Expression of Oxidative Enzymes in Cotton Plant under Biotic Stress

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Abstract: Cotton leaf curl disease caused by the Cotton Leaf Curl Gemini Virus (CLCuV) is limited to Asia and Africa. This study was conducted in green house to check the role of antioxidant enzymes under diseased conditions. Four cotton cultivars (Gossypium hirsutum) were planted with two replications to check the expression in healthy and disease infected plants. The estimations of total soluble protein contents and the activities of peroxidases (POX), catalases (CAT), proteases and superoxide dismutase (SOD) were studied in leaves with 8 genotypes of cotton, four susceptible and four are disease infected. Disease symptoms were shown less intense in disease infected genotypes but severe in highly susceptible genotypes. The results showed that protein, POX, CAT, proteases and SOD played major role in disease resistance against CLCuV. The SOD, protein contents and CAT activities showed lower values in disease infected genotypes while they decreased significantly in susceptible genotypes. The CAT was found to be increased in both genotypes with high percentage in infected genotypes as compared to healthy. The results showed that significantly higher concentrations of total phenols and higher activities of protease the SOD, CAT and TFC in disease infected genotypes after infection with CLCuV. Statistically the ANOVA was performed to check the levels of above mentioned enzymes against plant resistance. These findings could be considered as biochemical markers for studying plant-virus compatible and incompatible interactions.

Key words: Cotton leaf curls virus, G. hirsutum, disease index, disease incidence, whitefly, antioxidant enzymes, and biochemical compounds.

INTRODUCTION

1.1 Introduction of cotton

Cotton is a well-known and most important industrial crop worldwide which is grown more than 80 countries in tropical and subtropical regions. Cotton word derived from Arabic word “qutun”.It is also known as “Kapas” in Pakistan and India which is derived from a Sanskrit and “Karparsa” (Lee, 1984; Smith, 1995). Cotton plays a vital role in agronomy and agriculture economy. The total world cotton production almost 50% comes from two countries China and India (Alkudssi et al., 2013).

Table 1.1: List of top 10 cotton producer countries

Top 10 Cotton Producing Countries (in metric tonnes)				
Rank	Country	2010	2011	2012
1	China	5,970,000	6,588,950	6,840,000
2	India	5,683,000	5,984,000	5,321,000
3	United States	3,941,700	3,412,550	3,598,000
4	Pakistan	1,869,000	2,312,000	2,215,000
5	Brazil	973,449	1,673,337	1,638,103
6	Uzbekistan	1,136,120	983,400	1,052,000
7	Turkey	816,705	954,600	851,000
8	Australia	386,800	843,572	973,497
9	Argentina	230,000	295,000	210,000
10	Turkmenistan	225,000	195,000	198,000

Cotton production in different countries

Cotton is commercially developed in the tropical and mild regions of more than 100 countries. Cotton in Pakistan, India, USA and China are the best production. Cotton has an extended commerce around the worlds of hundreds billions. China has an important source of income from cotton and constitutes 100 million agricultures and in textile industry 19 million labors work and in related industries (Zhu *et al*., 2011). Fiber exchange is not as it were thing on cotton business based; in oil de-lined cotton seed are handled, bodies and super (up to 41% of protein), (Campbell *et al*., 2010).

Kinds of Cotton

Four types of cotton are discussed in this dissertation:

- Gossypium arboreum
- *Gossypium barbadense*
- *Gossypium herbaceum*
- *Gossypium hirsutum*

Cotton (*Gossypium spp.)* is a primary source of meal, protein, natural oil and textile fiber. Many countries are producing high yields of cotton. Large scale producers of cotton including Pakistan, United States, India and China around the globe more than 70% of the total cotton production contribute. Amongst the 50 *Gossypium* species, two diploid species (*G. arboreum and G. herbaceum)* and two tetraploid species (*G. barbedense* and *G.hirsutum*) are universal, but overall cotton production for *G. hirsutum* is more than 90% of the rest of all species (Lee *et al.*, 2007). Also called "white gold" cotton could be a delicate, staple fiber that develops in a form known as boll around the seeds of the cotton plant *Gossypium sp.* In the middle of the 18th century Gossypium named by Linneaus and it is related to the Malvaceae family (Smith, 1995). Its global distribution induced an impressive diversification in terms of morphology (seeds, trichome), physiology and ecology. This diversification led to a remarkable evolution at the chromosome level (substantial evolution of chromosome size and structure) (Endrizzi *et al*., 1985; Wendel and Grover, 2015).

Fig. No 1. White- gold cotton

The industrial and agricultural economy of the country cotton plays a vital role in fiber and cash crops. It provides the basic raw material (cotton fiber) to cotton textile industry.

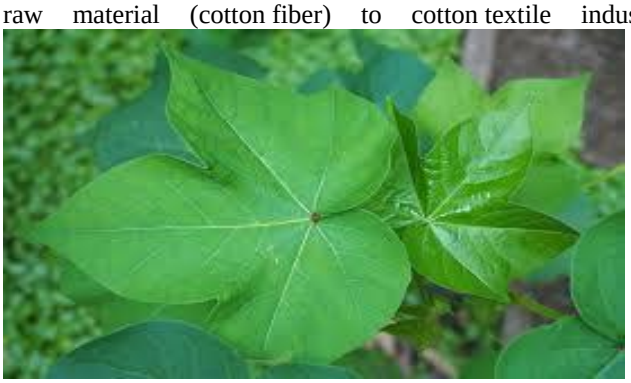


Fig. No 2. Leaves of tetraploid cotton species

The *Gossypium* genus contains about 45 diploid species with 26 chromosomes and 5 tetraploid species and in which 52 pairs of chromosomes (Brubaker *et al.*, 1999). The reason for the development of different number of sets of chromosomes

seems to be due to different origins of these organisms. *G. hirsutum* originated from Central America and *G. barbadense* originated from Peru (Lubbers *et al.,* 2009*)*. Cotton plant has remained very useful in the history as it was the source of food and fiber (Cantrell, 2005). There are almost 51 species of the genus *Gossypium* (Hendrix *et al.,* 2005). Different species of the genus *Gossypium* are having different number of sets of chromosomes, like *Gossypium arboreum* L. being diploid have two sets (2n) and *Gossypium hirsutum* L. being tetraploid have four sets (4n) and *Gossypium barbadense* L. is allotetraploid (8n) (Wendel and Cronn, 2003).

The most developed species from these two forms is *G. hirsutum* because it produces quality fibers in all respects. The local species of cotton *G. herbaceum* and *G. arboretum* are both diploid which do not produce as much quality fiber as tetraploid species. The interesting fact about diploid forms of cotton plants is that these are resistant to CLCuV and these have adapted themselves to the hot and dry climate which increases their demand in the cotton business (Akhtar *et al.,* 2010). In any case the yield of cotton is disadvantageous influenced by biotic and abiotic stresses. Therefore, an effort to examine the molecular adjustment mechanisms of stresses and to reinforce push resistance in this plant is of essential importance to upgrade cotton production (Bota *et al*., 2004).

Gossypium Arboreum

Gossypium is a blooming plant within the tribe Gossipieae of the mallow family, Malvacea from which cotton is harvested. It is local to tropical and subtropical areas of old and unused world.

Fig. No 3. Leaves of *Gossypium arboreum*

G. arboreum L. is not only to make sure a importance of hypothetical vile for preserving the Asiatic cotton germplasm assests, while moreover to recognize and appropriate progress , perfect characteristic in which yield and different plant structures that cutting edge of cotton plant that can be misused (Verma *et al*., 2014).

Gossypium hirsutum

Cotton *Gossypium hirsutum* L is a plant fiber of major financial significance, with seeds giving an extra source of protein in human and instinctive nourishment. Flavonoids play a dynamic role in maintaining plant health and much research has examined the part of flavonoids in plant protection and plant energy and the impact these have on cotton generation (Nix *et al*., 2017). *Gossypium hirsutum* is the most abundant species of all other kind of cotton species. It shares about 90% of the world's cotton. *Gossypium barbadense* is on second position which shares 8% of the all while *Gossypium herbaceum* and *Gossypium arboreum* share only 2% in the world's cotton (Jiang *et al*., 1998).

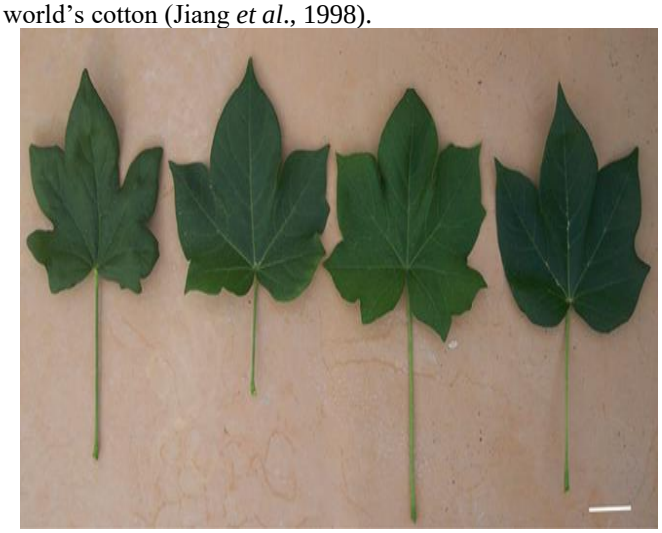


Fig. No 4. *Gossypium hirsutum* samples from NIBGE

In spite of the fact *Gossypium arboreum*, that it provides small surrender while it has numerous imperative characteristics for example various disease ,many insects tolerant and illness and high fiber quality and extraordinary versatility, which are missing in *G. hirsutum* ,while close to *G. arboreum* can be developed beneath modest conditions and less overseen range. Cotton is infected by more than a few insects, pests and pathogens inducing diverse infections. Cotton leaf curl virus (CLCuV) is the greatest harmful infection, causing colossal injured the cotton plant in among them (Khan and Ahmad, 2005). All provinces of the Pakistan CLCuV have spread out with the passage of time (Tariq, 2005). Whitefly *Bemisia tabaci* is a cause of transmission of cotton leaf curl disease and the causal agent may also reside in other insects (Khan and Ahmad, 2005). As described by many model studies by controlling the attack of Whitefly *Bemisia tabaci* which is the main vector of the infection, subsequent onset of disease-CLCuD can be checked (Holt *et al*., 1999).

Fig. No 5. Whitefly *Bemisia tabaci* vector

Leaf curl is a widely recognized and damaging disease of the upland cotton within the Africa and in Indian subcontinent. Its ingenious mediator has been described from India, Pakistan and Sudan (Azhar *et al*., 2010). Whitefly Bemisia tabaci is

transmitted by single-stranded satellite DNA β portion and a small side impact directing ,illness to be caused by a complex of monopartite begomoviruses (Akhtar *et al.,* 2013). The *begomovirus*– beta satellite edifices that reason cotton leaf curl virus ailment in Africa and Asia are particular. Amidst of 1990s, the complex of different *begomovirus* species appeared in Pakistan and India (regularly contaminating every plant for one or more infection), and caused an illness in which particularly beta satellite (Cotton leaf curl Multan beta satellite [CLCuMuB]) was associated (Tahir *et al.,* 2011). In 2001, intensity of infection of a begomovirus was increased and the existing strain defeated existing resistance which was generally accounted for in Pakistan (Akhtar *et al.,* 2002; Mansoor *et al.,* 2003). Reported that after studying the DNA sequences one comes to the conclusion that there was only one type of *begomovirus* that developed it to act as infectious agent (Tahir *et al.,* 2011). In a huge number of Begomo viruses consisted of two genomic fragments known as DNA-*A* and DNA-*B*, which are required for illness that is spread by whitefly Bemisia tabaci (Monga *et al.,* 2011).

Here is a large number from the ancient World which have as it were a single fragment comparable to DNA-A, which has been isolated and displayed to bring on illness indications (Navot *et al.,* 1991; Dry *et al.,* 1993). In Pakistan, the most important crop is cotton, over 60% cotton is exported to foreign contraries for earnings (Briddon *et al.,* 2000). Cotton is major fiber crop in Pakistan and it only only share 1.2% in of its production in local market and rest of the amount is exported worldwide. Cotton leaf curl disease (CLCuD) could be a major risk to the production of cotton in Pakistan (Azam *et al.,* 2013). CLCuD has particular symptoms in which swelling, blackening and thickening of veins, downward or upward rolling of leaves, and cognation (Akhtar *et al*., 2002).

The fibers of the cotton plant are single cells. An ovule epidermal cell on differentiation and growth produces a cotton fiber. When the cell is alive it elongates and has normal set of cellular structures. New growing fiber cell have only primary cell wall and continues to grow further up to 39 days and develops secondary cell wall at the 17th day of its differentiation (Pesquet *et al.,* 2011). On the deposition of secondary cell wall the thickness of the fiber increases. In cotton plant both primary and secondary cell walls are made up of cellulose. The deposition of the cell wall requires a unique set of enzymes with specific conditions. Cotton plant provides us an array of other textile products such as clothing, animal feed, oil and various other industrial products. As we can define species of virus and is all terms definitions can be devised but what is a virus and what is the entity it is still questionable (Van Regen mortel *et al*., 2006). We find different meanings of virus by different authors, it is a thing that different scientists understand by their experiments as they used to stay it causes infections in body and potentially very strong pathogenic parasite in living bodies. It is sometimes can be defined as a parasite that is small and obligate intracellular either it contains a DNA or RNA genome that is surrounded by a virus coded protein that is protective for it and structure of nucleoprotein is a type of nucleic acid as name has sowed us, it too contain either DNA or RNA (Roossinck *et al*., 2011).

Later on it was found that there are certain other genotypes; cotton leaf curl Multan virus (CLCuMuV), cotton leaf curl Burewala virus (CLCuBuV) and cotton leaf curl Kokhran virus (CLCuKV) cause CLCuV disease. Just one sort of DNA β satellite, generally beginning from CLCuMuB DNA β segregated from tomato, is observed to be related with the disease that was observed to be related but now clearly deficient with regards to an alpha satellite (Tahir *et al*., 2011).

As of late, CLCuBuV has been accounted for India and commanding in numerous crops (Kumar *et al*. 2011; Rajagopalan *et al*., 2012). These viruses are transferred to the plant by whitefly (*Bemisia tabaci*). Different pathogens, insects and pests cause various disorders in which cotton is infected (Khan and Ahmad, 2005). With the passage of time CLCuV has spread in all areas of Pakistan (Tariq, 2005). During the last decade, 9.45 million bales of cotton production decrease due to CLCuV virus (Hussain, 1995; Khan *et al.,* 2001). For the textile sector, higher strength of fiber is very important. The high fiber strength does not have its availability in commercial kinds in Pakistan. For extra fiber strength starting Culp and Harrel (1973), Green and Culp (1990), and Culp and Green (1992) starting in 1946,focused on transferring genes, which was based on Beasley (1940) triple hybrid to develop cotton plant (Ahmad *et al.,* 2010). If plants are infected early in their life, right after germination, and then the symptoms are more severe with no harvestable lint. On the other hand if the plant was infected very late in his life then the symptoms will be milder (Sattar *et al.,* 2013).

The inverse relation between fiber strength and lint yield was terminated and fiber strength was increased. Mostly cotton produce in Punjab, Rajasthan, Haryana, Gujarat, Andhra Pradesh, Maharashtra states in India. Those all areas in which grow cotton are infected by whitefly-transmitted begomovirus. The primary record of CLCuD in India than Sri Ganganagar, Rajasthan in 1993 (Ajmera *et al.,* 2004).

In 1978, a period of Gemini virus was noticed on distinctive plants with different features of size and structure and was at last demonstrated to be single stranded deoxyribonucleic acid virus (Mathews, 1987). During the year 1997 outburst of disease was recorded in all these three states and people were infected with the disease roughly about 2.19 lakh. Including biotic and abiotic stresses the environmental calamities are the major threats to food security and agriculture. A number of crucial plant diseases are due to biotic stresses including viruses and are liable for a large number of deficits of yield production and its nature in all segments of the world also in Pakistan. The family Gemini viridae comprises of three genera i.e. Mastrevirus, Curtovirus and Begomovirus. A well-known bad gather of these infections are related to sort Begomovirus, a source of great danger to cotton yield which is known to us as Cotton leaf curl infection malady (CLCuD) and is spreaded from one to another by whitefly i.e. *Bemisia tabaci* complex (including *B. argentifolii*) in a ceaseless way (Brown *et al.,* 1995). There is a contaminated plant of cotton from which DNA is separated with CLCuD exposed a great ranging similarity with the Begomoviruses and other DNA-A fragments and Indian sub-continent (Zhou *et al*., 1998**).**

In Vidarbha region, the most crucial cash crop is cotton, raised on an area of 13.00 lacks hectors with of 27 lack cotton bales

(2008-09). Decreasing in the output of the cotton is due to diseases. Most of the disease is present on cotton plant leaves about 80% to 90% (Azam *et al.,* 2013). The point to ponder here is the cotton tree leaf rather than a complete cotton leaf plant is mainly borne from various infections like fungus, foliar leaf spot of cotton. Various negative impacts have been seen on the morphology of the infected plants and it has been seen that the disease have cancerous effect on the cotton filaments (Ahmad *et al.,* 2002). Among the symptoms of cotton leave curl virus disease appearance of different colors on the leaves is an important one. To control the disease it is the most effect technique to pick up the infected leaves out from the rest of growing crop (Agrions, 1978).

According to the studies of Iqbal *et al*., (2006) Seed cotton production has some significant and positive relationship with sympodial per plant, bolls per plant and boll weight. (Kale *et al*., 2007). Infections are able to contaminate all sort of living beings including plants, creatures, microscopic organisms, and Archaea infections (Huang *et al*., 2010) are ubiquitously show in all biological systems around world (Edwards *et al.,* 2005). In Pakistan, one within the India and other within the Sudan begomo infections having seven species detailed so distant while five out of seven have been recognized (Amin *et al.,* 2006; Sharma and Rishi, 2007). A latest recombinant strain of A begomovirus derivative from cotton leaf curl Multan called (CLCuMV) and Kokhran virus (CLCuKV) has been found in existing cotton assortments with the breakage of resistance to be related with a latest recombinant strain (Akhtar *et al*., 2010). Cotton leaf curl Burewala virus (CLCuBV), having recombinant strain infection which is present in Pakistan whose commonly growing in cotton. One of the premium ways to conflict CLCuV, especially all endeavors in which treatment have ineffectual and when there is high inoculum pressure by the assortment of breeding resistant.

Different attempts have been made to exchange resistant genes through interspecific hybridization between diploid and tetraploid species have been developed by cotton plant. Clear (1963) exchanged unaffected genes caused by Puccinia cacabata against cotton rust (Bao-Liang *et al*., 2003).

Qualities for resistance against illness and dry season have been exchanged among Gossypium hirsutum and Gossypium arboretum (Amin, 1940). Interspecific introgression has moreover been done during the Gossypium hirsutum and Gossypium arboretum. Essentially, safe qualities against bacterial curse of cotton display in G. Arboretum have been introgressed into *G. Barba* thick (2009) against *Rotylenchulus reni* shape is from 2n to 3n cotton (Knight, 1957; Brinkerhoff, 1970). Among the population of peoples in which the sum of genetic variability and genetic diversity can be characterized and the betterment of crop and also hereditary for supportability. It is a zone of interest. Gossypium arboreum L. has various promising characteristics such as dry season resilience and tolerate to insects, pests etc., which are not present in the G. hirsutum L (Mehetree *et al*., 2003). In any case, for the final two decades the cotton production has remained quiet in which both biotic and abiotic stresses containing a few components in which cotton has great yield quality and fourth biggest producer in Pakistan than others (Saeed *et al*., 2014). Cotton leaf curl disease (CLCuD) is one and only the main restricting components producing vast crop damages (Farooq *et al.,* 2011). Numerous efforts by creating resistance/tolerant variabilities using diverse conservative breeding approaches have been made by the breeders to overcome this issue but these assortments ended up helpless after two to three a long time due to changes happening within the viral strains causing CLCuD.

In this manner, appropriate genetic changeability, proper misuse of the polyploidy creation and presentation of misuse exotic germplasm are exceptionally critical and existing various varieties through hybridization (Ismail *et al.,* 2008).

Different levels of chromosomal variety can be assessed utilizing different mathematical methods such as fundamental factors analysis (Li Zheng *et al.,* 2008). Cluster examination and most important methods are organized (Brown *et al*., 2000). Among these biometrical strategies, the major prerequisite of central component in which study can be allocated in each genotypes as it were bunch only and it too imitates the importance of biggest supporter to full inconstancy in every point of separation (Sharma *et al*., 1998).

The effect of cotton leaf curl virus (CLCuV) which causes cotton leaf curl disease (CLCuD), on the expression of oxidative enzymes of cotton plants (Genus: *Gossypium*) has extensively been studied. Generally, this disorder prevails in many plants of the family malvaceae (Azhar *et al*., 2013). In the structure of leaves virus causes very unusual changes in the cotton plant such as the formations of outgrowths and enations on veins and the underside the leaf. The growing enations appear leaf-like and can be the size of the leaves on which they are present (Akhtar *et al.,* 2000). The effects of this virus are very unusual, such as the plants infected with this virus have more amount of chlorophyll in their leaves than the normal plants. The distribution of chlorophyll is more in CLCuV infected plants however based on the age and certain other morphological features of the plant; hence symptoms may vary accordingly (Sattar *et al*., 2013).

Application of pesticide have very harmful impacts caused on the environment in which whitefly may cultivate the resistance of pesticide (Pico *et al*., 1998; Palumbo *et al.,* 2001). The foremost important aspect of control of illness is utilization and documentation of solid sources of plant resistance. The use of it is simple, very effective and environmentally friendly as compared to depending on pesticides. From the past 20 years, many influential steps have been taken to produce CLCuD-resistant cultivars in Pakistan. As the time passed, the infection was strongly manage by producing CLCuD-resistant variety. As of late, a strain of the infection that overcame this resistance appeared and rendered already different safe assortments susceptible (Akhtar *et al.,* 2008).This consider was started to recognize sources of resistance in developed and wild genotypes indicating five Gossypium species. Many present researches have an objective to explore the genetic potential of different cotton cultivars and relation of seed cotton generation with different precedence, production and fiber related traits under CLCuV serious states.

MATERIAL AND METHODS

Plant material

Eight cotton cultivars, each with two replications with known CLCuV-resistance and vulnerability levels were taken and grown in green house.

Source of viral inoculums

A susceptible inoculated cotton plant was serving as a source of CLCuV source. These plants are inoculated artificially which were put in their favorable environment in the green house (Akhtar et al., 2013).

Virus transmission and identification

Newly growing cotton plants were inoculated with the virus strain (CLCuV). Data were recorded on the ratio of effective grafts, rate of illness transmission, inactive sum (aver-age time needed for initial symptom look once grafting), and sort of indications created ninety days post vaccination (Akhtar et al., 2015).

Total soluble protein content

Mature leaves were used for extraction of protein contents by grinding them in extraction tube. Involvement of protein components in plant infections resistance has been reported in numerous plant pathogenic intuitive (Tornero et al., 2002; Carballo *et al*., 2006). In non-inoculated plants, total soluble protein substances was essentially higher in leaves of vulnerable genotype-I but was comparative in vulnerable genotypes-II and both of the resistant genotypes. As a rule contaminated, plants appear high protein content, which could be due to both the activation of the host defense mechanisms and the pathogen attack components (Agrios, 1997). The high level of susceptibility of these genotypes in which possible explanation for significant decrease in total soluble proteins in susceptible genotypes after CLCu-BuV injected. Comparative results have been detailed in maize, tomato, grapevine, and apple contaminated with mollicutes (Favali et al., 2001; Bertamini et al., 2002, 2002; Musetti et al., 2010).

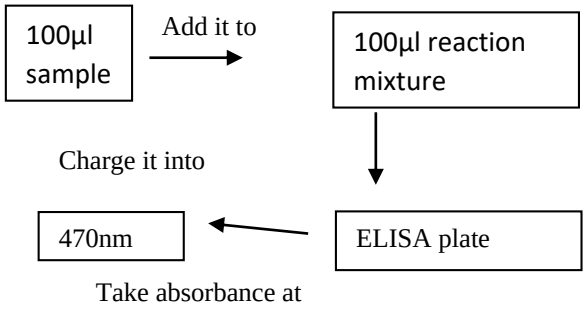


Fig. No 6: Flow chart of peroxidase activity

Peroxidase (POD)

POD estimation was done with the help of potassium phosphate buffer, 100µl reaction mixture, and 100µl samples. The reaction was initiated by adding the enzyme extract. At 470 nm increased the absorbance of the reaction solution after every 20s was recorded. POD activities were recorded based on the definition that one unit of activity of POD is 0.01 unit per minute of rate of change of absorbance (Gogai *et al.*, 2011).

(2, 2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) Activity

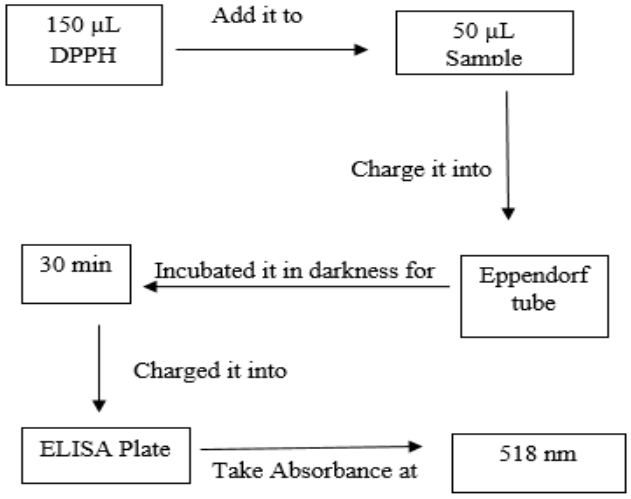


Fig. no 7: Flow chart of DPPH activity

Sample stock solution in which 150ul DPPH added it to 50 µl sample were diluted to final concentrations then charge it into Eppendorf tube. After the mixtures were vigorously shaken they were incubated in dark for 30 min after incubation again charged it into ELISA plate in which different values of sample solutions, and then take at room emperature for 30 mints the absorbance values were measured after 30 mins were recorded at 518 nm and change over into the rate antioxidant activity (AA) utilizing the following formula: **A 100 = Abs sample-Abs blank /100Abs control**

They must take blank absorbance in which we take Ethanol (150 mL) plus plant extract solution (50 mL). For negative control DPPH solution was used and the standard solutions using the positive controls.

Total Flavonoid Content

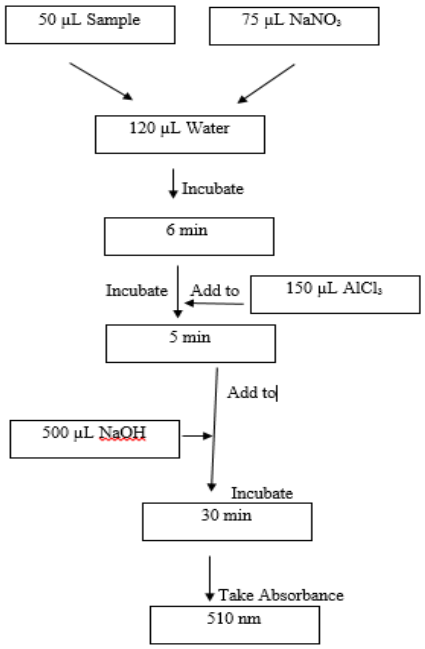


Fig. No: 8 Flow chart of Total flavonoid content

Aluminum chloride colorimetric assay was used to measure by total flavonoid contents. An adding 50 µl sample of extract and 75 µl NaNO3 was put to 10ml flask having 120 µl of distilled water then incubate for 6 minutes. After 6 minutes, 150 µl $AlCl_3$ was added than incubate for 5 min. After incubation we were added, 500 µl NaOH and the total concentrations was made up to 10 ml with distilled water than incubate for 30 min and in the last step absorbance was taken by ELISA reader at 510nm. Through this method a blank sample was also run (Georgleva *et al*., 2011).

Total protein content

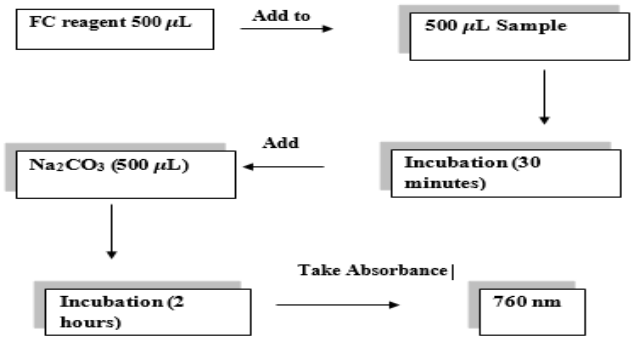


Fig. No 9: Flow chart of total protein content.

Protein content of 8 samples (500 µl each) was determined by above mentioned protocol. A blank sample was also run through this procedure as a control.

According to the literature total phenolic contents were determined (Singleton & Rossi, 1965. Added 500 mL of the diluted sample into diluted Folin– Ciocalteu reagent (the most common usage of FC reagent for determining protein concentrations in Lowry method) then incubation for 30 minutes. After 30 min, we were added 500 µl of saturated sodium carbonate solution. The mixture absorbance was measured at 760 nm after incubation for 2 hours at room temperature. At the end absorbance was taken by ELISA reader (Song *et al*., 2010).

Catalase (CAT)

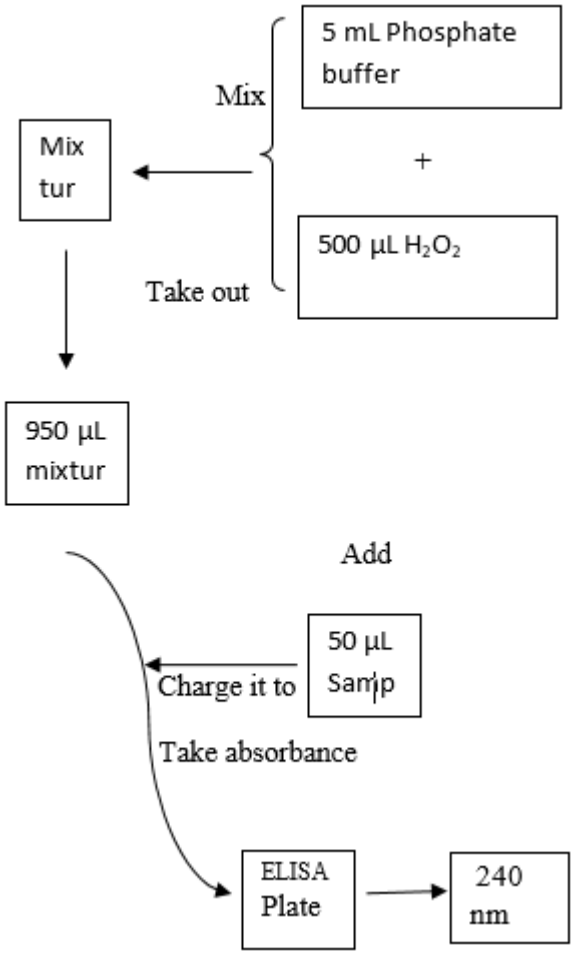


Fig. No 10: Flow chart of catalase activity.

Catalase activity was by 5ml potassium phosphate and 500ul water for some modification of CAT action. Extract from both CLCuV-inoculated and non-inoculated plants were completely blended in a medium composed of 50 mM potassium phosphate buffer, 50µl sample then shake vigorously at pH 7.0 for measurement of CAT activity. The absorbance was recorded at 240 nm after every 20 s and reduction in reaction solution. CAT activity were recorded based on the definition that one unit of activity of CAT is 0.01 units'min−1 of rate of change of absorbance. And enzyme activity was expressed on the base of fresh weight (Hameed et al., 2011).

Protease activity

Protease activity was obtained from CLCuV inoculated and non- inoculated plants, treated with potassium phosphate buffer solution of casein and TCA solution. Enzyme activity was done by ELISA reader. To all the tubes, 100 µl of protease extracts was added and mixed well. A blank sample was also run through this method. Exactly after 10 min added

sample, reaction was stopped. 2.0 ml TCA solution and mixed well. Tubes were then allowed to stand for 10 min, and then reaction solution was filtered to remove the precipitate formed during reaction. The filtrate absorbance was measured at 280 nm. By this method, protease activities were recorded based on the definition that one unit of activity of protease is 0.001 unit per minute is that amount of enzyme, A280 per min at 37°C and pH 7.8. On protein basis enzyme activity was also expressed (Hameed et al., 2013).

Superoxide dismutase (SOD)

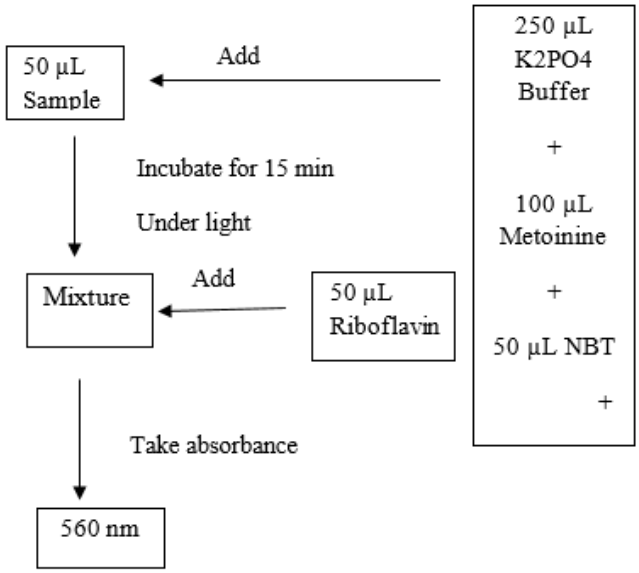


Fig. No 11: Flow chart of Superoxide dismutase (SOD)

Leaves extracts of CLCuV cotton plants were tested with 50 µl sample and 250 potassium phosphate buffer, 100 µl methionine, 100 µl Triton X ,50 µl NBT ,400 µl distilled water all are charged into Eppendorf then take under light for 15 min and take out and add 50 µl Riboflavin and then take absorbance at 560nm. While nitro blue tetrazolium (NBT) will be reduced photo chemically (Dixit *et al*., 2001).

For the assessment of SOD activity, leaflet was homogenized in a medium solution composed of 50 mM potassium phosphate buffer. To inhibit the photochemical reduction of nitro blue tetrazolium (NBT) and measuring its ability and the activity of SOD was assayed. One unit of activity of SOD was determined which caused 50% inhibition of photochemical reduction of NBT due to the quantity of enzymes.

RESULTS AND DISCUSSION

CLCuV cotton plant

Disease response

CLCuV inoculated plants of all tested genotype showed a wide range of symptoms depending on their genetic makeup. From *G. hirsutum* developed disease symptoms 10 days post inoculation and showed sever symptoms like leaf rolling & curling, swelling and darkening of veins, and leaf-like out-growths called 'enations' within 30 days post inoculation of susceptible genotypes. All the grafting inoculated plants of resistant genotypes from *G. herbaceum* (Co Tiep Khac) initiated disease at 30 days post inoculation, respectively.

On these genotypes CLCuD symptoms started as slightly vein swelling/darkening and 'enations'on the veins on the undersides of leaves and *G. herbaceum* (Co Tiep Khac) leaves of the grafted plants on few symptoms were developed. The end of the experiment (90 days post inoculation) no disease severity was observed. Through PCR CLCuBuV was readily detected in both symptomatic and symptom-less leaves of these plants. The lower disease severity in the leaves of CLCuBuV-inoculated plants of genotypes and (*G. herbaceum*) (Akhter *et al*., 2013). . Involvement of protein components in plant infections resistance has been reported in numerous plant pathogenic intuitive (Tornero *et al*., 2002; Carballo *et al*., 2006). In non-inoculated plants, total soluble protein substances was essentially higher in leaves of vulnerable genotype-I but was comparative in vulnerable genotypes-II and both of the resistant genotypes. As a rule contaminated, plants appear high protein content, which could be due to both the activation of the host defense mechanisms and the pathogen attack components (Agrios, 1997). The high level of susceptibility of these genotypes in which possible explanation for significant decrease in total soluble proteins in susceptible genotypes after CLCu-BuV injected. Comparative results have been detailed in maize, tomato, grapevine, and apple contaminated with mollicutes (Favali *et al*., 2001; Bertamini *et al*., 2002, 2002; Musetti *et al*., 2010).

The present study indicated that the two enzymes (SOD and POD) reacted in the same way to the disease (a decline of their activities with the disease aggravation) which may be explained by their co-regulation. Previous studies also reported the simultaneous induction or decline of the two enzymes (Shigeoka *et al*., 2002; Abedi and Pakniyat 2010) .

The CAT activity was found to be positively correlated with the disease thus negatively correlated with the resistance. The increase of the CAT can be explained by the decline of the POD activity leading to an increase of hydrogen peroxide which must be scavenged by the CAT. CAT is enzymes with the potential to directly dismutase hydrogen peroxide into H_2O and O_2. They play an important role in the plant cells detoxification during oxidative stress. The level of CAT has been shown to increase in varieties sensitive to a stress (sarvista *et al*., 2002).

One of the main effects of CLCuV disease is the restriction of the water movement due the vessels occlusion in the infected plants. An important adaptation of plants to water stress is the increase in the concentration of intracellular solutes, such as proline and total soluble sugars (TSS), which facilitate the maintenance of cell pressure potential (Osakabe *et al*., 2014). The level of TSS was found to increase with the aggravation of the disease most probably due to the mentioned reason. Similarly the TSS show a significant buildup in leaves of inoculated paper plants from 20day (Osakabe *et al*., 2014) which tends to confirm our results. The proline content was not significantly correlated with the disease parameters in our study although it was reported to increase in infected plant in most of the reports (Siddique *et al*., 2014). Again in this study, the lack of correlation can be explained by the different level of the disease. In our study the augmentation of the protein

content is positively correlated with the disease; this fact can be explained by the activation of the host defense mechanism and the pathogen attack mechanism which both required the production of new proteins (Siddique *et al*., 2014).

In the field, only three parameters were tested for two years experiments: one antioxidant enzyme (SOD) and the content of proline and total soluble sugar. The correlation of the biochemical parameters with the disease parameters differed in the two years. The 2015 SOD activity was not correlated with any of the same year disease parameter however, in 2014, a correlation was observed between the disease incidence and the SOD activity. The TSS content had shown a negative correlation with the two disease parameters in 2014 but was only positively correlated with the disease incidence in 2015. The proline content had shown a negative correlation with the disease index in 2014. These observed variations of the SOD activity and in the content of TSS and proline can be explained either by the difference level of reactivity of our population lines to the disease from year to year, the instability of the environmental conditions and the level of virulence of the pathogens present in the field. The decrease of the total soluble sugar with the aggravation of the disease observed in 2014 can be explained by the use of the total soluble sugars to maintain the cell pressure potential.

In any case, protein substance was found to be diminished essentially in CLCuBuV-inoculated plants of both susceptible genotypes (CIM-496, 20.39% and NIAB-111, 23.64%), whereas it was found to alter non-significantly in leaves of vaccinated plants of both resistant genotypes Ravi (9.41%) and Co Tiep Khac (11.04%), as compared to their non-inoculated plants. In this study, aside the negative correlation between SOD and CAT and the SOD and the POD, no other correlation was observed between the antioxidant enzymes. However moderate correlations were found between antioxidant enzymes and the other biochemical compounds. In a study on tomato's antioxidant response to salt stress (Frary *et al*., 2010).

Another interesting remark is that except for one trait (TSS), no correlation was obtained between the trait measured in the greenhouse and those measured in the field. This lack of correlation can be explained by the non-controlled environment in the field (other diseases, drought, and insects).

Levels of POD in Cotton Plant

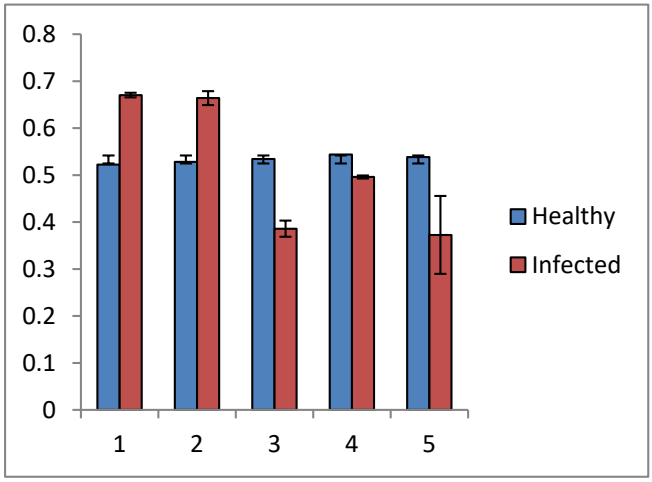


Fig. No.12: Levels of peroxidase enzyme in cotton plant (cotton leaf curl virus) under biotic stress, showing that the expression of POD in two of the infected samples got over expressed and rest of the 3 out of 5 got suppressed.

Peroxidase is an enzyme during a wide selection of organisms, from plants to human to bacterium. Its major function is to breakdown peroxide that is one in every of the toxins produced as a byproduct of using oxygen for respiration. It is a chemical compound and antioxidant resistant. Expression of levels is checked in IU/ml of protein.

Peroxides are also one of the primary responding enzymes giving quick defense against plant pathogens. As reported by Siddique *et al*., 2014,resistance against pathogens and in the regulation of the cell wall elongation wound healing they are involved (Zhou et al.,2012).

Cotton leaf curl virus is not a seed borne disease while it is transmitted by the Whitefly *Bemisia tabaci* and some virus lives in alternate hosts. After infection of three plants with cotton leaf curl virus expression of peroxidase enzyme was studied in terms of enzyme activity. The expression levels of peroxidase are measured in IU/ml. In first two infected cotton plant, graph shows that there are higher expression of POD in infected plant than healthy plants. The rest of three plants are showing lower expression of POD in infected plants. Expression levels of peroxidase in which results were significant (1.352± 0.548), (2.971±1.18) and (0.969±0.387) were recorded with extract. So, predominantly the infection causes the suppression POD expression as the graph suggests the value of POD activity of 1.337±0.538 and 0.6703±0.017 for healthy plants.

In non-inoculated plants POD activity was significantly higher of both susceptible genotypes, as compared to resistant genotypes plants was observed in NIAB-111 followed by CIM-496, Ravi, and Co Tiep Khac in which highest POD activity was non- inoculated. POD activity significantly decreased in CLCuBuV-inoculated plants of susceptible genotypes CIM-496 (31.54 %) and NIAB-111 (26.497 %) compared to the non-immunized plants, whereas it changed none essentially in CLCuBuV-inoculated takes off of both resistant genotypes (Mydlarz & Harvell 2006).

Levels of CAT in Cotton Plant

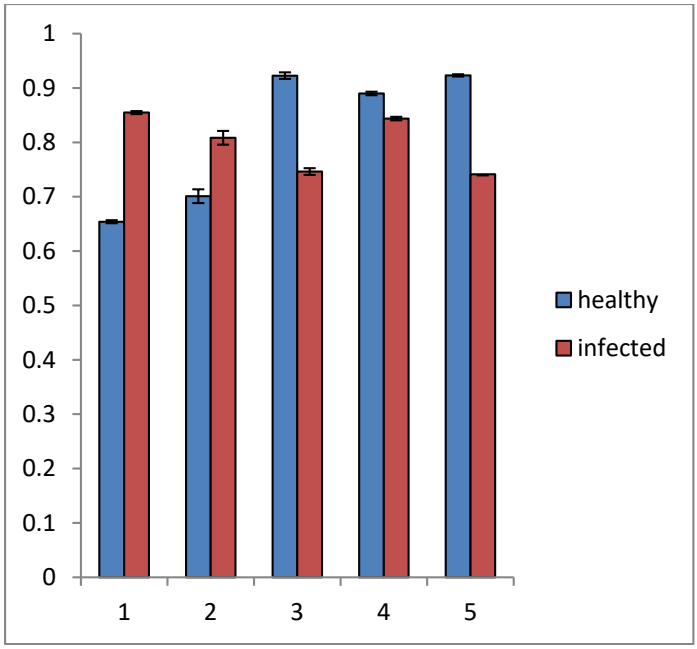


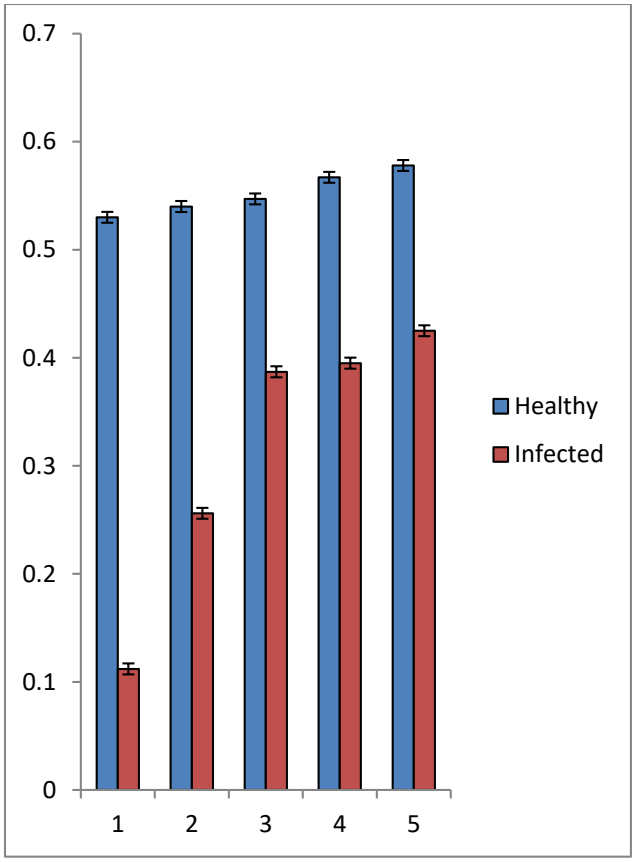
Fig. No.13: Levels of catalase (CAT) in cotton plant (cotton leaf curl virus CLCuV) under biotic stress, showing that the expression of CAT in two of the infected samples got over expressed and rest of the 3 out of 5 got suppressed.

$2\ H_2O \longrightarrow 2\ H_2O + O_2$

It is also measured in international unit (IU/ml) of protein.

Cotton leaf curl virus is not a seed borne disease while it is transmitted by the Whitefly *Bemisia tabaci* and some virus lives in alternate hosts. After infection of five plants with cotton leaf curl virus, the expression of catalase enzyme was studied in terms of enzyme activity these cotton plants graph shows that 2 plants are having higher activities for catalase on infection while the activities of catalase for 3 of these plants were suppressed. The expression levels of CAT are measured in IU/ml. It has been observed that expression levels of CAT (8.312±0.058) of this group were dominantly suppressed by infection than the healthy plants (8.226±0.0031). The effect of cotton leaf curl virus, (CLCuV) which causes cotton leaf curl disease, (CLCuD) on the expression of oxidative enzymes of cotton plants. The results were significant as expression levels of catalyze (8.586±0.006), (7.556±0.0021) and (1.764±0.121) were recorded with extract. The CAT activity was found to be positively correlated with the disease thus negatively correlated with the resistance. The increase of the CAT can be explained by the decline of the POD activity leading to an increase of hydrogen peroxide which must be scavenged by the CAT. CAT is enzymes with the potential to directly dismutase hydrogen peroxide into H_2O and O_2. They play an important role in the plant cells detoxification during oxidative stress. The level of CAT has been shown to increase in varieties sensitive to a stress (sarvista *et al*., 2002).

LEVELS OF (SOD) IN COTTON PLANTS



reported to increase in resistant cultivars with the resistance (Kumari *et al*., 2015). The results were significant as expression levels of sodium oxide dismutase (19.54± 0.005), (0.664±0.082) and (0.385±0.006) of protein were recorded with extract.

LEVELS OF TOTAL PHENOLIC CONTENTS

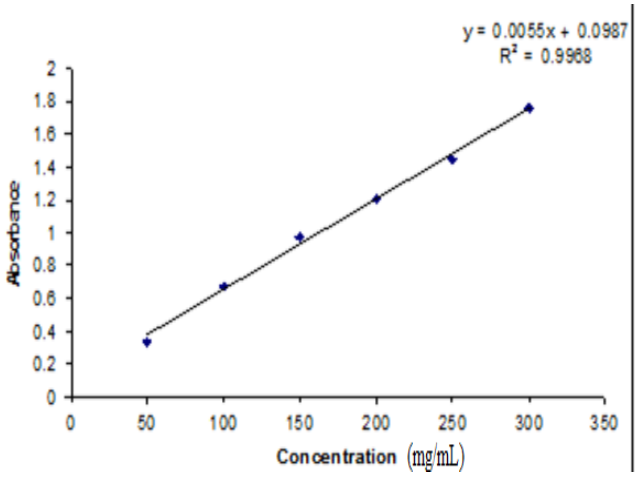


Fig. No.15: Graph shows that there is a strong linear correlation between absorption and concentration of phenolic contents over the defined limit of concentration. So based on the value of absorbance we can easily calculate the concentration at any other value of absorbance.

LEVELS OF TOTAL PHENOLIC CONTENTS IN PLANTS COTTON LEAF CURL VIRUS (CLCuV) UNDER BIOTIC STRESS

The total phenolic contents can be measured using the Folin-ciocalteu reaction. In which results are typically expressed as Gallic acid equivalents. Cotton leaf curl virus is not a seed borne disease while it is transmitted by the Whitefly *bemisia tabaci* and some virus lives in alternate hosts. After infection of three plants with cotton leaf curl virus expression of total phenolic contents was studied in terms of enzyme activity in first infected cotton plant graph shows are higher expression in healthy plants. The expression levels of TPC are measured in mg of GA/g of extract. It has been observed that expression levels of total phenolic contents (60.43±1.346) of this group were much higher than the infected (28.85±1.449) group. The effect of (CLCuV) which causes cotton leaf curl disease, (CLCuD) on the expression of oxidative enzymes of cotton plants.

The results were significant as expression levels of total phenolic contents (15.59±2.021), (20.023±1.008), (32.21±1.361) of protein were recorded with extract.

LEVELS OF TOTAL FLAVONOID CONTENT (TFC)

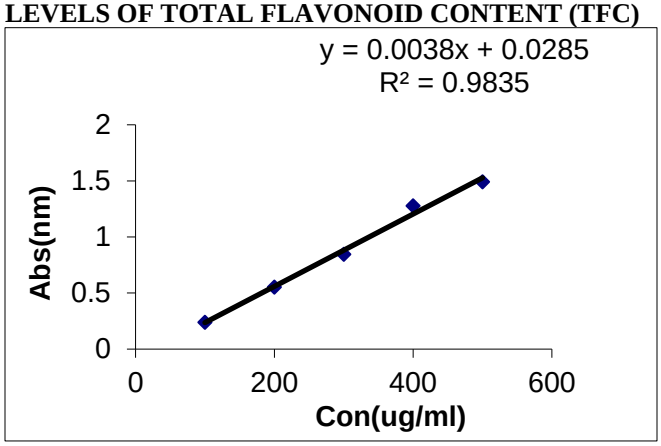


Fig. No.16: Graph shows that there is a strong linear correlation between absorption and concentration of flavonoid contents over the defined limit of concentration. So based on the value of absorbance we can easily calculate the concentration at any other value of absorbance.

The standard curve equation: Y =0.038x – 0.0285 with r^2=0.9835. The total flavonoid content was calculated from a calibration curve, and the result was expressed as mg equivalent per gram dry weight**.**

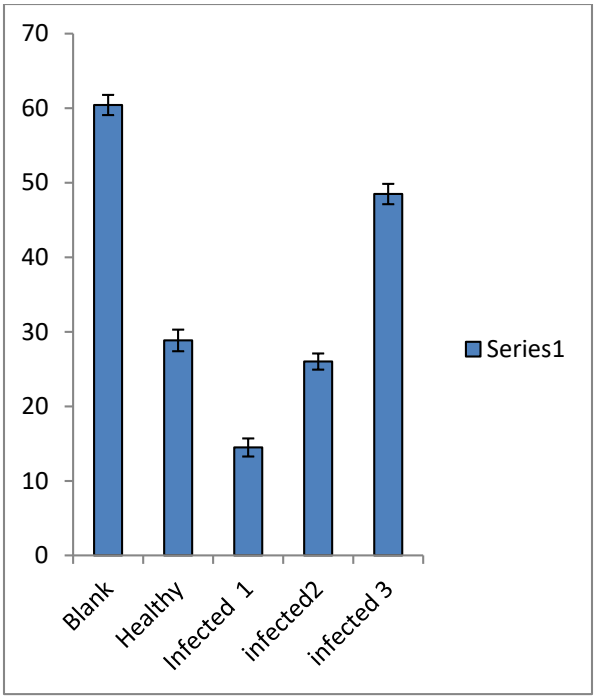


Fig. No.17: Levels of (TFC) In Plant (Cotton Leaf Curl Virus) Under Biotic Stress. This graph shows that TFC in two out of three infected plants got decreased and one of the infected plants showed higher level of TFC as compared to the healthy plant.

The total flavonoid content of fluid extract as determined by the aluminum chloride colorimetric method .The total flavonoid content was calculated from a calibration curve, and the result was expressed as mg rutin equivalent per g dry weight. Cotton leaf curl virus is not a seed borne disease while it is transmitted by the Whitefly *bemisia tabaci* and some virus lives in alternate hosts. After infection of three plants with cotton leaf curl virus expression of total flavonoid contents was studied in terms of enzyme activity in first infected cotton plant graph shows are higher expression than healthy plants. The expression levels of total flavonoid contents are measured in mg ruitn per day. It has been observed that expression levels of TFC (28.8±1.449) of this healthy group were much higher than the infected (14.48±1.203) group. The effect of cotton leaf curl virus, (CLCuV) which causes cotton leaf curl disease, (CLCuD) on the expression of oxidative enzymes of cotton plants.

The results were significant as expression levels of total flavonoid contents (26.013± 1.008), (48.43± 1.361) and (32.42±1.563) of protein were recorded with extract.

LEVELS OF (DPPH) IN COTTON PLANT

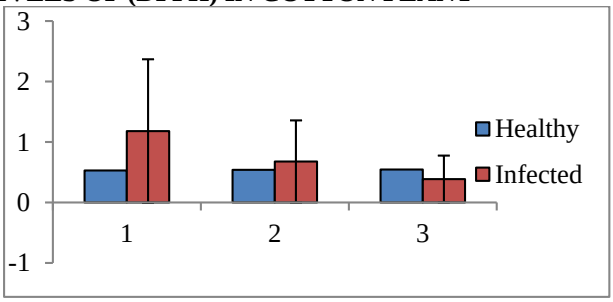


Fig. No.18: Graph of Levels of (DPPH) In Cotton Plant (Cotton Leaf Curl Virus) Under Biotic Stress shows that the level of DPPH in two out of three infected samples got suppressed and one of them showed enhanced level of DPPH after infection.

Cotton leaf curl virus is not a seed borne disease while it is transmitted by the Whitefly *Bemisia tabaci* and some virus lives in alternate hosts. After infection of three plants with cotton leaf curl virus expression of DPPH was studied in terms of enzyme activity in first infected cotton plant graph shows are higher expression than healthy plants. The expression levels of DPPH are measured in Iu. It has been observed that expression levels of DPPH (7.303±0.25) of this group were much higher than the infected (4.123±1.02) group.

The results were significant as expression levels of DPPH (6.023±2.22), (4.502±0.09) and (5.023±2.04) of protein were recorded with extract cotton leaf curl virus.

Table No.2: Levels of different parameters in expression of oxidative enzymes in cotton plant under biotic str

Groups	POD	SOD	TFC
Healthy 1	1.337±0.538	25.54±0.0049	28.85±1.449
Healthy 2	1.352±0.548	30.12±0.0147	32.45±1.563
Infected 1	2.971±1.188	0.6703±0.017	14.48±1.203
Infected 2	1.697±0.678	0.664±0.082	26.013±1.088
Infected 3	0.969±0.387	0.385±0.006	48.43±1.361

POD for Peroxidase, SOD for Superoxide dismutase, and TPC for Total protein content.

Statistical analysis

Statistical analysis was done by ANOVA along with Tukey's HSD (Honestly Significant Differences) test for all collected information. In triplicate manner all experiments were accomplished .Mean, standard deviation (SD) is used for the results and all the results are written according to this. For comparison between two means we used Student's t-test and if we wanted comparison of more than two means and one-way analysis of variance (ANOVA) was used (Runyon and Haber, 1988).

Conclusion

Important characteristics of plant resistivity to pathogens are from early and heavy levels of explaining different shield proteins .We need resistance for disease against CLCuBuV and many studies have explained those proteases. SOD, TFC POX, CAT, Protein, TFC and phenolic compounds play active part in its resistance. Exposed kinds after polluting with CLCuBuV and proteases, total phenolic and SOD were found to vary considerably in resistant due to the presence of high level of phenolic pre-infection and post-infection happens and additional studies are subjected to characterized the phenolic compounds and proteases involved and post-infection significant increase in resist squash yellow blotch tent cultivar Ravi may have further role in disease resistance.

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