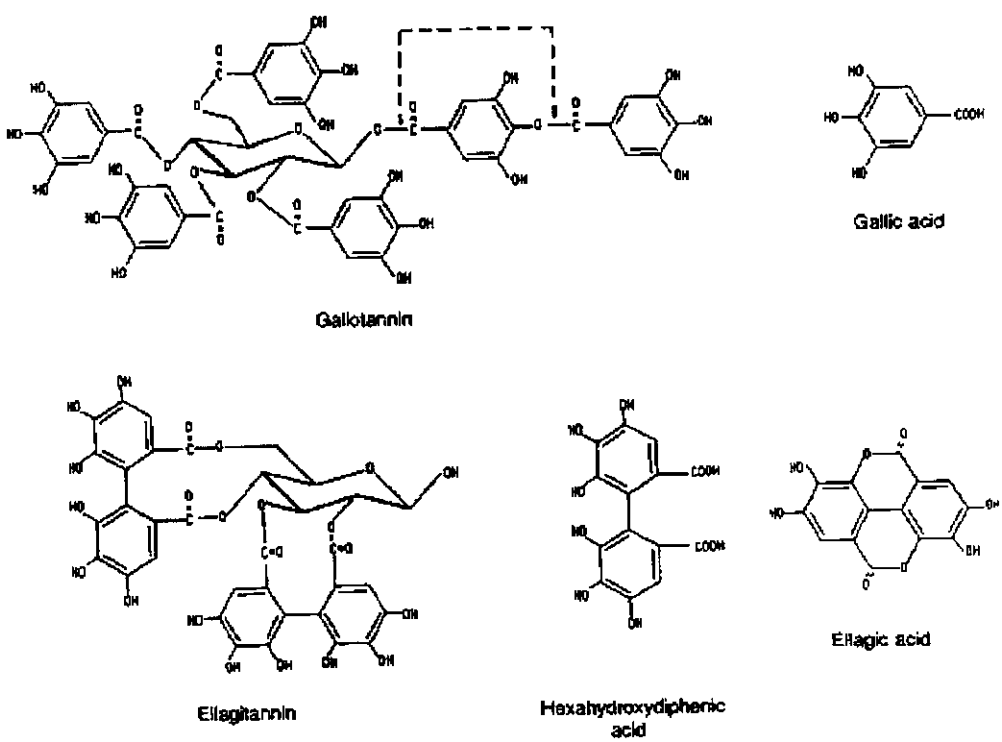
Tannin are usually divided into two types

1. Hydrolysable tannins
2. Condensed tannins (proantho cyanidins)

Hydrolysable Tannins:

Hydrolysable Tannins are molecules with a polyol (generally D-glucose) as a central core. The hydroxyl groups of these carbohydrates are partially or totally esterified with phenolic groups like gallic acid (gallotannins) or ellagic acid (ellagitannins). Hydrolysable Tannins are usually present in low amounts in plants.

Fig 2.7: Structure of Hydrolysable Tannins

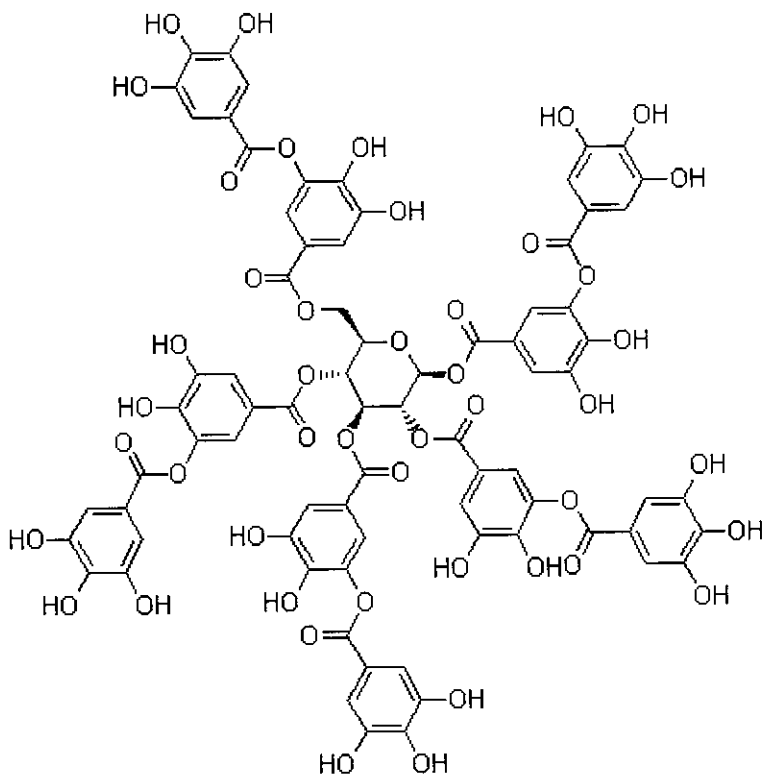


The properties of hydrolysable tannins include:

- Hydrolyzed by mild acids or mild bases to yield carbohydrate and phenolic acids

- Under the same conditions, proanthocyanidins (condensed tannins) do not hydrolyze.
- Hydrolysable tannins are also hydrolyzed by hot water or enzymes (i.e. tannase).

Fig 2.8: Structure of Tannic acid



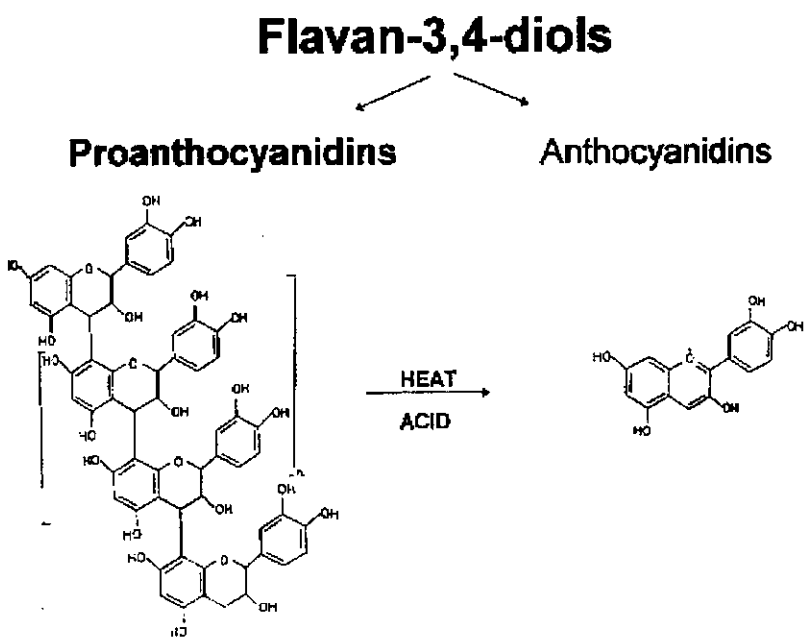
Proanthocyanidins (Condensed Tannins)

Proanthocyanidins are more widely distributed than hydrolysable tannins. They are oligomers or polymers of flavonoid units (i.e. flavan-3-ol) linked by carbon-carbon bonds not susceptible to cleavage by hydrolysis.

- Proanthocyanidins are more often called condensed tannins due to their condensed chemical structure.

- The term, proanthocyanidins, is derived from the acid catalyzed oxidation reaction that produces red anthocyanidins upon heating proanthocyanidins in acidic alcohol solutions.

Fig 2.9: Structure of condensed tannins



The most common anthocyanidins produced are cyanidin (flavan-3-ol, from procyanidin) and delphinidin (from prodelphinidin)

- Proanthocyanidins may contain from 2 to 50 or greater flavonoid units; Proanthocyanidins polymers have complex structures because the flavonoid units can differ for some substituents and because of the variable sites for interflavan bonds.
- Anthocyanidin pigments are responsible for the wide array of pink, scarlet, red, mauve, violet, and blue colors in flowers, leaves, fruits, fruit juices, and wines. They are also responsible for the astringent taste of fruit and wines.
- Proanthocyanidins carbon-carbon bonds are not cleaved by hydrolysis.

- Depending on their chemical structure and degree of polymerization, Proanthocyanidins may or may not be soluble in aqueous organic solvents.

2.6 Occurence

Tannins are widely distributed in the plant kingdom. Condensed tannin (proanthocyanidins) are the most widely distributed in vascular plants and are made up by condensation of hydroxyflavans, leucoanthocyanidin (flavan-3,4-diol) and catechin (flavan-3-ol). Hydrolysable tannins are restricted to angiosperm dicotyledons and usually contain glucose as a central core.

They are common both in Gymnosperms and Angiosperms. Within Angiosperms, tannins are more common in Dicotyledons than in Monocotyledons.

Examples of families of Dicotyledons rich in tannins are:

- Leguminosae : Acacia sp. (wattle); Sesbania sp.; Lotus sp. (trefoil); Onobrychis sp. (sainfoin);
- Anacardiaceae: Scinopsis balansae (quebracho)
- Combretaceae: myrobalan
- Rhizophoraceae : mangrove
- Myrtaceae: Eucalyptus sp., Mirtus sp. (Myrtle)
- Polinaceae: canaigre.

Other important tannin containing plants are *Quercus* sp. (oak), *Acer* sp. (maple), *Betula* sp. (birch), *Salix caprea* (willow), *Pinus* sp. (Pine), *Sorghum* sp. Tannins are located mainly in the vacuoles or surface wax of the plants. In these sites they do not interfere with plant metabolism. Only after cell breakdown and death can they act and have metabolic effects.

2.7 Biological Role and Effect on Other Systems

Tannins have major impact on animal nutrition because of their ability to form complexes with numerous type of molecules. Tannins negatively affect an animal's feed intake, feed digestibility and efficiency of production. These effects vary

depending on the content and type of tannin ingested and on the animal's tolerance, which in turn is dependant on characteristics such as type of digestive track, feeding behavior, body size, and detoxification mechanisms. Tannin toxicity to rumen micro organisms has been described for several bacterial species such as *Streptococcus bovis*, *Butyvibrio fibrosolvens*, *Fibrofacter succinogenes* and *Ruminobacter amylophilis*

Tannin act as defense mechanism in plants against pathogens, herbivores and hostile environmental conditions. Tannins provide important plant defense mechanism against animals, insects and microbes. Bacteria may be inhibited by tannins that bind to extracellular polymers, causing substrate and ion deprivation and by inhibiting enzymes (Krause *et al.*, 2001). Mechanisms used by different bacteria to tolerate tannins appear to differ, but these mechanisms are poorly understood (Asada., *et al.*, 1987). Their presence in trees and woody shrubs produces a bitter taste and astringency which may affect palatability and voluntary intake.

OBJECTIVES

3. OBJECTIVES:

The relevance of oxidative stress response under various environmental conditions has been extensively addressed in mammalian systems. Such a detailed knowledge is not available for the majority of plant species, particularly leguminous plants.

The model system - the germinating seedlings of *Vigna radiata* ,was chosen for the present study on account of its (1) high metabolic rate and, (2) short generation time. The present study aims to address the relevance of tannin exposure on oxidative stress response, as reflected in the ascorbate peroxidase activity of germinating seedlings of *Vigna radiata.*

The present study was carried out with the following considerations

- Optimize growth conditions and monitor growth parameters in germinating seeds of *Vigna radiata*
- Explore the biological effects of tannins on germinating seedlings of *Vigna radiata*
- To isolate hydrolysable and condensed tannins from sorghum seeds, a rich source of tannins
- Standardize various estimation procedures for hydrolysable and condensed tannins
- Study the effect if any, due to tannin exposure on ascorbate peroxidase activity in *Vigna radiata.*
- Partially purified one of the isoenzymes of ascorbate peroxidase of *Vigna radiata.*

MATERIALS AND METHODS

4. MATERIALS AND METHODS

4.1 MATERIALS:

All chemicals used for the study was analytical grade, unless specified otherwise. Deionised water was used for the experiments, unless specified otherwise. Seeds of *Vigna radiata, Sorghum* and *Maize* was purchased from a local dealer. The seeds of sorghum and maize were pulverised to a fine powder by a commercial cereal grinder.

4.2. METHODS:

4.2.1. Germination of seedlings:

Healthy seeds of *Vigna radiata* were chosen and were surface sterilized by 0.1% $HgCl_2$. The seeds were then washed with distilled water, and layered on cheese cloth stretched over petriplates. The control seeds were irrigated with distilled water, while the supplemented seeds were irrigated with tannic acid (or) isolated hydrolysable tannins at defined concentration.

4.2.2 Isolation of Subcellular Organelles

The seedlings were homogenized using the homogenization media, and the concentrations of various reagents of the homogenization medium are given in Appendix pg. 64. The ideal homogenization medium should be capable of maintaining the morphological and functional integrity of organelles. 0.3 M mannitol was used so as to be isotonic with the cytosol. The medium also contained potassium phosphate buffer pH 7.8. Mercaptoethanol was added at about 10mM concentrations to minimize the inactivation of enzymes. Mg^{2+} and Ca^{2+} was included at 10mM and 1mM to keep ribosomes intact and to prevent the clumping of nuclei respectively. Polyvinyl pyrrolidone (0.1%) (to precipitate out of tannins & phenolics.) and bovine serum Albumin (0.1-0.2%) was added.

The media was added in the ratio of 1:10 w/v to homogenize. The seeds were initially grinded for 10 to 15 mins at 4°C and were centrifuged for 3 mins at 400 rpm. The precipitated debris was discarded and the supernatant was transferred to a new tube, which were appropriately labeled. Then the supernatant was centrifuged at 1200 rpm for

5 mins to pellet the nucleus. Again the supernatant was transferred to a new tube and centrifuged at 6000 rpm for 20 mins, to pellet the mitochondria, the supernatant obtained was the soluble fraction.

4.2.3 Extraction of Tannins

a. Extraction of Hydrolysable Tannins

5 g of the powdered material was weighed and transferred to a 250 ml conical flask and 75 ml of water was added. The flask was gently heated and boiled for 30 min and centrifuged for 20 min at 2000 rpm. The supernatant was collected and made up to 100 ml in volumetric flask. 1 ml of sample extract was transferred to 100 ml volumetric flask containing 75 ml water. From this, 0.1ml was taken to estimate the amount of hydrolysable tannins present in the powdered material.

b. Extraction of Condensed Tannins

5 g of the powdered material was weighed and transferred to a 250 ml conical flask and 75 ml of methanol was added. The flask was gently heated and boiled for 30 min and centrifuged for 20 min at 2000 rpm. The supernatant was collected and made up to 100ml in volumetric flask. 1 ml of sample extract was transferred to 100 ml volumetric flask containing 75 ml methanol. From this, 0.1 ml was taken to estimate the amount of hydrolysable tannins present in the powdered material.

4.2.4. Protein Estimation by Folin-Lowry's Method

Protein concentrations were estimated by the procedure of Folin Lowry's. Various concentrations of reagents are given in Appendix pg. 62.

Aliquots of 0.4ml protein solution to be analyzed were added into appropriately labelled test tubes. The solutions were made up to 1ml with distilled water. A volume of 2.1 ml alkaline copper reagent was added to the test tubes and was incubated for room temperature for 10 min. After the incubation 0.2 ml of Folin's reagent was added to the test tubes and was kept at room temperature for 20 min. The intensity of the color developed was measured spectro photometrically at 660 nm. The intensity of the colour developed was directly proportional to the amount of protein present in it. The

concentration of protein solution was determined from a standard graph obtained by following the above procedure for pure crystalline bovine serum albumin in the concentration range of 40 μg to 200 μg.

4.2.5. Prussian Blue Method for Total Phenols

This is the original method described by Price and Butler. The concentrations of various reagents are given in Appendix pg 63. An aliquot (0.1 ml) of the was dispensed sample into a 125 ml Erlenmeyer flask .To it 50 ml of distilled water was added. A volume of 3.0 ml ferric ammonium sulphate was added at 1 minute intervals with swirling. Exactly 20 min after the addition of ferric ammonium sulphate, 3.0 ml of potassium ferric cyanide was added at timed intervals and swirled. Exactly 20 min after the addition of ferri cyanide, the absorbance was read at 720 nm, at 1 min intervals. The absorbance of the blank was subtracted from the absorbance obtained for each sample. Standardized against 0.01 M tannic acid monohydrate per 50 ml methanol. The concentration of total tannins present was determined from a standard graph obtained by following the above procedure for pure commercial tannic acid monohydrate in the concentration range of 5 μg/ml to 50 μg/ml.

4.2.6. Modified Prussian blue Assay for Total Phenols

The modified prussioan blue assay is the Price and butler method as modified by H D Graham to give greater color stability.

An aliquot of 0.10 ml of sample was taken in a test tube and 3 ml of distilled water was added. The concentrations of various reagents are given in Appendix pg. 63. The mixture was vortexed. To each sample, 1.00 ml of potassium ferric cyanide was added, followed immediately by 1 ml of ferric chloride and immediately vortexing of the mixture. The interval between handling each sample should be approximately 15 min after adding the reagents to sample, 5 ml of stabilizer was added to the sample and vortexed. The absorbance was read at 700 nm using appropriate blanks. The concentration of total tannin present was determined from a standard graph obtained by following the above procedure for pure commercial tannic acid in the concentration range of 5 μg/ml to 50 μg/ml

4.2.7. Folin-Ciocalteau Assay for Tannins

The phenolic content of water extract was measured by the Folin and Ciocalteau reagent (Julkunen – Tiitto, 1985). The concentrations of various reagents are given in Appendix pg. 64. 5ml of 70% acetone solution v/v was added to a test tube containing 100 mg freeze dried sample to extract phenolic compounds. 900 µl water, 0.5 ml 1M Folin-Ciocalteau reagent and 2.5 ml 20% sodium carbonate were added to 0.1ml extract. The mixture was vortexed after the addition of each reagent. It was kept for 30 mins at room temperature and the absorbance at 725 nm standard graph was determined using pure tannic acid.

4.2.8. Partial Purification of Ascorbate Peroxidase

(a) Isolation of Soluble Fraction

Tissue homogenization, ammonium sulphate precipitation and dialysis were performed at 4°C. Germinated seeds were homogenized in a pestle and mortar at the highest speed with 1l of ice-cold 100 mM potassium phosphate buffer (pH7.8), 2 mM ascorbate and 5mM EDTA. After filtration, the homogenate was centrifuged for 30 minutes at 4000 rpm. Ammonium sulphate granules were slowly added with constant stirring to the supernatant to achieve 50% saturation at 4°C and the resulting suspension was stirred for an additional 1 hr at 4°C.The pellet resulting from the centrifugation at 1000 rpm for 20 minutes was resuspended in 30 ml of 10 mM potassium phosphate buffer (pH 7.8) and 1mM EDTA and dialysed for 16 hrs against 4l of the same buffer changed twice.

(b) Ammonium Sulphate Fractionation of Proteins

Ammonium sulphate is a particularly useful salt for the fractional precipitation of proteins. It is available in a highly purified form, has great solubility and is inexpensive. Changes in the ammonium sulphate concentration of a solution can be brought about either by adding solid crystals or by adding 100% saturated solution. At lower salt concentrations, solubility of proteins is very high. However with increasing the salt concentrations, proteins begins to precipitate. It is observed that hydrophobic proteins

precipitate first with hydrophilic proteins precipitating at a much higher salt concentrations. In laboratory scale purification strategies, ammonium sulphate precipitation is often used as the first purification procedure. To purify the cytosolic APX ammonium sulphate crystals were added to the supernatant. Ammonium sulphate concentration was used in the range from 0 – 80%. Transfer the supernatant to a beaker and slowly add ammonium sulphate in the different ranges and each range was tested for the activity of ascorbate peroxidase.

(c) Dialysis of Protein Solution

Dialysis bags of suitable pore size and length was cut for the volume required. Concentrations of various solution are given in Appendix pg no 64. Bags were soaked in a solution of 2% sodium bicarbonate and 0.005% EDTA. The solution was boiled for 10 minutes. The bags were washed again in distilled water. The bags were then preserved in a suitable solution containing 0.1% sodium azide.

The dialysis bag was filled with a suitable volume of partially purified enzyme by ammonium sulphate precipitation. The ends of the bags were sealed tightly with the clips. The bag was then dialysed against 20mM glycine-NaOH buffer for the dialysis to occur. It was incubated till the final desired concentration is reached.

(d) Ultrafiltration of protein sollutions

The ultrafiltration equipment used was manufactured by Pall India Pvt. Limited, Mumbai (Model number 77200–52). The membrane used was the 50 KDa omega membrane. The time taken to run the process was 1 hr. The sample was allowed to run through the omega membrane (50 KDa). The filtrate contained proteins whose molecular weights exceeded 50 kDa and the retentate those whose molecular weight were less than 50 kDa. With the help of the peristaltic pump the filtrate and the retentate was separated.

4.2.9 Polyacrylamide Gel Electrophoresis (PAGE)

PAGE was carried out by the procedure of Lamelli. Concentrations of various solutions are given in Appendix pg. 62.

The glass plates and spacers were thoroughly cleaned and dried and assembled properly. The assembly was held firmly together with clips and clamped in an upright position. A solution of 2% agar (melted in a boiling water bath) was then applied around the edges of the spacers to hold them in place and to seal the chamber between the glass plates.

A 10% gel was prepared by mixing the following. Stock acryl amide solution-13.3ml; Tris-HCl (pH8.8)-8ml; Water-18.1ml; ammonium Persulphate solution-0.2ml and TEMED-20µl. The mixture was poured into the chamber between the glass plates distill water was layered on top of the gel and was left to set for 30-60 mins.

The stacking gel was prepared (4%) by mixing the following solutions: Stock acrylamide solution-1.35ml; Tris-HCl (pH 6.8) - 1ml; Water-7.5ml; Ammonium Persulphate-50µl and TEMED-10µl.

The layered water was removed from the top of the gel and washed with a little stacking gel solution. The stacking gel was poured into the slab spacer and the comb was positioned appropriately in the stacking gel. The gel was allowed to set for a period of 30-60 minutes. After the stacking gel has polymerized, the comb was removed without distorting the wells. The gel was carefully installed in the electrophoresis apparatus. The apparatus was filled electrode buffer and trapped air bubbles were removed at the bottom of the gel. The cathode was connected at the top and the power was turned on briefly to check the electrical circuit. The samples were prepared for electrophoresis, following suitable extraction procedures. The protein concentration of each sample was adjusted using the 5-strength sample buffer and water in such a way that the same amount of protein is present per unit volume. The concentration should be such as to give a sufficient amount of protein (50-200µl) in a volume (25-50µl) not greater than the size of the sample well. The required volume of sample solutions containing the marker dye bromophenol blue was taken in a microsyringe and carefully injected into a sample well through the electrode buffer. The current was maintained at 10-15 mA for initial 10-15 min until the samples travel through the stacking gel. The stacking gel helps to

concentrate the protein in the samples. The run was continued at 30 mA until the bromophenol blue reaches the bottom of the gel. After the run was complete, the gel was carefully removed and immersed in staining solution (coomassie brilliant blue.) for at least 3 hours or overnight with uniform shaking. The proteins are stained by coomassie brilliant blue. The gel was transferred to a suitable container with atleast 200-300 ml of destaining solution and shaked gently and continuously. Dye that is not bound to protein was removed. The destainer was changed frequently, particularly during initial periods, until the background of the gel is colorless. The proteins fractionated into band were seen coloured blue. The gel was photographed using a A400 powershot cannon of 3.2 megapixel digital camera.

4.2.10. Ascorbate Peroxidase Assay

Ascorbate peroxidase was assayed by the amount of ascorbate oxidized at 290 nm.Concentrations of various solutions are given in Appendix pg no 64. The reaction mixture contains 1.2 M H_2O_2, 50 mM potassium phosphate buffer (pH 7.4), water, enzyme extract, 40 mM ascorbate. The compositions of the buffer are given in the Appendix Pg.57. The total reaction volume was 3 ml. Initially 1.5 ml of 50 mM potassium phosphate buffer (pH 7.4) and 45 µl of H_2O_2 was added. To this, 60 µl of ascorbate was added. Finally, 1.3 ml of water and 80 µl of enzyme extract was added and the volume was made up to 3 ml. The blank contained 1.5 ml of water and 1.5 ml of buffer. The activity of the enzyme was observed by the decrease in absorbance at 290 nm due to oxidation of ascorbate.

4.2.11. Other Methods

Seedlings of *Vigna radiata* were hand picked and were weighed at regular growth periods to obtain wet tissue weights. The seedlings were then dried overnight at 60° C to obtain dry weights.

RESULTS AND DISCUSSIONS

5. RESULTS AND DISCUSSION

Seed development and maturation is a highly orchestrated multi-step process during which embryos are formed and supplied with the carbohydrates, proteins and lipids needed for the subsequent germination. During seed development the increase in size is due to cell division and expansion, followed by a progressive accumulation of storage compounds.

Table 5.1:Comparision of wet weight and dry weight of seedlings

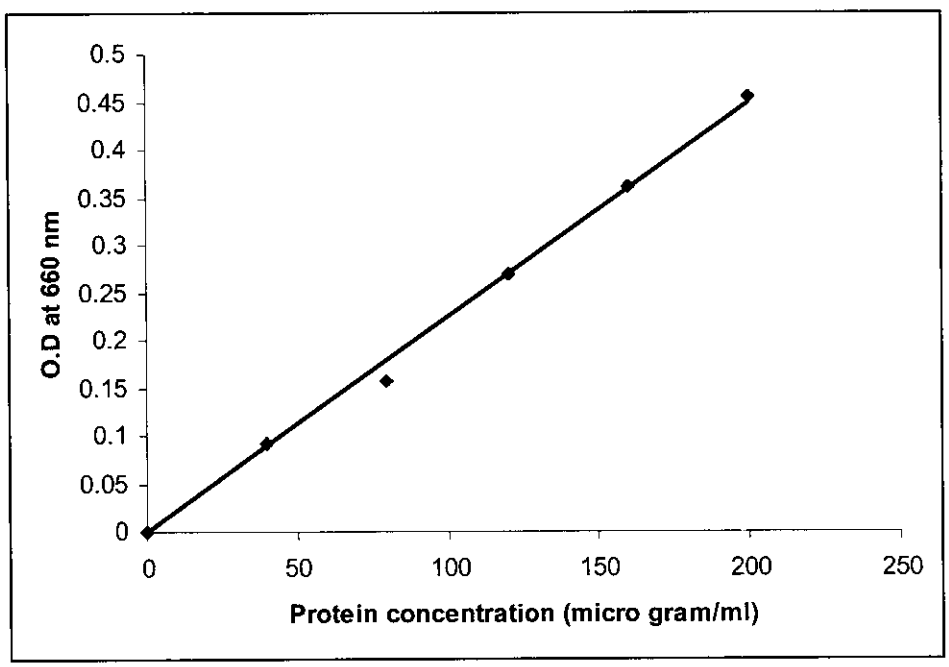
Growth period (h)	Weight (g per 100 seedlings)	
	Wet weight	Dry weight
24	9.23	7.77
48	10.12	8.54
72	10.98	9.03

In the present study, the growth of seedlings was monitored at 24-hour intervals up to 72 hours. The wet weight show dramatic increases of 60 and 70% at 48 and 72 hours growth periods .The dry weight though increased significantly was not as profound as seen in wet weight.

Figure 5.1: Germinating seedlings of *Vigna radiata*.

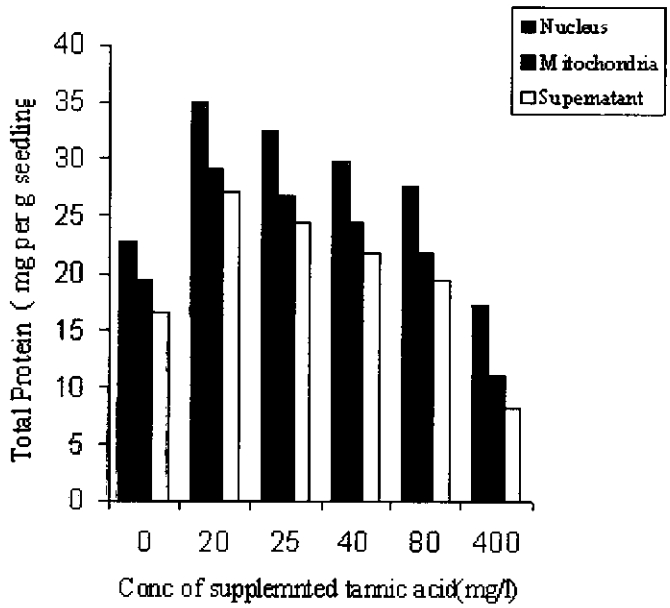
The protein concentration were prepared in the concentration range 50 – 200 µg/ml, using bovine serum albumin. A linear relationship was obtained using folin's - lowry as the reagent (Fig. 5.2).

Fig. 5.2 Protein standardization graph



Supplementation of tannic acid had a profound influence on the total protein content of various subcellular fractions (Fig. 5.3). A dose dependent effect were observed with enhancements in the protein levels at lower concentrations. Supplementations of tannic acids at progressively higher concentration resulted in lesser enhancements in protein levels.

Fig 5.3: Effect of supplemented tannic acid on total protein content of germinating seedlings of *Vigna radiata* - 24 hr growth period



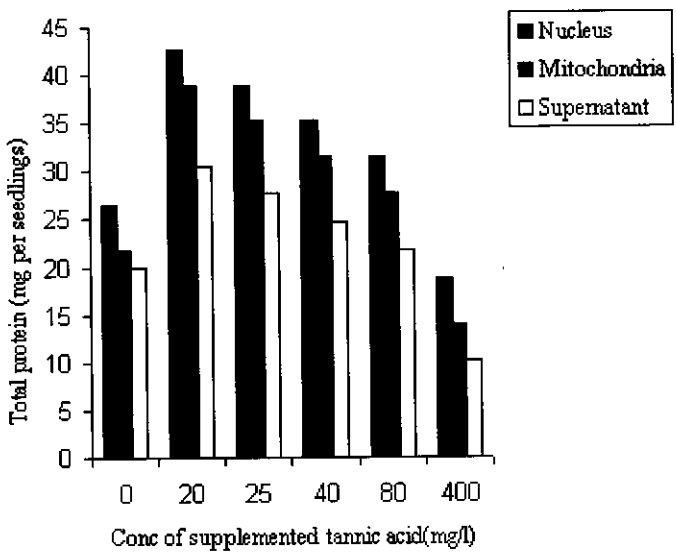
At the supplementations level of 20 mg/l. A 50 % increases was noted in nuclear and mitochondrial fractions. Increasing concentrations of up to 80 mg/l resulted in only about 10 to 20% enhancement in protein levels .In other words increasing concentrations of tannic acids seems to be growth inhibitory.

Maximum growth enhancements at 24 h growth period was seen in the soluble fraction thus tannic acid supplementation seems to preferentially influence the proteins of the soluble fractions.

Supplementation of tannic acid at borderline toxic levels of 400 mg/l resulted in dramatic decreases in the total protein levels of all sub cellular fractions tested. Notably maximal inhibitory effect of 45 to 50 % was seen in the mitochondrial and supernatant fractions.

At further growth up to 48h, supplementation of tannic acid showed differential response with respect to protein levels of the sub cellular fractions (Fig. 5.4).

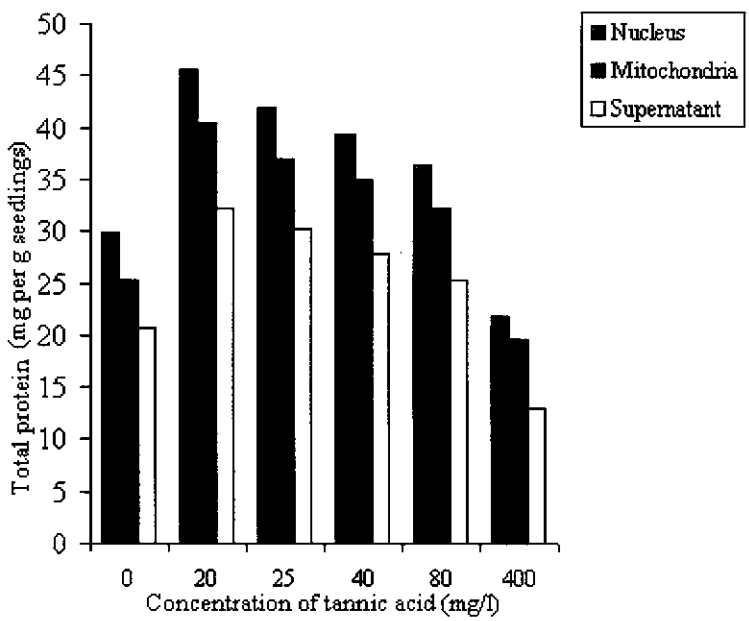
Fig 5.4: Effect of supplemented tannic acid on total protein content of germinating seedlings of *Vigna radiata* - 48 hr growth period



In contrast to the effect seen at 24 h growth period, wherein maximal growth enhancement was observed in soluble fractions at 48h growth period, maximal growth enhancement was seen with mitochondrial fractions. However the borderline toxic concentrations of tannic acid showed a growth inhibition which followed a trend similar to that seen at 24 h growth period.

The differential response of tannic acid supplementation in differential subcellular fractions observed at 24 and 48 h growth periods disappeared at 72h growth period i.e, supplementation at 20mg/l and 80mg/l elicited about 55 to 60% and 20 to 25% growth enhancements respectively (Fig.5.5). The mitochondrial or solute fractions showed differing response with respect to tannic acid supplementation.

Fig 5.5: Effect of supplemented tannic acid on total protein content of germinating seedlings of *Vigna radiata* - 72 h growth period



Control seedlings were grown in the absence of supplemented tannic acid. The ascorbate peroxidase activity of the soluble fractions increased by 20% at 72 h growth period compared to the level of 24 h growth period (Fig. 5.6). The supplemented seedlings however showed increase in total activity of about 40 to 50% at 72 h growth period. The increasing concentration of tannic acids at all growth periods showed a similar trend of decrease in the total activity. At the supplemented level of 80 mg/l maximum decrease of about 30% was observed at 24 h growth period. Border line toxic level of supplementation at 400 mg/l invariably showed marked decrease in total activity of ascorbate peroxidase.

Recent studies have focused on the changes in activities of ascorbate peroxidase isoenzymes in higher plants subjected to several environmental stresses such as ozone, high light, extremes of temperature, salt, and paraquat (Tanaka *et al.*, 1985 Mittler and Zilinskas, 1994; Prasad *et al.*, 1994; Conklin and Last, 1995; Rao *et al.*, 1996; Dat *et al.*, 1998; López *et al.*, 1996; Donahue *et al.*, 1997;Yoshimura *et al.*, 2000). Interestingly, ascorbate peroxidase activities generally increase along with activities of other antioxidant enzymes like catalase, SOD, and GSH reductase in response to various environmental stress factors, suggesting that the components of Active oxygen species-scavenging systems are co-regulated. Drought-resistant maize shows greater induction of ascorbate peroxidase activity than sensitive plants, in addition to a significant increase in GSH reductase activity (Pastori and Trippi, 1992)

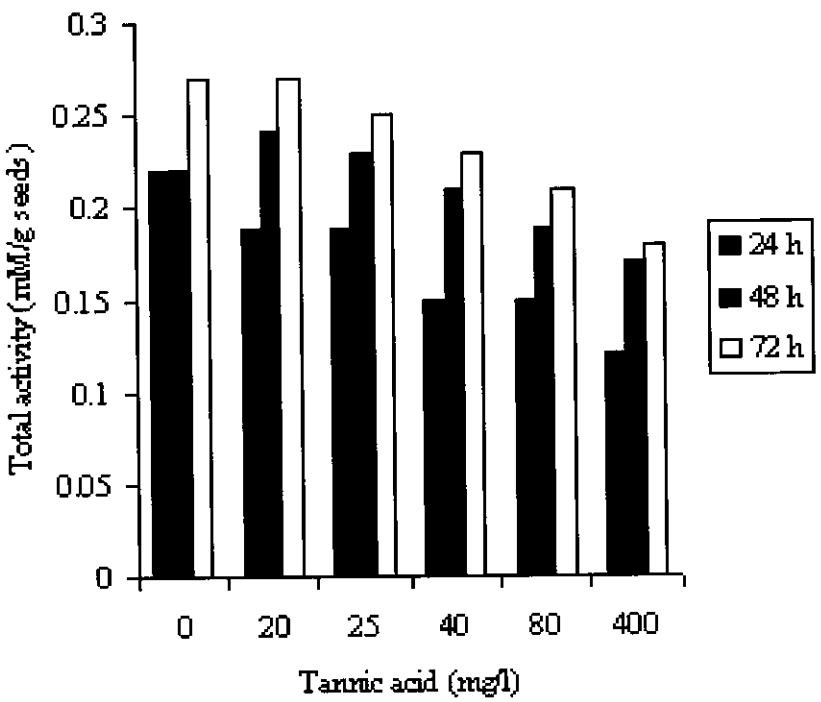
The distribution of Ascorbate Peroxidase activity in various sub-cellular frations of *Vigna radiata* is shown in Table 5.2. Maximal activity was noted in the soluble fraction and subsequent studies on ascorbate peroxidase activity were carried out with the soluble fractions.

Table 5.2. Comparison of specific activity of ascorbate peroxidase from various sub-cellular organelles of *Vigna radiata*

Growth Period (h)	Specific Activity (µM/g seeds)		
	Homogenate	Mitochondria	Soluble fraction
24	0.29	0.27	24
48	0.71	0.62	29
72	0.96	0.81	32

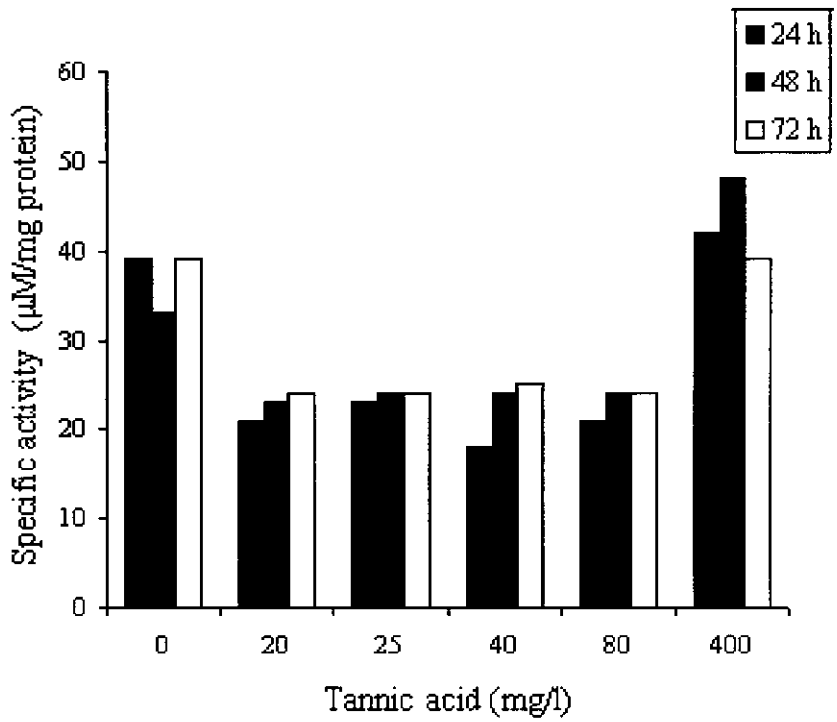
Seedlings was grown on deionized water.

Fig 5.6: Effect of supplemented tannic acid on total activity of ascorbate peroxidase of germinating seedlings of *Vigna radiata*



Transgenic tobacco plants expressing antisense RNA for cytoplasmic ascorbate peroxidase showed 45–55% less activity than the non-transgenic plants, and increased susceptibility to ozone (Övar and Ellis, 1997)

Fig 5.7: Effect of supplemented tannic acid on specific activity of ascorbate peroxidase of germinating seedlings of *Vigna radiata*



The specific activity of control seedlings during growth remained more or less unchanged (Fig. 5.7). However, supplementation of tannic acid at all levels had the influence on specific activity during growth. In general, supplementation of tannic acid up to 80mg/l resulted in increasing levels of activity during growth, whereas at the borderline toxic level of 400mg/l the specific activity decreases marginally. Supplementation of tannic acid at 40mg/l showed maximal increase of about 40%. In general,

- Control seedlings had maximal specific activity,
- Specific activity of control seedlings remain constant during growth,
- Compared to activity of the control seedlings, specific activity decreased upon tannic acid supplementation at all concentration levels and at all growth periods,
- Maximal increases in specific activity during growth was observed at the supplementation level of 40 mg/l.

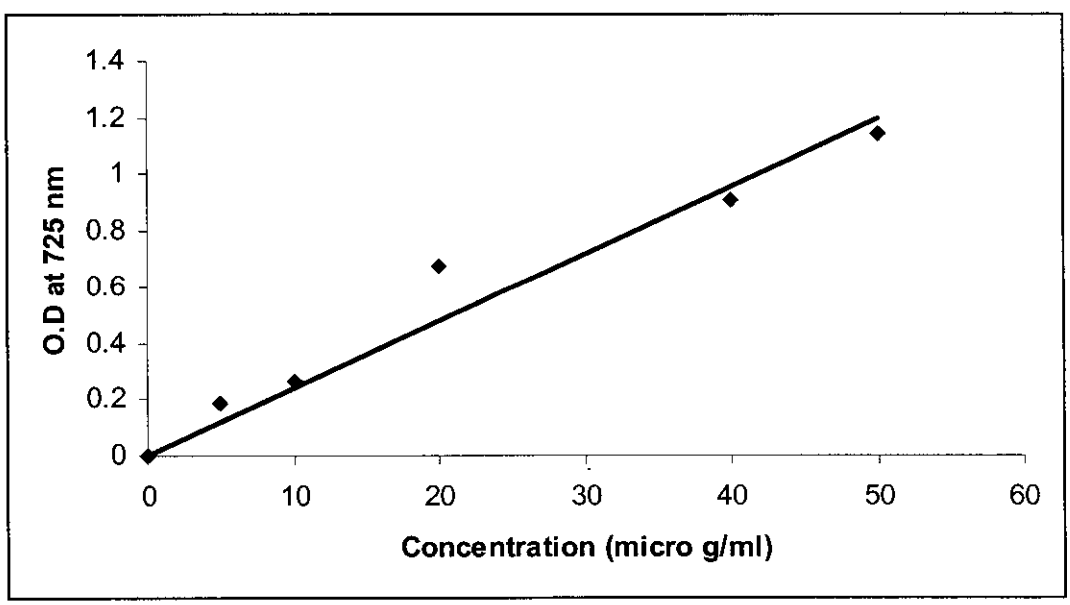
Recent studies have focused on the changes in the cytoplasmic ascorbate peroxidase expression level under environmental stresses such as ozone, UV-B radiation, low temperature, high-light stress, salinity, water stress including drought, and pathogen infection (Tanaka *et al.,* 1985; Schöner and Krause, 1990; Mittler and Zilinskas, 1992, 1994; Mishra *et al.,* 1993; Willekens *et al.,* 1994; Conklin and Last, 1995; Hernández *et al.,* 1995; Kubo *et al.,* 1995; Rao *et al.,* 1996; Örvar *et al.,* 1997; Mittler *et al.,* 1998).

Cytoplasmic ascorbate peroxidase induction by methyl viologen or high-light stress were reported in pea, maize, rice, and Arabidopsis (Mittler and Zilinskas, 1992; Pastori and Trippi, 1992; Donahue *et al.,* 1997; Karpinski *et al.,* 1997; Storozhenko *et al.,* 1998; Morita *et al.,* 1999).

Over expression of c ascorbate peroxidase in tobacco plants provides increased resistance against mv treatment (Allen *et al.,* 1997).Wang *et al.* (1999) indicated that the protective action provided by the expression of mAPX seems to be specific against oxidative stress originating from microbodies.

Standards of tannic acid in the concentration range 5 – 50 μg/ml were prepared and the color was developed using Folin's – Ciocalteau reagent. A linear relation obtained is shown in Fig. 5.8.

Fig. 5.8 Tannic acid standardization graph



Sorghum is a rich source of both hydrolysable and condensed tannins. For the present study we have limited ourselves to study the effect of hydrolysable tannins. Condensed tannins are water insoluble and therefore creates practical difficulties for supplementation. The tannin contents of sorghum was compared with that of closely related maize seeds (Table 5.3).

Table 5.3: Comparison of amount of type of tannin in sorghum and maize

Type of tannin	Tannin content in sorghum(mg/l)	Tannin content in maize(mg/l)
Condensed tannin	13	3
Hydrolysable tannin	40	4

The sorghum seedlings had four fold higher concentration of condensed tannin contents compared to that of maize, while the hydrolysable tannin levels were remarkably higher by 10 fold justifying our choice of sorghum seeds as a source of hydrolysable tannins.

To determine the effect of hydrolysable tannins on growth, isolated hydrolysable tannins was supplemented at a concentration range of 2 to 40 mg/l. Supplementation of hydrolysable tannins even at the lowest concentrations of 2mg/l resulted in dramatic increase of 80 to 90% in the protein levels of various subcellular fractions (Table 5.3).

Table 5.4: Effect of isolated hydrolysable tannins on total protein content of germinating seedlings of *Vigna radiata* – 24 hr growth period

S.No	Concentration of tannic acid (mg/l)	Total protein content (mg per g seedlings)		
		Nucleus	Mitochondria	Supernatant
1.	Control	22.70	19.46	16.51
2.	2	44.25	34.32	29.70
3.	3	41.73	32.01	27.09
4.	4	39.12	29.18	24.48
5.	8	36.64	26.68	22.89
6.	40	21.76	19.87	15.59

Seedlings of *Vigna radiata* were grown with supplemented isolated hydrolysable tannins at the indicated concentration levels. Control group seedlings was grown on deionized water.

The maximal increase of about 95% was observed in the nuclear fractions. Further studies are needed to address the underlying cause. Similar to the effects seen with supplemented tannic acids, increasing concentrations of isolated hydrolysable tannins, resulted in progressive decrease in protein levels. Thus the tannic acids and hydrolysable tannins act similarly i.e, at lower concentrations they are growth enhancing, while at increasingly higher concentrations they become progressively growth inhibitory. At the highest concentration level tested i.e 40 mg/l marginal decrease in protein levels was observed in all subcellular fractions.

The trend observed at 24h growth period continued at 48 h growth period (Table 5.5). However protein levels of mitochondria and soluble fractions was preferentially enhanced by 30% compared to the levels at 24h.

Table 5.5: Effect of isolated hydrolysable tannins on total protein content of germinating seedlings of *Vigna radiata* - 48 h growth period

S.No	Concentration of tannic acid (mg/l)	Total protein content (mg per g seedlings)		
		Nucleus	Mitochondria	Supernatant
1.	Control	26.56	21.76	19.98
2.	2	50.54	42.69	38.50
3.	3	47.92	39.86	34.95
4.	4	44.78	37.25	32.22
5.	8	42.38	34.32	29.40
6.	40	28.35	21.76	16.53

Seedlings of *Vigna radiata* were grown with supplemented isolated hydrolysable tannins at the indicated concentration levels. Control group seedlings was grown on deionized water.

The levels of the nuclear fractions however remained constant. Notably at the border-line toxic level of 40 mg/l the supernatant showed about 20% decrease in protein levels, an effect not seen in any other cellular fractions.

More pronounced effects upon supplementation of hydrolysable tannins at 72h growth period (Table 5.6).

Table 5.6: Effect of isolated hydrolysable tannins on total protein content of germinating seedlings of *Vigna radiata* - 72 hr growth period

S.No	Concentration of tannic acid (mg/l)	Total protein content (mg per g seedlings)		
		Nucleus	Mitochondria	Supernatant
1.	Control	29.92	25.21	20.71
2.	2	53.16	45.62	40.90
3.	3	51.69	43.32	38.29
4.	4	48.76	40.60	34.95
5.	8	45.83	37.56	31.18
6.	40	33.79	30.97	27.31

Seedlings of *Vigna radiata* were grown with supplemented isolated hydrolysable tannins at the indicated concentration levels. Control group seedlings was grown on deionized water.

The proteins of the soluble fraction were differentially enhanced by hydrolysable tannins upto 98%. The effect of hydrolysable tannins was distinctly different from that of tannic acid supplementation to seedlings of *Vigna radiata* 72h growth period. While the effect of tannic acid was normalized among the various subcellular fractions, the effect of hydrolysable tannins was distinctly more profound in the soluble fraction, mitochondrial and nuclear fractions respectively.

Hydrolysable tannins influence the total activity of ascorbate peroxidase greatly in a dose dependent manner (Table 5.7). The total activity upon supplementation of hydrolysable tannins decrease by 30%, at 24h growth period and about 15% at 48 and 72h growth period.

Table 5.7: Effect of isolated hydrolysable tannins on total activity of ascorbate peroxidase of germinating seedlings of V*igna radiata*

Concentration of Hydrolysable tannins (mg/l)	Total activity (mM/g seeds)		
	Growth period		
	24 h	48 h	72 h
Control	0.22	0.22	0.27
2	0.23	0.21	0.25
3	0.19	0.21	0.27
4	0.13	0.21	0.23
8	0.15	0.19	0.22
40	0.11	0.15	0.23

Seedlings of *Vigna radiata* were grown with supplemented isolated hydrolysable tannins at the indicated concentration levels. Control group seedlings was grown on deionized water.
One unit of activity corresponds to 1 µmol ascorbate oxidase per min.

The trend of decreases continued with border line toxic level of 40 mg/l. Remarkably at 72 h growth period a 40% increase in total activity was seen at 3 mg/l and 77% increase at 4 mg/l and a dramatic two-fold increase at 40 mg./l. In summary, hydrolysable tannins elicited more pronounced increases on the total activity during growth and this effect increased linearly with higher concentrations of hydrolysable tannins.

The treatment of cultured rice cells with hydroxyurea, a suicide inhibitor of ascorbate peroxidase, or aminotriazole, an effective inhibitor of catalase, led to increased cellular H_2O_2 content and a large increase in the cAPX transcript level (Morita *et al.*, 1999)

Changes in the ASC–GSH cycle also occur in developmental processes such as seed germination (Tommasi *et al.*, 2001). Catalase is mainly present in the microbodies (Scandalios, 1994), organelles that, being involved in the fatty acid metabolism, are particularly active in lipid synthesizing cells. This could explain an increase in Catalase

activity during the first period of kernel development, when the synthesis of the storage lipids also increases (Morrison, 1988). On the other hand, ascorbate peroxidase , being more widely shared out in cell organelles (De Gara and Tommasi, 1999), could intervene in the removal of H_2O_2 produced by metabolic pathways with different timing during kernel maturation. Moreover, ascorbate peroxidase is always very active in dividing cells and tissues undergoing differentiation (De Gara *et al.*, 1996; de Pinto *et al.*, 2000), whereas it decreases in senescent tissues (Borraccino *et al.*, 1994)

As mentioned previously, specific activity of control seedlings remained unchanged during growth at lowest level of supplemental of 2 mg/l. Supplementation of hydrolysable tannins at 72 h growth period decrease in about 15% specific activity (Table 5.7). Such a decrease disappeared at the next higher level supplementation of 3 and 4 mg/l. Further supplementation of 8 mg/l lead to the reversal. in the response, that is an increase of 15%. This trend continued at higher level of 40 mg/l with 30% increase in specific activity. As was the case with supplementation of tannic acid, isolated hydrolysable tannins decreases the specific activity of ascorbate peroxidase in the soluble fraction of germinating seedlings of *Vigna radiata*. The decreases were about 45 to 50% at all growth periods, (i.e. tannic acid elicited decrease of 30 to 40%).

The transcript level of pea cytoplasmic ascorbate peroxidase also increases 4-fold in response to drought stress, but is even more dramatically enhanced (15-fold) following recovery from stress (Mittler and Zilinskas, 1994. The analysis of the protein level and activity of ascorbate peroxidase indicates that, during recovery from drought stress, cytoplasmic ascorbate peroxidase expression in pea is regulated at the post-transcriptional level, at least in part at the level of protein synthesis. Furthermore, many environmental factors such as high light, salt, wounding, pathogen infection, fruit ripening, and paraquat affect the steady-state transcription level of cytoplasmic ascorbate peroxidase (Mittler and Zilinskas, 1992; Pastori and Trippi, 1992; Schantz *et al.*, 1995; Karpinski *et al.*, 1997, 1999; Övar *et al.*, 1997; Biemelt *et al.*, 1998; Mittler *et al.*, 1998, 1999; Morita *et al.*, 1999; Yoshimura *et al.*, 2000)

Table 5.8: Effect of isolated hydrolysable tannins on specific activity of ascorbate peroxidase of germinating seedlings of *Vigna radiata*

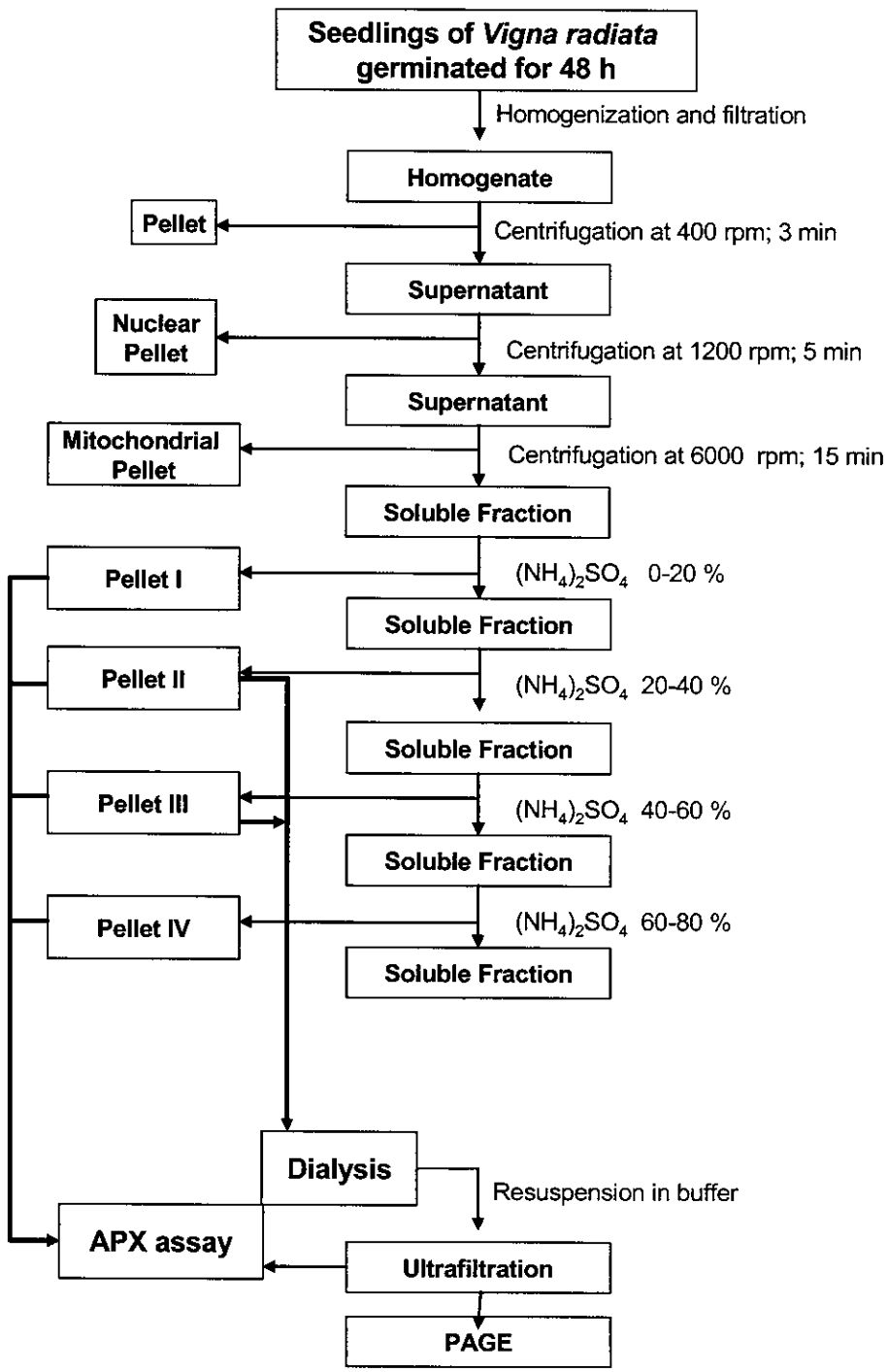
Concentration of Hydrolysable tannins (mg/l)	Specific activity (μM/mg protein)		
	Growth period		
	24 h	48 h	72 h
Control	39	33	39
2	21	18	18
3	21	18	21
4	18	18	18
8	18	18	21
40	18	27	24

Seedlings of *Vigna radiata* were grown with supplemented isolated hydrolysable tannins at the indicated concentration levels. Control group seedlings was grown on deionized water.
One unit of activity corresponds to 1 μmol ascorbate oxidase per min.

These results indicate that the genes for chlorophyll ascorbate peroxidase and m ascorbate peroxidase are constitutively expressed for the immediate and efficient detoxification of H_2O_2 under normal and stress conditions, while the gene expression for c-ascorbate peroxidase is responsive to environmental changes, resulting in the protection of important cellular compartments from oxidative stress and in strict control of the level of H_2O_2 in intracellular signaling.

The isoenzymes of the soluble fractions of the Vigna radiata was chosen for purification for the following reasons: The activity of the isoenzymes was higher in these fractions and the isoenzymes was relatively stable and also stable in the absence of Ascorbate. Ascorbate Peroxidases are highly labile enzymes. They are easily inactivated in the absence of substrate, Ascorbate. In the present study, keeping in view of the above mentioned instability of the enzyme, partial purification of the enzymes was attempted in the presence of added Ascorbate.

Fig.5.9 Purification chart for ascorbate peroxidase of *Vigna radiata*



Ammonium Sulphate fractionation of the soluble fractions from both tannic acid and isolated hydrolysable tannins revealed maximal activity in the 40-60% fractions (Table 5.9; 5.10) The control seedlings however showed reasonable Ascorbate Peroxidasae activity at other fractions as well. i.e., 20-40% and 60-80% (Table 5.11). The gel electrophoretic profile is in agreement with this observation (Fig. 5.9). It might be possible that these isoenzymes are the ones that are modulated by tannin exposure. Further studies are needed to ascertain this fact.

Table 5.9 Total activity of ascorbate peroxidase of *Vigna radiata* supplemented with isolated hydrolysable tannins.

Ammonium Sulphate fraction	Total activity (mM/ml)
Before addition	0.021
0-20%	0.093
20-40%	0.008
40-60%	0.334
60-80%	0.06
80-100%	0.061

Table 5.10 Total activity of ascorbate peroxidase of *Vigna radiata* supplemented with tannic acid

Ammonium Sulphate fraction	Total activity (mM/ml)
Before addition	0.107
0-20%	0.093
20-40%	0.08
40-60%	0.32
60-80%	0.12
80-100%	0.09

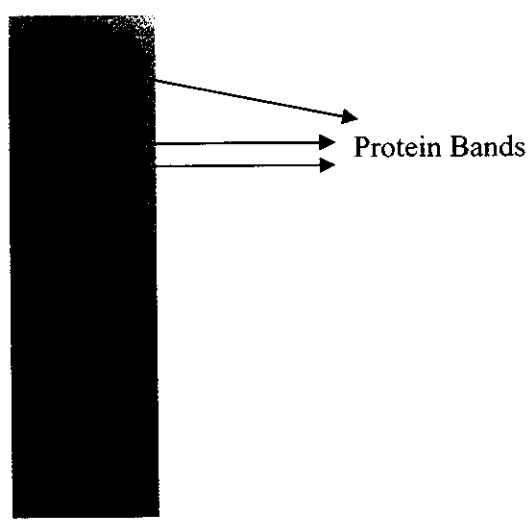
Table 5.11 Total activity of ascorbate peroxidase of control seedlings of *Vigna radiata*

Ammonium Sulphate fraction	Total activity (mM/ml)
Before addition	0.093
0-20%	0.093
20-40%	0.107
40-60%	0.27
60-80%	0.107
80-100%	0.085

Table 5.12. Partial purification of ascorbate peroxidase by ultrafiltation

Protein Fraction	Total activity (mM/ml)
Before ultrafiltration	0.27
After ultrafiltration	0.532

Figure 5.10. Electrophoretic profile of proteins



The instability of APX isoenzymes seems to be one of the reasons for the difficulty in obtaining large amounts of highly purified APX isoenzymes. Therefore, advances in the expression of the recombinant APX isoenzymes utilizing cDNA clones have provided new approaches for characterizing the structure and function of each APX isoenzyme (Raven, 2000).

One of the characteristic properties of the chlAPX isoforms is rapid inactivation when the level of ascorbate is too low for the operation of the catalytic cycle of the APX isoenzymes (Miyake and Asada, 1996). It has been suggested that the level of ascorbate in chloroplasts affects the stability of the chlAPX isoforms under oxidative stress conditions.

One of the specific properties of APX isoenzymes is rapid inactivation in an ascorbate-depleted medium. This is especially true for chloroplastic APX isoforms, whose half-inactivation time is only 15 s (Miyake and Asada, 1992; Yoshimura *et al.*, 1998). Both the cAPX and the mAPX isoenzymes are less sensitive to depletion of ascorbate than the chloroplastic APX isoforms, so the half-inactivation times of cAPX and mAPX were approximately 60 min and over 24 h, respectively (Chen and Asada, 1989; Miyake and Asada, 1992; Ishikawa *et al.*, 1998).

CONCLUSIONS

CONCLUSIONS

It has emerged from the present study that tannin has a profound influence on the ascorbate peroxidase activity. Supplementation of tannic acid and isolated hydrolysable tannins had a profound influence on the total protein content of various sub cellular fractions. A dose dependent effect was observed with enhancements in the protein levels at lower concentrations. Supplementations of tannic acid at progressively higher concentration resulted in lesser enhancements in protein levels.

In addition to distribution of activity in the different sub cellular fractions, varied during growth the total activity of ascorbate peroxidase in the soluble fraction was maximal in control seedlings. Supplementation of tannins partially reversed the stress conditions as seen by the lowered levels. The specific activities clearly confirmed the above observation.

Partial purification of the soluble fraction isoenzyme was attempted. An interesting observation was the presence of more than one isoenzyme in the soluble fraction. Further studies will shed more light on this aspect.

APPENDIX

APPENDIX

I. Reagents for Estimation of Protein by Folin-Lowry's Method

Alkaline copper reagent (Lowry's reagent):

Solution A: 1% Copper Sulphate

-In a clean dry 100ml standard flask weigh 1g of $CuS0_4$ and make up to the mark with distilled water.

Solution B: 1% Sodium Potassium Tartarate

-In a clean dry 100ml standard flask weigh 1g of sodium potassium tartarate and make upto the mark with distilled water.

Solution C: 2% Sodium Carbonate in 0.1N NaOH

-In a clean dry 100ml standard flask weigh 2g of sodium carbonate and make uoto the mark with 0.1N NaOH.

Mix 1ml of Solution A, 1ml of Solution B and 98ml of Solution C.

Folin- Ciocalteau Reagent:

The composition of Folin-Ciocalteau reagent is 100ml Sodium Tungstate, 25g sodium molybdate, 500ml distilled water, 50ml of 65% phosphoric acid and 100ml of conc. HCl. The reagent should be prepared freshly.

II. Reagents for Poly Acrylamide Gel Electrophoresis

1. Stock Acrylamide Solution

Acrylamide 30%	30g
Bisacrylamide 0.8%	0.8g
Water	100ml

2. Separating Gel Buffer

1.875M Tris-HCl	22.7g (pH 8.8)
Water	100ml

3. Stacking Gel Bufffer

0.6M Tris-HCl	7.26g (pH 6.8)
Water	100ml

4. Polymerizing Agents

a) Ammonium persulphate 5%	0.5g/10ml, prepare freshly before use
b) TEMED	fresh from the refrigerator

5. Electrode Buffer

0.05M Tris	12g
0.192M Glycine	28.8g (pH 8.2-8.4)
Water	2L

6. Sample Buffer (5X concentration)

Tris-HCl Buffer (pH 6.8)	5ml
Sucrose	5g
Bromophenol Blue (0.5% W/V solution in water)	1ml

III. Reagents for Prussian blue assay for total phenols:

0.1M $FeNH_4(SO_4)_2$ in 0.1M HCl

Dilute concentrated HCl to 0.1M by bringing 8.3ml of concentrated acid to 1l of distilled water.

Make the ferric ammonium sulphate by dissolving 48.2g of dodecahydrate salt $FeNH_4(SO_4)_2.12H_2O$ in 1l of the 0.1M HCl. This will make a pale yellow solution.

0.008M $K_3Fe(CN)_6$

Dissolve 2.63g of potassium ferricyanide in 1l of distilled water. This will make a yellow solution.

IV. Reagents for modified Prussian blue assay for total phenols:

0.02M Ferric chloride in 0.1M HCl

Dilute concentrated HCl to 0.1M by bringing 8.3ml of concentrated acid to 1l of distilled water. Make the ferric chloride by dissolving 3.24g of anhydrous ferric chloride in 1l of the 0.1M HCl. This will make a pale yellow solution.

0.016M $K_3Fe(CN)_6$

Dissolve 5.26g of potassium ferricyanide in 1l of distilled water. This will make a yellow solution.

V. Solutions for Dialysis of protein solutions:

100 mM Tris (pH 7.5)

200 mM NaCl

5 mM $MgCl_2$

Sodium azide, Sodium bicarbonate

EDTA, Glycine and NaOH.

VI. Solutions for preparation of homogenization media:

0.3M Mannitol

Tris buffer (pH7-8)

10mM Mercaptoethanol

10mM $Mg2^+$

1mM $Ca2^+$

Poly vinyl pyrrolidone and BSA (0.1-0.2%)

VII. Reagents for Folin - Ciocalteau reagent:

5ml of 70% acetone solution

0.5ml 1M Folin-Ciocalteau reagent

2.5ml 20% sodium carbonate

100mg phenolic compounds.

VIII. Solutions for ascorbate peroxidase assay:

50mM Potassium phosphate buffer (pH7.4)

40mM Ascorbate

45µl of H_2O_2

80µl of enzyme extract

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REFERENCES:

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