STUDIES ONOXALICACIDANDOXALATE OXIDASE ENZYME IN *Costus pictus* D.Don

By

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THESIS

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DECLARATION

I hereby declare that the thesis entitled "Studies on oxalic acid and oxalate oxidase enzyme in *Costus pictus* D.Don" is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, fellowship, associateship or other similar title, of any other university or society.

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CERTIFICATE

Certified that the thesis entitled "Studies on oxalic acid and oxalate oxidase enzyme in *Costus pictus* D.Don" is a record of research work done independently by Mr.R.Sathishraj under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to him.

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ABBREVIATIONS

ALP	Alkaline phosphatase
ALT	Aminotransferases
AST	Aspartate aminotransferase
BSA	Bovine Serum Albumin
BLAST	Basic Local Alignment Search Tool
β	Beta
b.w	Body weight
CE	Capillary Electrophoresis
$CaCl_2$	Calcium chloride
^oC	Degree Celsius
Da	Dalton
D.Don	David Don
EC	Enzyme commission
ECB	European corn borer
EDTA	Ethylenediaminetetraacetic acid
GC	Gas Chromotography
g	Gravity
g	Gram
h	Hour(s)
HPLC	High Performance Liquid Chromotography
H_2O_2	Hydrogen peroxide
KAU	Kerala Agricultural University
kDa	kilo Dalton
Kg	Kilogram
K_m	Michaelis constant
m	Meter
M	Molar
MECP	Methanol Extract of *Costus pictus*
MS	Mass Spectrometer

mg	Milligram
min	Minute(s)
ml	Millilitre
mm	Millimeter
mM	Millimolar
nmol	Nanomole
MW	Molecular weight
µg	Microgram
µl	Microlitre
µM	Micromole
nm	Nanometer
N	Normality
NaOH	Sodium hydroxide
OXO	Oxalate oxiase
PSI-BLAST	Position Specific Iterated BLAST
pKa	Acid dissociation constant
po	By mouth (orally)
pH	Hydrogen ion concentration
rpm	Rotations per minute
R_f	Retention factor
S	Substrate
Sec	Second (s)
TCA	Trichloro acetic acid
TLC	Thin Layer Chromatography
V	Velocity
V_{max}	Velocity maximum
v/v	Volume by volume
λ_{max}	Lamda maximum
%	Percentage
V	Five

INTRODUCTION

1. INTRODUCTION

Oxalic acid is a ubiquitous constituent of plants, and several species, including some crop plants, accumulate high levels of the simplest dicarboxylic acid (Libert and Franceschi, 1987). Oxalates have been studied in the basic and applied sciences for centuries. In biology, oxalates have usually been regarded as inert end products of carbon assimilation that, in the forms of their insoluble calcium salts, are well-tolerated 'storage forms' of calcium in plants, and serious contributors to chronic, sometimes acute, 'stone diseases' in humans (Holmes *et al.*, 2001).

Entrenched and traditional views of oxalate as a 'static substance' were challenged by the discovery that germin, long known as a marker of growth onset in germinating cereals (Lane, 1994) and long suspected of being an agent in host-plant resistance to disease (Lane *et al.*, 1986), is an oxalate oxidase (Dumas *et al.*, 1993) conveniently called germin oxalate oxidase to distinguish them from oxalate oxidase in other plants (e.g., moss, beet, spinach, banana). Germins are a small group of homologous proteins first found, and still only known to occur, in "true cereals": barley, maize, oat, rice, rye and wheat.

Oxalate oxidase is widespread in nature and has been found in bacteria (Koyama 1988), fungi (Escutia *et al.*, 2005) and various plant tissues (Bernier and Berna, 2001). In plants, oxalate oxidase appears to play important roles in antimicrobial defense and signaling. A number of fungal pathogens of plants produce millimolar concentrations of oxalic acid during infection, etching the cell surface and interfering with guard cell function (Livingstone *et al.*, 2005). Plant cells express oxalate oxidase as an important counter measure to fungal invasion, eliminating the oxalic acid and producing hydrogen peroxide, which can serve both as a fungicidal agent and as a signal for plant defenses and development (Lane, 2002).

Several species of plants have been rendered fungus-resistant by heterologous expression of wheat oxalate oxidase (Welch *et al.,* 2007). In addition to these biological roles, oxalate oxidase has significant bio-analytical applications and is routinely used in the clinical determination of oxalate in biological fluids (Li and Madappolly, 1989), and has potential applications in treatment of calcium oxalate kidney stones (Svedruzic *et al.,* 2005). Other applications of oxalate oxidase include the prevention of formation of calcium oxalate deposits in paper manufacture (Cassland *et al.,* 2004).

On the other hand, oxalic acid is a strong chelating agent and forms salts with multivalent cations. Oxalic acid has been considered as an antinutrient due to its inhibitory effect on mineral bioavailability and to its formative effect on calcium oxalate urinary stone. In man, oxalate is a metabolic end product with no enzyme present to act on it (Scarpellini *et al*., 2008). Hence excess oxalate contributed by intake of oxalate rich foods leads to nephrolithiasis. It is a condition in which oxalate crystallises to form stones in the kidney, bladder and urethra. But in plants, oxalic acid exists along with its metabolising enzyme oxalate oxidase. The oxalate oxidase from plants can be effectively used to reduce the oxalate level in humans. Oral administration of oxalate oxidase crystals effectively reduces the oxalate content in humans (Shenoy *et al.,* 2006).

The detection of oxalate in urine and blood is important in the diagnosis and management of clinical disorders caused by oxalates. A number of methods are available to detect oxalate, the most common methods being amperometry, chromatography and spectrophotometry. Chromatography involves the detection of oxalate using HPLC, GC and CE. In clinical diagnosis, rapidity, reliability, reproducibility and cost are all important factors. A number of established enzymatic assays have been optimised and investigated in detail by many research groups detecting oxalate utilising oxalate oxidase.

Oxalate oxidase enzyme is commercially available from Sigma Aldrich Ltd. and was also obtainable from Boehringer Mannheim until it was withdrawn in 1998. Sigma oxalate oxidase is purified from barley roots and has been reported to be insoluble, relatively impure (75 per cent) and varies in activity from batch to batch (Dawson, 1998), posing questions regarding its quality.

The sour taste of *Costus pictus* is due to the presence of oxalic acid in the leaves (Benny, 2006). If *Costus pictus* is used as an anti-diabetic drug source, the oxalate value of this plant should be considered for safe use of the plant as such and as a cheap source of oxalate oxidase enzyme for clinical oxalate determination. The present study entitled "Studies on oxalic acid and oxalate oxidase enzyme in *Costus pictus* D.Don" was carried out at the Centre for Plant Biotechnology and Molecular Biology with the following objectives:

1. To estimate the oxalic acid and oxalate oxidase enzyme activity of *Costus pictus* in leaf samples at different stages of maturity.

2. To study the effects of drying and extraction with various solvents on oxalic acid content and oxalate oxidase enzyme activity.

3. To study the effect of substrate concentration, enzyme concentration, pH, temperature on oxalate oxidase enzyme activity.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

The data available on *Costus pictus*, oxalic acid and oxalate oxidase is discussed below in different subtitles. The medicinal properties of *Costus pictus* are discussed. Occurrence of oxalate in plants, its localization, synthesis, storage in plants, its metabolism, possible functions, methods of isolation and detection are discussed. The occurrence of oxalate oxidase, its properties, function and structure are discussed.

2.1 Costaceae

Costaceae is a pantropical family of monocots, one of the most easily recognizable groups within the order Zingiberales. It is distinguished from other families by its well-developed and sometimes branched aerial shoots that have a characteristic spiral monistichous (one-sided) phyllotaxy (Kirchoff and Rutishauser, 1990). Its close relationship with Zingiberaceae is evidenced by its former placement as a subfamily within the larger Zingiberaceae family.

The placement of Costaceae within Zingiberaceae was largely based on broad similarities of inflorescence and floral characters. Tomlinson (1962) suggested that, although these types of characters may indicate common ancestry, they are not sufficient to overcome the morphological and anatomical differences that warrant independent familial rank of the two lineages which had been proposed by Nakai (1941).

Costaceae was separated from Zingiberaceae based on the distinctive chemistry, presence of monistichous phyllotaxy and based on floral characters (Ogden, 2007). The floral structure of Costaceae is also unique within the Zingiberales in that only a single fertile stamen develops while the remaining five infertile stamens fuse together to form a large, petaloid labellum that dominates

the floral display (Kirchoff, 1988). The labellum can be open or tubular, and when tubular can be modified to accommodate either bee or bird pollination.

2.2 Morphology of *Costus pictus* "Red stemmed form"

Costus pictus in the usual form, with the patterns on the stems, sometimes called *Costus heiroglyphica*. The flower and inflorescence is virtually identical on this plant, but the stems are a striking red color instead of the patterned stem of the more common form (Ogden, 2007).

Maas (1972) says *Costus pictus* can be recognized by its reddish yellow flowers, green inflorescence, callus absent, and short ligule. Placed between *Costus laevis* and *Costus malortienaus*, differs from *Costus laevis* by shorter ligule, shorter petiole, smaller leaves, and smaller flowers and differs from *Costus malortienus* by leaf shape and indument.

Red form of *Costus pictus* has broader leaves and red stems do not have the markings as the one common in horticulture. Flower parts are also mostly shorter and wider. This is the easiest of all the spiral gingers to grow, as it can be able to handle a wide range of soil and sunlight. It can be propagated very easily from stem cuttings.

2.3 Phytochemical analysis

George *et al*. (2007) investigated *Costus pictus* for phytochemicals. The preliminary physio - chemical analysis of the air dried leaves is given in table 1. The fibre content of the leaves is 21.1 per cent, which indicates that the leaves contain more fibres which are essential for diabetic patients. Qualitative ash analysis showed that it contains carbonate, oxalate, chloride, phosphate, and sulphate as anion and sodium, potassium, magnesium and iron as cations.

Successive extraction of leaves gave 5.2 per cent extractive in petroleum ether, 1.06 per cent in cyclohexane, 1.33 per cent in acetone and 2.95 per cent in ethanol. Analysis of successive extracts showed the presence of steroids in all extracts. The ethanol extract contained alkaloid also. These extracts were subjected to TLC analysis using different solvent systems. In *n*-hexane:acetone (9:1) system the petroleum ether extract gave 6 spots (R_f : 0.12, 0.27, 0.35, 0.54, 0.65, and 0.98) while acetone gave 3 spots (R_f : 0.13, 0.54 and 0.92) and in ethanol only 1 spot, (R_f : 0.52).

The MS fragmentation patterns of the compounds are assigned and are given in table 2. From the chromatogram, it was evident that the major component in the ether fraction is *bis*(2'-ethylhexyl)-1,2-benzene dicarboxylate (59.04 per cent). Presence of α-tocopherol in this fraction may be the cause of the antioxidant property of the leaf extract. A steroid, ergastanol present in the ether fraction supports the TLC analyses of the successive extracts.

The analysis of acid fraction gave ten peaks in the chromatogram and the compounds present in this fraction are assigned and given in table 3. From the data obtained, the major components in the acid fraction are Hexadecanoic acid (44.53 per cent) and 4,8,2,16-tetramethyl-heptadecan-4-olide (27.86 per cent). This fraction also contains decosanoic acid. Presence of octacosanoic acid and tetracosanoic acid were reported from *Costus specious* and *Costus tonkinensis*, respectively.

Jothivel *et al*. (2007) investigated the dried eaves of *Costus pictus* by sequential extraction process using different solvents such as petroleum ether, chloroform, methanol, and water. These sequential extracts were subjected to preliminary phytochemical screening for the presence of different chemical groups and the data was tabulated in table 4. Of all extracts tested, methanol extract was found to contain the highest number of phytochemicals such as carbohydrates, triterpenoids, proteins, alkaloids, tannins, saponins, and flavonoids.

Table 1: Analytical data of air dried leaves of *Costus pictus*

(George *et al*., 2007)

Constituent		Per cent
Moisture content		10.00
Fibre content		21.10
Sugar content	Reducing	10.40
	Total	16.77
Ash content		15.02
Water soluble extractives		15.93

Table 2: GC-MS analytical data of the ether fraction of *Costus pictus*

(George *et al*., 2007)

Retention time (min)	%	M.W.	Base peak	Compounds assigned
40.073	1.99	296	71	Phytol
50.579	1.58	332	271	Xanthene-3-one
51.632	59.04	390	149	*Bis*(2'-ethylhexyl)-1,2- benzene dicarboxylate
54.335	1.55	394	71	Octacosane
55.775	2.25	400	135	α-ergastanol
59.494	5.09	-	71	Derivatives of octacosane
61.206	8.16	402	402	α-tocopherol
68.292	20.34	436	71	Derivatives of octacosane

Table 3: GC-MS analytical data of the acid fraction of *Costus pictus* (George *et al*., 2007)

Retention time (min)	%	M.W.	Base peak	Compounds assigned
20.237	1.20	228	73	Tetradecanoic acid
21.277	1.19	242	73	Pentadecanoic acid
22.380	44.53	256	73	Hexadecanoic acid
23.233	1.30	270	73	Heptadecanoic acid
23.985	13.39	282	69	Heptadeccene-[8]-carbonic aid [1]
24.171	5.47	284	73	Octadecanoic acid (Stearic acid)
25.864	27.86	324	99	4,8,12,16-tetramethyl-heptadecan-4-olide
27.036	1.50	198	113	7-Tridecanone
27.484	1.40	340	73	Docosanoic acid (Behenic acid)
29.009	1.35	368	73	9-Octadecenoic acid (Oleic acid)

Table 4: Qualitative phytochemical analysis in different extracts of leaves of *Costus pictus* (Jothivel *et al*., 2007)

Plant Constituents	Extractive solvents of			
	Petroleum ether	Chloroform	Methanol	Water
Carbohydrate	Absent	Absent	Present	Present
Protein	Absent	Absent	Present	Present
Steroids	Present	Present	Absent	Absent
Alkaloids	Absent	Present	Present	Absent
Tannins	Absent	Absent	Present	Present
Glycosides	Absent	Absent	Absent	Present
Saponins	Absent	Absent	Present	Present
Flavonoids	Absent	Absent	Present	Absent
Fixed oils	Present	Absent	Absent	Absent

The pentacyclic triterpenoids such as α- and β-amyrin and related compounds occur especially in waxy coatings of the leaves. Mostly the terpenic compounds were successfully isolated from leaves.

2.4 Toxicity studies

Toxicity was studied by Benny (2005), Camargo *et al.* (2006) and Moron *et al.* (2007) none of them reported that *Costus pictus* extract produced toxicity.

Benny (2005) used methanol extract of *Costus pictus* for conducting toxicity studies. Oral feeding of *Costus pictus* extract up to a dose of 1g/kg body weight was found to be non toxic and even up to 200 mg/kg body weight for 3 months did not produce any toxic effects in sub-acute toxicity studies. Observation of haematological and biochemical parameters of experimental animals was in normal range as in the control group. Normal level of AST, ALT and ALP indicates that *Costus pictus* extract has no hepatotoxic effect. Histological studies of liver and pancreas in the extract administered group and control group were similar. Administration of high dose did not produce hypoglycaemia. And also the overdose of extract did not result in hypoglycaemia unlike insulin and other common hypoglycaemic agents.

Camargo *et al.* (2006) conducted acute toxicity study of aqueous extract of *Costus pictus* using adult female wistar rats. Aqueous extract up to 200 mg/Kg body weight did not produce mortality or any behavioural disorders.

Moron *et al.* (2007), evaluated the acute toxicity of aqueous extract of *Costus pictus* using Acute Toxic Classes (ATC) model in wistar rats, the decoction was administered in doses peak (2.0 ml 100 g^{-1} po) to wistar rats (150-200 g) (3 males and 3 females in each group).There were no signs of acute toxicity or deaths in the study of acute toxicity to the highest dose administered.

2.5 Diuretic effect

Herbal diuretics produce very little acute toxicity and in general they can be considered potentially useful measures, when compared to conventional precipition diuretics. Diuretic activity of the aqueous extract of the traditional medicine *Costus pictus* was demonstrated by measuring increase in the excreation of the both K^+ and Na^+ in the urine of treated rats. *Costus pictus* induced a natriuretic effect similar to furosenide at the dose of 200 mg kg b.w.$^{-1}$ of adult female wistar rats. Water excretion was not modified to the same degree as that of Na^+ and K^+ extract, the aqueous extract induces an increment in sodium and potassium clearance similar to the one obtained with furosemide suggesting that it represents significant diuretics. In contrast to other herbal species used for diuretic, where the extract does not affect potassium clearance to the same degree as measured here, thus use of this herbal should be accomplished by potassium in take (Camargo *et al.*, 2006).

2.6 Analgesic effect

Decoction of fresh leaves and stems of "Mexican cane" is widely used by the Cuban population as a traditional medicinal remedy for urinary problems like stones, pain and sepsis.

Moron *et al.* (2007) evaluated the analgesic effect of aqueous extract of *Costus pictus* using writhing test induced by administration of acetic acid 0.75 per cent at doses of 0.1 ml 10 g^{-1}) intraperitoneally and the tail flick test by immersion in hot water at 55ºC, both models were performed in male mice OF-1 (20-25 g). The doses were administered orally in decoction equivalent to 0.5, 1.0 and 5.0 grams of plant material kg-^{1} and controls received distilled water at peak volume.

Decoction at doses of 0.5 and 1.0 g kg^{-1} significantly decreased withdrawal in acetic acid-induced mice model and the withdrawal of the tail were

only significantly with the higher dose. The results of this study indicate that traditional use of decoction from fresh leaves of *Costus pictus* is due to the analgesic effect.

2.7 Antidiabetic property

The hypoglycaemic properties of the plant have been reported both in streptozotocin and alloxan induced diabetic rats. Oral feeding of *Costus pictus* crude extract up to 1 g kg b.w^{-1} did not produce any toxic effect (Benny, 2004; Benny *et al.*, 2004a&b).

Balaji (2005) reported that the active constituents in the leaf extract of *Costus pictus*, which are soluble, both in water and alcohol medium could help in controlling hyperglycemia in alloxan induced albino rats. He noted that the plant extract not only effective in preventing the loss of body weight but also in increasing the body weight of diabetic rats. It increased the hemoglobin content (35 per cent) but decreased the glycosylated haemoglobin (34 per cent) after 28 days of treatment. Plasma glucose was found to decrease from the pre-treatment value in all the treatment groups (74 per cent). He also reported that the plant extract also possess hypolipidaemic property. There was a substantial reduction in plasma total cholesterol level (42 per cent). All the treatment groups showed an enhancement in plasma protein content over 60 days of treatment (34 per cent).

Jothivel *et al*. (2007) reported that the methanol extract of *Costus pictus* (MECP) leaf was investigated for its anti-diabetic effect in Wistar Albino rats. Diabetes was induced in Albino rats by administration of single doses of alloxan monohydrate (120 mg kg^{-1}).The methanol extract of *Costus pictus* at a dose of 120 mg/kg was administered as single dose per day to diabetes-induced rats for a period of 21days. The effect of MECP leaf on blood glucose, plasma insulin, serum lipid profile (cholesterol, triglycerides, phospholipids, Very Low Density Lipoprotein (VLDL), Low Density Lipoprotein (LDL), and High Density

Lipoprotein (HDL), serum enzymes, total protein and liver glycogen were measured in the diabetic rats. Histopathological studies of liver, pancreas and kidney were also carried out. MECP elicited significant reductions of blood glucose, lipid parameters except HDL and serum enzymes and significantly increased HDL level. MECP also caused significant increases in plasma insulin levels in the diabetic rats. It significantly increased total protein and liver glycogen in diabetic rats. Histopathological observations revealed that leaf is nontoxic and regenerates the toxic effect of alloxan. From the above results, it was concluded that MECP possesses significant anti- diabetic effects in alloxan induced diabetic rats.

It has been well documented that methanol leaf extract of *Costus pictus* exhibited strong glucose-lowering activity. Jothivel *et al.* (2007) presumed that pentacyclic triterpene compounds such as β-amyrin might be the active principle contributing to the anti-diabetic actions. This was supported by TLC separation technique and histopathological observations of different tissues such as liver, pancreas and kidney. Thus in conclusion, the methanol leaf extract of *Costus pictus* had anti-diabetic effects and improved hyperlipidemia consequent upon diabetes.

Gireesh (2007) examined the regulatory role of *Costus pictus* in insulin secreation by examination of neurotransmitters produced after treatment of *Costus pictus* aqueous extract to Wistar Albino rats. It was concluded that *Costus pictus* leaf extract had a regulatory role in the insulin secreation and glucose haemostesis through muscarinic (sub type M1) receptor.

2.8 Oxalate

Oxalate is a common constituent of plants, and several species, including some crop plants, accumulate high levels of this C_2 dicarboxylic acid anion. Dependent upon species, oxalate accumulates primarily as soluble oxalate,

insoluble calcium oxalate, or a combination of these two forms. Oxalate is involved in several metabolic processes, possibly even within the same species. Most of the extreme oxalate accumulators (more than 5 per cent oxalate by dry weight) belong to the three families Caryophyllaceae, Chenopodiaceae, and Polygonaceae. The relationship between these families (all within the subclass Caryophyllidae) suggests that similar biosynthetic pathways and functional roles for oxalate exist within this group.

Oxalic acid is the simplest of the dicarboxylic acids with pKa values of 1.25 and 4.14. Besides being a relatively strong acid it is also a reducing agent. The acid normally crystallizes as a dihydrate. The oxalate ion is a very strong chelating agent, and salts formed with divalent cations are, as a rule, sparingly soluble. An important example is the calcium salt, which is soluble in water to only 6.0 mg l^{-1} at 18°C. The solubility and instability of calcium oxalate increase with the level of hydration, the trihydrate being most soluble and unstable, followed in order by the dihydrate and the monohydrate.

2.8.1 Occurrence in plants

Oxalic acid has been detected in various organisms, including animals, plants and fungi. The occurrence and the distribution of oxalate vary enormously among organisms. For instance, in plants the highest oxalate concentrations commonly occur in the leaves and the lowest in roots. Meanwhile, the oxalate content of plants can vary according to their age, the season, the climate and the type of soil. In some plants, such as rhubarb, oxalate content tends to increase as the plants mature, whereas, in other plants, e.g. spinach, sugar beet leaves, and bananas, there is a large increase in oxalate content during the early stages of development, followed by a decrease as the plants mature.

According to Zindler-Frank (1976) the majority of the species of only 11 out of 93 orders of higher plants do not store oxalate. In three of the orders

accumulation of water-soluble oxalate is a prominent feature: polygonales, caryophyllales (including chenopodiales), and Begoniales (Zindler-Frank, 1976).

2.8.2 Oxalate in metabolism

Many plants and animals produce oxalic acid, and it is of interest that they share some common pathways of oxalic acid synthesis. Oxalate may be present as the free oxalic acid, as soluble sodium and potassium salts or as insoluble calcium oxalate crystals. Unlike plants, calcium oxalate crystal formation in animals is generally considered to be pathological. In a related context, most analytical studies that have dealt with the occurrence and distribution of oxalates in plants have focused on their possible anti-nutritive calcium-sequestering influence in the human diet (Fassett, 1973). High oxalate contents of rhubarb and spinach are of particular interest, and in extreme cases, oxalate-rich rhubarb leaves are acutely toxic for humans just as high oxalate pasture grasses are acutely toxic for grazing cattle (Libert and Franceschi, 1987). The pathological role of oxalic acid in the formation of urinary stones in animals and humans has been known since the early 18th century. For example, excess consumption of oxalate-rich foods leads to hyperoxaluria which is recognised as a key risk factor for calcium oxalate stone formation (Sharma *et al.*, 1991).

Furthermore, there is evidence that high ascorbic acid intake increases urinary oxalate levels which could lead to the formation of calcium oxalate stones in the kidneys and other regions of the urinary system (Obzansky and Richardson, 1983). Oxalic acid is regarded as an undesirable component of our food not only because it raises the risk of urinary stones but also because it sequesters calcium, which is one of the essential ions, as insoluble calcium oxalate. The toxicology of oxalic acid in humans has been reviewed by several researchers (Holmes and Assimos, 2004).

In medicine, the knowledge of the oxalate concentration in blood and other body fluids can be very important in certain clinical situations such as primary hyperoxaluria (Petrarulo *et al.*, 1998). Oxalic acid has usually been seen as an inert end product of the metabolism and only plants have been reported to be able to metabolise oxalic acid and oxalates. However, the levels of oxalate are too high for the substance to only be an end-product of the metabolism in animals (Emsley, 1994). Therefore, it has been suggested that there could be an oxalate oxidase pathway in animals which uses oxalate to produce H_2O_2, which could then be used to promote a "burst" of phagocytes: cells that engulf and break down foreign particles, cell debris and disease-producing micro-organisms.

Unlike animals, plants are highly tolerant of oxalic acid and oxalates. Oxalic acid and oxalates have been detected in varying quantities in all parts of most plants' leaves, leaf stalks, flowers, tubers and roots (Srivastava and Krishnan, 1959). It is well established that plants are capable of metabolising oxalate by observing fluctuations in oxalate concentrations under certain conditions (Franceschil and Nakata, 2005), and enzymes degrading oxalic acid have been detected in numerous plants (Koyama, 1988; Caliskan and Cuming, 1998).

Several functions have been proposed for the presence of oxalic acid in plants. It has been implied that oxalic acid might be related to ionic balance, since it can combine with various plant ions to form soluble or insoluble compounds. It was suggested that oxalate synthesis occurred to balance the excess of inorganic cations (represented by K^+, Na^+, NH_4^+, Ca^{2+} and Mg^{2+}) over anions (represented by NO_3^-, Cl^-, $H_2PO_4^-$, SO_4^{2-}) normally present in the plant-the ability of nitrate ions and chloride ions to inhibit oxalic acid oxidase activity in *Beta vulgaris* results in an accumulation of oxalate.

Calcium oxalate crystals were amongst the first objects observed in plants in the early days of light microscopy in the late 17th century. Insoluble calcium

oxalate formation enables plants to control the concentration both of ionically active oxalic acid and calcium. Both of these molecules might have a toxic effect when accumulating in excess quantities. Thus plants could induce calcium oxalate crystal formation to remove excess oxalic acid or calcium. Although calcium is essential to biological growth and development, free calcium at high concentrations is toxic to cells.

So it was suggested that calcium oxalate precipitation serves to sequester excess calcium and remove it from the active metabolism (Webb *et al.*, 1995). The rapid induction of calcium oxalate crystal formation by calcium in Lemma plants suggests that the crystals may serve as a storage form for calcium for future needs (Hepler and Wayne, 1985). Further support for this came from the observation that in some plants the crystals appear to be dissolved during calcium deficient conditions, presumably to supply calcium for growth and cell maintenance (Franceschi and Nakata, 2005). Calcium is required for the activation and/or stabilisation of certain enzymes; for example plant cells need calcium to release peroxidases which are related to the control of cell elongation since they can rigidify walls by their cross linking activity and their ability to participate in the formation of lignin. Thus, for this role they are under the control of cellular calcium levels (Sticher *et al.*, 1981).

One of the major roles of calcium in plant cells is its action in the formation of the middle lamella where Ca^{2+} ions from stabilising ionic bridges between pectin chains. Although it has been suggested that calcium oxalate crystals are a means of detoxifying excess oxalic acid, the fact that many plants are able to retain high concentrations of soluble and free oxalic acid within their vacuoles indicates that oxalic acid may not be particularly toxic to plant tissues. However, accumulation of oxalic acid may have some toxic consequences (for example in causing osmotic problems and destabilisation of cells), unless it is readily metabolised (Raven and Smith, 1976).

2.8.3 Localisation of synthesis and transport

Information on localization of oxalate biosynthesis and oxalate transport in plants is scarce. Considering the various pathways possible, synthesis could occur in peroxisomes or the cytoplasm. Most labeling experiments have been made with cell-free extracts, detached leaves, and germinating seedlings. As for oxalate transport, it can be deduced from the low levels of Ca^{2+} in the phloem sap (Raven, 1977) that transport of oxalate in the phloem is possible.

2.8.4 Synthesis and storage of oxalate

Early studies on plant organic acids, including oxalic acid, yielded evidence that the production of these acids was related to photosynthesis and carbohydrate metabolism. Myers (1947) noted that oxalate concentration in rhubarb leaves increased in parallel with the growing seasons, being correlated with the seasons of most active photosynthesis. Later, experiments on rhubarb and Begonia indicated that oxalic acid was not a direct product of photosynthesis but it was synthesised from precursors synthesised in the photosynthetic pathway (Stutz and Burris, 1951).

It is well known that oxalic acid is synthesised via several major pathways. Glyoxylate and L-ascorbic acid appear to be the major precursors of oxalic acid in plants (Yang and Loewus, 1975; Davies and Asker, 1983). Glucose, acetate and some acids of the tricaboxylic acid cycle were determined to be involved in oxalate biosynthesis in red beetroots and young spinach leaves (Chang and Beevers, 1968). Moreover, glycolic and isocitric acids (Millerd *et al.*, 1963), and oxaloacetic acid (Davies and Asker, 1983) are known to donate carbon to oxalic acid in plants. The relative significance of these metabolites as precursors of oxalic acid has not been established (Wagner, 1981).

The enzymes involved in the synthesis of oxalic acid are of interest. In lettuce, two enzymes identified to have a role in the oxidation of glycolate and glyoxylate to oxalic acid were lactate dehydrogenase and glycolate oxidase (Davies and Asker, 1983). Since oxalic acid could be formed from several precursors, there is no clear evidence of its synthesis and deposition site. However, it was observed that the primary site of deposition of oxalic acid formed from L-ascorbic acid was the vacuole in barley which is a low level oxalic acid accumulator (Wagner, 1981).

2.8.5 Possible functions of oxalate

2.8.5.1 Protection of plants

As early as 1888, Stahl (in Libert and Franceschi, 1987) proposed that oxalate provided protection for plants against foraging animals in as much as oxalate-rich plants tended not to be protected by hairs, thorns, etc… Ecological factors behind evolutionary changes of metabolic character are difficult to prove. Besides the observations of Stahl, there is some circumstantial evidence that a high oxalate level may be of some importance for protection of plants against insects and foraging animals. In rice (*Oryza sativa*), genes for resistance to the brown plant hopper appear to be associated with an elevated oxalate level of the leaf sheath (Yoshihara *et al*., 1979). Furthermore, low concentrations of oxalate in test solutions inhibit sucking by the plant hopper (Yoshihara *et al*., 1980). Similar results were obtained by Massonie (1980) in experiments with the *Myzus persicae* (aphid).

The background of a possible protective effect of oxalate is clearly not the same for foraging animals, insects, and microbial pathogens. For foraging animals, preference for an oxalate-rich plant may be affected by taste or texture, rather than oxalate toxicity. As for taste, acidity may be influenced by the oxalate level. Further, texture can be strongly affected by calcium oxalate crystals

(Franceschi and Horner, 1980). Microorganisms are more likely to be affected by the presence of soluble oxalate and its effects on calcium availability and metabolism. For insects both preference and toxicity may play a significant role. Some plants may themselves show wilting symptoms when fed with oxalate, and there are examples of plant pathogens where isolates inducing oxalate accumulation are more virulent than other isolates (Noyes and Hancock, 1981; Punja *et al*., 1985).

2.8.5.2 pH regulation and osmoregulation in plants

Cellular processes are pH sensitive, and it is essential that mechanisms for pH regulation in sub cellular compartments are developed. Nitrate reduction and excess cation over anion uptake are examples of important processes that consume H^+ ions, while organic acids can serve as a source of H^+ ions (Raven and Smith, 1974; 1976). A homeostatic relationship between nitrate uptake/reduction and organic anion accumulation has long been recognized (Ergle and Eaton, 1947).

Malate is the dominant anion in some plants (Kirkby and Knight, 1977). Raven and Smith (1976) proposed that oxalate has the same function with the further advantage that deposition of calcium oxalate in the vacuole will not influence the osmotic potential of the cell. A metabolically plausible pH-stat involving oxalate was suggested by Davies and Asker (1983). Oxalate has also been found to be an important counter ion to inorganic cations such as sodium and potassium (Osmond, 1963; 1967; Karimi and Ungar, 1986). Soluble salts of oxalate may play an important role in turgor generation and osmoregulation.

In a number of plant species, nitrate as the source of nitrogen as opposed to ammonium lead to a higher oxalate content of the plant (Clark, 1936; Gilbert *et al*., 1951; Grutz, 1956; Joy, 1964). Higher oxalate levels with ammonium as the nitrogen source were, however, found by Crombie (1954) and Olsen (1939) (in

Libert and Franceschi, 1987). The source of nitrogen has a great effect on aspects of plant metabolism other than nitrate reduction. Meeuse and Campbell (1959) identified nitrate as a potent inhibitor of oxalic acid oxidase in *Beta vulgaris*.

If photorespiratory glyoxylate would be a precursor of oxalate, the supply of nitrogen could influence whether glyoxylate will accumulate and be further oxidized or be transaminated to glycine (Kpodar *et al.*, 1978). Moreover, the source of nitrogen appears to affect the aggregation state of glycolate oxidase, which may have physiological significance (Emes and Erismann, 1982). It should be emphasized that relatively high levels of oxalate are found in oxalate-accumulating plants even when ammonium is the sole source of nitrogen. Also, Roughan and Warrington (1976) observed that oxalate accumulation continued in older tissue of *Setaria sphacelata* after nitrate reduction activity had ceased. However unclear the picture remains, the role of oxalate accumulation in pH regulation and osmoregulation of the cell appears quite real.

2.8.5.3 Accumulation of calcium ions in plants

Two explanations for high levels of calcium oxalate in plants have been offered:

1. Oxalate synthesis has evolved as a means of neutralizing the negative effects of excessive calcium uptake (Libert and Franceschi, 1987).
2. Oxalate, which is a toxic product for the plant, is made harmless by precipitation as the highly insoluble calcium salt. (Libert and Franceschi, 1987).

Kinzel's (1963) (in Loetsch and Kinzel, 1971) observation that oxalate-accumulating plants tend to occur on non calcareous soils can be interpreted as an indication that oxalate is not produced for decalcification primarily. Examples can also be found where oxalate production induces calcium deficiency in the plant

(Brumagen and Hiatt, 1966; Loetsch and Kinzel, 1971). On the other hand, the existence of species with very high levels of water-soluble oxalate proves that oxalate can be efficiently "detoxified" without calcium oxalate precipitation in some species.

A positive correlation between calcium content of the growth medium and oxalate formation (Gilbert *et al.*, 1951; Grutz, 1956; Rasmussen and Smith, 1961; Bornkamm, 1965; Zindler-Frank, 1975) appears to be related to processes involving chemical regulation: Uptake of calcium leads to calcium oxalate precipitation, altering the equilibrium of oxalate synthesis and/or breakdown in the plant. In some investigations on plants with a high level of water-soluble oxalates (Chenopodiaceae and Polygonaceae), little or no correlation was found between calcium in the medium or plant and oxalate in the plant (Wittwer *et al.*, 1946; 1947; Osmond, 1967).

It must be pointed out that calcium oxalate formation is not a simple accumulation phenomenon. Before the calcium oxalate crystals are formed, specialized membranes develop within the cell called ioioblast (Franceschi and Horner, 1980). Often cells that produce crystals are formed in very specific regions of the plant and appear modified in morphology and cytology so that they are specialized for this particular function. The number of crystal-forming cells produced is related to calcium availability (Frank, 1972; Franceschi and Horner, 1979; Van Balen *et al.*, 1980). It is clear that the plant controls the precipitation process and so exerts some control over soluble and insoluble levels of calcium and oxalate.

2.8.6 Isolation methods of oxalate

Traditional methods of oxalate isolation from plants can be divided in to three groups.

1. Extraction of oxalic acid from plants with cold or hot hydrochloric acid, and then precipitating as the calcium salt (Gregoire and Carpiaux, 1912; Kohman, 1939; Andrews and Viser, 1951).

2. Digestion of plant sample with hot concentrated sodium carbonate solution to convert oxalic acid to the soluble salt and then acidification with hydrochloric acid and precipitation as calcium salt (Arbenz, 1917; Talapatra *et al*., 1948).

3. Esterification of oxalic acid in presence of methanol or ethanol in hot acid solution, and then distillation and precipitation as the calcium salt (Dodds and Gallimore, 1932; Lehman and Grutz, 1953).

Recently, a number of *in vitro* assays have been proposed (Chidambaram, *et al*., 1989; Beer *et al*., 1997; Kennefick and Cashman, 2000) but the protocol outlined by Versantvoort *et al*., (2005) appears to follow the human digestive process most accurately and this method was modified by Catherwood (2005) to give a measure of oxalates solubilized in the stomach and the small intestine. The *in vitro* digestion method proposed by Versantvoort *et al*., (2005) is based on the transit times, temperatures, constituents, concentrations and pH of normal physiological values reported in the literature. It is a static gastrointestinal model based around the addition of four solutions, saliva, gastric and duodenal juices and bile solution to the test sample incubated at 37°C.

2.8.7 Methods for detecting oxalate

Prior to 1977, the most commonly used procedures were permanganate titration, various colorimetric methods, and paper chromatography, while radioisotope and gas chromatographic methods, though more accurate, were used to a lesser extent (Hodgkinson, 1977). Since 1977, improvements in enzymatic (Potezny *et al*., 1983) and gas chromatographic (Sarkar and Malhotra, 1979)

methods, as well as development of high pressure liquid chromatographic analyses for oxalate (Libert, 1981; Wilson *et al.*, 1982; Bushway *et al.*, 1984), have made these the preferred procedures.

At present the most common methods being used are amperometry, chromatography and spectrophotometry. Chromatography involves the detection of oxalate using HPLC, GC and CE. The oxalate oxidase activity-based determination of oxalate has become very popular and is used widely because of its simplicity, specificity and sensitivity (Pundir *et al.*, 1985).

2.9 Oxalate oxidase

Oxalate oxidase (oxalate: oxygen oxidoreductase, EC 1.2.3.4) is a member of the cupin superfamily of proteins (Donaldson *et al.*, 2001) catalysing the oxidation of oxalate to form carbon dioxide and hydrogen peroxide.

An enzyme with oxalate oxidase (ox-ox) activity that converts oxalic acid to carbon dioxide and hydrogen peroxide was first reported by Zaleski and Reinhard in 1912 from studies on powdered wheat grains. After the first report, the enzyme was studied in both wheat flour and bran by Paladin and Lovchinovsaka in 1916 and in 1928 Zaleski and Kukharkova concluded that this enzyme should be considered as dehydrogenase to use only oxygen as a hydrogen acceptor.

Mean while Staehelin in 1919 had reported a similar enzyme from leaves of higher plants, including some known to contain oxalic acid, such as *Rumex*, *Rheum* and *Spinacia*. However the most spectacular case of oxalic acid degradation was found in mosses – a source first discovered by Houget *et al.*, in 1927 in *Hypnum triquetrum*, *Hypnum cupressiforme*, *Thudium abietinum* and *Polytrichum juniperum*. Subsequently Franke and Hasse in 1937 characterized the enzyme from *Hyalocomium umbratum* and noted its remarkable thermo stability.

Thereafter the presence of oxalate oxidase has been reported in numerous plants. It is presumed that the production of hydrogen peroxide during the oxidation of oxalate by oxalate oxidase destroys fungal toxins and microbes, serving as a defence mechanism (Dumas *et al.*, 1995; Keates *et al.*, 1996; Piquery *et al.*, 2000), or it could be used in peroxidase catalysed cross linking reactions, strengthening cell walls (Davoine *et al.*, 2001).

Oxalate oxidase has been purified and characterised from a variety of sources, ranging from fungi to plants. Oxalate oxidase has been purified from three classes of organisms: fungi, bacteria and plants, with the majority of oxalate oxidase sources being plants. A summary of oxalate oxidase isolated from different sources is shown in table 5.

Pundir (1991b) investigated the presence of oxalate oxidase in three genotypes of sorghum. These studies led to the finding that all three genotypes contained oxalate oxidase, however, the location of oxalate oxidase varied in the order of leaves > stems > roots in PC-6 and PC-1 sorghum varieties, but the order was reversed in CSH-5.

2.9.1 Oxalate oxidase assays

A number of established enzymatic assays have been optimised and investigated in detail by many research groups for assaying. The general principle of oxalate oxidase assay involves the oxidation of oxalate by oxalate oxidase, generating hydrogen peroxide. The resultant hydrogen peroxide reacts with dye precursor(s) and horseradish peroxidase (HRP) yielding a coloured dye during the coupled assay:

$$\text{Oxalate} + O_2 \xrightarrow{\text{Oxalate oxidase}} H_2O_2 + 2CO_2$$

$$H_2O_2 + \text{Dye precursor(s)} \xrightarrow{\text{Horseradish peroxidase}} \text{Coloured dye} + H_2O$$

Table 5: Sources of reported oxalate oxidase enzyme

Sources	Reference
Amaranthus Leaves (*Amaranthus spinosus*)	Goyal *et al*. (1999); Chandran *et al*. (2001)
Banana Peel (*Musa paradisiaca* var. plantain)	Raghavan and Devasagayam (1985); Inamdar *et al*. (1986); Inamdar *et al*. (1991)
Barley Leaves (*Hordeum vulgare*)	Zhang *et al*. (1996)
Barley Roots (*Hordeum vulgare*)	Skotty and Nieman (1995); Kotsira and Clonis (1997); Woo *et al*. (1998); Kotsira and Clonis (1998); Requena and Bornemann (1999)
Barley Seedlings (*Hordeum vulgare*)	Chiraboga (1963; 1966); Sugiura *et al*. (1979)
Beet Shoots, Stems and Leaves (*Beta vulgaris*)	Leek *et al*. (1972); Obzansky and Richardson (1983); Varalakshmi and Richardson (1992); Azarashvili *et al*. (1996); Woo *et al*. (1998)
Bougainvillea spectabilis Leaves	Srivastava and Krishnan (1962)
Ceriporiopsis subvermispora	Aguilar *et al*. (1999)
Maize Roots (*Zea mays*)	Vuletic and Šukalovic (2000)
Moss (*Minium affine*)	Datta and Meeuse (1955); Suzuki and Meeuse (1965)
Pseudomonas sp. OX-53	Koyama (1988)
Ryegrass (*Lolium perenne*)	Davoine *et al*, (2001)
Sorghum Leaves (*Sorghum vulgare* var. CSH-5, PC-6 and PC-1)	Pundir and Nath (1984); Pundir (1991a); Pundir, (1991b); Satyapal and Pundir (1993); Pundir and Satyapal (1998); Thakur *et al*. (2000); Thakur *et al*. (2001)
Sorghum Roots (*Sorghum vulgare* var. CSH-5)	Pundir and Kuchhal (1989); Pundir, (1991b)
Tilletia controversa	Vaisey *et al*. (1961)
Thale Cress (*Arabidopsis thaliana*)	Membré *et al*. (1997)
Wheat Grains (*Triticum aestivum*)	Lane (2000)
Wheat seedling (*Triticum aestivum*)	Zhenfei-Guo (2008)

The four most common dye precursor based enzymatic assays used to detect oxalate oxidase are ABTS (2, 2'-azino-bis (3-ethylbenz-thiazoline-6-sulphonic acid)), aminophenazone (4-Aminophenazone), σ-diansidine and MBTH (3-methyl-2-benzothiazolinone hydrazone) assays. Each oxalate oxidase assay employs different dye precursor(s) and is monitored at ranging λ max, shown in table 6. The coloured dye generated during assay is directly proportional to the concentration of hydrogen peroxide evolved which in turn directly proportional to the oxalate oxidase activity in the assay mixture.

The ABTS and the MBTH assays were more sensitive than the aminophenazone assay; however, all three assays generated reproducible, repeatable and reliable.

2.9.2 Purification of oxalate oxidase

Most research groups isolated oxalate oxidase by employing only one isolation technique: subcellular fractionation (Vaisey *et al.,* 1961; Srivastava and Krishnan 1962; Leek and Butt 1973; Raghavan and Devasagayam 1985; Pundir, 1991a; Vuletic and Šukalovic, 2000), whereas others isolated the enzyme by more complex four step protocols (Thakur *et al.,* 2000).

Later, from the 1980s, efforts were concentrated on the purification of oxalate oxidase from a number of sources. The protocols available vary greatly, for example, Azarashvili *et al.* (1996) purified oxalate oxidase from beet shoots employing a three step purification protocol, whereas, Requena and Bornemann (1999), and Koyama (1988) purified oxalate oxidase from barley roots and *Pseudomonas* sp. OX-53 respectively using six step protocols. The principle procedures employed to purify oxalate oxidase are subcellular fractionation (SF), thermal treatment, ammonium sulphate fractionation (ASF), affinity chromatography, ion exchange chromatography (anion and cation), gel filtration chromatography, chromato focussing and isoelectric focussing.

Table 6: Assays for the detection of oxalate oxidase

Oxalate oxidase assay	Dye Precursor(s)	λ max (nm)	Reference
ABTS	ABTS (2,2'-azino-bis(3-ethylbenzthiazoline- 6-sulphonic acid)	650	Azarashvili *et al.* (1996); Requena and Bornemann (1998); Requena and Bornemann (1999); Whittaker and Whittaker (2002); Malpass *et al.* (2002)
Aminophenazone	4-Aminophenazone and Phenol	520	Pundir, (1991a&b); Satyapal and Pundir (1993); Yamanaka *et al.* (1996); Pundir and Satyapal (1998); Goyal *et al.* (1999); Goyal *et al.* (2000); Thakur *et al.* (2000); Thakur *et al.* (2001); Chandran *et al.* (2001)
σ-Diansidine	σ-Diansidine	500	Aguilar *et al.* (1999)
MBTH	MBTH (3-methyl-2-benzothiazolinone hydrazone) and DMA (N,Ndimethylaniline)	578	Laker *et al.* (1980); Obzansky and Richardson 1983; Raghavan and Devasagayam (1985); Wilson and Liedtke (1991); Varalakshmi and Richardson (1992); Yamanaka *et al.* (1996); Kotsira and Clonis (1997); Kotsira and Clonis (1998); Thakur *et al.* (2000)

2.9.3 Characterisation of oxalate oxidase

Extensive research has been conducted with purified oxalate oxidase. The properties and characteristics of purified oxalate oxidase with respect to molecular mass, pH optima, thermal stability and effect of cations are discussed below.

2.9.3.1 Molecular mass and subunit composition of oxalate oxidase

Purified oxalate oxidase from different sources varies in molecular mass, Pundir isolated oxalate oxidase from sorghum leaves with a molecular mass of 62 kDa (Pundir 1991a) and Aguilar *et al.* (1999) with a molecular mass of 400 kDa. The number of subunits comprising oxalate oxidase differs from two (Sugiura *et al.,* 1979; Pietta *et al.,* 1982; Pundir and Satyapal 1998; Goyal *et al.,* 1999) to 8 (Koyama 1988; Requena and Bornemann 1999). Table 7 gives an overview of the reported molecular masses and subunit composition of purified oxalate oxidase from different sources.

The molecular mass of oxalate oxidase subunits varies from 25 kDa (Requena and Bornemann 1999) to 75 kDa (Sugiura *et al.,* 1979; Pietta *et al.,* 1982) for eight and two subunits respectively. Dissimilarities in molecular mass not only exist in oxalate oxidase purified from different sources, but also exist in the material from the same sources. For example, the molecular mass of oxalate oxidase purified from barley roots by Kotsira and Clonis (1998) was reported to be 125 kDa comprising five subunits, however, oxalate oxidase isolated from the same source by Requena and Bornemann (1999) was 200 kDa composed of 8 subunits.

2.9.3.2 pH optima of purified oxalate oxidase

Extensive information regarding the optimum pH of purified oxalate oxidase from different sources is available. A reduction or increase in pH may

Table 7: The molecular masses of purified oxalate oxidase

Source	Molecular Mass (kDa)	Subunits and Molecular Mass (kDa)	Reference
Amaranthus Leaves	130	2 subunits of 65	Goyal *et al.* (1999)
Barley Roots	200	8 subunits of 25	Requena and Bornemann (1999)
Barley Roots	125	5 subunits of 26	Kotsira and Clonis (1998)
Barley Seedlings and Roots	150	2 subunits of 75	Sugiura *et al.* (1979); Pietta *et al.* (1982)
Ceriporiopsis subvermispora	400	6 subunits of 65.5	Aguilar *et al.* (1999)
Pseudomonas sp. OX-53	320	8 subunits of 38	Koyama (1988)
Sorghum Leaves	120	2 subunits of 62	Satyapal and Pundir (1993)
Wheat seedling	170	5 subunits of 32.6	Zhenfei-Guo (2008)

lead to lower rates of reaction. Table 8 summarises the optimum pH of purified oxalate oxidase from different sources.

The optimum pH of purified oxalate oxidase ranges from pH 2.6 to pH 6.8, with most being towards the acidic range. oxalate oxidase purified from fungi (Vaisey *et al.,* 1961) possesses a more acidic pH of 2.6, in comparison to oxalate oxidase purified from plants where the pH optima ranges from pH 3.5 to pH 5.0, with *Bougainvillea spectabilis* leaves being an exception in which maximum activity was observed at pH 6.8 (Srivastava and Krishnan, 1962).

2.9.3.3 Thermal properties of purified oxalate oxidase

There has been disagreement regarding the thermal stability of oxalate oxidase from different sources. Oxalate oxidase from barley seedlings (Chiraboga, 1966; Sugiura *et al.,* 1979) and barley leaves (Zhang *et al.,* 1996) are reported to be stable at 80°C for 3 minutes. Barley roots (Kotsira and Clonis, 1997; Requena and Bornemann, 1999) have also been reported to be thermally stable, however, at a lower temperature of 60°C for 10 minutes. Banana peel oxalate oxidase isolated by Inamdar *et al.* (1986) retained 70 per cent of its activity after being thermally treated at 60°C. Chiraboga (1966) also found barley seedling oxalate oxidase to be thermally stable at 60°C for 3 minutes; however, treating at 89°C for 3 minutes resulted in the loss of 51 per cent of the initial oxalate oxidase activity.

Bougainvillea spectabilis leaves oxalate oxidase (Srivastava and Krishnan, 1962) has been reported to be thermally unstable at 60°C for 15 minutes since approximately 60 per cent of the activity was reported to be lost. Further thermal treatment at 80°C to 100°C led to the complete loss of activity. Additionally, Varalakshmi and Richardson (1992) purified oxalate oxidase from beet stems and reported a loss of 50 per cent of its activity at 60°C and 70 per cent at 70°C.

Table 8: The optimum pH of purified oxalate oxidase

Source	pH Optima	Reference
Amaranthus Leaves	3.5	Goyal *et al.* (1999)
Banana Peel	5.2	Inamdar *et al.* (1986)
Barley Roots	3.5	Chiraboga (1966)
Barley Roots	4.0	Kotsira and Clonis (1997)
Barley Seedlings	3.2	Sugiura *et al.* (1979)
Barley Seedlings	3.5	Chiraboga (1963; 1966)
Beet Shoots and Stems	4.0	Obzansky and Richardson (1983); Woo *et al.* (1998)
Beet Stems	4.5	Varalakshmi and Richardson (1992)
Bougainvillea spectabilis Leaves	6.8	Chiraboga (1966)
Ceriporiopsis subvermispora	3.5	Aguilar *et al.* (1999)
Maize	3.2	Vuletic and Šukalovic (2000)
Moss	4.0	Datta and Meeuse (1955)
Pseudomonus sp. OX-53	4.8	Koyama (1998)
Sorghum Leaves	4.3	Pundir (1991a)
Sorghum Leaves	5.0	Pundir and Nath (1984); Pundir and Satyapal (1998)
Sorghum Leaves	4.0	Pundir (1991b)
Sorghum Leaves	4.5	Pundir (1991b)
Sorghum Roots	5.0	Pundir and Kuchhal (1989); Pundir (1991b)
Tilletia controversa	2.6	Vaisey *et al.* (1961)
Wheat seedling	3.5	Zhenfei-Guo (2008)

Oxalate oxidase purified from moss by Datta and Meeuse (1955), and Suzuki and Meeuse (1965) were reported to be thermally stable at 100°C for 15 minutes with only a slight loss in oxalate oxidase activity. In contrast, oxalate oxidase purified from sorghum leaves (Pundir, 1991a) and three genotypes of sorghum (Pundir, 1991b) are not thermally stable above temperatures of 45°C.

2.9.3.4 Effect of cations on oxalate oxidase activity

In Moss (Suzuki and Meeuse 1965), sorghum leaves (Pundir and Nath 1984) and *Amaranthus* leaves (Goyal *et al.*, 1999) oxalate oxidase activity was insignificantly affected by K^{+}, Ba^{2+}, Ca^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Pb^{2+}, Sn^{2+}, VO^{3-} and Zn^{2+}. Conversely, Pundir and Kuchhal (1989) and Satyapal and Pundir (1993), demonstrated oxalate oxidase from sorghum leaves and roots were strongly activated by only Cu^{2+} by 210 per cent and 200 per cent respectively.

Oxalate oxidase from barley seedling was found to be very sensitive to changes in ionic strength; the divalent affects of the cations could not be explained by Chiriboga (1966). This led to the suggestion that the inhibition was likely to be caused by a mixture of reactions between the metal, protein, substrate and the enzyme-substrate complex. It was proposed the divalents Cu^{2+} and Ca^{2+} had acted on the substrate as well as the enzyme causing the adverse effects (Chiriboga 1966).

The effect of bivalent metal ions on *Bougainvillea spectabilis* leaves oxalate oxidase activity investigated by Srivastava and Krishnan (1962) demonstrated Ca^{2+} inhibited the activity by 40 per cent, whereas Mn^{2+}, Co^{2+}, Zn^{2+}, Mg^{2+} and Ni^{2+} had no effect. Goyal *et al.*, (1999) performed similar studies with Cd^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Sr^{2+} and Zn^{2+}, however, only Fe^{2+} activating oxalate oxidase activity purified from *Amaranthus* leaves.

Vuletic and Šukalovic (2000) studies with Al^{3+}, Cu^{2+}, Fe^{2+}, Mg^{2+} and Zn^{2+} on maize oxalate oxidase demonstrated these divalent metals did not affect the activity. Similar findings were obtained by Requena and Bornemann (1998) during the investigation of the effect of Fe^{2+}, Fe^{3+} and Zn^{2+}on commercially available oxalate oxidase from Boehringer Mannheim.

The effect of manganese ions on oxalate oxidase activity was of great interest since studies performed by Requena and Bornemann (1999) suggested oxalate oxidase from barley roots contains a manganese cofactor. Manganese ions was not affected oxalate oxidase activity in barley seedlings (Sugiura *et al.,* 1979), maize (Vuletic and Šukalovic, 2000) and Boehringer Mannheim oxalate oxidase (Requena and Bornemann, 1998). In contrast, manganese stimulated oxalate oxidase activity in *Pseudomonas* sp. OX-53 by 75 per cent; however, Koyama (1988) concluded Mn^{2+} only slightly activated oxalate oxidase activity.

Requena and Bornemann (1999) preincubated barley root oxalate oxidase with manganese chloride and observed no increase in oxalate oxidase activity. This led Requena and Bornemann to suggest either the enzyme had its full complement of Mn^{2+}, or it had not reversibly bound the ion in its fully folded form. They concluded that since extensive dialysis performed in the absence of Mn^{2+} did not lead to the loss of oxalate oxidase activity. The summary of the effect of cations on purified oxalate oxidase is given in the table 9.

2.9.4 Immobilization of oxalate oxidase

Enzymic colorimetric determination of urinary oxalate with oxalate oxidase requires bulk quantities of the enzyme for a large number of clinical samples, which makes it expensive. To overcome these difficulties and to make the cost of the assay cheaper immobilation of oxalate oxidase was done. Table 10 shows the list of immobilized oxalate oxidase enzyme from different source on different matrices.

Table 9: The effect of cations on purified oxalate oxidase

Source	Cation	Conc. (mM)	% Activity	Reference
Barley Seedlings	Ag^{+}	1	-22	Sugiura *et al.* (1979)
	Ca^{2+}	1	+14,-3	Sugiura *et al.* (1979); Chiriboga (1966)
	Cu^{2+}	1,7.5	+36,-59	
	Hg^{2+}	1,7.5	-73	Sugiura *et al.* (1979)
	Mn^{2+}	1	-3,-44	Sugiura *et al.* (1979); Chiriboga (1966)
	Na^{+}	1,7.5	-11	Sugiura *et al.* (1979)
	Ni^{2+}	1	+51	
	Pb^{2+}	1	+12	
	Zn^{2+}	1	+36	
Barley Seedlings	Ba^{2+}	7.5	-32	Chiriboga (1966)
	Fe^{2+}	7.5	-100	
	Sr^{2+}	7.5	-2	
Barley Roots	Ca^{2+}	1	+71	Kotsira and Clonis (1997)
	Cu^{2+}	1	+10	
	Fe^{2+}	1	-99	
	Pb^{2+}	1	+157	
Bougainvillea Leaves	Ca^{2+}	1	-40	Srivastava and Krishnan (1962)
Pseudomonus sp. OX-53	Cu^{2+}	0.1	-36	Koyama (1988)
	Fe^{2+}	0.1	-6	
	Hg^{2+}	0.1	-47	
	Mg^{2+}	0.1	-3	
	Na^{+}	0.1	-10	
	Mn^{2+}	0.1	+75	
	Zn^{2+}	0.1	-12	
Sorghum Roots	Cu^{2+}	0.5	+210	Pundir and Kuchhal (1989)
Sorghum Leaves	Cu^{2+}	0.5	+200	Satyapal and Pundir (1993)

Note: Stimulation (+) or Inhibition (-)

Table 10: Immobilization of oxalate oxidase

Plant	Support	Reference
Barley seedlings	Nylon tubing	Potezny *et al.* (1983)
	Collagen membrane	Amini and Vallan (1994)
	Gelatin	Dinckaya and Telefoncu (1993)
	Polyamise membrane	Assolant *et al.* (1987)
	Alkylamine glass	Pundir *et al.* (1993)
		Chandran *et al.* (2001)
Banana peel	Immune complex	Inamdar *et al.* (1986)
	Ethyl maleic anhydride	Lathika *et al.* (1995)
Beet stem	Polyethylene glycol	Varalakshmi *et al.* (1995)
Sorghum leaf	Alkylamine glass	Thakur *et al.* (2000)
Amaranthus leaf	Arylamine glass	Sharma *et al.* (2007)
	Alkylamine	Godara and Pundir (2008)

2.9.5 Oxalate degradation and bioremediation

An interesting, non medical application of oxalate degradation is provided by the example (Morton *et al.,* 1994) of using *Pseudomonas oxalaticus* (Chandra and Shethna, 1975), a bacterium found in rhubarb patches, to digest sodium oxalate, a hazardous byproduct of the Bayer process (Fraquharson *et al.,* 1995) utilized in aluminum smelters.

2.9.6 Germins and germin like proteins in plants

2.9.6.1 Studies on oxalate oxidase activity

The onset of growth in germinated wheat embryos was accomplished by the production of a soluble, pepsin-resistant homopentameric glycoprotein (oligomer approximately 125kD) that was initially called 'g' and then named 'germin' (Grzelczak and Lane, 1984; McCubbin *et al.,* 1987). In subsequent studies, the sequence of two very similar germin genes designated *gf-2.8* and *gf 3.8* were obtained by Dratewka-Kos *et al.* (1989) and Lane *et al.* (1991). The isoforms of germin were discrete temporal markers of wheat embryo development; wall-associated germin accounts for about 40 per cent of total germin in germinating wheat embryos, with the appearance of germin in the apoplast being the most conspicuous germination – related change in the distribution of cell wall proteins (Lane *et al.,* 1992).

Although these studies revealed much about details of the germin protein and its expression, its function remained unknown till the sequence of barley oxalate oxidase became available was it revealed that germin *gf 2.8* was a protein with oxalate oxidase activity (Lane *et al.,* 1993; Dumas *et al.,* 1993). It was thought that the closely related *gf-3.8* is monomeric and does not exhibit oxalate oxidase activity (Nowakowska, 1998). This difference creates a potential problem in nomenclature; in those similar genes encode protein with quite different

properties and therefore different functions. It was proposed to restrict the term germin to the cereal genes and to stress that the gene products may have a range of functions (Nowakowska, 1998).

2.9.6.2 Germins in cereals

It was discovered at an early stage in the study of germin that an immunologically similar protein could be detected in all cereals tested, i.e. in the stems of barley oats and rye (Grzelczak *et al.*, 1985) and in maize and rice (Lane *et al.*, 1991). Of all cereals tested to date, the largest number of germin-like gene sequences has been found in rice. This comprises one 20 AA fragment (gi348652) from a 25 kD protein and 20 ESTs. The ESTs sequences are from 8-day old shoots, rather green or etiolated or from the panicle at the ripening stage. Translations and manual editing of obvious sequencing errors revealed a possible total of eight distinct proteins which was divided in to three classes. The first was most similar (approx. 70 per cent) to the Prunus ABP 20 protein (gi1916809), the second was related to the barley X93171 protein, and the third was similar to the *Arabidopsis* GLP 2b germin like protein. Interestingly, there was no similarity to the wheat germin or barley root oxalate oxidase.

Oxalate oxidase is localized in the epidermal cells of the mature region of the primary roots of 3-day old barley seedlings and 10 days old coleorhiza, (Dumas *et al.*, 1995).

2.9.6.3 Functional properties of oxalate oxidase

2.9.6.3.1 Development

Two features of wheat germin had been linked to specific roles in plant development (Lane, 1991), particularly in the modification of the extra-cellular matrix (Lane, 1994). First, the tight adhesion of germin to the highly substituted

glucuronogalactoarabinoxylans (GGAX) suggested a possible role in the incorporation of these compounds in to the growing wall. Secondly, the generation of H_2O_2 was required for peroxidase mediated reactions such as lignification and the cross linking of coumarates, extensions and ferulates to cell wall hemicellulose and pectin. These reactions would stop the process of wall extension. The presence of several auxin responsive elements in the promoter of germin *gf-2.8* (Berna and Bernier, 1997) was additional evidence for a link with cell wall pH and germin activity (Lane *et al.,* 1992).

2.9.6.3.2 Plant pathogen interactions

In a study of defense related gene expression in barley, Wei (1994) identified a cDNA clone (gi1070358, X93171) with approximately 45 per cent identity to barley root oxalate oxidase. It was known to be most similar (90 per cent) in sequence to a reconstructed protein encoded by the fusion of two rice ESTs, gi1632028 and gi1632551. later two significant publications (Dumas *et al.,* 1995; Zhang *et al.,* 1995) describing the involvement of ox-ox in the response of barley plant to powdery mildew infections. In the former study, it was shown by immunoblots and direct assays that the enzyme was induced in leaves between three and five days after inoculation and was localized mainly along the vascular bundles. There was no induction by wounding. The latter investigation demonstrated a much more rapid response, with colorimetric assay and activity blots showed a detectable increase after 24 h and a 10-fold increase after 48 h. These increases appeared 1-3 days earlier than PR-1 accumulation and it was suggested that the activity results from *de novo* protein synthesis, though pathogen induced enzyme activation was also possible. It was not known whether the H_2O_2 generated by the enzymatic process was used as a signal for the induction of other components of the defence response, for lignification, or directly to inhibit pathogen development (Thordahl-christensen *et al.,* 1997; Wojtaszek, 1997). Similar results were reported by Hurkman and Tanaka (1996a) for wheat leaves inoculated with the same pathogen.

2.9.6.3.3 Salt stress

There had been several related studies on the effects of salt stress on the expression of germin genes in barley (*Hordeum vulgare* L. cv CM72) seedlings (Hurkman and Tanaka, 1996b). In control seedlings, germin mRNA levels were regulated developmentally, with the highest amount in roots and the lowest in shoots. These levels were maximum 2 days after imbibitions, before declining. In presence of 200mM salt, the level remains high for one more day for seedlings. Expression patterns in salt stressed roots were more complicated, with levels rising only transiently when 6-day old roots were exposed to 200 mM NaCl (Hurkman and Tanaka 1996b).

2.9.6.4 Expression oxalate oxidase in transgenic plants

The practical application of germins in transgenic crops is based their activity as an oxalate oxidase, and its potential use as an antifungal reagent. Oxalic acid is produced by several plant pathogenic fungi (Loewus *et al.,* 1995) and is considered to play a major role in the pathogenicity of *Sclerotinia sclerotiorum* (Noyes and Hancock, 1981; Rowe, 1993), a particularly important pathogen of sunflower (Masirevic and Gulya, 1992; Sackston, 1992) and many other dicot crops. Evidence for the role of oxalic acid came from studies on mutant strains of the fungus which were deficient in oxalic acid production and also avirulent. Revertants for acid production regained their virulent nature (Godoy *et al.,* 1990).

The proposed mode of action of oxalic acid in producing disease symptom was first the chelation of calcium from pectate fraction of the xylem and associated pit vessels, secondly the entry of air leading to xylem embolisms and wilting (Sperry and Tyree, 1998), and finally the reduction in pH which stimulates the activity of the fungal enzymes such as polygalacturonase, methyl esterase and cellulose (Marciano *et al.,* 1983).

Analysis of oxalic acid in disease development led to the strategy of introducing oxalate degrading enzymes in to plants as a mean of protecting them against the fungal toxin (Thompson *et al.,* 1995). This transgenic strategy, which had some parallels with the work on human gene therapy, had utilized available genes, the ox-ox from barley or wheat and the fungal oxalate decarboxylase. A similar transgenic approach utilizing bacterial genes for oxalate degradation (Koyama, 1988), had been suggested in a study of *Sclerotinia* infection of *Arabidopsis* (Dickman and Mitra, 1992).

Transgenic plants had also been used in a series of more fundamental studies in which the wheat *gf-2.8* germin gene introduction either as an intact gene or as promoter-GUS fusions into transgenic tobacco (Berna and Bernier, 1997). Heterologous gene expression was monitored *in vitro* by GUS or oxalate oxidase activity and shown to occur in developing seeds and seedlings. This transcription was stimulated by auxin, probably owing to the presence of several auxin-responsive elements (e.g. TGTCCCAT and TGTCTC) in the promoter sequence. Interestingly this sequence also contains nine legumin boxes scattered between -1530 and -1142 upstream. Analysis of the transgenic protein itself revealed two enzymatically active isoforms, corresponding to the wheat germins G and G' which differed by the presence of antennary N-acetylglucosamines (Jaikaran *et al.,* 1990). This analysis suggested that the post translational modifications of the protein (oligomer assembly, stability, glycosylation) were very similar, if not identical, in the homologous and heterologous hosts.

The expression of the wheat oxalate oxidase gene in tomato plants was demonstrated. Molecular and biochemical analyses indicated successful incorporation of the gene into the plant genome and the expression of oxalate oxidase activity in the transgenic tissue. Transgenic plants were more tolerant towards different concentrations of oxalic acid *in vitro* than WT plants. Furthermore, reduced disease severity was observed in transgenic tissue relative to controls, after inoculation with either *B. cinerea* or *S. sclerotiorum*, two

necrotrophic oxalic acid producing fungal pathogens. These results also support the proposal that oxalic acid production is an important determinant of pathogenicity (Walz *et al.*, 2008).

Transformation of maize with the wheat oxalate oxidase gene regulated by a constitutive rice actin promoter pAct1-D induced oxalate oxidase enzymatic activity and elevated H_2O_2 levels in leaf tissue. This leads to alteration of secondary metabolism in two biosynthetic pathways (shikimate pathway and Hydroxamic acid biosynthesis pathway), leading to the enhancement of the phenolic acid synthesis, which resulted in a significant increase in all soluble phenolic acid contents, especially free phenolics. Generally, free phenolic acid content in maize was low, but in maize transformed with oxalate oxidase the, free phenolics accounted for 92.5 per cent of soluble phenolics, which was 14-fold higher than the control. The hyper acidification of insect gut from high contents of free phenolic acids was a direct way for secondary compounds to discourage insects from consuming tissue because ECB maintained a basic gut for optimal protease activity. Alternatively, H_2O_2 produced by oxalate oxidase has direct effects on ECB growth (Mao *et al.*, 2007).

Lou and Baldwin (Gibeaut and Carpita, 1991a) examined the use of antisense RNA technology to silence the endogenous oxalate oxidase gene in *Nicotiana attenuate*. They found that defensive responses such as diterpene glycosides and proteinase inhibitors were reduced and that two native herbivorous insect larvae performed significantly better on the transformed foliage as compared to controls. These results confirmed the role of oxalate oxidase in establishing insect defensive responses in another plant.

Castanea dentata (American chestnut) has been decimated by chestnut blight which was caused by an exotic, oxalate producing pathogen; *Cryphonectria parasitica*. American chestnut wild type callus tissue was transformed with oxalate oxidase gene from wheat. It was cultured on media containing various

concentrations of oxalic acid along with control. Thermo gravimetric analysis (TGA) was employed to test the cellulose, hemicellulose, and lignin content of the calli. In the presence of oxalic acid, wild type tissues demonstrated a significant decrease in lignin content and increase in cellulose content, while transformed tissues did not. Transforming American chestnut with an oxalate oxidase gene proved to be useful in preventing changes in the cell wall composition caused by the oxalate produced during *Cryphonectria parasitica* infection and could possibly enhance resistance (Welch *et al.*, 2007).

2.9.6.5 Reporter gene

A wheat germin gene, with oxalate oxidase activity used as a sensitive reporter gene in both monocot and dicot transformations studies, provided that appropriate controls for native oxalate oxidase activity and non-specific activity were carried out. The discrete association of oxalate oxidase activity with germination of monocots and the weak non-specific activity in soybean stems and roots, provided the basis for using oxalate oxidase as a reporter protein in both monocot and dicot transformations (Simmonds *et al.*, 2004).

Detection of H_2O_2 generated by oxalate oxidase oxidation with oxalate provides simple, rapid detection of gene expression. Inexpensive substrates were required for both assays. Oxalate oxidase activity, could be detected histochemically with in minutes, without the removal of chlorophyll. This assay was used to optimize transformation procedures and to track stable transgene expression in breeding populations over many generations. A simple spectrophotometric quantitative enzyme activity assay was used to select lines with various levels of transgene expression and to monitor transgene silencing phenomena. The quantitative oxalate oxidase assay was used as an internal DNA delivery standard with a second reporter gene used in gene expression studies. The simplicity of the assay is ideal for screening large populations to identify primary transgenics, for monitoring transgene segregation in large populations in

field studies and for assessing stability of transgene expression over numerous generations.

2.9.6.6 Oxalate oxidase structure

The primary structures of the oxalate oxidase present in wheat (Hamel *et al.,* 1998), barley (Zhou *et al.,* 1998), maize (Vuletic and Sukalovic, 2000), and ryegrass (Deunff *et al.,* 2004) had been deduced by gene cloning. Sequence alignment studies, using PSI-BLAST (Altschul *et al.,* 1997), placed oxalate oxidase in the functionally diverse cupin superfamily (Dunwell and Gane, 1998; Dunwell *et al.,* 2004), which also includes seed germination and storage proteins (Dunwell *et al.,* 1998). In an interesting study (Gane *et al.,* 1998), homology-based methods (Sanchez and Sali, 1997; Sali and Blundell, 1997) were employed to obtain the first structural model of oxalate oxidase using X-ray crystal structures of *Canavalia ensiformis canavalin* (Ko *et al.,* 1993) and *Phaseolus vulgaris phaseolin* (Lawrence *et al.,* 1990; Lawrence *et al.,* 1994).

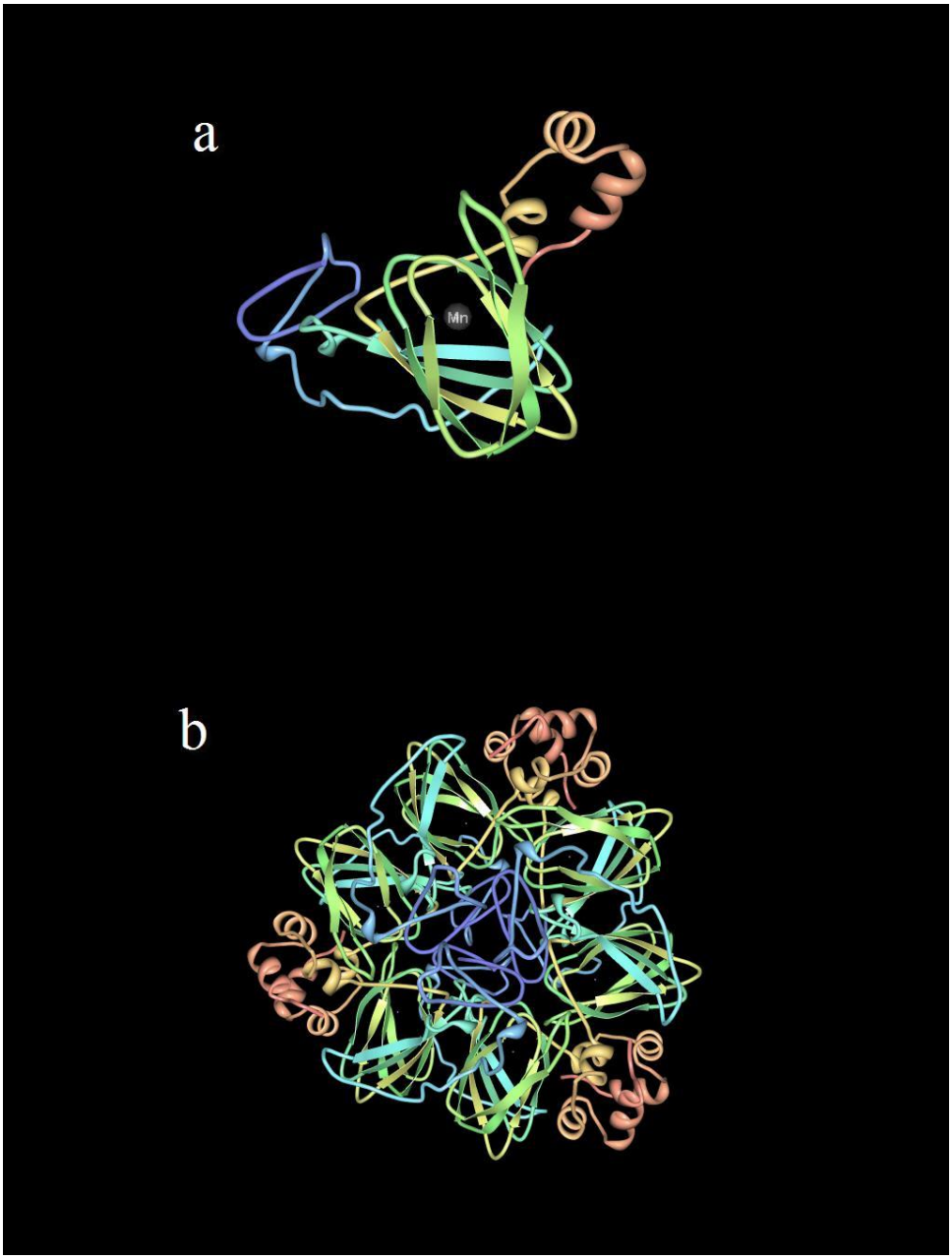
A cluster of three histidine residues, two of which are present in the GSKTLLHKHYTSEEIYYILEGRG sequence motif defining members of the germin/oxalate oxidase family (Dunwell *et al.,* 2004), was proposed to form the active site of oxalate oxidase in these modeling studies (Gane *et al.,* 1998) on the basis of their three dimensional structural similarity to metal-binding sites observed in other proteins (Regan, 1995). With the development of a rapid, three-step purification procedure to obtain oxalate oxidase from barley roots, however, the activity of the homogeneous enzyme was shown to be unaffected by riboflavin or FAD (Kotsira and Clonis, 1997), and subsequent electron paramagnetic resonance (EPR) studies confirmed the presence of Mn(II) in the resting enzyme (Whittaker and Whittaker, 2002).

The hypothesis that the three conserved histidines form a Mn-binding site (Gane *et al.,* 1998) was confirmed by X-ray crystallography of the native, barley

oxalate oxidase, the structure of which was solved to 1.6 A˚ resolution (Woo *et al.,* 2000). Each oxalate oxidase monomer possesses the predicted jellyroll β-barrel that is characteristic of the cupin superfamily (Dunwell and Khuri, 2000; Dunwell *et al.,* 2004) followed by a C-terminal domain comprised of three α-helices (Plate 1a). Although there were two potential asparagine residues that could participate in N-linked glycosylation, electron density consistent with the presence of bound carbohydrate was associated only with Asn-47 (barley numbering) (Woo *et al.,* 2000).

The enzyme itself forms strongly bound dimers, an arrangement that results in extensive burial of the monomer surfaces and hydrophobic residues. These dimeric units then associate via their C-terminal domains to give a hexamer, which is believed to be the biologically active form of the enzyme (Plate 1b). This quaternary structure has been hypothesized to be the basis for the resistance of oxalate oxidase to degradation by proteases or heat (Woo *et al.,* 2000). Each of the barley oxalate oxidase monomers contains Mn bound to the side chains of conserved glutamate and histidine residues in a site that is located towards the narrow end of the barrel-like domain, in agreement with the predictions of the homology-derived model (Gane *et al.,* 1998). In contrast to other enzymes that reduce dioxygen to H_2O_2, such as copper-dependent amine oxidases (Dawkes and Phillips, 2001) and galactose oxidase (Whittaker, 2003), there are no modified active site residues in oxalate oxidase.

Spectroscopic studies demonstrated that oxalate oxidase requires manganese for catalysis (Requena and Bornemann, 1999) and subsequent crystallographic studies on the barley enzyme revealed the structure of the hexamer and confirmed the presence of a mononuclear manganese center buried deep within its jellyroll β-barrel domain (Woo *et al.,* 2000) (Plate 2a). The manganese was bound by the side chains of three histidines and one glutamate residue, as well as two water molecules that occupy adjacent positions in the roughly octahedral metal complex (Plate 2b). Based on the lack of obvious optical

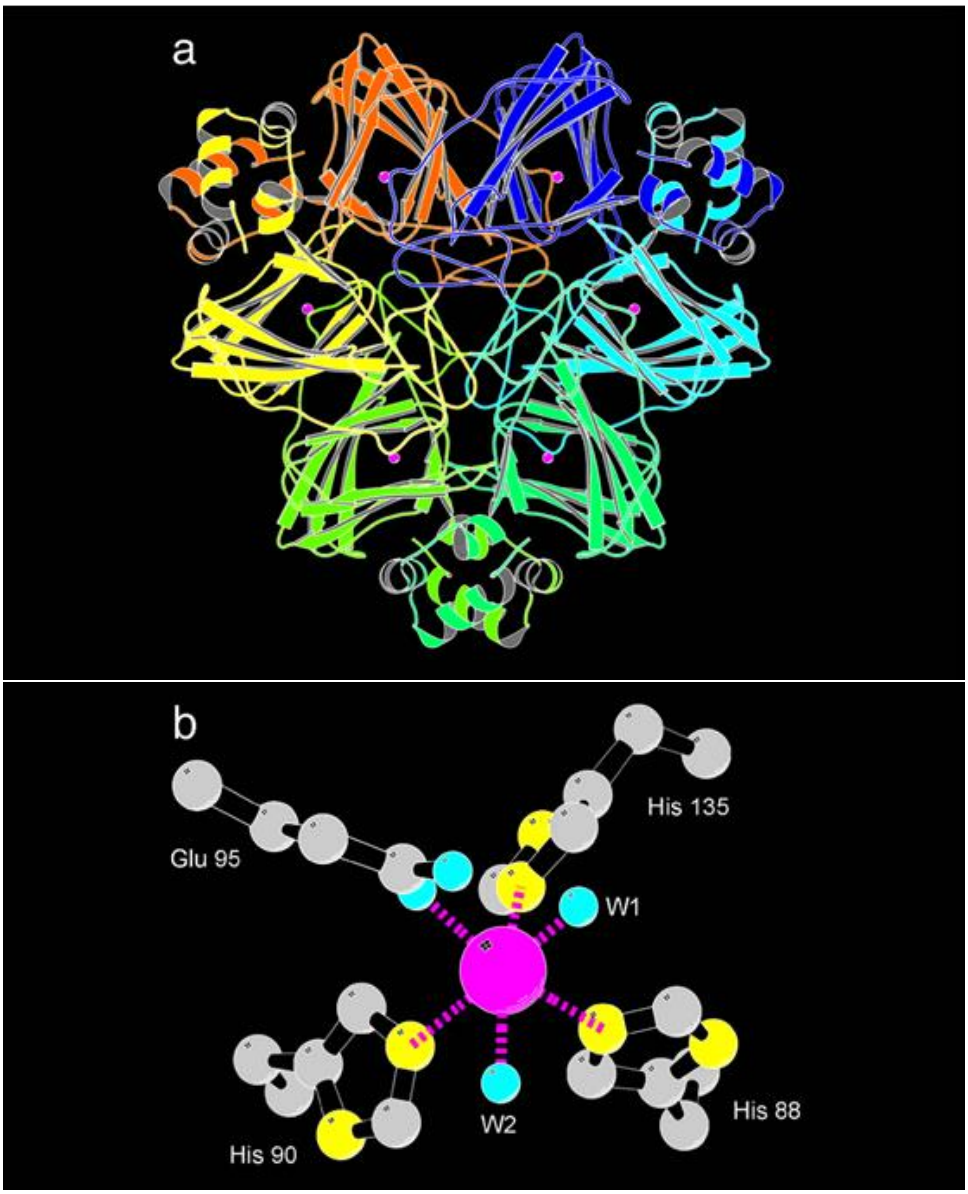


a - Secondary structure of oxalate oxidase monomer showing jellyroll β-barrel and three α-helices with manganese ion.

b - Homohexameric structure of oxalate oxidase comprising of trimer of dimers

This figure was prepared using MOLSCRIPT

Plate 1: Oxalate oxidase structure (PDB ID - 1FI2)



a - The oxalate oxidase homohexamer with six manganese ions indicated (pink spheres). The α-helical clasps bind the dimers into the trimer of dimers and the tight packing around the three-fold axis

b - The molecular model showing octahedral coordination of the manganese ion at the catalytic center of the oxalate oxidase subunit is by three histidines, one glutamate, and two water molecules.

This figure was prepared using MOLSCRIPT

Plate 2: Octahedral coordination of the manganese ion at the catalytic center of the oxalate oxidase (PDB ID-1FI2)

absorption and the presence of a characteristic EPR spectrum, the manganese ion had been assigned as the reduced Mn(II) oxidation state in the resting enzyme (Requena and Bornemann, 1999).

Spectroscopic studies using recombinant OXO expressed in *Pichia pastoris* confirmed the presence of Mn(II) in the resting recombinant enzyme and provided the first spectroscopic evidence for oxalate binding to the manganese (Whittaker and Whittaker, 2002). Oxalate oxidase binds the singly ionized oxalate monoanion as substrate at the optimum pH for the enzymatic reaction (pH = 4), which lies between the two pKa values for oxalic acid dissociation (pKa_1 = 1.25, pKa_2 = 4.14).

Overall structure of the oxalate oxidase comprised of disc shaped homohexamer organized as a trimer of dimers with the dimers arranged tightly around the three-fold symmetry axis. The monomers in each of the three dimers were related by three two-fold symmetry axes perpendicular to the three-fold symmetry axis. Each homohexamer comprised of 1,206 amino acids, six manganese ions and 1,512 water molecules and each monomer was composed of an irregular N-terminal extension, a classic jellyroll β-barrel domain, and an α-helical C-terminal domain comprised of three α-helices. The core β-barrel comprised two five-stranded β-sheets, with the eight central β-strands.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The investigation entitled 'studies on oxalic acid and oxalate oxidase enzyme in *Costus pictus* D. Don' was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara during the period 2006-2008. Materials and methodologies are described below:

3.1 MATERIALS

3.1.1 Plant materials

Sample plants of *Costus pictus* were maintained in the CPBMB field. Fully opened leaves from top 1st to 3rd, 4th to 6th and 7th to 9th were collected for analysis of oxalic acid and oxalate oxidase enzyme. The samples were designated as stage one, stage two and stage three respectively. The characteristics of the *Costus pictus* used for the present analysis is given section 4.1.

3.1.2 Chemicals, glass and plastic ware

Chemicals of good quality (AR/GR grade) procured from Merck India Ltd., Sisco Research Laboratories, Himedia and Sigma-Aldrich, were used for the study. Dialysing tube for desalting of enzyme sample were obtained from Sigma-Aldrich, USA. The glassware required for biochemical studies were purchased from Vensil and Borosil India Ltd. The plastic wares were supplied by Tarson India Ltd. and Axygen, USA.

3.1.3 Laboratory equipments

The equipments available at CPBMB were utilized for the present study.

3.2 METHODS

3.2.1 OXALATE ASSAY

Oxalate from *Costus pictus* was isolated by the method proposed by Burrows (1950) and Baker (1952) with slight modifications. The method of estimating oxalate content was adopted from Burrows (1950) with slight modifications.

3.2.1.1 Oxalate isolation

In Burrows method oxalate was isolated as total oxalate but in Baker method the oxalate was isolated as soluble and total oxalate.

3.2.1.1.1 Burrows method

The method of isolating total oxalate from fresh and dried leaf sample of *Costus pictus* was adopted from Burrows (1950) with slight modifications.

Reagents

Citric acid reagent

Citric acid reagent was prepared by dissolving 2 g of citric acid and 5 g of anhydrous calcium chloride in minimum quantity of distilled water and made up to 100 ml. It was heated to boiling. Saturated ammonium oxalate solution was added drop by drop until a permanent turbidity was formed then it was boiled for 10 minutes and kept overnight. It was filtered through Whatman No. 42 filter paper before use.

HCl 0.4 N

Hydrochloric acid 0.4 N was prepared by dissolving 3.5 ml of concentrated hydrochloric acid (min. assay - 35 per cent; Sp.gr. - 1.18 kg) in minimum quantity of distilled water and made up to 100 ml with distilled water.

Protocol for fresh leaf sample

1. Five grams of leaf sample was ground with 10 ml of distilled water and 10 ml of citric acid reagent in a pestle and mortar at room temperature.
2. The extract was filtered by Whatman No. 42 filter paper.
3. The precipitate in the filter paper was dissolved in 50 ml of 0.4 N HCl.
4. The dissolved precipitate was again filtered by Whatman No. 42 filter paper.
5. The filtrate was saved and used for the estimation of total oxalate by iron ferron method.

Protocol for dry leaf sample

1. A 10 ml of distilled water and 10 ml of citric acid reagent were added to 1 gram dried leaf sample in a 100 ml volumetric flask.
2. The sample was extracted by shaking for 10 minutes at room temperature.
3. The extract was filtered by Whatman No. 42 filter paper.
4. The precipitate in the filter paper was dissolved in 50 ml of 0.4 N HCl.
5. The dissolved precipitate was again filtered by Whatman No. 42 filter paper.
6. The filtrate was saved and used for the estimation of total oxalate by iron ferron method.

3.2.1.1.2 Baker method

The method for isolating soluble and total oxalate from fresh and dried leaf sample was adopted from Baker (1952) with slight modifications.

Reagents

Ammonium hydroxide

Concentrated ammonium solution with specific gravity 0.880 was dissolved in distilled water at 1:1 v/v ratio.

Diluted hydrochloric acid

Concentrated hydrochloric acid (min. assay - 35 per cent; Sp.gr-1.18 kg) was dissolved in distilled water in the ratio of 1:1 v/v.

Phosphoric-tungstate reagent

Sodium tungstate 2.4 g was dissolved in 4 ml of orthophosphoric acid and made up to 100 ml with distilled water.

Calcium chloride buffer

Anhydrous calcium chloride was dissolved in 50 ml of 50 per cent v/v glacial acetic acid and this solution was added to the solution of 66 g of sodium acetate in minimum quantity of distilled water and it was made up to 100 ml with distilled water.

Wash solution

Acetic acid solution 5 per cent v/v kept over calcium oxalate at room temperature for saturation and used as a wash solution. The solution was stirred periodically and filtered before use for complete saturation.

Protocol

In the isolation of oxalate by Baker method, there was a difference in the protocols for extracting total and water-soluble oxalate. Total oxalate is isolated with strong-acid solution leading to the dissolution of crystalline calcium oxalate. Isolation of tissues with water removes free oxalic acid as well as potassium oxalates and sodium oxalates but does not remove calcium oxalate

Isolation of total oxalate

1. Five gram of fresh leaf sample/one gram of dried leaf sample was homogenised with 25 ml of diluted hydrochloric acid (1+1) in pestle and mortar at room temperature.
2. The homogenate was transferred to a 50 ml beaker was boiled for 10 minutes and then 10 ml of diluted hydrochloric acid (1+1) was added and kept aside overnight at room temperature.
3. The extract was filtered through Whatman No. 42 filter paper.
4. Phosphoric - tungstate reagent was added to the filtrate and mixed by vortexing and kept aside for 5 h at room temperature.
5. It was centrifuged at 5000 rpm for 10 minutes at 4^{o}C.
6. The supernatant was transferred to a fresh tube and added ammonium hydroxide solution drop by drop until a slight precipitate was formed.
7. Then 5 ml calcium chloride buffer was added and mixed by vortexing and kept aside overnight at 4^{o}C.
8. It was centrifuged at 5000 rpm for 10 minutes at 4^{o}C.

9. The supernatant was discarded and the precipitate was dissolved in 20 ml of wash solution and centrifuged at 5000 rpm for 10 minutes at 4°C.
10. The supernatant was discarded and the precipitate was dissolved in 10 ml of 0.4 N HCl and used for the estimation of total oxalate by iron ferron method.

Isolation of soluble oxalate

1. Five gram of fresh leaf sample/one gram of dried leaf sample was homogenised with 25 ml of distilled water in pestle and mortar at room temperature.
2. The homogenate was transferred to a 50 ml beaker, boiled for 10 minutes, and then 10 ml of diluted hydrochloric acid (1+1) was added and kept aside overnight at room temperature.
3. The extract was filtered through Whatman No. 1 filter paper.
4. Phosphoric - tungstate reagent was added to the filtrate and mixed by vortexing and kept aside for 5 hr at room temperature.
5. It was centrifuged at 5000 rpm for 10 minutes at 4°C.
6. The supernatant was transferred to a fresh tube and added ammonium hydroxide solution drop by drop until a slight precipitate was formed.
7. Then 5 ml calcium chloride buffer was added and mixed by vortexing and kept aside overnight at 4°C.
8. It was centrifuged at 5000 rpm for 10 minutes at 4°C.
9. The supernatant was discarded and the precipitate dissolved in 20 ml of wash solution and centrifuged at 5000 rpm for 10 minutes at 4°C.
10. The supernatant was discarded and the precipitate dissolved in 10 ml of 0.4 N HCl and used for the estimation of soluble oxalate by iron ferron method.

3.2.1.1.3 Solvent extraction

To study the effect of solvents for the isolation of oxalate content in fresh and dry leaf samples, water, ethanol and methanol were used. Five gram of fresh/one gram of dry leaf sample was ground in 25 ml of corresponding solvent and centrifuged at 15,000 X g for 20 minutes. The supernatant was collected and stored at 4°C and used for the estimation of oxalate content by iron ferron method.

3.2.1.2 Oxalate estimation

Estimation of oxalate was done by iron ferron method of Burrows (1950). In iron ferron method, the fading effect of oxalate on the colour produced by iron with ferron reagent was used for the estimation of oxalate. The fading of colour was directly proportional to the concentration of ferron reagent and oxalic acid. The concentration of oxalic acid is determined by keeping the concentration of ferron reagent constant. The fading of colour was read at 450 nm.

Reagents

Iron ferron stock solution

Iron ferron reagent was prepared by dissolving 0.4 g of ferron in 50 ml of hot distilled water containing 0.1g of ferric chloride, 30 ml of 2 N HCl and 6.8 g of sodium acetate, and the mixture was allowed to cool to room temperature and then made up to 100ml.

Iron ferron working solution

Working solution was prepared by diluting 10 ml of the iron ferron stock solution to 100 ml with distilled water and it was used for oxalate estimation.

Standard stock solution

Stock solution was prepared by dissolving 1 g of commercially available calcium oxalate crystals in 100 ml of 0.4 N HCl

Standard working solution

Working standard solution was prepared by dissolving 1 ml of stock in10 ml of 0.4 N HCl. The solution was shook well before drawing the aliquot for calibration curve preparation.

Sample reading

Two ml of extract was added to 3 ml of iron ferron reagent and the fading of colour was read at 540 nm against zero setting blank of 0.4 N HCl. Three ml iron ferron reagent with 2 ml 0.4 N HCl was used as a sample blank.

Calibration curve

Standard curve was prepared using commercially available calcium oxalate dissolved in 0.4 N HCl. The concentration of oxalate in the sample was determined by extrapolating the standard curve prepared using calcium oxalate in the range of 0.1 to 0.5 mg.

Calcium oxalate concentration ranging from 0.1 to 0.5 mg was prepared by pipetting out 0.1, 0.2, 0.3, 0.4 and 0.5 ml of the working standard solution in a series of test tubes containing required quantity of 0.4 N HCl to make up the volume to 2 ml to each tube 3.0 ml of working iron ferron solution was added to make up the final volume to 5 ml. Absorbance was measured at 540 nm against the zero setting blank which contains 0.4 N HCl. A reagent blank was maintained essentially except adding working standard solution. Standard graph was drawn

by plotting the concentration of calcium oxalate in the x axis and absorbance in the y axis. From the standard graph, the amount of oxalate in the sample was calculated. The standard graph was given in the figure 1A.

The concentration of oxalate in different solvents was determined by extrapolating the standard curve prepared by dissolving the oxalic acid in the corresponding solvent in the concentration range of 0.1 to 0.5 mg. standard graph prepared in various solvents was given in figure 1B, 1C and 1D.

3.2.2 OXALATE OXIDASE ENZYME

Method for isolation and assay of oxalate oxidase was adopted from Sing *et al.* (2006) with slight modifications.

3.2.2.1 Isolation and assay of oxalate oxidase

Oxalate oxidase was isolated with 0.1 M phosphate buffer pH 7.0 at 4°C. Oxalate oxidase was assayed by 4-aminophenazone method. The hydrogen peroxide produced by the oxidation of oxalate by oxalate oxidase enzyme combines with 4- aminophenazone and phenol in the presence of peroxidase to form quinoeimine dye. The concentration of quinoeimine dye is directly proportional to the concentration of hydrogen peroxide which in turn proportional to the activity of oxalate oxidase enzyme. The colour produced due to quinoeimine dye formation was read at 520 nm.

$$\text{Oxalate} + O_2 \xrightarrow{\text{Oxalate oxidase}} H_2O_2 + 2\,CO_2$$

$$2H_2O_2 + \text{Phenol} + \text{4-aminoantipyrine} \xrightarrow{\text{Peroxidase}} \text{Quinoeimine dye} + 4H_2O$$

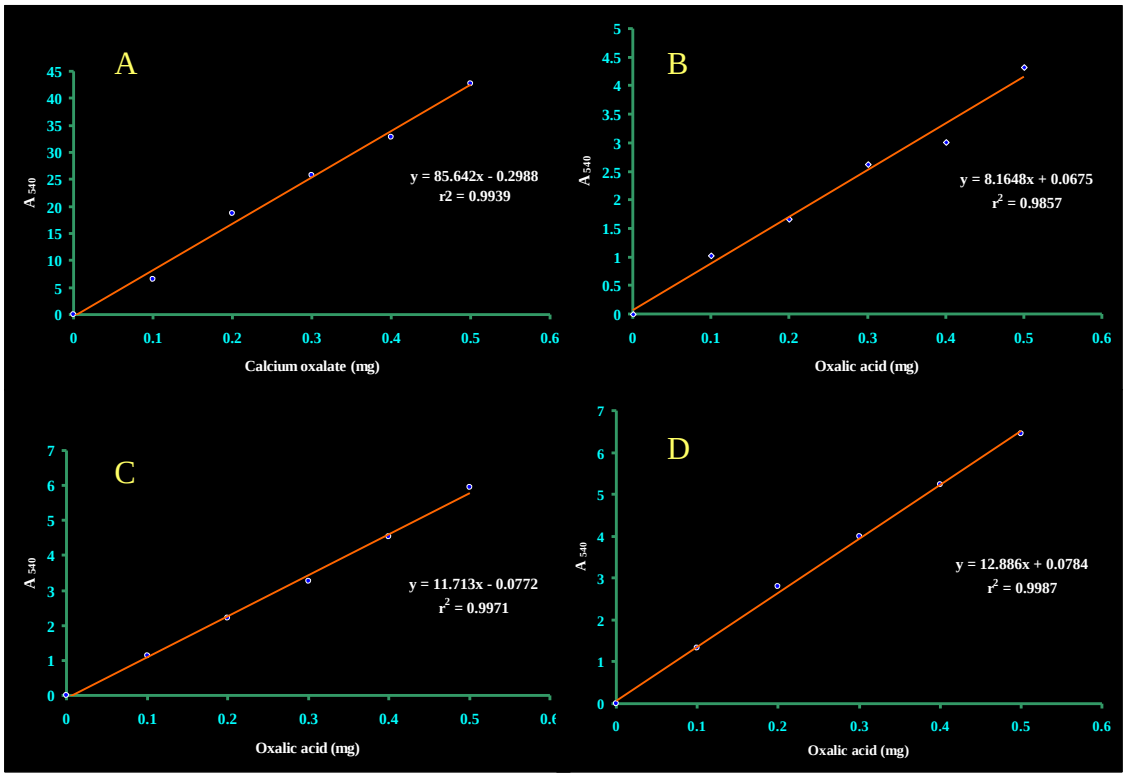


Figure 1A: Calibration curve for estimation of oxalate precipitated as calcium oxalate by standard isolation method

Figure 1B: Calibration curve for estimation of oxalate isolated with water

Figure 1C: Calibration curve for estimation of oxalate isolated with ethanol

Figure 1D: Calibration curve for estimation of oxalate isolated with methanol

Figure 1: Calibration curve for estimation of oxalate by iron ferron method

Reagents

Phosphate buffer

Phosphate buffer 0.1 M pH 7.0
Phosphate buffer 0.4 M pH 7.0

Composition of phosphate buffer is given in Appendix.

Succinate buffer

Succinate buffer 0.05 M, pH 5.0 was prepared by dissolving 5.9045 g of succinic acid (MW – 118.09 g $mole^{-1}$) in 500 ml of distilled water and adjusting the pH to 5.0 with 1N NaOH and made up to 1000 ml with distilled water.

Copper sulphate

Copper sulphate 0.01 M was prepared by dissolving 0.25 g of copper sulphate ($CuSO_4.5H_2O$, MW – 294.68 g $mole^{-1}$) in 100 ml of distilled water.

Oxalic acid

Oxalic acid 0.01 M was prepared by dissolving 0.216 g of oxalic acid (MW – 126.07 g $mole^{-1}$) in 100 ml of distilled water.

Colour reagent

The colour reagent was prepared by dissolving 50 mg 4-aminophenazone, 0.1 g solid phenol and 1 mg HOPD in 100 ml of 0.4 M sodium phosphate buffer (pH 7.0). It was stored in amber coloured bottles at 4°C and prepared for every 7 days.

3.2.2.1.1 Isolation of oxalate oxidase

1. Five grams of fresh leaf sample//one gram of dried leaf sample was homogenised with 0.1 M sodium phosphate buffer pH 7.0.
2. The homogenate was centrifuged at 15000 X g for 20 minutes.
3. Supernatant was collected as crude enzyme.
4. The crude extract was diluted to 100 times with 0.1 M sodium phosphate buffer pH 7.0. This diluent was used for oxalate oxidase assay.

3.2.2.1.2 Oxalate oxidase assay

1. The assay of oxalate oxidase was carried out in tubes wrapped with aluminium foil.
2. To each tube 1.7ml succinate buffer (0.05 M, pH 5.0), 0.1ml $CuSO_4$ (0.01 M) and 0.1ml crude extract were added.
3. The reaction mixture was pre-incubated at 40^oC for 2 minutes.
4. The reaction was started by adding 0.1ml oxalic acid (10 mM) to each tube.
5. After incubating it at 40^oC for 5 minutes, 1.0 ml colour reagent was added to each tube and kept at room temperature for 30 minutes in dark to develop the colour.
6. Absorbance was read at 520 nm against the zero setting blank of 0.05M succinate buffer pH 5.0. A sample blank was maintained which contains all the reagents except the enzyme extract.
7. The amount of H_2O_2 generated during the reaction was determined from a standard curve of H_2O_2 prepared in 0.05 M succinate buffer, pH 5.0.
8. The protein in the sample was estimated by Bradford method.
9. One unit of oxalate oxidase was defined as the amount of enzyme required to produce 1 nmole of H_2O_2 per five minute under standard assay conditions.

Calibration curve

Standard curve was prepared using commercially available hydrogen peroxide solution dissolved in succinate buffer 0.05 M, pH 5.0. The amount of hydrogen peroxide produced from the sample due to oxalate oxidase activity on oxalic acid was determined by extrapolating the standard curve prepared using hydrogen peroxide in the range of 0.01µmol to 0.30 µmol.

Calibration curve was prepared by pipetting out 0.1 of Hydrogen peroxide ranging from 0.01µmol to 0.30 µmol prepared in 0.05 M succinate buffer pH 5.0 in to a series of test tubes containing 1.7 ml of succinate buffer, 0.1 ml of $CuSO_4$, 0.1 ml of oxalic acid and 1 ml of colour reagent and it is incubated for 30 minutes in dark to develop colour. A reagent blank was maintained essentially except adding hydrogen peroxide solution. Absorbance was measured at 520 nm against the reagent blank. A standard graph was drawn by plotting the concentration of hydrogen peroxide in the x axis and the absorbance in the y axis. From the standard graph, the amount of hydrogen peroxide produced in the sample due to oxalate oxidase activity on oxalic acid was calculated. The standard graph for hydrogen peroxide was given in figure 2.

3.2.3 PROTEIN ESTIMATION

The soluble protein in the *Costus pictus* leaf extract was determined by the method proposed by Bradford (1976) with slight modification.

This method is based on the principle that protein binds to coomassie brilliant blue G-250 in acid solution and forms a blue complex whose extension coefficient (λ_{max} = 595 nm) is much greater than the free dye (λ_{max} = 465 nm) itself. The dye binds strongly to positively charged groups of proteins and also hydrophobic regions in proteins. As a result, a blue colour is formed with a λ_{max} at 595 nm (on binding to proteins the λ_{max} is shifted from 465 to 595 nm).

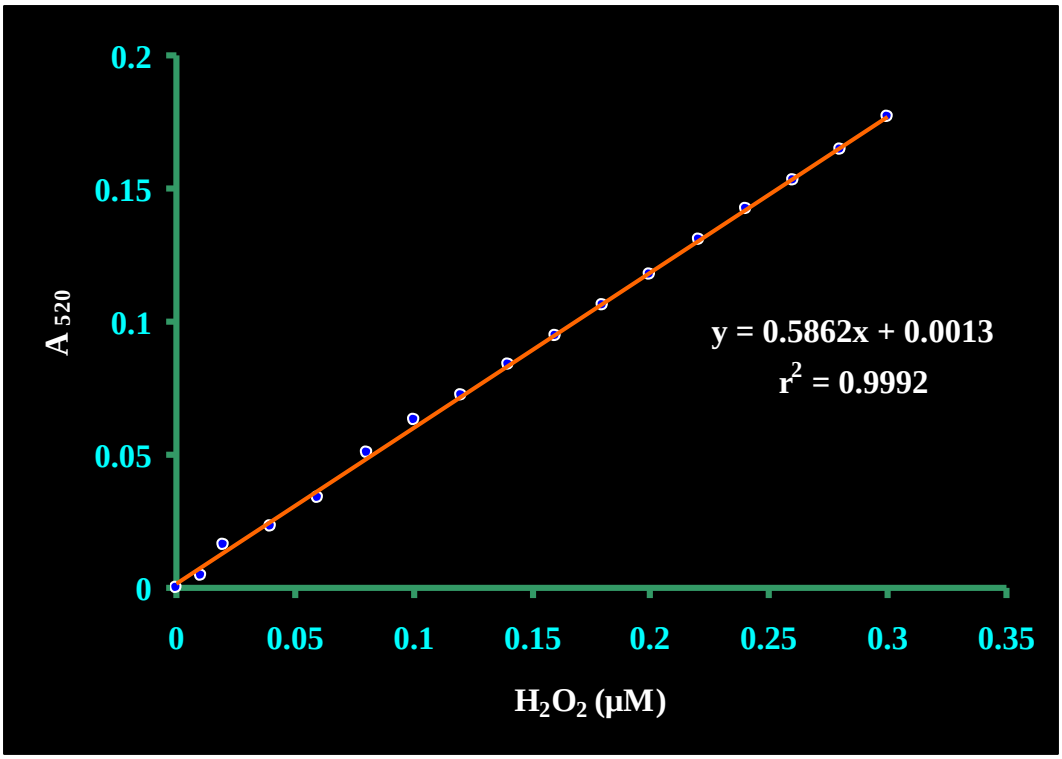


Figure 2: Calibration curve for the estimation of H_2O_2 evolved during oxalate oxidase enzyme assay

Reagents

Bradford stock dye solution

Bradford stock dye solution was prepared by dissolving 100 mg of comassie brilliant blue G 250 in 50 ml methanol (95 per cent v/v) and 100 ml of concentrated orthophosphoric acid and the volume was made up to 1000 ml with distilled water and stored at 4°C.

Bradford working dye solution

Bradford working dye solution was prepared by dissolving 100 ml of Bradford stock solution in 400 ml of distilled water and filtered before use.

Standard stock solution

Standard stock solution was prepared by dissolving 100 mg of Bovine Serum Albumin (BSA) fraction V in 100 ml of 0.1 N NaOH.

Standard working solution

One ml of stock solution was diluted 10 times to get a working solution containing 100µg ml^{-1}.

Protein assay

Protein concentration in the enzyme extract was determined by pipetting out 0.2 ml of the extract in to a tube containing 0.8 ml of NaOH and 5.0 ml of Bradford working dye solution shook well and allowed to stand for 5 minutes. Absorbance was measured at 595 nm against the zero setting blank of 0.1 N

NaOH. A sample blank was maintained essentially except adding the enzyme extract. The amount of protein was calculated from the standard graph prepared.

Calibration curve preparation

Protein concentration ranging from 10-100 µg were prepared by pipetting out 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 ml of the working standard solution in a series of test tubes containing required quantity of 0.1 N NaOH to make up the volume to 1.0 ml. In each tube 5.0 ml of working dye solution was added shook well and allowed to stand for 5 minutes. Absorbance was measured at 595 nm against the zero setting blank of 0.1 N NaOH. A reagent blank was maintained essentially except adding working standard solution. A standard graph was drawn by plotting the concentration of protein in the x-axis and the absorbance in the y-axis. From the standard graph, the amount of protein in the sample was calculated. The standard graph for Bradford method was given in figure 3.

3.2.4 PARTIAL PURIFICATION

The method for partial purification of the enzyme was adopted from Goyal *et al.* (1999) with slight modification. All the steps were carried at 4°C.

3.2.4.1 Preparation of saturated ammonium sulphate solution

Crystalline ammonium sulphate was added part by part to about 1000 ml of distilled water with stirring to maximum saturation. It was kept hot and added more ammonium sulphate till the crystal became insoluble. Cooled solution to room temperature and filtered. The pH was adjusted to 7.0 with ammonia solution.

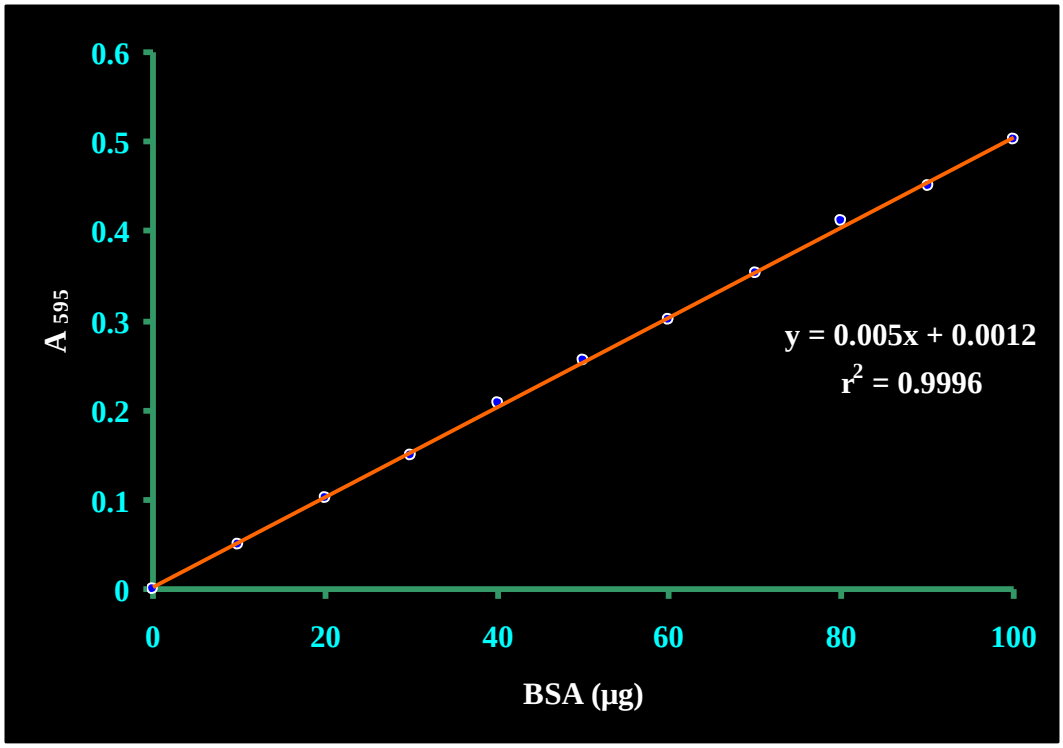


Figure 3: Calibration curve for the estimation of protein by Bradford method

3.2.4.2 Preparation of sample

Ten gram leaf sample was collected (4th to 6th leaf from top) ground in ice-cold mortar and pestle with 50 ml 0.1 M phosphate buffer. The extract was centrifuged at 15000 X g for 20 minutes. The supernatant was collected and 25 ml of extract was used for partial purification and the rest is saved as such.

3.2.4.3 Precipitation of enzyme using saturated ammonium sulphate solution

Hundred ml of saturated ice cold ammonium sulphate solution pH 7.0 was added drop by drop to the 25 ml of supernatant prepared at 4°C so as to get 80 per cent saturation. The mixture was kept at 4°C overnight for precipitation of proteins. On the next day, it was centrifuged at 10000 X g for 30 minutes. The precipitates were collected and dissolved in 15 ml 0.1 M phosphate buffer pH 7.0 and dialysed against the same buffer (0.05 M, pH 7.0) at 4°C with four changes of buffer at an interval of six hours for each change.

3.2.4.4 Dialysis

3.2.4.4.1 Pretreatment of dialysis membrane

The dialysis membrane was cut in to small pieces of about 10-15 cm and placed inside a beaker containing 500 ml of 2 per cent $NaHCO_3$, 10 mM EDTA and boiled for 20 minutes. (The tubes should not touch the walls of the beaker). After 20 minutes boiling the inside and outside of the tubes were washed with distilled water using a squeeze bottle. The tubes were again boiled for 10 minutes in 1mM EDTA to remove excess $NaHCO_3$. The inside and outside of the tubes were again washed thoroughly with distilled water and stored in 10 percent ethanol at 4°C.

3.2.4.4.2 Procedure

To dialyse the sample 10 to 15 cm tube was taken and inside and out side of the tube was rinsed with distilled water. One end of the tube was tied securely with a thread. Using the open end, the tube was filled with distilled water. Holding the tube securely at the top, the tube was gently squeezed to check weather there is any leakage on the membrane or on the closed end. After verifying for the leakage the water was removed and filled the sample using a pipette. The open end is also closed securely by tying with a thread. Now the membrane was placed in 1000 ml of 0.05 M phosphate buffer pH 7.0 at 4°C. The buffer was changed regularly at six hours intervals.

3.2.5 ENZYME KINETICS

Effect of pH, temperature, substrate concentration and enzyme concentration on enzyme activity were studied for both crude and partially purified samples.

3.2.5.1 Effect of pH on oxalate oxidase enzyme activity

To find out the optimum pH for the enzymatic activity, 0.05 M succinate buffer of various pHs from 3 to 8 was prepared with in increment of 1 pH units and subsequently the increment was reduced to 0.1 pH units for the pH range 4 to 6.

To the series of tubes 1.7 ml succinate buffer 0.05 M of varying pHs, 0.1 ml $CuSO_4$ (0.01 M) and 0.1 ml of enzyme extract added serially. The reaction mixture was pre-incubated at 40°C for 2 minutes and reaction was started by adding 0.1ml oxalic acid (10 mM) to each tube. After incubating at 40°C for 5 minutes, 1.0 ml colour reagent was added to each tube and kept at room temperature for 30 minutes in dark to develop the colour. Absorbance was read at

520 nm against the zero setting blank of 0.05 M succinate buffer pH 5.0. A separate sample blank was maintained for each reaction mixture for each pH of all the reagents except enzyme extract.

3.2.5.2 Effect of temperature on oxalate oxidase enzyme activity

To find out the optimum temperature of the enzyme activity the reaction mixture containing 1.7 ml succinate buffer (0.05 M, pH 5.0), 0.1 ml $CuSO_4$ (0.01 M), 0.1 ml oxalic acid (10 mM) and 0.1 ml of enzyme extract was incubated at various temperatures from 10 to 100°C with the increment of 10°C and 5°C.

To the series of tubes 1.7 ml succinate buffer (0.05 M, pH 5.0), 0.1 ml $CuSO_4$ (0.01 M) and 0.1 ml of enzyme extract were added. The series of tubes containing reaction mixture was pre-incubated at varying temperatures starting from 10 to 100°C for 2 minutes. The reaction was started by adding 0.1ml oxalic acid (10 mM) to each tube. After incubating at varying temperatures from 10 to 100°C for 5 minutes, 1.0 ml colour reagent was added to each tube and kept at room temperature for 30 minutes in dark to develop the colour. Absorbance was read at 520 nm against the zero setting blank of 0.05 M succinate buffer pH 5.0. A separate sample blank was maintained for reaction mixture for each temperature of all the reagents except enzyme extract.

3.2.5.3 Effect of substrate on oxalate oxidase enzyme activity

To find out the effect of substrate concentration on the enzyme activity, the enzyme is assayed with increasing concentration of substrate from 0.1 mM to 20 mM under standard assay conditions.

To the series of tubes 1.7 ml succinate buffer (0.05 M, pH 5.0), 0.1 ml $CuSO_4$ (0.01 M) and 0.1 ml of enzyme extract was added. The reaction mixture was pre-incubated at 40°C for 2 minutes. The reaction was started by adding

0.1ml oxalic acid from 0.1mM to 20 mM to the series of tubes. After incubating it at 40°C for 5 minutes, 1.0 ml colour reagent was added to each tube and kept at room temperature for 30 minutes in dark to develop the colour. Absorbance was read at 520 nm against the zero setting blank of 0.05 M succinate buffer pH 5.0. A separate sample blank was maintained for each substrate concentration where all the reagents except enzyme extract were there.

3.2.5.4 Effect of enzyme concentration on oxalate oxidase enzyme activity

To find out the effect of enzyme concentration on the enzyme activity, the assay was carried out with increasing concentration of enzyme from 0.1 ml to 1 ml under standard assay conditions.

To the series of tubes required succinate buffer (0.05 M, pH 5.0), 0.1 ml $CuSO_4$ (0.01 M) and varying concentration of enzyme extract from 0.1 ml to 1 ml was added. The reaction mixture was pre-incubated at 40°C for 2 minutes. The reaction was started by adding 0.1ml oxalic acid (10 mM) to the series of tubes. After incubating it at 40°C for 5 minutes, 1.0 ml colour reagent was added to each tube and kept at room temperature for 30 minutes in dark to develop the colour. Absorbance was read at 520 nm against the zero setting blank of 0.05 M succinate buffer pH 5.0. A separate sample blank was maintained where all the reagents except enzyme extract was there.

3.2.5.5 Effect of metal ions on oxalate oxidase enzyme activity

To find out the effect of copper and manganese ion on enzyme activity, the assay was carried with different concentrations of copper sulphate and magnesium sulphate.

To the series of tubes 1.7 ml succinate buffer (0.05 M, pH 5.0), 0.1 ml $CuSO_4$ (0.005 M, 0.01 M and 0.20 M) and MnSO4 (0.005 M, 0.01 M and 0.20 M)

and 0.1 ml of enzyme extract was added. The reaction mixture was pre-incubated at 40°C for 2 minutes. The reaction was started by adding 0.1ml oxalic acid (10 mM) to the series of tubes. After incubating it at 40°C for 5 minutes, 1.0 ml colour reagent was added to each tube and kept at room temperature for 30 minutes in dark to develop the colour. Absorbance was read at 520 nm against the zero setting blank of 0.05 M succinate buffer pH 5.0. A control was maintained with out adding any metal. A separate sample blank was maintained where all the reagents except enzyme extract was added.

RESULTS

4. RESULTS

The results of "Studies on oxalic acid and oxalate oxidase enzyme in *Costus pictus* D.Don" at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara are presented in this chapter under different subheads.

4.1 Characteristics of *Costus pictus*

The stem is purplish-red at the base changing to green towards the upper parts. The ligule is short and truncate, short stiff hairs are absent. The petiole is green with pink tinge and glabrous. The leaves are green and glabrous on both sides, edges of leaves have a characteristic undulation. Leaf shape is narrowly obovate to narrowly elliptical. Base is slightly cordate and apex is acuminate. The leaves are arranged sprially on the stem which is the characteristics of the plant. The morphological characters of *Costus pictus* are given in plate 3. The Inflorescence is terminal large arching from bracts, overall appearance is yellow. Bracts are green with dark green callus in the exposed parts and red, glabrous, ovate in the covered parts. The bracteole is red with few sparse hairs. The calyx is very small, red and glabrous. The corolla is bright yellow and glabrous. Labellum is yellow with red stripes and yellow on center lobe. The stamens are yellow, elliptical in shape, dark red and apex is triangular. The floral characters of *Costus pictus* are given in plate 4.

4.2 Isolation and estimation of oxalates

Isolation of oxalates from fresh and dry leaf sample was carried by two methods. In Baker method oxalate was isolated as water soluble oxalate and total oxalates whereas in Burrows method it was total oxalate.

Plate 3: Morphology of *Costus pictus*

Bract – outer view | **Bract – inner view** | **Bracteole - Calyx** | **Bract - Bracteole - Calyx**

Plate 4: Floral characters of *Costus pictus*

Iron ferron method was adopted to determine the oxalate content isolated by two different methods. This method was standardized to estimate oxalate content in *Costus pictus* leaf sample. Different concentrations and volume of iron ferron solution was tried and finally 3 ml of 1:10 diluted iron ferron solution was selected for oxalate determination in *Costus pictus*.

4.3 Oxalate content

4.3.1 Baker method

In the baker method of isolation a different protocol was adopted to isolate water soluble oxalate and total oxalate from three different leaf stages of fresh and dried leaf samples. The oxalate content of fresh and dried leaf sample isolated by baker method is given in table 11.

Fresh samples

In fresh samples the water soluble content was more or less same for first and second leaf stages which correspond to 10.75 and 10.83 mg g^{-1} of leaf sample. The third leaf stage was having slightly lower oxalate value of 9.67 mg g^{-1} compared to other two stages. The total oxalate content of three different stages was 10.77, 11.06 and 10.18 mg g^{-1} of leaf sample respectively. The total oxalate content was slightly higher in second leaf stage compared to other two leaf stages. Generally oxalate content was high in the first two leaf stages compared to third leaf stage.

Dry leaf sample

In dry sample the oxalate content was lower than that of the fresh sample and the same trend was obtained in the oxalate content of different leaf stages. The water soluble oxalate content for three different stages was 6.09, 6.19 and

5.06 mg g^{-1} of leaf sample respectively. The total oxalate content in the dry sample for three different stages was 6.34, 6.47 and 5.26 mg g^{-1} of leaf sample respectively. The loss of oxalate content due to drying was approximately 4.6 mg g^{-1}.

4.3.2 Burrows method

In burrows method, the oxalate was isolated from both fresh and dry sample of three different leaf stages. In this method total oxalates was isolated by a single procedure not as baker method where different procedures were adopted to isolate water soluble and total oxalate. The oxalate content of fresh and dried leaf sample isolated by burrows method is given in table 12.

Fresh sample

In fresh sample the total oxalate content was 16.41, 16.50 and 15.82 mg g^{-1} of leaf sample for three different leaf stages respectively. The total oxalate content was slightly higher in second leaf stage compared to other two stages of leaf samples. Generally oxalate content was high in the first two leaf stages compared to third leaf stage.

Dry leaf sample

In dry sample, the total oxalate content was 12.10, 12.21 and 11.47 mg g^{-1} of leaf sample for three different stages of leaf sample. The dry sample contains less oxalate content compared to fresh leaf samples. The total oxalate content was slightly higher in second stage compared to other two stages of leaf sample. Generally oxalate content was high in the first two stages compared to third stage of leaf sample. Generally oxalate accumulation was high in the first two leaf stages compared to that of the third stage.

Table 11: Oxalate content in various leaf stages of *Costus pictus* isolated by Baker method

Sample	Stage	Soluble oxalate (mg g^{-1})	Insoluble oxalate (mg g^{-1})	Total oxalate (mg g^{-1})
Fresh	1st to 3rd	10.75	0.02	10.77
	4th to 6th	10.83	0.23	11.06
	7th to 9th	9.67	0.5	10.18
	Mean	10.42	0.25	10.67
Dry	1st to 3rd	6.09	0.25	6.34
	4th to 6th	6.19	0.28	6.47
	7th to 9th	5.06	0.2	5.26
	Mean	5.78	0.24	6.02

Table 12: Total oxalate content in various leaf stages of *Costus pictus* isolated by Burrows method

Leaf	Stage	Oxalate content (mg g^{-1})	Mean
Fresh	1st to 3rd	16.41	16.24
	4th to 6th	16.50	
	7th to 9th	15.82	
Dry	1st to 3rd	12.10	11.93
	4th to 6th	12.21	
	7th to 9th	11.47	

4.3.3 Effect of solvents on the recovery of oxalates

The oxalate was isolated from fresh and dried leaf samples with water, ethanol and methanol at three different leaf stages. The oxalate content in various stages of leaf is given in the table 13.

Fresh leaf sample

In fresh leaf sample, high oxalate content was found in water extract of second stage which corresponds to 8.70 mg g^{-1}. The first and third leaf stage contains 8.53 and 8.35 mg g^{-1} respectively. Ethanol extract contains 3.81, 4.29 and 3.69 mg g^{-1} of oxalate in three different leaf stages respectively. Methanol extract contains 4.17, 5.01 and 3.52 mg g^{-1} of oxalate in three different leaf stages respectively.

Dry leaf sample

In dry leaf sample, high oxalate content was found in water extract of second stage which corresponds to 3.87 mg g^{-1}. The first and third stage contains 3.73 and 3.61 mg g^{-1} of leaf sample respectively. Ethanol extract contains 2.90, 3.72 and 2.64 mg g^{-1} of oxalate in three different leaf stages respectively. Methanol extract contains 1.11, 1.76 and 0.56 mg g^{-1} of oxalate in three different leaf stages respectively.

High oxalate content was found in water extract followed by methanol and ethanol extract. Fresh leaf sample contains more oxalate than dry leaf samples. Generally oxalate accumulation was high in the first two leaf stages compared to third leaf in both fresh and dry samples.

Table 13: Effect of solvents on oxalate content in various leaf stages of *Costus pictus*

Solvent	Leaf	Stage	Oxalate (mg g^{-1})	Mean
Water	Fresh	1st to 3rd	8.53	8.53
		4th to 6th	8.70	
		7th to 9th	8.35	
	Dry	1st to 3rd	3.73	3.74
		4th to 6th	3.87	
		7th to 9th	3.61	
Ethanol	Fresh	1st to 3rd	3.81	3.93
		4th to 6th	4.29	
		7th to 9th	3.69	
	Dry	1st to 3rd	2.90	3.08
		4th to 6th	3.72	
		7th to 9th	2.64	
Methanol	Fresh	1st to 3rd	4.17	4.23
		4th to 6th	5.01	
		7th to 9th	3.52	
	Dry	1st to 3rd	1.11	1.14
		4th to 6th	1.76	
		7th to 9th	0.56	

4.4 Oxalate oxidase assay

4.4.1 Preparation of crude sample

Oxalate oxidase was isolated with 0.1 M phosphate buffer at pH 7.0 from fresh and dry leaf samples of three different stages and assayed for activity. The undiluted crude extract shows no oxalate oxidase activity due to the presence of high oxalate content in the extract. Hence the extract was diluted 100 times with 0.1 M phosphate buffer at pH 7.0.

Increase in the oxalate oxidase activity was recorded in the diluted samples in the absence of oxalic acid addition. The substrate inhibition of oxalate oxidase activity was confirmed by the decrease in the activity with the addition of substrate to the diluted samples. The activity of crude enzyme at different dilutions with and with out substrate is given in the table 14 and figure 4. It was observed that the rate of decrease of activity was directly proportional to the concentration of the sample. It was also observed that the same trend in the addition of oxalic acid. The difference in the activity with and with out addition of oxalic acid had also showed the same effect.

4.4.2 Oxalate oxidase activity

The oxalate oxidase activity at different stages of fresh leaf sample was 93.17, 125.58 and 84.81 units g^{-1} of leaf sample respectively. In fresh sample oxalate oxidase activity was found high in second leaf stage followed by first and third leaf stages. The activity of fresh and dry leaf samples of different stages along with protein content is given in table 15. No oxalate oxidase activity was found in dry leaf samples.

Table 14: Effect of dilution on oxalate oxidase enzyme activity in *Costus pictus*

Dilution	Units (nmol g^{-1} 5 min^{-1})		Difference in activity (nmol g^{-1} 5 min^{-1})
	0.01 M oxalic acid	0.0 M oxalic acid	
1:50	41.79	67.67	25.88
1:100	73.53	125.95	52.42
1:200	112.42	186.65	74.23
1:300	170.09	248.21	78.12
1:400	236.88	324.18	87.30
1:500	318.14	438.39	120.25

Table 15: Oxalate oxidase activity in crude extract of different stages of fresh and dry leaf samples of *Costus pictus*

Stage	Fresh			Dry		
	Protein mg g^{-1}	Units (nmol H_2O_2 g^{-1} 5 min^{-1})	Specific activity (Units mg^{-1})	Protein (mg g^{-1})	Units (nmol H_2O_2 g^{-1} 5 min^{-1})	Specific activity (Units mg^{-1})
1st to 3rd	0.93	93.17	100.18	0.69	0	0
4th to 6th	0.67	125.58	187.43	0.51	0	0
7th to 9th	0.52	84.81	163.10	0.41	0	0

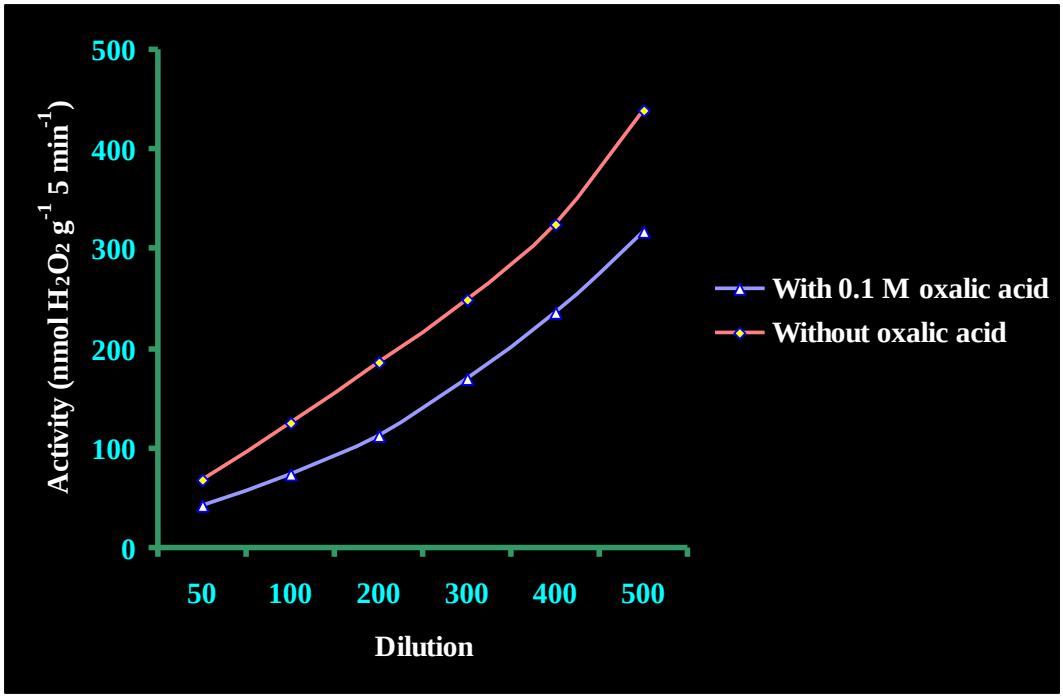


Figure 4: Effect of dilution on oxalate oxidase enzyme activity with and with out substrate

4.4.3 Effect of solvents on oxalate oxidase enzyme activity

Oxalate oxidase was isolated with different solvents and assayed for its activity. The water extract of fresh leaf sample has the activity of 89.19, 96.75 and 74.44 units g^{-1} of leaf sample for three different leaf stages respectively. The ethanol extract of fresh leaf sample has the activity of 453.97, 549.30 and 381.13 units g^{-1} of leaf sample for three different leaf stages respectively. The methanol extract of fresh leaf sample has the activity of 80.66, 101.02 and 53.17 units g^{-1} of leaf sample for three different leaf stages respectively.

Ethanol extract showed maximum activity compared to water and methanol extracts. Generally the oxalate oxidase activity was more in the second leaf stage followed by first and third leaf stages. No oxalate oxidase activity was found in dry leaf samples. The activity of oxalate oxidase in various solvents is given in the table16.

4.5 Protein estimation

Soluble protein in the enzyme extract was estimated by Bradford method. Different concentrations of Bradford dye were tried for the estimation of leaf protein with and without TCA precipitation. It was observed that the difference in the quantity of protein was insignificant in the both methods. The protein quantity increased with increase in concentration up to 1: 5 dilutions of Bradford reagent and then decreased. The protein content at different concentration of Bradford reagent with and without TCA precipitation was given in table 17. So, 1:5 diluted Bradford reagent without TCA precipitation of sample was used for determination of proteins.

Table 16: Effect of solvents on oxalate oxidase enzyme activity in crude extract of various leaf stages of *Costus pictus*

Solvent	Leaf	Stage	Protein (mg g^{-1})	Units (nmol H_2O_2 g^{-1} 5 min^{-1})	Specific activity (Units mg^{-1})
Water	Fresh	1st to 3rd	0.47	89.19	189.77
		4th to 6th	0.44	96.75	219.89
		7th to 9th	0.35	74.44	212.29
	Dry	1st to 3rd	0.37	0	0
		4th to 6th	0.35	0	0
		7th to 9th	0.29	0	0
Ethanol	Fresh	1st to 3rd	1.24	453.97	366.10
		4th to 6th	1.18	549.30	465.51
		7th to 9th	1.04	396.38	381.13
	Dry	1st to 3rd	0.90	0	0
		4th to 6th	0.85	0	0
		7th to 9th	0.76	0	0
Methanol	Fresh	1st to 3rd	0.56	80.66	144.04
		4th to 6th	0.39	101.02	259.03
		7th to 9th	0.29	53.17	183.34
	Dry	1st to 3rd	0.44	0	0
		4th to 6th	0.32	0	0
		7th to 9th	0.25	0	0

4.5.1 Protein content of *Costus pictus* leaf sample

The soluble protein in the enzyme extract was determined for three different leaf stages for fresh and dry leaf samples during the assay of oxalate oxidase.

Fresh leaf sample

The phosphate buffer extract of fresh leaf sample contains 0.93, 0.67 and 0.52 mg g^{-1} of protein for three different stages respectively. The protein content of water extract was 0.47, 0.44 and 0.35 mg g^{-1} of protein for three different leaf stages. The protein content of ethanol extract was 1.24, 1.18 and 1.04 mg g^{-1} of protein for three different leaf stages respectively. The protein content of methanol extract was 0.56, 0.39 and 0.29 mg g^{-1} of protein for three different leaf stages respectively.

Dry leaf sample

The phosphate buffer extract of dry leaf sample contains 0.69, 0.51, and 0.41 mg g^{-1} of protein for three different stages respectively. The water extract of dry sample contains 0.37, 0.35 and 0.29 mg g^{-1} of protein for three different leaf stages respectively. The ethanol extract of dry leaf sample contains 0.90, 0.85 and 0.76 mg g^{-1} of protein for three different stages respectively. The methanol extract of dry sample contains 0.44, 0.32 and 0.25 mg g^{-1} of protein for three different leaf stages respectively. The ethanol extract showed high quantity of protein in the leaf sample. Generally the protein content was high in first stage followed by second and third stage.

4.6 Specific activity of oxalate oxidase

Effect of different extraction system such as phosphate buffer, water, ethanol and methanol on specific activity was recorded. The specific activity of oxalate oxidase isolated with buffer for three different stages was 100.18, 187.43 was 163.10 units mg^{-1} of protein respectively. The specific activity with water for three different stages was 189.77, 219.89 and 212.29 units mg^{-1} of protein respectively. The Specific activity with ethanol for three different leaf stages was 366.10, 465.51 and 381.13 units mg^{-1} of protein respectively. The specific activity with methanol for three different stages was 144.04, 259.03 and 183.34 units mg^{-1} of protein respectively.

Highest specific activity was recorded with ethanol extract. Generally second leaf stage was recorded highest specific activity in all the solvent systems followed by third and first leaf stages.

4.7 Partial purification of crude extract

The crude extract was partially purified by 80 per cent ammonium sulphate precipitation with and with out heat treatment. For heat treatment the crude extract was incubated at 80^{o}C for 3 minutes with continuous agitation and then quickly chilled on ice. The table 18 shows the activity of crude and ammonium sulphate precipitated samples. Maximum protein content was recorded in crude (0.81 mg g^{-1}). With 80 percent ammonium sulphate precipitation the protein content was recorded 0.74 mg g^{-1} (with out heat treatment) and 0.48 mg g^{-1} (with heat treatment). In heat treated sample, considerable loss of activity was observed but the specific activity was 1.1 times more compared to that of the sample with out heat treatment. The ammonium sulphate precipitation gives four fold increase in activity compared to crude extract.

Table 17: Effect of Bradford reagent concentration and TCA precipitation

Bradford reagent dilution	Protein content (mg g^{-1})	
	TCA precipitate	No TCA precipitate
No dilution	0.15	0.23
1:1	0.21	0.32
1:2	0.35	0.39
1:4	0.42	0.46
1:5	0.52	0.56
1:10	0.49	0.52

Table 18: Oxalate oxidase enzyme activity in crude and partially purified sample of stage two leaves of *Costus pictus*

	Crude	Ammonium sulphate precipitation	
		Without heat treatment	With heat treatment
Protein (mg g^{-1})	0.81	0.744	0.48
Units (nmol H_2O_2 g^{-1} 5 min^{-1})	34.69	155.52	110.03
Specific activity (mg g^{-1})	42.83	209.03	229.23

4.8 Effect of pH on oxalate oxidase enzyme activity

Oxalate oxidase from crude extract and partially purified extract (diluted and undiluted) were assayed at varying pH (3 to 8 with the increment of 1 pH unit). The activity at different pH was given in table 19a. The oxalate oxidase from crude extract showed maximum activity at pH 5.0 while the partially purified showed maximum activity at pH 6.0. The assay was repeated with the pH range of 4 to 6 for crude extract and 5 to 8 for partially purified (diluted and undiluted) with the increment of 0.1 pH unit. The activity at various pH was given in table 19b. Crude extract showed maximum activity at pH 5.2, while the partially purified (diluted and undiluted) showed maximum activity at pH 5.8. The activity at different pH is given in figure 5.

4.9 Effect of temperature on oxalate oxidase enzyme activity

Oxalate oxidase from crude extract and partially purified extract (diluted and undiluted) were assayed by incubating at different temperatures from 10 to 100^{o}C at an increment of 10^{o}C under standard assay conditions. The activity at varying temperatures was given in the table 20a. The crude and partially purified oxalate oxidase showed the maximum activity at 50^{o}C. The activity was recorded at an interval of 5^{o}C from 40 to 60^{o}C. The recorded observations are given in table 20b. The crude and partially purified oxalate oxidase showed maximum activity at 45^{o}C. Further increase in temperature, the enzyme showed decrease in activity sharply. The activity of the enzyme at different temperatures is given in figure 6.

4.10 Effect of substrate concentration on oxalate oxidase enzyme activity

Oxalate oxidase from crude extract and partially purified extract (diluted and undiluted) were assayed with varying substrate concentrations from 0 to 20 mM. No activity was recorded at 0 mM (without substrate) of substrate in partially purified extract but in crude, activity was recorded at 0 mM of substrate.

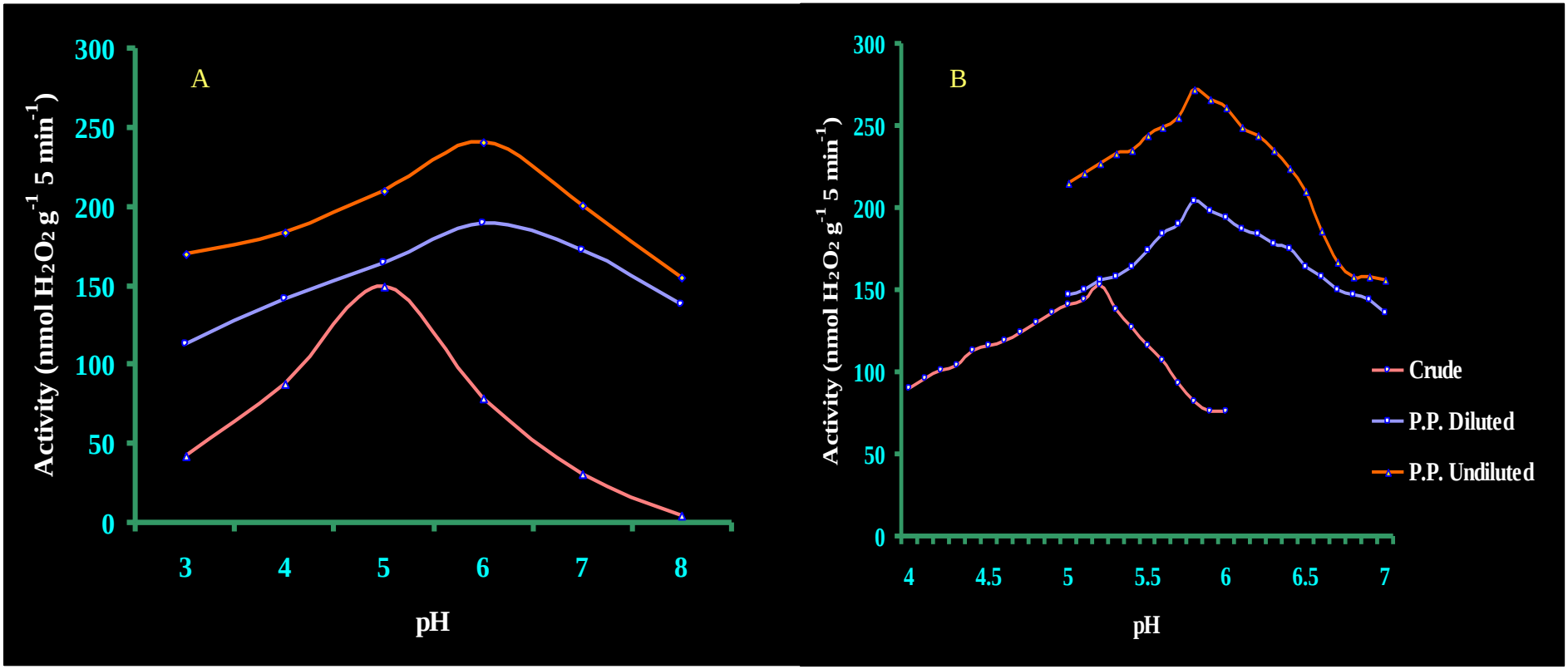
Table 19a: Effect of pH on oxalate oxidase enzyme activity in crude and partially purified sample of stage two leaves of *Costus pictus*

pH	Units (nmol H_2O_2 g^{-1} 5 min^{-1})		
	Crude	P.P Diluted	P.P Undiluted
3.0	41.79461	112.8739	169.7373
4.0	87.28534	141.3056	183.9531
5.0	149.8351	164.0509	209.5417
6.0	78.75583	189.6395	240.8166
7.0	30.42193	172.5805	201.0122
8.0	4.83339	138.4624	155.5214

Table 19b: Oxalate oxidase enzyme activity at pH range of 4 to 6 for crude and 5 to 7 for partially purified sample

Crude		Partially purified		
pH	Activity (nmol H_2O_2 g^{-1} 5 min^{-1})	pH	Units (nmol H_2O_2 g^{-1} 5 min^{-1})	
			Diluted	Undiluted
4.0	90.13	5.0	147.00	215.23
4.1	95.81	5.1	149.84	220.91
4.2	101.50	5.2	155.52	226.60
4.3	104.34	5.3	158.36	232.70
4.4	112.87	5.4	164.05	235.13
4.5	115.70	5.5	173.60	243.66
4.6	118.56	5.6	183.95	249.35
4.7	124.25	5.7	189.64	255.03
4.8	129.93	5.8	203.86	272.09
4.9	135.62	5.9	198.17	266.41
5.0	141.31	6.0	193.80	260.72
5.1	144.15	6.1	186.80	249.35
5.2	152.68	6.2	183.95	243.66
5.3	138.46	6.3	178.27	235.40
5.4	127.09	6.4	175.42	223.76
5.5	115.73	6.5	164.05	209.54
5.6	107.19	6.6	158.36	185.80
5.7	92.97	6.7	149.84	166.90
5.8	81.60	6.8	146.99	158.36
5.9	75.91	6.9	144.15	158.36
6.0	75.91	7.0	135.62	155.52

Note: P.P – Partially purified



A - Effect of pH on oxalate oxidase enzyme activity in crude and partially purified sample of stage II leaf of *Costus pictus* with the increment of 1.0 pH unit from pH 3 to 8

B - Effect of pH on oxalate oxidase enzyme activity in crude and partially purified sample of stage II leaf of *Costus pictus* with the increment of 0.1 pH unit from pH 4 to 6 for crude and 5 to 7 for partially purified sample

Figure 5: Effect of pH on oxalate oxidase activity

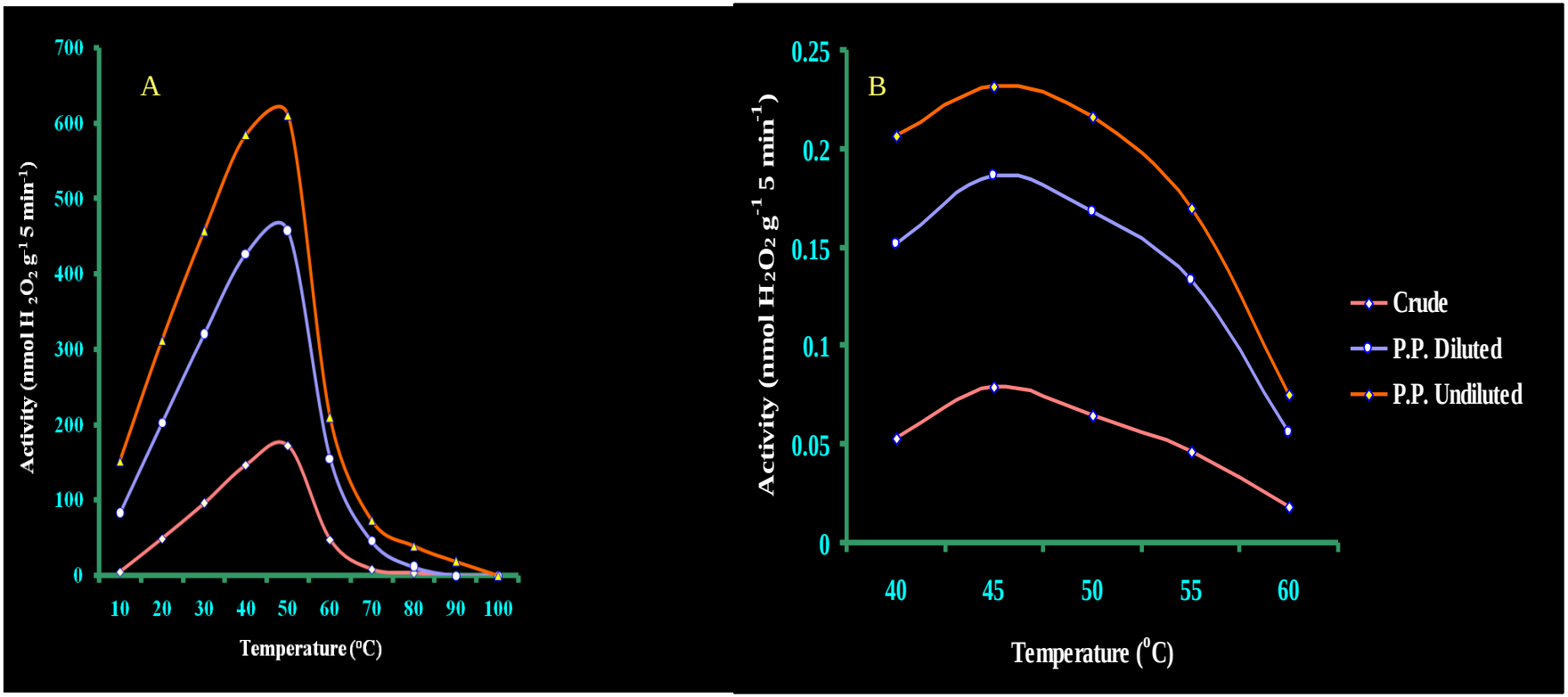
Table 20a: Effect of temperature on oxalate oxidase enzyme activity in crude and partially purified sample of stage two leaves of *Costus pictus*

Temp °C	Units (nmol H_2O_2 g^{-1} 5 min^{-1})		
	Crude	P.P Diluted	P.P Undiluted
10	4.55	82.45	151.83
20	49.19	202.72	311.90
30	95.81	320.14	456.90
40	146.71	427.04	584.84
50	172.58	456. 90	610.43
60	47.48	155.52	209.54
70	8.53	46.06	73.07
80	3.41	11.66	38.95
90	0	0	19.05
100	0	0	0

Table 20b: Oxalate oxidase enzyme activity in crude and partially purified sample at optimum temperature range

Temp °C	Units (nmol H_2O_2 g^{-1} 5 min^{-1})		
	Crude	P.P Diluted	P.P Undiluted
40	146.71	427.04	584.84
45	220.35	525.13	655.92
50	180.83	473.96	610.43
55	128.23	374.45	479.64
60	47.49	155.52	209.54

Note: P.P – Partially purified



A - Effect of temperature on oxalate oxidase enzyme activity in crude and partially purified sample of stage II leaf of *Costus pictus* with the increment of 10°C from 10 to 100°C

B - Effect of temperature on oxalate oxidase enzyme activity in crude and partially purified sample of stage II leaf of *Costus pictus* with the increment of 5°C from 40 to 60°C

Figure 6: Effect of temperature on oxalate oxidase activity

The activity has steadily increased from 0.1 to 0.8 mM of substrate in partially purified extract; further increase in the substrate concentration decreased the activity. Crude extract has recorded steady decrease in activity in various substrate concentrations from 0 to 20 mM of substrate. Figure 7 shows the substrate inhibition of oxalate oxidase at higher concentrations of substrate

The partially purified enzyme both diluted and undiluted showed same trend in different substrate concentrations which showed variation only in the activity. The partially purified undiluted sample showed high activity (398.90 units/g of leaf sample) at 0.8 mM while partially purified diluted sample showed 238.54 units/g of leaf at 0.8 mM. The activity of oxalate oxidase at various substrate concentrations are given in the table 21. The Lineweaver-Burk plots showed V_{max} of 427.2/5min and K_m of 0.065 mM (Figure 8).

4.11 Effect of enzyme concentration on oxalate oxidase enzyme activity

Oxalate oxidase from crude extract and partially purified extract (diluted and undiluted) were assayed with the increment in the enzyme concentration from 0.1 to 1 ml under standard assay conditions. The activity of oxalate oxidase in the crude and partially purified extract increased with the increase in enzyme concentration. The partially purified diluted sample and the crude extract showed same trend in the enzyme concentration. The partially purified undiluted sample showed increase in activity with increase in the enzyme concentration. The activity of oxalate oxidase at varying enzyme concentrations was given in the table 22. The activity at different enzyme concentrations is given in figure 9.

4.12 Effect of metal ion on oxalate oxidase enzyme activity

Oxalate oxidase assay was carried out with copper sulphate and manganese sulphate at different concentrations. The maximum activity was recorded in 10 mM copper sulphate which corresponds to 152.68 units g^{-1} of leaf

Table 21: Effect of substrate concentration on oxalate oxidase enzyme activity in crude and partially purified sample of stage two leaves of *Costus pictus*

Substrate mM	Units (nmol H_2O_2 g^{-1} 5 min^{-1})		
	Crude	P.P Diluted	P.P Undiluted
0	147.00	0.0	0.0
0.1	142.73	163.20	248.21
0.2	121.69	205.56	334.64
0.4	104.34	224.90	374.16
0.6	90.00	237.69	392.64
0.8	75.34	238.54	398.90
1.0	60.56	223.19	384.12
2.0	49.47	199.31	348.86
4.0	37.81	169.17	302.23
6.0	27.86	136.76	247.36
8.0	17.91	110.03	192.48
10.0	9.38	87.85	150.12
12.0	5.69	67.67	114.58
14.0	5.69	50.04	92.69
16.0	4.26	40.66	75.91
18.0	3.70	34.97	65.68
20.0	3.13	33.55	62.27

Note: P.P – Partially purified

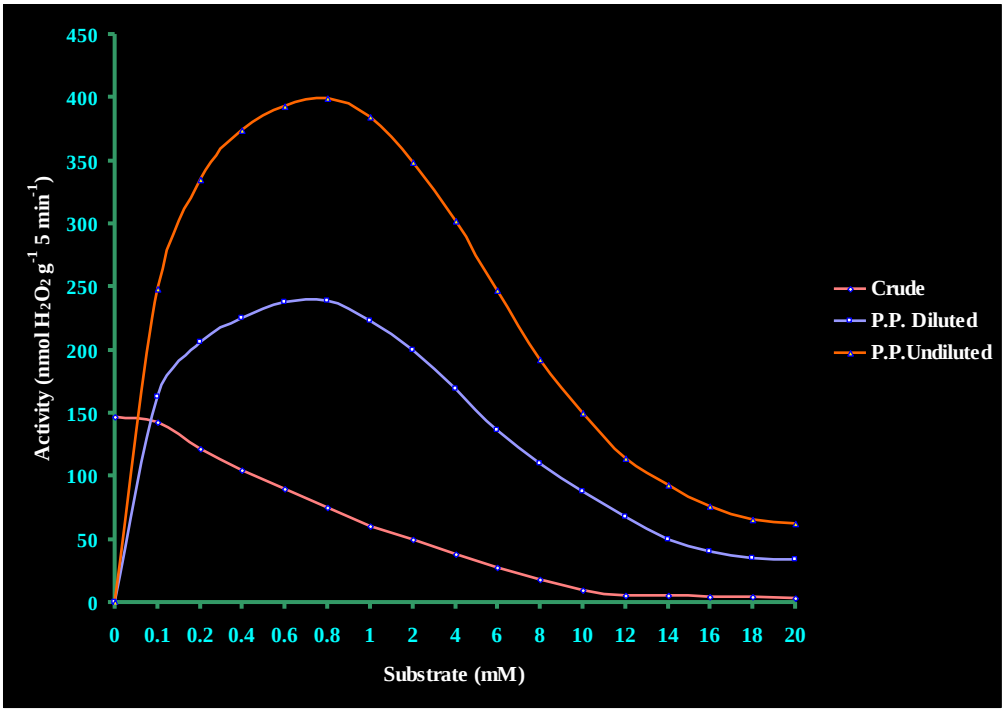


Figure 7: Effect of substrate concentration (from 0 to 20 mM of oxalic acid) on oxalate oxidase enzyme activity in crude and partially purified sample of stage II leaf of *Costus pictus*

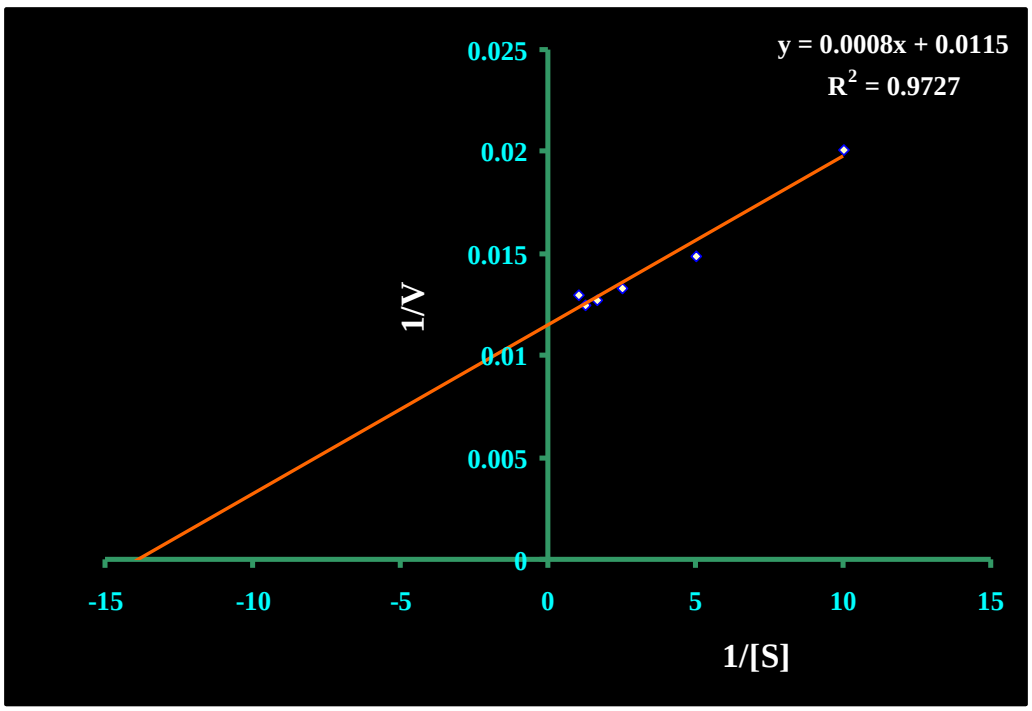


Figure 8: Lineweaver-Burk plot

sample which is 10 times more when compared to control which has the activity of 15.26 units g^{-1} of leaf sample. Above or below 10 mM copper sulpahte showed decrease in activity. Activities of oxalate oxidase at different concentrations of copper sulphate and manganese sulphate are given in the table 23.

4.13 Effect of solvents on oxalate oxidase enzyme activity during storage

The oxalate oxidase isolated with 0.1 M phosphate buffer pH 7.0 and with various solvents was stored at 4°C assayed at regular intervals to study the effect of storage of leaf extract on oxalate oxidase activity. Oxalate oxidase of ethanol extract and methanol extract were least stable and lost complete activity after 15 days. Partially purified stored in phosphate buffer is comparatively more stable than other solvent systems. The activity of oxalate oxidase in different solvents at different time intervals is given in table 24 and figure 10.

Table 22: Effect of enzyme concentration on oxalate oxidase enzyme activity in crude and partially purified sample of stage two leaves of *Costus pictus*

Extract volume (ml)	Units (nmol H_2O_2 g^{-1} 5 min^{-1})		
	Crude	P.P Diluted	P.P Undiluted
0	0	0	0
0.1	40.94	44.64	140.17
0.2	75.91	87.29	236.27
0.4	102.92	124.82	312.18
0.6	124.82	149.27	369.04
0.8	140.74	174.57	425.05
1	152.68	187.36	481.07

Note: P.P – Partially purified

Table 23: Effect of metal ion on oxalate oxidase enzyme activity in partially purified sample of stage two leaves of *Costus pictus*

Metal	Concentration (mM)	Units (nmol H_2O_2 g^{-1} 5 min^{-1})
Copper sulphate	0	15.26
	5	33.27
	10	152.68
	20	44.64
Manganese sulphate	0	15.00
	5	42.74
	10	55.06
	20	38.00

Table 24: Effect of solvents on oxalate oxidase enzyme activity during storage

Day	Units (nmol H_2O_2 g^{-1} 5 min^{-1})				
	Ethanol	**P.P**	**Crude**	**Methanol**	**Water**
1	549.26	240.19	156.88	109.38	85.98
8	278.55	174.38	100.15	50.59	48.63
15	119.26	121.23	52.47	14.74	20.45
22	0	65.12	28.57	0	14.18
30	0	27.18	15.32	0	0

Note: P.P – Partially purified

: Partially purified and crude extract was stored in 0.1 M phosphate buffer, pH 7.0

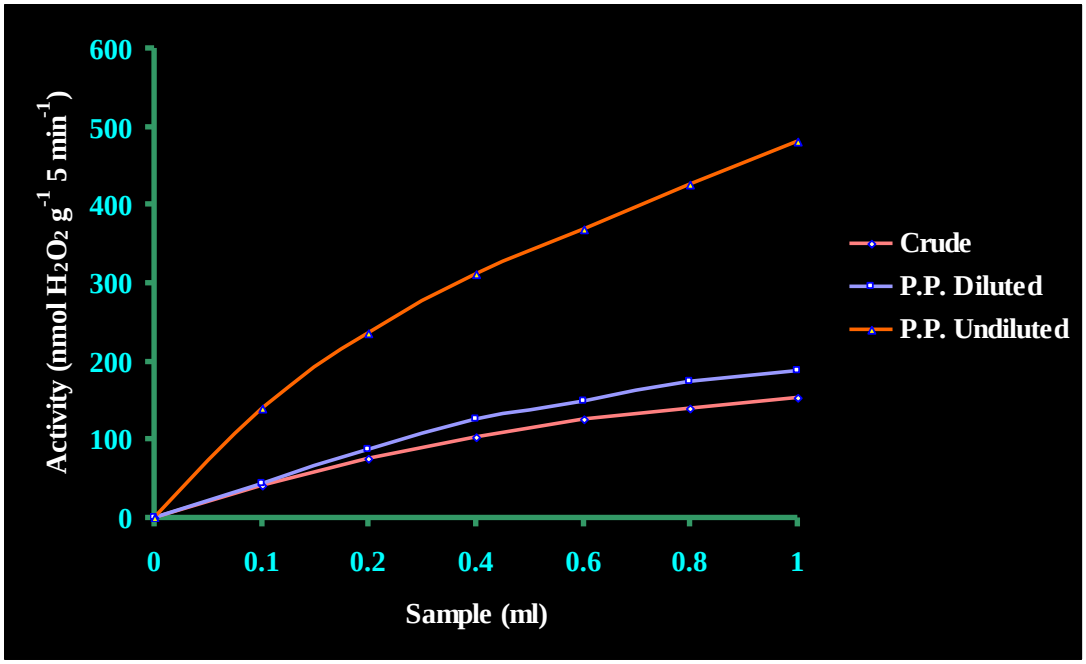
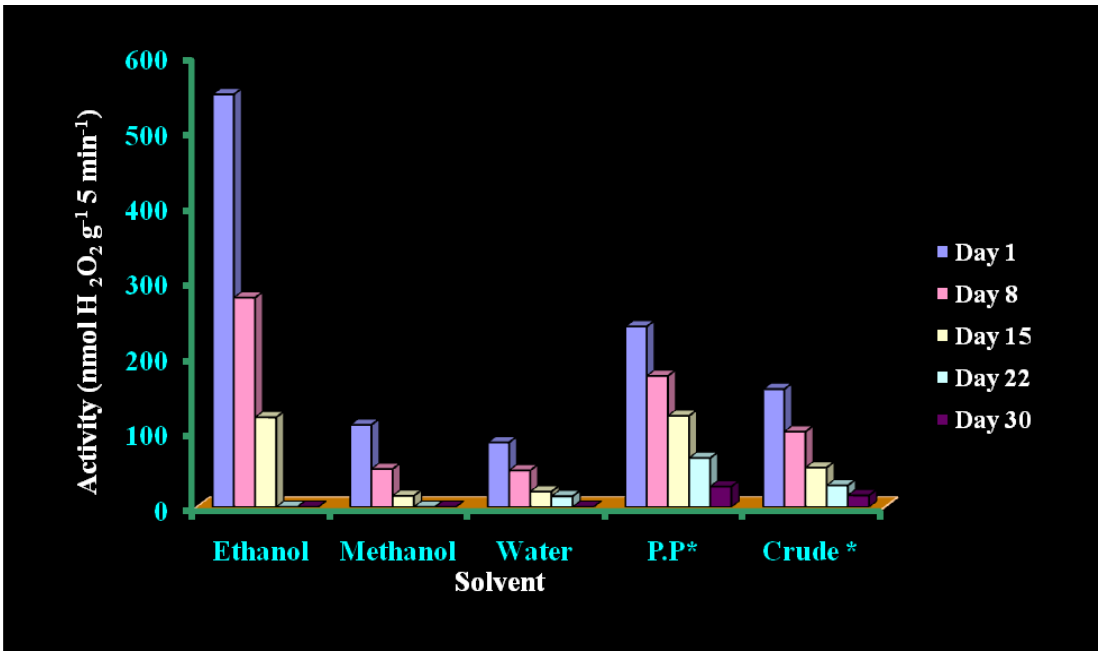


Figure 9: Effect of enzyme concentration on oxalate oxidase enzyme activity in crude and partially purified sample of stage II leaf of *Costus pictus*



Note: Partially purified and crude was stored in phosphate buffer

Figure 10: Effect of solvents on oxalate oxidase enzyme activity during storage

DISCUSSION

5. DISCUSSION

Costus pictus D.Don is native of Mexico and known as Mexican cane or spiral ginger. It is commonly known as insulin plant in Kerala. The hypoglycemic property of *Costus pictus* was well established. "Preparation process and a regenerative method and technique for prevention, treatment and glycemic control of diabetic mellitus using *Costus pictus* extract" was patented by Merina Benny in 2007. Administration of *Costus pictus* extract about 500 to 2000 mg day^{-1} brings down the blood glucose level to normal in diabetic patients (Benny, 2007). The aerial part of *Costus pictus* is used in Mexican folk medicine to treat renal diseases. The aqueous extract of *Costus pictus* at 200 mg kg^{-1} body weight induces a natriuetric response to that of the flurosemide (Camargo *et al*., 2006). Meanwhile the decoction of fresh leaves and stems of *Costus pictus* is widely used by the Cuban population as a traditional medicinal remedy for urinary problems like stones, pain and sepsis. Aqueous extract of *Costus pictus* at a dose of 0.5 to 1.0 g kg^{-1} b.w induces analgesic effect (Moron *et al.,* 2007).

The sour taste of *Costus pictus* is due to the presence of oxalic acid in the leaves (Benny, 2006). Excess oxalate intake through oxalate rich foods leads to nephrolithiasis, a condition in which oxalate crystallises to form stones in the kidney, bladder and urethra (Siener *et al.,* 2005). But *Costus pictus* is used to treat kidney problems even though it contains substantial quantity of oxalic acid (Camargo *et al*., 2006; Moron *et al.,* 2007). In effect the presence of oxalic acid is nullified by the presence of oxalate oxidase. In plants oxalic acid exist along with its metabolising enzyme oxalate oxidase (Caliskan, 2000). The oxalate oxidase from plants is effectively used to reduce the oxalate level in humans. Oral administration of oxalate oxidase crystals effectively reduces the oxalate content in humans (Shenoy *et al.,* 2006). Based on the above facts, a basic study on *Costus pictus* is required for extending the application in other fields. In this context, the present investigation entitled "Studies on oxalic acid and oxalate

oxidase enzyme in *Costus pictus* D.Don" was carried out at Centre for Plant Biotechnology and Molecular Biology.

5.1 Morphology of *Costus pictus*

Costus pictus in the usual form, with the patterns on the stems, sometimes called *Costus heiroglyphica*. The flower and inflorescence are virtually identical on this plant, but the stems are in striking red color instead of the patterned stem of the more common form (Ogden, 2007). Placed between *Costus laevis* and *Costus malortienaus*, differs from *Costus laevis* by shorter ligule, shorter petiole, smaller leaves, and smaller flowers and differs from *Costus malortienus* by leaf shape and indument.

Red form of *Costus pictus* had broader leaves and red stems do not have the markings as the one common in horticulture. Flower parts were also mostly shorter and wider. This was the easiest of all the spiral gingers to grow, as it can be able to handle a wide range of soil and sunlight. It can be propagated very easily from stem cuttings.

5.2 Oxalate

The oxalate was isolated by Burrows and Baker method and estimated by iron ferron method. Three different stages of fresh and dry leaf samples were used for analysis. The effect of solvents on the recovery of oxalate content were also studied

5.2.1 Oxalate content in *Costus pictus*

Burrows (1950) and Baker method (1952) were used for isolating the oxalate from *Costus pictus* leaf samples and the oxalate content was estimated by iron ferron method. The analysis was carried out at three stages of leaf maturity,

fully opened from top 1st to 3rd, 4th to 6th and 7th to 9th leaf. In Baker method, the total oxalate content of fresh sample at different stages of leaf were 10.77, 11.06 and 10.18 mg g^{-1} and the soluble oxalate were 10.75, 10.83 and 9.67 mg g^{-1}. The total oxalate content of dry sample was 6.34, 6.47 and 5.26 mg g^{-1} and soluble oxalate content was 6.09, 6.19 and 5.06 mg g^{-1} (table 11). In dry and fresh sample the oxalate content is high in first two leaf stages compared to third stage in both cases. In Burrows method the total oxalate content in fresh samples was 16.41, 16.50 and 15.82 mg g^{-1} whereas in dry samples oxalate content was 12.10, 12.21 and 11.47 mg g^{-1} (table 12).

Hoover and Karunaratnam (1965) reported that in plant sample, oxalate was stable at 1.5 N HCl for 12 h above which the oxalic acid slowly degrades. But in Baker method the oxalate was isolated using (1+1) diluted HCl with boiling for 15 minutes as a result reduction in oxalic acid content occurred. Moreover a series of steps were involved in Baker method which contributed to the loss of oxalate during isolation. In Burrows method, simple procedure was followed to isolate oxalic acid which reduced the loss of oxalate during isolation. Hence oxalate content was high in Burrows method compared to that of Baker method.

Oxalate was less in dry sample compared to fresh sample due to the chemical degradation of oxalate during drying (Baker, 1952). The oxalate loss was about of 4.53 mg/g during the sun drying of two weeks.

The oxalate content in *Costus pictus* was moderately high compared to oxalate rich plants like spinach, rhubarb, rumex etc... Rhubrarb contains 18-216 mg g^{-1} of oxalate in the petiole in fresh sample. Spinach contains 10.14 to 13.7 mg g^{-1} of oxalate in leaf. In spinach, greater variability of oxalate occurs between species. The highest oxalate was recorded in New Zealand spinach leaf (*Tetragonia tetragonioldes*) which has 117 mg g^{-1} (Hui *et al.*, 2001). The plant which we use regularly in our daily life also contains considerable amount of oxalate. For example, curry leaf contains 13.52 mg g^{-1} of oxalate; *Piper betel*

(betel leaf) contains 13.5 mg $^{-1}$g of oxalate; *Coriander sativum* (coriander) leaf contains 12.68 mg g^{-1} of oxalate (Hui *et al.,* 2001).

5.2.2 Forms of oxalate in *Costus pictus*

Depending upon the plant species, oxalate accumulates primarily as soluble oxalate, insoluble oxalate or a combination of these forms. In *Costus pictus*, the oxalate accumulates primarily as soluble oxalate which is also observed in spinach in which 98 per cent oxalate accumulation as soluble oxalates (Siener *et al.,* 2006). In *Costus pictus*, the soluble oxalate decreases with the maturity. In first leaf stage, 100 per cent oxalate accumulated as soluble oxalates. In second leaf stage 98 per cent oxalate accumulated as water soluble oxalates. In third leaf stage, 95 per cent oxalate accumulated as soluble oxalate. In other words, the insoluble oxalate formation increased with maturity of leaf (Table 11). The insoluble salt formed primarily as magnesium or iron oxalate because calcium oxalate crystals are absent in Costaceae (Tomlinson, 1969).

In plants, high oxalate content occurs in leaves than in roots. Oxalate content also varies according to the age of the plant, season, climate and type of soil. In rhubarb, oxalate content tend to increase as the plant matures, whereas in leaves of spinach, sugarbeet, banana and *Colocasia esculenta* a decrease in oxalate content was observed during the course of maturity. Diurnal variation of oxalate content was also observed in *Oxalis corniculata* by Seal and Sen (1970) and in *Setaria sphacelata* by Jones and Ford (1972).

Ontogenetic stage, light condition, temperature and nutrient status will influence oxalate content. The relationship of ontogenetic stage to oxalate concentration in the plant tissue is complex and may even differ between studies on the same crop. The total oxalate and soluble oxalate concentration of spinach has been found to decrease (Sengbusch *et al.,* 1965; Bengtsson *et al.,* 1966) as well as increase (Sing and Saxena, 1972) with ontogenetic stage. With low growth

rate, oxalate concentration in plant tissue increases with maturity, with high growth rate it decreases with maturity. *Costus pictus* multiply at a faster rate (Orden, 2007) hence the total oxalate content decreases with maturity.

5.2.3 Presence of oxalate and oxalate oxidase enzyme in *Costus pictus*

Even though the quantity of oxalate varied at different stages of maturity, the maximum quantity recorded was 16.5 mg g^{-1} in fresh leaves (Table 12). The lethal dose of oxalic acid for human varies from 2 to 30 g (Hui *et al.*, 2001). According to this data 0.12 to 1.82 kg of *Costus pictus* should be taken to reach the lethal dose which is practically impossible. Hence the oxalate in *Costus pictus* won't cause any problem. Traditionally the decoction of *Costus pictus* leaves is used by the Cuban population to treat urinary problems like stones, pains and sepsis. The property of *Costus pictus* to dissolve stones in kidney may be due to the high activity of sturdy oxalate oxidase in the plant.

5.2.4 Effect of solvents on the recovery of oxalate content

Oxalate was isolated with water, ethanol and methanol from three different stages of fresh and dry samples. The fresh sample recorded more oxalate than dry sample which was due to degradation of oxalic acid during drying as mentioned earlier. Water extract contains more oxalate in fresh and dry sample. The oxalate content of different solvents were low compared to the oxalate content isolated by standard methods but the trend of oxalate variation at different stages of maturity were same for all (Table 13). The first two stages of leaf sample expressed more oxalate content than the third leaf stage. The reduction in oxalate content in solvents was due to poor solubility of oxalate in the solvents (Benjamin, 2005). Extraction with water followed with solvent extraction for the active principles can eliminate the oxalate content in *Costus pictus*.

5.3 Nature of oxalate oxidase enzyme

In enzymology, an oxalate oxidase (EC 1.2.3.4) is an enzyme that catalyzes the oxidation of oxalate to carbon dioxide and hydrogen peroxide. This enzyme belongs to the family of oxidoreductases, specifically those acting on the aldehyde or keto group of donor with oxygen as acceptor. The systematic name of this enzyme class is oxalate:oxygen oxidoreductase. Other names in common use include aero-oxalo dehydrogenase and oxalic acid oxidase. This enzyme participates in glyoxylate and dicarboxylate metabolism.

Oxalate oxidase is used for the clinical detection of oxalate in urine and plasma (Sharma *et al.*, 2007). The enzyme activity has been detected in several species, with the barley enzyme being the best characterized (Sugiura *et al.*, 1979; Lane, 1994). Oxalate oxidase is a member of cupin super family which comprises six β-jellyroll monomers locked into a homohexamer (a trimer of dimers), this structure accounts for its remarkable stability to various denaturing agents; proteases, heat, SDS and extreme pH (Lane *et al.*, 1993; Lane, 1994; Carter and Thornburg, 2000).

5.4 Effect of extract dilution on oxalate oxidase enzyme activity

In the crude extract, the oxalate oxidase was inhibited or regulated by the oxalate present in the extract itself. Though the activity was inhibited or regulated, the enzyme was not denatured. So the inhibition of oxalate oxidase in *Costus pictus* can be considered as competitive inhibition or allosteric regulation. The activity of oxalate oxidase in the diluted samples increased, with the increase in dilution, with out addition of external substrate (oxalic acid 0.01 M). With the addition of oxalic acid, the activity of oxalate oxidase decreased. The difference in the activities with substrate and with out substrate are steadily increased with dilution (Table 14). So the enzyme was not denatured by the substrate, instead there was an equilibrium between the enzyme and substrate in the activity and

substrate utility. The substrate regulated the activity of the enzyme. At the same time the oxalate oxidase regulated the presence of oxalic acid, to maintain the concentration of oxalic acid in the plant for regulating the metabolism which in turn maintained the growth and development of the plant.

5.5 Oxalate oxidase enzyme activity at different stages of leaf maturity

Oxalate oxidase from three stages of leaf sample was isolated with 0.1 M phosphate at pH 7.0. Table 15 shows the activity of oxalate oxidase enzyme at different leaf stages. The activity was higher in second leaf stage (125.22 units) followed by first stage (93.17 units) and third leaf stage (84.81 units).

In *Costus pictus,* oxalate content was high in the first two leaf stages compared to third leaf stage. Oxalate oxidase is also high in the first two stages of leaf samples compared to third leaf stage. This indicates that the oxalate and oxalate oxidase enzyme exists in an equilibrium and the oxalate oxidase enzyme regulated the oxalate content.

In fast growing plants, oxalate accumulates more in immature leaves than in mature leaves (Libert, 1987). Same trend was recorded in *Costus pictus*. The oxalate is generating H_2O_2 by oxalate oxidase activity. This H_2O_2 is the substrate for peroxidase to lignify the cell wall. Hence the enzyme activity as well as oxalate content are high in the developing stage which is essential for developmental process till attain maturity (Lane, 1994). *Costus pictus* was not an exception to this. It also showed that oxalate content and oxalate oxidase enzyme activity were high in the growing stages.

The specific activity was high in the second leaf stage (187.43 units mg^{-1}) followed by third leaf stage (163.10 units mg^{-1}) and first leaf stage (100.18 units mg^{-1}). The trend in the specific activity indicated that oxalate oxidase helps in the lignification of cell wall in the immature stage and in the full maturity it was a

source of free radical which causes senescence because the protein content in the third leaf stage was half of that in the first leaf stage. Even though the protein content was less in the third leaf stage, the specific activity was more which indicated that the oxalate oxidase was high with in the low protein content. It is in aggrement with the report of Deunff *et al.* (2004).

Oxalate oxidase synthesis was reported to be closely associated with cell wall extension in cereals and grasses (Gibeaut and Carpita, 1991b). Upon this observation, it was suggested that germin-like oxalate oxidase might play a crucial role in early plant development by controlling integration of cell wall extension. Germin oxalate oxidase synthesis also appears to be auxin responsive (Caliskan and Cuming, 1998). Typically, auxins stimulate cell wall loosening and bring about cell wall expansion. On the other hand, germin oxalate oxidase activity produces hydrogen peroxide which is required for the peroxidase mediated cross-linking reactions in the cell wall. Thus, it was suggested that germin oxalate oxidase synthesis is associated with both initiation and termination of cell wall expansion in early development (Lane, 1994).

The high oxalate oxidase activity at early stage of development in *Costus pictus* revealed the close association of the enzyme with cell wall development. The high rate of oxalate oxidase enzyme activity at early stage and high specific activity at later stage throw light to the different roles of the oxalate oxidase enzyme. The activity of the enzyme at early stage is much concerned with the developmental process and defense against pathogens and predators. Whereas, high specific activity at later stage was more concerned with senescence process and defense against pathogen and predators. In brief it can very well presume that defense against pathogen and predators seem to be common factor of the oxalate and oxalate oxidase enzyme at all the stages of development of *Costus pictus*. The following review contains implication of the above facts in brief manner.

In rye grass, cutting of leaf blades induced a two-step oxidative stress. An early H_2O_2 burst just after wounding and a second one several hours later. The early burst, which involved O^{2-} and SOD-dependent H_2O_2 production, was detected only at neutral pH (i.e. above pH 6). The subsequent induction of the synthesis of wound proteins was detected at acidic pH (Schaller and Oecking, 1999). Such an effect of wounding was also obtained *in plants* with wheat germin *gf-2.8* after cutting of leaf blades (Berna and Bernier, 1999). The early H_2O_2 burst, which was derived from the disproportion of O^{2-}, might be used for the peroxidase-dependent wound healing, which occurred within the first few hours after wounding (Sasaki *et al.*, 2002). The delayed production of H_2O_2, which derived from germin oxalate oxidase activity, would be involved in long-lasting defences against pathogens and predators.

A parallel may be drawn between the hypersensitive response induced by pathogens (Lamb and Dixon, 1997) and the wound-induced oxidative burst (Orozco-Cardenas *et al.*, 2001). In both cases, H_2O_2 production is biphasic (Wojtaszek, 1997) and originates from distinct sub-cellular sources (Allan and Fluhr, 1997). Furthermore, H_2O_2 is involved in related processes such as tissue necrosis and reinforcement of cell walls. The capacity of germin oxalate oxidase to generate H_2O_2 favours hypersensitive responses and protein cross linking in papillae of infected wheat leaves, thus hindering penetration of pathogens (Thordal-Christensen *et al.*, 1997).

Evidence for the role of germin oxalate oxidase in the mechanism of cross protection against pathogens is provided by observations of improved resistance against a broad spectrum of pathogens after insertion of germin oxalate oxidase into various plants (Liang *et al.*, 2001; Schweizer *et al.*, 1999). The concept of cross tolerance in which exposure to one stress can induce tolerance to other stresses (Bowler and Fluhr, 2000) is widely accepted, and it is increasingly obvious that H_2O_2 plays a pivotal role in cross tolerance (Desikan *et al.*, 2001; Pastori and Foyer, 2002). Exposure of wheat roots to aluminium induces, *via*

germin oxalate oxidase synthesis, an over production of H_2O_2 (Delisle *et al.,* 2001), which has been suggested to be implicated in the defence against pathogens (Hamel *et al.,* 1998). Berna and Bernier (1999) suggested that germin oxalate oxidase is involved in stress-induced signalling in oxalate-producing plants.

It has been reported that senescence is characterized by an increase in H_2O_2 production and by germin oxalate oxidase activity (Davoine *et al.,* 2001), and the function of the germin oxalate oxidase/oxalate system in the outer leaf sheaths would be to set up a sterile shield around the basal leaf meristem. Similarly, Lane (2000, 2002) reported that germin oxalate oxidase was involved in programmed cell death of surface structures of cereals, suggesting a role of germin oxalate oxidase in producing refractory barriers against invasion by predators. Expression of germin oxalate oxidase after wounding or during senescence might confer protection against fungi and herbivorous predators as suggested by transgenic studies (Donaldson *et al.,* 2001; Liang *et al.,* 2001; Ramputh *et al.,* 2002). H_2O_2 might therefore be considered as an alarm signal involved in both constitutive defences (senescence) and adaptative defences (wounding) against pathogens.

5.6 Effects of solvents on the oxalate oxidase enzyme activity

Oxalate oxidase was isolated with water, ethanol and methanol from fresh and dried leaf samples. The enzyme activity in the order of ethanol > methanol > water. But the trend in the oxalate oxidase activity at different stages was same in all solvent systems. High activity was recorded in the second leaf stage followed by first stage and third stage (Table 16).

Ethanol was extracting only selected proteins. Oxalate oxidase enzyme was one of the protein in which active site was exposed to substrate. So ethanol increases the sensitivity of oxalate oxidase (Zhang, 1996). Hence the activity was

high in the ethanol extract compared to methanol and water extract. The stability of oxalate oxidase at 4°C is poor in ethanol extract. As enzyme is more active in ethanol, it looses its activity rapidly over time. About 78 per cent of its initial activity was lost with in 15 days, after which complete loss of activity was observed. In methanol extract, the activity was comparatively less than ethanol and 86 per cent of its activity was lost with in 15 days. The activity of water extract was low compared to ethanol and methanol extract but the loss of activity was also low compared to ethanol and methanol. After 22 days, only 83.5 percent of its activity was lost (Table 24 and fig.10).

Extraction and storage of oxalate oxidase enzyme with solvents like ethanol and methanol were not advisable. But there are reports of storing oxalate oxidase enzyme in an immobilized condition with little loss of activity. The oxalate oxidase from sorghum immobilized on alkylamine glass beads retained 90 per cent of its activity when stored at 4°C in water (Thakur *et al.,* 2000). The oxalate oxidase from *Amaranthus* immobilized on arylamine glass beads did not show any noticeable change in the activity up to 60 days during its regular use (150 times) and lost 50 per cent of its activity after four months when stored at 4°C in 0.05 M sodium citrate buffer, pH 3.5 (Sharma *et al.,* 2007).

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5.7 Protein content and oxalate oxidase activity

Protein was estimated by Bradford method which is one of the established methods for small quantity of protein available in plants (Jones *et al.,* 1989). It is based on absorbance shift of coomassie dye when the previously red form coomassie dye changes and stabilizes into coomassie blue by the binding of protein. It essentially measures the amount of arginine and hydrophobic amino acid residues (Bradford, 1976).

Based on the analytical results, it was observed that first stage of leaf expressed high quantity of protein followed by the second and third stage. Even

though the protein content was high at the first leaf stage, oxalate oxidase activity was lower than in the second leaf stage. It was an indication of the presence of other enzymes/proteins contributing to the growth and development of the plant. The partially purified sample with heat treatment at 80°C for 3 minutes recorded substantial loss of protein content. The protein available after heating showed oxalate oxidase activity in which specific activity was higher than the recorded specific activity of other samples. The protein content was reduced due to degradation of proteins to simple biomolecules and secondary products. Bradford method also supporting this concept because binding of dye is only to proteins which will give blue complex. The presence of oxalate oxidase enzyme even after heating recorded the stability of the enzyme under stress condition. Heat stable proteins with oxalate oxidase activity can be exploited for medical and pharmaceutical purpose.

5.8 Partial purification of oxalate oxidase

The crude extract (Section 3.2.4) of *Costus pictus* was partially purified by ammonium sulphate precipitation (80 per cent) with and with out heat treatment.

Purification of oxalate oxidase employing heat treatment and ammonium sulphate precipitation was done in barley seedlings (Chiraboga, 1966; Kotsira and Clonis, 1998; Requena and Bornemann, 1999), sorghum (Pundir, 1991a) and wheat (Zhenfei-Guo, 2008). Purification of oxalate oxidase employing ammoium sulphate precipitation with out heat treatment was done in *Amaranthus* (Mongkolisirikieat and Srisawan, 1987; Goyal *et al.*, 1999), maize (Vuletic and Šukalovic, 2000), barley (Thakur *et al.*, 2000) and sorghum leaves (Sing *et al.*, 2006).

Partial purification yielded 4.5 fold increased purification compared to crude extract of *Costus pictus* (Table 18). The heat treated sample shows decrease in activity (29 per cent) compared to heat untreated ammonium sulphate

precipitated sample. But the specific activity was 1.1 times more compared to heat untreated ammonium sulphate precipitated sample, which indicates that even though there was decrease in activity (29 per cent) after heat treatment, the enzyme was stable. More discussions on the effect of temperature are given separately in 5.10.

Partial purification along with heat treatment reduced the protein content by 40.7 per cent compared to crude but in partial purified without heat treated sample the protein content was reduced only by 8.1 per cent compared to crude. Specific activity of oxalate oxidase in partial purified heat treated sample is 1.1 times higher than the partial purified without heat treated sample and 5.4 times higher than the crude.

The activity of partial purified enzyme with out heat treatment was 155.52 units which is 4.5 times higher than crude. The specific activity of partial purified enzyme with out heat treatment is 209.03 units mg^{-1} which is 4.9 times higher than the crude. The protein content of partial purified sample with out heat treatment was 0.744 mg g^{-1}. The protein content in the partial purified sample with heat treatment was 0.48 mg g^{-1} which is 1.5 times lesser than the normal and 1.7 times lesser than crude.

5.9 Effect of pH on oxalate oxidase enzyme activity

The oxalate oxidase was assayed at varying pHs from 3 to 8. The enzyme showed maximum activity at pH 5.2 for crude extract and 5.8 for partially purified extract when incubated at 40°C (table 19b and fig.5). The pH optima in the acidic range has been reported for oxalate oxidase from various sources such as *Bougainvillea spectabilis* leaves pH at optimum 6.8 (Srivastava and Krishnan, 1962), sorghum roots pH 5.0 (Pundir, 1991b; Pundir and Kuchhal, 1989), sorghum leaves (Pundir and Nath 1984; Pundir and Satyapal, 1998) and banana peel pH 5.2 (Inamdar *et al.*, 1986). The optimum pH of the enzyme at acidic pH

indicates the involvement of imidazole groups of histidine at the catalytic centre of the enzyme (Gane *et al.,* 1998). The increase in pH from 5.2 (crude) to 5.8 (partially purified) was due to the availability of organic acids and other biomolecules which contribute to the pH at acidic range in the crude extract. The sensitivity of the enzyme to pH was high in the crude sample than the partially purified sample. The trend of activity at different pH of partially purified diluted and undiluted sample was same. The acidic pH could also be attributed to the anionic effect of oxalate, the major substrate for the enzyme.

5.10 Effect of temperature on oxalate oxidase enzyme activity

The enzyme showed maximum activity at 45°C for crude and partially purified (diluted and undiluted) above which it showed a rapid decline due to the denaturation of the enzyme (table 20b and fig.6). The temperature for maximum activity at 45°C was reported for *Amaranthus* leaves (Goyal *et al.*, 1999). Barley root (Kotsira and Clonis, 1997), Bougainvillea leaf (Srivastava and Krishnan, 1962), *Pseudomonas* (Koyama, 1988) and barley seedlings (Sugiura *et al.,* 1979) had shown maximum activity at 37°C. The oxalate oxidase enzyme from *Costus pictus* retained 6.4 per cent of its maximum activity (partially purified undiluted), 2.6 per cent of its maximum activity (partially purified diluted) and 2 per cent of its maximum activity (crude) on heating for 5 min at 80°C in 0.05 M succinate buffer, pH 5.0, whereas sorghum leaf retained 10% of the initial activity when heated at 80°C for 3 min. The *Pseudomonas* oxalate oxidase retained only 9 per cent activity after heating at 65°C for 10 min (Koyama, 1988) and beet stem oxalate oxidase retained 30 per cent activity after heating at 60°C for 10 min but became inactive at temperature higher than 60°C (Obzansky and Richardson, 1983). However, barley enzyme has been reported to be more thermostable than *Costus pictus* enzyme, since it retained 60% activity on heating at 80°C for 5 min at its optimal pH (Kotsira and Clonis, 1997).

The fairly thermal stability of oxalate oxidase was due to the hexameric structure of oxalate oxidase. The oxalate oxidase enzyme from *Costus pictus* might have formed strongly bound dimers, an arrangement that results in extensive burial of the monomer surfaces and hydrophobic residues. These dimeric units then associate via their C-terminal domains to give a hexamer, which is believed to be the biologically active form of the enzyme (Plate 1b). This quaternary structure has been hypothesized to be the basis for the resistance of oxalate oxidase to degradation by proteases or heat (Woo *et al.*, 2000).

5.11 Effect of substrate concentration on oxalate oxidase enzyme activity

The activities of the crude, partially purified enzyme (diluted and undiluted) were measured with increasing concentration of oxalic acid from 0.1 mM to 20 mM. The activity of the crude extract was steadily decreased with increase in substrate concentration. The activity of the partially purified enzyme showed a hyperbolic relationship with oxalic acid concentration only up to 0.8 mM above which the enzyme showed decrease in activity due to substrate inhibition (table 21 and fig.7)

Earlier hyperbolic relationship between the initial velocity and the substrate concentration was reported up to 4 $X10^{-3}$ M for grain sorghum leaf (Satyapal and Pundir, 1993), barley seedlings (Sugiura *et al.*, 1979) and barley root (Kotsira and Clonis, 1997). A Lineweaver-Burk plot of 1/S Vs 1/V revealed an K_m of 0.065 mM and V_{max} of 427.5 nmol of H_2O_2 g^{-1} 5 min^{-1} at 40°C, pH 5.0 (fig.8). Lower the K_m value higher the affinity towards the substrate. The affinity of oxalate oxidase enzyme for oxalic acid can be exploited for clinical purposes.

Substrate inhibition of oxalate oxidase is also reported in various plants. Barley root oxalate oxidase showed substrate inhibition when oxalic acid reached to 4 mM and had K_m of 0.27 (Chiraboga, 1966) and 0.42 mM (Kotsira and Clonis, 1997). In contrast, oxalate oxidase from barley seedling did not exhibit substrate

inhibition until oxalic acid was up to 200 mM and has a Km of 1.3 mM (Requena and Bornemann, 1999), 0.42 mM (Sugiura *et al.*, 1979). The oxalate oxidase from *Costus pictus* had the K_m of 0.065 mM which is 20 (K_m 1.3 mM) and 6.5 (K_m 0.42) times lesser than barley seedling. This comparison leads to speculation that the use of oxalate oxidase from *Costus pictus* in enzymatic determination of oxalate in biological fluids could enhance the sensitivity of the method over the barley seedling enzyme.

5.12 Effect of enzyme concentration on oxalate oxidase enzyme activity

The oxalate oxidase from crude and partially purified extract was assayed at varying concentration of enzyme extract. The activity of the oxalate oxidase enzyme increases with increase in the concentration of the enzyme. But the crude and the partially purified diluted extract showed similar trend with concentration of enzyme. In both the cases the activity got reduced. The partially purified undiluted extract showed increase in activity with increase in the enzyme concentration. It was due to enzyme concentration and the substrate availability was low so the increase in the enzyme concentration increased the activity (table 22 and figure 9). It may be due to the following reasons; the crude sample contained excess oxalate. The partially purified enzyme extract contained 4.5 fold enzyme than the crude extract with no oxalate content. When the partially purified enzyme extract was diluted, the activity assay showed same trend as that of the crude extract. Crude extract was having substantial quantity of oxalate whereas partially purified diluted extract had lower concentration of enzyme and substrate.

5.13 Effect of metal ion on oxalate oxidase enzyme activity

The oxalate oxidase enzyme activity of partially purified undiluted sample with 5 mM, 10 mM, and 20 mM of copper sulphate and manganese sulphate were measured. The 5 mM, 10 mM and 20 mM of copper sulphate increased the activity by 2.2, 10 and 2.9 times compared to control. The 5 mM, 10 mM and 20

mM of manganese sulphate increased the activity by 2.8, 3.7 and 2.5 times compared to control.

From the data it could be concluded that oxalate oxidase in *Costus pictus* was a copper/manganese dependent enzyme. Sorghum oxalate oxidase (Pundir and Kuchhal, 1989; Pundir, 1991a&b; Satyapal and Pundir, 1993) was a copper dependent enzyme. But barley oxalate oxidase is a manganese dependent enzyme (Requena and Bornemann, 1998; Requena and Bornemann, 1999). Role of metal ions are highly dependent on the nature and properties of the enzyme from different sources. Some of the germin-like proteins in the cupin superfamily are thought to bind other metal ions (Dunwell and Gane, 1998), it is possible that all members of the superfamily are capable of binding specific metal ions that have distinct catalytic and structural roles. For example, the iron-containing 3-hydroxyanthranilate-3,4-dioxygenases have an aspartate residue in place of one of the highly conserved histidine residues (Requena and Bornemann, 1999). Further detailed investigation is required to establish the unique requirement of metal for oxalate oxidase activity in *Costus pictus*.

Future line of studies

1. The enzyme can be immobilised and used to detect oxalate in the biological fluids.
2. The oxalate oxidase gene from *Costus pictus* can be isolated and characterised. It can be used to produce transgenics for biotic and abiotic stress tolerance (Cross tolerance).
3. The oxalate oxidase gene can be used as a reporter gene in monocot and dicot transformations.

SUMMARY

6. SUMMARY

The study entitled 'studies on oxalic acid and oxalate oxidase enzyme in *Costus pictus* D.Don' was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara during the period 2006-2008. The salient findings of the study are given below.

1. Oxalate in *Costus pictus* accumulated primarily as a water soluble oxalate.

2. The second leaf stage accumulated maximum oxalate compared to other two stages.

3. Fresh leaves contain more oxalate compared to dry leaves. Drying causes loss of oxalate in *Costus pictus*.

4. Oxalate degraded when higher concentration of hydrochloric acid was used. Diluted hydrochloric acid 0.4 N is suitable for isolating oxalate from *Costus pictus*.

5. Recovery of oxalate was more in water than the methanol and ethanol.

6. The undiluted sample of *Costus pictus* showed no oxalate oxidase activity due to inhibition of substantial concentration of oxalate present in the extract itself.

7. TCA precipitation was not required for the estimation of proteins in *Costus pictus* by Bradford method.

8. The 1:5 diluted Bradford reagent can be satisfactorily used for the estimation of proteins in *Costus pictus*.

9. The fresh leaf showed oxalate oxidase whereas the dry sample was not having oxalate oxidase activity.

10. The second leaf stage showed maximum oxalate oxidase activity as well as specific activity compared to other two leaf stages.

11. Ethanol extract showed maximum oxalate oxidase activity than the methanol and water extract but the storability of oxalate oxidase was poor in ethanol.

12. The buffer extract was suitable for assaying oxalate oxidase enzyme in *Costus pictus*.

13. Partial purification of oxalate oxidase yielded 4.5 times increase in activity compared to crude extract.

14. Heat treatment can also be employed for purification of oxalate oxidase in *Costus pictus* to some extent.

15. The crude oxalate oxidase was having an optimum pH of 5.2 where as the partially purified oxalate oxidase had optimum pH of 5.8 in the activity expression.

16. The optimum temperature of crude and the partially purified oxalate oxidase was at 45^{o}C which can be exploited for clinical purposes.

17. The partially purified oxalate oxidase showed hyperbolic relationship with substrate concentration up to 0.8mM above which it showed substrate inhibition.

18. The Lineweaver-Burk plots showed V_{max} of 427.2/5min and K_m of 0.065 mM. affinity towards substrate was indicated by lower K_m value.

19. The oxalate oxidase activity increases with increase in the enzyme concentration.

20. Oxalate oxidase from *Costus pictus* was a Cu^{2+}/Mn^{2+} dependent enzyme.

21. The sensitivity of the oxalate determination in biological fluids employing oxalate oxidase from *Costus pictus* will be more as it had K_m value 20 times lesser than the commercially available oxalate oxidase from barley.

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APPENDIX

APPENDIX

Phospahate buffer 0.1 M pH 7.0

Stock solution A 0.2 M (Monobasic)

27.598 g of sodium dihydrogen phosphate ($NaH_2PO_4.H_2O$ MW - 137.99 g/mole) dissolved in minimum quantity of distilled water and made up to 1000 ml with distilled water and stored at 4°C.

Stock solution B 0.2 M (Dibasic)

28.392 g of disodium hydrogen phosphate (Na_2HPO_4 MW – 141.96 g/mole) dissolved in minimum quantity of distilled water and made up to 1000 ml with distilled water and stored at 4°C.

Working solution

Stock solution A 39 ml and stock solution B 61 ml was mixed and the pH was adjusted to 7.0 and made up to 200 ml with distilled water to get 0.1 M phosphate buffer pH 7.0.

Stock solutions were stored at the maximum of 30 days and prepared fresh every 30 days. Working solution was prepared afresh.

Phospahate buffer 0.4 M pH 7.0

Stock solution A 0.5 M (Monobasic)

68.995 g of sodium dihydrogen phosphate ($NaH_2PO_4.H_2O$ MW - 137.99 g/mole) dissolved in minimum quantity of distilled water and made up to 1000 ml with distilled water and stored at 4°C.

Stock solution B 0.5 M (Dibasic)

70.98 g of disodium hydrogen phosphate (Na_2HPO_4 MW – 141.96 g/mole) dissolved in minimum quantity of distilled water and made up to 1000 ml with distilled water and stored at 4°C.

Working solution

Stock solution A 78 ml and stock solution B 122 ml was mixed and the pH was adjusted to 7.0 and made up to 250 ml with distilled water to get 0.4 M phosphate buffer pH 7.0.

Stock solutions were stored at the maximum of 30 days and prepared fresh every 30 days. Working solution was prepared afresh.

STUDIES ON OXALIC ACID AND OXALATE OXIDASE ENZYME IN *Costus pictus* D.Don

By

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ABSTRACT OF THE THESIS

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ABSTRACT

Costus pictus D.Don is a native of Mexico and referred as Mexican cane or spiral ginger. It is commonly known as insulin plant in Kerala. The hypoglycemic property of *Costus pictus* was well established. The aerial part of *Costus pictus* is used in Mexican folk medicine to treat renal diseases. The decoction of fresh leaves and stems of *Costus pictus* is widely used by the Cuban population as a traditional medicinal remedy for urinary problems like stones, pain and sepsis.

The sour taste of *Costus pictus* is due to the presence of oxalic acid in the leaves. Excess oxalate intake through oxalate rich foods leads to nephrolithiasis, a condition in which oxalate crystallises to form stones in the kidney, bladder and urethra. Based on the above fact, a basic study on *Costus pictus* is required for safer use of this plant in medical preparations. In this context the present study entitled "Studies on oxalic acid and oxalate oxidase enzyme in *Costus pictus* D.Don" was carried out at Centre for Plant Biotechnology and Molecular Biology. The objective of the study was to estimate oxalic acid and oxalate oxidase enzyme activity of *Costus pictus* D.Don leaf sample at different stages of maturity and to study the effect of drying and extraction with various solvents on oxalic acid content and oxalate oxidase enzyme activity.

Fully opened leaves from top 1st to 3rd, 4th to 6th and 7th to 9th were collected for analysis of oxalic acid and oxalate oxidase enzyme. The samples were designated as stage 1, stage 2 and stage 3 respectively. Oxalate was isolated by two different methods, Baker and Burrows method. Oxalate content was determined by iron ferron method. Oxalate oxidase enzyme was assayed by 4-aminoantipyrine method and protein content was determined by Bradford method.

The study revealed that oxalate and oxalate oxidase activity was maximum in second leaf stage followed by first leaf stage and third leaf stage. Drying causes

substantial loss of oxalate content and complete loss of oxalate oxidase activity. With various solvents water recovered more oxalate followed by methanol and ethanol while oxalate oxidase activity was maximum in ethanol followed by methanol and water. The storability of oxalate oxidase was poor in ethanol followed by methanol and water.

The crude oxalate oxidase enzyme showed maximum activity at pH 5.2 while the partially purified enzyme showed maximum activity at pH 5.8. The crude and partial purified enzyme showed maximum activity at 45^{o}C. The activity of the partially purified enzyme showed a hyperbolic relationship with oxalic acid concentration only up to 0.8 mM above which the enzyme showed decrease in activity due to substrate inhibition. The activity of the crude enzyme was steadily decreased with increase in oxalic acid concentration due to excess availability of oxalate in the crude extract which inhibits the oxalate oxidase activity. The K_m and V_{max} of partially purified oxalate oxidase was 0.065 mM and 427.5 units g^{-1} 5 min^{-1} at 40^{o}C respectively. Oxalate oxidase in *Costus pictus* requires Cu^{2+}/Mn^{2+} for its activity.

The ethanol or methanol extract of second leaf stage of *Costus pictus* can be used for isolating active principles. The oxalate oxidase from *Costus pictus* can be used as a cheap source of oxalate oxidase enzyme which is used in oxalate determination in biological fluids. More over the sensitivity of oxalate determination employing oxalate oxidase from *Costus pictus* will be more as oxalate oxidase in *Costus pictus* has K_m 20 times lesser than the commercially available barley oxalate oxidase enzyme.