Some Effects of Metabolic Changes Induced in Etrog Citron by Three Isolates of Exocortis Virus

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ABSTRACT

Results of metabolic changes in 'Etrog' citron plant infected with three different isolates of exocortis virus were examined. A positive correlation was found between symptom severity, oxygen uptake, and respiration. An inverse relationship was observed between symptom severity and total nitrogen in citron leaves. Marked changes in several free amino acids were noted with all three isolates. Changes were observed in alanine, arginine, aspartic acid, glutamic acid, glycine, and proline. Plants chronically infected with the severe isolate of exocortis showed an increase in total and reducing sugars. Peroxidase activity in the infected plants was at a higher level than noninoculated controls. Catalase activity increased only with the severe and moderate isolates. No differing nucleic acid species were observed in extracts from infected leaves.

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Virus infections usually induce biochemical and physiological changes in plants. In some *Citrus* spp. infected with viruses, the physiological aberrations are tolerated to the extent that symptoms are often delayed or fail to develop. Comparatively few investigations have been conducted on the physiology of citrus plants infected with citrus exocortis virus (CEV). Rossetti et al. (27) reported an increase in lysine and a decrease in arginine in young twig bark of exocortis-affected *Poncirus trifoliata* [L.] Raf. and 'Rangpur' and 'Kusaie' lime (*Citrus limonia* Osb.) and 'Red Ling Mung' mandarin (*C. reticulata* Blanco). They observed an increase in citric, malonic, oxalic and glycolic acids and a decrease in fumaric, succinic, lactic, and malic acids. Feldman and Hanks (8) observed approximately 50% reduction in the total

amount of free amino acids in leaves of CEV-infected trees. Monselise and Goren (24) using the level of hesperidine in leaves as a function of phenolic changes associated with virus infection, found reduction of this flavanone in exocortis as well as xyloporosis-affected 'Shamouti' sweet orange (*C. sinensis* [L.] Osb.). They also observed a decrease in peroxidase activity in these trees. Feldman and Hanks (9) isolated a total of 39 phenolics, free and bound, from the roots and leaves of healthy and CEV-infected 'Valencia' sweet orange trees on *P. trifoliata* rootstock and found essentially similar composition in healthy and CEV-infected trees although differences in amounts of certain individual phenolics were observed.

This paper reports some metabolic changes in Etrog citron (*C. medica* L.) plants infected with different isolates of CEV.

MATERIALS AND METHODS. – Three isolates provided by E. C. Calavan served as sources of exocortis virus. The three isolates were differentiated on the basis of severity of symptoms in 'Arizona 861' Etrog citron plants (15).

Healthy Etrog citron cuttings, approximately 30.5 cm (12 in) high, were singly inoculated with the three isolates. Plants were inoculated by grafting as well as by razor slashing the stems.

Disks 1.0 cm in diam were cut with a sharp cork-borer from the fourth leaf from the apex of at least 10 inoculated seedlings at the time of sampling and the composite sample of leaf discs was used for the various studies which follow. Each experiment was repeated at least twice with a different composite sample.

Oxygen uptake was measured in a Gilson differential respirometer. Samples of each isolate consisted of 30 leaf disks. Fresh discs were placed in the main compartments of respirometer flasks to which 1 ml of distilled water had previously been added. Filter paper wicks and 0.5 ml of 10% KOH were added to the center compartment of each respirometer flask before placing them in the water-bath maintained at 27 C. Flasks were allowed to equilibrate for 30 min. Oxygen uptake was measured in the dark at 15-min intervals over a period of 1 h. Results were calculated as μl of 0_2 absorbed per hr per mg dry wt.

Total nitrogen was estimated by the standard micro-Kjeldahl method and results are expressed as percent total nitrogen on a dry wt basis.

To extract amino acids and sugars for analyses, 2 g of fresh leaf tissue was triturated in liquid nitrogen and then extracted with 20 ml 80% ethanol for 5 min in a 'Virtis 45' homogenizer. The homogenate was filtered through sintered glass and rinsed with three washings of 5-ml aliquots of ethanol. The filtrate was taken to dryness and then resuspended in 2 ml of water. For deproteinization 15 ml of 1% picric acid were added to the above filtrate which was then allowed to stand for 1 h before it was passed through a column of Dowex 1-X8 anion exchange resin. Effluents were taken to dryness and redissolved in 2 ml of water or 2 ml of 0.2M citrate buffer (pH 2.2) for sugar and amino acid analyses, respectively.

Amino acid analyses were performed in a Beckman, Model 120 C, automatic amino acid analyzer.

Total- and reducing sugars were determined by phenol-sulfuric acid (7) and dinitrosalicylic acid (22) methods, respectively, using glucose standards. Results are expressed as mg glucose equivalents per g freshwt leaf tissue.

For enzymatic studies, one g of leaf disks was ground with a mortar and pestle in 10 ml 0.05M phosphate buffer (pH 6.8) and centrifuged at 5,000 rpm for 20 min at 4 C. The supernatant fluid served as the crude enzyme extract.

Peroxidase activity was measured by the method described by Loebenstein and Linsey (18). Enzyme extract (0.2 ml) was added to a colorimeter tube containing 5 ml pyrogallol reagent. The colorimeter was adjusted to 0 optical density at 420 nm with this mixture and then one-half ml of 1% H_2O_2 was added rapidly to the tube. The time required to move from 0.1 OD to 0.3 OD was measured and results expressed as (min/g fresh wt^{-1}).

Catalase activity was determined by the method of Feinstein as described by Dekock et al. (4). Activity was calculated as the amount of sodium perborate consumed per g fresh leaf tissue.

Total nucleic acids were examined from healthy citron leaves and leaves singly infected with different isolates every 10 days after inoculation over a period of 50 days. Extraction of cellular nucleic acids was performed by the method of Pring (26) using the extraction buffer of Brakke and Van Pelt (2). These were compared by centrifugation followed by fractionation on linear-log sucrose gradients.

Sucrose density-gradient columns were prepared for the Spinco SW 41 Ti rotor as specified by Brakke and Van Pelt (3). One-half ml was removed from the top of the gradient, 0.5 ml of solution containing 1.0 OD/260 unit of nucleic acid preparation was floated on the column, and the gradient columns centrifuged at 40,000 rpm and 10 C for 7.5 h in a Beckman Model L2 65 B ultracentrifuge. Centrifuged gradients were analyzed in an ISCO density gradient fractionator with an external recorder.

RESULTS. – *Oxygen uptake.* – The rate of 0_2 uptake by leaf disks from citron plants inoculated with the severe isolate of CEV showed an increase 10 days after inoculation, while infection with the moderate isolate caused an increase around 20 days after inoculation (Fig. 1). In both cases, however, 0_2 uptake reached its maximum 30 days after inoculation and declined thereafter. The mild isolate did not influence the rate of respiration over a period of 60 days.

Total nitrogen.–Changes in total nitrogen of leaves of plants infected with the different isolates of exocortis virus were similar. Each decreased during the time of infection as compared to the check (Fig. 2).

Free amino acids. – Infection with CEV greatly

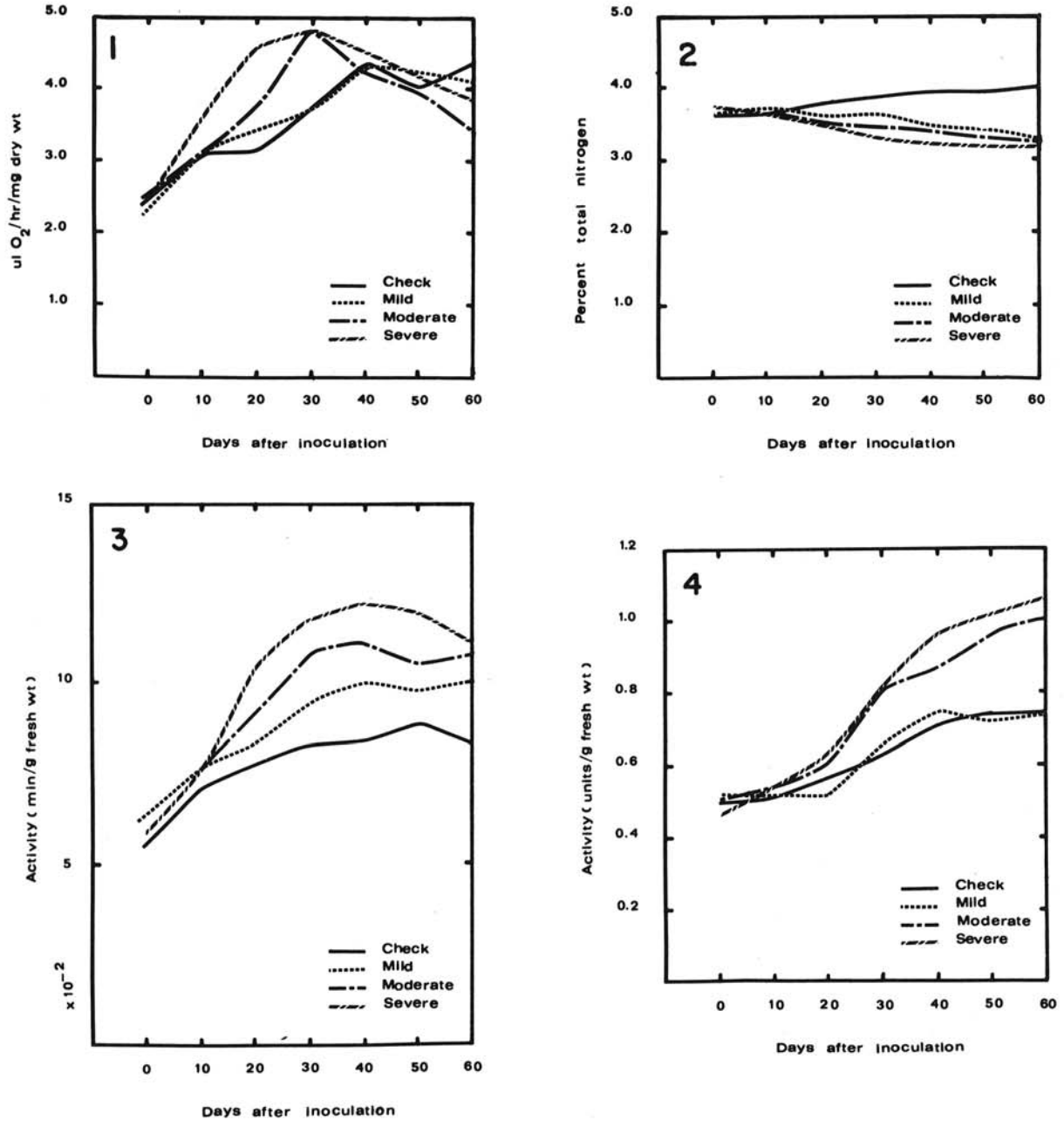

Fig. 1-4. Effect of three isolates (mild, moderate, and severe) of exocortis virus upon leaves of Etrog citron: **1)** oxygen uptake; **2)** total nitrogen content; **3)** peroxidase activity; and **4)** catalase activity.

affected free amino acid composition of citron leaves compared to healthy leaves. Changes were observed in alanine, aspartic acid, glutamic acid, glycine and proline (Tables 1, 2, 3, 4). Marked decreases were recorded in aspartic acid, glutamic acid, glycine, and proline. On the 40th and 60th day, an increase in alanine, aspartic acid, glutamic acid, and proline was observed. Arginine could not be detected in either infected or noninfected leaves on the 60th day.

Analyses of extracts from leaves infected with the moderate isolate showed a decrease in glutamic acid, and an increase in alanine and glycine. Increases were observed in aspartic acid, arginine, and proline 40 and 60 days after inoculation.

Amino acid compositions of leaves infected with the severe isolate were similar to those of the moderate isolate. A decrease in alanine and glutamic acid was observed on the 20th day after inoculation

TABLE 1. Free amino acids in healthy citron leaves and leaves infested with isolates of citrus exocortis virus (CEV) (Day of inoculation)

	μmoles/g fresh wt			
		CEV isolate		
Amino acid	Healthy	Mild	Moderate	Severe
Alanine	1.89	1.78	1.83	1.70
Arginine	0.05	0.04	0.04	0.04
Aspartic acid	0.97	1.04	0.84	0.90
Glutamic acid	0.98	1.02	0.95	0.77
Glycine	0.47	0.54	0.41	0.41
Isoleucine	0.05	0.05	0.04	0.04
Leucine	0.09	0.08	0.09	0.07
Lysine	0.12	0.14	0.12	0.09
Phenylalanine	0.10	0.14	0.13	0.11
Proline	4.45	4.04	4.20	3.89
Tyrosine	0.07	0.07	0.08	0.08

TABLE 2. Free amino acids in healthy citron leaves and leaves infected with isolates of citrus exocortis virus (CEV) (20 days after inoculation)

	μmoles/g fresh wt			
		CEV isolate		
Amino acid	Healthy	Mild	Moderate	Severe
Alanine	0.89	0.62	1.14	0.42
Arginine	0.03	0.06	0.24	0.18
Aspartic acid	0.72	0.47	0.62	0.84
Glutamic acid	0.59	0.29	0.24	0.10
Glycine	0.54	0.13	0.81	0.32
Isoleucine	0.05	0.04	0.05	0.05
Leucine	0.10	0.09	0.09	0.08
Lysine	0.13	0.14	0.16	0.11
Phenylalanine	0.15	0.11	0.13	0.24
Proline	3.70	2.93	3.25	3.75
Tyrosine	0.10	0.09	0.09	0.09

although alanine increased on the 40th and 60th days. There was an increase in aspartic acid, arginine, glycine, and proline in the final analyses. As a result of consistently poor chromatographic separation of methionine, serine, threonine, and valine from the samples, their exact quantities could not be determined.

Total and reducing sugars. – Differences in total and reducing sugars from healthy and infected citron leaves were observed only with the severe infection. There was an increase in both at the first appearance of symptoms. No increase or decrease was observed in sugars in leaves of plants infected with moderate or mild isolates.

Enzyme activity. – Each CEV isolate caused a more rapid increase in peroxidase acitivity then noninoculated controls during the first 40 days of the experiment (Fig. 3). Differences in peroxidase activity attributed to each isolate paralleled differences in severity of symptoms induced by each isolate.

Catalase activity was increased in plants infected with severe and moderate isolates (Fig. 4). Catalase activity in all plants, unlike peroxidase, continued to increase until the termination of the studies.

Total nucleic acids. – Generally, no appreciable differences could be detected in the levels and sedimentation rates of the nucleic acids from CEV-infected citron leaves.

DISCUSSION. – Severe and moderate isolates of exocortis virus had a marked influence on the oxygen uptake of Etrog citron leaves. The mild isolate appeared to have no effect on O_2 uptake. A positive correlation seems to exist between symptom appearance and ultimate symptom severity and oxygen uptake. Singh (28) obtained similar results with 'Key' lime tissue infected with citrus tristeza virus. Increased respiratory activity in systemically infected plants has been reported for several virus-host combinations (5, 13, 14, 23, 33, 35).

An increased rate of respiration in the early stages of infection should not be expected. This is the period of intensive virus synthesis, and energy is directed toward this process (23). In rapidly growing

TABLE 3. Free amino acids in healthy citron leaves and leaves infected with isolates of citrus exocortis virus (CEV) (40 days after inoculation)

	μmoles/g fresh wt			
		CEV isolate		
Amino acid	Healthy	Mild	Moderate	Severe
Alanine	0.63	0.85	0.73	1.43
Arginine	0	0	0.37	0.26
Aspartic acid	0.50	0.94	0.76	1.28
Glutamic acid	1.17	1.51	0.88	1.29
Glycine	0.58	0.40	0.66	0.38
Isoleucine	0.11	0.04	0.04	0.05
Leucine	0.06	0.05	0.05	0.07
Lysine	0.10	0.07	0.09	0.09
Phenylalanine	0.13	0.18	0.12	0.11
Proline	4.07	4.36	4.74	6.75
Tyrosine	0.07	0.06	0.05	0.06

TABLE 4. Free amino acids in healthy citron leaves and leaves infected with isolates of citrus exocortis virus (CEV) (60 days after inoculation)

	μmoles/g fresh wt			
		CEV isolate		
Amino acid	Healthy	Mild	Moderate	Severe
Alanine	0.65	1.03	1.35	1.23
Arginine	0	0	0.35	0.33
Aspartic acid	0.72	1.00	1.21	1.13
Glutamic acid	0.42	0.51	0.31	0.23
Glycine	0.20	0.20	0.62	0.67
Isoleucine	0.03	0.04	0.06	0.07
Leucine	0.06	0.06	0.12	0.10
Lysine	0.07	0	0.15	0.03
Phenylalanine	0.07	0.10	0.15	0.11
Proline	3.73	5.01	9.47	12.06
Tyrosine	0.05	0.04	0.11	0.09

tissue, and during intensive virus synthesis, there is a rapid ATP utilization and regeneration of high-energy phosphate acceptors (10). In later stages, infection with virus causes premature senescence of tissue (5). Anabolic reactions and ATP turnover would presumably be at a minimum which should induce a decreased respiration rate. Decline in respiratory rates during senescence of leaves has been reported (21).

The correlation of low nitrogen to increased severity of symptoms is in accordance with reports of other workers with phloem-inhabiting viruses (12, 25, 28, 29, 30).

Wynd (35) postulated that variation in the amount of total nitrogen in infected plant results from: (i) disturbed translocation; and (ii) dilution effect of accumulating carbohydrates without a corresponding change in total nitrogen. Low contents of total nitrogen and protein nitrogen could also be caused by a destruction of the photosynthetic centers (25) or reduced photosynthetic area, conditions known to exist in epinastic and curled leaves.

Amino acid analyses of extracts from healthy and diseased citron leaves infected with mild, moderate, and severe isolates of CEV revealed marked changes in several amino acids. The active involvement of alanine, aspartic acid, and glutamic acid may reflect the highly active metabolic nature of these acids, since these are primary amino acids from which, directly or indirectly, most other amino acids are synthesized (17).

Infection with moderate and severe isolates of CEV resulted in the accumulation of arginine and proline in affected citron leaves. A possible relationship between symptom production and accumulation of these amino acids is given credence by the report of Stewart (31), who showed that leaves of girdled citrus plants accumulated arginine and proline, and the report of Fudl-Allah et al. (11) who showed that CEV induces a phloem necrosis in citron. The disease syndrome of western X disease in peaches could be simulated by injection of pipecolic acid and proline into healthy trees (6).

Increases in total and reducing sugars were observed only in the leaves of plants chronically infected with the severe isolate of the virus. Such an increase reflects a faster breakdown of carbohydrates which serve as substrates for increased respiration observed in early stages of infection.

Accumulation of carbohydrates in plants systemically infected with viruses has long been known (12, 19, 29, 30, 32, 34, 35), and various theories have been advanced to explain this accumulation. True et al. (32) claimed that carbohydrates accumulated because of slower growth of diseased plants, with consequent diminished use of manufactured food. Another suggestion has been made that accumulation of carbohydrates result from distributed translocation caused by phloem necrosis which occurs in CEV infected citron (11). According to Wynd (35), infection by viruses causes an alteration in permeability of cytoplasm so that soluble substances diffuse more slowly in infected cells than in uninfected cells.

Peroxidase activity started increasing 10 days after inoculation and the period of maximum activity coincided with symptom severity. This suggests that changes in peroxidase activity are more likely associated with development of symptoms, and are thereby a result of virus infection rather than being the cause of any particular symptom.

A clear-cut relationship between severity of symptoms and the increase in catalase activity became evident in the chronic stage of infection and continued until the termination of the experiment.

Several therories summarized by Menke and Walker (20), have been proposed to explain the increase of peroxidase and catalase activity in diseased plant tissue. Activation of flavoprotein enzymes which are responsible for production of H_2O_2 could be one reason for increased peroxidase activity. Higher level of peroxide required for oxidation of various substrates by peroxidase is another. It has also been suggested that high peroxidase activity in virus-diseased plants might be the consequence of a greater breakdown of carbohydrates through the hexose monophosphate shunt. This cycle also produces precursors of phenolic compounds which can be oxidized by peroxidase in the presence of hydrogen peroxide. The role of catalase is probably to remove H_2O_2 produced in the respiratory reactions which might otherwise accumulate to toxic levels in the tissue (1). High activity of catalase results in quick removal of H_2O_2 thereby making the plant more susceptible to infection (16). Severe and moderate isolates of exocortis virus induced increased activity of the enzyme in the plants after inoculation. Differences in severity of symptoms might therefore be related to increased activity of this enzyme.

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