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1 **Alterations in the Antioxidant Metabolites in Cotton Leaf Curl Disease Infected and**

2 **Healthy Cotton Leaves**

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9 # Abstract

10 The upland cotton (*Gossypium hirsutum*) belongs to the family *Malvaceae* and cultivated

11 since ancient times in warmer parts of the world. Pakistan is the 4th largest cotton producing

12 country and prominent cotton yarn exporter of the world. Cotton is the major source of natural

13 fiber and significantly contributes to the economy of Pakistan. There are many factors (biotic and

14 abiotic) affecting the cotton yield in Pakistan. The cotton leaf curl disease (CLCuD) is one of the

15 biotic factors and endemic in the most cotton growing areas of the country. The antioxidant

16 enzymes and antioxidant biochemical metabolites play important role during stress. The present

17 study was planned to compare the antioxidant enzymes and metabolites from healthy and CLCuD

18 infected cotton leaves. Antioxidant enzymes activities including catalase, peroxidase and

19 peroxidase were measured through different antioxidant assays and different antioxidant

20 metabolites were also determined. During stress condition antioxidant metabolites serve as signal

21 for the activation of antioxidant enzymes. Present study revealed that antioxidant enzymes were

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22 significantly higher in the infected cotton leaves as compared to the healthy cotton leaves. While
23 the amount of antioxidant metabolites like total phenolic contents (TPC) and total flavonoid
24 contents (TFC) were higher in healthy cotton leaves as compared to the infected leaves.

25 **Keywords:** Cotton leaf curl disease, Antioxidants, Catalase, Peroxidase, Superoxide dismutase

26 Introduction

27 The upland cotton belongs to the family Malvaceae and cultivated since ancient times in
28 warmer parts of the world. The crop is mainly cultivated for its precious fiber but it also contributes
29 80% share to oil production of Pakistan in addition to its lint [1]. Pakistan is the 4th largest cotton
30 producing country of the world and prominent cotton yarn exporter of the world. The cotton crop
31 is considered backbone of national economy as it is cultivated on the area of 3 million hectares in
32 the country [2]. The economy of Pakistan greatly dependents on cotton and cotton products. It has
33 less than 10% share in agriculture value but provide about 2% share to the GDP of the country [3].
34 It is economically most important crop of the country contributing up to 60 % of total exports and
35 having largest value chain employing 50% of total industrial labor from field to the final valuable
36 product [4]. The single crop provides the livelihood to the millions of farmers, agricultural labor,
37 industrial labor and traders [5]. There are multiple biotic and abiotic factors that affect the yield of
38 cotton. Among biotic factors, insect pests and plant diseases are leading challenges to the
39 sustainable cotton cultivation. In Indian subcontinent especially the Pakistan "the cotton leaf curl
40 disease" is the major dilemma to cotton crop since last few decades. The disease also deteriorate
41 the quality of fiber by affecting traits like percentage of ginning out turn, fiber fineness, staple
42 length, fiber uniformity index, fiber bundle strength and maturity ratio due to the changes in
43 metabolites (cellulose, pectin, waxes and proteins) composition in fiber in addition to the effect on
44 total crop yield [6].

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45 The reactive oxygen species (ROS) are not always harmful and toxic for the plants but also
46 perform special assigned role during development and regulation of metabolism of plant. ROS
47 may also act as indispensable signal molecules in plant biological system such as in response to
48 high light [7], cell cycle regulation [8], germination of seed [9], development of root system [10]
49 and during pathogenic infection [11]. The aerobic organisms to counteract oxidative stress caused
50 by ROS produce antioxidant enzymes and antioxidant compounds. ROS comprises of molecular
51 oxygen ($^{\cdot}O^{-}$), superoxide radical ($^{\cdot}O2^{-}$), hydroxyl radical ($^{\cdot}OH$) and hydrogen peroxide (H_2O_2)
52 produced during oxidation/reduction (redox) reactions of aerobic metabolism as bi-products [12].
53 To detoxify ROS, there are three antioxidant enzymes with key roles in plants. The superoxide
54 dismutase (SOD) converts $^{\cdot}O_2^{-}$ to O_2 and H_2O_2. The H_2O_2 is then degraded by catalase and
55 peroxidases [13]. Catalases have higher rate of reaction and low affinity to H_2O_2 as compared to
56 that of peroxidases which have higher affinity and can even detoxify H_2O_2 in lower concentrations
57 [14].

58 Phenolic metabolites are important plant constituents including phenolic acids, flavonoids,
59 stilbenes, lignins, lignans and tannins etc. [15]. These compounds are very good electron donors
60 and contribute their role as antioxidants [16]. Flavonoids are versatile secondary metabolites with
61 polyphenolic molecular structure and low molecular weight. They are widely distributed in plants
62 and perform diverse physiological functions in plants especially under biotic and abiotic stress.
63 The diverse functions perform by these biomolecules are protective role against salt stress [17],
64 drought stress [18], partial detoxification of ROS [19, 20]. Enormous flavonoids have also been
65 characterized with diverse biological roles for example, shield against ultra violet radiation,
66 signaling during plant microbe interaction for nodulation [21], defense against pest and
67 phytopathogens [22], male fertility of plant, production of visual signals by coloring the flowers

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68 to attract pollinators [23] and in transportation of hormones from one part of plant to the other.
69 Few additional functions are protection against photo-radiation and enhancement of nutritional
70 retrieval efficiency during senescence [24].

71 The plant response to elicitors is the developing field of research in plant physiology.
72 Among commonly used elicitors are salicylic acid, methyl salicylate, benzothiadiazide, benzoic
73 acid and chitosan etc. which activate different defense related enzymes and alter the production of
74 phenolic compounds [25]. These compounds stimulate the interaction between pathogens and
75 plants that subsequently triggers plant's defense system which constrain the invasion of pathogenic
76 viruses. The tannins and phenols are well studied examples and these biomolecules control
77 resistance genes regulation [26]. The role of these biochemical metabolites is crucial in infection
78 of CLCuD and thus survival of plant under stress. The study of these important biomolecules can
79 provide useful information regarding host defense mechanism against viral infection. In the present
80 study, we have compared the activity of antioxidant enzymes as well as flavonoids and total
81 phenolic contents in the leaves of healthy cotton plants and CLCuD infected cotton plants.

82 Materials and Methods

83 This research work was carried out at Medicinal Biochemistry Lab, Department of
84 Biochemistry, University of Agriculture, Faisalabad.

85 Sample collection

86 The leaf samples were collected randomly from eight locations of cotton growing areas of
87 Punjab, Pakistan, during field survey in September 2019. Total six sample (three infected and three
88 healthy) from each location were collected. The samples were collected in labeled polyether zipper

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89 bags and preserved in thermophore box containing ice bricks. The samples were stored in -70˚C
90 freezer for long term storage.

91 **Quantification of Antioxidant Enzymes**

92 The leaf samples (10 mg) of each plant were taken in 2 mL tubes and 1 ml of pre-cooled
93 50 MM potassium phosphate buffer was add to the tubes containing leaf samples and samples were
94 grounded using bead beater (Omni bead rupture, Canada). The samples were centrifuged for 10
95 minutes at 13000 rpm to pellet the leaf contents. The supernatant containing the cell lysate were
96 collected in fresh prelabelled eppendorf tubes.

97 **Catalase Estimation**

98 The catalase is present in almost every aerobic cell and detoxify the H_2O_2. The enzyme
99 activity was determined in the leaf samples using Amplex Red Catalase Assay kit (A22180).

100 **Reaction Mixture and Standard curve**

101 The reaction mixture was prepared using solution provided in the kit and according to
102 instructions of manufacturer. The 20 MM H_2O_2 was prepared by diluting 23 µl of 3 % H_2O_2 in 977
103 µl of deionized water (dH_2O) and for final use 40 µM H_2O_2 was prepared using 10 µl of 20 MM in
104 490 µl of 1X reaction buffer. The 260 µg Amplex Red reagent was dissolved in 100 µl of
105 dimethylsulfoxide (DMSO) to prepare the working solution of Amplex Red. 100 µl of 1 X reaction
106 buffer was added to the horseradish peroxide (HRP) vail to get 100 U/ml solution. The working
107 reaction mixture of Amplex Red and HRP 100 µM and 400 mU, respectively was prepared from
108 previously prepared solutions. To prepare standards, 100 µl of dH_2O was added to the vail
109 containing catalase and eight standards ranging from 0 to 4 U/ml were prepared using 1 X reaction
110 buffer. The standard curve was plotted using these standard solutions of catalase (Supplementary

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111 Figure S1). 25 µl of each standard was pipetted in three wells of ELISA plate and 25 µl of 40 µM
112 H_2O_2 was added to each well using multichannel pipette. The mixture was allowed to react for 3
113 minutes at 37˚C followed by addition of 50 µl Amplex Red reagent and horseradish peroxidase
114 working solution to make the reaction mixture to 100 µl. The plate was incubated at 25 ˚C for 5
115 minutes to complete the reaction and the incubation time was optimized after multiple trails.

116 The absorbance of the reaction plate was measured at 560 nm by using micro well plate
117 reader (BioTek, µ-QuantTM, USA). The plant samples were arranged in the racks according to
118 descending order and kept at room temperature before use. The assay was carried out using 25 µl
119 sample and 25 µl of 40 µM H_2O_2 and plate was placed at 37 ˚C for 3 minutes. Apmlex Red and
120 HRP solution were added and plate was placed at 25 ˚C for 5 minutes before measurement of
121 absorbance.

122 **Calculation of Enzyme activity**

123 To calculate the activity of catalase in our samples linear regression equation (Y=-0.0002X
124 + 0.8033) obtained from standard curve was used (Supplementary Figure S1). The U/mL units
125 were converted to the U/g of sample weight by multiplying with dilution factors and expressed in
126 (Table 1). 1 U of catalase is described as the quantity of enzyme which decomposes 1.0 µM H_2O_2
127 per minutes at optimum conditions of pH and temperature.

128 Peroxidase Estimation

129 Peroxidase (PODs) detoxify the H_2O_2 and other hydro peroxides by catalyzing the
130 reduction of ascorbate (AsA), monodehydroascorbate (MDHA), glutathione (GSH) or
131 dehydroascorbate (DHA) depending upon the peroxidase and shielding cells from oxidative
132 damage [27]. PODs were estimated as described by Siddique and co workers in cotton leaf samples

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133 as with slight modification. In this assay guaiacol is used which produce brown color upon
134 oxidation [28].

135 **Reaction Mixture**

136 The reaction mixture was prepared in 50 MM potassium phosphate buffer. The calculated
137 volume of H_2O_2, and guaiacol were added to the buffer to make reaction mixture with final
138 concentrations 40 MM and 20 MM, respectively. The 240 µl of reaction mixture was added to each
139 well of micro well plate and the samples to be estimated were pored to another plate in predefined
140 order. The samples were added to the reaction mixture with the help of multichannel pipette and
141 time was recorded on the mobile stopwatch. Few wells were loaded with only phosphate buffer
142 instead of sample to get the blank values. The absorbance was measured at 470 nm after every 60
143 sec using ELISA plate reader (BioTek, µ-QuantTM, USA**)** and 6 reading were recorded.

144 **Calculations**

145 The PODs activity was estimated by the difference of absorbance. The first minutes
146 readings were used after subtracting from blank reading. The ΔA_{470} /min is the absorbance after
147 first minute minus absorbance of blanks. The following formula was used to calculate enzyme
148 activity in U/mL. Finally the values are expressed as U/g of fresh weight and given in (Table 1**)**.

149

150
151
152

$$\text{Peroxidase activity in U/mL} = \frac{(\Delta A_{470}/\text{min})\ \text{x}1000}{26.6 \quad \text{x } 10/240}$$

153

154 The 26.6 is H_2O_2 extinction coefficient, and 10/240 is dilution factor as 10 µl of sample
155 was added to 240 µl of reaction mixture and there are 1000 µl in 1 mL.

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156 **Superoxide Dismutase (SOD) Determination**

157 Superoxide dismutase assay was performed on the basis of NBT reduction inhibition as
158 described by Siddique and coworkers with little modifications [28].

159 **Reaction mixture and Enzyme Assay**

160 The master reaction mixture was prepared by addition of 50 MM potassium phosphate
161 buffer (pH-7.0), 100 MM Ethylene diamine tetra acetate (EDTA), 1.5 MM Nitro blue tetrazolium
162 (NBT) and 0.1 MM riboflavin in the end according to these ratios 53:4:2:1, respectively. This is
163 light sensitive solution and used immediately after formation. The master mixture was prepared
164 according to the number of samples and each well was loaded with 230 µl master mix. 10 µl of
165 sample was added to each well according to predefined well schedule and few wells were loaded
166 with dH_2O as control. After addition of all the samples blank reading was recorded using ELISA
167 plate reader (BioTek, µ-QuantTM, USA) at 560 nm wavelength. The ELISA plate was placed in
168 light for the reaction and absorbance was recorder after every 30 sec and took 5 reading. The
169 difference in absorbance was observed to check the pattern of reduction of NBT in controls
170 samples suggests a linear reduction pattern only in first reading after 30 Sec. The enzyme activity
171 was calculated using the following equation to calculate the percentage inhibition by SOD in each
172 sample. The enzyme activity was converted to the weight of the enzyme present in the each gram
173 of sample through appropriate calculation. The values are expressed as U/g of fresh weight and
174 given in (Table 1).

175–177

$$\%\text{ age inhibition of reduction} = \frac{(\text{Blank Absorbance} - \text{Sample Absorbance})}{\text{Blank Absorbance}} \times 100$$

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178 Where, sample absorbance is after 30 sec so the values obtained were multiplied by 2 to
179 obtain 1 minute activity of the enzyme.

180 Estimation of Non-Enzymatic Metabolites

181 The fresh leaves of healthy and virus infected cotton leaves were used to estimate the non-
182 enzymatic antioxidant metabolites (total phenolic compounds and total flavonoid contents).

183 Determination of Total Phenolic Contents (TPC)

184 The TPC of cotton leaves (infected and healthy) collected from different locations were
185 determined as described by [29] with minor modifications. The leave samples (10mg each) were
186 homogenized in 80% acetone in bead beater (Omni Bead Ruptor 24, by Omni International
187 Canada) in 2 mL tubes and homogenates were centrifuged at 10000 rpm for 10 minutes.
188 Supernatant was collected in new eppendorf and use for TPC estimation.

189 Reaction Mixture and Standard Curve

190 The reaction mixture was prepared by dilution 2 mL of Folin-Ciocalteau's reagent in 16
191 mL of distilled water. The each well of ELISA plate were loaded with 50 µl of reaction mixture
192 and 5 µl of Gallic acid standers were added and mixed in 3 consecutive wells each to get the mean
193 value for standard curve. The plate was incubated at room temperature for 15 minutes. The 200 µl
194 saturated solution of sodium carbonate (Na_2CO_3) was added to the each well and incubated at room
195 temperature for half hour. The absorbance was measured at 750 nm by ELISA plate reader
196 (BioTek, µ-QuantTM, USA).The standard curve was obtain using the mean value of these readings
197 (Supplementary Figure S2). The TPC of cotton leave samples were determined in the same way
198 using 5 µl of supernatant. The value of TPC in our samples linear regression equation (Y= 0.0025

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199 X + 0.0206) obtained from was standard curve was used (Supplementary Figure S2). The values
200 are expressed as mg/g of fresh weight Gallic acid equivalence (GAE) and given in (Table 1).

201 **Determination of Total Flavonoid Contents (TFC)**

202 Flavonoids are phenolic compounds have antioxidant properties. The flavonoids contents
203 were estimated in the cotton leaves as described by Chanda and Dave with few modifications. The
204 fresh leave samples (10mg each) were homogenized in (200 µl) 50 to 80% ethanol in bead beater
205 (Omni Bead Ruptor 24, by Omni International Canada) in 2 mL tubes and homogenates were
206 centrifuged at 10000 rpm for 10 minutes. Supernatant was collected in new eppendorf and used
207 for TFC estimation and stored in freezer [30].

208 **Reaction Mixture and Standard Curve**

209 The reaction mixture was prepared by adding 70 µl sample and standard extract each well
210 of ELISA plate followed by addition of 70 µl of sodium Nitrite, $NaNO_2$ (5%) and incubated
211 mixture for 5 Minutes. The 15 µl of aluminum chloride, $AlCl_3$ (10%) was added to each well and
212 incubated at room temperature for 10 mins followed by addition of addition 70 µl of 1M sodium
213 hydroxide (NaOH) to each well. The plates were incubated for 10 minutes before recoding
214 absorbance at 415 nm by ELISA plate reader (BioTek, µ-QuantTM, USA). Quercetin standers
215 were used to get standard curve (Supplementary Figure S3). The standard curve was obtained
216 using the mean value of these readings. The value of TFC in our samples was determined using
217 linear regression equation (Y= 0.0005X + 0.0055) obtained from standard curve (Supplementary
218 Figure S3). The values are expressed as mg/g and given in (Table 1).

219 **Results and Discussion**

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The homeostasis of ROS is dependent on its production and scavenging activity of anti-oxidant enzymes and other anti-oxidant compounds [31]. There are two types of defense mechanism against ROS in nature, enzymatic and non-enzymatic. The enzymatic defense system has variety of scavenger enzymes in plants but there are three main enzymes responsible for enzymatic defense against ROS viz. catalase, peroxidases and superoxide dismutase. The non-enzymatic defense includes compounds with intrinsic antioxidant properties and these compounds are either water soluble such as phenolic compounds, flavonoids, ascorbate glutathione or fat soluble like carotenoids and tocopherols. These antioxidant compounds act as electron donor to reduce ROS to less reactive molecules which are not that harmful to cell. Under stress conditions, the ROS homeostatic defense system becomes disturbed due to increased ROS production. To minimize the damaging effect of ROS, the aerobic organisms up regulate the both enzymatic and non-enzymatic antioxidant defense systems [32]. Present study compared the activity of antioxidant enzymes and some antioxidant compounds under stress caused by cotton leaf curl disease.

Catalase

Catalase is an antioxidant enzyme present in almost every aerobic cell in abundance and detoxify hydrogen peroxide (H_2O_2) a toxic product of aerobic metabolism and ROS produced by pathogens or response to pathogens. This enzyme protect cells from toxicity of H_2O_2 which can be lethal if not degraded [33]. It converts two molecules of H_2O_2 into water and molecular oxygen [34].

$$H_2O_2 + H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

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241 Catalase is tetrameric protein of 240 kDa and each polypeptide of 60 kDa in weight and
242 similar to each other containing a single ferriprotoporphyrin [35]. In the cell it scouts for hydrogen
243 peroxide and have highest turnover number and a single tetramer can convert millions of H_2O_2 to
244 water and oxygen in one second. This reaction took place in two steps during first step H_2O_2
245 molecule oxidizes the heme and one porphyrin radical generated and in second step another H_2O_2
246 molecules reduces the porphyrin radical to enzyme resting state producing one molecule of oxygen
247 and two water molecules [36]. In this study the catalase activity in infected cotton leaves is
248 significantly higher than that of healthy leaves as shown in Figure 1 which suggests the increase
249 of ROS in infected cell as compared to the normal and healthy cells.

250 Catalase activity was recorded increased in cotton leaf curl infected leaves and these
251 findings are in agreement with that of Siddique et al. [28]. These finding are in contrast with
252 Riedle-Bauer [37] findings that cucumber mosaic virus does not affect catalase activity in
253 cucumber plants. [38] Ahammed et al. also reported the enhanced activity of catalase under iron
254 stress which is in accordance with our results that the under stress the activity of catalase increases.
255 The increased catalase activity in rice under drought stress is also reported by the [39] which is
256 accordance with our results in this study. [40] Hasanuzzaman et al. also reported the significant
257 increase in catalase activity under abiotic stress which supports our results that the activity of
258 catalase enhanced during stress. These finding suggests that during virus infection the ROS
259 synthesis increases and subsequently plants enzymatic antioxidant defense system also activated
260 to cope with the stress condition.

261 **Peroxidase**

262 Peroxidases (PODs) are the antioxidant enzymes present in almost every aerobic cell and
263 among the first enzymes respond to phytopathogens in addition to detoxification of hydrogen

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264 peroxide (H_2O_2) [41]. PODs are involved in Lignification, regulation of cell wall elongation,
265 suberification, wound healing and resistance against phytopathogens [42]. The reaction of PODs
266 are given below

267

$$H_2O_2 + R(OH) \xrightarrow{\text{Peroxidase}} 2H_2O + R(O)_2$$

268 Where, "R" may be Ascorbate (AsA), monodehydroascorbate (MDHA), glutathione
269 (GSH) or dehydroascorbate (DHA) depending upon the peroxidase. Peroxidases counteract the
270 increased hydrogen peroxide along with catalases and plays critical role in the antioxidant defense
271 response of plant [43, 44]. The protective role of peroxidases have been reported against plant
272 disease and tissue injuries [45]. These have greater affinity for H_2O_2 than that of catalase and
273 present in cytoplasm and mitochondria. In this study the catalase activity in infected cotton leaves
274 is significantly higher than that of healthy leaves as shown Figure 1 which suggests that, this group
275 of enzymes have role in plant defense against pathogens.

276 These finding of enhanced PODs activity in infected cells is in accordance with the finding
277 of [46] who observed immediate increase of PODs level after inoculation of virus. [47] also
278 observed increased PODs activity after inoculating tomato leaves with *A. alternate* leaf blight
279 causing pathogen which is also in accordance with our results the higher expression of PODs after
280 infection. Our results are also in agreement with [28] for the increased activity of PODs in plants
281 grafted with virus infected scion. The increased PODs activity in rice under drought stress is also
282 reported by the [39] which is accordance with our results in this study. These finding suggests that
283 during virus infection the activity of PODs also increases as defense against pathogens.

284

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Superoxide Dismutase (SOD)

Superoxide dismutase are the metalloproteins which catalyze the dismutation of superoxide (O_2^-) radicals to molecular oxygen or H_2O_2. The SODs are classified into three types according to the metal ion association and important part of cells antioxidant defense system [48]. The H_2O_2 - produce during this reaction is catalyzed to water and oxygen by catalases and peroxidases. The reaction scheme is given below.

$$2O_2^- + 2H \xrightarrow{\text{superoxide dismutase}} H_2O_2 + O_2$$

SODs are the first line defense of cell against ROS and also plays important role against the pathogenic infections. The SOD activity in infected cotton leaves is significantly higher than that of healthy leaves as shown in Figure 1. These findings suggests that, this enzyme have role in plant defense against pathogens. These finding of enhanced SOD activity in infected cells is in contrast with the finding of [46] who observed decrease of SODs activity after inoculation of virus and with [28] for the increase activity of SODs in plants grafted with virus infected scion. These findings are in accordance with [39] who reported increased SOD activity in rice under drought stress. [40] also reported the significant increase in SOD activity under abiotic stress which supports our results that the activity of SOD enhanced during stress. We can conclude that SOD activity altered during stress.

Total Phenolic Contents (TPC)

Polyphenols of plants are distinct group of phenolic compounds (phenolic acids, flavonoids, stilbenes, lignins etc.) having ideal structure for scavenging of free radicals. Their property of electron or hydrogen donation makes them good antioxidant compounds [49]. These phenolic compounds enhance the mechanical strength of cell wall of the host cells may be by

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308 synthesis of lignin and suberin and form a physical barrier for the pathogens [50, 51]. In this study
309 the amount of Phenolic compounds is significantly reduced in infected plants shown in Figure 1.
310 A significant difference of total phenolic contents observed between the samples from different
311 locations this might be due the difference of cultivars or climatic conditions affected accumulation
312 of these compounds. These results are in accordance with the results of [28] who reported that
313 reduction of phenolic compounds in infected/inoculated cotton cultivars as compared to the
314 healthy/non inoculated plants. Chatarjee [52] reported that virus infected Mesta plants have lower
315 total phenolic contents as compared to that of healthy plants and also observed that the resistant
316 cultivars have high level of total phenolic compounds as compared to the susceptible varieties the
317 first part is in accordance with our results of this study. (Chaubey & Mishra, 2020) also reported
318 that the leaf curl disease to chili also reduced the total phenolic contents in the infected leaves as
319 compared to the healthy leaves which is in agreement with our findings in this study. It can be
320 concluded that in the infected leaves the level of total phenolic compounds drop significantly as
321 compared to that of healthy plants. Moreover, the climatic condition also effect the level of these
322 compounds.

323 **Total Flavonoid Contents (TFC)**

324 Flavonoids are phenolic compounds have antioxidant properties and can scavenge free
325 radicals as well as chelate the metal ions (Iron, copper etc.**)** subsequently inhibiting the enzymes
326 responsible for generation of free radicals [53]. These compounds occur in the form of glycosides
327 in plants and hypothesized to perform several roles like photoproduction, hormonal translocation
328 modulation and sequestration of ROS [54]. Flavonoids can scavenge all known ROS depending
329 on their structure. During stress condition the level of TFC may be altered as in our study. The
330 level of flavonoids in CLCuD infected plants has dropped little as compared to healthy leaves as

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331 shown in Figure 1. There might be difference of verities which express different flavonoid contents
332 and the information of the cotton varieties is not available. The difference might be due to the
333 different climatic condition as the samples were collected from different locations. The overall
334 results shows that the flavonoid content in the infected leaves is lower than that of healthy leaves
335 from the same field. These results are in accordance with that of [28] who perform the assay for
336 the cotton leaf curl disease infected plants and healthy plants in addition to that of disease resistant
337 cotton cultivars which express more flavonoid contents than that of susceptible cultivars. It can be
338 concluded that in the infected leaves the level of total flavonoid contents drop significantly as
339 compared to that of healthy plants. Moreover, the climatic condition also effect the level of these
340 compounds.

341 **Conclusion**

342 These findings suggests that antioxidant enzymes catalase, peroxidases and superoxide
343 dismutase along with antioxidant compounds constitue the first line of defense with key role in
344 total defense machanism of biological system. The activity of all antioxidant enymes during cotton
345 leaf curl disease increases and the level of antioxidant compounds decreased.

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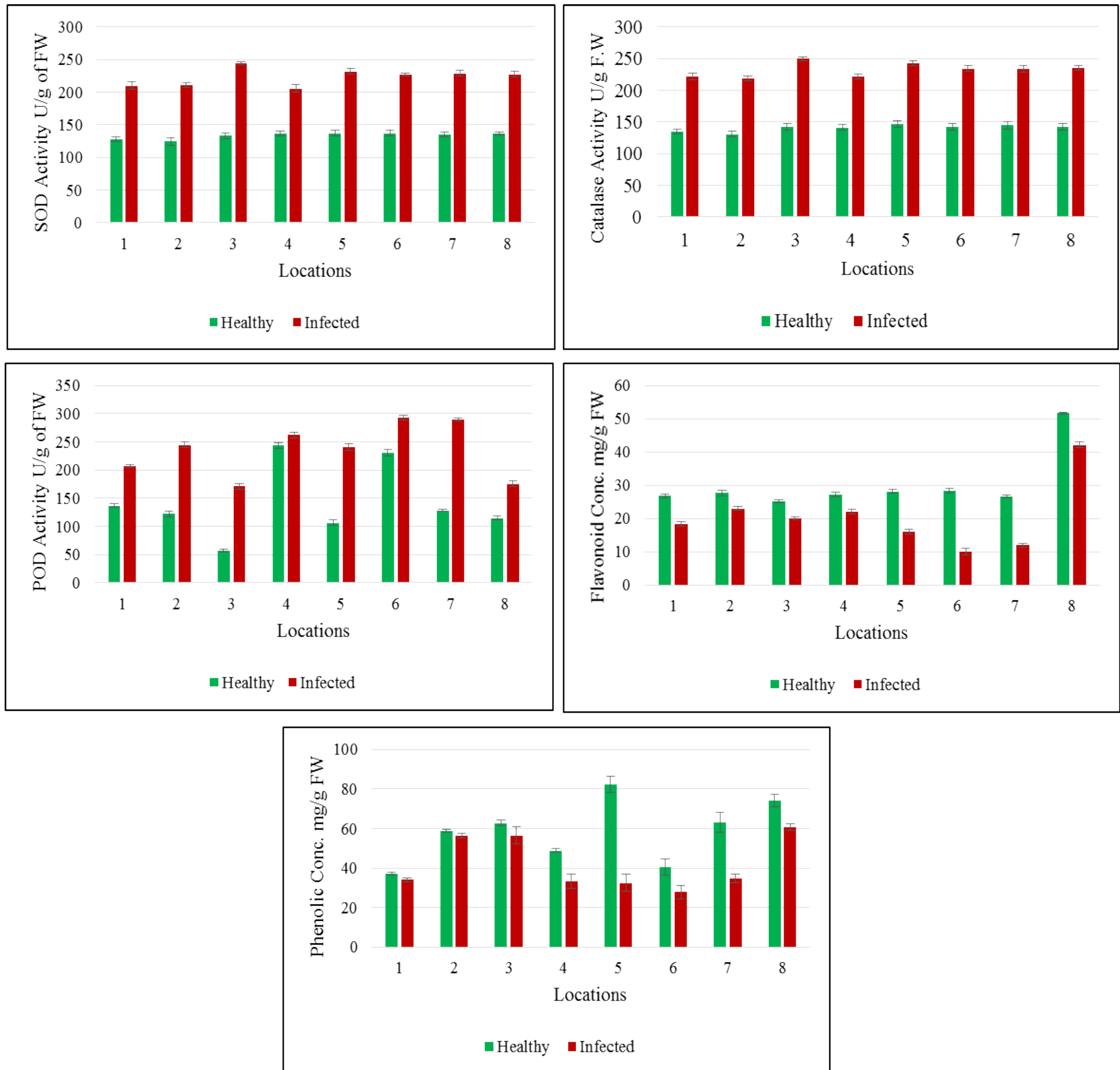

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499 Figure 1. The graphical representation of antioxidant enzymes super oxide dismutase, catalase, peroxidase
500 activity and antioxidant metabolites total flavonoids contents and total phenolic contents in the healthy and
501 CLCuD infected leaves of cotton.

502

Table 1. Location of collected sample, physical condition of the plant, antioxidant enzymes superoxide dismutase, catalase and peroxidase in unit/g of fresh weight and antioxidant metabolites, total phenolic contents in equivalence of gallic acid (GAE) and total flavonoid contents in equivalence of quercetin (QE) from eight different locations in Punjab, Pakistan.

Sample No.	**Location**	**Physical condition of cotton sample**	**Superoxide Dismutase (SOD) (Unit/g of FW)**	**Catalase (CAT) (Unit/g of FW)**	**Peroxidases (PODs) (Unit/g of FW)**	**Total phenolic contents (mg GAE/g. FW)**	**Total flavonoids contents (mg QE/g. FW)**
1	District Faisalabad 31°23'53.3"N 73°02'01.0"E	healthy	123.62	129.58	132.81	35.60	25.62
2		healthy	134.62	141.58	133.52	38.68	27.22
3		healthy	125.16	133.12	143.46	37.28	27.52
4		infected	215.76	226.53	209.49	32.10	18.42
5		infected	216.57	228.34	210.51	35.25	16.92
6		infected	197.53	210.30	201.81	35.04	19.52
7	District Multan	healthy	114.16	124.65	120.75	58.91	26.52

8	30°14'55.3"N 71°35'20.3"E	healthy	124.46	128.40	132.50	57.72	29.22
9		healthy	135.13	140.07	113.86	59.82	27.12
10		infected	205.37	211.31	237.45	54.08	23.32
11		infected	217.25	224.19	244.58	58.00	21.92
12		infected	211.65	219.59	252.33	57.58	23.62
13	District Multan 29°34'33.2"N 71°38'51.3"E	healthy	124.46	132.69	59.90	60.66	25.52
14		healthy	134.86	144.09	52.38	65.42	24.43
15		healthy	140.71	150.94	60.91	62.13	25.62
16		infected	240.47	244.63	167.20	48.13	19.98
17		infected	245.35	250.51	164.85	60.73	20.62
18		infected	248.75	254.91	181.84	61.08	19.42
19	Matila chowk 29°59'52.4"N 71°49'36.4"E	healthy	142.94	150.10	237.45	47.15	25.82
20		healthy	133.46	141.62	239.41	49.67	27.42
21		healthy	123.80	132.96	253.97	49.81	28.22
22		infected	205.09	215.25	264.00	38.68	23.32
23		infected	195.54	206.70	253.19	27.06	20.62

24		infected	215.31	227.47	268.78	34.20	21.92
25	District Multan 30°06'19.0"N 71°37'41.3"E	healthy	145.36	155.96	115.97	83.62	29.22
26		healthy	136.54	148.14	102.74	88.66	26.82
27		healthy	124.26	136.86	101.17	74.66	28.42
28		infected	228.61	240.05	248.81	26.64	14.62
29		infected	239.74	251.18	244.97	40.85	16.12
30		infected	216.86	228.30	229.23	29.79	17.32
31	District Lodhran 29°40'25.2"N 71°35'25.1"E	healthy	123.23	133.07	241.29	38.12	27.12
32		healthy	142.91	151.36	222.57	34.76	29.82
33		healthy	133.17	142.62	225.78	48.55	27.92
34		infected	215.36	225.81	293.06	31.82	9.42
35		infected	225.13	236.58	285.22	30.91	12.02
36		infected	232.19	241.16	300.89	21.11	8.82
37	District Lodhran 29°34'53.7"N 71°38'09.2"E	healthy	124.41	134.38	126.39	72.14	26.42
38		healthy	142.29	153.26	129.68	61.92	26.02
39		healthy	134.53	146.50	129.44	54.92	27.22

40		infected	212.48	224.78	288.44	38.82	12.22
41		infected	236.69	243.07	286.79	32.59	10.92
42		infected	228.39	235.77	294.62	32.52	12.72
43	District Jhang 30°37'00.9"N 71°40'28.5"E	healthy	124.47	131.85	107.91	68.64	52.02
44		healthy	135.62	144.00	116.05	73.89	51.02
45		healthy	140.88	150.26	119.42	79.70	51.82
46		infected	206.53	216.91	169.31	64.16	40.72
47		infected	235.74	241.38	185.05	58.70	42.32
48		infected	220.57	227.21	171.82	58.98	43.32

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Supplementary Data

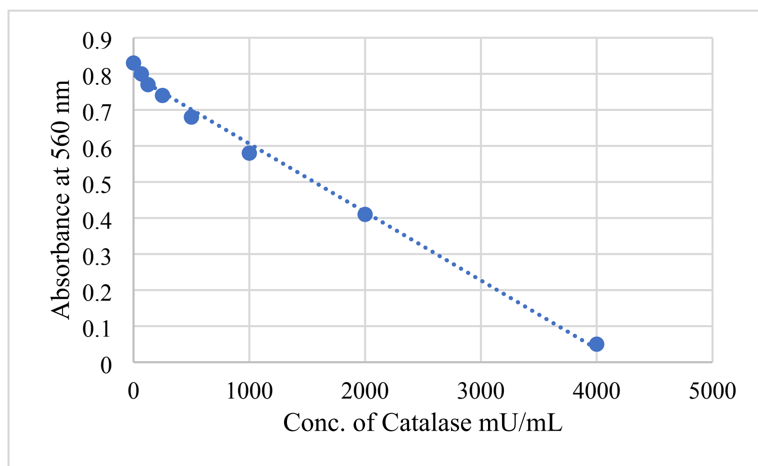

Figure S1. The Standard Curve for Catalase activity

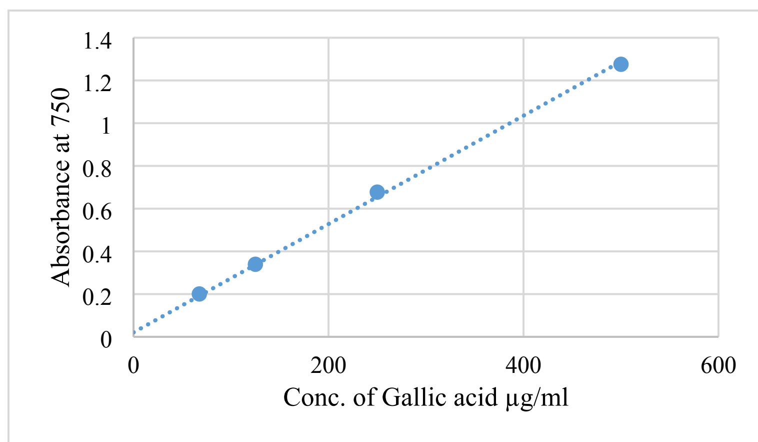

Figure S2. The Standard Curve for Gallic acid (GA) for quantification of total phenolic contents.

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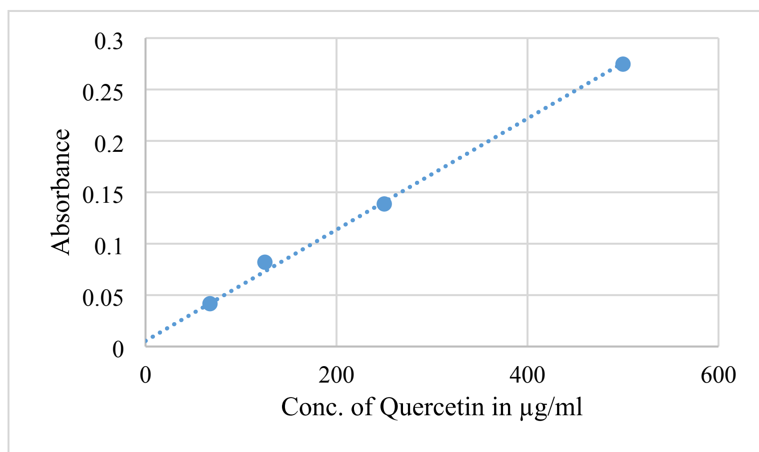

Figure S3. The Standard Curve for Quercetin for quantification of total flavonoid contents