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Role of secondary metabolites biosynthesis in resistance to cotton leaf curl virus (CLCuV) disease

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Disease percentage on six cotton varieties with respect to time for cotton leaf curl virus (CLCuV) was evaluated. In August 2007, the maximum disease was observed in CIM-506, CYTO-89 and BH-118 (susceptible), whereas CIM-443 was resistant with lower disease percentage. It was found that the leaf area, fresh weight and dry weight were more in healthy sample of leaves as compared to diseased samples of same varieties. Maximum leaf area was observed in BH-118 in healthy sample as compared to diseased ones and minimum leaf area was observed in CYTO-89. Maximum fresh weight and dry weight were present in NIAB-111 and CIM-443 in healthy sample as compared to diseased sample, respectively. Secondary metabolites production in healthy and diseased sample of leaves of cotton varieties after the attack of CLCuV found maximum phenolics, carotenoids, chlorophyll a, chlorophyll b and total chlorophyll a and b in healthy sample and minimum contents present in diseased sample. CIM-446 was the best variety to resist the cotton leaf curl disease. CIM-446 had maximum chlorophyll contents as compared to diseased leaves, and maximum phenolics were present in BH-118 and carotenoids in NIAB-111 in healthy sample in resistance to CLCuV. So, CIM-446, CIM-443, NIAB-111 and BH-118 were the best varieties in resistance to cotton leaf curl disease. The productions of secondary metabolites were high in healthy leaves in resistance to cotton leaf curl disease.

Key words: Cotton varieties, cotton leaf curl disease percentage, leaf area, fresh and dry weight, chlorophyll a, b, a + b, carotenoids, phenolics.

INTRODUCTION

Cotton is the most important natural fiber crop in the world. During 1992, Pakistan ranked third in production after China and USA. Other important countries in cotton production are India, Russia, Mexico, Brazil, Egypt and Turkey. The world production of cotton is nearly 50 million bales grown on more than 80 million acres. In Pakistan, cotton crop is cultivated in Punjab and Sindh. The share of two provinces in cotton production is 81%. The average yield ranged from 488 kg/ha in 1992/93 and 601 kg/ha in 1997/98 (Alam, 2000).

Cotton leaf curl virus (CLCuV) belongs to the genus *begomovirus* of the family *Geminiviridae* (Kirthi et al., 2004). Cotton leaf curl is a serious disease of cotton and several other Malvaceous plant species that is transmitted by the whitefly, *Bemisia tabaci* (Briddon, 2003). Cotton is the main cash crop of Pakistan and cotton leaf curl virus disease causes 30% losses due to the cotton leaf curl disease (Asad et al., 2003). In Pakistan, cotton leaf curl disease was first observed near Multan in 1967 and first reported in 1985 (Briddon and Markham, 2000). The CLCuV affected area accounted for 97,580 ha with a loss of 543,294 bales of cotton during 1992 to 93 in Punjab only (Idris, 1990). In cotton cultivars, the average reduction was in plants height (40.6%), boll weight (33.8%), number of bolls per plant (72.5%),

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Abbreviation: CLCuV, Cotton leaf curl virus.

ginning outturn (3.9%), and fiber length (0.7%) (Mahmood et al., 1996).

Plants have been known to produce a large number of secondary metabolites such as alkaloids, terpenoids, polyphenols, quinines and combined structures of these groups. The role of metabolites for plants is still under investigation (Yazaki, 2006).

Secondary metabolites are not directly essential for basic photosynthesis or respiratory metabolism in plants (Theis and Lerdau, 2003), although they are the major component present in plants.

They play a role in defense against herbivores, microbes, viruses or competing plants to attract pollinating or seed dispersing animals. They are important for the plant survival and reproductive fitness. They also protect plants from physical stresses like ultraviolet light and heat (Yazaki, 2006). Secondary metabolites mixture in high concentration provides a more effective protection against herbivores than a single compound or low diversity mixture in both low and high concentration (Castellanos and Garcia, 1997). Secondary metabolites represent adaptive characters that have been subjected to natural selection during evolution (Micheal, 2003).

Those metabolites containing nitrogen and sulphur are essential for protein synthesis. Chlorophyll, carotenoids (CAR) and phenolics (PHE) are commonly studied metabolites of plant kingdom. Secondary metabolites synthesis increase under stress condition and is believed to protect the cellular structure oxidative damage (Buchanan et al., 2000).

The soluble phenolics (PHE) produced by the phenyl-propanoid or shikimate pathways are powerful antioxidant in plant tissues under stress (Dixon and Paiva, 1995).

Unfortunately, no study documents were found on the synthesis of these metabolites after the attack of CLCuV. Demain and Fang (2000) studied the secondary metabolites produced in nature that belongs to heterogeneous group and served as competitive weapons against microorganisms. Secondary metabolites can slow down the infection of pathogens and clean the immediate environment of competing microorganism's infections. Therefore, to establish the possible role of secondary metabolites in resistance development against CLCuV, this study was carried out to determine the varietals differences in cotton (*Gossypium hirsutum L.)* for the biosynthesis of phenolics, carotenoids and chlorophyll. The objectives of these studies were;1) To determine the varietals differences in cotton for the biosynthesis of soluble phenolics, chlorophylls and carotenoids 2) To determine the change in some diseased and healthy plants morphological characters such as leaf area, fresh and dry weight of leaves.

MATERIALS AND METHODS

Six cotton varieties viz. CIM-443, CIM-506, CIM-446, NIAB-111, BH-118 and CYTO-89 were collected from CCRI Multan and

University of Agriculture Faisalabad. These were sown on May 16th in the experimental area of the Department of Plant Pathology, Central Cotton Research Institute (CCRI), Multan, during 2007 to 2008. These varieties seeds were not acid delinted and all agronomic practices were performed properly for healthy growth of plants. Each variety was sown in six lines with plant to plant distance 6 inches and row to row distance 30 inches.

Experiment was conducted in randomized complete block design with three replications. After two months of germination, leaves from healthy and diseased plants were taken separately from each line and data were recorded for the following parameters.

Physical parameters

Disease (CLCuV) % on the infected plants

From experimental plot, disease affected plants and total number of plants were counted to find out disease percentage by the following formula;

$$\text{Disease (\%)} = \frac{\text{Infected plants}}{\text{Total plants}} \times 100$$

Fresh/dry weight of sample leaf

Fresh and dry weight of sample leaves were weighed and noted.

Area of sample leaf

Leaf area was measured with a leaf area machine.

Biochemical parameters

Quantification of pigments

The chlorophyll a and b were determined as follows; 0.25 g of leaf sample and added 10 ml 80% acetone ground in the presence of sand in pestle and mortar and filtered. The absorbance of extract was taken at 633 and 645 nm wavelength for chlorophyll a and b respectively. Chlorophyll a, b and total chlorophyll were calculated using the following formula described by Arnon (1949):

Chlorophyll a (mg/g) = [12.7(OD663)-2.69(OD645] × v/1000× w

Chlorophyll b ((mg/g) = [22.9(OD645)-4.639(OD633] × v/1000 ×w

Total chlorophyll = chlorophyll a+ chlorophyll b

Carotenoid was determined by measuring the aforementioned extract at 480 nm and calculated by formula described by Davies (1976):

Carotenoid (mg/g) = (A car/ EM)

Where A car = OD480 + 0.114 (OD663) - 0.638 (OD645), and EM 100% = 2500. The OD indicates optical density; V, volume sample; W, weight of sample.

Secondary metabolites

Total soluble phenolics

Total soluble phenolics were determined by the method of

Table 1. Mean of different secondary metabolites in different cotton varieties.

Variety	Mean of disease (%)	Mean of Leaf area (cm$^{2)}$	Mean of fresh weight (g/plant)	Mean of dry weight (g/plant)	Mean of chlorophyll (a) content (mg/g)	Mean of chlorophyll (b) content (mg/g)	Mean of total chlorophyll content (mg/g)	Mean of phenolics (ug/g)	Mean of carotenoids (mg/g)
BH-118	41.014[a]	62.333[a]	69.286[c]	23.41[b]	0.00883[d]	0.00816[d]	0.0162[e]	0.3068[a]	0.068[a]
CYTO-89	40.60[ab]	46.167[b]	75.36[ab]	25.10[b]	0.014[c]	0.0326[b]	0.043[c]	0.2698[ab]	0.0425[d]
NIAB-111	39.42[abc]	48[b]	77.0266[a]	25.13[b]	0.02016[b]	0.0326[b]	0.053[b]	0.1937[bc]	0.0715[a]
CIM-506	37.85[bc]	48.583[b]	72.467[b]	27.335[a]	0.01483[c]	0.0123[c]	0.0272[d]	0.1959[bc]	0.01482[e]
CIM-446	37.25[c]	56.333[ab]	72.421[b]	27.245[a]	0.04416[a]	0.063[a]	0.1072[a]	0.2637[ab]	0.0563[b]
CIM-443	33.68[d]	56.083[ab]	74.303[ab]	24.94[b]	0.01916[b]	0.031[b]	0.049[b]	0.1927[c]	0.0485[c]
LSD value	2.8084	10.04887	2.87622	1.9498	0.002148	0.0022	0.00613	0.084787	0.003618

Julkenen-Titto (1985). Fresh material (0.05 g) was ground in 1 ml 80% acetone at 50°C for 1 h. This was centrifuged and supernatant were taken in microfuge tubes and then stored at –20°C. An aliquot (100 µL) was taken and diluted in 2 ml distilled water into 10 ml test tubes. Furthermore, 1 ml of Folin-ciocalteu and phenol reagent were added and shaken vigorously, after which it was immediately added with 5 ml of 20% Na_2CO_3 and volume made to 10 ml, and then vortexed vigorously for 5 to 10 s. After 20 min, absorbance was measured at 750 nm using spectrophotometer set at zero using 80% acetone.

Statistical analysis

Various treatments' means were compared by applying Duncan's multiple range (DMR) test at 0.05-level of significance (Steel and Torrie, 1980).

RESULTS

CLCuV disease percentage on the infected plants

The maximum mean disease percentage (41.014) was observed in BH-118 followed by CYTO-89 and NIAB-111 (Table 1), while the minimum mean disease percentage (33.68) was in CIM-443, a resistant variety.

The rest of the varieties, CIM-506 and CIM-446, were also affected by CLCuV disease. The maximum disease was observed in August and the minimum was in July. All the means were statistically significant.

Leaf area of sample leaf (cm^2)

There was a significant difference in the leaf area of healthy and diseased plants. There was highly significant difference among the varieties with respect to effect of disease on leaf area of the cotton varieties.

Disease reduced the leaf area of the cotton. The maximum mean value of leaf area was 62.333 cm^2 in BH-118 and minimum was 46.16 cm^2 in CYTO-89 (Table 1). Maximum mean value showed that the leaf area was reduced in this variety. The minimum mean value was in CYTO-89, which was less susceptible to disease. The rest of the varieties were statistically non-significant.

Fresh weight of sample leaf (g/plant)

The maximum mean value was 77.02 g in NIAB-111 and lower value was 69.286 g in BH-118, which showed that there was a significant difference in fresh weight of leaves in NIAB-111 as compared to the control (Table 1). There was also a minimum difference in fresh weight of healthy and diseased sample of BH-118 (Table 2).

Dry weight of sampled leaves (g/plant)

The dry weight of diseased leaves was less than healthy leaves (Table 2). This might be due to lower water contents and lower leaf area than the healthy sample. The maximum mean value 27.335 and 27.245 g was observed in CIM-506 and CIM- 446. Minimum mean was 23.41 g in BH-89 (Table 1). Dry weight of diseased leaves of BH-118 was less, which showed that BH-118 was a susceptible variety.

Chlorophyll a content (mg/g of fresh weight)

Chlorophyll a content was more in all healthy

Table 2. Mean of different secondary metabolites in diseased and healthy leaves of different cotton varieties. Means sharing same letters are statistically similar at 5% probability.

Mean of leaves	Leaf area	Fresh weight	Dry weight	Chlorophyll (a) content	Chlorophyll (b) content	Total chlorophyll content	Phenolics	Carotenoids
Healthy leaves	59.7222[a]	75.511[a]	26.785[a]	0.026611[a]	0.042111[a]	0.06833[a]	0.3077[a]	0.061277[a]
Diseased leaves	46.111[b]	71.443[b]	24.275[b]	0.013777[b]	0.017833[b]	0.030138[b]	0.1498[b]	0.039277[b]
LSD value	5.80172	1.66059	1.1257	0.00124	0.00132	0.003542	0.0489	0.002089

leaves sample as compared to CLCuV affected leaves (Table 2). The maximum mean value was 0.04416 mg/g in CIM-446, while the minimum mean value was 0.0083 mg/g in BH-118 (Table 1). Chlorophyll a content was higher in CIM-446 (resistant) and minimum in BH-118 (susceptible) variety.

Chlorophyll b content (mg/g of fresh weight)

There was difference in chlorophyll b contents in diseased sample as compared to healthy leaves in two way interactions. The chlorophyll b content was more in healthy leaves as compared to diseased leaves (Table 2). The maximum mean value of chlorophyll b was 0.063 mg/g in CIM-446 and the minimum mean value was 0.008 mg/g in BH-118 (Table 1), which meant that the chlorophyll b was maximum in CIM-446 healthy sample as compared to diseased while the minimum in BH-118 as compared to the diseased sample.

Total chlorophyll contents

Total chlorophyll contents was highly significant in individual effect of variety and type which means that there was difference in chlorophyll contents of healthy and diseased samples. The total chlorophyll content was more in healthy as compared to

diseased sample (Table 2). The maximum mean value was 0.107 mg/g in CIM-446 and minimum mean value was 0.01625 mg/g in BH-118 revealed that the total chlorophyll content was minimum in BH-118 and high content was present in CIM-446 (Table 1).

There was maximum total chlorophyll in healthy sample of CIM-446 as compared to diseased and minimum content was present in BH-118.

Total soluble phenolics (µg/g fresh weight)

The maximum phenolics mean was 0.084 µg/g in BH-118 and minimum value was 0.1427 µg/g in CIM-443. This revealed that phenolic content was maximum in BH-118 in healthy sample as compared to diseased and it was found minimum in CIM-443 (Table 1).

Phenolics compounds did not play any role in resistance against CLCuV.

Carotenoid contents (mg/g)

The maximum mean value was 0.0715 mg/g in CIM-446 and minimum mean value was 0.0148 mg/g in CIM-506, which showed that maximum carotenoids was present in CIM-446 in healthy sample and minimum content was in CIM-506. The rest of varieties were found statistically similar (Table 1).

DISCUSSION

Studies on physiological and biochemical parameters of cotton were carried out. Cotton shows symptoms of disease (cotton leaf curl virus disease) such as leaf curling and enation of veins. Disease percentage of different cotton varieties in different dates against CLCuV was evaluated. In August, the maximum disease was observed on different cotton varieties. CIM-443 had lower disease percentage so; it is a resistant variety, while BH-118 and CYTO-89 were susceptible varieties. While studying the effect of disease on leaf area, fresh and dry weight of leaves of cotton, it was found that the leaf area, fresh weight and dry weight were greater in healthy sample of leaves as compared to diseased sample. Maximum leaf area was observed in BH-118 in healthy sample as compared to diseased and minimum leaf area was observed in CYTO-89. Maximum fresh weight was present in NIAB-111 in healthy sample as compared to diseased sample. Maximum dry weight was present in CIM-506 as compared to diseased sample.

In order to determine the secondary metabolites effect on healthy and disease sample of leaves of cotton after the attack of CLCuV, various values were evaluated. It was found that maximum phenolics, carotenoids, chlorophyll a, chlorophyll b and total chlorophyll were present in healthy sample and minimum contents present in diseased sample after the attack of CLCuV. CIM-

446 was the best variety to resist the cotton leaf curl disease. CIM-446 has maximum chlorophyll contents as compared to diseased and maximum were phenolics present in BH-118 and carotenoids in NIAB-111 in healthy sample in resistance to CLCuV. So, CIM-446, BH-118 and NIAB-111 are best varieties in resistant to cotton leaf curl disease. CLCuV appeared on all available commercial varieties with varying degree of occurrence during 1999 and 2000 (Perveen et al., 2004; Perveen and Khan, 2005). The production of secondary metabolites was greater in healthy leaf in resistance to cotton leaf curl disease.

Disease had adverse effect on morphology, yield component and fiber quality of cotton varieties (Ahamad et al., 2002). Infected plants had a lower leaf water contents than healthy plants (Harrison, 1970). According to Sultana et al. (2000), the rate of photosynthesis is lower in susceptible varieties, which might be due to the low chlorophyll a contents of the CLCuV affected leaves. Felton et al. (1997) also found that plant phenolics are not a determining factor for host plants resistance against insects. Secondary metabolites therefore protect the plant against herbivores, pathogen and from physical stress (Yazaki, 2006).

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REFERENCES

Asad S, Haris WAA, Bashir A, Zafar Y, Malik KA, Lichtenctein (2003). Resistant to the serious viral pathogen causing cotton leaf curl disease. Arch. Virol. (148): 2341-2352.

Ahamad G, Malik SA Mahmood Z, Iqbal MZ, Ahamad S (2002). Effect of cotton leaf curl virus disease on morphology, yield and fiber on susceptible lines/cultivars of cotton (Gossypium hirsutum L.) Asia. J. Plant Sci., 1: 705-707.

Arnon DI (1949). Copper enzyme is isolated chloroplasts. polyphenoloxidose in Beta vulgaris. Plant Physiol. 24: 1-15.

Alam SM (2000). Cotton: An important cash crop. Cotton is providing livelihood for Over 5 million people at the form and industry assed on www.pakistaneconomist.com/issue2000/issue 18/ise2.htm.

Briddon RW, Markham PG (2000). Cotton leaf curl virus Res. 71 (1-2): 151-159.

Briddon RW (2003). Cotton leaf curl disease, a multicomponent begmovirus Complex. J. Mol. Pl. Pathol. 6(4): 427-434.

Buchanan BB, Gruissem W, Jones R, (2000). Biochemistry and molecular biology of Plants, Maryland: Am. Soc. Plant Physiol. Rockville, MD, pp. 610-628.

Felton GW, Murphy JB, Hawles PA, Dixon RA, Lamb CJ (1997). Do plant phenolics confer resistance to specialist and Generalist Insect Herbivores? J. Agric. Food Chem.45 (11): 4500-4504.

Castellanos I, Espinose-Garcia FJ (1997). Plant secondary metabolite diversity As a resistance trait against Insects: a test with Sitophilus Granararius (coleopteran:curculionidae) and secondary Metabolites Biochemical systematics and Ecology. Elsev, 7(25): 591-602.

Dixon RA, Pavia NL (1995). Stress induced phenylpropanoid metabolism. Plant Cell.7:1085-97.

Davies BH (1976). Carotenoids. In: Good win TW (ed). Chemistry and Biochemistry of plant pigments, 2nd ed., Academic press London. pp. 38-165.

Demain AL, Fang A (2000). The natural functions of secondary metabolites. Adv. Biochem. Eng. Biotechnol. 69: 1-39.

Idris A (1990). Cotton leaf curl virus disease in Sudan. Med.Fac. Lanbow, Rikksunir. Gennet. 55(2).

Julkenen-Titto R (1985). Phenolic constituents in the leaves of northern willows: Methods for the analysis of certain phenolics. Agric. Food Chem. 33: 213-217.

Harrison JAC (1970). Water deficit in potato plants infected with Verticillium alboatrum. Ann. Appl. Biol. 66: 225-231.

Kirthi N, Priyadarshinic CGP, Sharma P, Maiya SP, Hemalatha V, Sivaraman P, Dhawan P, Rishi N, Savithri HS (2004). Genetic Variability Of begmoviruses associated with cotton leaf curl disease originated From India. Arch. Virol. 149(10): 2047-2057.

Mahmood T, Tahir M, Tanveer A, Mirza MB (1996). Effect of cotton leaf curl virus on yield components and fibre properties of four commercial Varieties. Pak. J. Phytophathol. 8: 68-70.

Micheal W (2003). Evolution of secondary metabolites from an ecological and molecular phylogenetic perstive. J. Phytochem. 64(1): 3-19.

Perveen R, Khan SM, Khan MA (2004). Reactions of cotton germplasm to Cotton leaf curl Begomovirus (CLCuV) disease. Pak. J. Phytopathol. 16(2): 90-94.

Perveen R, Khan SM (2005). Screening of cotton germplasm against Cotton leaf curl Begomovirus (CLCuV). Pak J. Phytopathol. 17(1): 56-60

Sultana N, Ikeda AM, Kashen (2000). Effect of NaCl salinity on photosynthesis and dry matter accumulation in development of rice grain. Environ. Ex. Bot. 46:129-140.

Steel RGD, Torrie JH (1980). Principles and procedure of statistics approach. 2nd edition. McGraw Hill company, New York. p. 597.

Theis N, Lerdau M (2003). The evolution of function in plant secondary metabolites. Int. J. Plant Sci. 164(3): 93-102.

Yazaki K (2006). ABC transporters involved in the transport of plant secondary Metabolites. FEBS Letters, 580(4): 1183-1191.