2002

A Comparative Study on The Nutritional Quality of four Varieties of Healthy and Disease Affected Mulberry Leaves and Determination of Factors Associated with the Disease

Tang, Md. Abul Kashem

University of Rajshahi

A COMPARATIVE STUDY ON THE NUTRITIONAL QUALITY OF FOUR VARIETIES OF HEALTHY AND DISEASE AFFECTED MULBERRY LEAVES AND DETERMINATION OF FACTORS ASSOCIATED WITH THE DISEASE

SUBMITTED BY

MD. ABUL KASHEM TANG
B. Sc. (Hons.), M. Sc.

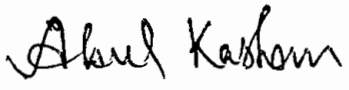
THESIS SUBMITTED TO THE UNIVERSITY OF RAJSHAHI, BANGLADESH, IN THE FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF PHILOSOPHY IN BIOCHEMISTRY AND MOLECULAR BIOLOGY

BATCH:JULY, 1998 ROLL NO. 55 JANUARY, 2002	PROTEIN AND ENZYME RESEARCH LABORATORY DEPARTMENT OF BIOCHEMISTRY & MOLECULAR BIOLOGY UNIVERSITY OF RAJSHAHI RAJSHAHI-6205 BANGLADESH

DEDICATED TO MY
PARENTS

DECLARATION

I hereby declare that the materials included in this thesis are the original research works and have not previously been submitted for the award of any degree or diploma and dose not contain any materials previously published or written by another person except when due reference is made in the text of this thesis.



(Md. Abul Kashem Tang)

Signature of the candidate

CERTIFICATE

This is to certify that the materials included in this thesis are the original research works conducted my supervision and have not previously been submitted for the award of any degree or diploma by the candidate.

To the best my knowledge and believe, this thesis does not contain any material previously published or written by another person except when due reference is made in the text of the thesis.

Nurul Absar

(Professor Nurul Absar)

Supervisor
Department of Biochemistry & Molecular Biology
University of Rajshahi
Rajshahi-6205
Bangladesh

CONTENTS

Name of Chapter		Page no.
	Abstract	i
	List of Table	iii
	List of Figure	v
	Introduction	1
Chapter-1	Isolation, identification and control of causative organisms responsible for leaf spot and powdery mildew diseases of mulberry leaves.	
	Introduction	15
	Materials and Method	17
	Results and Discussion	23
	Materials and Method of powder mildew	36
	Results and Discussion of powder mildew	39
Chapter-2	A comperative study on the nutrient compositions of different varieties of healthy and diseases infected mature mulberry leaves	
	Introduction	53
	Materials and Method	54
	Results and Discussion	89
Chapter-3	Analysis the activities and characteristics of some hydrolytic and oxidative enzymes present in different varieties of healthy and diseases infected mature mulberry leaves	
	Introduction	110
	Materials and Method	112
	Results and Discussion	130
	Conclusion	165
	References	168

ABSTRACT

The causal organisms, *Cercospora moricola* Cooke and *Phyllactinia corylea* Pers Karst responsible for leaf spot and powdery mildew diseases of mulberry leaf respectively were isolated, identified and characterized from the following observation, organisms are obligate parasite under the form of class Deuteromycetes and Ascomycetes, optimum temperature 28-30°C and 20-25°C; Optimum pH 5.0-6.0 and 5.0; optimum relative humidity 90-100% and 70% for conidial germination respectively. The conidium of C. moricola germinates better in rain water and 2.0-2.5% glucose solution whereas *P. corylea* germinates better in dew and 2.0% glucose solution. Out of the four varieties of the mulberry leaves examined, BM-2 is more resistance of leaf spot disease while BM-4 is more resistance of powdery mildew. The disease severity of leaf spot increases during the month of August while powdery mildew increases during the month of February. Among the six fungicides tested, Dithane M-45 and Bayleton-25wp were the most suitable fungicide for the control of leaf spot and powdery mildew disease respectively.

The physiochemical compositions such as TTA, pH, moisture, ash, chlorophyll, protein, lipid, total sugar, reducing sugar, non-reducing sugar, starch, crude fibre, phenol, vitamin-c, vitamin-B_1, vitamin-B_2, β-carotene and minerals as well as content of enzymes such as amylase, protease, invertase, cellulase, polyphenol oxidase, ascorbic acid oxidase and peroxidse in four varieties of healthy, tukra disease affected, leaf spot disease affected and powdery mildew disease affected mulberry leaves at mature stage were compared.

In general healthy mulberry leaf contained about 322-255 mg% chlorophyll, 71-74% moisture, 3.08-3.40% ash, 4.08-4.62% protein, 1.97-2.10% lipid, 2.7-3.0% total sugar, 0.59-0.71% reducing sugar, 2.06-2.31% non-reducing sugar, 6.38-7.22% starch, 6.08-7.04% crude fibre, 145-160 mg% phenol, 116-132 mg% vitamin-c, 98-120 mg% β-carotene, 44-60 mg% vitamin B_1 and 0.47-0.53 mg% vitamin B_2. The present data indicated that the contents of most of the chemical compositions such as ash (10-15%), chlorophyll (50-60%), lipid (25-30%), total sugar (40-50%), reducing sugar (40-50%), non-reducing sugar (40-50%), starch (35-40%), crude fibre (25-40%), vitamin-C (20-35%), vitamin-B_1 (25-35%), Vitamin-B_2 (25-30%), β-carotene (30-45%), calcium (35-40%), phosphorous (35-40%) and iron (40-50%) were decreased while the contents of protein (25-30%) and phenol (30-50%) were increased significantly after infection of healthy mulberry leaves with diseases.

The enzyme contents some hydrolytic enzymes such as amylase (45-55%), invertase (30-40%), protease (40-45%) and cellulase (50-55%) were decreased while that of the oxidative enzymes such as polyphenol oxidase (50-80%), ascorbic acid oxidase (85-125%) and peroxidase (45-75%) were increased remarkably after infection of mulberry leaves with diseases. The above mentioned crude enzymes were also characterized with respect to their temperature optimum, pH optimum, heat stability, K_m and V_{max}.

LIST OF TABLE

No. of Table	Name of the Table	Page No.
CHAPTER-1		
Table-1.1	Amount of H_2SO_4 and water for preparation of RH.	21
Table-1.2	Incidence of disease severity in four high mulberry yielding varieties.	23
Table-1.3	Artificial inoculation of leaf spot disease during the month of July, August and September.	26
Table-1.4	Effect of water from different sources and different concentrations of glucose solution on the conidial germination of *C. moricola.*	28
Table-1.5	Effect of temperature on the conidial germination of *C. moricola* after different hours of incubation.	31
Table-1.6	The effect of different relative humidity on conidial germination of *C. moricola* after different hours of incubation at 28°C.	32
Table-1.7	Effect of different pH of 2.5% glucose solution on the conidial germination of *C. moricola* after 24 hours of incubation at 28°C.	33
Table-1.8	Effect of different fungicides on conidial germination of *C. moricola*	35
Table-1.9	Assessment of Powdery mildew disease severity (Disease Index) of four high yielding varieties.	39
Table-1.10	Artificial inoculation of powdery mildew disease during the month of December, January and February.	43
Table-1.11	Effect of water from different sources as well as different concentrations of glucose solutions on the conidial germination of *P. corylea*	45

Table-1.12	Effect of temperature on the conidial germination of *P corylea* after different hours of incubation under identical condition.	46
Table-1.13	Effect of different relative humidity and pH values on conidial germination of *Phyllactinia corylea* under identical condition.	48
Table-1.14	Effect of different fungicides on conidial germination of *P. corylea*	50
Chapter-2		
Table-2.1	Estimation of pH and TTA of healthy and disease infected mulberry leaves at mature stage	89
Table-2.2	Chlorophyll content of mature healthy and disease infected mulberry leaves	91
Table-2.3	Moisture and ash content of mature healthy and disease infected mulberry leaves	93
Table-2.4	Total Protein, Water soluble protein and lipid content of mature healthy and disease infected mulberry leaves	96
Table-2.5	Total sugar, Reducing sugar, Non reducing sugar and starch content of mature healthy and disease infected mulberry leaves	99
Table-2.6	Crude fibre, Vitamin-C and Phenol content of mature healthy and disease infected mulberry leaves	102
Table-2.7	β-carotene, Vitamin-B_1 and Vitamin-B_2 content of mature healthy and disease infected mulberry leaves	105
Table-2.8	Calcium, Phosphorus and Iron content of mature healthy and disease infected mulberry leaves	107

CHAPTER-3		
Table-3.1	The contents of crude amylase, invertase, protease and cellulase of healthy and disease infected mature mulberry leaves	150
Table-3.2	The contents of crude polyphenol oxidase, ascorbic acid oxidase and peroxidase of healthy and disease infected mature mulberry leaves	163

LIST OF FIGURE

No. Of Figure		Page No.
Figure-1.1	Photograph of Mulberry field.	24
Figure-1.2	Photograph of Healthy Mulberry leaf.	24
Figure-1.3	Photograph of leaf spot infected Mulberry leaf	27
Figure-1.4	Microphotograph of conidiophore of *Cercospora moricola*	27
Figure-1.5	Microphotograph of conidia of *Cercospora moricola*	29
Figure-1.6	Microphotograph of Germinating conidia of *Cercospora moricola*	29
Figure-1.7	Photograph of powdery mildew infected Mulberry leaf	40
Figure-1.8	**Microphotograph of spores of** *Phyllactinia corylea*	40
Figure-1.9	Microphotograph of spore with germ tube of *Phyllactinia corylea*	41
Figure-1.10	Microphotograph of brusting stage of clestothesia of *Phyllactinia corylea*	41
Chapter-2		
Figure-2.1	Standard Curve for estimation of protein	63

Figure-2.2	Standard Curve of glucose for estimation of total sugar and starch	67
Figure-2.3	Standard Curve of glucose for estimation of Reducing sugar	69
Figure-2.4	Standard Curve of Catechol for estimation of phenol	75
Figure-2.5	Standard Curve of Phosphorus	79
Figure-2.6	Standard Curve of Iron	81
Figure-2.7	Standard Curve of β-carotene	84
CHAPTER-3		
Figure-3.1	Effect of pH on the activities of crude amylase isolated from healthy and disease infected mulberry leaves.	131
Figure-3.2	Effect of temperature on the activities of crude amylase isolated from healthy and disease infected mulberry leaves.	132
Figure-3.3	Heat stability curve of crude amylase isolated from healthy and disease infected mulberry leaves.	133
Figure-3.4	Lineweaver-Burk double reciprocal plots for the determination of K_m and V_{max} values of crude amylase isolated from healthy and disease infected mulberry leaves.	134
Figure-3.5	Effect of pH on the activities of crude invertase isolated from healthy and disease infected mulberry leaves.	136
Figure-3.6	Effect of temperature on the activities of crude invertase isolated from healthy and disease infected mulberry leaves.	137
Figure-3.7	Heat stability curve of crude invertase isolated from healthy and disease infected mulberry leaves.	138
Figure-3.8	Lineweaver-Burk double reciprocal plots for the determination of K_m and V_{max} values of crude invertase isolated from healthy and disease infected mulberry leaves.	139
Figure-3.9	Effect of pH on the activities of crude protease isolated from healthy and disease infected mulberry leaves.	141

Figure-3.10	Effect of temperature on the activities of crude protease isolated from healthy and disease infected mulberry leaves.	142
Figure-3.11	Heat stability curve of crude protease isolated from healthy and disease infected mulberry leaves.	143
Figure-3.12	Lineweaver-Burk double reciprocal plots for the determination of K_m and V_{max} values of crude protease isolated from healthy and disease infected mulberry leaves.	144
Figure-3.13	Effect of pH on the activities of crude cellulase isolated from healthy and disease infected mulberry leaves.	146
Figure-3.14	Effect of temperature on the activities of crude cellulase isolated from healthy and disease infected mulberry leaves.	147
Figure-3.15	Heat stability curve of crude cellulase isolated from healthy and disease infected mulberry leaves.	148
Figure-3.16	Lineweaver-Burk double reciprocal plots for the determination of K_m and V_{max} values of crude cellulase isolated from healthy and disease infected mulberry leaves.	149
Figure-3.17	Effect of pH on the activities of crude Ascorbic acid oxidase isolated from healthy and disease infected mulberry leaves.	152
Figure-3.18	Effect of temperature on the activities of crude Ascorbic acid oxidase isolated from healthy and disease infected mulberry leaves.	153
Figure-3.19	Heat stability curve of crude Ascorbic acid oxidase isolated from healthy and disease infected mulberry leaves.	154
Figure-3.20	Effect of pH on the activities of crude Polyphenol oxidase isolated from healthy and disease infected mulberry leaves.	156
Figure-3.21	Effect of temperature on the activities of crude Polyphenol oxidase isolated from healthy and disease infected mulberry leaves.	157

Figure-3.22	Heat stability curve of crude Polyphenol oxidase isolated from healthy and disease infected mulberry leaves.	158
Figure-3.23	Effect of pH on the activities of crude Peroxidase isolated from healthy and disease infected mulberry leaves.	160
Figure-3.24	Effect of temperature on the activities of crude Peroxidase isolated from healthy and disease infected mulberry leaves.	161
Figure-3.25	Heat stability curve of crude Peroxidase isolated from healthy and disease infected mulberry leaves.	162

INTRODUCTION

INTRODUCTION

Mulberry, a member of family "Moraceae" is recognized under genus *Morus*. It is mainly cultivated for its foliage to feed silkworm, *Bombyx mori* L. Silkworm takes necessary nutrients for its growth only from mulberry leaves. The nutritive value of mulberry leaves as the food for silkworm is due to the food value habit of the silkworm. Any shortage of nutrients can not be filled up with others food. Silkworm and mulberry plant are an excellent example for insect-plant interaction. The mulberry leaf's quality and quantity influence the growth and development of silkworm and quality of cocoon production (Thangavelu, 1990).

The silkworm exclusively feeds on the leaves of mulberry plants. It is well recognized that the growth and development of the silkworm larvae and their cocoon are greatly influenced by the quality of the mulberry leaves (Krishnaswami *et. al.*, 1971). Why silkworm feed only on mulberry leaves is a mysterious fact. It seems that two types of factors can be distinguished in mulberry serving as food for silkworms, the physical feeding factors include taste, dietetic texture like thickness of cuticle, waxy layer of the leaf, hair prevailing on the surface etc. (Raghuraman, 1989) while the chemical feeding factors are attracting factors, bitting factors and swallowing factors. Continuous eating occurs only when all the three chemical factors are present. These factors are stimulated by the chemical substances contained in the leaves of the host plant (Tribhuwan and Mathur, 1989). The chemicals associated with bitting factors are sucrose, inositol and sitosterol, which act as feeding stimulants. Morin and isoquecitrin together with essential oils in the leaf provide the specific basis for attracting silkworm to the food source. Cellulose, silicate and phosphate provide necessary stimulus to swallowing action (Ravidra, 1987). Two volatile

compounds β-hexenol and β-rhexrnol are isolated from mulberry leaves which are the attractants for silkworm larvae (Watanabe, 1958).

Now a days artificial diet is used in Japan to rear silkworm but artificial diet is very costly and the quality and the quantity of cocoons produced are inferior to that of cocoons produced on feeding mulberry leaves. Mulberry is practically the sole food and nutrient of the silkworm *Bombyx mori* L. Deficiency of a certain nutrient or imbalance of nutrients in a diet causes some changes in the composition of larval body or in metabolic activity (Ito, 1972). The nutrient requirements of young-age worms differ from those of the late-age worms. The former requires comparatively tender, soft and succulent leaves having higher moisture, protein and sugar content and less starch and fibre. On the other hand, late-age worms require higher protein and carbohydrate content and less moisture for silk production (Sarker, 1989).

Leaf colour as well as leaf position can be taken as the criteria for leaf selection while feeding silkworm larvae. For the first instar, leaf colour is light green, for the second instar leaf colour is turn to about dark green, while at the fourth and fifth instar, leaf colour is green (Sarker, 1989).

Chlorophyll content of the fresh mulberry leaves ranges from 0.15-0.35% in weight and varies according to growth of leaves. Chloroplast pigments are combination of chlorophyll, carotene and xanthophyll. Chloroplast in leaves performs photosynthesis i.e. carbohydrate such as glucose or starch are synthesized by using carbon-dioxide gas from air and water is absorbed by roots from the soil through the action of chlorophyll under the sunlight. The pigments of the blood colour of white and yellow cocoon race originates from the

xanthophyll of mulberry leaves (Anon, 1975). Leaves qualities are decided by the physical and chemical properties, particularly the chemical nature of leaves. Chemical composition of mulberry leaves varies due to variety season, nature of soil, fertilization, irrigation, pruning, maturity of leaf etc. (Bose, 1989). In order to achieve a good harvest of mulberry leaves and high yield of cocoons, it is necessary to create favorable condition suitable for its biological characteristics during the establishment of the mulberry field. Fertilization and cultural management must be providing to meet the needs of plants and silkworms.

I-1. Origin

Man's interest in mulberry grow with the growth of civilization, his fascination with quality fabric led him to silk the "Queen of Textiles". Queen Shilling Sharee of China has been credited the discovery of silk 4600 years ago. China is the first country in the world to know how to rear silkworms with mulberry leaves, how to rear cocoons, how to reel cocoons and how to weave silk fabrics. That is saying China is the cradle of sericulture.

The silk industry originated in the province of Chan Tong and the Chinese jealously guarded to secret it and they were success for about 3000 years. Chin-Nong, one of the successors of Emperor Fo-Hi, taught cultivation of mulberry in China (Rangaswami, 1976). Mulberry cultivation spread to Tibet when a Chinese carrying silkworm eggs and mulberry seeds in her head dress. From Tibet the industry spread slowly to India and Persia by "Silk Road."

I-2. Classification of mulberry

There are more than thousand varieties including wild and cultivated forms of mulberry plant. Mulberry tree belongs to the family Moraceae. Linnaeus (1707-1778) classified mulberry tree in the family Moraceae. The genus *Morus* includes five important species, which named by Linnaeus. They are *Morus alba* Linn, *M. nigra* L., *M. rubra* L., *M. tartarca* L. and *M. indica* L. About 560 varieties are available in India. Mainly *Morus alba* and *M. indica* varieties are available in Bangladesh.

I-3. Soil and Environmental Condition

Mulberry trees grow in nature, so there is a relationship among growth development and the environmental conditions. External environmental condition consists of a number of factors such as illumination, temperature, air, water and soil.

Selection of soil is very important in moriculture. Quality of mulberry leaves depends on the soil of the mulberry field. For these the soil is to supply

i) Essential major and minor nutrients
ii) Oxygen for root respiration and
iii) Mechanical support for anchorage and act as a storehouse of water.

Although, tolerant to a wide range of soil condition, mulberry grows well on loamy soil, sandy loam or silt loam's. On the contrary sand clay are undesirable. The suitable pH range for mulberry cultivation is 6.2-7.5; however, the optimum pH is 6.8 for luxuriant growth of mulberry plant.

Mulberry trees grow in various climatic conditions ranging from temperate to tropical. During winter season mulberry does not sprout in the temperate climate but the growth is continuous in the tropical. Mulberry requires a strong sunshine for its normal growth and synthesizing chlorophyll.

Optimum temperature for the growth of mulberry tree is about 25-28°C and it varies according to mulberry genotypes, day length and rainfall (Anon, 1975). Growth and sprouting of bud can not be obtained at temperature below 13°C and above 38°C. Mulberry requires 600-2500 mm rainfall annually. Under low rainfall condition the growth of mulberry is limited due to shortage of moisture. On an average 50 mm rainfall once in a ten days is considered ideal for mulberry. Humidity range of 65-80% is ideal for its growth (Rangaswami, 1976).

I. 4. Mulberry Growing Countries

Mulberry is a woody plant and has wide range of adaptability in various edaphic and climatic conditions. So, mulberry plants are cultivated more than 60 countries of the world. The main cultivating regions are distributed in the temperate and subtropical zone in Asia, 20-40° North latitudes. Geographical position of the mulberry growing countries of the world indicates that all of the countries except Brazil are located in the North of the equator. Countries which are located in the high latitude range of 28°N are Japan, China, USSR, Korea, Yugoslavia, France, Spain, Italy, Greece, Cyprus, Turkey, Hungary, Syria, Poland, Bulgaria Iran, Lebanon, Afghanistan, Rumania etc. Countries, which are located at lower latitude range of 5-28°N are India, Srilanka, Thailand, Egypt, The Republic of South Vietnam, Indonesia, Barma etc. (Rangaswami, *et. al.*1976).

Important mulberry growing countries of the world are as follows:

Name of the country	Hectare
China	716,000
India	241,600
Japan	88,500
USSR (Former)	80,000
South Korea	39,300
Thailand	36,421
Brazil	25,600
Vietnam	6400

Bangladesh is situated within subtropical region in the north-eastern part of South Asia between 23°34-26°38 North latitude and between 80°01-92°41 of East latitude. The general topography of the country is low, flat and fertile except some hilly regions in the north, northeast and in the southeast, including some old alluvial lands in the north and north-western part. Subtropical monsoon climates with flat and fertile alluvial lands are suitable for mulberry cultivation. The temperature in Bangladesh varies between 11-29°C in winter and 21-38°C in summer and average rainfall is 127-508 cm. All of these climatic factors and the fertile alluvial soil constitute very favorable for the promising growth of mulberry in Bangladesh. However, till now the leaf production in our country are far behind the international standard of 30 metric Ton per hectare (Bari *et. al.* 1988). Their remains some critical constrain behind this poor performance in yield. These are:

i) Lack of adaptation of improved varieties and replacement of local cultivars in the traditional area

ii) Inadequate use of fertilizer

iii) Unscientific pruning and leaf harvest

iv) Lack of knowledge on Scientific Sericulture.

In Bangladesh, mulberry plants are mostly cultivated in the districts of Rajshahi, Nawabganj, Natore, Bogra, Pabna, Jessore, Rangpur, Dinajpur, Thakurgaon, Manikganj, Tangail, Mymensingh, Comilla and Bandarban. About 60% of total production of Bangladesh come from Bholahat area under Nawabganj District.

I-5. Plantation

Planting material is obtained from stem cutting, root grafts and seedling. Seedling can not be used for making plantation because heterogenetic character of mulberry trees. Grafting require much labour and cost. Stem cuttings are preferred for propagation of mulberry.

Mulberry is generally planted in bush, low-cut and tree forms depending on the soil and climatic condition.

(a) Bush: It is planted through stem cutting and pruned at the bottom of the plant. Generally, it is cultivated in plain and high lands.

(b) Low cut: This is planted through saplings and pruned at the height of 150-200 cm from the ground. It is cultivated in hilly area.

(c) Tree: It is also planted through saplings and 240-360 cm height is maintained. The roadside, homestead, pond side and institutional premises cultivate it.

I-6. Nutritive value

Mulberry leaves form the basic food for silkworm. The silkworm, through digestion and absorption takes nutrients from mulberry leaves to ensure its normal growth and development. Main constituents of the mulberry leaves are moiture, protein, carbohydrate, fat, organic acid, minerals and vitamins. All these components are essential for the physiological function of the silkworm which is characteristic for each of the tissue, organ and haemolymph.

I-7. Important uses of mulberry

Mulberry is grown almost exclusively for rearing of silkworms. Besides its foliage use, its root, bark stem, etc. are used in many ways such as fodder, fuel, in paper industry, for the manufacture of sports items and fancy goods as well as in medicine. The mulberry leaf stalk remnants left after feeding silkworm, can also be used for feeding cattle without any adverse effect on their health and performance and also used as fish food (Dwivedi, 1988).

Mulberry wood is highly shock resistant and not liable to split highly elastic flexible, so it is preferred in the manufacture of sports articles like hockey sticks, cricket bats, stumps, tennis rackets etc. Mulberry woods can be also used in prepare bobbins, tool handles, toys, firewood, timber of building houses and boats, furniture etc. (Dandin and Ramesh, 1987). In North America mulberry is known mainly as fruit bearing tree. Ripe fruits of mulberry are sweet, well flavoured and are eaten fresh or made into a jam, jelly etc. In Europe, a wine is prepared by the fermentation of the fruits. Fruits also contain organic acids which stimulate appetite and help in proper digestion (Philip, 1989).

Mulberry plant possesses the medicinal property as it also carries some complex chemical substances like alkaloids, glucosides, steroids, essential oils, flavonoids etc. in different parts of leaf, fruit, root and the bark. Mulberry leaves are diaphoretic, emollient and its extract can be used to reduce arterials pressure and as a gargle to sooth inflammations of the throat. The root is reported to possess anithelmintic and astringent properties. The bark is used as a purgative and vermifuge (Bose, 1987). The fruit is also used for medical purposes. They can be used in febrile disease except when the patient suffering from diarrhoea. The fruit has a cooling effect and is a laxative. It is used in treating sore throat, dyspepsia and melancholia. It checks thirst and cools the blood (Philip, 1989). Mulberry has turned up with trumps in treating AIDS, that recently invited the Biochemists interest around the potential alkaloid deoxynojirimycin (DNJ) a root bark extracts of *M. nigra*. It was revealed that the interesting property of DNJ is selectively inhibiting enzymes that normally breakdown long chain of sugar into glucose lies behind its action against the scourge of AIDS. The glycoprotein 120 at the very surface of the human immune deficiency virus (HIV) carrying an unusually large number of sugar side chain becomes inert and looses its ability to invade new cells in the presence of alkaloid DNJ (Ray, 1990).

I-8. Infection of Mulberry plants with diseases

Sericulture Industry is dependent primarily on mulberry (*Morus sp.*) leaf crop. The silkworm takes all of their nutrients e.g. protein, carbohydrate, lipid, vitamins, minerals etc. for their growth only from mulberry leaves. Protein provides the chief structural elements of the muscle, glands and other tissues. Mc. Ginnis and Kasting (1972) reported that all insects require proteins for their

optimum growth, development and reproduction. Apart from all the agronomic factors the leaves of mulberry are to be protected from infection by various pathogens. Amongst which fungal and viral pathogens are most important. Various microorganisms seriously infect the mulberry leaves. Virus is one of them and the disease is called "Tukra" occurs on mid February to mid April. Virus rapidly grows in early age of leaf. High temperature and dry environment increase this disease rapidly. In this condition the leaves are contract into wrinkes and sized are smaller. When temperature reduces, the disease decreases. This disease makes great harms mulberry leaves as well as silk industry. Powdery mildew, caused by the members of the family *Erysiphaceae* and leaf spot, caused by *Cercospora* decreased the production of quality leaves by fifty percent and in turn affected the silkworm rearing (Anonymous, 1990; Anonymous, 1969; Nomani *et. al.* 1970; Krisnaprasad and Siddaramaiah, 1979; Sullia and Padma, 1987; Jeyarajan, 1986; Sikdar *et. al.*, 1979 and Gavindaiah *et. al.* 1990). This causes great loss to the sericulture Industry.

Besides fungal, bacterial and viral infection, some harmful insect pests such as nematodes also cause damage to mulberry plant and plant parts. During the growth period pathogenic agent come in contact with the mulberry plant under existing environmental conditions and cause diseases by interfering with the normal physiological functions of the plants. Net result is the decrease of quality and quantity of leaves.

Jeyarajan (1986) reported that mulberry plant is affected by six species of fungal pathogens namely *Phyllactinia corylea, Cercospora moricola, Aecidium mori, Colletortrichum moricola, Phyllosticta moricola* and *Sclerotium rolfsii.* He also mentioned that the first five pathogens infect mulberry leaves reducing not only

the yield but also nutritional values, thus making the leaves unsuitable for silkworm feeding. Sharma *et. al.* (1993) reported leaf spot, powdery mildew, leaf rust, leaf blight and bacterial blight are the most serious diseases of mulberry in India.

There is no doubt that these diseases, powdery mildew, leaf spot and tukra caused great loss to the Sericulturists, leading to the damage to the Sericulture Industry. In Bangladesh, tukra disease occurs during the month of February-April, powdery mildew occurs during the month of October-February and leaf spot disease occurs during the month of April to September which cause serious problem resulting in the production of poor quality leaves of mulberry. These leaves when fed to silkworm can not produce good quality cocoons and thus causing a serious set back to the sericulture Industry.

Phyllactinia corylea parasite affected mulberry leaves in most part of India and Bangladesh (Bakshi *et. al.*, 1972) during dry season from September to February.

Cercospora moricola was found to produce leaf spot disease on mulberry (*Morus alba* L. and *Morus rubra* L.) with the development of disease symptoms leading to extreme damage to the foliage during the months of monsoon beginning from July (Patel et. al. 1949 and Dayakar *et.al.*1993). *Cercospora* as well as *Pseudocercospora* infects a wide range of host species including mulberry in Bangladesh (Khan and Shamsi, 1983). Chupp (1953) recorded four species of *Cercospora:* i) *Cercospora missonariensis* Winter, ii) *Cercospora morina* Chupp, iii) *Cercospora mori* Hara and iv) *Cercospora moricola* Cooke which infected mulberry leaves.

I-9. Aim of the present study

The rural people of India and Bangladesh till now consider Sericulture as their pastime work and earn an extra money to help their family. If we want to save the Sericulture Industry, we must produce quality & quantity leaves for the silkworm. Sericulture Industry is dependent primarily on mulberry (*Morus sp.*) leaf crop. There is no doubt in it that the fungal diseases mainly leaf spot and powdery mildew and viral (Tukra) diseases has been causing great loss to the sericulturists and finally great damage to the Sericulture Industry. To have good cocoon harvest during all the season it is very essential to protect the mulberry leaves from leaf spot, powdery mildew and tukra diseases otherwise all attempts of harvesting good cocoon crop will fail and Sericulture Industry will suffer as a whole. To control these diseases and to protect the Sericulture Industries from this great damage, careful and thorough investigation of these diseases and its development and spread is urgently needed. In this system proper disease management programme is very much urgent to save the sericulture Industry in Bangladesh.

The information available in the literature support the contention that these diseases affecting the normal growth and leaf yield of mulberry and feeding of diseased leaves greatly reduced the yield of quality silk. Biochemical studies of mulberry have revealed that diseased leaves are poor in nutritive value. When these leaves are fed, silkworm can not produce good quality cocoons and thus causing a serious set back to the Sericulture Industry as well as silk sector of Bangladesh.

The present research study has designed to achieve the following objectives:

1. Investigation of the development of leaf spot and powdery mildew disease symptoms in field and analysis of disease incidence and disease index.
2. Morphological and pathological studies of the causal organism of leaf spot and powdery mildew diseases.
3. Biochemical changes in the mulberry leaves due to host-pathogen interaction.
4. Dose evaluation of some fungicides and plant extract in controlling the leaf spot and powdery mildew diseases of mulberry.
5. Changes in some enzyme content of mulberry leaves after infection with diseases.

CHAPTER ONE

ISOLATION, IDENTIFICATION AND CONTROL OF CAUSATIVE ORGANISMS RESPONSIBLE FOR LEAF SPOT DISEASE AND POWDERY MILDEW DISEASE MULBERRY LEAVES

INTRODUCTION

Diseases are one of the most limiting factors in successful mulberry cultivation. In the list of fungal diseases of mulberry the leaf spot disease has occupied an important part and this is a chronic disease in rainy season from April to September in Bangladesh. Mulberry plants produce maximum leaves in this season. During the growth period pathogenic agents come in contact with mulberry plant under the existing environmental conditions and cause diseases with interfering with the normal physiological functions of the plants. As a result it causes extensive damage to the foliage resulting in defoliation as well as in reduction of the nutritional value of the leaves (Patel *et. al.*, 1949; Jeyrajan, 1986 and Sharma *et. al*, 1993).

Symptoms of leaf spots vary depending upon the causal agent. Although leaf spots can be caused by air pollutants, insects, bacteria et al., most are a result of infection by pathogenic fungi. Once into the leaf, the fungi continue to grow and leaf tissue is destroyed. Resulting spots vary in size from that of a pinhead to spots that encompass the entire leaf. Dead areas on the leaves are usually brown, black, tan or reddish in color. Occasionally the necrotic areas have a red or purple border.

Many of the leaf spot fungi have a similar life cycle. The causal fungus over winters on fallen leaves. In the spring, during or following a rain, spores produced by the fungus are discharged and carried by wind and splashing rain to newly emerging leaves. If a film of water is present on the leaf, the spore germinates and penetrates the leaf, causing infection. In a few days to several weeks, depending

on temperature, small spots appear on the leaves. As the fungus grows, the spots enlarge. In general, the leaf spot fungi are favored by wet weather early in the growing season. Leaf spot diseases are seldom a problem following warm, dry weather in the spring.

Leaf spot disease caused is the second major fungal disease of mulberry spreads very quickly after the first appearance forming the characteristic spots on the mulberry leaves. Leaf spot infection render the mulberry leaves also unsuitable for silkworm consumption and decreases the production per unit area of land leading to the great loss of the sericulture Industry. Gavindrah *et. al* (1990) reported the leaf spot disease as one of the three major fungal disease in India.

Powdery mildew is most likely to attack in the late autumn and the fall when the weather is cool and the relative humidity is low. Most of the powdery mildew develops as thin layers of ***mycelium*** on the plant's surface. *Spores* or ***resting bodies*** make up the bulk and are the primary means of dispersal. The wind and rain carry powdery mildew spores to new hosts. Excess water on the plant's surface can kill spores and inhibit growth of mycelia, and both spores and mycelia are sensitive to extreme heat and direct sunlight. Powdery mildew is an important disease of mulberry. The disease can be devastating on susceptible varieties under the proper environmental conditions. Unlike black rot and downy mildew, the powdery mildew fungus does not require free water on the plant tissue surface to infect. Powdery mildew can result in reduced vine growth, yield, leaves quality, and winter hardiness.

MATERIALS AND METHODS

1.1: Identification of leaf spot disease of mulberry

Leaf spot is the most common and widespread disease of mulberry leaf. The symptoms of this disease of mulberry are so clear and distinct that one can easily distinguish the infected leaf from the healthy leaves. The disease is characterized by the appearance of dark brown spot on the surface of mulberry leaf at the final stage it look like black spot in the leaf and the spot may be from 0.3-0.75 cm in diameter and irregularly appeared (Fig.-1.3).

1.2: Assessment of leaf spot disease severity of four high yielding varieties

A field containing mulberry plants of four varieties (BM-1, BM-2, BM-3 and BM-4) was selected for experimental purposes in Bangladesh Sericulture Research Institute, Rajshahi. The disease severity was calculated (Disease Index) following the method of James (1971). The leaf spot infected leaves of five plants of each variety were collected for analysis during the season May-September of 1999 –2001.

1.3: Isolation of the Pathogen from Leaf spot disease

The leaf spot diseased infected mulberry leaves were collected from the experimental field and brought into the Laboratory and the diseased portions were then cut into pieces and selected for surface sterilization. The diseased samples were washed in running tap water, dipped in 100 ml of 0.1% mercuric chloride solution contained in a petridish. Surface sterilization of the materials were continued for 2-3 minutes by rotating the samples frequently with a needle and then washed several times by sterilized distilled water to remove mercuric

chloride from the samples. Excess water from surface of the samples was removed by gently pressing them between two flapes of sterilized filter paper. The samples were placed on the petridish containing PDA medium. Then the petridishes were incubated at 28±2°C and examined periodically for upto 10 days.

1.4: Study of Morphology of Pathogen

The leaf spot infected mulberry leaf was collected from the infected mulberry field of Bangladesh Sericulture Research Institute Campus, Rajshahi. The fungal mycelium and conidia were taken out from the leaf spot portion with the help of forceps and needle and put in one or two drops of lactophenol on clean slide. A clean cover glass was then placed over the material and excess fluid was removed by soaking with blotting paper. Finally the prepared slide was examined under a microscope.

1.5: Artificial inoculation of the pathogen

Fresh conidia of *Cercospora moricola* was collected from the infected leaf with the help of needle in distilled water with approximately 15 conidia per optical field of 400x power of a compound microscope. The conidial suspension was then sprayed on the healthy leaves of the plants grown in earthen tub. The inoculated plants were incubated in out door environmental condition for 15 days. Sufficient water was supplied to the tub to maintain required moisture as well as to raise the relative humidity level above 90% for the incubated plants. After the appearance of the dark brown or black spot on the inoculated leaf surface, the reappearance of *Cercospora moricola* was confirmed under high resolving power

microscope and all the pathological tests were then done in the laboratory from this artificially inoculated diseased sample.

Preparation of Lactophenol

The composition of lactophenol solution, used as mounting medium was prepared as reported (Ainsworth, 1963) using the following constituents:

Phenol crystal	20gm.
Lactic acid	20ml
Glycerol	40ml
Distilled water	20ml

All of these reagents were taken in a flask and shaken well till a homogeneous solution was obtained.

Preparation of lactophenol-cotton blue strain

One gram of cotton blue was added in 100ml of lactophenol and shaken well till it dissolved and the solution was stored at a cool and dark place in an amber coloured bottle.

1.6: Germination of Conidia

1.6.1: Effect of different substances on conidial germination

Fresh conidia of *Cercospora moricola* were collected from the artificially inoculated diseased leaves of 20 days old and conidial suspensions were made separately in different substances e.g. tap water, distilled water, pond water, rain water, river water and different concentrations of glucose solutions. Conidial suspension was made with approximately 20-30 conidia per optical field of 400x resolving power of a compound microscope. Two drops of each suspension were

taken in grooved slides separately in three replications and were kept them at 28±2°C in the petridish moist chamber. The groove slides were then removed periodically (4, 6, 8, 12 and 24 hours) from the petridish moist chamber and a drop of lactophenol was put to the spore suspension of each slide and covered them with cover glasses and the slides were examined under high resolving power (400x) microscope for recording the percentage of germination.

1.6.2: Effect of Temperature on conidial germination

In order to study the effect of temperature on germination of conidia of *C. moricola*, the conidia were collected from the artificially infected mulberry leaves and were placed on the grooved slides containing 2.5% glucose solution and cover slips were placed on the slides. The slides were then incubated at 10°C, 15°C, 20°C, 25°C, 28°C, 30°C, 35°C and 40°C in the moist chamber. The slides were removed after 4, 8, 12 and 24 hours from the moist chamber and a drop of Lactophenol was put on the slide to kill the conidia for microscopic observation. The slides were examined under high power microscope (400x) for recording the percentages of germination.

1.6.3: Effect of Relative Humidity on conidial germination

The effect of relative humidity on germination of conidia of *C. moricola* was studied using modified Bonner's (1948) technique. To maintain the desired constant relative humidities (RH) Buxton and Mellanby's (1934) method was used. Dry and clean grooved slides were filled with glucose solution (2.5%) and conidia were placed on them. The slides were placed in the dessicators of different humidities. Then the dessicators were incubated at 28°C in an inccubator

for 4, 8, 12 and 24 hours. The slides were removed periodically and conidial germination was observed in three replications under high power microscope (400x) by putting a drop of Lactophenol in the slides.

The required relative humidity inside the dessicators was maintained by mixing stock solution and distilled water in different amounts, as given in the Table below:

Table-1.1: Amount of H_2SO_4 and water for preparation of RH.

Relative Humidity (%)	Stock Solution in ml prepared by mixing equal volume of Conc. H_2SO_4 and water	Distilled Water (ml)
40	539	306
50	415	420
60	374	496
70	348	510
80	297	690
90	161	712
100	0	1000

1.6.4: Effect of pH on conidial germination

In order to study the effect of pH on the conidial germination of *C. moricola,* conidia were collected from infected mulberry leaves and were placed on the grooved slides containing 2.5% glucose solution of different pH ranges (2-10). pH adjustment was made by adding 0.1N HCl and 0.1N KOH in glucose solution. All the slides were incubated in moist chamber for 24 hours and then a drop of Lactophenol was added on the slide for observing germination. By placing cover slips on the slide the percentage of conidial germination was recorded by

observing under high power microscope. The experiment was conducted for three replications and mean values were calculated.

1.7: CONTROL MEASURES

Evaluation of the efficacy of six fungicides against *Cercospora moricola* (*in vitro*)

Reagents

1. Dithane M-45, Thiovit 80WP, Cupravit 50WP, Antrocol 50WP, Macupras 65WP and Bayleton-25WP solutions of different concentration (500ppm, 1000ppm, 1500ppm, 2000ppm and 2500ppm).
2. 2.5% glucose solution.

Method

Cercospora moricola infected mulberry leaves were brought into the laboratory and diseased portions were cut into pieces and dipped them into different concentration of fungicides for 5, 10, 20 and 30 minutes. The diseased portion were removed periodically and washed in distilled water. Conidia of *Cercospora moricola* from the diseased portion were separated and placed on grooved slides containing 2.5% glucose solution and then it was covered with cover slip. The slides were kept into petridish moist chamber and the petridishes were incubated at 28±2°C. After 24 hours incubation the slides were removed and observed the conidial germination under high power microscope.

RESULT AND DISCUSSION

R-1.2: Assessment of leaf spot disease severity

Severity of leaf spot disease was studied on four varieties of high yielding mulberry plants during May-September in 1999-2001 and the results are shown in the Table-1.2. Of the varieties examined, BM-4 variety is highly infected, followed in decrease order BM-3, then BM-1 and BM-2. The result also showed an increase in disease severity from May onwards with a peak during the month of September.

Table-1.2: Incidence of disease severity in four high mulberry yielding varieties.

	Disease severity in terms of disease index (DI)				
Varieties	May	June	July	August	September
BM-1	5.45	8.25	12.50	20.38	24.28
BM-2	4.55	8.05	12.50	18.85	22.56
BM-3	6.25	10.25	14.55	22.25	28.36
BM-4	7.05	9.85	15.05	24.64	30.45

Sulaiman and Agashe (1965) stated that maximum pre-disposing factor for leaf spot disease caused by *Cercospora* development occurs in the month of September. In India the considerable loss of mulberry leaf due to *Cercospora moricola* was observed during August-October (Sikder, 1987). He also reported that 35% disease incidence due to *C. moricola.* Ali and Qaiyyum (1993) also reported similar result.

Fig.-1.1: Photograph of Mulberry field.

Fig.-1.2: Photograph of Healthy Mulberry leaf.

R-1.3: Isolation of the Pathogen

For the study the morphology the causal organism of leaf spot infected leaves were cultured on PDA media. It was found that the organism did not germinate and no mycellium was observed on the PDA media. From these findings, it may suggest that the organism is an obligate parasite. Sikder (1987) reported that most of the species of the genus *Cercospora* are obligate parasite and seldom grow in culture media.

R-1.4: Morphology of the causal Organism

The morphology of the causal organism was studied after transferring the organism directly on the slide from the infected mulberry leaf and the characteristics are summarized below:

The fungus produces a compact mass of interwoven cushion like hyphae. The hyphae are septate, intercellular and entirely internal. The haustoria are unbranched and are visible in the host cells when stained. Conidiophores are olive brown geniculate and septate (Fig.-1.4) which produce conidia singly and successively on the tips. Conidia are obclavate to cylidrical, 4-7 septate and 36-100μ x 3.0-4.5μ in size. From the above data it can be concluded that the causal organism for leaf spot disease of mulberry leaf is *Cercospora moricola.*

Taxonomic Position of the isolated organism

Division	Mycota
Sub-division	Eumycota
From Class	Deuteromycetes
From Order	Moniliales

From Family	Moniliaceae
From Genus	*Cercospora*
From Species	*moricola* (cooke) Sacc.

Patel *et. al.* (1949) first reported that the leaf spot disease in mulberry was caused by *Cercospora moricola,* while Sharma *et. al.* (1993) also reported the leaf spot disease as one of the most serious disease of mulberry caused by *Cercospora moricola.*

R-1.5: Artificial inoculation of leaf spot disease

Data of the artificial inoculation of leaf spot disease on the mulberry leaves by inoculating *Cercospora moricola* is presented in the Table-1.3. The result indicated that 44%, 56% and 52% successful inoculation of the leaf spot disease on the leaf during July, August and September when average outdoor temperature and relative humidity were 26°C and 90% respectively.

Table-1.3: Artificial inoculation of leaf spot disease during the month of July, August and September.

Month	No. of leaf inoculated	No. of leaf infected	Successful infection (%)
July	50	22	44
August	50	28	56
September	50	26	52

[The result of means value of three replications].

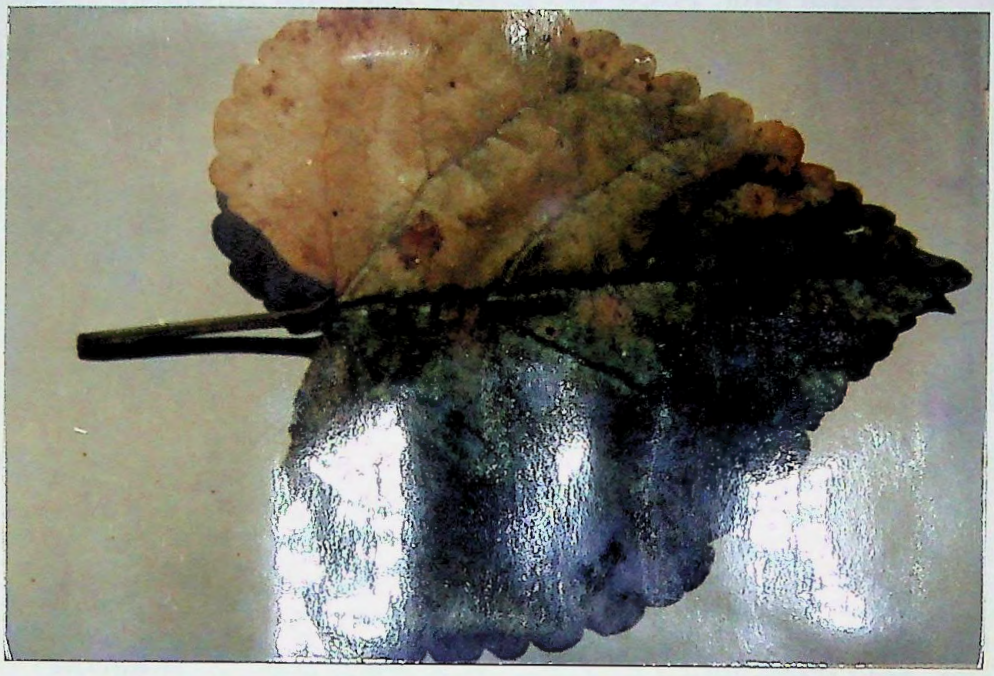


Fig.-1.3: Photograph of leaf spot infected Mulberry leaf

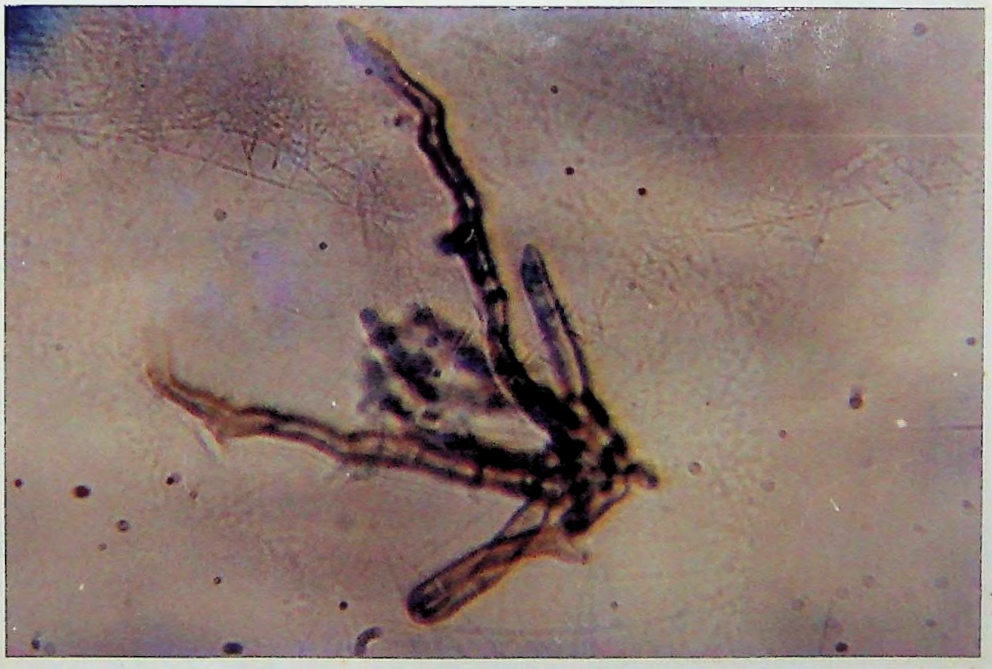


Fig.-1.4: Microphotograph of conidiophore of *Cercospora moricola*

R-1.6: Germination of Conidia

R-1.6.1: Effect of different sources of water and different concentration of glucose on conidial germination.

The conidia of *C. moricola* was germinated under identical conditions in water from different sources as well as in different concentration of glucose solution and the results were summarized in Table-1.4.

Table-1.4: Effect of water from different sources and different concentrations of glucose solution on the conidial germination of *C. moricola*.

Medium	Percentage of conidial germination after incubation in different hours					
	4	6	8	10	12	24
Tap water	0.00	2.00	8.00	18.00	43.33	90
River water	0.00	1.66	7.33	15.00	38.35	85.00
Rain water	0.00	4.33	12.33	22.33	47.66	94.66
Pond water	0.00	2.66	8.33	20.66	42.66	92.00
Distilled water	0.00	2.33	9.33	20.00	40.33	90.66
1% glucose	0.00	5.33	12.66	24.00	48.66	96.33
1.5% glucose	0.00	6.66	14.33	28.66	50.33	98.33
2% glucose	1.00	10.33	20.33	32.33	60.66	100
2.5% glucose	2.66	12.33	23.33	42.66	65.33	100
3% glucose	0.00	8.33	20.66	28.33	52.33	96.66

[Represent mean of three replications.]

Fig.-1.5: Microphotograph of conidia of *Cercospora moricola*

Fig.1.6: Microphotograph of Germinating conidia of *Cercospora moricola*

Among the different sources of water used for germination, rainwater was found to be best, followed by pond water and so on in decreasing; suggesting that difference in composition of water may have some effect on the conidial germination of the *C. moricola.* Comparatively less germination was found in river water, which might be due to higher salt concentration of river water. Sukumar and Ramalingam (1987) reported rainfall as the sole agent in the initiation and the spread of the disease by way of splash mechanism. Dayakar *et.al.* (1993) also reported that distributed rainfall over a period of 8 days in a month was more effective in the spread and development of leaf spot in mulberry. Again, among different concentration of glucose used for conidial germination, 2.0-2.5% glucose concentration was found to be best since 100% conidial germination was obsereved in these concentrations after 24 hours of incubation. Chuang (1986) and Yarwood (1954) reported that glucose and asparagine favours conidial germination in host cell with temperature range of 20-25°C.

R-1.6.2: Effect of temperature on conidial germination

The conida of *C. moricola* was incubated at different temperatures for various time intervals and the effects of temperature on the conidial germination are summarized in the Table-1.5. The conidial germination was found to be influenced greatly with the changes of temperature and after 24 hr. incubation 100% conidial germination was recorded around 28-30°C. The conidial germination was reduced drastically at or below 20°C as well as at or above 35°C. On the other hand, no conidial germination was observed at 10°C and 40°C even after incubation of 24 hr.

Table-1.5: Effect of temperature on the conidial germination of *C. moricola* after different hours of incubation.

Temperature	Percentage of conidial germination after different hours of incubation					
	4	6	8	10	12	24
10°C	-	-	-	-	-	-
15°C	-	1	5	11	14	18
20°C	1	4	12	20	28	54
25°C	2	15	30	44	62	98
28°C	3	18	32	50	75	100
30°C	3	15	28	46	70	100
35°C	2	12	18	25	25	25
40°C	-	-	-	-	-	-

It may be concluded from the results that the optimum temperature for conidial germination of C*ercospora moricola* was 28-30°C. Patel reported that 26°-28°C as optimum temperature for the conidial germination of *Colletotrichum sp.* The optimum growth temperature of *Cercospora gloeosporioides* was reported to be 25-30°C by Ahmed (1985). Bedi and singh (1972) reported the optimum temperature was 30°C for conidial germination of *A. alternata.* Dayakar *et. al* (1993) reported that 28°C temperature was most suitable for leaf spot disease.

R-1.6.3: Effect of Relative Humidity on conidial germination

The effect of relative humidity on conidial germination of *C. moricola* is shown in Table-1.6. The result indicated that germination initiated after 4 hours at relative humidity of 90-100%. The result also pointed out that higher percentage of RH favour the better conidial germination of *C. moricola* and 85-100% humidity was considered to be the best for conidial germination. Further, no conidial germination was recorded at RH of 40% even after incubation of 24hrs.

Table-1.6: The effect of different relative humidity on conidial germination of *C. moricola* after different hours of incubation at 28°C.

Relative Humidity (%)	Conidial germination in (%) after different hours incubation					
	4	6	8	10	12	24
40	-	-	-	-	-	-
50	-	-	-	-	-	12
60	-	-	-	8	15	26
70	-	10	22	25	42	68
80	-	15	24	35	56	92
90	10	23	42	56	77	100
100	15	22	40	55	82	100

Khare and Neema (1982) reported that 100% humidity was best for spore germination of *Alternaria poori*. Lim and Tang (1985) reported the effect of RH on growth and sporulation of *C. gloeosporioides* and the optimum RH was 80-90%. Kore and Kharwade (1987) studied the effect of relative humidity on the growth and sporulation of *Fusarium oxysporum* on PDA and observed that RH of

70-90% showed best growth of the fungus. They also observed that below 50% RH resulted poor growth of the fungus.

R-1.6.4: Effect of pH on conidial germination

As shown in Table-1.7, the conidial germination of *C. moricola* was affected greatly with the changes of pH and about 100% conidial suspension was obtained around pH 5.0-6.0. The percentage of germination was decreased beyond these pH ranges. Further only 18% and 10% germination was recorded at pH 3.0 and 10.0 respectively. Ganacharya (1976) reported that fungal spores were germinated a wide ranges but better at pH 4.5-5.5. Monga and Grover (1991) reported best growth of fungal mycelium and spore germination at pH 6.0-7.0.

Table1.7: Effect of different pH of 2.5% glucose solution on the conidial germination of *C. moricola* after 24 hours of incubation at 28°C.

pH Value	Percentage of conidial germination after 24 hours incubation
2	0
3	18
4	58
5	95
6	100
7	86
8	53
9	28
10	10

R-1.7: Control Measures

Among the objectives of the present study, disease management was the most important one as leaf spot disease reduces the nutritional value of mulberry leaves, as a result the leaves completely unsuitable for consumption by the silkworm larvae and losses the yield. So, proper disease management programmed is very essential to save the mulberry leaves from leaf spot disease. Six fungicides namely Dithane M-45, Thiovit 80WP, Cupravit, Antracol, Macuprax and Bayleton-25 WP in five different concentrations (500ppm, 1000ppm, 1500ppm, 2000ppm and 2500ppm) were tested for inhibition of the conidial germination of *Cercospora moricola* and the results are shown in the table-1.7. The results indicated that out of six tested fungicides, Dithan M-45, Antracol 50WP and Cupravit 50WP were found to be effective in inhibiting the conidial germination of *Cercospora moricola* and among them Dithane M-45 was the most effective fungicide which inhibited almost completely the conidial germination of *C. moricola* at all concentration during the observing time of 30 minutes. Kanit (1975) reported that the conidial germination of *C. moricola* were inhibited completely by Dithane M-45 of 0.05%, 0.1% and 0.2% concentrations while Siddaramaiah and Krisnaprasad (1978) reported that Dithane M-45 at 400 ppm completely inhibited the growth of *C. moricola in laboratory.* Sanaullah and Alam (1989) reported that Dithane-M-45 was best fungicide for both *in vitro* against *C. moricola.* Ismail and Wadud (1981) reported that Dithene M-45 as the effective fungicide against leaf spot of peanut caused by *Cercospora moricola.*

Table-1.8: Effect of different fungicides on conidial germination of *C. moricola*

Fungicides	Concentrations in ppm	Percentage of Conidial germination after treating the conidia in fungicidal solution for different times intervals (min)			
		5	10	20	30
	500	26	8	00	00
Dithane M-45	1000	14	00	00	00
	1500	00	00	00	00
	2000	00	00	00	00
	2500	00	00	00	00
	500	100	100	100	90
	1000	100	88	76	75
Thiovit 80WP	1500	90	78	74	73
	2000	80	72	60	58
	2500	80	68	53	46
	500	75	52	42	26
	1000	50	35	18	00
Cupravit 50WP	1500	26	12	00	00
	2000	00	00	00	00
	2500	00	00	00	00
	500	74	62	44	28
	1000	46	32	26	10
Antracol 50WP	1500	32	22	14	5
	2000	18	10	00	00
	2500	00	00	00	00
	500	100	100	95	90
	1000	100	100	90	85
Macuprax 65WP	1500	90	90	75	55
	2000	90	71	62	42
	2500	80	60	44	20
	500	100	100	92	92
Bayleton-25WP	1000	100	100	84	80
	1500	100	82	73	56
	2000	90	74	52	45
	2500	80	64	34	22

[Mean of three replications]

Study on powdery mildew disease
Materials and methods

1'.1: Identification of Powdery mildew disease of mulberry leaves

The powdery mildew fungus can infect all green tissues of the plant. The symptoms of powdery mildew disease of mulberry are so clear and distinct that one can easily distinguish the infected leaf from the healthy leaves. Powdery mildew is the most common and major epidemic and widespread disease of mulberry leaf. The disease is characterized by the appearance of patches of white to grayish powdery growth on the dorsal surface of mulberry leaf (Fig.-1.7). These patches usually enlarge until the entire upper leaf surface has a powdery, white to gray coating. The patches may remain limited through out most of the season. Severely affected leaves may curl upward during dry weather. Expanding leaves that are infected may become distorted and stunted.

At the initial stage, some scattered and ash coloured patches forms under the surface of the leaf with slight yellowing on the upper surface just opposite on the powdery patches. The powdery patches may be from 0.5-2.5 cm in diameter and irregularly appeared. As the disease advances, the infected leaf turn yellowish browns and finally becomes coarse, lethery and fall off from the attachments of branches. Sometimes at the late season of infection, some orange yellowish spot become prominent, many in number and their colour change from orange yellow to brown and brown to black, these are the cleistothecia of the causal fungus (Fig.1.10). Powdery mildew infection by *Phyllactinia corylea* of mulberry that

occurs exclusively on the lower surface and usually the older leaves becomes infected.

1'.2: Assessment of leaf spot disease severity of four high yielding varieties

A field at Bangladesh Sericulture Research Institute, Rajshahi containing BM-1, BM-2, BM-3 and BM-4 plant has selected and analyzed the disease severity (Disease Index) following the method of James (1971). The leaves of five plants of each variety during the season November-February of 1998–2001 were selected.

1'.3: Isolation of the Pathogen of Leaf spot disease

Procedure was same as described above (1.3)

1'.4: Study of Morphology of Pathogen

Procedure was same as described above (1.4)

1'.5: Artificial inoculation of the pathogen

Powdery mildew infected mulberry leaf was collected from the infected mulberry field. Conidia of *Phyllactinia corylea* were collected from the infected leaf and were dusted with the help of camel hairbrush on the healthy leaves of the plants grown in earthen tub. The inoculated plants were then incubated in the Campus of the Institute in dry condition for 15 days. All the laboratory tests of the pathogenic fungus were done by the conidia collected from artificially inoculated plants.

1'.6: Germination of Conidia

1'.6.1: Effect of different substances on conidial germination

The procedure was same as described above (1.6.1)

1'.6.2: Effect of Temperature on conidial germination

The procedure was same as described above (1.6.2)

1'.6.3: Effect of Relative Humidity on conidial germination

The procedure was same as described above (1.6.3)

1'.6.4: Effect of pH on conidial germination

The procedure was same as described above (1.6.4)

1'.7: CONTROL MEASURES

Evaluation of the efficacy of six fungicides in vitro against *Phyllactinia corylea*

The procedure was same as described 1.7.

RESULT AND DISCUSSION

R-1'.2: Assessment of Powdery mildew disease severity

Severity of powdery mildew disease of four high yielding mulberry varieties during November-February in 1998-2001 was studied and data are shown in the Table-1.9. The result indicated that out of the varieties examined BM-3 showed the maximum disease severity during the experimental periods but BM-4 was found to be slightly more disease resistance comparison with other varieties. The results also indicated that the disease severity increased during January and February.

Table-1.9: Assessment of Powdery mildew disease severity (Disease Index) of four high yielding varieties.

Varieties	Disease severity in terms of disease index (DI)			
Month	November	December	January	February
BM-1	4.45	10.45	25.50	32.38
BM-2	5.75	12.40	26.50	35.85
BM-3	6.25	16.25	31.55	37.25
BM-4	0.00	8.85	22.05	30.64

Pasha and Barman (1990) reported that in Bangladesh above 54% mulberry leaves are infected by powdery mildew disease but Siddaramaiah *et.al* (1978) recorded 92% in India. Anonymous (1969); Sullia and Padma (1987); Krisnaprashad and Siddaramaiah (1979) and Nomani *et.al.* (1970) studied the outbreak of powdery mildew disease of mulberry in India and they also assessed the qualitative effects of powdery mildew infected mulberry leaves on silkworm.

Fig.-1.7: Photograph of powdery mildew infected Mulberry leaf

Fig.-1.8: Microphotograph of spores of *Phyllactinia corylea*

Fig.-1.9: Microphotograph of spore with germ tube of *Phyllactinia corylea*

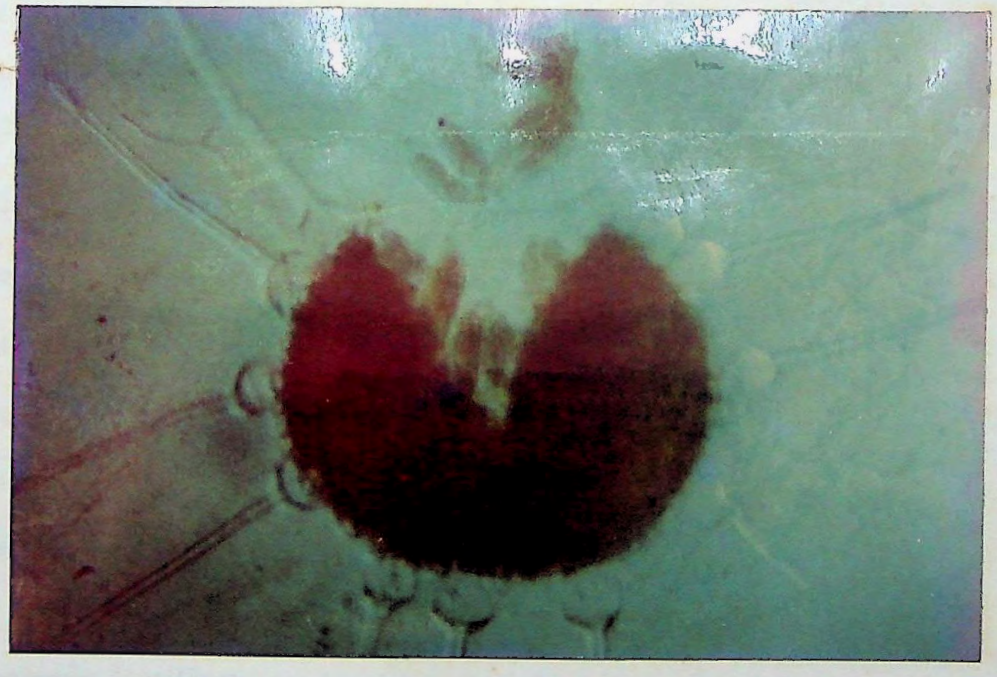


Fig.1.10: Microphotograph of brusting stage of clestothesia of *Phyllactinia corylea*

R-1'.3: Isolation of the Pathogen

To study the morphology the causal organism from the infected leaves was tried to grow on PDA media. It was found that organism did not germinate and no mycellium was observed on the PDA media. From these findings, it may suggest that the organism is an obligate parasite. Sikder (1987) also reported that obligate parasite organisms seldom grow in culture media.

R-1'.4: Morphology of the causal Organism

The fungal mycelium is white, superficial and intercellular. The fungus produces white superficial conidia and dark superficial cleistothecia, which are the identical characters of the fungus. Fungal mycelium cris-crossed to form a net like mat and depends on to the haustoria to stick to leaf epidermis. Haustoria arise from the hyphae in the intercellular space and draw nourishments from the host tissue. Erect colourless conidiophores are produced from the hyphae with 3-4 septa and are of 150-250μ x 4.0-8.5μ in size. Conidiophore produces conidia by dividing the top cell. The conidida are colourless unicellular and clavate, mostly single, seldom in chain and are of 50-80μ x 15-20μ in size. Flat and oval shaped cleistothecia are found to form without ostoles, yellow when young, yellow browns to black when mature (Fig.-1.3). ascospore are formed inside the asci similar in structure to conodia and 2-4 in number in each asci.

Taxonomic Position

Division	Mycota
Class	Ascomycetes
Sub-class	Euascomycetidae

Series	Pyrenomycetes
Order	Erysiphales
Family	Erysiphaceae
Genus	*Phyllactinia*
Species	*corylea* (Pers) Karst

Sitarama Iyenger (1975) concluded that powdery mildew disease of mulberry due to *Phyllactinia corylea* is the most common and important disease.

R-1'.5: Artificial inoculation of powdery mildew disease

Data on the artificial inoculation of powdery mildew disease on the mulberry leaves by inoculating *P. corylea* is presented in the Table-1.10.

Table-1.10: Artificial inoculation of powdery mildew disease during the month of December, January and February.

Month	No. of leaf inoculated	No. of leaf infected	Successful infection (%)
December	100	75	75
January	100	83	83
February	100	90	90

Data Represent three replications

The result indicated that 75%, 83% and 90% successful inoculation of the disease on the leaf during December, January and February when average outdoor temperature and relative humidity were 20°C and 78% respectively. Similar results reported by Ali (1995)

R-1'.6: Germination of Conidia

Factors affecting conidial germination

Environmental factors influence directly on the germination of fungal conidia e.g. nutrition, temperature, pH of the growing media, relative humidity etc. are most important factors. Conidia of *Phyllactinia corylea* was allowed to germinate separately in different growing substrates, under different temperature ranges and different pH values of 2% glucose solution and at different RH as described in materials and methods.

R-1'6.1: Effect of water from different sources as well as different concentrations of glucose on conidial germination of *Phyllactinia corylea*.

The conidia of *P. corylea* was germinated under identical condition in water from different sources as well as different concentration of glucose solutions and the results were summarized in Table-1.11.

Among the sources of water used for germination of conidia, pond and rain water were found to be better followed by tap water and so on in decreasing order. Dew was found to be best suitable for conidial germination. It may be suggested from the result that differences in compositions of water might have some effect on the conidial germination of the organism. Again, among the different concentrations of glucose used, 2.0-2.5% glucose concentrations might be the best as 90% conidial germination was observed in these concentrations after 24 hour of incubation. Yarwood (1936) observed that increased of conidial germination in sugar solution.

Table-1.11: Effect of water from different sources as well as different concentrations of glucose solutions on the conidial germination of *P. corylea*.

Medium	Percentage of conidial germination after incubation for different hours					
	4	6	8	10	12	24
Tap water	00	2.00	8.00	15.33	40.33	75.00
River water	00	1.66	7.66	15.00	30.33	65.00
Rain water	00	3.00	10.66	20.00	45.33	80.50
Pond water	00	3.00	10.33	18.33	42.66	80.66
Dew	00	4.00	12.25	24.66	48.33	90.00
Distilled water	00	2.33	8.66	20.33	40.33	75.33
1% glucose	00	4.33	12.66	20.00	40.66	75.00
1.5% glucose	00	6.66	14.33	22.66	45.50	80.33
2% glucose	2.00	10.33	15.66	30.33	50.66	90.00
2.5% glucose	2.00	10.00	14.33	28.66	45.33	90.00
3% glucose	00	8.33	15.00	25.33	45.33	85.66

Data represent three replications.

R-1'.6.2: Effect of temperature on conidial germination

Temperature influenced greatly the conidial germination of different fungi. Plant pathogenic fungi differ in their optimum temperature for conidial germination and infection (Bruehl and Cunfer, 1971). Effect of temperature on conidial germination of *Phyllactinia corylea* is shown in the Table-1.12. The results indicated that germination of conidia initiated at 10°C within 24 hours on the

other hand germination was initiated at 15°C, 20°C, 25°C, and 30°C after incubate after of 4 hours. Further maximum germination (85-89%) was observed around 20°C-25°C. Present investigation also indicated that no germination was found at 40°C temperature.

Table-1.12: Effect of temperature on the conidial germination of *P corylea* after different hours of incubation under identical condition.

Temperature	Percentage of conidial germination after different hours in incubation				
	4	6	8	12	24
10°C	00	00	00	00	02.00
15°C	00	02.00	08.00	25.33	52.00
20°C	2.00	10.33	25.66	54.00	85.00
25°C	02.00	12.00	32.00	57.00	89.00
30°C	4.00	15.00	28.00	34.00	60.00
35°C	00	5	10	10	15
40°C	00	00	00	00	00

Data represent in three replications.

It is evident from the result that optimum temperature for conidial germination of *Phyllactinia corylea* was 20-25°C and the maximum incubation time was 24 hours. Yarwood *et. al.* (1954) reported that the optimum temperature is 22°C for conidial germination of powdery mildews of different species. Etoi *et. al.* (1960) indicated that the most suitable temperature for conidial germination of *Phyllactinia corylea* is 30°C on the other hand Leu and Lee (1982) found that 24-

28°C temperature with variable relative humidity are most congenial for conidial germination. Probhu *et. al.* (1962) observed 15-20°C temperature as optimum for conidial germination of *Erysiphae graminis* while Sing (1978) reported 19°C and 22-25°C as optimum temperature for the conidial germination of *Podosphaera leucotrica* and the family of *Erysiphaeceae.* It might be suggested from the results that above 30°C, conidia of *P. corylea* may lost its germinability and therefore poor and zero percent germination possibly recorded at temperature above 35°C. At very low temperature perhaps conidial germination did not activate properly and at high temperature were harmful for conidial germination. Khare and Neema (1982) reported that the optimum temperature of spore germination of *Alternaria poori* at 22-25°C.

R-1'.6.3: Effect of Relative Humidity on conidial germination

Effect of relative humidity on conidial germination of *Phyllactinia corylea* is shown in Table-1.13. The result indicated that germination initiated at relative humidity of 40-100% after 24 hours incubation under identical condition 20, 52, 80, 90, 75, 62 and 50% of conidial germination were obtained at RH of 40, 50, 60, 70, 80, 90 and 100% respectively.

The result also pointed out that at 60-80% of RH is very favourable for conidial germination of *Phyllactinia corylea.* Conidial germination did not occur well at the higher relative humidity above 80%. Etoi *et.al.* (1960) indicated that 80% RH was found to be most suitable for conidial germination in *P. corylea.* Yarwood (1936) indicated that low RH is injurious to conidial germination in *Erysiphae*

polygoni. Clayton (1942) reported that even at high humidity spores of some fungi could not germinate. Arya and Ghemawat (1954) obtained no germination below 85% RH in the powdery mildew of wheat.

Table-1.13: Effect of different relative humidity and pH values on conidial germination of *Phyllactinia corylea* under identical condition.

Relative Humidity (RH) (%)	Conidial germination (%)	pH Value	Conidial germination (%)
40	20	2	-
50	52	3	18.66
60	80	4	35
70	90	5	90
80	75	6	75
90	62	7	45
100	50	8	10
		9	0
		10	0

R-1'.6.4: Effect of pH on conidial germination

Fungal spores were found to germinate in a wide range of temperature but spores of most fungi are found to germinate better at pH ranging from 4.5-6.5. The percentages of conidial germination in different pH levels are shown in the Table-1.13. The result indicated that conidial germination starts from pH level of 3.0-8.0 with an optimum pH of 5.0. At pH 2.0, 9.0 orabove, conidia did not germinate, indicating the lethal effect on conidial germination. Goose and Tschirsch (1962) observed optimum pH 6.0 for *Gloeosporium musarum.* Satya and Grewal (1972) found *F. caeruleum* to grow at different pH level from 2.0-8.0. Smart (1973) reported spores of *Fuligo saptica* germinated at pH level of 2.0-10.0. pH below

3.0 and above 8.0 conidial germination was inactivated because alkaline and highly acidic pH level was found to be harmful for the conidial germination of *P. corylea.*

R-1'.7: Control measure

Six fungicides, Dithane M-45, Thiovit 80WP, Cupravit, Antracol, Macuprax and Bayleton-25WP in five different concentrations (500ppm, 1000ppm, 1500ppm, 2000ppm and 2500ppm) were tested in the laboratory (*in vitro)* for inhibition of the conidial germination of *Phyllactinia corylea,* the causal organism of powdery mildew disease and the results are given in the table-1.14 The results indicated that conidial germination were inhibited slightly or significantly by all the fungicides. Further, Bayleton-25WP completely inhibited of germination of conidia during the experimental periods while Dithan M-45, Thiovit-80WP also showed complete inhibition of germination in presence of 1500ppm or higher concentration after 24 hours incubation. On the other hand, Macuprax-65WP, Antracol 50WP and Cupravit 50WP were found to be not so much effective at low concentration but at high concentration slightly inhibition of conidial germination was observed.

Table-1.14: Effect of different fungicides on conidial germination of *C. moricola*

Name of Fungicides	Concentrations in ppm	% of Conidial germination after soaking the conidia in fungicidal solution for different times (minute)			
		5	10	20	30
	500	55	36	20	8
	1000	36	22	00	00
Dithane M-45	1500	00	00	00	00
	2000	00	00	00	00
	2500	00	00	00	00
	500	64	42	25	10
	1000	50	25	00	00
Thiovit 80WP	1500	00	00	00	00
	2000	00	00	00	00
	2500	00	00	00	00
	500	85	70	65	55
	1000	65	58	45	42
Cupravit 50WP	1500	68	52	44	42
	2000	60	50	35	30
	2500	50	40	25	20
	500	100	90	75	65
	1000	88	72	55	54
Antracol 50WP	1500	75	60	50	42
	2000	75	50	50	50
	2500	60	54	40	36
	500	100	85	80	75
	1000	90	75	65	50
Macuprax 65WP	1500	85	75	50	40
	2000	78	60	54	40
	2500	75	54	48	35
	500	00	00	00	00
	1000	00	00	00	00
Bayleton-25WP	1500	00	00	00	00
	2000	00	00	00	00
	2500	00	00	00	00

Ali (1995) reported that the Bayleton-25wp was effective to control of conidial germination of *P. corylea*. He also reported that lower dose (0.2-0.3%) of Bayleton-25wp control of the powdery mildew disease in the field condition. Sitarama Iyerngер (1975) reported that Morestan was effective fungicide for the control of powdery mildew disease in mulberry.

CHAPTER TWO

A COMPERATIVE STUDY ON THE NUTRIENT COMPOSITIOS OF DIFFERENT VARIETIES OF HEALTHY AND DISEASE INFECTED MATURE MULBERRY LEAVES

INTRODUCTION

Silkworm (*Bombyx mori*) requires certain essential sugars, proteins, amino acids, fatty acids, vitamins and micro nutrients for its growth, survival as well as for growth of silkgland and higher production of good quality silk. Supplementation with minerals plays a vital role in the larval development and cocoon characters. All of these substances are supplied by mulberry leaf. So, growth development and cocoon productions of the silkworms are greatly influenced by the quality of mulberry leaves (Basavajappa and Savanurmath, 1997; Hugar and Kaliwal, 1997; Loknath *et. al.*, 1986). Nutritional composition and moisture content of mulberry leaves influence the growth of the silkworm larvae and good quality of cocoons. Protein is the main constituent of mulberry leaves. Rangaswami *et. al.*, (1976) reported that nearly 70% of silk protein produced by silkworm directly derived from mulberry leaves. Qader *et. al.* (1992, 95) reported that quality of mulberry leaves greatly affects larval growth, silkgland development, production of good cocoons and finally affects on the economy of the Sericulture Industry. Chlorophyll and total sugar content directly related to the photosynthetic quantity of the plants. The increase in chlorophyll and total sugar content is attributable to the enhancing trend of photosynthesis and photosynthetic metabolism. Depletion of chlorophyll ratio between healthy and diseased leaves, which is directly related to the biochemical changes. (Mahadevan and Sridhar, 1982).

In this chapter, the changes in the nutritional composition of mulberry leaves due to host pathogen interaction were studied.

MATERIALS AND METHOD

Collection of leaves

For the studied in the nutritional composition of mulberry leaves, freshly harvested healthy and diseased infected mature mulberry leaves were collected from the Bangladesh Sericulture Research Institute, Rajshahi, Bangladesh and brought into the Protein and Enzyme research laboratory of Biochemistry & Molecular Biology Department, Rajshahi University, Rajshahi, Bangladesh.

2.1: Determination of pH

Extraction of mulberry leaves juice

About 2 gram of mulberry leaves were taken in a mortar. The leaves were crushed thoroughly in a mortar with a pestle and homogenized well with 30ml of distilled water and then filtered through two layers of muslin cloth. The filtrate was then centrifuged for 10 min. at 5000 g and the clear supernatant was collected.

Standard buffer solution

Buffer tablet of pH 7.0 and 4.0 (BDH Chemicals Ltd. Poole, England) was dissolved in distilled water and made upto the mark of 100ml with distilled water.

Procedure

The electrode assembly of the pH meter was dipped into the standard buffer solution of pH 7.0 taken in a clean and dry beaker. The temperature correction knob was set to 28°C and the fine adjustment was made by asymmetry potentially knob to pH 7.0. After washing the electrode assembly it was then dipped into a solution of standard pH 4.0 and adjusted to the required pH by fine asymmetry potentially knob. The electrode assembly was raised, washed twice with distilled water, rinsed off with juice of the cultivars and then dipped into the juice of the mulberry leaves. The pH of the juice was noted.

2.2: Determination of total titratable acidity (TTA)

The total titratable acidity was determined by Folin's method (Oser, B.L., 1965).

Reagents

a) Standard NaOH solution (0.I N).

b) 1% Phenolphthalein solution.

Extraction of mulberry leaves juice

The mulberry leaf juice was extracted by the procedure as described before.

Procedure

Mulberry leaf juice was taken in a conical flask. Two to three drops of phenolphthalein indicator was added and shaked vigorously. It was then titrated immediately with 0.IN NaOH solution from a burette till a permanent pink colour was appeared. The volume of NaOH solution required for titration was noted.

2.3: Extraction and estimation of chlorophyll

Chlorophyll is the green pigment universally present in all photosynthetic tissues. Chlorophyll-a and chlorophyll-b occur in higher plants. Chlorophyll content of the healthy and diseased mulberry leaf tissues were estimated following the method as described in below (Mahadevan and Shidhar, 1982).

Materials

a) Mortar and pestle
b) Buchner funnel
c) Whatman No. 42 filter paper
d) Volumetric flasks (50ml and 100ml)
e) Pipette (5ml and10ml)
f) Colorimeter

Reagent

80% Acetone

Procedure

One gram of mulberry leaf was cut into small pieces and homogenized well with excess 80% acetone in a mortar with pestle and then filtered the extract through a buchner funnel using Whatman no. 42 filter paper. Then sufficient amount of 80% acetone was added and repeated the extraction. The extract was then transferred to the Buchner funnel and washed with 80% acetone until colourless. The filtrate was pooled and made upto 100ml in a volumetric flask with 80% acetone. The absorbance of this extract was measured at 645nm and 663nm for determination of chlorophyll-a and chlorophyll-b.

The chlorophyll contents were calculated on fresh weight basis employing the following formula as described in below (Mahadevan and Shidhar, 1982), using the specific absorbtion coefficient for chlorophyll-a and chlorophyll-b at 663nm and 645nm in 80% acetone respectively.

Calculation

$$\text{Total chlorophyll (mg/gm)} = \frac{20.2A_{645} + 8.02A_{663}}{l \times 1000 \times w} \times v$$

$$\text{Chlorophyll-a (mg/gm)} = \frac{12.7A_{663} - 2.69A_{645}}{l \times 1000 \times w} \times v$$

$$\text{Chlorophyll-b (mg/gm)} = \frac{22.9A_{645} - 4.68A_{663}}{l \times 1000 \times w} \times v$$

Where, A= Optical density in each case

l = Length of light path in the cell (usually 1cm)

v = Volume of the extract in ml and

w = Fresh weight of the sample in gram.

2.4: Determination of moisture content

Moisture content was determined by the conventional procedure.

Materials

a) Porcelain crucible.

b) Electrical balance.

c) Oven.

d) Dessicator.

Procedure

Four gram of leaves were weighed in a porcelain crucible (which was previously cleaned and heated to 100°C, cooled and weighed). The crucible containing the sample was heated in an electrical oven for about six hours at 100°C. It was then cooled in a dessicator and weighed. The process was repeated until constant weight was obtained.

Calculation

Percentage of moisture content (gram per 100 gram of mulberry leaves)

$$= \frac{Amount\ \ of\ \ moisture\ \ obtained}{Weight\ \ of\ \ mulberry\ \ leaf} \times 100$$

2.5: Determination of ash content

Ash content was determined following the method of A.O.A.C. (1980).

Materials

a) Porcelain crucible.
b) Electrical balance.
c) Dessicator.
d) Muffle furnace.

Procedure

Four gram of healthy and infected mulberry leaves were weighed separately in a porcelain crucible (which was previously cleaned and heated to about 100°C, cooled and weighed). The crucible with its content was placed in a muffle furnace

for four hours at about 600°C. These were then cooled in a dessicator and weighed. To ensure completion of ashing, the crucible was heated at the same temperature in the muffle furnace for half an hour, cooled and weighed again. This was repeated until constant weight was obtained.

Calculation

Percentage of ash content (gm per 100 gram of mulberry leaves)

$$= \frac{Amount\ \ of\ ash\ obtained}{Weight\ \ of\ mulberry\ \ leaf} \times 100$$

2.6: Determination of protein

Protein content of fresh and diseased mulberry leaves were determined by the method of Micro-Kjeldahl (Wong, 1923).

Reagents

a) Solid potassium sulphate
b) Concentrated sulphuric acid
c) 5% $CuSO_4.5\ H_2O$ in distilled water
d) 0.01N H_2SO_4 solution
e) Concentrated sodium hydroxide solution (5N approximately)
f) Boric acid solution containing bromocresol green (receiving fluid): 10 gram of boric acid was dissolved in hot water (250 ml) and cooled. 1ml of 0.1% bromocresol green in alcohol was added and diluted to 500 ml with distilled water.
g) Few quartz chips

h) Nitrogen determination apparatus according to Paranas-Wagner, made of JENA glass. All connections with inter-changeable ground joints.

Procedure

a) **Digestion:** Four gram of mulberry leaves, 8-10 ml of concentrated H_2SO_4, 1.0 gm of K_2SO_4, one to two drops of 5% $CuSO_4$ solution used as a catalyst and some quartz chips were added (to avoid bumping) in a Kjeldhal flask and mixed. The mixture was heated till it became light green (2-3 hours). The light green solution was taken in a 100 ml volumetric flask and made upto 100 ml with distilled water.

b) **Collection of ammonia:** The digestion was carried out in the steam distillation chamber of the nitrogen determination apparatus. The chamber is designed to act as a Micro-Kjeldhal flask and can be easily detached, when needed. After completion of digestion, the steam distillation chamber containing the digested mixture was fitted back to the nitrogen determination apparatus. Boric acid solution (15 ml) taken in a small flask was placed, so that the condenser outlet dipped below the surface of boric acid solution. Sufficient amount of concentrated sodium hydroxide solution (approximately, 30-40 ml) was added to the digest in the chamber to neutralize the amount of acid present. Steam was generated from the steam-generating flask and the sample in the chamber was steam distilled until 20 ml of distillate was collected in boric acid solution. The condenser outlet was then rinsed with little distilled water and the receiving flask was removed.

c) **Estimation of ammonia:** The ammonia in boric acid solution was titrated with 0.01N H_2SO_4 until the solution had been brought back to its original

yellow green colour. The titration was repeated with a control containing 20 ml boric acid solution and diluted approximately to the final volume of 40 ml. The two differences gave the volume of acid required for titration.

The total nitrogen content was calculated by using the formula given below:

1ml of 0.01N H_2SO_4 = 140μg of nitrogen in NH_3

Thus from the volume of standard sulphuric acid (H_2SO_4) used for titration, the amount of nitrogen in sample was calculated. This value multiplied by 6.25 give the approximate protein content of the sample used.

Percentage of protein content of mulberry leaf (gram per 100 gram of mulberry leaves)

$$= \frac{Amount\ of\ prtein\ obtained}{Weight\ of\ mulberry\ leaf} \times 100$$

2.7: Determination of Protein concentration by the Folin-lowry Method (Lowry *et al.* 1951).

Materials

(i) Alkaline sodium carbonate solution (20 gm/litre Na_2CO_3 in 0.1M NaOH solution)

(ii) Freshly prepared Copper sulphate and Sodium potassium tartrate solution (5 gm/litre $CuSO_4.5H_2O$ in 10 gm/litre Na-K tartrate).

(iii) Alkaline solution: Mixture of solution 1&2 in the proportion of 50:1 respectively.

(iv) Folin-Ciocolteau's reagent (Diluted with equal volume of H_2O, just before use).

(v) Standard protein (Bovine serum albumin 0.1mg/ml in dist. H_2O) solution.

Method

From standard protein solution of 0.0, 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 ml containing 0.0, 0.01, 0.02, 0.04, 0.06, 0.08 and 0.1 mg of protein were taken in different test tubes and made upto the volume 1ml by distilled water. Then 5ml of the alkaline solution (solution-iii) was added to the protein solution in different test tubes and mixed thoroughly. It was allowed to stand at room temperature for 10 minutes. Then 0.5 ml of diluted Folin-Ciocalteau's reagent was added rapidly with immediate mixing and left for 30 minutes. The dark blue color formed was measured at 650nm against the appropriate blank. By applying the same procedure described above the absorbance of purified protein solution was measured and the concentration of protein was determined from the standard curve (Fig. 2.1).

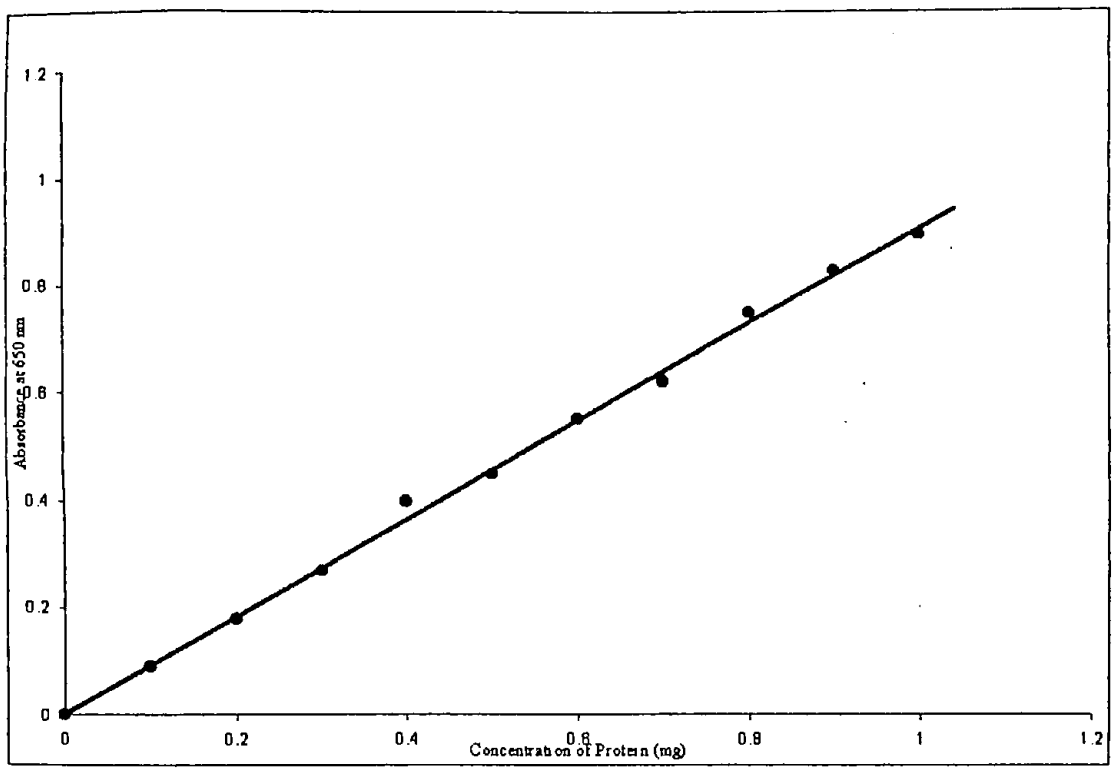


Fig. 2.1: Standard curve of protein

2.7. Determination of lipid content

Lipid content of mulberry leaf was determined by the method of Bligh and Dyer (1959).

Reagent

A mixture of chloroform and ethanol (2:1 v/v)

Procedure

Two gram of mulberry leaves was first grounded in a mortar with about 10 ml of distilled water. The grounded flesh was transferred to a separating funnel and 30 ml of chloroform-ethanol mixture was added. The mixture was mixed well and then kept overnight at room temperature in the dark. At the end of this period 20

ml of chloroform and 20 ml of water were further added and mixed well. Generally, three layers were seen. A clear lower layer of chloroform containing all the lipids, a colored aqueous layer of ethanol with all water soluble materials and a thick pasty inner phase were seen.

The chloroform layer was carefully collected in a pre-weighed beaker (50 ml) and then placed on a steam bath for evaporation. After evaporation of the chloroform, the weight of the beaker was determined again. The difference in weight gives the amount of lipid.

Calculation

Percentage of lipid content (gram per 100 gram of mulberry leaves)

$$= \frac{Amount\ of\ lipid\ obtained}{Weight\ of\ mulberry\ leaf} \times 100$$

2.8: Determination of total sugar content of mulberry leaves

Total sugar content of mulberry leaf was determined colorimetrically by the anthrone method (Jayaraman, 1981).

Reagents

a) **Anthrone reagent:** The reagent was prepared by dissolving 2 gram of anthrone in one litre of concentrated sulphuric acid (H_2SO_4).

b) **Standard glucose solution:** A standard solution of glucose was prepared by dissolving 10 mg of glucose in 100 ml of distilled water.

Preparation of sugar extract from mulberry leaves

Sugar extract of mulberry leaves was prepared by the following method (Loomis and Shull, 1937)

Four gram of fresh healthy and infected mulberry leaves were cut into small pieces and grinded in a mortar and immediately plunged into boiling ethyl alcohol and allowed to boil for 10 minutes (8 ml of alcohol was used for every gram of mulberry leaves). The extract was cooled and crushed thoroughly in a mortar with a pestle. Then the extract was filtered through two layers of muslin cloth and re-extracted the ground tissue for three minutes in hot 80% alcohol, using 2-3 ml of alcohol for every gram of tissue. This second extraction ensured complete removal of alcohol soluble substances. The extract was cooled and passed through muslin cloth. Both the extract were filtered through Whatman no. 41 filter paper.

The volume of the extract was evaporated to about ¼ th the volume over a steam bath and cooled. This reduced volume of the extract was then transferred to a 100 ml volumetric flask and made upto the mark with distilled water. Then 1 ml of the diluted solution was taken into another 100 ml volumetric flask and made upto the mark with distilled water.

Procedure

Aliquot of 1 ml of extracted solution was pipetted into test tubes and 4ml of the anthrone reagent was added to each of this solution and mixed well. Glass marbles were placed on top of each tube to prevent loss of water by evaporation. The tubes were placed in a boiling water bath for ten minutes, then removed and cooled. A reagent blank was prepared by taking 1 ml of water and 4ml of

anthrone reagent in a test tube and treated similarly. The absorbance of the blue green solution was measured at 620 nm in a spectrophotometer.

A standard curve of glucose was prepared by taking 0.0, 0.1, 0.2, 0.4, 0.6, 0.8 and 1 ml of standard glucose solution in different test tubes containing 0.0, 0.01 mg, 0.02 mg, 0.04 mg, 0.06 mg, 0.08 mg and 0.1 mg of glucose respectively and made the volume upto 1.0 ml with distilled water. Then 4 ml of anthrone reagent was added to each of the test tubes and mixed well. All these solutions were treated similarly as described above. The absorbance was measured at 620 nm using the blank containing 1 ml of water and 4 ml of anthrone reagent.

Calculation: The amount of total sugar present in the extract was calculated from the standard curve of glucose (Fig-2.2). Finally, the percentage of total sugar present in mulberry leaf was determined using the formula given below:

Percentage of total sugar (gram per 100 gram of mulberry leaves)

$$= \frac{Amount\ of\ total\ sugar\ obtained}{Weight\ of\ mulberry\ leaf} \times 100$$

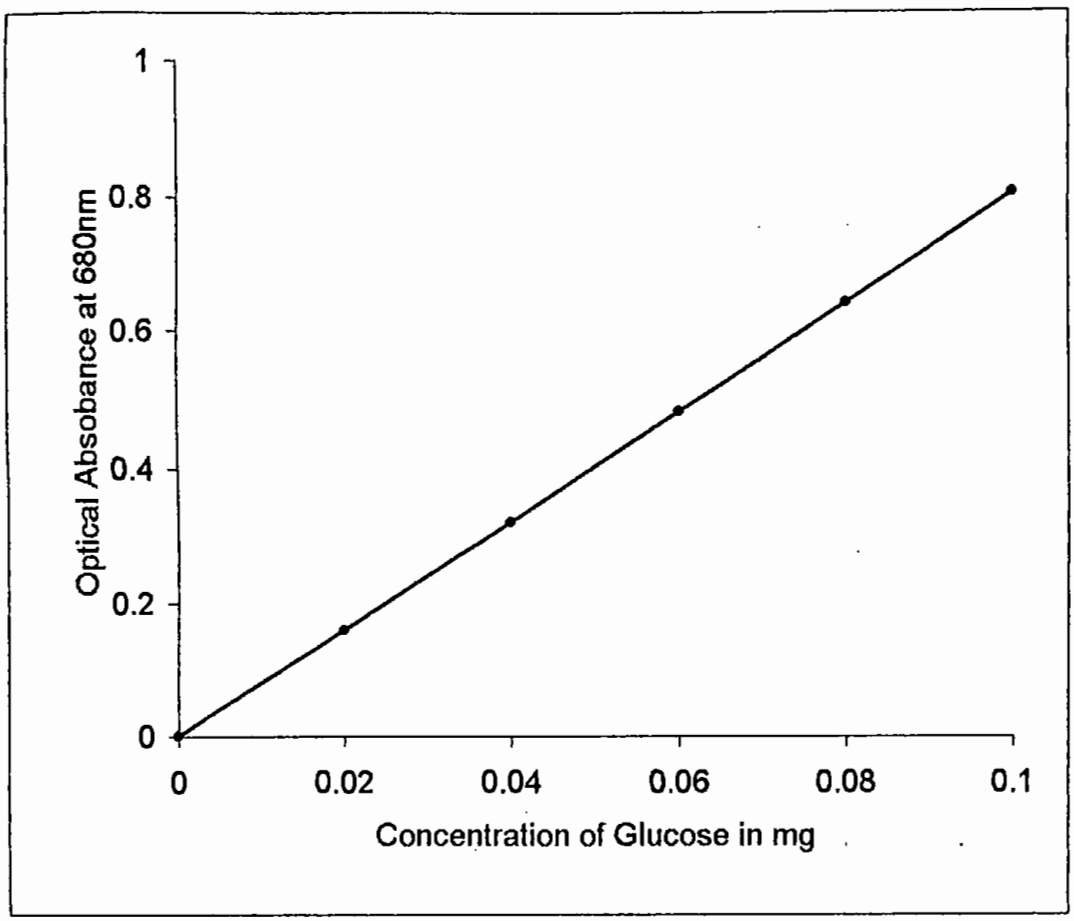


Fig.-2.2: Standard curve of glucose for estimation of total sugar and starch.

2.9. Determination of reducing sugar

Reducing sugar content of the healthy and diseased mulberry leaves were determined by dinitrosalicylic acid method (Miller, 1972).

Reagents

a) **Dinitrosalicylic acid (DNS) reagent:** Simultaneously 1gram of DNS, 200 mg of crystalline phenol and 50 mg of sodium sulphite were placed in a beaker and mixed with 100ml of 1% NaOH solution by stirring. If it is stored then sodium sulphite is added just before use.

b) **40% Rochelle salts.**

c) **Standard glucose solution:** A standard glucose solution was prepared by dissolving 50 mg of glucose in 100 ml of distilled water.

Preparation of reducing sugar extract of mulberry leaves

Reducing sugar extract of mulberry leaves was prepared following the procedure as described previously (2.8).

Procedure

Aliquot of 3 ml of the extract was pipetted into test tubes and 3 ml of DNS reagent was added to each of the solution and mixed well. The test tubes were heated for 5 minutes in a boiling water bath. After the color has developed, 1 ml of 40% Rochelle salt was added to each of the tubes, when the contents of the tubes were still warm. The test tubes were then cooled under a running tap water. A reagent blank was prepared by taking 3 ml of water and 3 ml of DNS reagent in a tube and treated similarly. The absorbance of the solution was measured at 575 nm in a colorimeter.

A standard curve of glucose was prepared by taking 0.0, 0.3, 0.6, 0.9, 1.2, 1.5 and 1.8 ml of standard glucose solution in different test tubes containing 0.0, 0.15 mg, 0.3 mg, 0.45 mg, 0.60 mg, 0.75 mg and 0.9 mg of glucose respectively and made the volume upto 3.0 ml with distilled water and 3 ml of DNS reagent in a tube and treated similarly. The absorbance of the solution was measured at 575 nm in a colorimeter.

Calculation: The amount of reducing sugar present in the extract was calculated from the standard curve of glucose (Fig-2.3). Finally, the percentage of total sugar present in mulberry leaf was determined using the formula given below:

The percentage of reducing sugar (gram per 100 gram of mulberry leaves)

$$= \frac{Amount\ of\ reducing\ sugar\ obtained}{Weight\ of\ mulberry\ leaf} \times 100$$

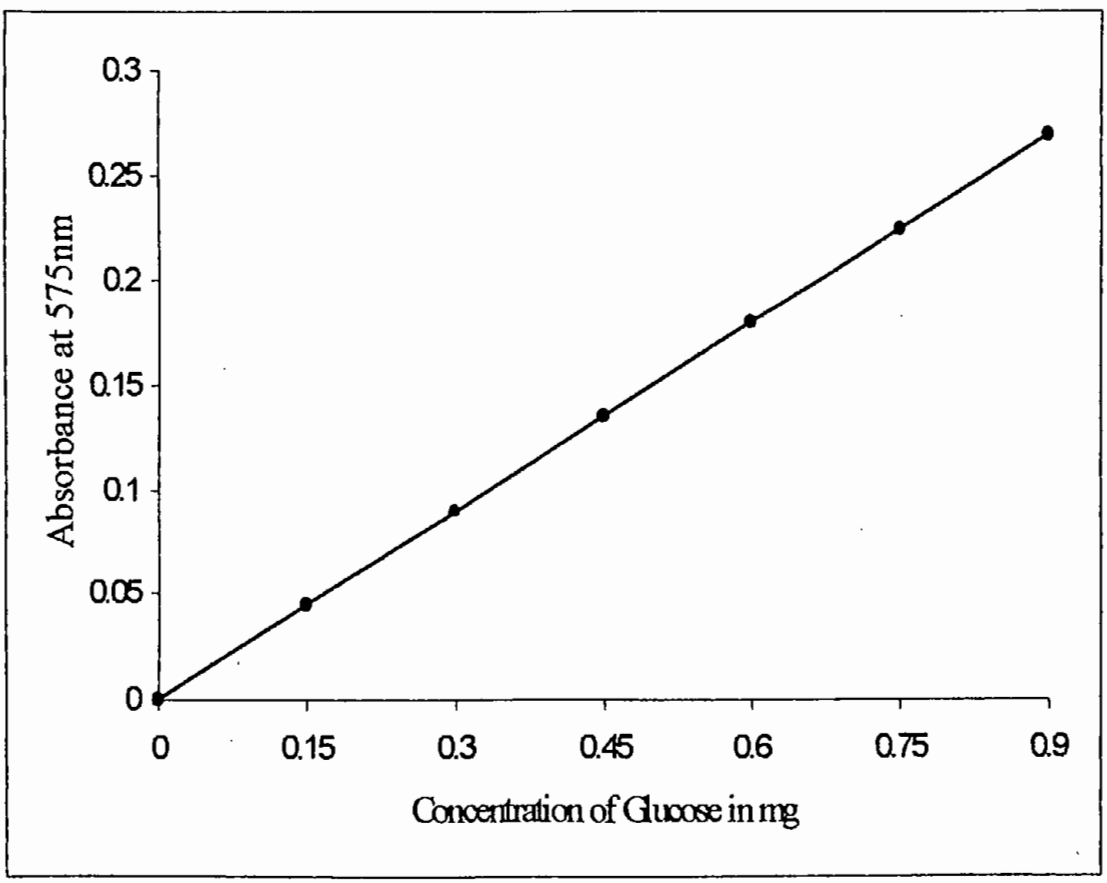


Fig.-2.3: Standard curve of glucose for estimation of reducing sugar

2. 10: Determination of non-reducing sugar content

Non reducing sugar content was calculated from the following formula (Ranganna, 1979)

% Sucrose or non reducing sugar = (% Total sugar- % Reducing sugar) × 0.95

2. 11: Determination of vitamin-C content

Vitamin-C content of mulberry leaf was determined by the Bessey's titrimetric method (1933).

Reagents

a) **Dye solution:** 200 mg of 2,6 dichlorophenol indophenol and 210 mg of sodium bicarbonate were dissolved in distilled water and made upto 1000 ml. The solution was filtered.

b) **3% metaphosphoric acid reagent:** 3 gram of meta-phosphoric acid was dissolved in 80 ml of acetic acid and made up to 100 ml with distilled water.

c) **Standard Vitamin-C solution (0.1 mg/ml):** 10 mg of pure vitamin-C was dissolved in 3% metaphosphoric acid and made upto 100 ml with 3% metaphosphoric acid.

Method

Four gram of healthy and infected mulberry leaves were separately cut into small pieces and homogenized well with 3% metaphosphoric acid (approximately 20 ml) and filtered through double layer of muslin cloth. The filtrate was centrifuged at 3,000g. for 10 minutes and the clear supernatant was taken in a conical flask and titrated with 2,6 dichlorophenol indophenol solution. Standard vitamin-C solution was taken in a conical flask and titrated with the same dye solution. The

amount of vitamin-C present in the extract was determined by comparing with the titration result of standard vitamin-C solution.

Calculation

Percentage of vitamin-C content (mg per 100 gram of mulberry leaves)

$$= \frac{Amount\ of\ vita\ \min - C\ of\ obtained}{Weight\ of\ mulberry\ leaf} \times 100$$

2.12: Estimation of Crude fibre

Crude fibre was determined by the following method (AOAC, 1980).

Reagents

a) H_2SO_4 (0.26N)
b) NaOH (1.25%)
c) Ethanol
d) Ether.

Procedure

Three gram of fat free mulberry leaves were taken into 500 ml beaker and 200 ml of boiling 0.26N H_2SO_4 was added. The mixture was boiled for 30 minutes, keeping the volume constant by the addition of water at frequent intervals (a glass rod inserted in the beaker help smooth boiling). At the end of this period, the mixture was filtered through a muslin cloth and the residue was washed with hot water till free from acid.

The extract was then transferred into the same beaker and 200 ml of boiling 1.25% NaOH was added. After boiling for 30 minutes (keeping the volume constant as before) the mixture was filtered through muslin cloth. The extract was washed with hot water until free from alkali, followed by washing with some ehtanol and ether. It was then transferred to a crucible dried overnight at 80°C-100°C and weighed.

The crucible was then heated in a muffle furnace at 600°C for three hours, cooled and weighed again. The difference in the weight represented the weight of crude fibre. The percentage of crude fibre (on dry basis) was calculated from the formula given below.

Calculation

Crude fibre content (gram per 100 gram of mulberry leaves)

$$= \frac{Weight\ of\ dried\ extract}{Weight\ of\ mulberry\ leaf} \times 100$$

2.13: Determination of Starch

The starch content of the mulberry leaves was determined by the anthrone method (Morse, 1947 and Loomis, 1937)

Reagents

a) Anthrone reagent (0.2% in concentrated H_2SO_4).

b) Standard glucose solution (10 mg/100 ml)

Procedure

Four gram of mulberry leaves were homogenized well with 20 ml of distilled water and the content was filtered through double layer of muslin cloth. To the filtrate, twice the volume of ethanol was added to precipitate the polysaccharide, mainly the starch. After kept it overnight in cold, the precipitate was collected by centrifugation at 3000 g for 15 minutes. The precipitate was dried over a steam bath, then 40 ml of 1M hydrochloric acid was added to the dried precipitate and heated to about 70°C. It was then transferred to a volumetric flask and diluted to 100 ml with 1M HCl. Diluted solution (1 ml) was taken in another 100 ml volumetric flask and made upto 100 ml with 1M HCl.

Aliquots of 1 ml of the mulberry leaf extract from each cultivar were pipetted into different test tubes in duplicate and treated in the same manner as described for free sugar estimation, previously.

A standard curve of glucose was prepared and the amount of starch in the mulberry leaf was calculated from the curve (Fig-2.2).

Calculation

Percentage of starch content (gram per 100 gram of mulberry leaves)

$$= \frac{Weight\ of\ starch\ obtained}{Weight\ of\ mulberry\ leaf} \times 100$$

2.14: Estimation of phenol

Estimation of phenols with Folin-Ciocalteu reagent is based on the reaction between phenols and an oxidizing agent phosphomolybdate, which results in the

formation of a blue complex. So, total phenol content of the mulberry leaf was determined colorimetrically by Folin-Ciocalteu method (Bray and Thorpe, 1954).

Reagents

a) Folin-Ciocalteu reagent
b) 20% Sodium carbonate
c) Catechol (0.1mg/ml)

Extraction of phenol

Extractions of phenols from mulberry leaves were done following the method as described before (2.8).

Method

Aliquot of 1 ml of the extract was pipetted into test tubes and 1 ml of Folin-Ciocalteu Reagent followed by 2 ml of sodium carbonate (Na_2CO_3) solution were added to each tube and mixed well. The test tubes were placed in a boiling water bath for exactly 2 minutes, then removed and cooled. The blue solution was then transferred to a 25 ml volumetric flask and made upto the mark with distilled water. The solution was filtered. A reagent blank was prepared by taking 1 ml of water and 1 ml of Folin-Ciocalteu reagent in a test tube and treated similarly. The absorbance of the blue solution was measured at 650 nm in a colorimeter. The amount of total phenol was calculated from the standard curve of catechol (Fig-2.4).

Calculation

Percentage of total phenol (mg per 100 gram of mulberry leaves)

$$= \frac{mg\ of\ total\ phenol\ obtained}{Weight\ of\ mulberry\ leaf} \times 100$$

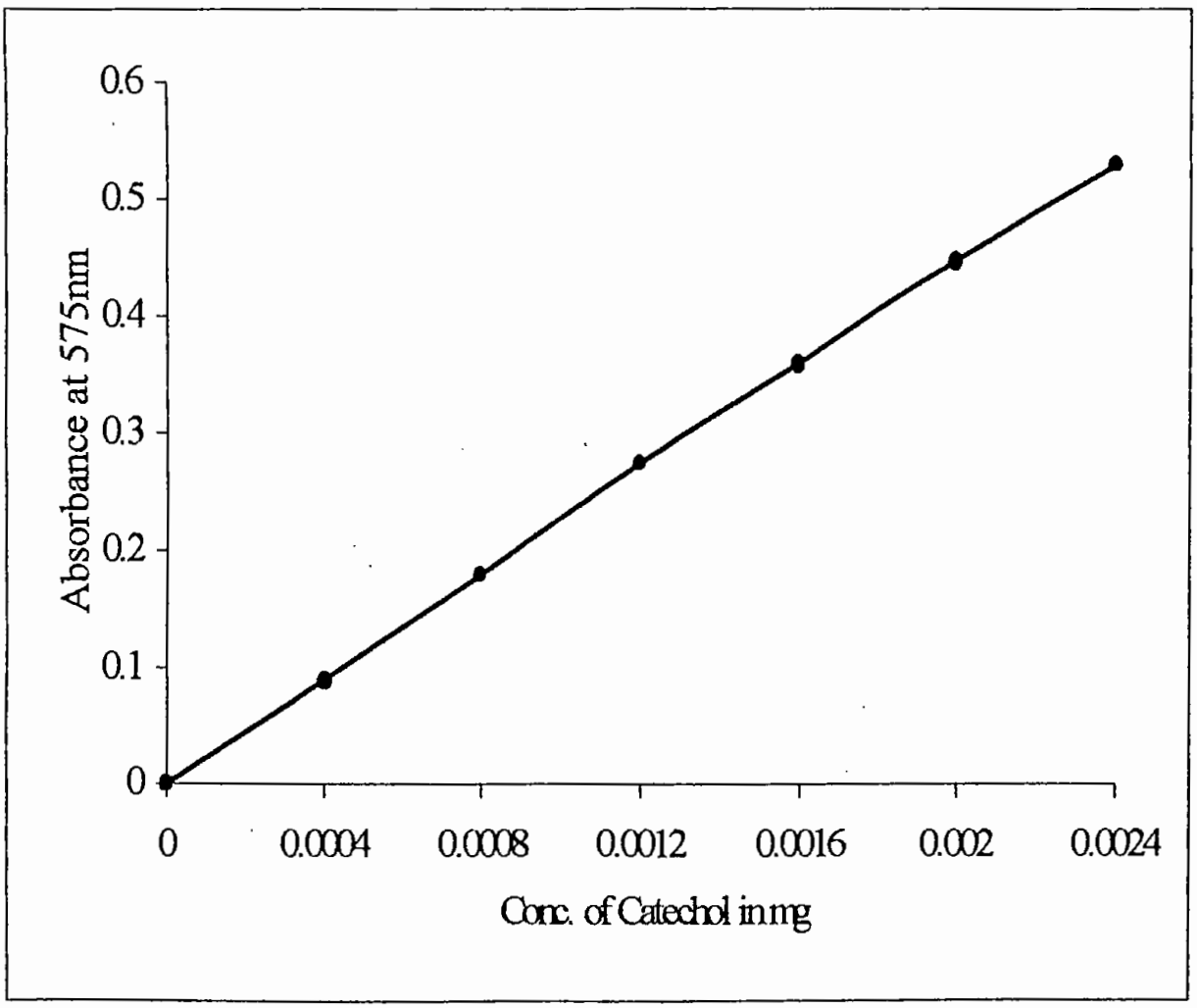


Fig. 2.4: Standard curve of catechol for estimation of total phenol

2.15: Determination of calcium content

Calcium content was determined by titrimetric method (Bernard, 1965).

Reagents

a) Hydrochloric acid (concentrated).
b) Ammonium oxalate (6%).
c) Methyl red indicator
d) Dilute sulphuric acid (2N).
e) Strong ammonia.
f) Potassium permanganate solution (N/100).

Preparation of stock solution

The ash obtained as described earlier was moistened with a small amount of distilled water (0.5-1.0 ml) and then 5 ml of concentrated HCl was added to it. The mixture was evaporated to dryness on a boiling water bath. 5 ml of concentrated HCl were added again and the solution was evaporated to dryness. The residue was dissolved in about 4 ml of HCl and a few ml of water, and the solution was warmed on a boiling water bath. The warmed solution was then filtered using Whatman No. 40 filter paper. After cooling, the volume was made upto 100 ml with distilled water and suitable aliquots were used for the estimation of calcium.

Procedure

25 ml of the stock solution was taken in a conical flask and 125 ml of double distilled water was added to it. A few drops of methyl red indicator was added and the mixture was neutralized with ammonia, till the pink colour changed to yellow. The solution was heated to boiling and 10 ml of ammonium oxalate was

then added. The mixture was allowed to boil for a few minutes and then glacial acetic acid was added to it till the colour became distinctly pink. The mixture was kept in dark at room temperature for an hour. When the precipitate was settled down, the supernatant was tested with a drop of ammonium oxalate solution to ensure the completion of the precipitation. The precipitate was then filtered through Whatman no. 40 filter paper and washed with warm water till the precipitate became free of oxalate (tested with $CaCl_2$). The precipitate was transferred to a beaker by piercing a hole in the filter paper and about 5 to 10 ml of dilute H_2SO_4 (2N) was poured over it. The solution was then heated to about 70°C and titrated with N/100 $KMnO_4$ solution.

Calculation

1ml of N/100 $KMnO_4$ solution ≡ 0.2004 mg of calcium

Amount of calcium content (mg per 100 gm of mulberry leaves)

$$= \frac{mg\ of\ calcium\ obtained}{Weight\ of\ mulberry\ leaf} \times 100$$

2.16: Determination of phosphorus

Phosphorus content of mulberry leaves was determined by the method of Vogel (1961).

Preparation of molybdate solution

12.50 gm of sodium molybdate ($Na_2MoO_4.2H_2O$) was dissolved in 10N H_2SO_4 and diluted to 500 ml with 10N H_2SO_4.

Preparation of Hydrazine sulphate solution

0.75 gm of hydrazine sulphate was dissolved in deionized water and diluted to 500 ml.

Preparation of standard phosphate solution

Exactly 0.219 gm of potassium dihydrogen phosphate was dissolved in de-ionized water and diluted to one litre.

Then, 1 ml solution ≡ 0.05 mg of phosphorus.

Preparation of stock solution

Same as described previously in case of calcium. Suitable aliquots of stock solution were used for the estimation of phosphorus.

Procedure

One ml of mulberry leaf extract from each cultivars were taken separately in the 50ml volumetric flask and added 2 ml of hydrazine sulphate and 5 ml of molybdate reagent and made upto the mark with deionized water. The mixture was mixed well. The flask was kept immersed in a boiling water bath for ten minutes, then it was removed and cooled rapidly. The absorbance for each of the solutions was measured at 830 nm against reagent blank.

Construction of calibration curve

A calibration curve (Fig-2.5) was constructed in the usual process by using six standard phosphorus solution containing 1, 2, 4, 6, 8, and 10 ppm of phosphorus. The mg percentage of phosphorus present in each different mulberry leaf extract was calculated by using the standard curve of phosphorus.

Calculation

Amount of phosphorus present (mg per 100 gm of mulberry leaves)

$$= \frac{mg\ of\ phosphorus\ obtained}{Weight\ of\ mulberry\ leaf} \times 100$$

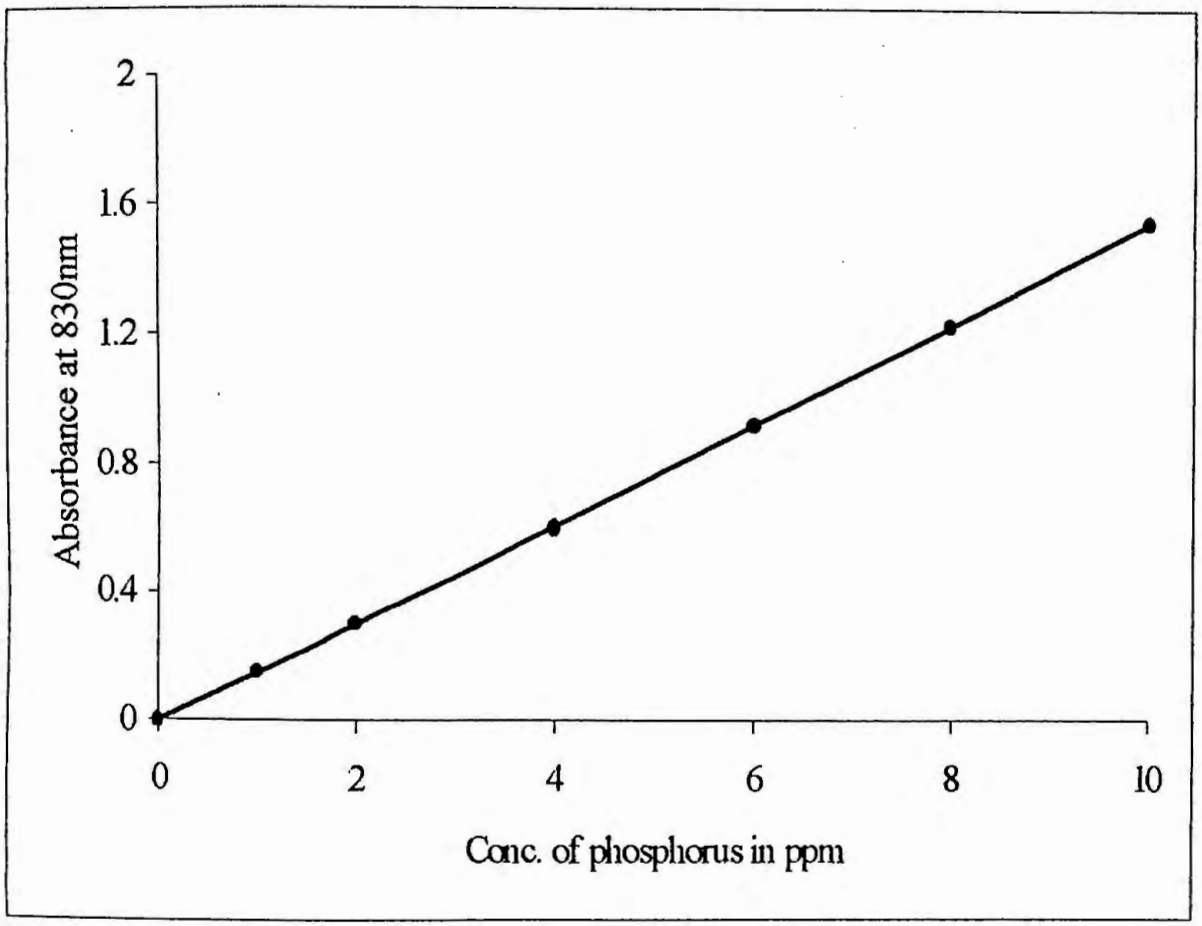


Fig. 2.5: Standard curve of phosphorus.

2.17: Determination of iron

Iron (Fe) content of mulberry leaves was determined spectrophotometrically by thiocyanate method (Vogel, 1961).

Reagents

a) 4N HCl

b) Potassium thiocyanate solution (20%)

c) **Preparation of Fe (III) standard solution:** Exactly 0.702 gm of ferrous ammonium sulphate was taken in a 1 litre volumetric flask and dissolved in 100 ml (2 gm/l.) distilled water. A dilute solution of potassium permanganate was run slowly until a faint pink colouration remained after stirring well. The resulting solution was then made upto mark with distilled water.

Then, 1 ml solution contains ≡ 0.1 mg of Fe (III).

Procedure

10 ml of mulberry leaf extract from each cultivars were taken separately in 50 ml volumetric flask and 2 ml of 4N HCl and 5 ml of 20% potassium thiocyanate solution were added in each of the flask. Then each flask was made upto the mark with de-ionized water. The absorbance for each of the solution was measured at 480 nm against a reagent blank.

Iron content of this solution was determined by constructing a standard curve.

Construction of standard curve

A standard curve of iron (Fig-2.6) was prepared by taking 0.5 ml, 1.0 ml, 1.5 ml, 2.0 ml, 2.5 ml and 3.0 ml of the standard (0.1 mg/ml) ammonium iron (III) sulphate solution. To the flask, 2 ml of 4N hydrochloric acid and 5 ml of 20%

potassium thiocyanate solution were added and diluted upto the mark and shaken slowly for uniform mixing. The absorbance for each of the solution was measured at 480 nm against a reagent blank.

The mg percent of iron present in each different variety of healthy and disease infected mulberry leaf extract were calculated by using the standard curve.

Calculation

Amount of iron present (mg per 100 gm of mulberry leaves)

$$= \frac{mg\ of\ iron\ obtained}{Weight\ of\ mulberry\ leaf} \times 100$$

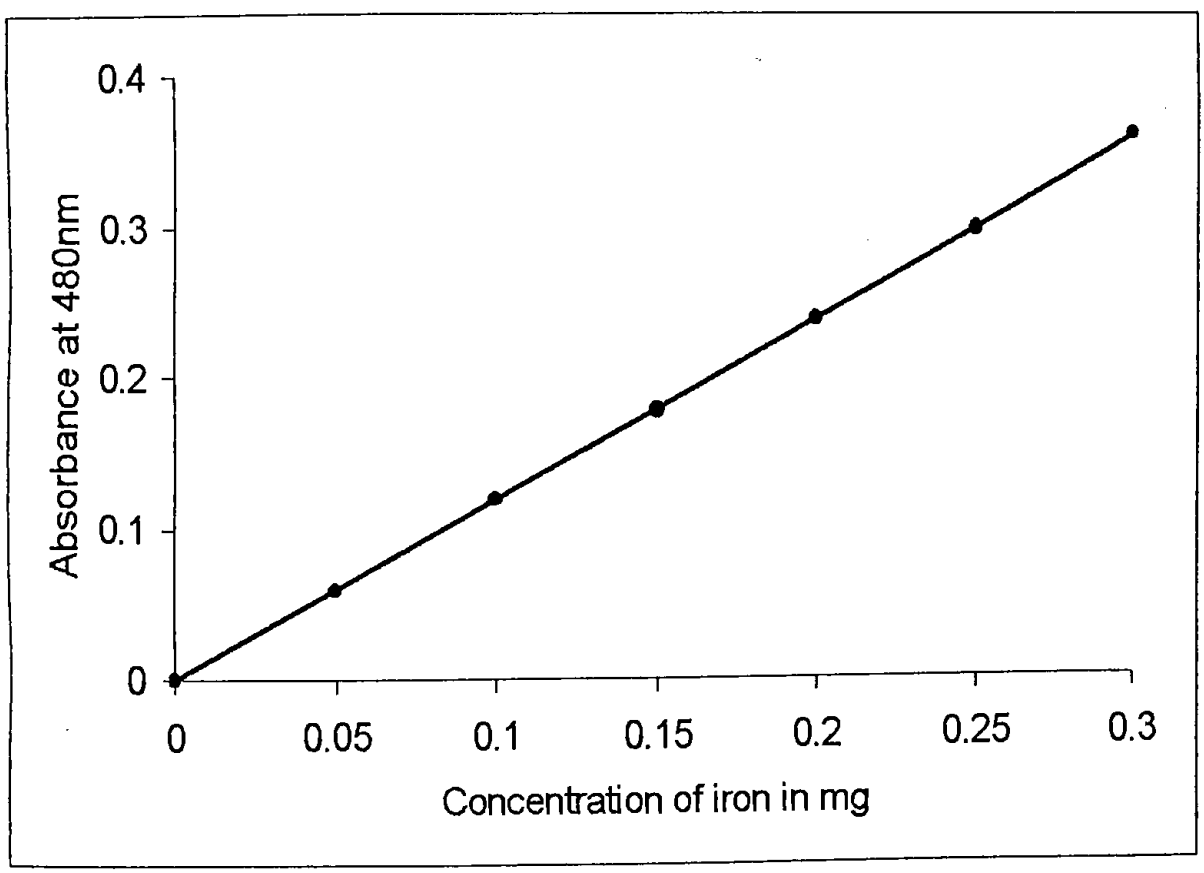


Fig.2.6: Standard curve of iron.

2.18: Estimation of β-carotene

β-carotene content of mulberry leaves were determined according to the procedure described in the method of Vitamin Assay (Anon,1960) and method of Biochemical analysis (Glick, 1957)

Reagents

a) Ammonium sulphate
b) Acetone
c) Petroleum ether (40-60°C)
d) n-Hexane
e) Activated alumina (BDH chemicals Ltd.)
f) Standard solution of β-carotene: A standard solution of β-carotene (BDH chemicals Ltd.) was prepared by dissolving 50 mg of β-carotene in 100 ml of petroleum ether.

Column preparation

A column (400mm × 2.5mm) was prepared by using alumina as a packing material. 10% acetone in petroleum ether was used as an eluant buffer.

Procedure

Four gram of fresh healthy and diseased mulberry leaves and about four gram of ammonium sulphate were taken in a mortar, and rubbed to an even paste with pestle. The extraction was carried out with acetone and small amount of hexane. Extraction was continued until the acetone extract became colorless. Potassium hydroxide solution (10 ml, 5.6%) was added to the extract and it was kept in a dark place for half an hour. The mixture was then transferred to a separating

funnel, then 20ml of petroleum ether, a few ml of hexane and 10 ml of water were added to the separating funnel and shaked gently. The ether layer was collected and the process was repeated until the petroleum ether layer became colorless. The petroleum ether extract was concentrated by gentle heating. The concentrated extract (1-2 ml) was applied onto the top of the alumina column and eluted with 10% acetone in petroleum ether. The absorbancy of the eluant was taken at 440nm in a Coleman Junior-II spectrophotometer.

Construction of standard curve of β-carotene

A standard curve was prepared by taking 0.0, 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 ml of standard solution containing 0.0, 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 mg of β-carotene and the volume was made upto 5 ml with petroleum ether and mixed well. The absorbancy of the solutions were taken at 440 nm in a Coleman Junior II spectrophotometer and a standard curve of β-carotene was prepared by plotting the data.

The amount of β-carotene content in each cultivar of mulberry leaf was calculated by using the standard curve (Fig.-2.7).

Calculation

mg percent of β-carotene (mg per 100 gm of mulberry leaves)

$$= \frac{mg\ of\ \beta - carotene\ obtained}{Weight\ of\ mulberry\ leaf} \times 100$$

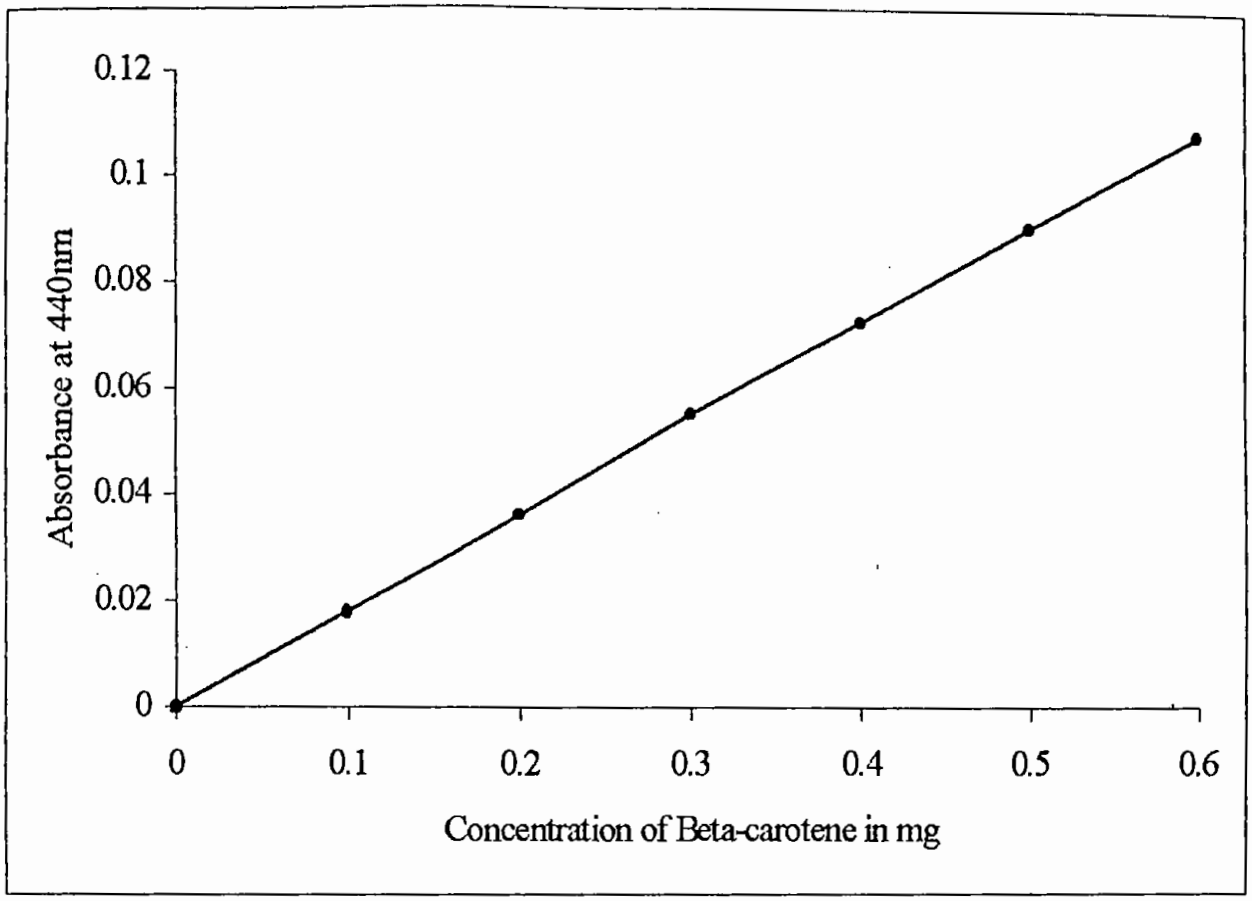


Fig.-2.7: Standard curve of β-carotene.

2. 19: Determination of vitamin-B_1 content

Vitamin-B_1 content of mulberry leaf was determined following the method of Anon (1965).

Reagents

a) **Potassium ferricyanide (2%)**

b) **Oxidizing reagents:** 10 ml of 2% potassium ferricyanide was mixed with 3.5N NaOH solution (90ml). This solution might be used within 4 hours.

c) **Quinine sulphate**

d) **0.2N HCl**

e) **Alcohol**

Preparation of standard thiamine hydrochloride solution

About 25 mg of thiamine hydrochloride was taken in 1000 ml volumetric flask and it was dissolved in 300 ml of dilute alcohol solution. The pH was adjusted to 4.0 with dilute HCl and stored in a light resistant container.

Procedure

Five to six gram of fresh healthy and disease infected mulberry leaves were cut into small pieces and homogenized well with 0.2N HCl. The mixture was heated on a steam bath and cooled. Standard thiamine-HCl solution (5 ml) was mixed rapidly with 3ml oxidizing reagent and then 20 ml of isobutyl alcohol was added within 30 seconds. The mixture was mixed vigorously for 90 seconds by shaking the tubes manually. A blank was prepared only by substituting the oxidizing reagent with an equal volume of 3.5N sodium hydroxide and proceed in the same manner. 2 ml of dehydrate alcohol was added, by swirling for few seconds, allowed the phase to be separate decanted and transferred into cuvettes for measurement of fluorescence. Mulberry leaves extract (5ml) were pipetted in different test tubes and treated in the same manner as described above.

Calculation

The amount (in milligram) of thiamine hydrochloride in each 5 ml of the mulberry leaf extract was calculated from the formula (A-b)/(S-d), in which A and S were the average fluorometer reading of the portions of mulberry leaf extract and standard preparation with oxidizing reagent, respectively, and b and d were the readings for the blanks of mulberry leaves extract and standard preparation respectively.

mg percentage of vitamin-B_1 (mg per 100 gm of fresh mulberry leaves)

$$= \frac{mg\ of\ vita\min B_1\ obtained}{Weight\ of\ mulberry\ leaf} \times 100$$

2. 20: Determination of vitamin B_2

Vitamin B_2 content of mulberry leaves was determined by the method of Anon (1965).

Reagents

a) 0.02N acetic acid
b) 0.1N H_2SO_4
c) 0.1N NaOH
d) 0.1N HCl
e) 4% Potassium permanganate
f) Hydrogen peroxide
g) Standard riboflavin solution.

Procedure

Preparation of standard riboflavin: 50 mg of riboflavin was mixed with 300 ml of 0.02N acetic acid and the mixture was heated on steam bath, with frequent agitation until the riboflavin was dissolved. Then cooled and made upto 500 ml with 0.02N acetic acid. This solution was diluted appropriately with 0.02N acetic acid to made final riboflavin concentration of 10 µg/ml.

Extraction of riboflavin from mulberry leaves

Fresh four gram of mulberry leaves were cut into small pieces and homogenized well with 0.1N H_2SO_4 (about 50 ml). The mixture was heated in an autoclave at 121-123°C for 30 minutes then cooled it and filtered through double layer of muslin cloth. The filtrate was made upto 100 ml with distilled water and 25 ml of this solution was taken in a beaker and 25 ml of water was added to it. The mixture was agitated vigorously and adjusted to the pH 6.0-6.5 with 0.1N NaOH. Immediately, 0.1N HCl was added until no precipitation occurs. The extract was again filtered and pH of the extract was adjusted to 6.6-6.8 with 0.1N NaOH.

Mulberry leaf extract (10 ml) was taken in the test tube and 1.0 ml of water plus 1.0 ml of glacial acetic acid were added to it. The mixture was then mixed with 0.05 ml of potassium permanganate solution and allowed to stand for two minutes. Then 0.5 ml of hydrogen peroxide solution was added, where upon the permanganate color was destroyed within 10 seconds. The tube was shaken vigorously until excess oxygen expelled. 1 ml of standard solution was pipetted in a test tube and treated in the same manner as that described for the leaf extract. The fluorescence of the solution was measured by a suitable flurometer. Then, to each tubes 20 mg of sodium hydrosulphite were added, mixed well and the fluorescence was measured, within 5 seconds.

Calculation

The quantity in milligram of each ml of the mulberry leaf extract was calculated by the formula, $0.0001 (I_u-I_S) (I_S-I_u)$

Where,

I_u = Average reading for mulberry leaf extract

I_S = Average reading for standard preparation

I_B = Average reading for mixed with sodium hydrosulphite

mg percentage of vitamin-B_2 content in mulberry leaf (mg per 100gm of mulberry leaves)

$$= \frac{mg\ of\ vitamin\ B_2\ obtained}{Weight\ of\ mulberry\ leaf} \times 100$$

RESULTS AND DISCUSSIONS

R-2.1: pH and Total Titratable Acidity (TTA) of Mulberry leaves

pH and TTA of healthy and disease infected Mulberry leaves at mature stage are given in the Table 2.1. It can be concluded from the results that the pH of the healthy mulberry leaf pulp was always in the moderately acid region.

Table-2.1: pH and TTA of healthy and disease infected Mulberry leaves at mature stage.

Variety of leaves	Types of leaves	pH	TTA (ml of 0.1N NaOH required/100 gm of leaf extract)
BM-1	Healthy	6.00 ±0.02	23.24±0.02
BM-1	Tukra	5.44±0.04	36.37±0.03
BM-1	Leaf spot	5.64±0.02	32.33±0.02
BM-1	Powdery mildew	5.75±0.05	29.30±0.02
BM-2	Healthy	6.24±0.01	18. ±0.02
BM-2	Tukra	5.46±0.04	36.56±0.03
BM-2	Leaf spot	5.48±0.02	35.44±0.01
BM-2	Powdery mildew	5.82±0.01	28.04±0.02
BM-3	Healthy	5.94±0.02	24.08±0.04
BM-3	Tukra (Viral)	5.22±0.04	41.21±0.01
BM-3	Leaf spot	5.40±0.03	37.82±0.03
BM-3	Powdery mildew	5.61±0.01	32.10±0.02
BM-4	Healthy	6.10±0.02	19.85±0.01
BM-4	Tukra	5.42±0.02	37.62±0.01
BM-4	Leaf spot	5.66±0.03	32.02±0.02
BM-4	Powdery mildew	5.72±0.01	30.92±0.01

Further, the leaf extract becomes slightly more acidic after infection of leaves with disease. These results were further confirmed from the results of TTA estimation as presented in the Table-2.1.

R-2.2: Chlorophyll content of mature Mulberry leaves

Disease development causes impairment in the photosynthetic pigments that affect the utilization of light energy by the plants. This interferes with the biochemical events occurring in the diseased tissue and culminates in reduced photosynthesis and their yield. Chlorophyll is the green pigment universally present in all photosynthetic tissues. Disease development affects not only the total chlorophyll content but also alters the ratio between chlorophyll a and chlorophyll b. Chlorophyll estimates may also be required to relate other biochemical changes in the plant tissue (Mahadevan and Sridhar, 1982).

Chlorophyll content of four varieties of healthy and disease infected mulberry leaves are given in the Table-2.2.

As given in the table, healthy mulberry leaves contained significant amount of chlorophyll. Of the varieties examined BM-3 variety contained highest amount of chlorophyll (322 mg%) followed by BM-1 (275 mg%) and so on in decreasing order. It can be concluded that the chlorophyll content of mulberry leaves decreased (50-60%) significantly when the leaves were infected with diseases. The results also indicate that not only decreases total chlorophyll but also decrease chlorophyll-a and chlorophyll b. Similar results were reported by Tofazzol *et. al.* (1999), Sasikumaran *et. al.* (1979), Borah *et.al.* (1978) and Subba *et.al.* (1979).

Table-2.2: Chlorophyll content of matures healthy and disease infected Mulberry leaves.

Variety of leaves	Types of leaves	Total Chlorophyll (mg%)	Chlorophyll a (mg%)	Chlorophyll b (mg%)
BM-1	Healthy	275.25±0.03	185.20±0.02	90.52±0.01
BM-1	Tukra	125.20±0.04	85.34±0.05	39.86±0.02
BM-1	Leaf spot	108.68±0.04	78.84±0.03	29.84±0.04
BM-1	Powdery mildew	110.75±0.02	79.95±0.05	30.81±0.03
BM-2	Healthy	255.45±0.04	175.25±0.02	80.20±0.05
BM-2	Tukra	128.35±0.03	87.45±0.01	40.96±0.01
BM-2	Leaf spot	110.18±0.05	75.68±0.03	34.50±0.05
BM-2	Powdery mildew	130.86±0.02	88.74±0.03	42.06±0.02
BM-3	Healthy	322.25±0.04	194.30±0.04	127.95±0.02
BM-3	Tukra (Viral)	195.25±0.05	121.73±0.03	73.52±0.04
BM-3	Leaf spot	135.40±0.02	91.42±0.02	43.98±0.03
BM-3	Powdery mildew	202.25±0.04	132.56±0.03	69.69±0.02
BM-4	Healthy	265.20±0.04	179.82±0.04	83.22±0.01
BM-4	Tukra	145.20±0.02	102.56±0.03	42.64±0.03
BM-4	Leaf spot	120.50±0.03	87.52±0.02	32.98±0.04
BM-4	Powdery mildew	150.28±0.04	106.56±0.03	43.72±0.05

R-2.3: Moisture content of mulberry leaves

Moisture plays an important role in the growth activities of plants, herbs etc. Water is indispensable to the absorption and transportation of food to carry on photosynthesis, metabolism of materials and the regulation of temperature.

Moisture is also essential for most of the physiological reaction in plant tissue and in its absence, life does not exist (Rangaswami, 1976).

It was found that the moisture content of mulberry leaves was affected after infection with diseases. As presented in Table-2.3. The moisture content in healthy leaves were found to be varied between 70-75% while that was increased moderately in viral infected leaves (74-76%).

On the other hand, the moisture content was found to be decreased remarkably after infection of healthy leaves with fungal diseases and its content was found to be varied between 58-69%. The increase in moisture content of viral infected leaves might be due to higher metabolic decomposition of solid materials by virus, which decreased the moisture content in fungal affected mulberry leaves. Hossain *et.al.*(1999) reported similar result.

R-2.4: Ash content of mulberry leaves

Mineral elements of mulberry leaves must plays important role as food for silkworm. Minerals of mulberry leaves has to do with physiological alkalinity of food, when mulberry leaves are ingested by silkworm (Anon, 1975).

Most of the inorganic constituent or minerals are present in ash. Ash content of four varieties of healthy and disease infected mulberry leaves are represented in the Table-2.3. The results indicated that the mineral contents of mulberry leaf decreased significantly after infected by disease. The ash content of healthy and disease infected mulberry leaves were varied between 3.0-3.4% and 2.6-2.8% respectively.

Table-2.3: Moisture and Ash content of mature healthy and disease infected Mulberry leaves.

Variety of leaves	Types of leaves	Moisture (%)	Ash (%)
BM-1	Healthy	71.96±0.14	3.18±0.10
BM-1	Tukra	76.47±0.21	2.74±0.08
BM-1	Leaf spot	64.55±0.11	2.83±0.12
BM-1	Powdery mildew	68.44±0.12	2.72±0.06
BM-2	Healthy	72.84±0.10	3.08±0.08
BM-2	Tukra	74.88±0.14	2.62±0.04
BM-2	Leaf spot	60.94±0.12	2.72±0.06
BM-2	Powdery mildew	66.23±0.22	2.81±0.05
BM-3	Healthy	74.86±0.18	3.05±0.09
BM-3	Tukra	76.46±0.14	2.74±0.05
BM-3	Leaf spot	58.35±0.12	2.83±0.06
BM-3	Powdery mildew	69.43±0.18	2.71±0.08
BM-4	Healthy	70.98±0.09	3.40±0.04
BM-4	Tukra	76.00±0.12	2.71±0.06
BM-4	Leaf spot	62.18±0.08	2.56±0.10
BM-4	Powdery mildew	67.31±0.12	2.86±0.08

R-2.5: Protein content of mulberry leaves

Protein plays an important role in all biological processes. The protein constituents of plants and herbs although occurring in low concentrations are of primary importance not only as component of nuclear and cytoplasmic structures but also including as they must be the full complement of enzymes involved in

metabolism during growth, development, maturation and the post harvest of the plant (Hansen, 1970).

Protein is indispensable for the body of mulberry plant as well as silkworm. Apart from building up the worm's body, 1/3 of the digested and absorbed nutrients from leaves is used for energy consumption and the consumption rate being most substantial for carbohydrate but least for protein. 70% of the protein absorbed from the mulberry leaves is used to produce silk (Rangaswami, 1976).

Protein content of healthy and disease infected mulberry leaves are given in the Table-2.4. As given in the table, the mulberry leaves are a very good sources of protein and the content of protein was found to be increased remarkably after infection of leaves with viral as well as fungal diseases. The protein content in healthy and disease infected mulberry leaves were varied between 4.2-4.6% and 4.8-5.3% respectively.

Among the four varieties of mulberry plants examined in the present study, BM-4 contained the highest amount of protein (4.62%), followed by BM-1 and so on in decreasing order. Further more than 3% of protein found in mulberry leaves are water-soluble. Similar results were also reported in purple vein virus infected tomato leaves by Nuhu Alam *et al.*, (1995). Singh (1987) also reported higher content of protein and nitrogen in infected rose leaves. According to Hayshi (1962) the accumulation of nitrogen due to increase in the activity of amino acid activating enzymes, which indicates that an increase in protein content in virus infected leaf is due to synthesis of viral protein.

R-2.6: Lipid content of mulberry leaves

Lipid is more useful in silkworm body than in the body of mulberry tree. Fat serves as efficient source of energy and insulating material. Dietary fat helps in the absorption of fat soluble vitamins, lipo-protein are important cellular constituents. In silkworm the growth and development depend on the dietary sterol. Phospholipids also proved to be effective for growth improvement (Ito, 1972). Lipids are essential components of cell membrane, source of metabolic energy for cell maintenance, flight, reproduction and embrayogenesis in insects (Patton *et.al.*, 1941).

Lipid content of four varieties of healthy and diseased mulberry leaves is given in the Table-2.4. As presented in the Table, the leaves of mulberry contained about 2% lipid but its content was found to be decreased by 25-35% after infection of mulberry leave with fungal and viral diseases.

R-2.7: Total sugar content

The total carbohydrate percentage reflects the physiological activity of the plant, whereas its total weight per leaf represents the accumulated results of the metabolic activity (Hassanein *et. al.*, 1962). Carbohydrates of mulberry leaves play an important role for the healthy growth of silkworm, especially for infant larval growth. Protein or other elements of mulberry are also synthesized from carbohydrate (Anon, 1975). The rate of fatty acid synthesis in silkworm larvae is regulated by the dietary levels of fatty acid and carbohydrate (Horie and Nakasone, 1971). The rates of carbohydrate utilization by the silkworm are co-related to a high degree with their nutritive value for this insect (Ito and Tanaka, 1961).

Table-2.4: Protein, water soluble protein and lipid contents of mature healthy and disease infected Mulberry leaves.

Variety of leaves	Types of leaves	Protein (%)	Water soluble protein (%)	Lipid (%)
BM-1	Healthy	4.12±0.08	3.12±0.12	1.97±0.09
BM-1	Tukra	5.18±0.11	3.94±0.14	1.42±0.06
BM-1	Leaf spot	5.58±0.10	4.48±0.11	1.28±0.07
BM-1	Powdery mildew	5.02±0.06	3.89±0.16	1.30±0.04
BM-2	Healthy	4.08±0.09	3.23±0.10	2.00±0.05
BM-2	Tukra	5.02±0.12	4.02±0.14	1.38±0.08
BM-2	Leaf spot	5.11±0.08	4.04±0.12	1.50±0.06
BM-2	Powdery mildew	5.09±0.06	3.97±0.09	1.32±0.08
BM-3	Healthy	4.12±0.08	3.22±0.08	2.18±0.05
BM-3	Tukra (Viral)	4.98±0.12	3.17±0.08	1.34±0.02
BM-3	Leaf spot	5.14±0.14	3.75±0.05	1.38±0.07
BM-3	Powdery mildew	5.02±0.10	3.72±0.04	1.42±0.04
BM-4	Healthy	4.62±0.12	3.32±0.06	2.10±0.08
BM-4	Tukra	5.22±0.08	4.18±0.09	1.48±0.10
BM-4	Leaf spot	5.46±0.10	4.24±0.04	1.32±0.06
BM-4	Powdery mildew	5.28±0.08	3.96±0.08	1.38±0.04

Total sugar content of four varieties of healthy and disease infected mulberry leaves at mature stage are given in the Table-2.5.

It can be concluded from the present data that the total sugar content of mulberry leaves decreased significantly (35-50%) after infected with microbial diseases. As shown in the Table-2.5, the total sugar content in healthy and disease infected

mulberry leaves were found to be varied between 2.7-3.0% and 1.4-1.6% respectively.

Hossain, *et. al.* (1999) reported that the total sugar, reducing sugar, non reducing sugar contents reduced of mango leaves when infected with fungus. Naik *et al.*, (1988) also reported that the total sugar content of betel vine leaf decreased when infected with *Colletotrichum gloeosporioides.* Anjana Singh and Sinha (1982) found that *Aspergillus flavus* and *A. parasiticus* caused considerable losses in the quantity of total sugar in tomato leaves.

R-2.8: Reducing and non-reducing sugar content

The present data also indicated that the content of reducing as well as non-reducing sugar in mature mulberry leaves were decreased remarkably (40-50%) after infected with diseases. The healthy mulberry leaves contained about 0.59-0.71% and 2.0-2.3% while the disease infected leaves contained 0.29-0.42% and 1.06-1.26% of reducing sugar and non-reducing sugar respectively (Table-2.5).

Sharma and Wahab (1975) reported the gradual reduction of reducing, non-reducing and total sugar in leaf spot infected barly tissue during the disease development. Shukla, *et.al.* (1972) reported the decrease in total sugar, reducing sugar and starch content in jute when inoculated and infected with *Macrophomina, olletorichum* and *Botrydiplodia* sp. Hedge and Monjul (1967) noted a considerable reduction of reducing sugar in pods infected with *C. Lindenullianum* while Anjana and Sinha (1983) reported that *A. flavus* and *A.*

parasiticus caused considerable losses in the quantity of reducing and non-reducing sugar in mulberry leaves. Naik *et al.*, (1988) stated that the percentage of reducing sugar content reduced in betel vine leaves after infected with *Colletotrichum gloeosporioides*. Mehta *et al.* (1995) also found that sugar content decreased due to fruit-rot disease of tomato.

R-2.9: Starch content of mulberry leaves

Starch is the store carbohydrate of chlorophyll containing plants. In plants, the starch is laid down in the cells in granules.

As presented in Table-2.5 The healthy mulberry leaves contained about 6.38-7.22% while disease infected mulberry leaves contained about 3.22-4.14% starch. It might be concluded from the results that the starch content of mulberry leaves decreased drastically after infection with diseases. The reduction of starch may be due to the hydrolysis of starch in diseased leaves by micro-organism for their carbon source. Similar results were also obtained by Gary and Mandar (1975), who noticed the decrease in starch in dowry mildew infected permillet leaves. Kumar *et al.*, (1977) reported that the starch content of early blight disease of potato.

Table-2.5: Total sugar, reducing sugar, non-reducing sugar and starch contents of mature healthy and disease infected Mulberry leaves.

Variety of leaves	Types of leaves	Total sugar (%)	Reducing sugar (%)	Non reducing sugar (%)	Starch (%)
BM-1	Healthy	2.82±0.04	0.62±0.04	2.20±0.06	6.46±0.11
BM-1	Tukra	1.40±0.06	0.32±0.03	1.08±0.04	4.15±0.08
BM-1	Leaf spot	1.46±0.02	0.36±0.01	1.10±0.08	3.82±0.04
BM-1	Powdery mildew	1.42±0.08	0.34±0.05	1.08±0.05	3.94±0.09
BM-2	Healthy	3.02±0.03	0.71±0.05	2.31±0.10	6.82±0.07
BM-2	Tukra	1.62±0.04	0.36±0.02	1.26±0.04	4.12±0.09
BM-2	Leaf spot	1.58±0.05	0.42±0.02	1.16±0.08	4.06±0.05
BM-2	Powdery mildew	1.46±0.03	0.38±0.01	1.08±0.06	4.11±0.08
BM-3	Healthy	2.82±0.06	0.59±0.02	2.23±0.04	7.22±0.04
BM-3	Tukra (Viral)	1.43±0.02	0.29±0.04	1.14±0.06	3.72±0.09
BM-3	Leaf spot	1.51±0.05	0.38±0.01	1.13±0.08	4.06±0.05
BM-3	Powdery mildew	1.44±0.06	0.37±0.03	1.07±0.10	4.14±0.08
BM-4	Healthy	2.74±0.03	0.68±0.03	2.06±0.03	6.38±0.05
BM-4	Tukra	1.44±0.05	0.38±0.04	1.06±0.06	3.52±0.09
BM-4	Leaf spot	1.46±0.02	0.38±0.02	1.08±0.05	3.68±0.06
BM-4	Powdery mildew	1.51±0.04	0.41±0.05	1.10±0.07	4.04±0.06

R-2.10: Crude fibre content of mulberry leaves

Silkworm larvae can not digest crude fibre. Cellulose, lignin and pentosan are the component of crude fibre of mulberry leaves (Anonymous, 1975). In mulberry leaves cellulose is present in cell wall, stimulate the swallowing factor (Ravindra,

1987). Crude fibre has pronounced effect on the digestion and absorption process of nutrients.

Crude fibre content of four varieties of healthy and disease infected mulberry leaves at mature stage are given in the Table-2.6.

The present data indicated that mature healthy and diseased infected mulberry leaves contained about 6-7% and 3.4-4.8% crude fibre respectively.

Wahab *et al.*, (1984) also found that crude fibre content of betel leaves decreased after infection with *C. gloeosporioides.*

R-2.11: Ascorbic acid content in mulberry leaves

Ascorbic acid is a strong phago stimulant for the silkworm *Bombyx mori* L. and necessary for the better growth and development of the silkworm (Ito, 1961). Without Vitamin-C no growth is obtained beyond the second instar. Nutritionist suggested the biosynthesis of ascorbic acid from D-glucose, Vitamin D, glucuronolactone and D-gulnolactone did not occur in the larval body (Ito, 1967). Synthesis of sericin (one of the silk protein) is stimulated by ascorbic acid (Fukuda, 1963). Fecundity of silkworm is increased with supplementation of ascorbic acid in mulberry, which is very important in silkworm egg production (Rahman, *et.al.* 1990).

Ascorbic acid contents of four varieties of healthy and disease infected mulberry leaves are given in the table-2.6.

Of the varieties examined, BM-3 variety contained the highest amount of vitamin-C (133 mg%) followed by BM-2 (127 mg%) and so on in decreasing order. Further the content of vitamin-C was decreased remarkably (35-45%) after infection of leaves with diseases. Tandom *et al.*, (1974) reported that fungal infection of plants and herbs reduced the concentration of ascorbic acid and thus lowered their nutrient value. Anjana Singh *et al.*, (1982) also found that *A. flavus* and *A. parasiticus* caused considerable losses in the quantity of ascorbic acid in musambi fruit.

Reddy *et al.* (1980) noticed the decrease in vitamin C content in diseased fruits of tomato with *R. solani, Phoma exigua* while Agarwal and Ghosh (1979) reported similar results in disease infected leaves.

R-2.12: Phenol content of mulberry leaves

Phenolic compounds enjoy a distribution in the plant kingdom and they are particularly prominent in plants where they are important in determining color and flavor (Buren, 1970).

Phenol contents of four varieties of healthy and diseased mulberry leaves are given in the table-2.6. Healthy mulberry leaves contained about 145-150 mg% while disease infected mulberry leaves contained about 170-225 mg% phenol. The present investigation concluded that the phenol content of mulberry leaves increased after infected (20-30%). Changes in phenolic substances in almost all the inoculated plants have been reported (Farkas *et al.*, 1962; Mahadevan, 1970). Ramkrishnan (1966) reported that the phenolic compound increased of rice after infetion blight disease.

Table-2.6: Crude fibre, Vitamin-C and phenol contents of mature healthy and disease infected Mulberry leaves.

Variety of leaves	Types of leaves	Crude fibre (%)	Vitamin-C (mg%)	Phenol (mg%)
BM-1	Healthy	6.12±0.12	124.95±0.14	145.24±0.18
BM-1	Tukra	3.78±0.09	98.64±0.12	180.86±0.12
BM-1	Leaf spot	4.64±0.06	76.93±0.16	215.25±0.16
BM-1	Powdery mildew	3.73±0.10	91.24±0.15	208.38±0.14
BM-2	Healthy	6.08±0.14	126.82±0.14	150.84±0.16
BM-2	Tukra	3.48±0.11	97.24±0.18	204.28±0.14
BM-2	Leaf spot	4.80±0.12	76.25±0.12	212.28±0.17
BM-2	Powdery mildew	4.08±0.08	89.28±0.16	208.28±0.18
BM-3	Healthy	7.04±0.10	132.75±0.14	148.25±0.14
BM-3	Tukra (Viral)	3.72±0.13	99.75±0.15	201.46±0.12
BM-3	Leaf spot	4.87±0.14	82.44±0.16	225.86±0.15
BM-3	Powdery mildew	4.28±0.09	94.22±0.12	216.34±0.11
BM-4	Healthy	6.78±0.12	116.00±0.18	160.86±0.18
BM-4	Tukra	3.38±0.11	84.25±0.09	210.26±0.15
BM-4	Leaf spot	4.48±0.13	74.25±0.10	225.98±0.14
BM-4	Powdery mildew	4.28±0.09	80.27±0.12	218.65±0.20

R-2.13: β-carotene content of mulberry leaves

β-Carotene is precursors of vitamin-A. In plants, it is very necessary for growth and development of soft tissue through its effect upon protein synthesis. Vitamin-A also plays a role in the maintenance of normal epithelial structure.

β-carotene content of both the healthy and diseased leaves of four varieties of mulberry leaves are given in the table-2.7.

As given in the result mulberry leaves contained comparatively significant amount of β-carotene. Of the varieties examined BM-4 variety contained the highest amount of β-carotene (120 mg%), followed by BM-3 (105 mg%) and so on in decreasing order. Further, β-carotene was found to be decreased significantly (17-32%) after infection of leaves with disease. Mahinder *et al.*, (1994) reported similar result when they worked on fungal infected betel leaf.

R-2.14. Vitamin-B_1 content of mulberry leaves

Thiamine exists in tissues mostly in the form of thiamine pyrophosphate known as co-carboxylase. TPP serves as co-enzyme in the metabolism of carbohydrate, fat and protein. In plants and herbs, it is also very essential. Vitmin-B_1 content of four varieties of healthy and diseased mulberry leaves are given in the Table-2.7.

The results indicated that mulberry leaves are also good sources of Vit-B_1. The amount of Vit-B_1 in healthy and disease infected leaves were varied between 44-60 mg% and 30-50mg% respectively. The present data clearly indicated that the vitmin-B_1 content in mulberry leaves decreased significantly (23-31%) after infection with diseases.

Shankar *et al.* (1996) reported that the decrease in vitamin-B_1 content is due to fungal infection.

R-2.15: Vitamin-B_2 content of mulberry leaves

Riboflavin combines in the tissue with phosphoric acid and become part of the structure of two flavin co-enzymes, FMN and FAD, which act as a co-enzymes in the enzymatic reaction. It is essential for normal growth and tissue maintenance.

Vitamin-B_2 (riboflavin) content of healthy and disease infected mulberry leaves are given in the Table-2.7. It can be suggested from the present finding that mulberry leaves are not good sources of vitamin-B_2, and its content was found to be varied variety to variety. The mulberry leaves contained about 0.47-0.52 mg% of Vit-B_2 and its content was decreased significantly after infection with disease. Ghosh *et al.* (1992) reported that riboflavin content of betel leaves decreased when infected with fungus.

R-2.16: Calcium content of mulberry leaves

Calcium plays an important role for the growth of silkworm. It is also a constituent of mulberry plant cell wall, being needed in large amount for cell division of the growing part of the plant. In extreme cases of calcium deficiency mulberry leaves become molted, finally leading to the death of their tissues (Rangaswami, 1976).

The amount of Calcium, present in different varieties of healthy and diseased infected mulberry leaves are given in Table-2.8. Of the varieties examined, BM-3 contained the highest amount (245 mg%) followed by BM-1 (240 mg%) and so on in decreasing order. The result clearly indicated that calcium content of mulberry leaves was decreased more remarkably after infected with disease (32-46%) and its content in diseased leaves were varied between 128-166 mg%.

Similar result was also reported by Ranghanath *et al.* (1989) that calcium content of betelvine leaves decreased due to fungal infection.

Table-2.7: β-carotene, Vitamin-B_1 and Vitamin-B_2 contents of mature healthy and disease infected Mulberry leaves.

Variety of leaves	Types of leaves	β-carotene (mg%)	Vitamin-B_1 (mg%)	Vitamin-B_2 (mg%)
BM-1	Healthy	102.86±0.10	44.23±0.05	0.480±0.012
BM-1	Tukra	68.32±0.14	30.76±0.03	0.326±0.016
BM-1	Leaf spot	72.05±0.08	33.33±0.04	0.272±0.010
BM-1	Powdery mildew	70.25±0.12	30.25±0.02	0.280±0.009
BM-2	Healthy	98.38±0.09	46.32±0.03	0.502±0.014
BM-2	Tukra	64.86±0.11	32.93±0.05	0.342±0.011
BM-2	Leaf spot	70.21±0.16	33.76±0.04	0.326±0.013
BM-2	Powdery mildew	69.86±0.10	32.06±0.02	0.305±0.016
BM-3	Healthy	105.32±0.09	60.79±0.01	0.468±0.014
BM-3	Tukra (Viral)	82.08±0.12	42.26±0.06	0.302±0.013
BM-3	Leaf spot	84.28±0.08	49.21±0.04	0.274±0.015
BM-3	Powdery mildew	83.79±0.09	50.36±0.02	0.265±0.012
BM-4	Healthy	120.23±0.10	54.28±0.03	0.523±0.010
BM-4	Tukra	86.37±0.12	40.06±0.06	0.346±0.011
BM-4	Leaf spot	88.29±0.14	42.38±0.02	0.306±0.014
BM-4	Powdery mildew	86.76±0.11	40.06±0.04	0.296±0.011

R-2.17: Phosphorus content of mulberry leaves

Phosphorus is one of the major nutrients for mulberry plant growth. It is the structural constituent of nucleotide (ATP), which is an energy carrier for all the metabolic activities. Phosphorus is also found in the form of phosphorylated sugar, alcohol and phospholipids in mulberry leaves (Sabtosh and Sehkar, 1989). Phosphorus is an important element for normal growth of silkworm and silkworm growth are directly proportional to the phosphorous content of the mulberry leaves (Sontosh, 1969).

Phosphorus content of four varieties of healthy and disease infected mulberry leaves are given in the Table-2.8. The result indicated that the healthy mulberry leaves contained 75-82 mg% phosphorus while diseased leaves contained 43-54 mg%. Further more like calcium, phosphorous content of mulberry leaves was also decreased very significantly (34-42%) after infection with disease. Similar result also found by Santosh and Sekhar (1989) on mulberry leaves.

R-2.18: Iron contents of mulberry leaves

Iron is essential to the development and activity of chlorophyll in mulberry plant. Iron deficiency result a very characteristic golden yellow colouration of the leaf known as chlorosis with termination of plant growth (Izuka, 1985). Iron is an essential element for the growth of silkworm. The blood colour of the cocoon is originated from the chlorophyll of mulberry leaves (Anon, 1975). Horie *et. al.* (1967) observed accelerated growth of larvae fed with iron sprayed leaves. Larval development showed significant difference with mature larvae fed with iron sprayed leaves giving maximum weight (Vishwanathan and Krishnamurthy,

1982-83), productivity of raw silk increased when larvae feed iron sprayed leaves (Lokanath *et. al.*, 1986).

Table-2.8: Calcium, Phosphorus and Iron contents of mature healthy and disease infected Mulberry leaves.

Variety of leaves	Types of leaves	Calcium (mg%)	Phosphorus (mg%)	Iron (mg%)
BM-1	Healthy	240±2	75.81±0.12	11.82±004
BM-1	Tukra	134±1	44.25±0.14	7.03±0.06
BM-1	Leaf spot	164±4	49.44±0.09	7.48±0.10
BM-1	Powdery mildew	152±1	44.25±0.10	6.44±0.05
BM-2	Healthy	225±3	81.89±0.11	12.34±0.06
BM-2	Tukra	128±2	54.12±0.06	6.46±0.08
BM-2	Leaf spot	146±3	50.02±0.08	8.04±0.04
BM-2	Powdery mildew	130±4	52.08±0.10	6.82±0.05
BM-3	Healthy	245±1	78.04±0.09	13.04±0.08
BM-3	Tukra (Viral)	130±2	48.26±0.11	7.03±0.04
BM-3	Leaf spot	166±3	52.23±0.12	7.26±0.06
BM-3	Powdery mildew	145±1	54.08±0.08	6.44±0.02
BM-4	Healthy	238±2	76.78±0.06	14.00±0.05
BM-4	Tukra	130±4	43.23±0.10	7.20±0.02
BM-4	Leaf spot	160±1	46.48±0.08	7.45±0.06
BM-4	Powdery mildew	152±2	50.42±0.06	6.75±0.04

The present results clearly indicated that mulberry leaves are not good sources of iron. As given in the Table-2.8, the healthy and disease infected mulberry leaves cotained 11-14 mg% and 6-8mg% of iron respectively. The results clearly indicated that like other minerals, iron content of mulberry leaves were also decreased remarkably after infection of viral and fungal diseases. Agarwal and Ghosh (1979) also reported potassium, iron and zinc contents of betel leaves decreased when infected with *C. gloeosporioides*. Izuka (1985) reported that iron content of mulberry leaves decreased when infected by fungus.

CHAPTER THREE

ANALYSIS ON THE ACTIVITIES AND CHARACTERISTICES OF SOME HYDROLYTIC AND OXIDATIVE ENZYMES PRESENT IN DIFFERENT VARIETIES OF HEALTHY AND DISEASE INFECTED MATURE MULBERRY LEAVES

INTRODUCTION

Various hydrolytic enzymes cleave the macromolecules of the tissues and these are importance in pathogenesis. These enzymes are secreted by the infecting parasite and/or activated in the host tissues during infection. Studies on the occurrence of these enzymes during disease development are helpful in understanding symptom development (Mahadevan & Sirdhar, 1982).

Cellulose is the major constituent of plant cell-walls in particular the secondary cell walls. Cellulose is a linear polymer of D-glucose units with β-1,4 glycosidic linkages. Hydrolysis of cellulose ultimately yields glucose, which is an important energy source for pathogenic microorganisms. Cellulases secreted by parasites are important in pathogenesis as by way of cellulose hydrolysis, cell-walls lose their strength and then collapse.

β-Glucosidases release reducing sugars from glucosides or oligosaccharides. This enzyme is particularly important in disease resistance mechanisms where phenolic compounds are released from phenolic glucosides. β-Glucosidases induced accumulation of flavonoid aglycones (Olah and Sherwood, 1973) may be related to disease resistance as phytoalexins in leguminous crops.

For direct cellular penetration, the parasite should elaborate besides cellulolytic and pectinolytic enzymes, proteolytic enzymes to depolymerize the proteins in the cell wall. The degradation products, amino acids and amides serve as nitrogen and carbon sources to the pathogen as many microorganisms utilize amino acids as carbon and nitrogen sources.

Mulberry leaf is the sole source of silkworm food. It was found that the physico-chemical properties of mulberry leaves are greatly affected with the disease condition. Proteolytic and hydrolytic enzymes may play some physiological roles during maturation and senescence of fruit (Hasinaga *et al.*, 1983; Desai and Deshpande; 1978; Mahadevan, 1970). They also reported that proteolytic enzymes play important role in disease development. P. K. Kharanath (1996) found that hydrolytic and proteolytic enzymes have great physiological roles during maturation and senescence of betel leaves.

In this study the contents of protease, amylase, cellulase, invertase, ascorbic acid oxidase, polyphenol oxidase and peroxidase were measured in healthy and disease infected mature mulberry leaves. Further, these crude enzymes were also characterized with respect to that pH optimum, temperature optimum, K_m etc.

MATERIALS AND METHODS

The fresh healthy and diseased infected mulberry leaves were collected from the experimental mulberry field. All the experimental results are presented as the mean value of three replications.

Preparation of crude enzyme extract

At first 10 gm of mulberry leaves were cut into small pieces, grinned in a mortar with pestle and then homogenized well with cold 0.1M phosphate buffer of respective pH (for amylase: pH 6.7, for invertase and protease: pH 7.0, for polyphenol oxidase, peroxidase and ascorbic acid oxidase: pH 6.0) while for the measurement of cellulase activity 0.1M sodium acetate buffer, pH 5.2 were used. Then the extract was filtrated through a double layer of muslin cloth. After centrifugation at 6,000g for 10 minutes the supernatant was used as crude enzyme extract.

3.1: Measurement of amylase activity

Amylase activity was assayed following the method as described in laboratory Manual in Biochemistry (Jayaraman, 1981) using 1% starch solution as substrate. The amylase activity was measured by estimating the release of maltose. The amount of maltose released was calculated from the standard curve (Fig.10) prepared with maltose. One unit of amylase activity was defined as the amount required for liberating 1 μg of maltose per minutes at 37°C.

Reagents

i) 0.1 M phosphate buffer, pH 6.7.

ii) 1% starch solution in 0.1 M phosphate buffer, pH 6.7

iii) 1% NaCl in distilled water

iv) 2 N NaOH

v) Dinitrosalicylic acid (DNS): Simultaneously 1 gm of DNS, 200mg of crystalline phenol and 50mg of sodium sulphite were taken in a beaker and mixed with 100ml of 1% NaOH solution by stirring. If it is needed to store, then sodium sulphite must be added just before use.

Procedure

Three sets of experiments (Blank, Control and Experimental) were performed for the measurement of amylase activity. The following different solutions were taken in different test tubes.

Substances	Blank (ml)	Control (ml)	Experimental (ml)
0.1M phosphate buffer, pH 6.7	2.5	2.5	2.5
1% starch solution	2.5	2.5	2.5
1% NaCl	1.0	1.0	1.0

The contents in the test tubes were mixed uniformly and the test tubes were incubated in a water bath at 37°C for 10 minutes. Then 0.5 ml of crude enzyme extract and 0.5 ml of distilled water were added to the sample and control tubes, whereas 1 ml of distilled water was added to the blank test tube. In control test tube, after the addition of crude enzyme extract immediately added 0.5 ml of 2N NaOH to stop the enzymatic reaction.

The rest of the test tubes were incubated at 37°C for 15 minutes and the reaction was then stopped by the addition of 0.5 ml of 2N NaOH. Then 0.5 ml of DNS reagent was mixed to all the tubes. The tubes were heated in a boiling water bath for 5 minutes. After cooling at room temperature the absorbance was measured at 520 nm.

3.2: Characteristics of crude amylase

3.2.1: Determination of the Activity of crude Amylase at Different pH values

The activity of crude amylase was measured at different pH values (pH 3.0-4.0 CH_3COONa-HCl; pH 5.0-7.0 NaH_2PO_4-Na_2HPO_4 and pH 8.0-10.0 Tris-HCl) at 37°C following the procedure as described above (3.1). Starch solution (1%) was made in the above-mentioned buffer of different pH values.

3.2.2: Determination of the Activity of crude Amylase at Different Temperatures

The crude amylase in 0.1M phosphate buffer, pH 6.7 was incubated at different temperatures (20–90°C) for one hour and the activity was measured following the procedure as described above (3.1).

3.2.3: Determination of Heat stability of crude Amylase

The crude amylase in 0.1M phosphate buffer, pH 6.7 was incubated at different temperatures (20–90°C) for one hour and cooled the enzyme at 4°C and the activity was measured following the procedure as described above (3.1).

3.2.4: Determination of Km Value of crude Amylase

The initial velocity (V_i) is equal to the amount of product formed per unit time and it was determined quantitatively by measuring the amount of one of the product at various time intervals (Robyt and White, 1990).

(i) 0. 1 M phosphate buffer, pH 6.7

(ii) 0.6 %; 0.8 %; 1.0 %; 1.2 % and 1.4 % of starch solutions in 0.1M phosphate buffer pH 6.7

(iii) 1% NaCl in distilled water

(iv) 2N Sodium hydroxide

(v) Dinitrosalicylic acid (DNS).

Method

2.5 ml of 0.6%, 0.8%, 1% 1.2% and 1.4% of starch solutions were taken in different test tubes (for each set of experiment, control, blank and sample). Then 2.5 ml of 0.1M phosphate buffer (pH 6.7) and 1 ml of 1% NaCl were added to all the tubes. After mixing uniformly, the test tubes were incubated in a water bath at 37°C for 10 minutes. Then 0.5 ml of crude enzyme extract and 0.5 ml of distilled water were added to the sample and control test tubes whereas 1 ml distilled water was added to the blank test tube. Immediately after the addition of crude enzyme extract and distilled water, 0.5 ml of 2N NaOH was added to the control test tube while the rest of the test tubes were incubated at 37°C for various times intervals (5 min, 10 min, 15 min, 20 min, 25 min and 30 min). The test tubes were removed from the water bath at appropriate time intervals and the reaction was

stopped by addition of 0.5 ml of 2N NaOH. Then 0.5 ml of DNS reagent was mixed to all the tubes. The tubes were heated in a boiling water bath for 5 minutes. After cooling at room temperature the absorbance was measured at 520 nm.

3.3: Measurement of protease activity

The protease activity was measured following the method of Kunitz (1947). The milk protein, casein was used as a substrate. The activity is determined by detecting the release of amino acid (tyrosine). The amount of tyrosine released was calculated from the standard curve constructed with tyrosine. One unit of protease activity was defined as the amount required for liberating 1μg of tyrosine per minute at 45°C.

Reagents

i) **1.2% casein solution:** 1.2 gm of casein was dissolved in 0.1M phosphate buffer, pH 7.0. Since casein is sparingly soluble in water, it was dissolved in a minimal quantity of 0.1N NaOH and the volume was raised to 100 ml with the buffer.

ii) 0.4M Trichloro acetic acid (TCA).

Procedure

2.5 ml of 1.2% casein solution was taken in different test tubes (No-1 for control, No-2 for blank and No-3 for experiment). Then 0.5 ml of crude enzyme extract was added to the control and experimental test tubes whereas 0.5 ml of buffer was added to the blank test tube. Immediately after the addition of the enzyme, 2.5 ml of TCA were added to the control tube to stop the reaction. The rest of the tubes

were incubated at 45°C for an hour and the reaction was stopped by the addition of 2.5 ml of TCA into the test tube. After cooling the reaction mixture was centrifuged at 5,000g for 7 minutes. The supernatant was collected and absorbance was taken at 275 nm against the reagent blank.

3.4: Characteristics of Crude Protease

3.4.1: Determination of the Activity of Protease at Different pH values

The activity of the protease was measured at different buffer (0.1M) of pH (pH 2.0-3.0; KCl-HCl, pH 4.0-5.0; CH_3COONa-CH_3COOH, pH 6.0-8.0; NaH_2PO_4-Na_2HPO_4, and pH 9.0-10.0; $Na_2B_4O_7$–HCl) following the procedure as described above. Casein solution (1.2%) was made in the above mentioned buffer of different pH values.

3.4.2:Determination of the Activity of Protease at Different Temperatures

The crude protease solution in 0.1M phosphate buffer, pH 7.0 was incubated at different temperatures (20-90°C) for 60 minutes and the activity was measured following the procedure as described above.

3.4.3: Determination of Heat stability of crude Protease

The crude Protease in 0.1M phosphate buffer, pH 7.0 was incubated at different temperatures (20–90°C) for one hour and cooled the enzyme at 4°C and the activity was measured following the procedure as described above (3.3).

3.4.4: Determination of Km Value of Protease

Reagents

(i) 0.8%, 1.0%, 1.2%, 1.4% and 1.6% of casein solution were prepared by dissolving casein in phosphate buffer, pH 7.0. Since casein is sparingly soluble in water, so it was necessary to heat the solution mildly to obtain a clear solution,

(ii) 0.4M Trichloro acetic acid (TCA)

Method

2.5 ml of 0.8%, 1.0%, 1.2%, 1.4% and 1.6% casein solution were taken in different test tubes (for each set of experiment control, blank. and experimental.). Then 0.5 ml of crude enzyme extract was added to the control and experimental tubes whereas 0.5 ml of buffer was added to the blank test tube. Immediately after the addition of enzyme, 2.5 ml of TCA was added to the control tube to stop the reaction. The rest of the tubes were incubated at 45°C for various time intervals (30 min, 40 min, 50 min, 60 min, 70 min and 80 min). The test tubes were removed from the water bath at appropriate time intervals and the reaction was stopped by addition of 2.5 ml TCA into the test tubes. After cooling the reaction mixture was centrifuge at 5000g for 5 minutes. The supernatant was collected and absorbance was taken at 275 nm against the reagent blank.

3.5: Measurement of invertase activity

Invertase activity was assayed following the modified method as described in methods in Physiological Plant Pathology (Mahadevan and Sridhar, 1982)

Sucrose was used as substrate. The invertase activity was measured by estimating the release of glucose. The amount of glucose released was calculated from the standard curve (Fig.-2.3) prepared with glucose. One unit of invertase activity was defined as the amount required for liberating 1μg of glucose per minute at 30°C.

Reagents

i) 0.1M phosphate buffer, pH 7.0

ii) 1% sucrose solution in 0.1M phosphate buffer, pH 7.0

iii) 1% NaCl in distilled water.

iv) 2N NaOH.

v) Dinitrosalicylic acid (DNS): Simultaneously 1gm of DNS, 200 mg of crystalline phenol and 50mg of sodium sulphite were taken in a beaker and mixed with 100 ml of 1% NaOH solution by stirring. If it is needed to store then sodium sulphite must be added just before use.

Procedure

Three sets of experiments (Blank, Control and Experimental) were performed for the measurement of invertase activity. The following different solutions were taken in different test tubes.

Substances	Blank (ml)	Control (ml)	Experimental (ml)
0.1M phosphate buffer, pH 7.0	2.5	2.5	2.5
1% Sucrose solution	2.5	2.5	2.5
1% NaCl	1.0	1.0	1.0

The contents in the test tubes were mixed uniformly and the test tubes were incubated in a water bath at 37°C for 10 minutes. Then 0.5 ml of crude enzyme extract and 0.5 ml of distilled water were added to the sample and control tubes, whereas 1 ml of distilled water was added to the blank test tube. Immediately after the addition of crude enzyme extract and distilled water, 0.5 ml of 2N NaOH was added to the control test tube to stop the reaction.

The rest of the test tubes were incubated at 37°C for 15 minutes and adding 0.5 ml of 2N NaOH stopped the reaction. Then 0.5 ml of DNS reagent was mixed to all the tubes. The tubes were heated in a boiling water bath for 5 minutes. After cooling at room temperature the absorbance was measured at 650 nm.

3.6: Characteristics of crude invertase

3.6.1: Determination of the Activity of Invertase at Different pH Values

The activity of invertase at different buffer (0.1M) of pH values (pH 2.0-3.0; KCl-HCl, pH 4.0-5.0; CH_3COONa-CH_3COOH, pH 6.0-8.0; NaH_2PO_4-Na_2HPO_4, and pH 9.0-10.0; $Na_2B_4O_7$–HCl) was measured at 37°C following the procedure as described above. Sucrose solution (2.5%) was made in the above-mentioned buffer of different pH values.

3.6.2: Determination of the Activity of Invertase at Different Temperatures

The crude enzyme extract in 0.1M Na-acetate buffer, pH 4.5 was incubated at different temperature (20-90°C) for 60 minutes and the activity was measured at these temperature following the procedure as described above.

3.6.3: Determination of Heat stability of crude Invertase

The crude Invertase in 0.1M Na-acetate buffer, pH 4.5 was incubated at different temperatures (20–90°C) for one hour and cooled the enzyme at 4°C and the activity was measured following the procedure as described above (3.5).

3.6.4: Determination of Km Value of the Invertase Enzyme

Reagent

(i) 0.1M Sodium acetate-acetic acid buffer pH 4.5

(ii) 5mM, 10mM, 20mM, 40mM and 80mM of Sucrose in Na-acetate-acetic acid buffer pH 4.5

(iii) 1% Sodium hydroxide (NaOH)

(iv) Dinitrosalicylic acid (DNS).

Method

1 ml of 5 mM, 10 mM, 20 mM, 40 mM and 80 mM of sucrose solutions were taken in different experimental test tubes (for each set of experiment, control, blank and experimental) Then 2 ml of sodium acetate buffer pH 4.5 was added to each test tube. The content in the test tubes were mixed uniformly and 1 ml of crude enzyme extract was added to the control and experimental tubes while 1ml buffer was added to the blank tube. Immediately after the addition of crude extract 0.5 ml of 1% NaOH was added to the control tubes to stop the reaction.

All the tubes except control tube were incubated at 37°C for various times intervals (10 min, 20 min, 30 min, 40 min and 50 min). The test tubes were removed from the water bath at appropriate time intervals and the reaction was stopped by addition of 0.5 ml of 1% NaOH. Then 4.5 ml of DNS reagent was mixed to all the test tubes. The tubes were heated in a boiling water bath for 5 minutes. After cooling in running tap water at room temperature the absorbance was measured at 575 nm.

3.7: Measurement of cellulase activity

The cellulase activity was measured following the procedure as described in method in Physiological Plant Pathology (Mahadevan and Sridhar, 1982). Carboxymethyl cellulose (CMC) was used as substrate. Cellulase activity was measured by estimating the release of reducing sugar (Fig.-2.3). The amount of reducing sugar released was determined by dinitrosalicylic acid method (Miller, 1972). One unit of cellulase activity was defined as the amount of enzyme required for liberating 1 µg of reducing sugar per minute at 37°C.

Reagents

i) **Sodium acetate buffer, pH 5.2**

ii) **0.5% Carboxymethyl Cellulose (CMC) solution:** 0.5 gm of CMC was dissolved in 100 ml of sodium acetate buffer, pH 5.2 at 50-60°C. It was taken in a blender and homogenized it for 3-5 minutes at low speed. The content was stirred with a glass rod and again homogenized at high speed for 3-5 minutes. Then it was filtered through a filter paper whatman N0-1. The supernatant was used as substrate.

Procedure

4 ml of 0.5% CMC solution, 1 ml of buffer and 2 ml of the crude enzyme extract were taken in a test tube. The content of tube was mixed uniformly and incubated at 37°C in a water bath with manual shaking. Then the tube was taken out and heated in boiling water bath for 2-3 minutes.

In a test tube 4 ml of CMC solution and 3 ml of buffer were taken as a blank and in another test tube 4 ml of CMC solution, 1 ml of buffer and 2 ml of crude enzyme extract were taken as control. Then the test tubes were heated in boiling water bath for 2 or 3 minutes.

Each of these test tubes were then cooled and clarified by centrifugation. From these clear solutions, 3 ml was taken in different test tube and 3 ml of DNS reagents was added to it. The tubes were then heated in a boiling water bath for 5 minutes and 1 ml of 40% Rochelle salt was added to each warmed test tube. The absorbance was taken at 575 nm by a colorimeter.

3.8: Characteristics of crude Cellulase

3.8.1: Determination of the Activity of Cellulase at Different pH Values

The activity of cellulase at different buffer (0.1M) of pH values (pH 2.0-3.0; KCl-HCl, pH 4.0-5.0; CH_3COONa-CH_3COOH, pH 6.0-8.0; NaH_2PO_4-Na_2HPO_4, and pH 9.0-10.0; $Na_2B_4O_7$–HCl) was measured at 37°C following the procedure as described above. CMC solution (0.5%) was made in the above-mentioned buffer of different pH values.

3.8.2: Determination of the Activity of Cellulase at Different Temperatures

The crude enzyme extract in 0.1M Sodium-acetate buffer, pH 5.2 was incubated at different temperature (20-90°C) for 60 minutes and the activity was measured following the procedure as described above (3.7).

3.8.3: Determination of Heat stability of crude Cellulase

The crude Cellulase in 0.1M Sodium-acetate buffer, pH 5.2 was incubated at different temperatures (20–90°C) for one hour and cooled the enzyme at 4°C and the activity was measured following the procedure as described above (3.7).

3.8.4: Determination of Km Value of the Cellulose Enzyme

Reagent

(i) 0.1M Sodium acetate-acetic acid buffer pH 5.2

(ii) 0.4%, 0.5%, 0.6%, 0.8%, 1% and 1.2% of CMC in Na-acetate-acetic acid buffer pH 5.2

(iii) Dinitrosalicylic acid (DNS).

Method

4 ml of 0.4%, 0.5%, 0.6%, 0.8%, 1% and 1.2% of CMC solutions were taken in different experimental test tubes. 3 ml of buffer was added in blank test tube while 1 ml of buffer and 2 ml of crude enzyme extract were added in control and experimental tubes and mixed uniformly. After addition of crude enzyme extract in the control tube, it was heated immediately in boiling water bath for 2-3 minutes. The blank and the experimental tubes were incubated at 37°C in a water bath with manual shaking for various time intervals (10 min, 20 min, 30 min, 40

min and 50 min). The test tubes were removed from the water bath at appropriate time intervals. Then the tubes were taken out and heated it at boiling water bath for 2-3 minutes.

Each of these test tubes were then cooled and clarified by centrifugation. From these clear solutions, 3 ml was taken in a test tube and 3 ml of DNS reagents were added to each tube. The tubes were then heated in a boiling water bath for 5 minutes and 1ml of 40% Rochelle salt was added in each warmed test tube. The absorbance was taken at 575 nm by a colorimeter.

3.9: Measurement of ascorbic acid oxidase activity

Ascorbic acid oxidase activity was measured following the procedure as described in method in Physiological Plant Pathology (Mahadevan and Sridhar 1982). In this process ascorbic acid was used as substrate. The enzyme activity was measured by determining the residual ascorbic acid in the reaction mixture. The enzyme activity was described as units/min/gm leaf under assay condition. An increase in absorbance by 0.001 in one minute was taken as one unit of enzyme activity (Kaul and Munjal, 1980).

Reagents

i) 0.1M phosphate buffer, pH 6.0.
ii) 0.01M ascorbic acid dissolved in 0.1M phosphate buffer, pH 6.0.

Procedure

The enzyme extract (0.1ml) was pipetted into a cuvette and then 1ml of 0. 1M phosphate buffer, pH 6.0; 1.8ml of distilled water and 0.1ml of 0.01M ascorbic acid in buffer, were mixed. Recording the change in the absorbance at 265 nm in

30 sec interval upto 3 minutes followed the reaction. The cuvette containing enzyme extract and buffer only was used to adjust the absorbance to zero. Average change in absorbance between 30 and 150 sec. was used to plot ascorbic acid oxidase activity. A control was prepared by taking 0.1 ml of boiled enzyme extract, 1 ml of phosphate buffer; 1.8 ml distilled water and 0.1 ml of ascorbic acid solution.

3.10: Characteristics of Crude ascorbic acid oxidase

3.10.1: Determination of the Activity of ascorbic acid oxidase at different pH values

The activity of ascorbic acid oxidase at different buffer (0.1M) of pH values (pH 2.0-3.0; KCl-HCl, pH 4.0-5.0; CH_3COONa-CH_3COOH, pH 6.0-8.0; NaH_2PO_4-Na_2HPO_4, and pH 9.0-10.0; $Na_2B_4O_7$–HCl) was measured following the procedure as described above.

3.10.2: Determination of the Activity of ascorbic acid oxidase at Different Temperatures

The crude enzyme extract in 0.1M phosphate buffer, pH 6.0 was incubated at different temperature (20-90°C) for 60 minutes and the activity was measured following the procedure as described above (3.9).

3.10.3: Determination of Heat stability of crude ascorbic acid oxidase

The crude ascorbic acid oxidase in 0.1M phosphate buffer, pH 6.0 was incubated at different temperatures (20–90°C) for one hour and cooled the enzyme at 4°C and the activity was measured following the procedure as described above (3.9).

3.11: Measurement of polyphenol oxidase activity

The polyphenol oxidase activity was measured following the procedure as described in method in Physiological Plant Pathology (Mahadevan and Sridhar, 1982). In this method catechol was used as substrate. One unit of enzyme activity was defined as a change in absorbance of 0.001 per min. per gm of leaf.

Reagents

i) 0.1 M phosphate buffer, pH 6.0.

ii) 0.01 M catechol in 0.1 M phosphate buffer, pH 6.0

Procedure

Aliquot of 2 ml of the crude enzyme extract and 3 ml of 0.1M phosphate buffer, pH 6.0 were pipetted into the cuvette. The contents were mixed by inverting, placed in a spectrophotometer by setting at 495 nm and the absorbance was adjusted to zero. The cuvette was removed and 1 ml of 0.01M catechol was mixed quickly by inversion. The cuvette placed in the spectrophotometer and the change in absorbance at 495 nm was measured for 3 minutes. A blank was prepared by taking 2 ml boiled enzyme, 3 ml of 0.1M phosphate buffer and 1 ml of 0.01M catechol in the cuvette and treated similarly.

3.12: Characteristics of Crude polyphenol oxidase

3.12.1: Determination of the Activity of polyphenol oxidase at Different pH Values

The activity of polyphenol oxidase at different buffer (0.1M) of pH values (pH 2.0-3.0; KCl-HCl, pH 4.0-5.0; CH_3COONa-CH_3COOH, pH 6.0-8.0; NaH_2PO_4-Na_2HPO_4, and pH 9.0-10.0; $Na_2B_4O_7$–HCl) was measured following the procedure as described above.

3.12.2: Determination of the Activity of polyphenol oxidase at Different Temperatures

The crude enzyme extract of polyphenol oxidase in 0.1M phosphate buffer, pH 6.0 was incubated at different temperature (20-90°C) for 60 minutes and the activity was measured following the procedure as described above (3.11).

3.12.3: Determination of Heat stability of polyphenol oxidase

The crude polyphenol oxidase in 0.1M phosphate buffer, pH 6.0 was incubated at different temperatures (20–90°C) for one hour and cooled the enzyme at 4°C and the activity was measured following the procedure as described above (3.11).

3.13: Measurement of peroxidase activity

The peroxidase activity was measured following the procedure as described in method in Physiological Plant Pathology (Mahadevan and Sridhar, 1982). In this method pyrogallol is used as substrate. In presence of H_2O_2 pyrogallol is oxidized to colour derivative. The amount of purpurogallin formed during the reaction can be followed in a spectrophotometer. One unit of peroxidase is defined as the amount of purpurogallin formed per minute per gm leaf under the assay condition.

Reagents

i) 0.05 M pyrogallol dissolved in 0.1M phosphate buffer, pH 6.0.

ii) 1% H_2O_2

Procedure

Aliquot of 3 ml of 0.05M pyrogallol solution (prepared in 0.1 M phosphate buffer, pH 6.0) and 0.1 ml of the crude enzyme extract were pipetted into a cuvette. The contents were mixed well, placed in a spectrophotometer by setting

at 420 nm and the absorbance was adjusted to zero. The cuvette was removed and 1 ml of 1% H_2O_2 was mixed quickly by inversion. The cuvette was placed in the spectrophotometer and the absorbance changes were measured at 20 seconds interval for 3 minutes.

3.14: Characteristics of Crude peroxidase

3.14.1: Determination of the Activity of peroxidase at Different pH Values

The activity of peroxidase at different buffer (0.1M) of pH values (pH 2.0-3.0; KCl-HCl, pH 4.0-5.0; CH_3COONa-CH_3COOH, pH 6.0-8.0; NaH_2PO_4-Na_2HPO_4, and pH 9.0-10.0; $Na_2B_4O_7$–HCl) was measured following the procedure as described above (3.13).

3.14.2: Determination of the Activity of peroxidase at Different Temperatures

The crude enzyme extract in 0.1M phosphate buffer, pH 6.0 was incubated at different temperatures (20-90°C) for 60 minutes and the activity was measured following the procedure as described above (3.13).

3.14.3: Determination of Heat stability of peroxidase

The crude peroxidase in 0.1M phosphate buffer, pH 6.0 was incubated at different temperatures (20–90°C) for one hour and cooled the enzyme at 4°C and the activity was measured following the procedure as described above (3.13).

RESULTS AND DISCUSSION

R-3.1: Activity of amylase in mulberry leaves

Amylase is a hydrolytic enzyme which hydrolyses starch to yield monomeric carbohydrate. The amount of amylase present in the healthy and disease infected mulberry leaves are presented in the Table-3.1.

The present finding indicated that the activity of amylase decreased abruptly after infection of mulberry leaves with disease indicating the involvement of amylase in starch degradation to produce monomeric carbohydrate (glucose) during disease condition of leaves, which is used by microorganisms as the sole sources of carbon, growth as well as their nutrients. Similar trends was also reported by Desai and Deshpande (1978b); Nabeesa and Unnikrishnan (1988); Mao and Kinsella (1981); and Garcia *et al.* (1988) in case of banana.

R-3.2: Characteristics of Crude Amylase

R-3.2.1: Effect of pH

As shown in Fig.-3.1, the activity of crude amylase was affected greatly with the changes of pH. The crude amylase, extracted from both healthy and diseased affected mulberry leaves, showed a characteristic bell shaped pH activity curve. It was found that the amylase from both healthy and diseased leaves showed almost similar pattern of pH-activity profile curves but activities were found to be very much lower after infection with disease.

Further, the amylases from both the sources gave maximum activities around pH 7.0.

The activity of crude amylase was decreased rapidly in the acidic as well as basic pH regions and its activity was lost completely at or below pH 3 and at or above pH 10. Very similar pattern of pH profile curve was reported for amylase from pear fruits (McArthur-Hespe, 1956) and from banana pulp (Mao and Kinsella, 1981).

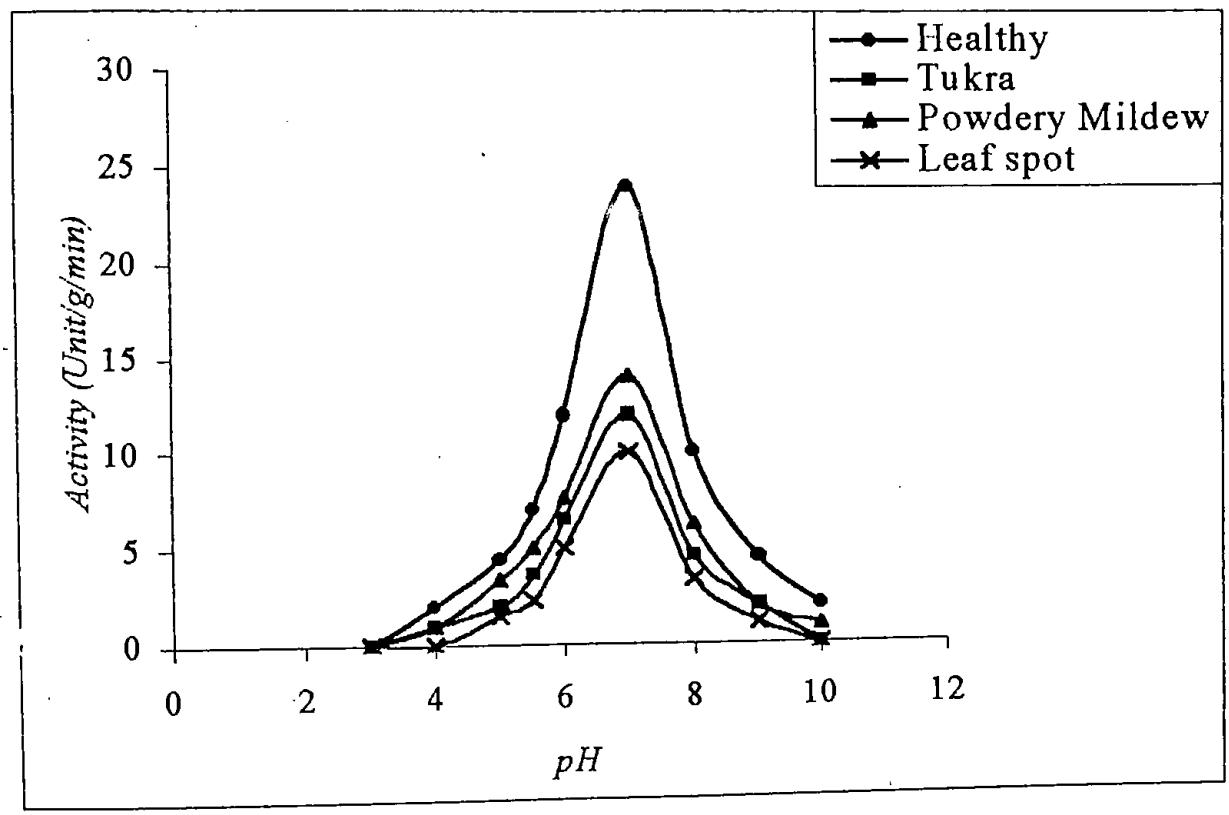


Fig.-3.1: Effect of pH on the activities of crude Amylase isolated from Healthy and disease infected mulberry leaves.

R-3.2.2: Effect of temperature

The effect of temperature on the activities of crude amylase, extracted from healthy and disease infected leaves, are shown in the Fig.-3.2. It was found that the activities were changed greatly with changes of temperature and the amylase showed maximum activities around 35°C to 40°C. Remarkably, the optimum temperature of amylase isolated from healthy leaves was found to be changed slightly as compared to those of isolated from disease affected leaves. A optimum temperature of crude amylase of banana pulp was reported 38°C (Mao and Kinsella, 1981).

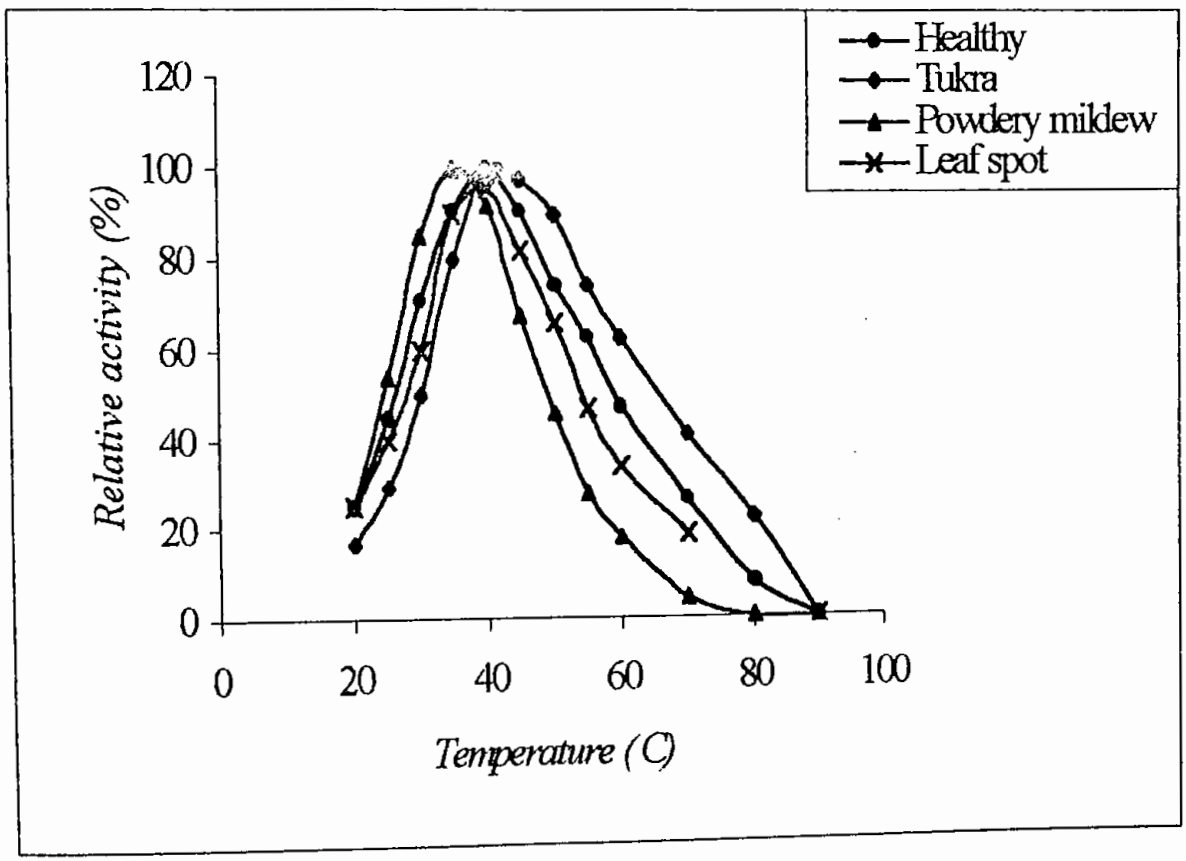


Fig.3.2: Effect of temperature on the activities of crude Amylase isolated from healthy and disease infected Mulberry leaves.

R-3.2.3: Heat stability

Heat stability of crude amylase are shown in the Fig.-3.3. The enzyme was stable at 60°C. At 80°C the enzyme, extracted from powdery mildew and healthy leaf completely lost their activities but the activities of enzyme extracted from leaf spot and tukra infected leaves lost completely at 90°C. The results also indicated that the enzyme isolated from tukra infected leaves were more stable than other.

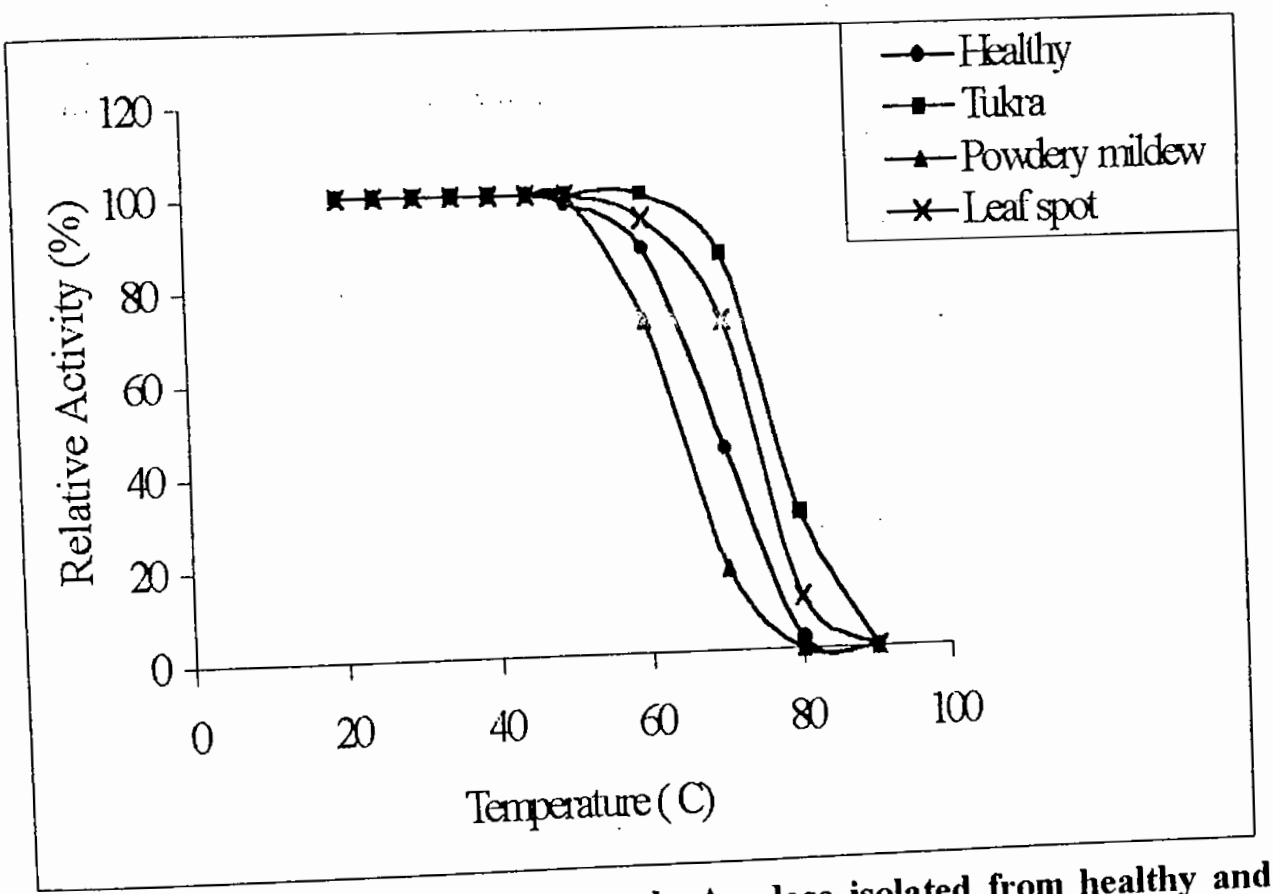


Fig. 3.3: Heat Stability curve of crude Amylase isolated from healthy and disease infected mulberry leaves.

R-3.2.4: Kinetic Parameters of Crude Amylase

The K_m value of crude amylase, isolated from healthy and disease infected leaves as determined by Lineweaver-Burk double reciprocal plot (Fig.3.4) was calculated to be 0.5% when starch was used as a substrate while the V_{max} was found to be 13.3 mg/min, 8.7 mg/min, 7.7 mg/min and 6.9 mg/min for crude amylase isolated from healthy, powdery mildew, viral infected (tukra) and leaf spot disease infected leaves respectively. Jain (1988) reported K_m value of 0.25-0.8% for amylase using starch as substrate.

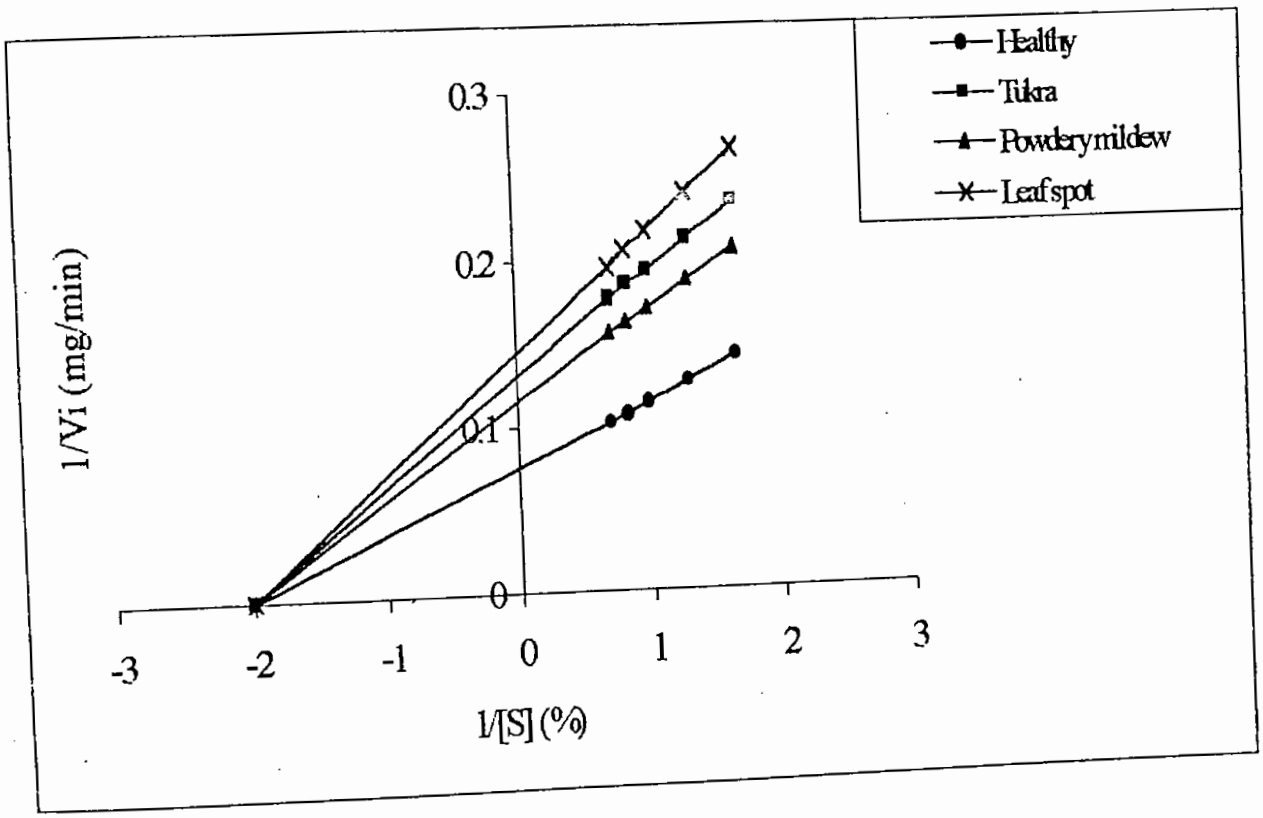


Fig.-3.4: Lineweaver-Burk double reciprocal plots for the determination of K_m value and V_{max} of crude amylase, isolated from healthy and disease infected mulverry leaves.

R-3.3: Activity of invertase of mulberry leaves

Like amylase, invertase is also a sugar hydrolytic enzyme, which hydrolyses sucrose to glucose and fructose. Invertase activities of healthy and disease infected mulberry leaves at mature stage are shown in Table-3.1.

The results indicated that mulberry leaves contained very low amount of invertase and its activity decreased significantly after infection of leaves with disease.

R-3.4: Characteristics of Crude Invertase

R-3.4.1: Effect of pH

As shown in Fig.-3.5, the activity of crude invertase was affected greatly with the changes of pH. The crude invertase, extracted from both healthy and diseased affected mulberry leaves, showed a characteristic bell shaped pH activity curve. It was found that the invertase from both healthy and diseased leaves showed almost similar pattern of pH-activity profile curves but activities were found to be very much lower after infection with disease.

Further, the invertases from all the sources gave maximum activities around the pH 4.0-5.0 and the optimum temperature of the enzyme isolated from healthy leaf was pH 5.0 but the disease infected were 4.5. The activity of crude invertase was decreased rapidly in the more acidic as well as basic pH regions and the enzyme activity was lost 65% at pH 3 but lost completely at or above pH 10.0 except that from tukra infected leaves whose activity was completely lost at pH 8.0. The result also indicated that the enzyme is more stable in acidic pH than basic pH region. Very similar pattern of pH profile curve was reported for crude invertase

from *Oryza sativa* (Maria *et. al.* 1995) and Potato invertase (Krishnan and Pueppke, 1990).

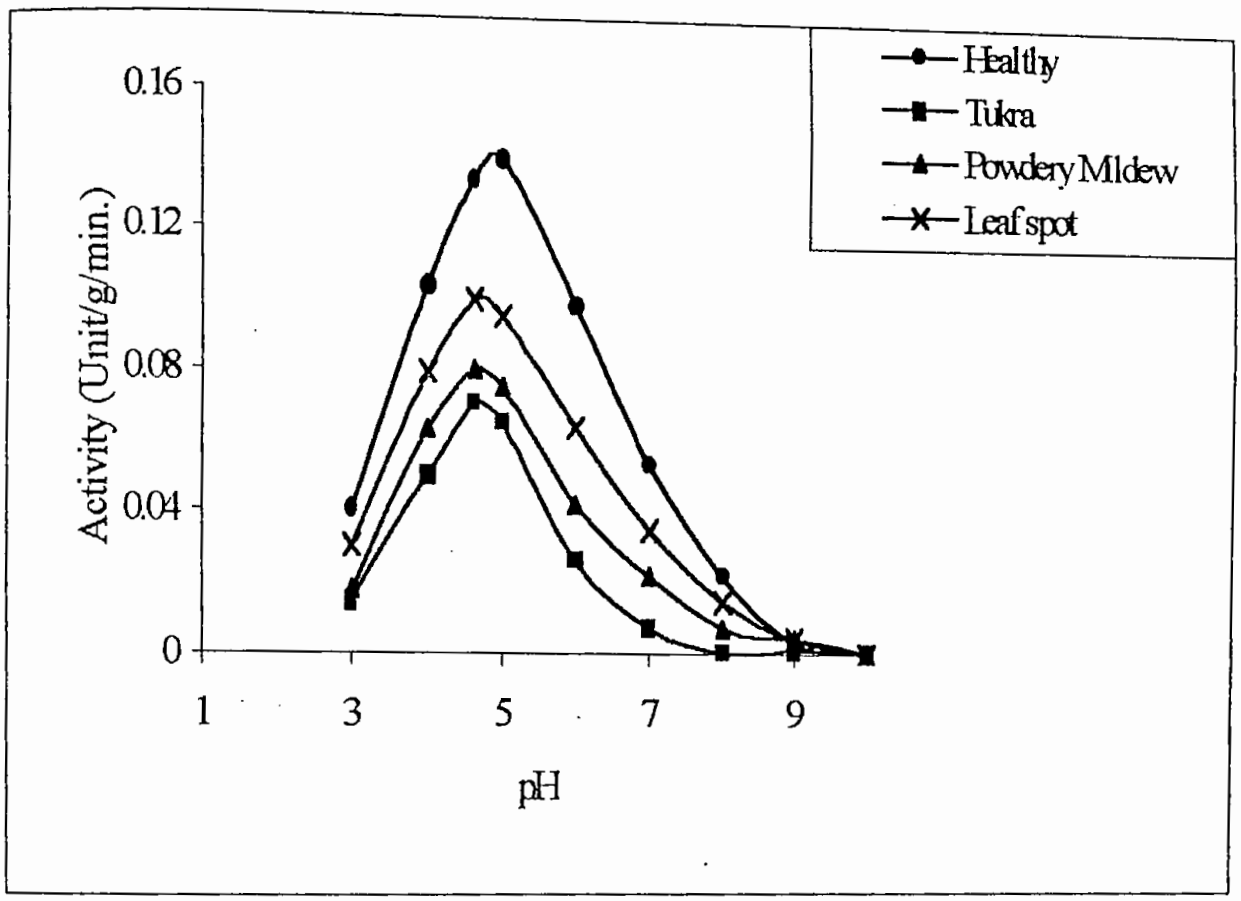


Fig.-3.5: Effect of pH on the activities of crude invertase isolated from Healthy and disease infected mulberry leaves.

R-3.4.2: Effect of temperature

The effect of temperature on the activities of crude invertase, extracted from healthy and disease-affected leaves are shown in the Fig.-3.6. It was found that the activities were changed greatly with changes of temperature and the invertase

showed maximum activities around 50°C to 55°C. Remarkably, the optimum temperature of invertase from healthy leaves was found to be changed slightly as compared to those of disease infected leaves.

The enzyme activity was decreased gradually above those temperature and the activity was lost about 90% at 90°C. Nakanishi and Yokotsuka (1990) also reported that invertase was active at high temperature.

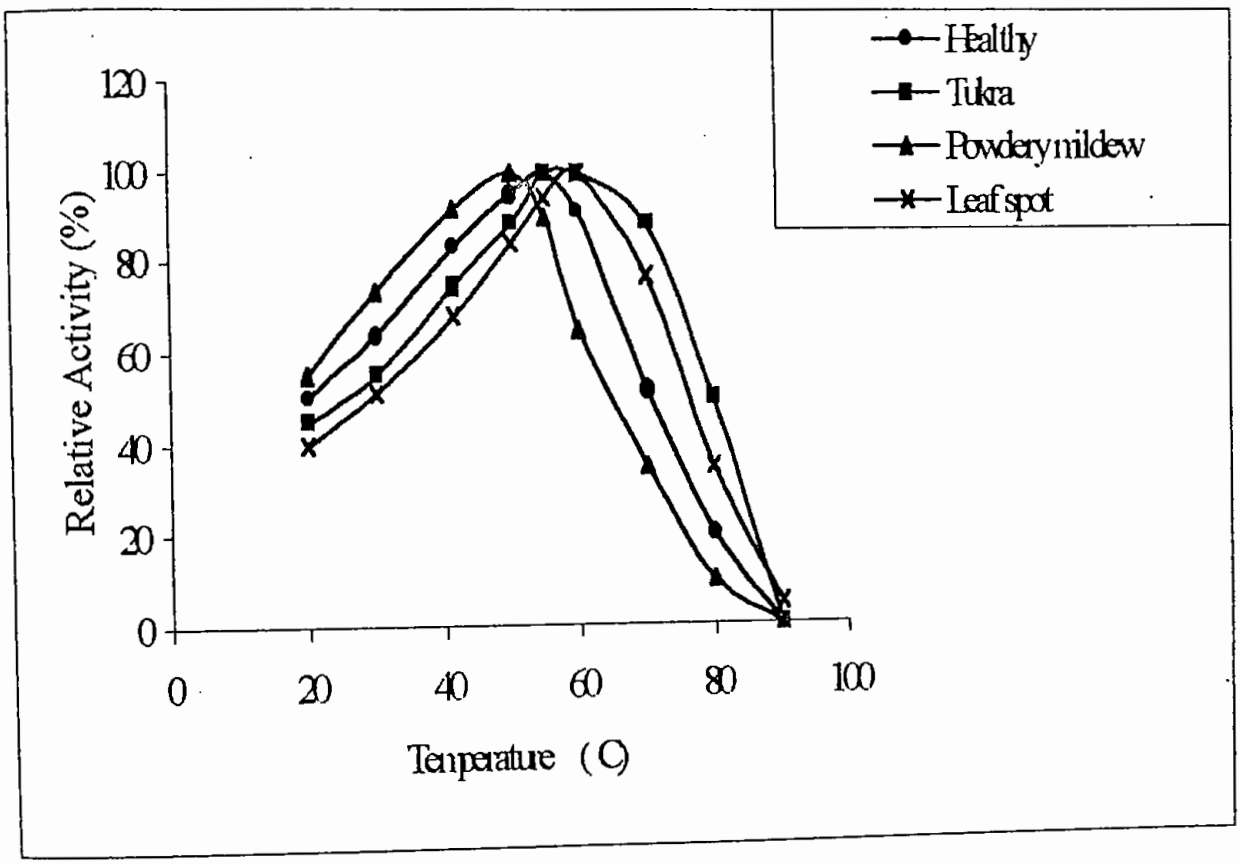


Fig.-3.6: Effect of temperature on the activities of crude Invertase isolated from healthy and disease infected mulberry leaves.

R-3.4.3: Heat stability

Heat stability of crude invertase isolated from healthy and disease infected mulberry leaves are shown in the Fig.-3.7. The enzyme was stable at 70°C. it can be concluded from the results that the enzyme, invertase isolated from the tukra and leaf spot disease infected leaves are more stable than those from healthy leaves.

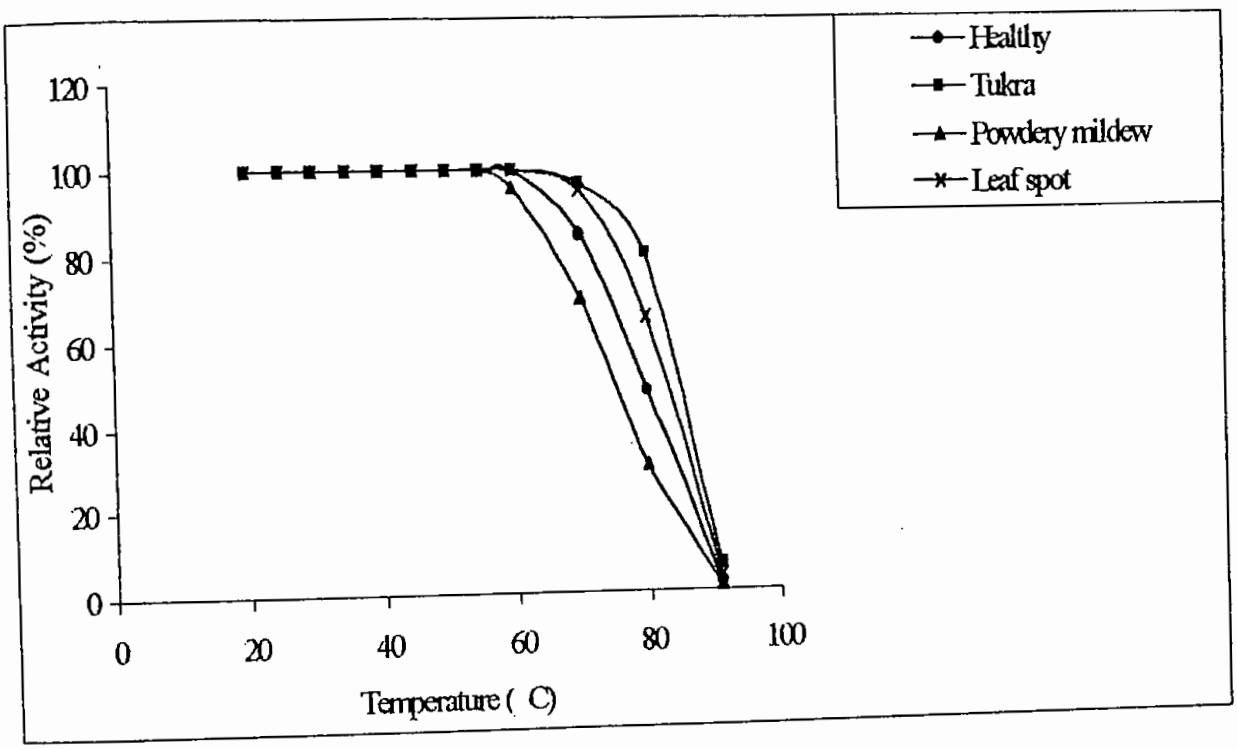


Fig.-3.7: Heat Stability curve of crude Invertase isolated from healthy and disease infected Mulberry leaves.

R-3.4.4: Kinetic Parameters of Crude invertase

The K_m of crude invertase, isolated from healthy and disease infected leaves as determined by Lineweaver-Burk double reciprocal plots (Fig.-3.8) were

calculated to be 4.54 mM when sucrose was used as a substrate and the V_{max} values were 1.33 mg/min, 0.9 mg/min, 0.72 mg/min and 0.83 mg/min for those from healthy, leaf spot, powdery mildew and tukra infected leaves respectively. Porntaveewat *et. al.* (1994) reported that the K_m value of invertase was 4.4 mM isolated from Bailey *A. Grapes* while Maria *et. al.* (1995) reported 6.6 mM for invertase from *Oryza sativa* using sucrose as substrate.

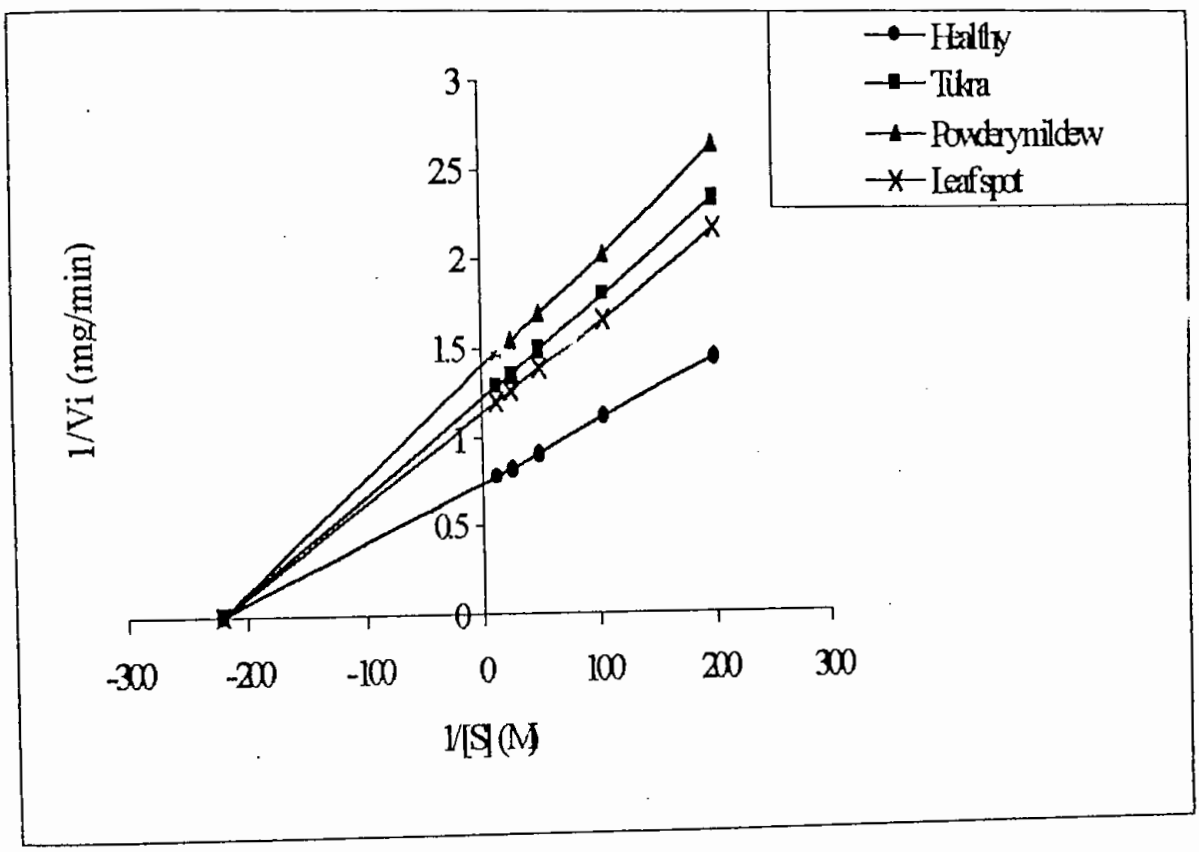


Fig.-3.8: Lineweaver-Burk double reciprocal plots for the determination of K_m value and V_{max} of crude invertase, isolated from healthy and disease infected mulberry leaves.

R-3.5: Activity of protease in mulberry leaves

Protease is protein hydrolyzing enzyme, which acts on protein in aqueous substances. The protease activity of four varieties of healthy and disease infected mulberry leaves at mature stage are given in the Table 3.1. The present data clearly indicated that the protease activities in disease infected leaves decreased remarkably and the activities were found to be 4-5 units/g and 2-3 units/g in healthy and disease infected leaves respectively. Similar result reported by Mahadevan (1970) and Balasubramania (1972).

R-3.6: Characteristics of Crude protease

R-3.6.1: Effect of pH

As shown in Figure-3.9, the crude protease, extracted from both healthy and disease infected mulberry leaves showed a characteristic bell shaped pH activity profile curve. It was found that the protease from both healthy and diseased leaves showed almost similar patter of pH-activity profile curves but activities were decreased significantly after infection with disease.

Further, the protease from both the sources gave maximum activities around the neutral pH. Beyond this pH ranges the activity of crude protease decreased rapidly in the acidic as well as basic pH and the enzyme activity was lost completely at pH 3. Very similar pattern of pH profile curve was reported for protease from tomato (Klapper *et. al.*, 1973).

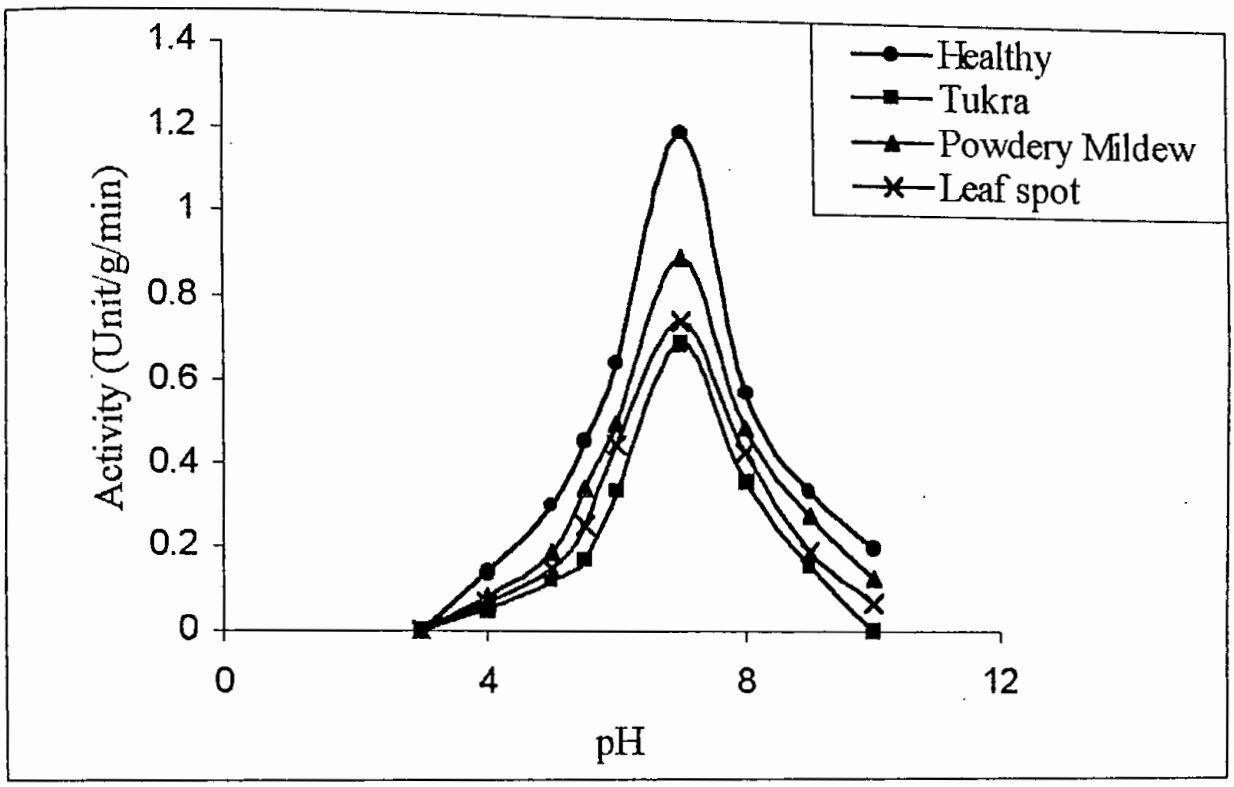


Fig.-3.9: Effect of pH on the activities of crude protease isolated from Healthy and disease infected mulberry leaves.

R-3.6.2: Effect of Temperature

The effect of temperature on the activities of crude protease, extracted from healthy and disease infected leaves are shown in the Fig.-3.10. It was found that the activities were changed greatly with changes of temperature and the amylase showed maximum activities around 40°C to 45°C. Remarkably, the optimum temperature of protease from healthy leaves was found to be changed slightly as compared to those of disease infected leaves.

The enzyme activity decreased rapidly with further rise of temperature and completely lost its activity at 90°C. Hashinaga (1983) reported that the optimum temperature of protease from passion fruit juice was 45°C.

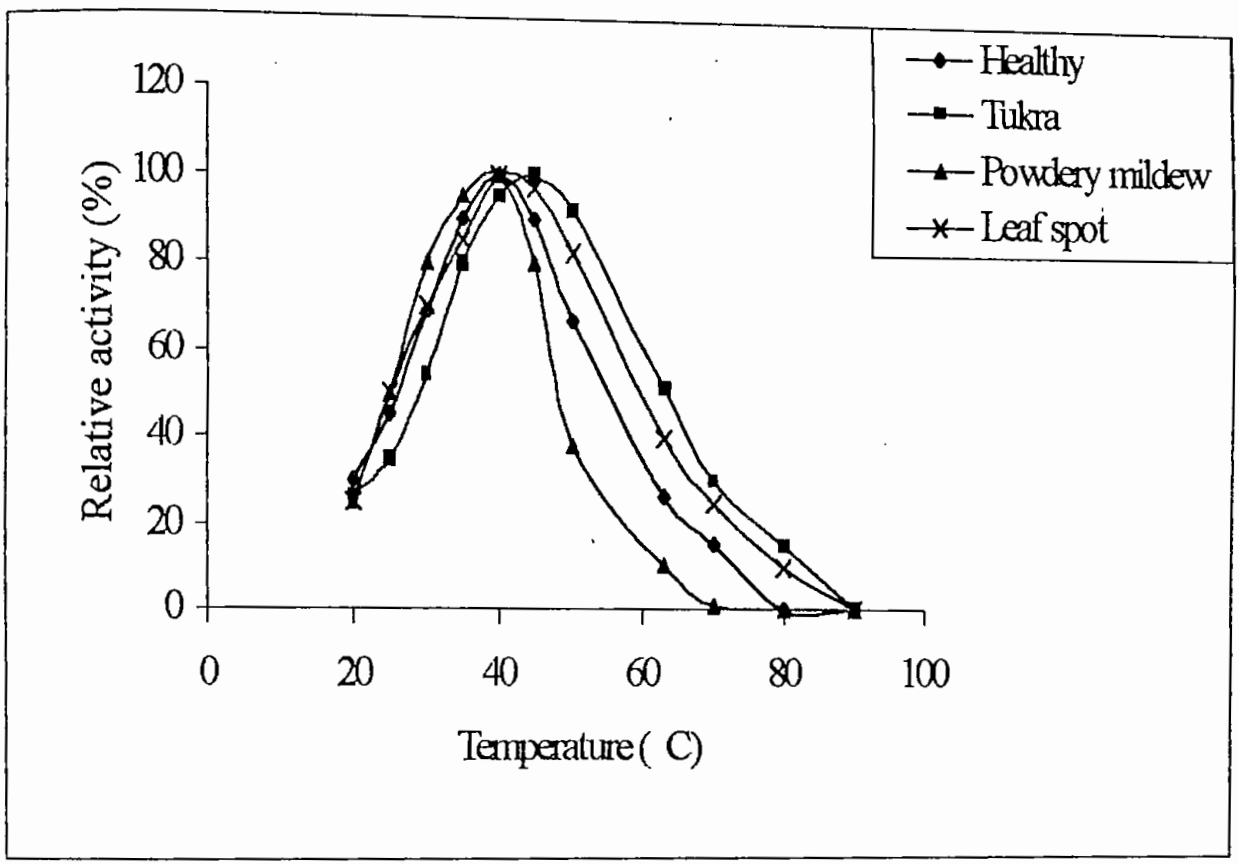


Fig.-3.10: Effect of temperature on the activities of crude protease isolated from healthy and disease infected mulberry leaves.

R-3.6.3: Heat stability

The crude protease was found to be stable upto 60°C (Fig.-3.11). The data also showed that protease of infected leaves were slightly more stable than that from healthy leaves.

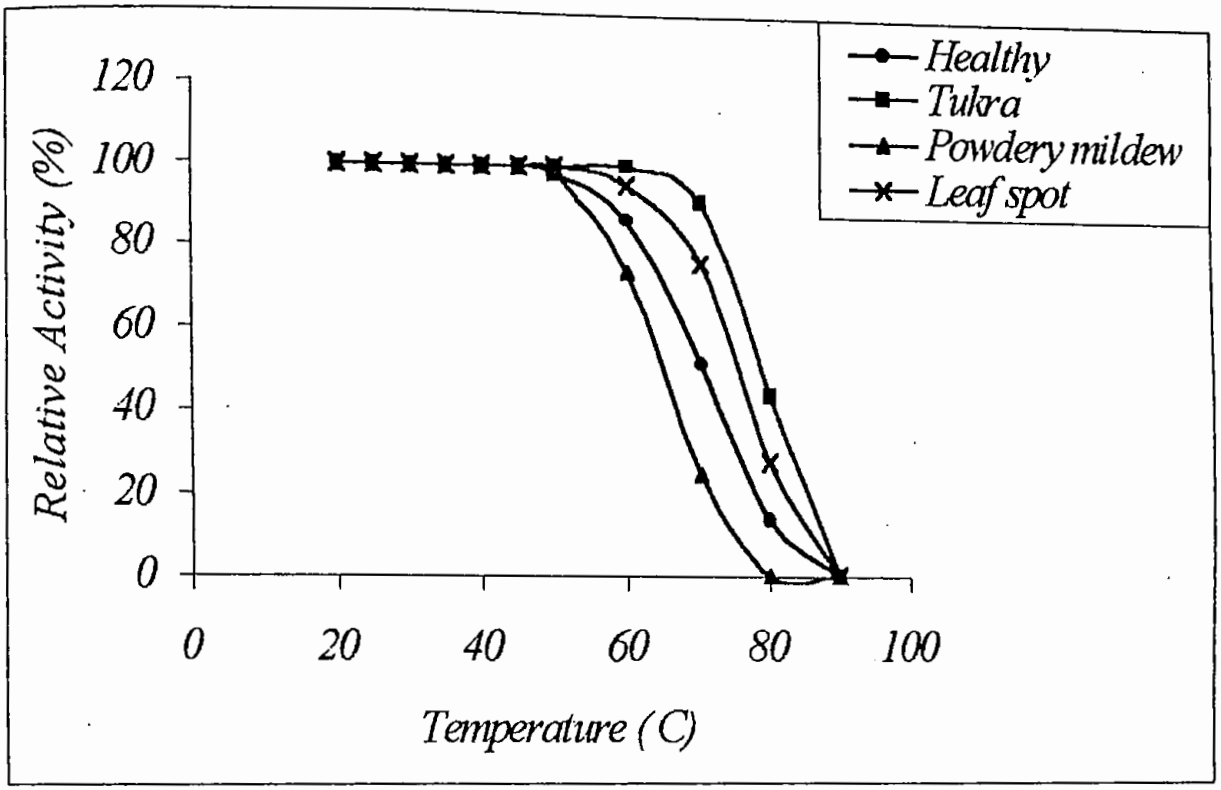


Fig.3.11: Heat Stability curve of crude protease isolated from healthy and disease infected Mulberry leaves.

R-3.6.4: Kinetic Parameters of Crude protease

The K_m value of crude protease, isolated from healthy and diseased affected leaves as determined by Lineweaver-Burk double reciprocal plots (Fig.-3.12) was 0.55% when casein was used as substrate and the V_{max} values were 1.66 mg/min, 1.0 mg/min, 0.93 mg/min and 0.83 mg/min for these from healthy, leaf spot, powdery mildew and tukra infected leaves respectively.

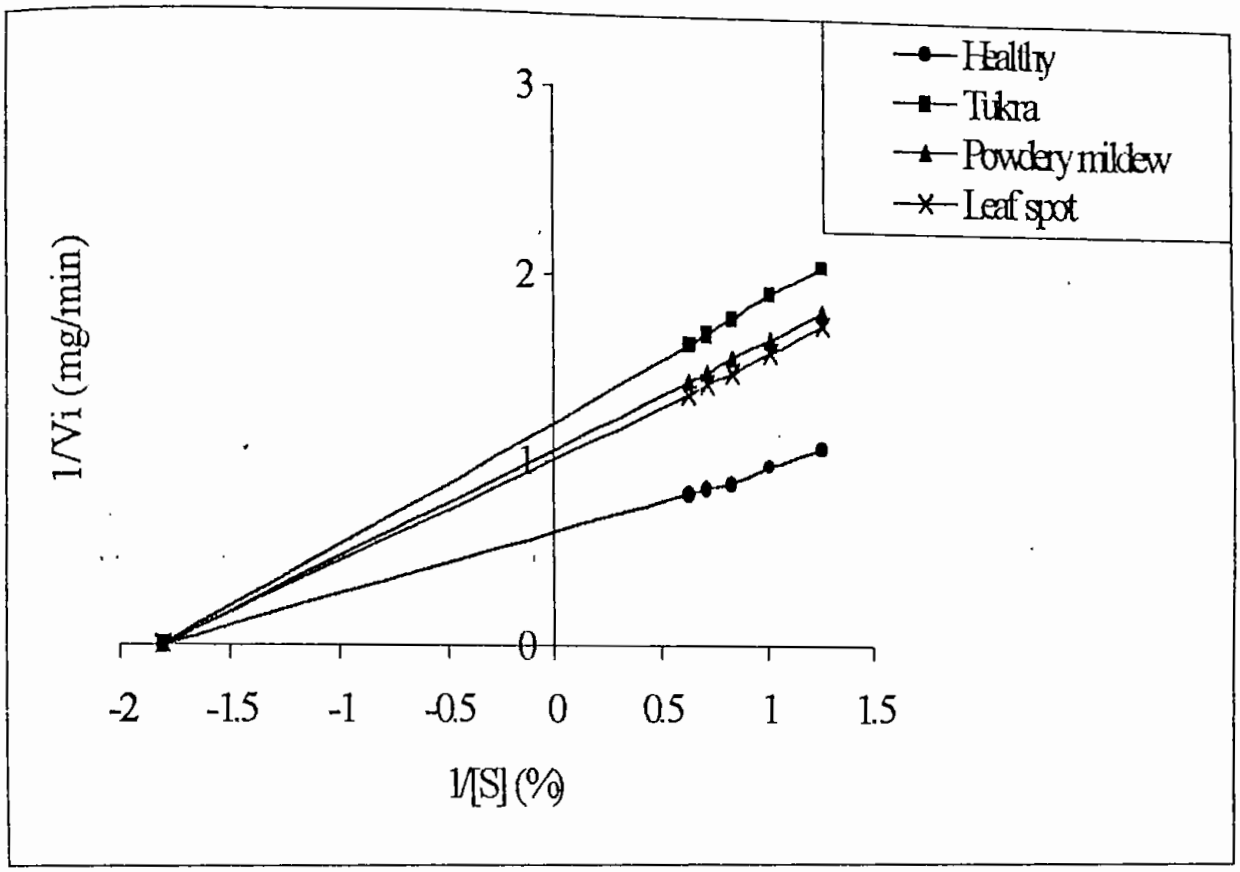


Fig.-3.12: Lineweaver-Burk double reciprocal plots for the determination of K_m value and V_{max} of crude protease, isolated from healthy and disease infected mulberry leaves.

R-3.7: Activity of cellulase in mulberry leaves

Cellulase is a hydrolytic enzyme, produced by the bacteria in the digestive tracts of animal, and is responsible for release of glucose from cellulose. Cellulose is the major constituent of plant cell walls, in particularly the secondary cell walls. The activities of cellulase in four varieties of healthy and disease infected mulberry leaves at mature stage are given in the Table-3.1.

It was found that healthy mulberry leaves contained 17-22 units whereas disease infected leaves contained 8-10 units of cellulase per gram of leaf at mature stage. The results indicated that the cellulase activities in infected leaves were much lower as compared to that in healthy leaves. Similar result was reported by Ramasamy (1980).

R-3.8: Characteristics of Crude Cellulase

R-3.8.1: Effect of pH

The crude cellulase, extracted from both healthy and disease infected mulberry leaves, showed a characteristic bell shaped pH-activity curve. As shown in Figure-3.13, the activity of crude cellulase was affected greatly with the changes of pH and the enzyme showed maximum activity at pH 5.5. Beyond this pH values the activity of crude protease decreased rapidly in the more acidic as well as in the mild basic pH region and the enzyme activity was lost completely at or above pH 10 and below pH 3.

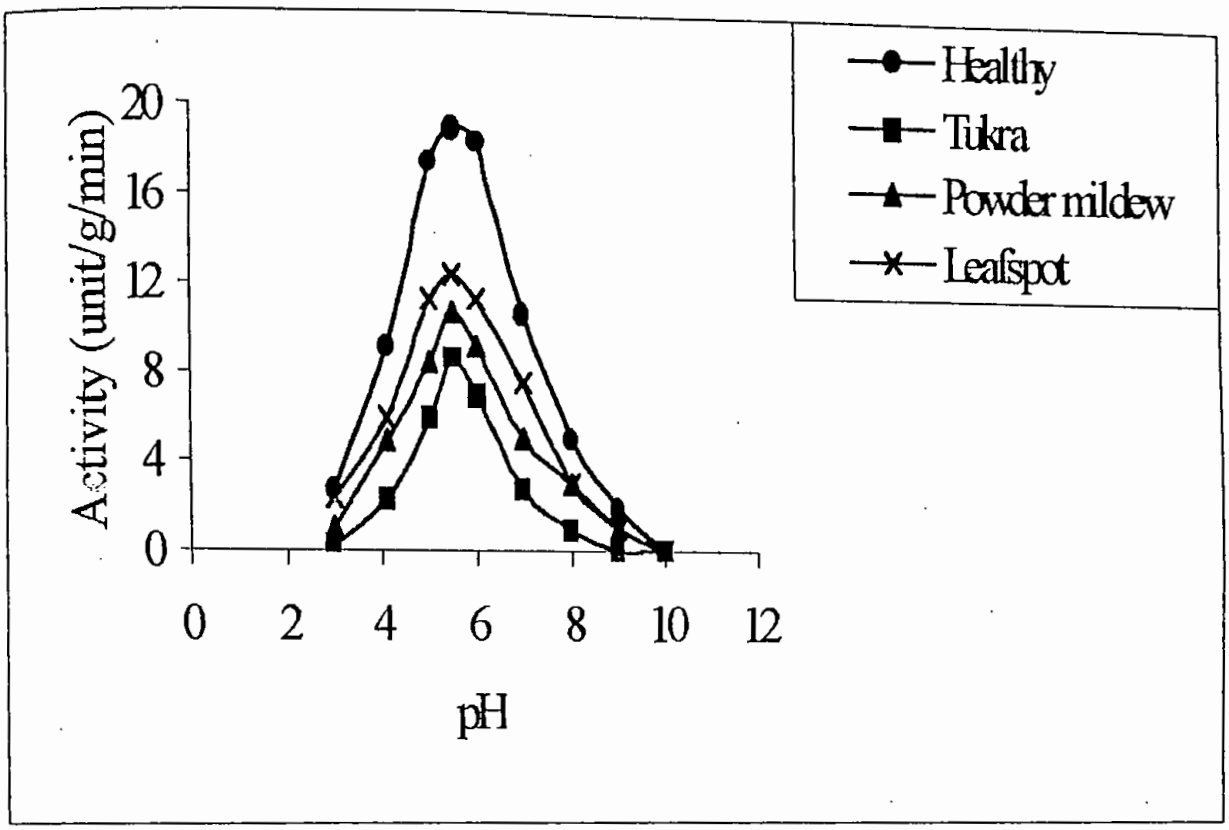


Fig.-3.13: Effect of pH on the activities of crude cellulase isolated from Healthy and disease infected mulberry leaves.

R-3.8.2: Effect of Temperature

The effect of temperature on the activities of crude cellulase, extracted from healthy and disease infected leaves are shown in the Fig-3.14. It was found that the activities were changed greatly with the changes of temperature and the enzyme showed maximum activities around 45° to 50°C. Remarkably, the optimum temperature of cellulase from healthy leaves was found to be changed slightly as compared to those from disease affected leaves. Above those temperatures the enzyme activity decreased rapidly and was lost completely at 90°C.

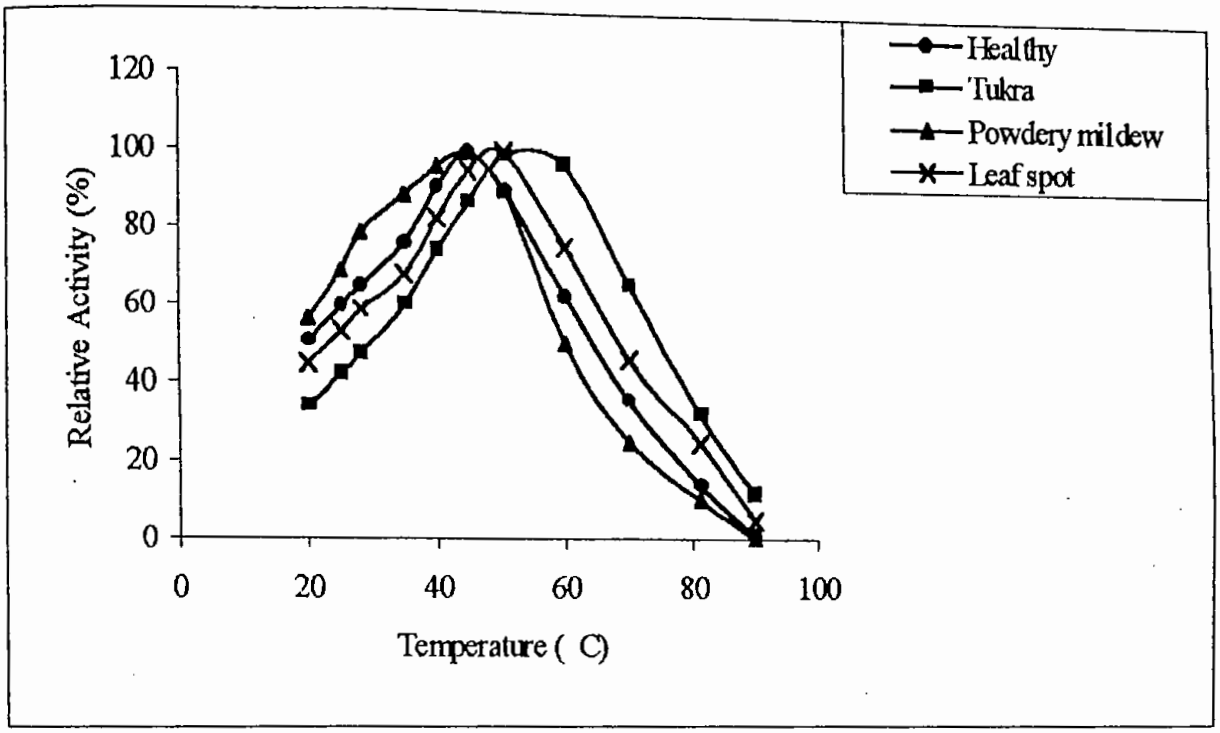


Fig.-3.14: Effect of temperature on the activities of crude cellulase isolated from healthy and disease infected mulberry leaves.

R-3.8.3: Heat stability

Heat stability curve of crude cellulase, isolated from healthy and disease infected mulberry leaves are shown in the Fig.-3.15. The data indicated that the cellulase isolated from disease infected leaves were slightly more stable that from of healthy leaves and the crude cellulase was more unstable.

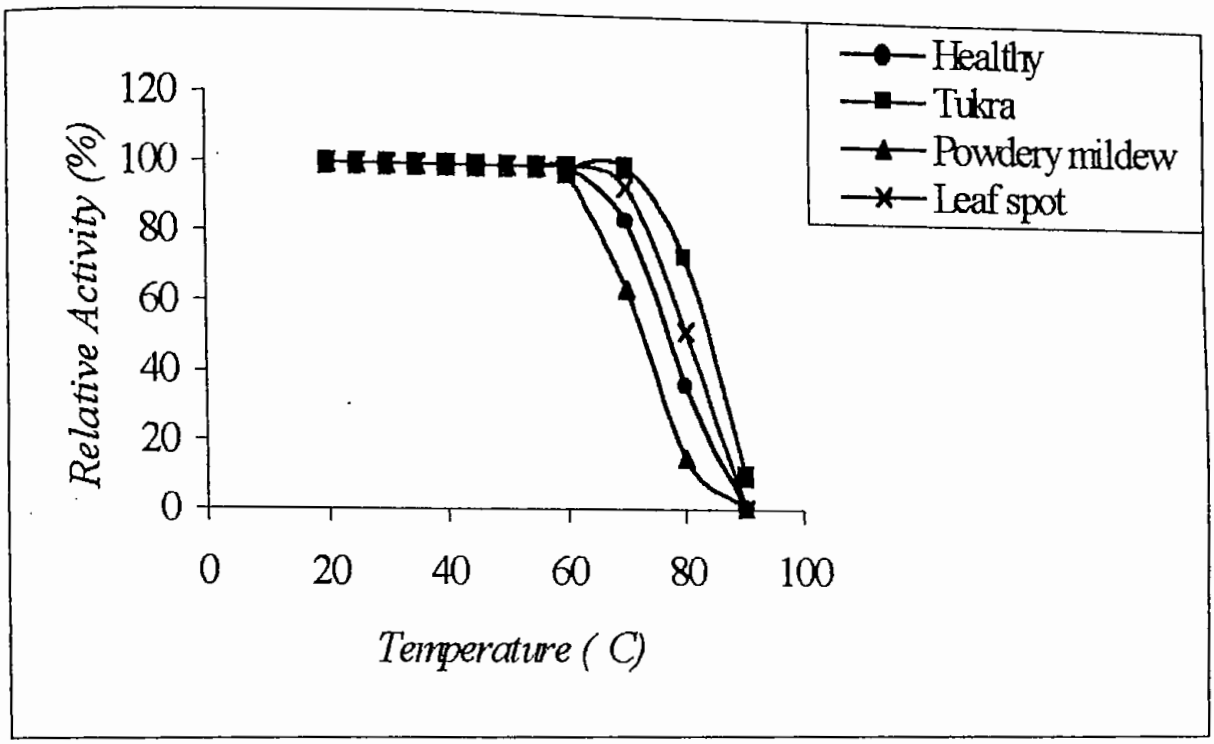


Fig.-3.15: Heat Stability curve of crude cellulase isolated from healthy and disease infected Mulberry leaves.

R-3.8.4: Kinetic Parameters of Crude Cellulase

The K_m value of crude cellulase, isolated from healthy and diseased affected mulberry leaves as determined by Lineweaver-Burk double reciprocal plots (Fig.-3.16) were calculated to be 0.598% and the V_{max} values were 33.33 mg/min, 25.00 mg/min, 22.22 mg/min and 18.18 mg/min for those from healthy, leaf spot, powdery mildew and tukra infected leaves respectively when CMC was used as substrate.

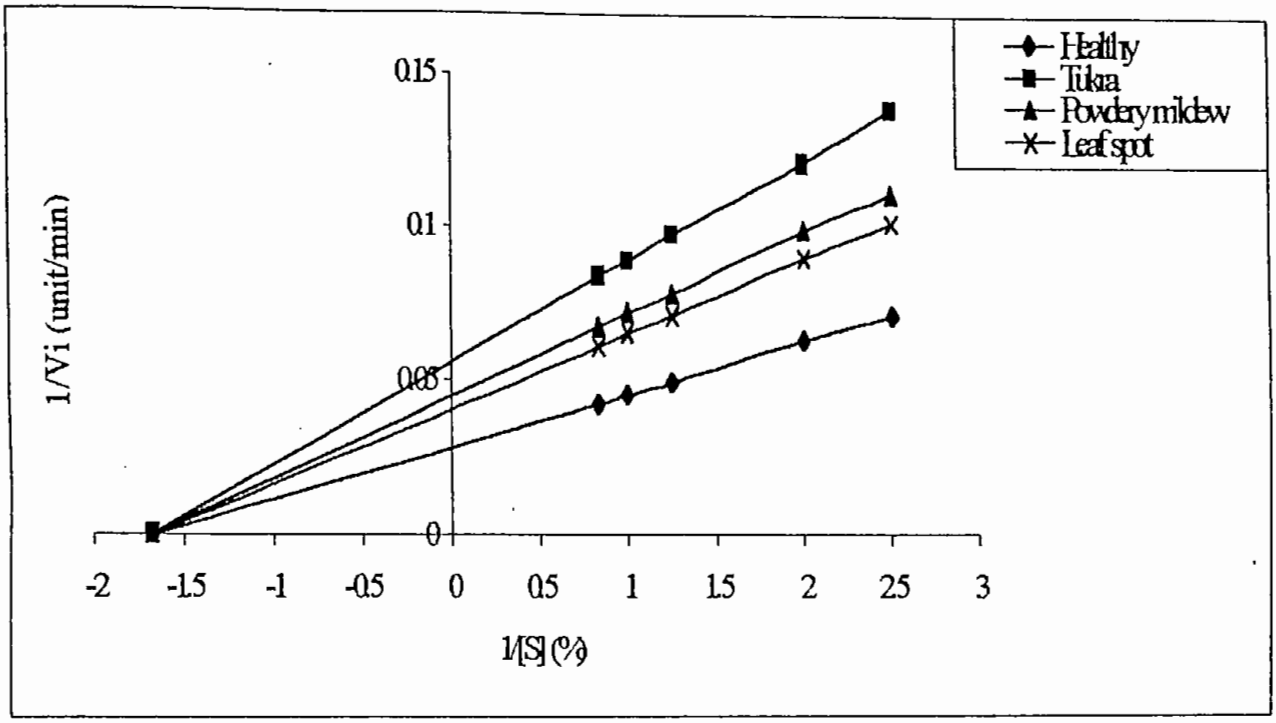


Fig.-3.16: Lineweaver-Burk double reciprocal plots for the determination of K_m and V_{max} values of crude cellulase, isolated from healthy and disease infected mulberry leaves.

Table:3.1: The activities of crude amylase, invertase, protease and cellulase isolated from healthy and disease infected mulberry leaves.

Variety of leaves	Types of leaves	Amylase (Unit/g leaf)	Invertase (Unit/g leaf)	Protease (Unit/g leaf)	Cellulase (Unit/g leaf)
BM-1	Healthy	24.20±0.02	0.11±0.002	5.18±0.05	17.58±0.01
BM-1	Tukra	13.23±0.04	0.062±0.001	2.75±0.03	7.75±0.03
BM-1	Leaf spot	10.85±0.01	0.078±0.003	2.44±0.02	9.44±0.01
BM-1	Powdery mildew	14.12±0.02	0.072±0.001	3.25±0.04	8.25±0.03
BM-2	Healthy	22.52±0.01	0.14±0.002	4.58±0.06	19.58±0.02
BM-2	Tukra	11.64±0.01	0.082±0.003	2.75±0.02	8.75±0.02
BM-2	Leaf spot	12.26±0.02	0.098±0.002	3.04±0.05	10.04±0.01
BM-2	Powdery mildew	13.08±0.04	0.082±0.003	3.22±0.04	9.22±0.04
BM-3	Healthy	24.52±0.02	0.184±0.001	5.28±0.02	22.28±0.02
BM-3	Tukra (Viral)	11.26±0.04	0.086±0.002	2.95±0.02	11.25±0.02
BM-3	Leaf spot	12.22±0.01	0.104±0.003	3.25±0.04	9.94±0.04
BM-3	Powdery mildew	14.16±0.03	0.092±0.001	3.25±0.03	10.25±0.02
BM-4	Healthy	18.44±0.02	0.138±0.002	4.88±0.05	20.88±0.02
BM-4	Tukra	10.24±0.02	0.074±0.001	2.85±0.03	8.50±0.03
BM-4	Leaf spot	9.56±0.04	0.092±0.003	3.24±0.04	11.24±0.04
BM-4	Powdery mildew	10.24±0.02	0.088±0.002	3.85±0.02	10.20±0.02

R-3.9: Activity of ascorbic acid oxidase in mulberry leaves

This enzyme is widely distributed throughout the plant kingdom. Ascorbic acid oxidase catalyzes the direct oxidation of ascorbic acid by molecular-oxygen, according to the equation.

$$\text{Ascorbic acid} + \tfrac{1}{2}\, O_2 \longrightarrow \text{Dehydro ascorbic acid} + H_2O.$$

The ascorbic acid oxidase content of mulberry leaves is shown in Table-3.2. It was found that ascorbic acid oxidase content of mulberry leaves increased rapidly after infection with disease and its content was found to be varied between 32-42 units and 52-74 units per gm in healthy and disease infected leaves. When microorganism infects leaves, the substrate ascorbic acid decreased and the enzyme became free form in the leaf because which might be responsible for increase in enzyme content. (Mahadevan and Sridhar, 1982).

R-3.10: Characteristics of Crude Ascorbic acid oxidase

R-3.10.1: Effect of pH

As shown in Figure-3.17, the activity of crude ascorbic acid oxidase, extracted from healthy and diseased infected mulberry leaves were affected greatly with the changes of pH. The crude ascorbic acid oxidase showed a characteristic bell shaped pH activity profile curve and gave the maximum activity at pH 6.0. Beyond this pH value the activity of crude enzyme decreased rapidly in the acidic as well as basic pH values and the activity was lost completely at or below pH 3 and at or above pH 10.

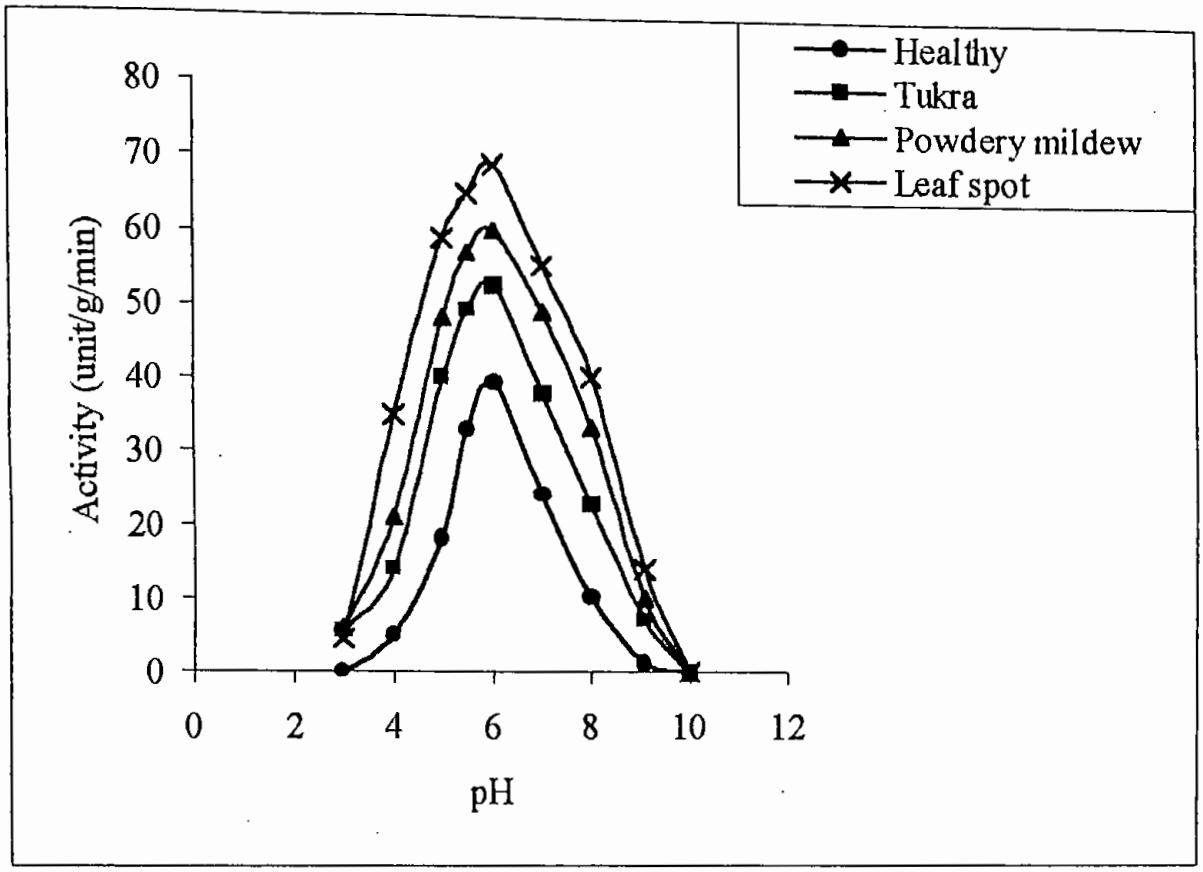


Fig.-3.17: Effect of pH on the activities of crude ascorbic acid oxidase isolated from Healthy and disease infected mulberry leaves.

R-3.10.2: Effect of Temperature

The activity of crude ascorbic acid oxidase at different temperatures are shown in the Fig.-3.18. Ascorbic acid oxidase activities increased gradually with the rise of temperature and optimum temperature was found at 30°C for powdery mildew and healthy leaves while that from leaf spot and tukra infected leaves gave maximum at 35°C. Again the enzyme activity decreased rapidly with increase in temperature and the activity was completely lost at 80°C.

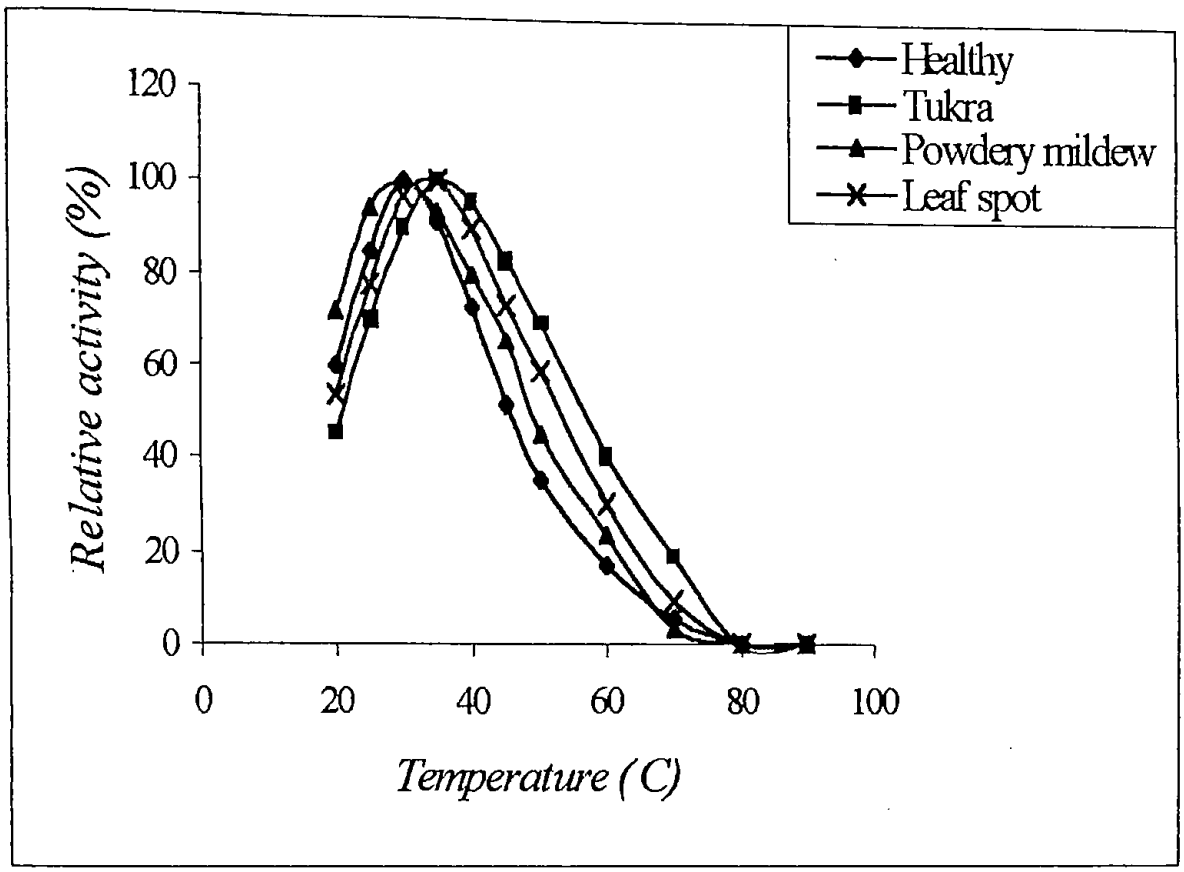


Fig.-3.18: Effect of temperature on the activities of crude Ascorbic acid oxidase isolated from healthy and disease infected mulberry leaves.

R-3.10.3: Heat stability

Heat stability of crude ascorbic acid oxidase, isolated from healthy and disease infected leaves are shown in the Fig-3.19. The enzyme isolated from tukra and leaf spot infected leaves were more stable than that from healthy leaves. The enzyme was stable at 60°C but denatured completely at 90°C.

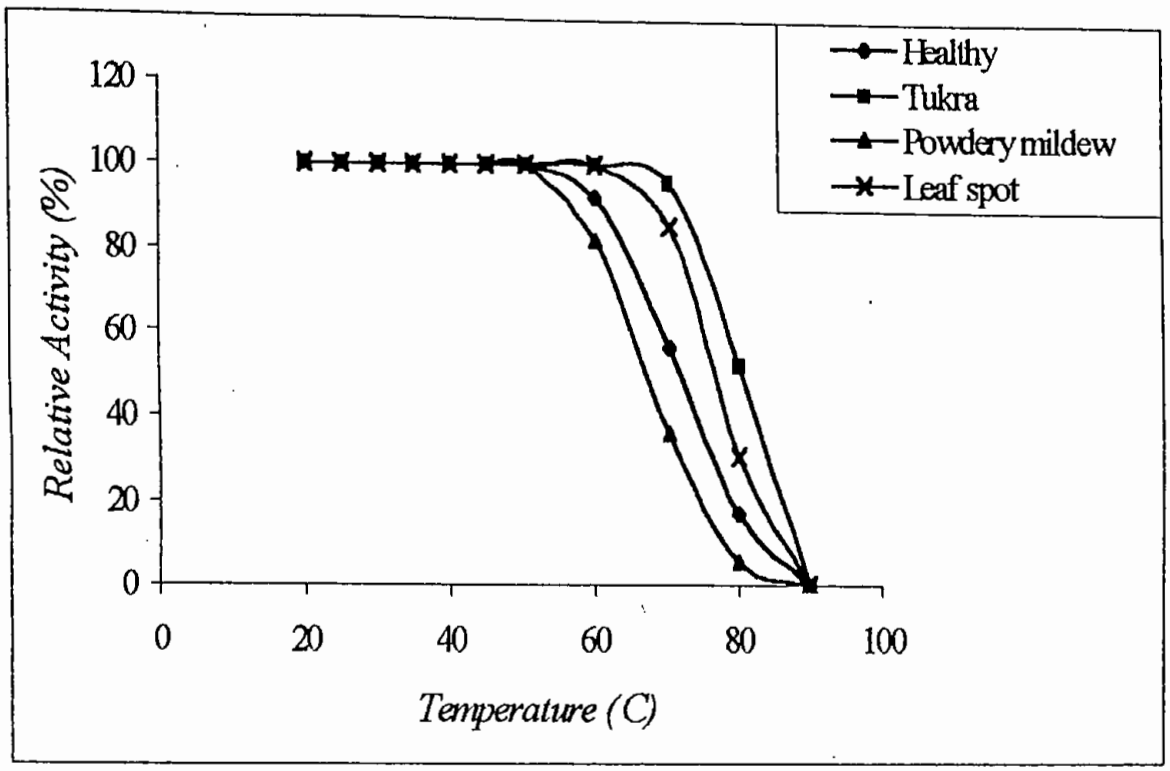
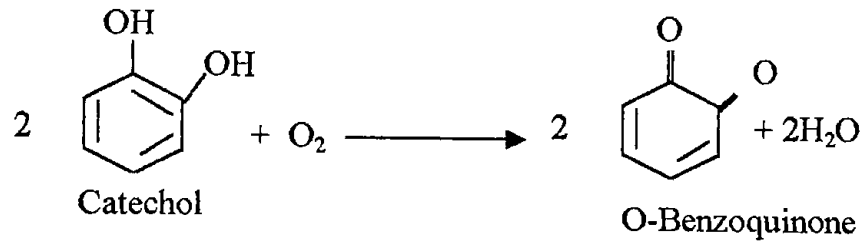


Fig.-3.19: Heat Stability curve of crude Ascorbic acid Oxidase isolated from healthy and disease infected Mulberry leaves.

R-3.11: Activity of Polyphenol oxidase of Mulberry Leaves

Polyphenol oxidase is also known as phenoloxidase, tyrosinase, dopaoxidase, catechol oxidase and potato oxidase. Polyphenol oxidase is a bifunctional, copper-containing oxidase having catecholase and cresolase activity. The enzyme catalyzes the oxidation of monophenols and orthodiphenols. Monophenols particularly tyronsine and ρ-cresol, orthodiphenols such as adrenaline, pyrogallol and substituted catechols are important substrates of the enzyme. These products are used as a anti fungal agents.

The equation, which catalyzed by polyphenol oxidase is:



Catechol O-Benzoquinone

Polyphenol oxidase content of four varieties of healthy and disease infected mulberry leaves at mature stage are shown in the Table-3.2. The polyphenol oxidase activity of mulberry leaves increased rapidly after infection with disease.

Nema (1991) reported that polyphenol oxidase activity was increased by 15-22% in betelvine leaves, when it was infected with *Xanthomonas campestns* Pv. Lovrekovich *et. al.* (1967) noted that polyphenol oxidase activity increased in *Erwina carolovora* infected potato leaves. Similar result was reported by Lovrekovich *et. al.* (1968) in tobaco leaves, infected with P. tobacco; Addy & Goodman (1972) observed that polyphenol oxidase activity increased when infection by *Erwina amylovora in* apple leaves.

R-3.12: Characteristics of Crude polyphenol oxidase

R-3.12.1: Effect of pH

The crude polyphenol oxidase, extracted from healthy and disease infected mulberry leaves, showed a characteristic bell shaped pH activity profile curve (Fig.-3.20). It was found that the polyphenol oxidase from both the sources showed almost similar pattern of pH-activity profile curve. Further, the polyphenol oxidase from healthy sources gave maximum activities at pH 7.0

while that from infected leaves gave maximum activity at pH 6.5. Beyond this pH values the activity of crude polyphenol oxidase decreased rapidly in the acidic as well as basic pH region and the enzyme activity was lost completely at pH 10. Similar results were reported by Duckworth and Coleman (1970) and by Jiang, Zauberman and Fuchs (1997) for polyphenol oxidase.

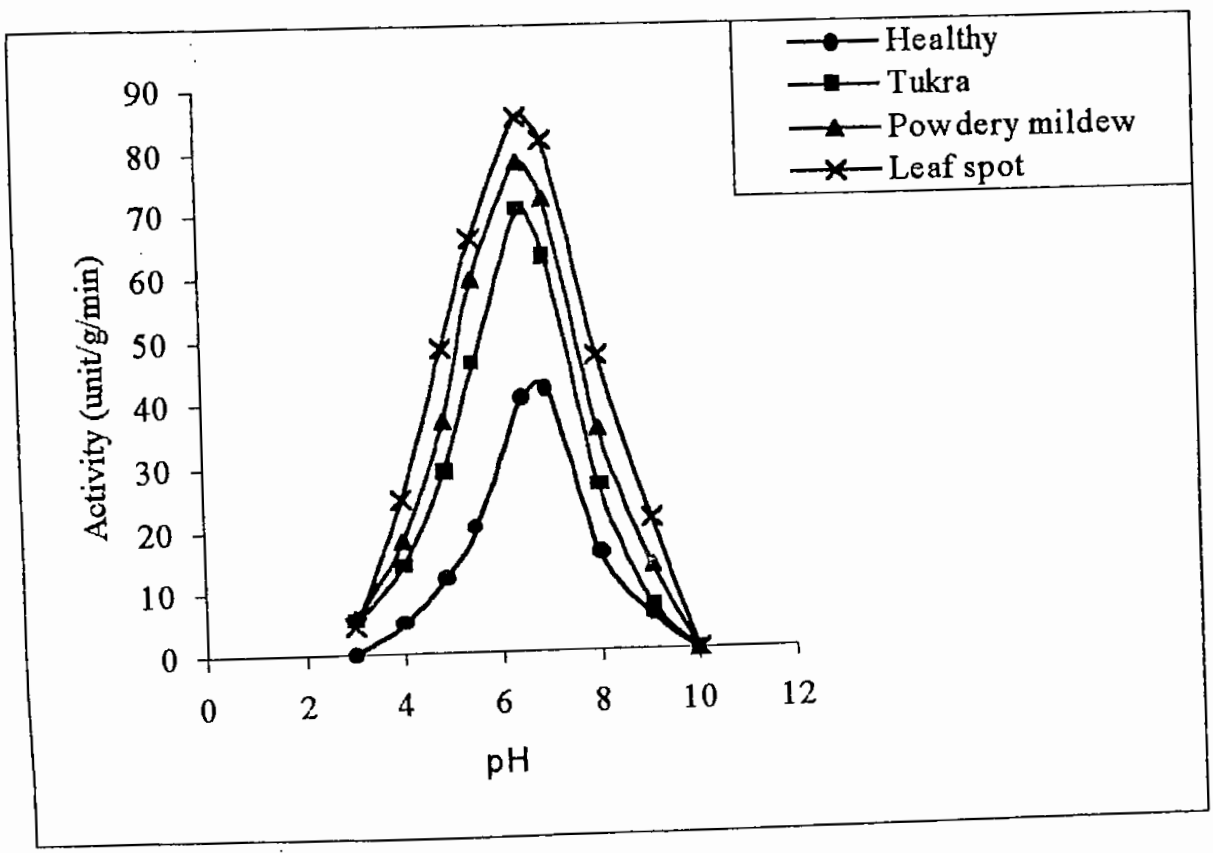


Fig.3.20: Effect of pH on the activities of crude polyphenol oxidase isolated from Healthy and disease infected mulberry leaves.

R-3.12.2: Effect of Temperature

The activity of crude polyphenol oxidase at different temperature are shown in the Fig.-3.21 The activities increased gradually with the rise of temperature and gave maximum activity at 30-35°C. It was also found that the enzyme activity decreased rapidly with further rise of temperature and lost completely around 80°C.

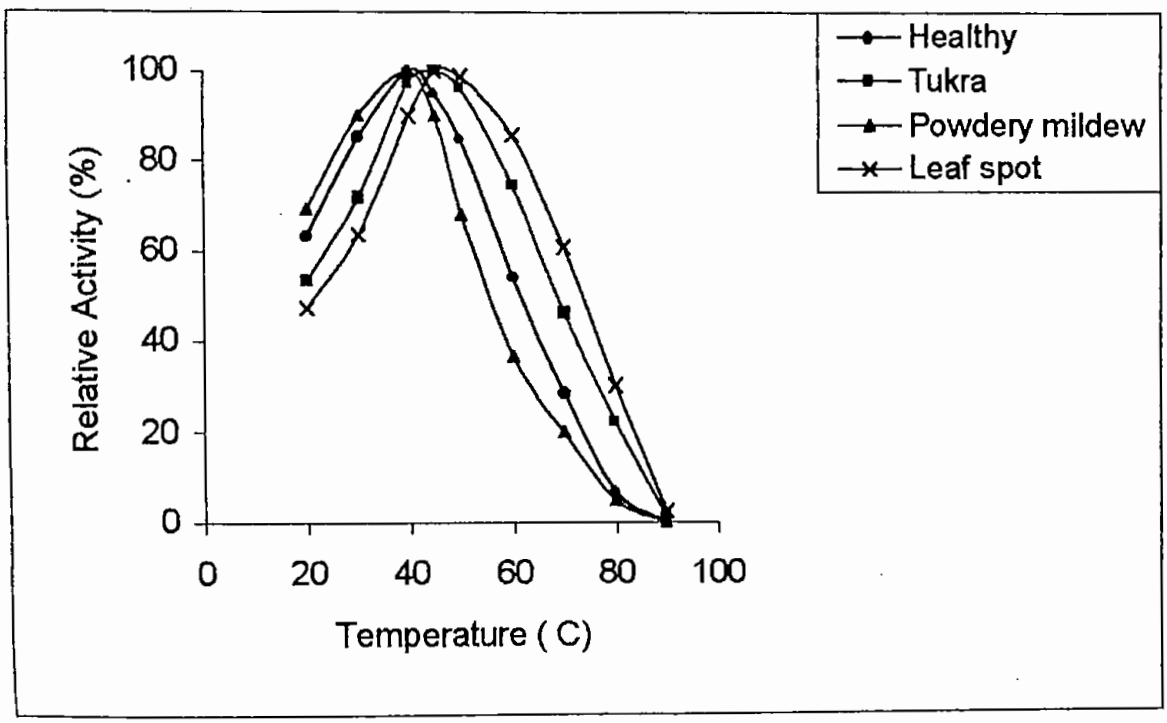


Fig.-3.21: Effect of temperature on the activities of crude polyphenol oxidase isolated from healthy and disease infected mulberry leaves.

R-3.12.3: Heat stability

As shown in the Fig.-3.22, the crude polyphenol oxidase isolated from leaf spot and tulira infected mulberry leaves are more stable than that from healthy and powdery mildew infected leaves and the enzymes were found to be stable at 60-80°C

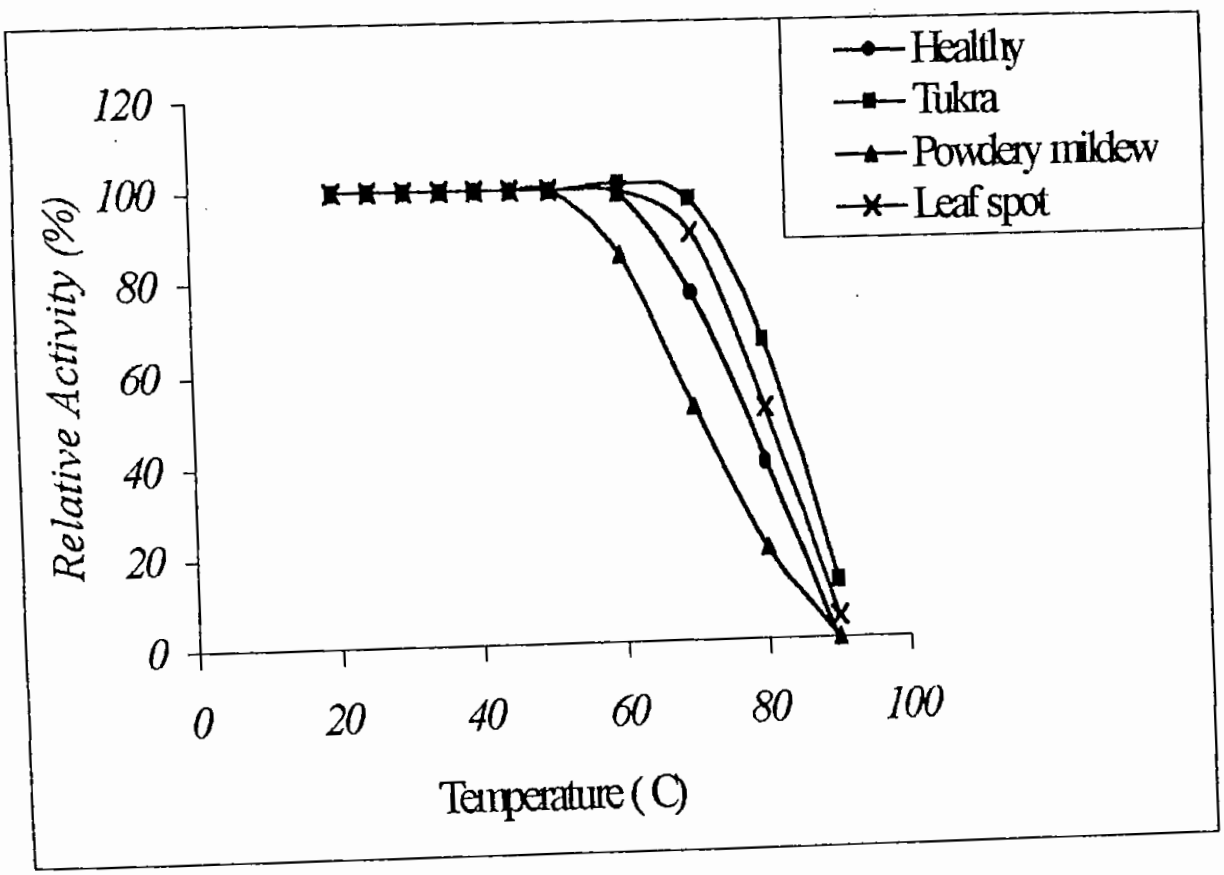


Fig.3.22: Heat Stability curve of crude Polyphenol Oxidase isolated from healthy and disease infected Mulberry leaves.

R-3.13: Activity of peroxidase in mulberry leaves

Peroxidases are widely distributed in the plant kingdom. It catalyzes the oxidation of various "hydrogen donors" like ρ-cresol, benzidine, ascorbic acid, nitrate and cytochrome in the presence of H_2O_2. The reaction may be represented as:

$$H_2O_2 + 2AH \longrightarrow 2A + 2H_2O$$

Peroxidase content of four varieties of healthy and disease infected mature mulberry leaves are given in the Table-3.2. The present results clearly demonstrated that the activity of peroxidase increased significantly when microorganisms infect leaves.

Nema (1991) reported that peroxidase activity was increased by 40-80% in betelvine leaves when it was infected with *Xanthomonas campestns* Pv. Similar result was reported by Lovrekovich *et. al.* (1968) in tobaco leaves, infected with tobacco; Addy and Goodman (1972) observed the increase of peroxidase activity when infection by *Erwina amylovora in* apple leaves while Kacel and Munjal (1980) reported that peroxidase activity in fungus infected apple fruits significantly higher than healthy fruits.

R-3.14: Characteristics of Crude Peroxidase

R-3.14.1: Effect of pH

As shown in Figure-3.23, the activity of crude Peroxidase was affected greatly with the changes of pH. All showed a characteristic bell shaped pH activity profile curve and gave maximum activity at pH 5.5. Further, the activity of the enzyme was lost completely at pH 10.

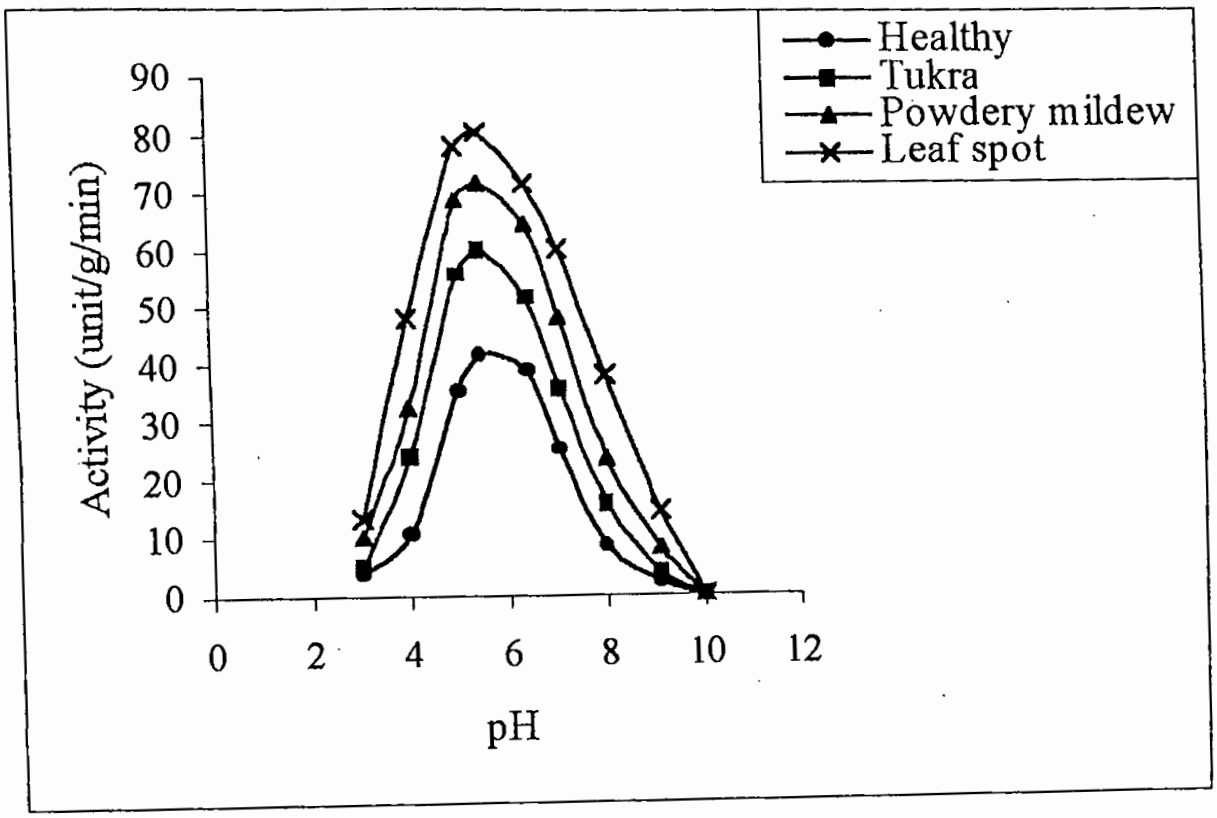


Fig.-3.23: Effect of pH on the activities of crude Peroxidase isolated from Healthy and disease infected mulberry leaves.

R-3.14.2: Effect of Temperature

The activity of crude peroxidase at different temperature are shown in the Fig.3.24. Peroxidase activity increased gradually with the rise of temperature. The crude enzyme from healthy, powdery mildew and leaf spot disease infected leaves gave maximum activity at 25°C while that from tukra infected leaf gave

maximum activity at 30°C. The enzyme activity decreased rapidly with further rise of temperature and lost its activity completely at 70°C.

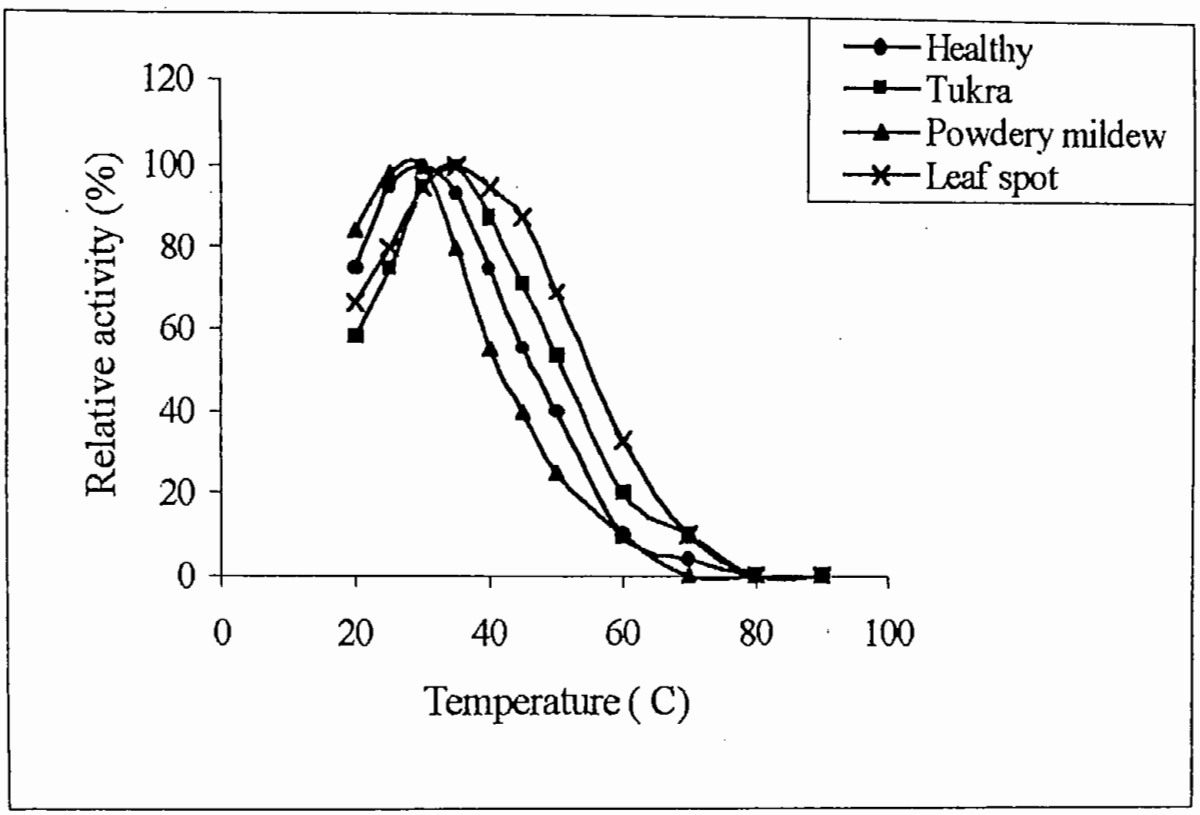


Fig.3.24: Effect of temperature on the activities of crude Peroxidase isolated from healthy and disease infected mulberry leaves.

3.14.3: Heat Stability

Crude peroxidase enzyme isolated from tukra infected leaf was more stable than that from other sources (Fig.-3.25). The crude peroxidase were 100% active at 60°C but completely denatured at 90°C.

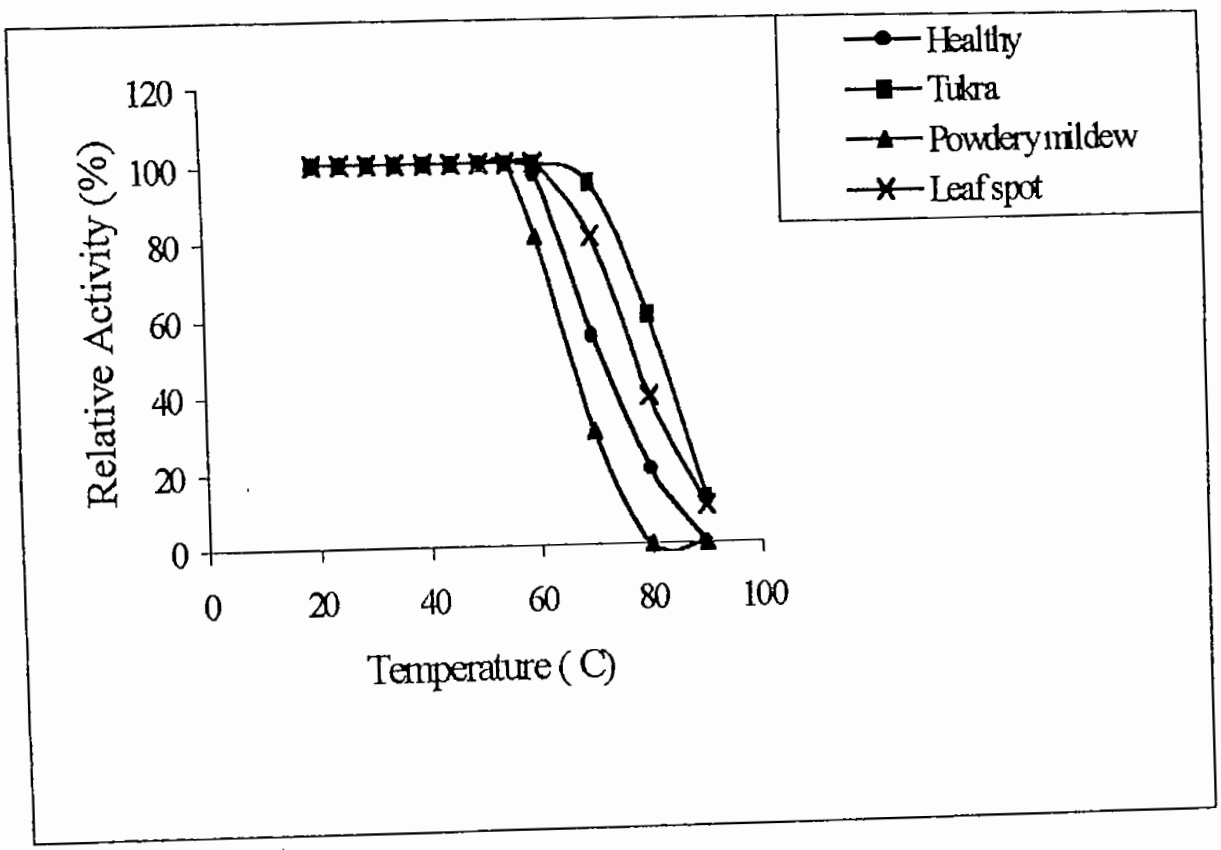


Fig.3.25: Heat Stability curve of crude Perxidase isolated from healthy and disease infected Mulberry leaves.

Table:3.2: The contents of crude polyphenol oxidase, Ascorbic acid oxidase and peroxidase in healthy and disease infected Mulberry leaves.

Variety of leaves	Types of leaves	Polyphenol oxidase (unit/g/min)	Ascorbic acid oxidase (Unit/g/min)	Peroxidase (Unit/g/min.)
BM-1	Healthy	44.80±0.02	28.58±0.01	33.11±0.02
BM-1	Tukra	66.23±0.04	52.75±0.03	60.08±0.05
BM-1	Leaf spot	72.85±0.01	64.44±0.01	48.58±0.03
BM-1	Powdery mildew	78.12±0.02	58.25±0.03	52.54±0.04
BM-2	Healthy	42.52±0.01	34.58±0.02	28.88±0.02
BM-2	Tukra	62.64±0.01	58.75±0.02	52.22±0.03
BM-2	Leaf spot	71.26±0.02	60.04±0.01	44.56±0.05
BM-2	Powdery mildew	70.08±0.04	74.22±0.04	40.26±0.03
BM-3	Healthy	48.52±0.02	34.28±0.02	35.25±0.04
BM-3	Tukra (Viral)	71.26±0.04	61.25±0.02	62.44±0.02
BM-3	Leaf spot	80.22±0.01	69.94±0.04	58.34±0.03
BM-3	Powdery mildew	75.16±0.03	74.25±0.02	64.25±0.05
BM-4	Healthy	44.80±0.02	40.88±0.02	32.86±0.02
BM-4	Tukra	62.24±0.02	58.50±0.03	56.24±0.04
BM-4	Leaf spot	74.56±0.04	58.50±0.04	61.22±0.03
BM-4	Powdery mildew	70.24±0.02	64.20±0.02	52.78±0.04

CONCLUSION

CONCLUSION

Increased production of mulberry leaf crop per hectare is the primary basis of expansion and development of Sericulture Industry. On the other hand increased leaf production enhance the cost-benefit ratio of the farmers. High nutritional quality leaf harvest ensures the production of good quality cocoons and these results encouraged more people to take Sericulture as their pastime profession. Although mulberry leaf production is not a problem in Bangladesh but important to control or protect from mulberry leaf diseases like powdery mildew, leaf spot and tukra disease. These diseases infect the leaves in the respective seasons of their occurrence leading to the degradation and defoliation of the leaves. The present research indicated that most of the nutrient compositions of mulberry leaf were decreased significantly which are required for production of good quality cocoons. Thus the disease hampers ultimately in the silk Industry.

Most of the experiment carried out under the present study are of applied in nature and the results obtained could be utilised for the production of increased amount of quality leaves and thus Sericulture Industry could get support for its further development. Besides, Scientists working in Sericulture Industry may be benefited for their future planning of research for the development of their industry.

From the findings of the present work more resistance variety could be recommended for mass scale cultivation. This will enable the farmer as well as field officers and the managers of this industry to decide the course of action to be followed against the serious leaf diseases in proper time and information regarding importance of fertiliser application in appropriate dose. The tukra disease spread so quickly during the drought season at high temperature (above 32°C). Irrigation

disease spread so quickly during the drought season at high temperature (above 32°C). Irrigation decreases spread of tukra disease. So, proper irrigation of the mulberry field during the drought seasons to improve the quality and quantity of leaves.

From the discussion of the present work, it is very easy to understand that to improve the Sericulture Industry and increase the productivity of silk cocoons and silk, following integrated approaches should be undertaken which include:

i) Selection of more disease resistance high yielding mulberry varieties for commercial cultivation.

ii) Take timely action against diseases and harmful pest.

iii) Proper irrigation during drought season.

iv) Application of appropriate dose of nitrogen fertiliser and avoiding high doses.

REFERENCES

REFERENCES

Addy, S.K. and Goodman, R.N. (1972). Polyphenoloxidase and peroxidase activity in apple leaves inoculated with a virulent or an avirulent strain of *Erwina amylovora*. *Indian Phytopath.* **25:** 575-579.

Agarwal M L and Tayal M S (1987) Gibberelline antagonizing the nematicidal effects on two hydrolyzing enzymes, protease and invertase and related chemical metabolites in *Lycopersicon esculentumm* and *Solanum melongena*. *Pesticide Biochemistry and Physiology*. **28 (3):** 297-300.

Ainsworth, G.C. (1971) Ainsworth and Bisby's Dictionary of fungi. 6 th ed. Commonwealth Mycological Institute, Kew, Surrey, England. pp. 663.

Ali, M.R. (1995). Studies on important fungal disease of mulberry leaf in Bangladesh and their control. Ph.D. thesis. Rajshahi University, Bangladesh. pp 1-224.

Ali, M.R. and Quiyyum, M.A. (1993). Incidence of leaf spot disease in different varieties of mulberry. Bull Sericult. Res. **4:** 74-76.

Anon (1960). Reports on determination of β-carotene, vitamin-B_1, vitamin-B_2 of betel leaves in this study. *Bio. In nature.* **46(3)**: 412.

Anonymous, (1950) Powdery mildew of cucumbers, pumlins and related plant . Agr. Gaz. New South Wales. 41:587-588.

Anonymous, (1969). Annual Administration and scientific report. Central Sericulture Research and Training Institute, Mysore.

Anonymous, (1975). Textbook of Tropical Sericulture. Japan Overseas Corporation volunteers. Tokyo, Japan. pp. 57-59, 114-116, 154-166, 375-439.

Association of Official Analytical Chemist (AOAC) (1980). Official Methods of Analysis, 13th Edition. Washington D C, USA.

Arya H.C. and Ghemawat, M.S. (1954). Occurrence of powdery mildew of wheat in the neighorhood of Jodpur. Ind. Phytopath. 6:123-130.

Bakshi, B.K. Reddy Ram, M.A., Puri, Y.N. and Singh, S. (1972). Survey of the diseases of important FRIC, Deharddun, India p. 93-98.

Balasubramanian, K.A. (1972) The possible role of proteolytic enzymes in the pathogenesis of *Rhizopus stololfer.Ind. Phytopath.* **25:** 475-477.

Bari, M.A. Qaiyyum, M.A. and Ahmed, S.U. (1988). Estimates of genetic variability for some quantitative characters in mulberry. Proc. Int. Congress on Tropical Sericulture practices. Bangalore, India. 18-23, Feb. p. 10.

Basavarajappa, S. and Savanurmath, C.J. (1997) Effect of feeding of mulberry (*Morus alba* L.) leaves of variable maturity on the economic characters in some breeds of silkworm *Bombyx mori* L. *Bull. Sercult. Res.* **8:** 13-17.

Bedi, P.S. and Singh, J.P. (1972) Leaf blight of rose in Panjab. Ind. Phytopath. **25:** 534-539.

Bernard, L. Oser (1965) Hawk's Physiological chemistry 14 th ed. Mc-Graw-Hill Book Company, New York. pp 1264-1265.

Bessey, O.A. and King, C.G. (1933) The distribution of vitamin C in plant and animal tissues and its determination. *J. Biol. Chem.* **103:** 687 - 698.

Bligh, E.G. and Dyer, W. (1959) A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37:** 911.

Bray, H.G. and Thrope, W.V. (1954) Analysis of phenolic compounds of interest in metabolism. *Meth. Biochem. Anal.* 1027-1052.

Bonner, J.T. (1948). A study of temperature and humidity requirents *Aspergillus niger*. Mycologia. **40:** 728-738.

Borah, R.C., Bajaj, K.L. and Bhatia, L.S. (1978). Biochemical changes in tea leaves after infection with rust (*Cephaleuros parasiticus*). Phytopath Z. **93:** 208-217.

Bose, P.C. (1987) Genetic Resources of mulberry and utilisation. Edited by Senguptan, K. and Dandin, S.B. Central Sericulture Board. Mysore, India, p. 184-185.

Bose, P.C. (1987). Mulberry is the medicine. Indian silk, **26**(7) Nov. p. 53-54.

Bruehl, G.W. and Confer,B. (1971). Physiological and environmental factors that affect the severity of snow mould to wheat. Phytopath. **61**: 792-799.

Buxton, P.A. and Mellanby, K. (1934). The measurement and control of humidity. Bull. Entamol. Res. **25:** 172-197.

Chung, T.Y. (1986). Studies on the soil suppressive to bananaFusarium wilt. I. Screening soils for suppressiveness to *Fusarium oxysporum* f sp. Cubens (E F Sen) Snyder & Hans. PI. Pro Bull. **28:** 345-352.

Chupp, C. (1953). A monograph of fungus genus *Cercospora.* pp. 667. Published by the Author, Ithaca, New York.

Clayton, C.N. (1942) the germination of fungal spores in relation to controlled humidity. Phytopathology. **32:** 921-943.

Dandin, B. and Ramesh, S.R. 91987) Akalpa vruksha called mulberry. Indian silk, 26(6)Oct. p 49-53.

Dayakar Yadab, B.R., Sukumar, J. and Prasad, K.V. (1993). Screening for potential resistance in the mulberry to leaf spot (*Cercospora moricola*) disease. Sericologis. 33(1): 81-90.

Desai B B and Deshpande P B (1978a) Effects of stage of maturity on some physical and biochemical constituents enzymes activities of banana. *Mysore J. Agric. Sci.,* **12:** 193-201.

Desai B B and Deshpande P B (1978b) hydrolytic and oxidative enzymes during banana ripening. *Scientia Horticulture* **9(2):** 147-153.

Duckworth, H.W., and Coleman, J.E.: Physicochemical and Kinetic Properties of Mushroom Tyrosinase, *J. Biol. Chem.*, *245*, 1613 (1970).

Dwivedi, N.K. (1988) Importance of white mulberry as a fooder tree. Indian silk, 27 (6), Oct. p. 18-19.

Etoi, S., Nakayama, K. and Kubomura, Y. (1960) Studies on the powdery mildew disease of mulberry tree caused by *Phyllactinia corylea* Karst. (II). On the germination and vitality of conidia. J. Seric. Sci. Jpn. **29:** 79-84.

Etoi, S., Nakayama, K. and Kubomura, Y. (1962) Studies on the powdery mildew disease of mulberry tree caused by *Phyllactinia moricola.* Bull. Seric. Exp. Stn. Japan. **17:** 433-445.

Etoi, S., Nakayama, K. and Kubomura, Y. (1964) The spread of mulberry powdery mildew and control of first outbreak in spring by eradicating perithecia. Bull. Seric. Exp.Stn. Japan. **18:** 545-558.

Farkas, G.L. and Kiraly, Z. (1962). Role of phenolic compounds in the physiology of plant diseases and disease resistance. *Phytopath. Z.* **44:** 105-150.

Fukuda, T. (1963). Studies on silk secretion of silkworm (*Bombyx mori*). *Indian . Seric.* **2(3)**: 4-5.

Ganacharya, N.M. (1976). Physiological requirments of *Fusarium oxysporum* causing wilt of potato. *Ind. Phytopath.* **30:** 532.

Garcia, E. and Lajola, F.M. (1988). Starch transformation during banana rpening: the amylase and glucosidase behavior. *J. Food Sci.* **53(4):** 1181-1186.

Garg, I.D. and Mandahar, C.L. (1975). Physiology of powdery mildew infected leaves of *Abelmoschus esculentus*.II. nitrigen metabolism. *Indian Phytopath.* **28:** 464-467

Gavindaiah, Philip, T., Sengupta, K., Girindhar and Suryanarayan, A.N. (1990). Incidence of leaf spot disease in relation to epidermal and cuticle thicness in some indigenous varieties of mulberry. Sericologia. 30(2):257-260.

Glick D (1957) *Methods of Biochemical Analysis.* Interscience publishers. London **4:**p. 2.

Goose, R.T. and Tschirsch, M. (1962) Effect of environmental factors on spore germination, spore survival and growth of *Gloeosporium musarum.* Mycologia. **54**: 353-367.

Hashinaga F, Yamato F and Itoo S (1983) Partial purification and characterization of protease from passion fruit juice. *Mem. Fac. Agr. Kagoshima Univ.* **19:** 73-79.

Hassanein, M.H. and EL Shaarawy, M.F. (1962). The nutritional value of certain mulberry varieties. Revue Duver A sole. *J. of silkworm.* **14:** 216.

Hayashi, Y. (1962). Amino acid activation in tobacco leaves infected with tobacco mosaic virus. *Virology* **18**: 140-142.

Hedge R.K. and Munjal, R.L. (1967). Physiolgical changes in the bean pods due to infection of *Collectotrichum lindemuthianum.Indian Phytopath.* **24:** 186-188.

Horie,Y. and Nakasone, S. (1971). Effect of the levels of fatty acid and carbohydrates in a diet on the biosynthesis of fatty acids in larvae of silkworm *Bombyx mori. J. Insect Physiol.* **17:** 1441-1450.

Hossain, T., Alam, Z. and Absar, N. (1999) Changes in different nutrients and enzyme contents in mango leaves infected with *Colletotrichum glaeosporioides*. *Indian Phyotopath.* **52(1)** : 75-76.

Hugar, I.I. and Kaliwal, B.B. (1997). Effect of nickel chloride supplementation on some economic trails of the bivoltne silkworm, *Bombyx mori* L. *Bull. Sericult. Res.* **8:** 23-27.

Ismail, H. and Wadud Mean, M.A. (1981). Comparative efficacies of five foliage fungicides in controling *Cercospora* leaf spot of peanut. *Bang. J. Bot.* **10(1):** 58-62

Ito, T. (1972) An approch to nutritional control mechanisms in the silkworm *Bombyx mori*. Israel. Journal of Entamology **7:** 1-4

Ito, T. and tanaka, S.M. (1961). Per as adminitration of the nutrients to the silkworm larvae, II. Effect of the administration of various sugar and their nutritive value.

Ito, T. (1961). Effect of dietary ascorbic acid on silkworm *bombyx mori* L. Sericultural experiments station Suginamiku. Tokyo. Decem. 9. Nature. pp 951.

Ito, T. (1967). Nutritional requirements of the silkworm *Bombyx mori*. Proc. Jap Acad. **43:** 57-61.

James, W.C. (1971) A manual of assessment keys for plant diseases. Can. Dep. Agr. Publ. 1458. pp 80

Jain J L (1988) Fundamentals of Biochemistry. Wiley Eastern Ltd. New Delhi, India.

Jayaraman. J (1981) *Laboratory Manual in Biochemistry.* New age International Lit, New Delhi. 180 pp.

Jeyarajan, R. (1986). Diseases mulberry and their control. National Seminar on Prospects and problems of Sericulture in India. March, 27-30. P. 37.

Jiang, Y-M., Zauberman, G., Fuchs, Y. (1997) Partial purification and some properties of polyphenol oxidase extracted from litchi fruit pericarp. *Postharvest Biology and Technology* **10:**221-228

Kanit, K.S.R. (1975). Studies on *Cercospora* leaf spot of mulberry (*Moris alba* L.) M.Sc. (Agric.) thesis, Uni. Of Agr. Sc. Hebbal, Banglore-24.

Khare U.K. and Neema, G.K. (1982). Factors affecting germination of spores af *Alternaria poori invitro. Ind. Phytopath.* **35:** 100-103.

Kore, S.S. and Kharwade, L.R. (1987). Fruit rot of round ground by *Fusarium oxysporum. Ind. Phytopath.* **40:** 251.

Klapper B R, Jameson D M and Mayer R M (1973) The purification and properties of an extra cellular protease from *Aspergillus oriza* NRRL 2160. **304:** 505-512.

Kunitz M (1947) Crystalline soybean trypsin inhibitor. *J. Gen. Physiol.* **30:** 291-310.

Khan, A.Z.M. Nowsher A. and Shamsi,S. (1983) *Cercosporae* from Bangladesh. I. Bang. J.Bot. **12:** 66-80.

Krisnaprasad, K.S. and Siddaramaiah, A.L. (1979) Studies on the development of powdery mildew disease of mulberry in Karnataka. Ind. J. Seric. **28:** 9-13.

Krisnaswami, S., Kumararaj, S. Vijayaraghavan, K. and Kasi Viswanathan, K. (1971). Silkworm feeding trials for evaluating the quality of mulberry leaves as influenced by variety, spacing and nitrogen fertilization, *India J. Seric.* **10:** 79-89

Krishnan, H.B. and Pueppke, S.G. (1990). Cherry fruit invertase: Partial purification, characterization and activity during fruit development. *J.Plant physiol.* **135:** 662-666.

Kumar, R. Gepta, J.S. and Shah, A. (1983). Effect of nutrition on the incidence of early blight disease and yield of potato. *Ind. Phytopath.* **36:** 405-406.

Leu, L.S. and Lee, C.C. (1982). Morphology and conidial germination of powdery mildew of mulberry. Plant Prot. Bull **24:** 27-36.

Lim, G. and Tang, M.C. (1985). Growth and sporulation in culture of *Colletotrichum gloeosporiodes* Penz. Sacc., the pathogen of pawpaw anthracnose. *Bull. Trimestriel de la Societe Myocologique de France.* **101:** 183-189.

Loknath, R., Shivashankar, K. and Kasivishwanath, K. (1986) Effect of foliar application of magnessium and micro nutrients to mulberry on the quality and production of cocoons. *Indian J. Seric.,* **25:** 40-41.

Lowry O H, Rosebrough N J, Farr A L and Randall R L (1951) Protein measurement with the folin phenol reagent. *J. Biol chem.* **193:** 265-275.

Loomis W E and Shull C A (1937) *Methods in Plant Physiology.* McGraw Hill, New York.

Lovrekovich, L., Lovrekovich, H. and Stahman, M.A. (1967). Inhibition of phenol oxidation by *Erwina caratovora* in potato tuber tissue and its significance disease resistance. *Phytopathology.* **57:** 737-742.

Lovrekovich, L., Lovrekovich, H. and Stahman, M.A. (1968). Tobacco mosaic virus induced resistance to *Pseudomonas tobaci* in tobacco. *Phytopathology.* **58:** 1034-1035.

Mahadevan A and Sriddhar R (1982) *Methods in Physiological Plant Pathology.* 2nd edition , Sivikami publication, Madras. India. 5-8, 25-30, 55-64, 115, 152-159, 205, 316 pp.

Mahadevan A. (1970). The role of proteolytic enzymes in disease development. In plant disease problems. *Ind. Phytopath,* New Delhi. pp 179-184

Mao, W W and Kinsella J E (1981) Amylase activity in banana fruit: Properties and changes in activity with ripening. *J. Food Sci.* **46:** 1400-1409.

Maria J L, Graciela S. Horacio P, Marta A V and Antonio R S. (1995) Purification and properties of the soluble acidic invertase from *Oryza sativa. Phytochemistry.* **38 (2):** 321-325.

Maris McArthur-Hespe G W F (1956) The activity of starch hydrolyzing enzymes in pears during the development and storage. Acta Botanic. Neerlandica 5:200 Cited from *J Food Sci.* **46:** 1403 (1981)

Mehta, P., Vyas, M. and Suksena, S. B. (1995) Metabolic changes during pathogenesis of fruit-rot disease of tamamto. *Indian Phytopath.* **28**: 253-255.

Miller G L (1972) Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* **31(3):** 426-428.

Monga, D. and Grover, R.K. (1991). Chemical control of root rot of cowpea in relation to altered pathogenicity of *Fusarium solani. Ind. Phytopath.* **44:** 462-469.

Nabeesa B and Unnikrishnan S (1988) Charbohydrates, amylase and invertase activity during ripening in redand green red banana. *Indian J. Plant Physiol.* **xxxi:** 28-37.

Nakanishi K and Yokotsuka K (1990) Characterization of thermostable invertase from wine grapes. *J. Ferment. Bioeng.* **69 (1):** 16-22.

Naik K C and Gangolly S R (1950) *A monograph on classification and nomenclature of South Indian mangoes.* Supdt. Govt. Press, Madras.

Naik, M.K., Hiremath, P.C. and Hiremath, S.V. (1988). Post infectional changes in the betel vine leaves infected with *Collectotrichum gloeosporioides. Indian Phytopath.* **41(3):** 370-372.

Nema, A.G. (1991). Changes of chlorophyll, nitrogen, protein, amino acid and some enzyme contents in betelvine leaves infected with *Xanthomonas compestris* pv. *Betlicola. Indian Phytopath.* **44(1):** 9-14.

Nomani, M.K.R., Mukheerjee, P.K. and Krishnaswami, S. (1970) Studies on the effect of feeding multivoltine silkworms (*B. mori*) larvae with mildew affected leaves. Ind. J. Seric. 9:49-52.

Nuhu Alam Akanda, A.M. and Khair, A. (1960). Biochemical changes of tomato due to purple vein virus. *Bangladesh J. Bot.* **25(1):** 321-349.

Olah, A.F. and Sherwwood. (1973). Glycosidase activity and flavonoid accumulation in alfalfa infected by *Ascochyta imperfecta. Phytopathology.* **63:** 739-742.

Oser, B.L., Hawk's physiological Chemistry, 14th Ed, McGraw-Hill Book Company, New York, 1965.

Pasha, M.K. and Barman, A.C. (1990). A survey on the incidence of powdery mildew disease of mulberry by *Phyllactinia corylea.* Bull. Seri. Res. **1**: 30-34.

Patel, M.K., Kamat, M.N. and Bhide V.P. (1949). Fungi of Bombay, Supplement 1. Ind. Phytopathol. 2: 142-155.

Patel, M.K. (1952) A new leaf blight of *Crossandra influndibuliformis* Ness. Ind. Phytopathol. 2:142-155.

Patten, M.B., Hitchock, F.A. and Haub, J.G. (1941). Interconversion of food stuffs in the blowgly phromia regina during metamophosis. II. Changes in composition as determined by the oxycalorimeter. *Ann. Pnt. Soc. Amer,* **34:** 26-31.

Philip, T. (1989). Mulberry fruit an ideal food. Indian silk, 28(8) Dec. p. 13-14

Porntaveewat, W., Takaynagi, T. and Yokotsuka, K. (1994). Purification and properties of invertase from Muscak Bailey *A.* grapes. *J. of ferment Bioeng* **78:** 288-292.

Probhu, A.S., Rajendran, V. and Prasada, R. (1962). Moiture requirement for the germinationof conidia of *Erysiphae graminis* tritici. Indian phytopath. **15:** 280-286.

Qader, M.A., Haque, R. and Absar, N. (1992). Nutritive effects of different types of mulberry leaves on larval growth and cocoon characters of *Bombyx mori* L. *Pakistan J. Zool.*, **24(4):** 341-345.

Qader, M.A., Haque, R. and Absar, N. (1992). Nutritive effects of bush, lowcut and tree mulberry leaves on economic characters of silkworm, *Bombyx mori* L. *Pakistan J. Zool.*, **27(1):** 77-81.

Rahman, S.M., Khanam, L.A.M. and Ferdous, J. (1990). Effect of various concentrations of ascorbic acid on the oviposition and hatchability of silkworm (*Bombyx mori*) *Bull. Sericult. res.* **1** : 47-50.

Rahman S H (1990) *Khadya-O- Pathya.* Ashish Publications, 339, Jafarabad, Dhaka-1207. pp. 18-21.

Ramakrishnan, L. (1966). Studies in the host –parasite relations of blast disease of rice II. Changes in the carbohydrates and phenolics. *Phytopath* Z. **56:** 279-287.

Ravindra, Sing. (1987) Why silkworm feed only on mulberry leaves. *Indian silk*, June, 25: 8

Raghuraman, R. (1989) Evaluation of mulberry varieties by bioassay studies. Genetics resources of mulberry and utilisation. Edited by Senguptan, K. and Dandin, S.B. Central Sericulture Board. Mysore, India.

Rangaaswami, G. (1976). Sericulture manual-1, Mulberry cultivation. Food and Agriculture organization of the united nation, Rome p. 2-5,55-56 and 72.

Rangaaswami, G. Narasimhanna, M.N., Kasiviswanathan, K. and Sastry, C.R. (1976). Sericulture manual-1. Mulberry Cultivation. FAO. Rome. P. 150.

Ranganna S (1986) *Handbook of Analysis and Quality Control for Fruit & Vegetable Products.* Tata McGraw-Hill Publishing Company Lit. New Delhi. 1101 pp.

Ray, Indrojit (1990) Defy Diabetes with mulberry. Indian silk, 28 (10): 34. Feb.

Reddy, S.M. Kumar,B.P. and Reddy, S.R. (1980). Vitamin-C changes in infected fruits of tomato. *Ind. Phytopath.* **33:** 511-512.

Robyt J F and White B J (1990) *Biochemical Techniques: Theory and Practice.* Waveland press, Inc. Illinois. USA. pp 295-296 and 304-305.

Sanaullah, A.A. and Alam, M.S. (1989). Studies on mulberry leaf spot disease caused by *Cercospora moricola* and *Alternaria* sp. In the greater Rajshahi district. M.Sc. thesis. Rajshahi uni. Bang. 126 p.

Saud, Z.H., Kashem Tang, M.A. and Absar, N. (1999) purification and characterization of amylase from Mango (*Mangifera indica)* leaves. *J. bio-sci.*7: 67-74.

Sarker, A.A., (1989) Requirement of silkworm nutrition and selection of mulberry leaves, Bangladesh Rasham. 1(3): 15-16 (Bangla).

Sarker, A.A., Quader, M. A., Ahmed, S.U., (1989) Effect of types and duration of storage on moisture content of mulberry leaves. Bangladesh J. Agri. 14(1): 37-44.

Sasikumaran, S., Kandaswamy, T.K. and Vidhyasekaran, P. (1979). Physiology of tomato plants affected by leaf curl virus. *Indian Phytopath.* **32:** 352-359.

Sharma, D.D. Gavindaiah, Misra, R.K., Chowdhury, P.C., Bajpai, A.K. and Datta, R.K. (1993). Influence of various agronomical practices and inputs on the incidence of mulberry diseases in chawki garden. Ind. J. Seric. 32(2): 150-155.

Sharma, B.B. and Wahab, S. (1975). Changes in sugar and protein contents of luffa cylindrica fruits infected with *Pythion ophanidermatum. Ind. Phytopath.* **28:** 308-311.

Shukla, B.B., Chand, J.N. and Kulkarni, S.N. (1972). Changes in sugar content of sesamum leaves infected with *Xanthomonas seasami. Ind. Phytopathology* **25:** 150-151.

Siddaramaiah, A.L. Krisnaprasad, K.S. and Padaganur. (1978). Field evaluation of fungicides in the control of mulberry powdery mildew. *Ind. J. Seric.* B17: **33-36.**

Sikder, A.K., Samson, M.V., Madhava Rao, Y.R., Murtuza, B. and Nataraju, B. (1979). Effect of feeding leaf spot affected and systemic fungicide sprayed leaves of mulberry (*Morus indica* L.). Ind. J. Seric. 28: 73-77.

Sikder, A.K., and Samson, M. (1980). Control of leaf spot disease of mulberry by systemic fungicides. *Indi. Phytopath.* **33(1):**38-41.

Sikder, A.K. (1987) Leaf spot disease of mulberry and its control with systemic fungicide. Ind. Silk. 25 (12): 34-36.

Singh, R.S. (1978). Plant diseases, 4 thed. Oxford and IBH Publishing Co. New Delhi, Bombay.

Singh, A. (1982). Total sugar, reducing sugar and non-reducing sugar content in plants. *Nattural Bio.* **34:** 145-162.

Singh, A. and Sinha, K.K. (1983). Biochemical changes and alfatoxin production in guava fruits by *Aspergillus flavus and A. parasiticus. Indian Phytopath.* **36:** 365-366.

Sitarama Iyenger M.N. (1975). *Ind.J. Seric.***14:** 1-5.

Smart, R.F. (1937). Influence of external factors on spore germination in the *Myxomycetes. Am. J. Botany.* **24:** 145-159.

Subba Roy, B.L. Ghosh, A. and John, V.T. (1979). Effect of rice tungro virus on chlorophyll and anthocyanin pigments in two rice cultivars. *Phytopath.* Z. **94:** 367-371.

Sukumar, J. and Ramalingam, A. (1987). Epidemiology of *Cercospora* leaf spot disease of mulberry II. Leaf age and disease susceptibilty. *Sericologia.* **27(4):** 685-688.

Sulaiman, M. and Agashi, N.G. (1965). Influence of climate on the incidence of tikka disease of groundnut. *Ind.oilseeds J.* **9:**176-179.

Sullia, S.D. and Padma, B.D. (1987) Acceptance of mildew affected leaves by silkworm (*B. mori)* and its effects on cocoon characteristics. Sercologia. 27(4): 693-696.

Tandon, R.N., Tandon, M.P. and Jamaluddin (1974). Studies on post harvest diseases of fruits and vegetables. In Raychaudhuri, S.P. and J.P. Verma (Ed) Current trends in plant pathology. p 209-220.

Thangavelu, K., (1990) Field problems in introducing new mulberry varieties in Tamil Nadu. Indian silk, March, 28 (II): 31.

Tribhuwan, S. and Mathur, S.M. (1989) The morin factor in mulberry that attracts the *Bombyx mori* L. silkworm. Indian Silk, Sept. 28(5): 39-40.

Vogel, A.I. (1961) A text book of quantitative inorganic analysis. 3 rd ed. Pub. Longmans. pp 787, 806, 808, 810.

Watanabe, T. (1958) Nature, 812: 325-326.

Wong S Y (1923) The use of per sulfate in the estimation of nitrogen by the arnold-gunning modification of kjeldahl's method. *J. Biol. Chem.* **55:** 427-430.

Wong S Y (1928) Colorimetric determination of iron and hemoglobin in blood. *J. Biol. Chem.* 77: 409-412.

Yarwood, C.E. (1954) Resistance of bean leaf pulvini to fungi and viruses. Phytopathology. 44: 64.

Yarwood, C.E. (1936) The diurnal cycle of the powdery mildew *Erysiphae potygoni. Jorn. Agr. Res.***52**:645-657.