Pak. J. Bot., 42(1): 447-454, 2010.

EFFECT OF URDBEAN LEAF CRINKLE VIRUS INFECTION ON TOTAL SOLUBLE PROTEIN AND ANTIOXIDANT ENZYMES IN BLACKGRAM PLANTS

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Abstract

Urdbean leaf crinkle virus (ULCV) is a common, wide spread, destructive and economically important disease causing systemic infection in blackgram (*Vigna mungo* (L.) Hepper), resulting in extreme crinkling, curling, puckering and rugosity of leaves, and yield reductions. Effect of viral infection was investigated on total soluble proteins and antioxidant enzymes activity in two genotypes viz., Mash-88-susceptible and CM-2002-resistant, at different growth stages under both the inoculated and un-inoculated conditions. ULCV infection resulted in significant increase in total soluble protein contents of the leaves in both genotypes. In healthy plant, superoxide dismutase (SOD), catalase (CAT) and peroxidase (PO) showed similar activity levels. In inoculated plants of Mash-88, SOD and PO activities decreased and increased non-significantly at all growth stages, respectively. The activities of PO and SOD increased and decreased significantly after 15 and 30 days of inoculation in resistant genotype, respectively. No significant changes in catalase (CAT) activity were detected in ULCV-infected leaves over the control. It was concluded that the superoxide dismutase and peroxidases might be associated with resistance/susceptibility to ULCV infection.

Introduction

Blackgram or Urdbean (*Vigna mungo* (L.) Hepper) is an important pulse crop grown all over the world including Pakistan. It is relatively more susceptible than other pulses to leaf crinkle disease caused by Urdbean leaf crinkle virus (ULCV) (Kadian, 1980; Rishi, 1990; Bashir *et al*., 2005; Ashfaq *et al*., 2007). It is 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.

Considerable progress has been made over the past few years in understanding the mechanisms of disease resistance or susceptibility (Ashraf & Zafar, 2000) and it has been reported that resistance to any virus depends on plant metabolism (Dawson & 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 effect of viral infection on the oxidases and total soluble protein contents in infected leaves of susceptible genotype of blackgram in comparison with resistant one.

Materials and Methods

Two genotypes of blackgram (*Vigna mungo*), one susceptible (Mash-88) and other resistant (CM-2002) used in the present study were the selection among genotypes screened against ULCV (Bashir *et al*., 2005; Ashfaq *et al*., 2007). Seeds of these genotypes were obtained from Ayub Agricultural Research Institute (AARI), Faisalabad and Barani Agricultural Research Institute (BARI), Chakwal. Twenty seeds were sown in each pot (30 cm diameter) filled with a mixture of soil and farm yard manure (10:1) and grown in a naturally illuminated greenhouse. After germination, only 10 seedlings per pot were maintained. The ULCV isolate was maintained in cv. Mash-3 (susceptible cultivar) and symptomatic leaves were freeze-dried and kept at –20°C. For virus inoculation, infected leaves were homogenized (1/3 w/v) in 0.05 M phosphate buffer, pH 7.2, containing 1% Na_2SO_3. The blackgram plants at the two leaf stage were rub-inoculated on the upper surface of the leaves with the slurry, using carborundum powder, 600 mesh as abrasive. After inoculation, the plants were rinsed with distilled water to remove superfluous inoculum and kept in an insect free glasshouse (25^{o}C temperature and 70% humidity). The un-inoculated plants (healthy plants) of each test cultivar were maintained as control. Leaf samples were harvested from both inoculated and control plants at 0, 15 and 30 days of inoculation and stored at –20°C to determine the superoxide dismutase (SOD), catalase (CAT) and peroxidase (PO) activities. A part of each lot was used for the determination of total soluble protein.

Virus identification: DAC-ELISA (Direct Antigen Coated ELISA) as described by Hobbs *et al*., (1985) was used with a polyclonal antiserum against ULCV. Leaf samples were ground in the extraction buffer in pestle and mortar and then filtered through the double layered muslin cloth and samples were charged into the wells @ 200 µl. Each step of ELISA was followed by overnight incubation at 4^{o}C by three washings with PBST buffer at 5 min., intervals. An aliquot of 200 µl of diluted 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. Substrate (p-nitrophenyl phosphate) was dissolved in substrate buffer @ 1 mg/ml, and 200 µl was added in each well. The plate was incubated at room temperature (25^{o}C) for about one hour. The colour development was observed visually and reaction was stopped by adding 50 µl of 3 M Sodium hydroxide.

Antioxidant enzyme activities: The extraction conditions were standardized using three different buffer systems. The plant samples were extracted in the following buffers: (i) Bf1: Phosphate buffer pH: 7 (10 mM Na_2HPO_4, 100 mM NaH_2PO_4, 100 mM KCl and 2 mM EDTA). (ii) Bf2: Na acetate buffer pH: 5.2 (100 mM Na acetate, 100 mM KCl, 0.1 % â- mercaptoethanol). (iii) Bf3: (10 mM Na_2HPO_4, 15 mM NaH_2PO_4, 100 mM KCl, 2 mM EDTA, 1.5% PVPP, 1mM PMSF and 2 mM Thiourea).

Blackgram leaves (young, older, mix) with viral symptoms were used for analyses. Samples were washed with tap water followed by distilled water, dried to remove excess

moisture and cut to a uniform small size pieces. These leaf samples (100 mg fresh weight) were ground to a fine powder and homogenized in 200 ml of extraction buffer. Homogenates were centrifuged at 10,000 rpm for 10 min at 40 °C and the supernatant was used to determine protein concentration (Bradford, 1976) and enzyme activity. Ten µl of each sample was taken in the sterilized test tube in triplicate and 1.0 ml of Bradford reagent was added to each test tube. All the samples were incubated at 37°C for 10-20 min. along with the blank. Bovine serum albumin (BSA) was used for constructing the standard curve. The absorbance was taken at 595 nm with a spectrophotometer (Hitachi U-2001). Total Superoxide dismutase (SOD) activity was assayed at 560 nm by measuring the inhibition of the photochemical reduction of nitro blue tetrazolium (NBT) (Giannopolitis & Ries, 1977). The reaction solution (3 ml) contained 50 µM NBT, 1.3 µM riboflavin, 13 mM methionine, 75 mM EDTA, 50 mM potassium phosphate buffer (pH 7.8) and 100 µl enzyme extract. Test tubes containing the reaction solution and leaves were irradiated under a light bank (15 fluorescent lamps) at 78 µmol m^{-2} s^{-1} for 15 min. One unit of SOD activity was defined as the amount of enzyme which causes at 50 % inhibition of the photochemical reduction of NBT and SOD specific activity was expressed as units per mg protein. Activities of catalase (CAT) and peroxidase (PO) were measured using the method of Chance & Maehly (1955) and modified by Liu & Huang (2000). The CAT reaction solution (3 ml) contained 50 mM potassium phosphate buffer (pH: 7.0), 5.9 mM H_2O_2, and 0.1 ml enzyme extract. The reaction was initiated by adding the enzyme extract. Changes in absorbance of the reaction solution at 240 nm were read after every 20 s. One unit CAT activity was defined as an absorbance change of 0.01 units per min. The PO reaction solution (3 ml) contained 50 mM potassium phosphate buffer (pH 5.0), 20 mM guaiacol, 40 mM H_2O_2, and 0.1 ml enzyme extract. Changes in absorbance of the reaction solution at 470 nm were determined at every 20 s. One unit PO activity was defined as an absorbance change of 0.01 units per min. The activity of each enzyme was expressed on protein basis.

Results and Discussion

All plants of both genotypes that were kept as controls 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 disease symptoms developed on only 10 % of CM-2002 plants. Leaf crinkling and puckering were severe in Mash-88 plants, whereas diseased plants of the CM-2002 genotype showed only slight crinkling and puckering of the leaves.

Total soluble protein: ULCV-infected plants, both susceptible and resistant, appeared to have increased total soluble protein contents at 15 and 30 days after inoculation (Fig. 1). Leaves from Mash-88 (susceptible genotype) had slightly higher protein content than the CM-2002 (resistant one) (Fig. 1). A decrease of total soluble proteins due to viral infection has been reported by several workers (Taiwo & Akinjogunla, 2006; Thind *et al*., 1996), but in the present study we observed higher total protein content that was more likely to be due to the increased level of viral proteins in the plant and this is in agreement with previous findings of other research workers (Shukla & Rao, 1994; Langhams & Glover, 2005; Yardimci *et al*., 2007).

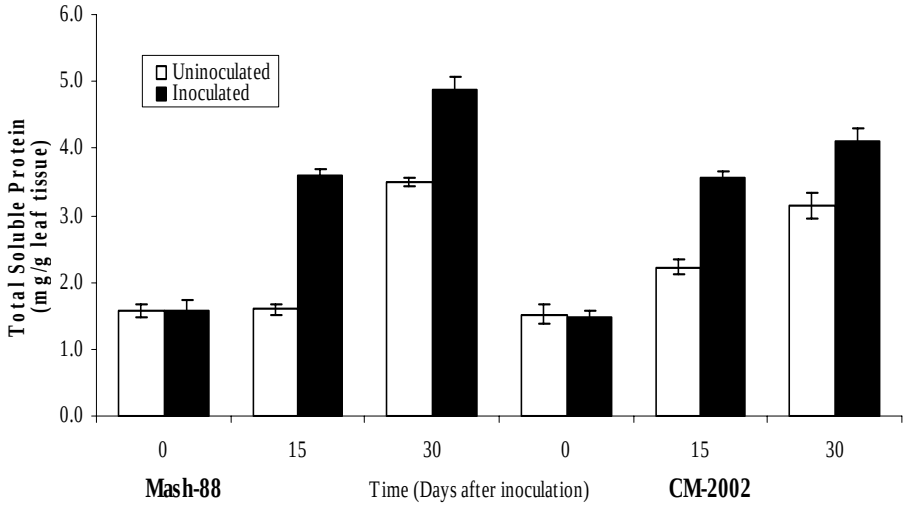

Fig. 1. Protein accumulation in leaves of blackgram genotypes Mash-88 and CM-2002 (Chakwal Mash-2002), at various stages of ULCV infection. Results are the mean ± SE of 10 plants.

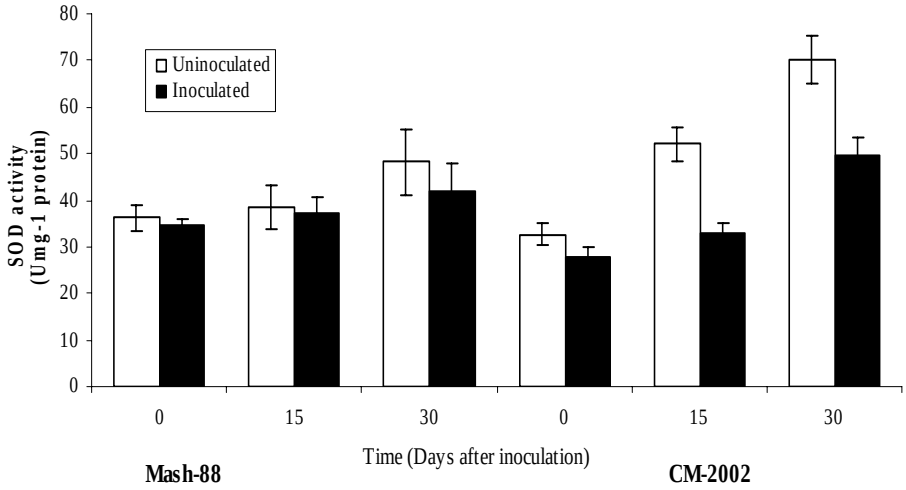

Fig. 2. Superoxide dismutase (SOD) specific activity in healthy and ULCV-infected plants of two blackgram genotypes Mash-88 and CM-2002 (Chakwal Mash-2002), at various stages of virus infection. Results are the mean ± SE of 10 plants.

Superoxide dismutase (SOD): In leaves of control plants, there was an increasing trend in the activities of superoxide dismutase (SOD) during plant development. Leaves from cv. Mash-88 inoculated with ULCV showed non-significant decrease in the enzymatic activities than in un-inoculated control plants up to 30 days post inoculation. Whereas, in resistant genotype (CM-2002), the SOD activity decreased significantly over the un-inoculated control plants after 15 and 30 days of inoculation ($p<0.05$) (Fig. 2). This is in agreement with Hernández *et al*., (2004) who also observed a significant decrease in SOD from *Plum pox virus* inoculated peaches and such type of results have also been

reported in WClMV-infected *Phaseolus vulgaris* L., plants (Clarke *et al*., 2002), PVY-infected tobacco plants (Buonaurio & Montalbini, 1993) and in resistant soybean infected with *Soybean mosaic virus* (Zhuang *et al*., 1993). On the other hand regarding susceptible cultivar, no significant change in SOD activity was observed. Similar responses have also been reported in CMV-infected cucumber plants (Riedle-Bauer, 1998).

Catalase (CAT): There was no significant change in catalase activities in ULCV-infected and control plants of both susceptible and resistant cultivars (Fig. 3). This is in agreement with previous findings of other research groups (Hernández *et al*., 2001; Riedle-Bauer, 1998; Fodor *et al*., 1997). They found no changes in tobacco infected by TMV or rather a non-significant decrease in CAT activity in cucumber plants infected with CMV (Riedle-Bauer, 1998) and in apricot infected with *Plum pox virus* (Hernández *et al*., 2001) . In contrast, CAT activity was significantly decreased in *P. vulgaris* infected with WClMV (Clarke *et al*., 2002) and in tobacco plants infected with TMV (Chen *et al*., 1993; Neuenschwander *et al*., 1995).

Peroxidase (PO): At all stages of sampling non- significantly higher PO activity was observed in healthy leaves of susceptible (Mash-88) genotype than in the resistant one. The activity of PO increased with the passage of time in both the genotypes (Fig. 4) but ULCV inoculation resulted in significant increase of total PO activity in resistant cultivar after 15 ($p<0.05$) and 30 ($p<0.05$) days of inoculation, whereas the increase remained non-significant in the diseased leaves of the susceptible one. The enhancement of PO might be responsible for the activation of resistance mechanism in virus-infected plants. These results are in an agreement with Clarke *et al*., (2002) and Karthikeyan *et al*., (2007) who also observed significant increase in peroxidase activity in *P. vulgaris* and *V.mungo* plants after inoculation with WClMV and ULCV, respectively. Van Loon (1976) and Nadlong & Sequeira (1980) suggested that the increased PO- activity following virus infection was a reflection of physiological changes associated with, but not responsible for, induced resistance whereas up-regulated peroxidases might be responsible for growth reductions and malformations in virus-infected plants (Riedle-Bauer, 2000). PO participates in a variety of plant defense mechanisms (Mareschbacher *et al*., 1986) in which H_2O_2 is often supplied by an oxidative burst, a common event in defense responses (Dixon & Lamb, 1990). The cell wall of plants appears to be a major site for defense-related peroxidase polymerization reactions such as lignification (Hammerschmidt & Kuc, 1982), suberization (Espelie *et al*., 1986) and cross-linking of structural cell wall proteins (Fry, 1986). The increased PO activity observed in the present study may be triggered by cellular damage caused by virus replication. Rathi *et al*., (1986) also reported non-involvement of PO and PPO in imparting resistance to pigeonpea against sterility mosaic virus.

Conclusion

In the present study we observed that ULCV infection resulted in higher total protein content in both susceptible and resistant genotypes of blackgram that was more likely to be due to the increased level of viral proteins in the plant. The activities of PO and SOD increased and decreased significantly after 15 and 30 days of virus-inoculation in resistant genotype, respectively. It is concluded that increase in PO activities interrupted the signals generated by the increase in ROS and thus might be involved in imparting resistance to blackgram against leaf crinkle virus. Support for these interpretations requires information on the behaviour of the plant defences in this pathosystem, and such research is underway.

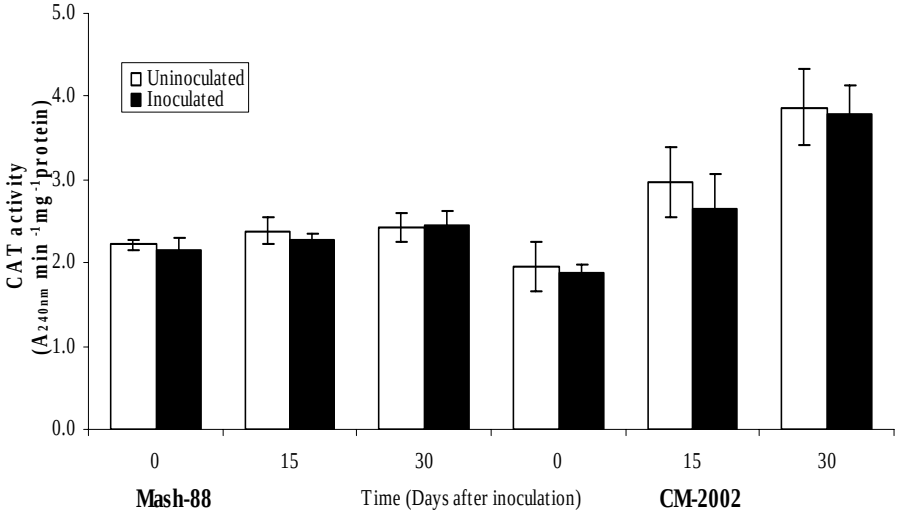

Fig. 3. Catalase (CAT) specific activity in healthy and ULCV-infected plants of two blackgram genotypes Mash-88 and CM-2002 (Chakwal Mash-2002), at various stages of virus infection. Results are the mean ± SE of 10 plants.

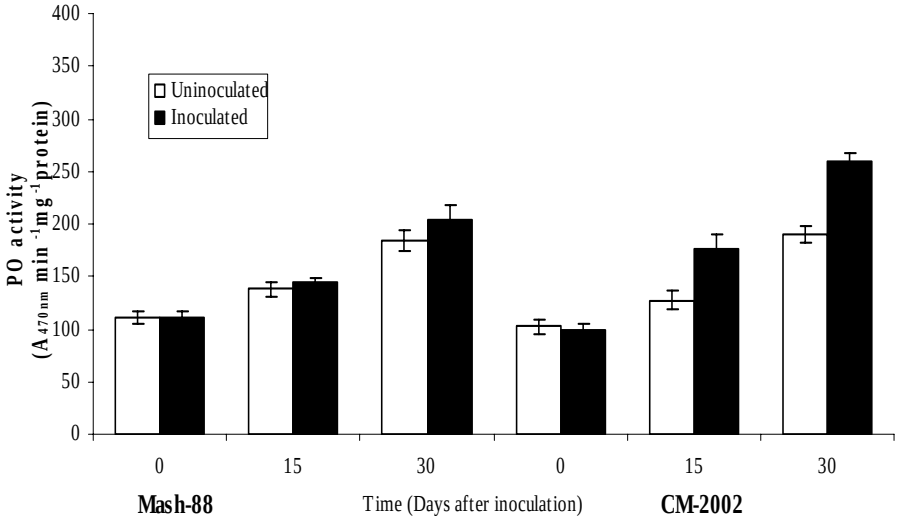

Fig. 4. Peroxidase (PO) specific activity in healthy and ULCV-infected plants of two blackgram genotypes Mash-88 and CM-2002 (Chakwal Mash-2002), at various stages of virus infection. Results are the mean ± SE of 10 plants.

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(Received for publication 22 January 2009)