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Full Length Article

Role of Mineral Metabolism and some Physiological Factors in Resistance against Urdbean Leaf Crinkle Virus in Blackgram Genotypes

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Abstract

This study was aimed to investigate the role of mineral metabolism, total soluble phenols, total chlorophyll and total soluble sugar in the defense of the blackgram genotypes: a susceptible (Mash-88) and a resistant (CM-2002). ULCV infection resulted in an invariable increase in N, Mg, Zn and Fe contents, whereas P, K, Na and Ca contents decreased in both resistant and susceptible genotypes. Results of the present study indicated that crinkling and puckering of ULCV-infected leaves was found more severe in those plants having highly significant reduction in leaf Ca content. Similarly, leaf K and Fe contents were also found positively associated with resistance to ULCV infection. ULCV infection also resulted in significant increment in total soluble phenols in susceptible genotype. However, disease had non-significant effects on foliar total soluble sugar and total chlorophyll contents of both susceptible and resistant genotypes but increment of these constituents in diseased leaves of susceptible plants was significantly higher than the increase in diseased leaves of resistant genotype.

Keywords: Urdbean leaf crinkle virus; Mineral contents; Phenol; Chlorophyll

Introduction

Urdbean leaf crinkle virus (ULCV), an unclassified virus, seed-borne with narrow host range and probably aphid-transmitted, is relatively a destructive and serious disease of blackgram (*Vigna mungo* (L.) Hepper) in Pakistan. It is relatively more susceptible than other pulses to leaf crinkle disease (Bashir *et al*., 2005; Ashfaq *et al*., 2007) and characterized by the appearance of extreme crinkling, curling, puckering and rugosity of leaves, stunting of plants and malformation of floral organs. Thus the disease is commonly, widespread, destructive and inflicts heavy losses annually. In Pakistan, the virus has been reported to decrease grain yield from 35 to 81% depending upon genotype and time of infection (Bashir *et al*., 1991). Research on epidemiological aspects also explains that ULCV disease incidence depends upon the host genotypes, growing seasons and suitable environmental conditions (Ashfaq *et al*., 2008). Certain resistant genotypes are now available to the breeders and farmers (Bashir *et al*., 2005; Ashfaq *et al*., 2007) but no information is available on the mechanism of disease resistance in these germplasms. Being dependent metabolic system, plant viruses cause disturbances in the physiology and anatomy of infected plants (Hussain, 1995; Ashraf and Zafar, 2000; Ashfaq *et al*., 2010). No generalization appears justified concerning the metabolic effects following infection with plant viruses. Most of the metabolic changes observed are probably indirect effects of viral infection as a result of interference with various metabolic processes and the transport of water, nutrients, and other substances. Minerals, apart from being a vital part of the plant nutrition, may manifest certain maladies in the plants either through disturbing normal metabolism and physiology of the plants or by favouring or by discouraging the plant pathogens, if in excess or otherwise deficient. Disturbance in growth regulation results in morphological abnormalities, ranging from a mosaic pattern on leaves and flowers to necrotic spots and streaks to leaf enation and tumours (Luria *et al.*, 1978).

Biochemical and physiological changes in plants due to either biotic or abiotic stress have been reported (Ashfaq *et al*., 2010; Montasser *et al*., 2012; Nafees *et al.*, 2013). Virus infection can be considered as a biotic stress to the host plant (Montasser *et al*., 2012). Infected blackgram plants with ULCV have been found to cause a remarkable increase in total soluble protein content in both susceptible and resistant genotypes (Ashfaq *et al*., 2010; Srivastava and Singh, 2010). The activities of peroxidase (PO) and superoxide dismutase (SOD) increased and decreased significantly after 15 and 30 days of ULCV-inoculation in resistant genotype, respectively (Ashfaq *et al*., 2010).

Considerable progress has been made over the past few years in understanding the mechanisms of disease resistance or susceptibility (Ashraf and Zafar, 2000; Ashfaq

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et al., 2010) and it has been reported that resistance to any virus depends on plant metabolism (Dawson and Hilf, 1992). Development of resistant varieties is the cheapest source for the management of plant viruses but for controls to be applied effectively, changes in biochemical processes due to viral infections must be recognized. Considering these observations, the present study was conducted to determine the role of macro- and micronutrients, total soluble sugars, total soluble phenols and total chlorophyll in imparting resistance/susceptibly against ULCV in blackgram genotypes.

Materials and Methods

Two genotypes of blackgram (*Vigna mungo*), CM-2002 (resistant) and Mash-88 (susceptible) used in the study against ULCV (Ashfaq *et al*., 2007). Seeds of these genotypes were obtained from Barani Agricultural Research Institute (BARI), Chakwal and Ayub Agricultural Research Institute (AARI), Faisalabad. Twenty seeds were sown in each earthen pot (30 cm diameter) and after germination only ten plants per pot were maintained. The ULCV isolate was maintained in cv. Mash-3 (susceptible genotype) and diseased (symptomatic) leaves were dried on silica gel at room temperature. For mechanical inoculation, 1 g of these dried leaf tissue was in 3 mL of 0.05 M potassium phosphate buffer (pH 7.2), containing 1% Na_2SO_3. The blackgram plants were dusted with carborundum powder (600 mesh) before inoculation with sap extract at the two leaf stage. After inoculation, the plants were rinsed with distilled water to remove any superfluous material and kept in an insect free glasshouse. The un-inoculated plants of each test genotype were also kept as control. Both healthy and diseased leaf samples of each genotype were harvested at 30 days of post-inoculation (dpi) and used as fresh to determine the ionic status, total soluble phenols, total chlorophyll and total soluble sugar.

All plants of both genotypes that were kept as control remained completely free from disease symptoms. The disease symptoms including wavy appearance on the third trifoliate leaves followed by crinkling, puckering and rugosity of leaves, shortening of petioles and crowding of leaves, first appeared in mechanically inoculated plants of Mash-88. All treated Mash-88 plants were affected by ULCV disease, whereas only seven percent of CM-2002 plants showed mild positive reaction for ULCV.

Virus Identification

ULCV was confirmed by Direct Antigen Coated ELISA (DAC-ELISA) following Hobbs *et al*. (1985). One gram leaves were ground in 3mL extraction buffer in pestle and mortar and then filtered through the double layered muslin cloth and samples were added into the wells @ 200 µL. Each step of ELISA was followed by overnight incubation at 4°C and three washings with PBST buffer at 5 min, intervals. An aliquot of 200 µL of diluted polyclonal antiserum was added to each well of plate. After incubation and washing as above, enzyme conjugate (IgG conjugated with alkaline phosphatase) was diluted at 1:200 and 200 µL was added in each well. p-nitrophenyl phosphate tablets were dissolved in substrate buffer @ 1 mg/mL, and 200 µL was added in each well. The plate was incubated at room temperature (25°C) for about one hour. The colour development was observed visually and 3 *M* NaOH was added @ 50 µL/well to stop the reaction.

Determination of Macro-and Micronutrients

Plant samples (comprising of leaves) were oven-dried at 70°C for 48 h until the dry weight became constant. These dried samples were ground and then boiled @ 100 mg/10 mL of 1.4 *N* HNO_3 on a hotplate (TH-550; Advantec, Japan) at 100°C for half an hour. The suspension was allowed to cool and then diluted with distilled water up to 250 times. Then this diluted suspension was subjected for the determination of N, P, K, Mg, Ca, Na, Zn and Fe following Bhargava and Raghupathi (1995). All of the elements were recorded as parts per million (ppm) of dry weight, while N and P content were recorded in percentage.

Determination of Total Soluble Phenols

Total soluble phenols were determined from leaf samples according to the method of Julkunen-Tiitto (1985). Fresh leaf samples were taken at 30 days post inoculation (dpi) and 0.5 g of each sample was powdered in liquid nitrogen in mortar and pestle. The powder was put into 5 mL polystyrene capped tubes having 2 mL of 80% acetone and extraction performed at 50°C in a water bath for 1 h. Then suspension was centrifuged at 12,000 rpm for 10 min and supernatant collected in centrifuge tube and kept at –20°C until used. Extract was diluted @ 50 µL/mL distilled water in a 10 mL capacity test tube, and thoroughly mixed with 2.5 mL of 20% Na_2CO_3 and 0.5mL of 2 *M* Folin-Ciocalteau' s phenol reagent (Sigma). The mixture was stored for 20 min at room temperature before recording the absorbance of the samples at 750 nm using the spectrophotometer (Hitachi U-2001, Model 121- 0032). The Gallic acid was used to prepare the standard curve to determine the concentration of total soluble phenols.

Determination of Total Chlorophyll

Extraction of chlorophyll from 9 cm^2 of leaf tissue was carried out in 9 mL of 80% acetone and then centrifuged at 12,000 rpm for 5 min to remove debris. Supernatant was diluted with 80% acetone up to 10 mL. The absorbance of each sample was taken at 663 and 645 nm for chlorophyll a and b respectively using the Hitachi U-2001 spectrophotometer (Model 121-0032). 80% acetone was used as a blank for all of these measurements. The total

chlorophyll was estimated, using the formulas as used by Inskeep and Bloom (1985).

Estimation of Total Soluble Sugar

Total soluble sugar (TSS) were quantified from leaves of both genotypes (resistant and susceptible) by the anthrone reagent method (Morris, 1948; Yemm and Willis, 1954).

Statistical Analysis

Data from this completely randomized experiments with three replications were analyzed by analysis of variance and means were compared by Duncan, New Multiple Range Test (DMT) at 5% probability (Steel *et al.,* 1997).

Results

Mineral Metabolism

The quantities of the mineral (N, P, K, Ca, Mg, Zn and Fe) contents in ULCV inoculated and un-inoculated plants are given in the Table1. It was observed that percent nitrogen was found slightly higher in the healthy blackgram susceptible genotype (Mash-88) than resistant one (Chakwal Mash-2002). Inoculation with the ULCV resulted in non-significant increase in percent nitrogen in resistant genotype from 2.68 to 2.77 and in susceptible genotype from 2.81 to 3.00. The phosphorus contents of un-inoculated and inoculated plants of susceptible genotypes were lower than those of resistant ones, respectively. The mean percent phosphorus in un-inoculated and inoculated susceptible plants was 0.69 and 0.48 respectively, while in case of un-inoculated and inoculated resistant plants, the mean percent phosphorus was 0.79 and 0.71. The content of P was decreased significantly in infected susceptible genotype, whereas this decrease was remained non-significant in resistant genotype. In case of inoculated susceptible genotype, there was significantly decrease (46.86%) in potassium content, while in case of resistant genotype there was only 11.83% decrease compared to healthy ones, respectively. Mean potassium content of diseased and healthy plants was 279.97 and 526.93 ppm respectively; while in case of resistant genotype the mean potassium content in diseased and healthy plants was 853.56 and 968.11 ppm, respectively. The concentration of sodium content in leaves of inoculated plants in both reaction groups was reduced but there was no statistically significant difference between healthy and diseased leaves in both genotypes regarding sodium accumulation. The susceptible genotype accumulated comparatively more sodium than did the resistant genotype. The susceptible genotype showed significant reduction in the accumulation of calcium in diseased leaves with severe symptoms like crinkling and puckering compared to that in healthy leaves. On the other hand the reduction in calcium accumulation in leaves of inoculated plants of resistant genotype was less than that observed for the susceptible genotype. In healthy plants of susceptible genotype, there was more concentration of magnesium than un-inoculated plants of resistant genotype. But as a result of inoculation, there was significantly increase in the magnesium content in both susceptible and resistant genotypes. In susceptible genotype, highly significant increase in zinc content was observed in inoculated plants over un-inoculated plants and increase was non-significant in resistant genotype. Iron content was observed significantly higher in case of resistant genotype as compared to the susceptible one. Increase in iron content in both ULCV infected susceptible and resistant genotypes was significant, being more pronounced in resistant genotype.

Physiological Factors

Total soluble phenols: The increase in total soluble phenols of both susceptible and resistant genotypes was statistically significant, though the total phenol content of the susceptible genotype increased more as compared to resistant one. The mean total phenols were 84.330 (73.87 to 100.11) mg/kg fresh weight in un-inoculated plants of susceptible genotype, which increased to 102.90 (91.32 to 116.62) mg/kg after ULCV infection, while in case of resistant genotype, there was non-significant increase in total phenols in leaves of inoculated plants (Fig. 1).

Total soluble sugar: The mean total soluble sugar was 206.68 (188.21 to 222.136) mg/kg fresh weight in un-inoculated leaves of susceptible plants, which non-significantly increased to 219.51 (205.83 to 229.36) mg/kg fresh weight in ULCV diseased leaves. Similarly, the TSS increased from 198.31 (183.75 to 221.34) mg/kg fresh weight in healthy leaves of resistant genotype to 205.36 (190.50 to 223.33) mg/kg in diseased leaves (Fig. 2).

Total chlorophyll content: A significant increase in total chlorophyll contents in leaves of inoculated plants was observed in both genotypes (Fig. 3). It was observed that the average increase in total chlorophyll content in diseased leaves of susceptible plants (average 1.661 mg/kg fresh weight) differed significantly from the increase in diseased leaves of resistant genotype (average 0.9426 mg/g fresh weight).

Discussion

The present study reports the impact of ULCV infection on the mineral metabolism and some of the physiological factors in infected blackgram (susceptible and resistant) plants. Biochemical and physiological changes in plants in response to viral infection have been reported (Ashfaq *et al*., 2010; Kandhasamy *et al*., 2010; Srivastava and Singh, 2010; Montasser *et al*., 2012). Nutrient uptake is one of the important processes that affect plant growth and metabolism. A pronounced effect of ULCV disease on the

Table 1: Effect of Urdbean leaf crinkle virus (ULCV) disease on mineral contents of blackgram genotypes

Nutrients (dry weight)	Genotypes	Group of genotype	Healthy plants	Diseased plants	% increase (+) or % decrease (-) over healthy
N(%)	Mash-88CM-2002	Susceptible Resistant	2.81±0.39; 2.68±0.3719	3.00±0.49; 2.77±0.51	0+6.95; +3.32
P (%)	Mash-88CM-2002	Susceptible Resistant	0.69±0.20; 0.79±0.30	0.48±0.32; 0.71±0.10	-29.94; -9.66
K(ppm)	Mash-88CM-2002	Susceptible Resistant	526.93±25.48; 968.11±74.38	279.97±24.08; 853.56±88.80	-46.86; -11.83
Na(ppm)	Mash-88CM-2002	Susceptible Resistant	371.70±60.09; 354.21±39.07	367.77±68.81; 349.14±40.68	-1.057; -1.43
Ca(ppm)	Mash-88CM-2002	Susceptible Resistant	3031.50±104.3; 3504.40±82.10	2027.60±73.3; 3029.80±56.3	-33.11; -13.54
Mg(ppm)	Mash-88CM-2002	Susceptible Resistant	1623.90±66.70; 1233.40±52.70	1845.30±94.26; 1380.20±77.50	+13.63; +11.90
Zn(ppm)	Mash-88CM-2002	Susceptible Resistant	332.28±32.72; 230.56±28.86	443.72±95.01; 245.49±26.95	+33.54; +6.47
Fe(ppm)	Mash-88CM-2002	Susceptible Resistant	353.08±91.14; 614.38±160.59	570.11±51.09; 775.23±139.76	+61.47; +26.18

Results represent the mean (±SD) of 10 plants in each replication

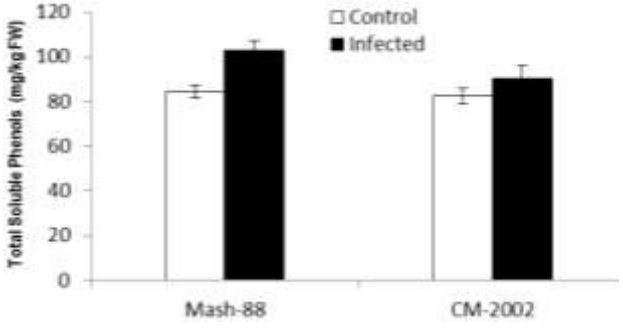

Fig. 1: Total soluble phenol content in leaves of healthy and ULCV-infected of the two blackgram genotypes (Mash-88 and Chakwal Mash-2002). Results represent the mean (±SD) of 10 plants in each replication

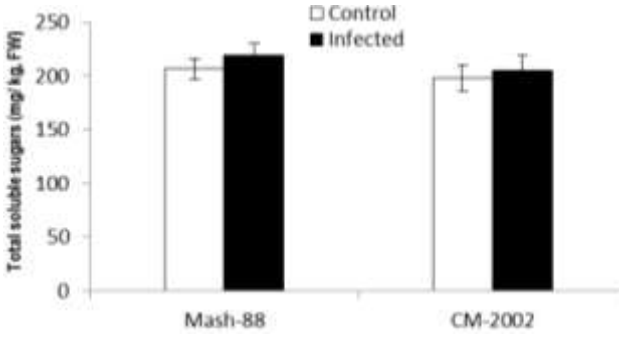

Fig. 2: Total soluble sugar content in leaves of healthy and ULCV-infected of the two blackgram genotypes (Mash-88 and Chakwal Mash-2002). Results represent the mean (±SD) of 10 plants in each replication

nutritional status of leaves was observed in the present study. The susceptible blackgram genotype contained more nitrogen in both healthy and diseased plants than the resistant one. Iqbal *et al.* (2006) also reported non-significant nitrogen increase in CLCuV-infected leaves. As the nitrogen is mobile within the plant (Devlin and Witham 1983; Yardimci *et al*., 2007), the younger leaves definitely contain more nitrogen than the older ones; the young leaves are more sensitive to viruses. The disease resistance and plant growth can adversely be affected by P deficiencies (Yardimci *et al*., 2007). ULCV adversely affected

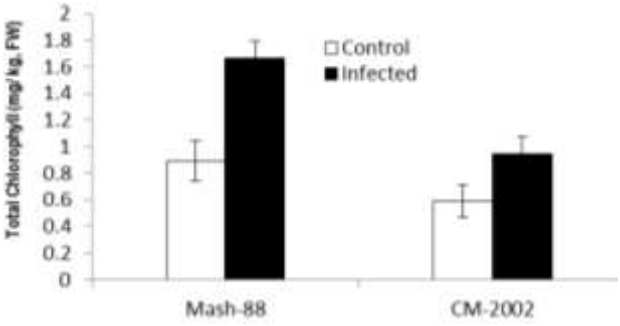

Fig. 3: Total chlorophyll content in leaves of healthy and ULCV-infected of the two blackgram genotypes (Mash-88 and Chakwal Mash-2002). Results represent the mean (±SD) of 10 plants in each replication

vegetative growth of plants (Bashir *et al*., 1991; Ashfaq *et al*., 2007) and leaves and sepals of flowers become thicker and greener than normal. In the present study, healthy and diseased plants in both resistant and susceptible groups did not differ significantly with regard to phosphorus accumulation, so it can be concluded that in ULCV-infected plants, the dark green leaves and sepals colour and stunted growth of plants are not associated with Phosphorus concentration.

Potassium has a major role in the activation of several enzymes that are involved mainly in the metabolism of carbohydrates. The reduction in leaf potassium concentration would also affect photosynthetic rate because potassium plays an important role in stomata opening (Salisbury and Ross, 1992). In the present study, it was observed that in both susceptible and resistant genotypes, there was considerably less potassium accumulation in diseased leaves than in healthy one. Iqbal *et al.* (2006) and Kandhasamy *et al*. (2010) also observed less accumulation of potassium in diseased leaves of cotton and finger millet plants, respectively, but Yardimci *et al*. (2007) observed no significant changes in AMV infected alfalfa leaves K content. As reduction in potassium concentration was greater in susceptible genotype than in the resistant one, so due to this disease severity was more pronounced in susceptible genotype. The decrease in potassium content in ULCV-infected leaves can be explained by the factor that it

is a mobile nutrient (Jones *et al.*, 1991) and is transported through the phloem from older to younger leaves (Yardimci *et al*., 2007). Ashraf *et al*. (1999) reported that phloem elements become clogged in response to CLCuV-infection owing to the proliferation of parenchymatous tissue. Because the transportation of potassium from older to younger leaves takes place through the phloem, a decrease of potassium concentration in leaves of viral infected plants would be expected. In the present study, sodium contents in diseased leaves were not significantly different from healthy one in both genotypes. Calcium, being a major constituent of the middle lamellae of the cell wall and plays an important role in maintaining cell integrity and membrane permeability (Devlin and Witham, 1983). Hence the calcium deficiency causes the curling of leaves and a decay of conductive tissues at the base of plants (Jones *et al*., 1991). Reduction in calcium concentration in ULCV-infected leaves was observed for both resistant and susceptible genotypes in the present study. Because the calcium concentration of severely infected leaves was much lower than that of normal leaves, it resulted in curling and puckering of ULCV-infected leaves. Magnesium also plays very important role in synthesis of chlorophyll and consequently in photosynthesis and carbohydrate metabolism (Devlin and Witham 1983; Kandhasamy *et al*., 2010). Upon inoculation, the magnesium increased in both genotypes but concentration of magnesium upon inoculation, suggests increased photosynthesis and carbohydrate metabolism in them due to increased photosynthetic area (Reddy *et al*., 2005). Zinc is an immobile element in the plant tissues and directly constitutes chlorophyll. This increase was significant only in case of susceptible genotype and ULCV-infected leaves become more greener than normal (Reddy *et al*., 2005) and chlorophyll contents also increased, so it can be concluded that zinc content might be directly or indirectly involved in chlorophyll formation.

Iron content increased in both resistant/susceptible genotypes upon inoculation with ULCV. The only justification may be the involvement of iron as a component of various flavoproteins (Metalloflavoproteins) active in biological oxidation (Devlin and Witham, 1983), which may increase as result of inoculation with the pathogen/ULCV (Ashfaq *et al*., 2010). Iron is also found in iron-porphyrn proteins, which include cytochromes, peroxidases and catalases. It may be assumed that the above-mentioned proteins may be responsible for increased catabolic activities in the ULCV-inoculated plants.

The results regarding total soluble phenols are in an agreement with Karthikeyan *et al.* (2007) and Rahioui *et al.* (2013) who also reported increase in phenol contents in blackgram and olive trees due to ULCV and *Spilocaea oleagina* infection, respectively. This can be justified as a failed attempt at resistance on the part of the host as it could be a consequence of successful invasion on the part of the virus. Accumulation of total soluble sugar in resistant and susceptible genotypes of blackgram was too little to be of any significance in providing resistance against ULCV. These results are not in any consistency with other those reports that have manifested pronounced influence of viral infection on host carbohydrate metabolism (Tecsi *et al*., 1994a; b; 1996; Kandhasamy *et al*., 2010). From the results of chlorophyll content it is apparent that the total chlorophyll contents were significantly increased in both genotypes due to ULCV-inoculation. These results are in an agreement with Brar and Rataul (1990) who also reported significant increment in total chlorophyll contents in ULCV-infected old leaves of urdbean.

In conclusion, an accumulation of both macro- and micronutrients in variable amounts in infected blackgram genotypes. More accumulation in magnesium in ULCV-infected genotypes suggested increased photosynthesis and carbohydrate metabolism in them due to increased photosynthetic area. As in the present study, it was also observed that the calcium concentration of ULCV infected leaves was much lower than that of normal leaves, so it can be assumed that curling and puckering of ULCV-infected leaves might be associated with Ca concentration. Moreover, leaf K, Fe contents and total soluble phenols were found positively associated with resistance to ULCV infection. Support for these interpretations requires information on the behavior of the plant defenses in this pathosystem, and such research is under way.

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