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RESPONSES OF TWO COTTON (*GOSSYPIUM HIRSUTUM* L.) CULTIVARS DIFFERING IN RESISTANCE TO LEAF CURL VIRUS DISEASE TO NITROGEN NUTRITION

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Abstract

Use of pesticides to eradicate pest attack on cotton crops has increased substantially during the past decade posing a serious threat to environment and human health. Application of nitrogenous fertilizers which modulates plant metabolism might reduce pest and pest-induced viral diseases. Understanding physiological basis of nitrogen nutrition on disease incidence in cotton may help in developing strategies to prevent, avoid, escape and control viral diseases. Thus, responses of two cultivars of cotton (*Gossypium hirsutum* L.), S-12 (CLCuV-susceptible) and CIM-448 (CLCuV-resistant), to varying concentrations of nitrogen were examined. Plants of both cotton cultivars were grown at varying concentration [224, 114 (control) and 56 mg N L^{-1}] of nitrogen supplied with Hoagland's nutrient solution. The virus resistant cultivar, CIM-448 remained free of all disease symptoms throughout the experiment, whereas in virus susceptible cultivar S-12 leaf curling and vein thickening occurred at all external nitrogen regimes. However, severity of disease symptoms decreased with decreasing external N supply. Growth of both cotton cultivars increased due to increasing external N supply. The CLCuV-resistant cultivar, CIM-448 had significantly greater fresh and dry biomass as compared to the virus susceptible cultivar S-12 at all external nitrogen regimes. Leaf epicuticular wax content was greater in CLCuV-resistant cultivar as compared to that of non-diseased leaves of CLCuV-susceptible cotton cultivar S-12. However, the diseased leaves of CLCuV-susceptible cultivar S-12 had higher epicuticular wax content as compared to those of healthy S-12 and CIM-448. Leaf K^+ decreased with decrease in N regimes in both cultivars. However, diseased leaves of S-12 had significantly higher leaf K^+ and Ca^{2+} as compared to those of healthy S-12 and CIM-448. Leaf Mg^{2+} concentration was higher in CIM-448 as compared to that in diseased or healthy leaves of S-12 at all N levels. Accumulation of N declined with decrease in N levels. However, CIM-448 had higher N content as compared to healthy or diseased leaves of S-12. Leaf P content was inconsistent in two cotton cultivars at different N levels. In conclusion, growth of both cotton cultivars increased with increase in N nutrition in growth medium, which in turn was associated with higher accumulation of N and K accumulation. However, vigorous growth of S-12 cultivar with high N supply enhanced the disease susceptibility due to change in pattern of N and K accumulation at different N levels, whereas the disease resistance of CIM-448 remained unchanged at changing N levels.

Introduction

During the last two decades, production and productivity have suffered a setback due to leaf curl disease (CLCuD), a disease caused by a whitefly-transmitted Gemini-virus (Briddon *et al*., 2001; Mansoor *et al*., 2003). Under the severe attack of the virus particularly at initial vegetative growth stages infected plants remain stunted causing considerable yield losses (Mahmood, 1999; Kirkpatrick & Rothrock, 2001). However, plant scientists have recommended some effective management practices which include cultivation of virus resistant varieties, effective management of causal agents and application of mineral nutrition (Akhtar *et al*., 2004).

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It has been reported that plants growing in a nutrient deficient environment are very weak and more vulnerable to a variety of diseases (Huber & Thompson, 2007). Although deficiency of all essential nutrients may facilitate in developing plant disease, macro-nutrients have a direct and greater impact on pest attack and plant disease incidences than others (Marschner, 1995). Although extent of disease resistance in plants is primarily determined by its genetic potential, it can be modulated by mineral nutrition (Huber & Graham, 1999). Highly resistant and highly susceptible varieties are less affected by changes in nutritional regimes than only resistant or susceptible varieties to diseases. From this, it can be hypothesized that plant diseases can be managed by proper nutrition (Huber & Thompson, 2007).

Of mineral nutrients, nitrogen (N) accounts for about 80% of the total mineral nutrients absorbed by the plants. In alfalfa, about 75% of leaf nitrogen is allocated to chloroplasts (Hak *et al*., 1993), most of which is used for the synthesis of compounds of photosynthetic apparatus. Appropriate N uptake is essential for growth and disease resistance (Marschner, 1995; Epstein & Bloom, 2005). It is widely accepted that low nitrogen levels in the growth medium may enhance the disease resistance, while high N nutrition causes vigorous growth with subsequent decrease in disease resistance (Marschner, 1995; Huber & Graham, 1999; Huber & Thompson, 2007). For example, it has been observed that vigorous growth stimulated by ample supply of N reduced the incidence of "take-all" (*Gaeumannomyces graminis*) in wheat (Brennan, 1980; Wrather *et al*., 1997). Moreover, attack of sucking insects, which are vectors for diseases such as whitefly, increased at high N supply that cause greater disease problems (Anon., 2004).

Knowledge about N fertilization that promotes or reduces the development of CLCuD will be useful for deciding management strategies of cotton cultivars and for optimizing the conditions for selection and breeding programs. The present study was conducted to appraise the role of N nutrition in the development of cotton leaf curl virus (CLCuV) disease incidence in two cotton cultivars differing in disease resistance. It was assumed that the cultivars differing in disease resistance have different patterns of nutrient accumulation in healthy and diseased plants. Thus, the main objective was to draw the relationship between pattern of ion accumulation and growth, and disease incidence in cotton cultivars at varying levels of nitrogen.

Materials and Methods

Seeds of two cultivars of cotton (*Gossypium hirsutum* L.), cv CIM-448 (CLCuV-resistant) and cv S-12 (CLCuV-susceptible) were obtained from the Central Cotton Research Institute (CCRI) Multan, Pakistan. The experiment was conducted in a glasshouse with mean day temperature of 38.6 ± 9.4ºC and night temperature of 22.3 ± 7.6ºC, and photoperiod from 12 to 14 h.

Thirty-six mosaic-cement pots of 28 cm diameter were filled with 16 kg of river sand thoroughly washed with tap water. The experiment was arranged in a complete randomized design with two cultivars, three N levels, and six replicates. Different levels of N in the Hoagland's nutrient solution (Epstein, 1972) were prepared as follows:

N 224 mg L^{-1} = Full strength Hoagland's nutrient solution

N 112 mg L^{-1} (Control) = Full strength Hoagland's nutrient solution without $Ca(NO_3)_2.4H_2O$ and amended with 4.0 mL/L 1*M* $CaCl_2.2H_2O$.

N 56 mg L^{-1} = Full strength Hoagland's nutrient solution except Ca $(NO_3)_2$. $4H_2O$ and the amendment in KNO_3 as follows: + 2.0 mL/L 1*M* KNO_3 + 4.0 mL/L 1*M* KCl + 4.0 mL/L 1*M* $CaCl_2.2H_2O$.

Seeds were surface sterilized in 5% Sodium hypochlorite solution for 10 min and washed with distilled water prior to experimentation. Three hundred seeds of each cultivar were sown in wet sand in plastic trays. Two weeks after sowing, four seedlings of comparable size were transplanted into each mosaic-cemented pot and allowed to establish/grow for one week and treatment solutions of N (224, 112 and 56 mg L^{-1}) were applied to all the pots and thereafter every week treatment solutions were applied to maintain the N concentration in sand. Two plants from each pot were harvested at the flowering stage and their fresh weights of shoots and roots recorded. Plants were oven-dried at 70ºC for 7 days and their dry weights recorded.

Polymerase chain reaction (PCR) detection for cotton Gemini virus: Total DNA was isolated from the leaves of two cotton cultivars, S-12 (healthy and infected with cotton leaf curl virus), and CIM-448. Fresh leaf tissue of these cultivars was ground in the buffer used for particle purification of cotton leaf curl virus. The composition of buffer was; 100 m*M* Sodium citrate pH 6.0, 18.5 m*M* ascorbic acid, 60 m*M* Sodium sulfite, 1% 2-mercaptoethanol and 5 m*M* EDTA. A small leaf (2 g) was ground in a pestle and mortar with 5 ml of extraction buffer. The extract was centrifuged in a microfuge tube for 10 min., at 10,000 *g*. Fifty µl of the extract was transferred to an autoclaved polypropylene PCR tube. The tube was incubated on ice for 20-30 min., and then washed three times with 200 µl of the same buffer and finally with distilled water. The PCR solution mixture was added to the dried tube and was directly used for PCR amplification using specific primers reported by Mansoor *et al*., (1999). PCR products were analyzed on 1 % agrose gel in TBE buffer in Central Cotton Research Institute, Multan, Pakistan.

Epicuticular wax content: Fresh leaves (2.0 g) of the same age and size were excised from healthy and diseased plants. The area of each leaf sample was first measured with a leaf area meter (Delta T Devices, Burwell, Cambridge, England). The leaf samples were then placed in weighed glass vials, and were washed with 40 mL of CCl_4 for 40 seconds three times. The extract was evaporated to dryness and wax was weighed, and wax content of leaf was expressed as per unit leaf (µg/cm^2)].

Macronutrients (K, Ca, Mg, N, and P): For the analysis of macronutrients a fully expanded youngest leaf from each plant was sampled. A diseased leaf of the comparable age was also sampled from each infected plant for analysis of macronutrients. The macronutrients in plant shoots and roots were measured by the methods as described by Allen et al. (1986). Dry ground leaf (100 mg) from each sample was digested in 2 mL of H_2SO_4-H_2O_2 digestion mixture until a clear and colourless solution was obtained. The volumes of the digested samples were made 50 mL with de-ionized water. K^+ in the shoots and roots was determined with a flame photometer (Jenway PFP7, Dunmow, Essex, UK), and Ca and Mg with an atomic absorption spectrometer (Perkin Elmer Analyst-100). P was estimated by the method described by Jackson (1958) using a spectrophotometer (Hitachi U-2000, Tokyo, Japan) and N by Kjeldahl method.

Statistical analysis of data: The data obtained were subjected to a 2-way ANOVA using a statistical computer package COSTAT (Cohort Software, Berkeley, USA) and means were compared with least significant difference following Snedecor & Cochran (1980).

Results

The DNA was tested for the presence of whitefly-transmitted geminivirus in cotton samples. Cotton leaf curl virus CLCuV) was detected in S-12, whereas it was absent in CIM-448. The disease incidence in each plant was recorded as described Ali *et al*., (1995) considering vein thickening and leaf curling as the selection criteria. The disease incidence on S-12 was more severe at higher levels of N as compared to lower N levels applied in the growth medium. Fig. 1. shows percentage of CLCuV incidence in S-12 at varying levels of N, which was the highest at the highest N level (26.09%).

Different N levels of the growth medium had a significant effect on fresh or dry matters of shoots of the two cultivars differing in resistance to CLCuV (Table 1; Fig. 2). Fresh and dry biomass of shoots of CIM-448 decreased with decrease in N regimes, whereas that of S-12 decreased only at the low N level (56 mg L^{-1}). Cultivar CIM-448 was generally better in growth than S-12 at all external N regimes.

Different N regimes of the growth medium had a significant effect on the epicuticular wax content of the two cultivars. Wax content in both cultivars were maximum at 112 mg L^{-1} of N, but it was low at high or low level of N. CIM-448 was higher in accumulation of leaf wax content than that in the non-infected leaves of S-12 at all N levels (Table 1; Fig. 2). The significant increase in wax content in infected leaves of S-12 may have been due to the curled and shriveled surface of the leaves with many slight grooves. Although it was tried to make the leaf surface plan and smooth by slightly pressing the leaf, it was not possible to make it fully smooth. Thus in view of this technical reason, inflated values of epicuticular wax content of diseased leaves are expected.

Potassium ($K^{+)}$ concentration in the non-infected leaves of S-12 was the lowest at 112 mg L^{-1} of N, whereas it was high at both high and low levels of external N. In contrast, in the leaves of infected S-12 plants it was the highest at 112 mg L^{-1} of N, whereas it was low at both high and low N levels. In CIM-448, K^{+} concentration decreased significantly with decrease in the external N concentrations (Table 2; Fig. 3).

Calcium (Ca^{2+}) concentration in the healthy leaves of S-12 was minimum at the highest N regime (224 mg L^{-1}), but it remained unaffected at all other external N regimes (Table 2; Fig. 3), whereas in the diseased leaves of S-12, it was maximum at the lowest N regime (56 mg L^{-1}), but remained unaffected at all other higher external N regimes. In contrast, in CIM-448, it was maximal at 224 mg L^{-1} of N and remained unaffected at the other two N regimes. However, cultivar difference was not consistent for this variable.

Magnesium (Mg^{2+}) concentration in the healthy leaves of S-12 decreased significantly at the lower external N regime, whereas in the diseased leaves of S-12 it was high at 112 mg L^{-1} of N and low at the two extreme N levels. In contrast, in CIM-448, Mg^{2+} concentration was low at 112 mg L^{-1} of N but high at the remaining two N levels (Table 2; Fig. 3). Cultivar difference was prominent at the low and high N regimes as compared to that at intermediate N regime. CIM-448 was generally higher in accumulation of Mg^{2+} as compared to S-12.

Nitrogen (N) concentration in the healthy leaves of S-12 and CIM-448 remained unaffected at the two higher N regimes, but it was low at the lowest N regime (56 mg L^{-1}). In the diseased leaves of S-12, N concentration decreased significantly with decrease in the external N regimes (Table 2; Fig. 3). Comparison of the varieties for this variable shows that S-12 had significantly lower concentration of N in its leaves, particularly in the healthy leaves, than that in CIM-448.

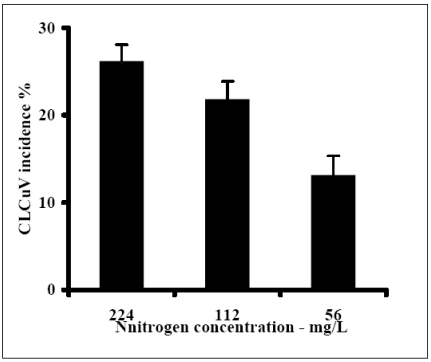

Fig. 1. Cotton leaf curl virus incidence (%) in S-12 at the flowering stage when grown at different N regimes.

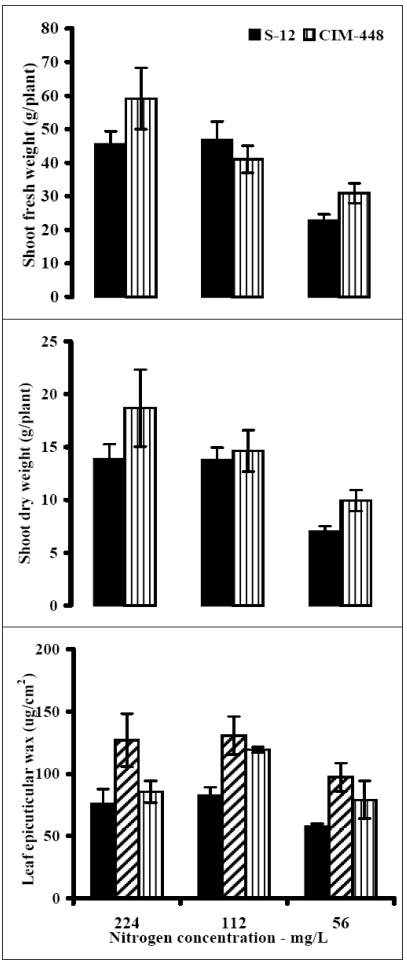

Fig. 2. Shoot fresh and dry weights (g/plant) and leaf epicuticular wax content (µg cm^{-2}) of two cotton cultivars at the flowering stage when grown at different N regimes.

Table 1. Mean squares from analyses of variance of data for shoot fresh and dry weights of two cotton cultivars at the flowering stage grown at different N regimes.

Source of variation	df	Shoot F. wt.	Shoot D. wt.	Wax content
Blocks	5	96.05 NS	17.27NS	11.60NS
Treatments (T)	2	2027.04***	195.92***	2434.91***
Cultivars (Cv)	1	248.74 NS	74.09NS	4787.89***
T × Cv	2	298.69NS	11.82NS	234.66NS
Error	25	137.22	18.33	162.63

*** = Significant at 0.001 level; NS = Non-significant

Table 2. Mean squares from analyses of variance of data for potassium, calcium, magnesium, nitrogen and phosphorus of healthy (H) and diseased (D) leaves of CLCuV-resistant or CLCuV-susceptible cotton cultivars/lines at the flowering stage grown at different N levels.

Source of variation	df	Leaf K^+	Leaf Ca^{2+}	Leaf Mg^{2+}	Leaf N	Leaf P
Blocks	5	670.1NS	6404.8 *	302.7NS	15435.6 NS	99.0 NS
Treatments (T)	2	27080.3***	7896.2 *	28081.9***	526028.4***	349.9 *
Cultivars (Cv)	2	70897.7***	146276.7***	75476.2***	221416.9***	3707.8***
T × Cv	4	13055.6***	24864.6***	14605.6***	38057.8**	1328.8***
Error	40	1167.1	1829.0	556.8	9194.3	75.9

*, **, *** = Significant at 0.05, 0.01 and 0.001 levels, respectively. NS = Non-significant.

Phosphorus concentration in the healthy leaves of S-12 was low at 112 mg L^{-1} of N, but it was high at the other two external N levels. In contrast, in the diseased leaves of S-12, it was high at 112 mg L^{-1} of N, but it was low at the two extreme N levels. In CIM-448, P concentration remained unaffected at higher N regimes, but it decreased at the lowest N regime (56 mg L^{-1}) (Table 2; Fig. 3).

Discussion

Mineral nutrients may either increase or decrease the resistance or tolerance of plants to pathogens and pests (Marschner, 1995). In the present study, the virus resistant CIM-448 produced significantly higher fresh and dry biomass as compared to the disease susceptible S-12 at all external N regimes. This differential response of the two cotton cultivars to external N supply can be explained in view of Daigger *et al.*, (1976) and Evans & Wardlaw (1996) that it is due to their variable physiological behavior that might have resulted in differences in growth. However, the severity of the disease incidence in S-12 increased with increase in external N regime, while disease resistance in CIM-448 was not changed with the change in N concentration. According to an internet report (Anonymous, 2004), disease resistance of highly disease resistant and susceptible cultivars do not change with the change in nutritional regimes and only moderately tolerant or susceptible to diseases respond to changing nutritional regimes. This view is supported by the fact that high N nutrition leads to production of amino acids and sugars, while defense related compounds such as tannins and phenolics are produced in less amount (Dudt & Shure, 1994; Peñuelas *et al*., 1997). This results in increase in feeding intensity and reproduction of sucking insects, like whitefly, thereby increasing disease susceptibility.

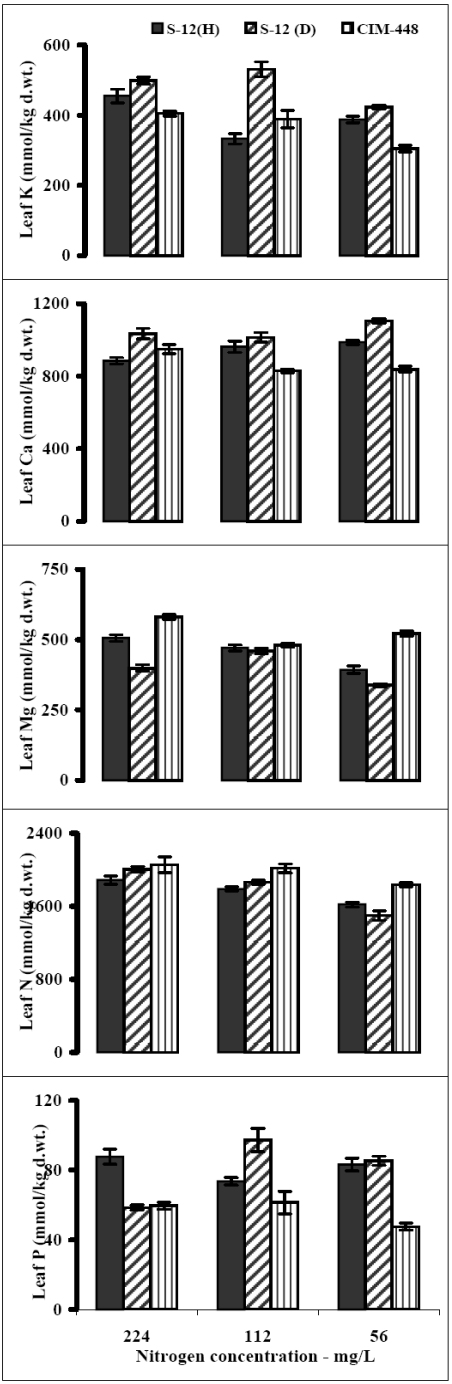

Fig. 3. Leaf K^+, Ca^{2+}, Mg^{2+}, N and P concentrations (mmol kg^{-1} d. wt.) in healthy (H) and diseased (D) leaves of CLCuV-resistant or CLCuV-susceptible cotton cultivars/lines at the flowering stage when grown in sand culture under different N regimes in Hoagland's nutrient solution.

Leaf epicuticular wax content of diseased leaves of S-12 was considerably higher than that of disease resistant CIM-448 and S-12 healthy plants. This may have been partly due to non-plan surface of the curled diseased leaves, the curling of which could not be eliminated during measurement of leaf area (Ashraf *et al*., 1999). The other possibility is that the diseased leaves might have secreted high amount of wax on their surface to avoid further attack of whitefly, the vector of CLCuV. However, CIM-448 had higher leaf epicuticular wax content as compared to that of healthy leaves of S-12, and this high amount of wax content in CLCuV resistant cultivar was related to its disease resistance in some earlier studies (Ashraf *et al.*, 1999; Ashraf & Zafar, 2000).

Appraisal of K^+ of the healthy and diseased leaves of both cultivars showed a differential pattern of accumulation of this nutrient. For instance, in the diseased leaves of S-12, K^+ accumulation was higher, whereas the reverse was true in the healthy leaves of CIM-448 and S-12. This could have been due to the argument that high amount of K^+ is needed to maintain N metabolism when large amounts of N are supplied in the external medium (Leigh & Wyn Jones, 1984; Ashraf & Rehman, 1999a).

Calcium is mainly required for cell wall synthesis and cell membrane integrity (Epstein & Bloom, 2005). It acts as a second messenger for various plant responses to environmental and hormonal signals (Marschner, 1995; Taize & Zeiger, 2006). However, high accumulation of Ca^{2+} in diseased leaves is not easy to explain in view of earlier findings (Ashraf & Zafar, 1999) in which it was reported that Ca^{2+} had a strong association with disease resistance. Comparison of Ca^{2+} and Mg^{2+} concentrations in both healthy and diseased leaves reveals that the two nutrients show antagonism in uptake (Ashraf & Zafar, 1999; 2000). Nitrogen and Mg^{2+} accumulations were generally higher in CIM-448, whereas the reverse was true for healthy and diseased leaves of S-12. Both N and Mg^{2+} play a vital role in chlorophyll biosynthesis (Walker & Weinstein, 1991), protein synthesis and photosynthesis (Ashraf & Rehman, 1999b). The results for N are consistent with those of Ashraf & Zafar (1999; 2000) who found that CLCuV-resistant cultivars accumulate higher N as compared to those of CLCuV-susceptible cultivars.

If parallels are drawn between data for biomass production and accumulation of different nutrients in the two cotton cultivars differing in resistance to CLCuV, it is clear that N and K accumulation in both cultivars were directly related to their growth. Vigorous growth stimulated by ample supply of N enhanced disease susceptibility in S-12. Although the pattern of nutrient accumulation in disease resistant CIM-448 was changed with the change in N supply, disease resistance remained almost unaffected.

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