Importance of Mesophyll in Mature-Leaf Resistance to Cancrosis of Citrus

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ABSTRACT

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Resistance in citrus leaves to cancrosis, caused by *Xanthomonas campestris* pv. *citri*, has been thought to be determined by stomatal structure. However, fewer lesions of cancrosis developed in mature citrus leaves than in immature leaves after inocula containing 10^3–10^4 cells per milliliter were infiltrated into the mesophyll. Mature-leaf resistance was not demonstrated at any inoculum level if an injury was used with inoculation. There was a positive correlation between inoculum dosage and numbers of lesions that developed in infiltrated leaves of grapefruit. The slope of the regression line decreased as age of leaves increased. Levels of resistance among some citrus species and cultivars in the field were distinguished after infiltration of 14- to 21-day-old leaves with inocula containing 5×10^3 cells per milliliter, but were not distinguishable by infiltrating leaves that were older. In all citrus types tested, resistance of the mesophyll to infection increased as leaves matured.

Additional key words: horizontal resistance.

Many workers have reported resistance among citrus species, hybrids, and cultivars to *Xanthomonas campestris* pv. *citri*, the causal agent of citrus cancrosis. These reports were based on field observations or artificial inoculations and resistance was expressed in degrees (8,9,10,16,17,21). Resistance may change with age of tree and maturity of the leaves (3,10).

The resistance in most commercial cultivars of citrus to cancrosis is not adequate for control of the disease. However, resistance in mature leaves is a beneficial supplement to control of the disease with protective sprays. One spray of copper on immature leaves is usually sufficient to protect them until they mature (14,15,19). Applications of copper to mature leaves are not necessary because resistance is strong enough to prevent the disease.

McLean and Lee (12,13) suggested that resistance to *X. campestris* pv. *citri* in leaves of a mandarin (*Citrus nobilis* Loureiro) was determined by the characteristics of stomata. The role of stomata in resistance was inferred after it was determined that resistance was absent if the leaf was injured by inoculation procedures. Stomata of a grapefruit (*Citrus paradisi* Macf.) (susceptible) and the mandarin (resistant) were similar in size and type, but the stomatal pore in grapefruit was larger than in mandarin owing to differences in formation of the cuticle of guard cells. The structural differences of stomata were significant because greater pressure was required to water-soak uninjured leaves of mandarin than those of grapefruit.

Goto (5) suggested that morphological differences of stomata also accounted for mature-leaf resistance of citrus. In young leaves the frontal cavity of stomatal pore was open, but in mature leaves only a narrow opening existed between ridges of the cuticle of the guard cells.

The purpose of this research was to investigate the dynamics of resistance to cancrosis in leaves of some citrus species and cultivars. In the experiments an inoculation procedure was used that bypassed the stomatal entrance. As a result, a resistance system in the mesophyll of mature leaves was discovered. The dynamics of the mesophyll resistance is reported.

MATERIALS AND METHODS

Inoculum preparation. An isolate of *X. campestris* pv. *citri* from a grapefruit leaf was used in all tests. The isolate (B-43) was

representative of the A-strain (2). Cultures were started by placing 0.5 ml of a stationary-phase culture of the bacterium on the surface of nutrient agar plates. After 24 hr of growth at 28 C, the bacteria were washed from the surface of plates with about 5 ml of sterile tap water. The resulting suspension was standardized to $A_{600nm} = 0.3$ with a Spectronic 20 spectrophotometer. This corresponded to about 5.0×10^8 cells per milliliter. Sterile tap water was used as the suspending medium for all inocula. Inocula of lower concentrations were prepared by proper dilutions. When inocula below 10^4 cells per milliliter were used, the actual concentration of bacteria was determined by placing 0.05 ml of suspension on each of three nutrient agar plates. The inoculum concentration was determined from the average number of colonies that developed.

Inoculation procedure. The infiltration technique described by Klement (7) was used. Suspensions of bacteria were injected into the leaves with a 2-ml hypodermic syringe and 0.47-mm-diameter (27-gauge) needle. The intercellular spaces of half of a leaf, divided by the midrib, were filled with inocula. The other half of each leaf served as a control.

Volume of intercellular spaces of leaves. Twelve leaves near 30 days of age were collected from trees of five orange (*Citrus sinensis* Osbeck) cultivars planted in the field. One-half of each leaf was injected with distilled water. Five disks, 1 cm in diameter, were cut from the water-soaked half and five from the untreated half of each leaf. The difference in weight between untreated and water-soaked disks was assumed to be due to the weight of water in the intercellular spaces. It was assumed that 1 ml of water weighed 1 g.

Infectivity titration. Duncan grapefruit seedlings were grown in 12-cm-diameter pots and were about 30 cm tall when inoculated. Twelve seedlings were selected from a group of plants when new shoots were about 1 cm long. Leaves were inoculated at 14, 21, and 28 days after the beginning of shoot development. One leaf per seedling was inoculated at each date and the leaf most basilar was inoculated first. Subsequently, the next leaf toward the apex was inoculated. Inocula was adjusted to about 5×10^3, 2.5×10^3, and 5×10^2 cells at each date and the actual concentration of inocula was determined as previously described. Test seedlings were kept at 28 C and under 16 hr of light and 8 hr of darkness.

Inoculation in the field. Leaves were inoculated on trees located at I.N.T.A., Bella Vista, Argentina, and cultivated in plots of H. M. Zubrzycki. The trees were 8 yr old and scions were on Rangpur lime (*Citrus limonia* Osbeck) rootstocks. Plots consisted of five trees and were randomly located in a block. Twelve shoots per citrus species or cultivar (four shoots per tree on three central trees of a

plot) were tagged when apical buds began to expand. Four leaves on each shoot were inoculated (one leaf on each of four dates) in a sequence as described above. Occasionally, leaves were lost in the field experiments, so data from 10 of the 12 inoculated leaves were used. Inocula were adjusted to about 5×10^3 cells per milliliter at each date. The actual numbers were determined as above.

Disease assessment. Lesions were counted from 3 to 6 wk after inoculation. The area of the inoculated portion of each leaf was determined by the dot method (11). The number of lesions per square centimeter of leaf area was calculated for each leaf and was adjusted to an equivalent inoculum of 5×10^3 cells per milliliter. The adjustment was necessary because the number of viable cells per milliliter in inoculum could not be determined immediately at the time of inoculation, and some variation occurred among inoculations. The adjustment was done by multiplying the number of lesions by the proportion of 5×10^3 over the number of viable cells in the inoculum. This was necessary because there was a linear relationship between inoculum doses and numbers of lesions formed.

RESULTS

Confluent necrosis occurred in leaves of Marsh grapefruit trees in the field that were about 14 days (young) and about 77 days old (old) after inocula containing 10^5–10^8 cells per milliliter were injected into them. Many distinct lesions developed in the young leaves inoculated with 10^3–10^4 cells per milliliter, but less than one lesion per square centimeter of leaf area developed in the old leaves with the lower concentrations of inocula.

Lesions developed at the injection points in all leaves and with all concentrations. They did not develop at injection points in control leaves injected with water only. This confirmed that old and young leaves were both susceptible if injury accompanied inoculation.

Volume of intercellular spaces in citrus leaves. The volumes of intercellular spaces of leaves of the same age of the different orange cultivars differed by a maximum of 3%, a difference that was considered insignificant. The average volume of intercellular space in leaf tissue with surface area of 1 cm² was calculated to be 0.0072 ml (6). Thus, an average of 36 viable bacteria were placed in each square centimeter of leaf tissue infiltrated with 5×10^3 cells per milliliter.

Infectivity titration. A linear relationship occurred between the number of bacteria placed in the mesophyll and the number of lesions that developed in leaves of grapefruit of the same age in the growth room (Fig. 1). The correlation coefficients for inoculum dose and quantitative response for 14-, 21-, and 28-day-old leaves were 0.976, 0.997, and 0.995, respectively. The regression lines calculated for 21- and 28-day-old leaves passed through the origin. However, the regression line for 14-day-old leaves did not pass through the origin when the 18.2 outlying point was included in calculations, but it did if the point was omitted. The value of 18.2 lesions probably was too low in relation to the inoculum used. When so many lesions developed, some probably coalesced and were counted as one lesion. The slopes for the regression lines were 0.75, 0.42, and 0.24 with 14-, 21-, and 28-day-old leaves, respectively.

Inoculations in the field. Leaves of nine orange cultivars that were known to differ in field resistance were infiltrated with one concentration of inoculum at 14, 24, 34, and 44 days after shoots began to grow. The actual inoculum concentration at each date was 5.5, 4.2, 6.4, and 6.0×10^3 cells per milliliter, respectively.

Significant differences (F-test, $P = 0.01$) occurred among the numbers of lesions that developed in leaves of different ages. The average adjusted-numbers of lesions per square centimeter that developed in leaves of all cultivars were 11.6, 4.4, 2.8, and 1.1 for the youngest to the oldest leaves, respectively (Fig. 2). The numbers of lesions after the second, third, and fourth inoculations were 38, 24, and 9% of the first inoculation. Thus, mesophyll resistance increased as leaves matured.

The nine cultivars of orange could be divided into susceptibility groups based on a Duncan's multiple range test analysis only with the data obtained at 14 days. The most susceptible leaves were those of cultivars Westin, Hamlin, and Petropolis; the next most susceptible included Sucral Vive and Natal; and the least susceptible were Enterprise, Valencia Wood, Valencia Frost, and Sanguinella Vil. Significant differences (F-test, $P = 0.05$) in lesions per square centimeter among cultivars did not occur after inoculations at 24, 34, and 44 days. As leaves increased in age, differences in resistance among the cultivars appear to be less.

Leaves of Hamlin, Natal, Valencia Wood, and Valencia Frost were inoculated with *X. campestris* pv. *citri* in another test. These cultivars represented the susceptibility groups determined
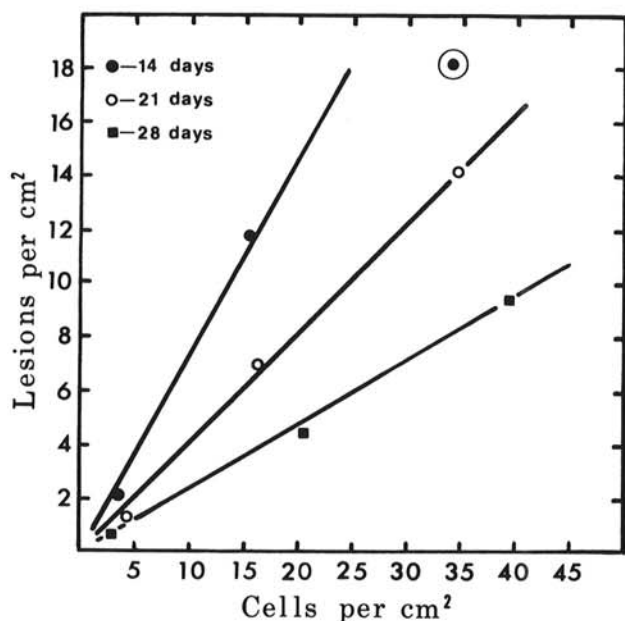

Fig. 1. Regression lines for cancrosis lesions per square centimeter in grapefruit leaves inoculated with three concentrations of *Xanthomonas campestris* pv. *citri* in three ages of leaves. The point in the circle was considered an outlier and was not used to determine the regression line for the 14-day-old leaves. The slopes for 14-, 21-, and 28-day-old leaves were 0.75, 0.42, and 0.25, respectively.
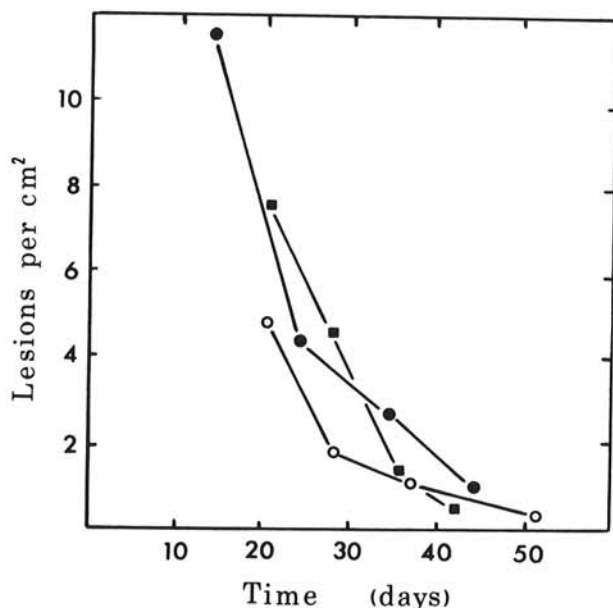

Fig. 2. The numbers of cancrosis lesions per square centimeter that developed in leaves of citrus after infiltration with suspensions containing 5 $\times 10^3$ cells of *Xanthomonas campestris* pv. *citri* per milliliter at indicated days after shoots began to expand: means of nine cultivars of orange (●—●), means of four cultivars of orange (○—○), and means of grapefruit, mandarin, and two cultivars of orange (■—■).

previously. The inoculations were made first when leaves were about 21 days old and were repeated when leaves were 28, 37, and 51 days old. The inoculum concentrations at those dates were 3.0, 4.8, 5.2, and 4.8×10^3 cells per milliliter, respectively.

Leaves of all cultivars again were significantly more resistant with increased age (Fig. 2). The average adjusted-numbers of lesions per square centimeter at each succeeding date for all cultivars were 4.8, 1.9, 1.2, and 0.3. The lesion numbers at the second, third, and fourth inoculations were 40, 25, and 6% of the first inoculation, respectively.

The numbers of lesions per square centimeter of leaf tissue at the first inoculation date in this test were relatively low but corresponded closely to the numbers of lesions that developed at the second inoculation in the preceding test. The leaves in the first inoculation in this test were only 3 days younger than those of the second inoculation in the preceding test. Significant differences among the cultivars were not observed at any inoculation date.

Marsh grapefruit, Navel and Valencia orange, and Intermedia Taller mandarin were inoculated at four ages, 7 days apart. The first inoculation date was about 21 days after the shoots began to develop. The inoculum concentration at each date was 5.1, 2.4, 4.2, and 2.1×10^3 cells per milliliter, respectively.

Fewer lesions again developed in leaves of all citrus types with increased age of leaves (Fig. 2). The average adjusted-numbers of lesions per square centimeter for all leaves at each successive inoculation date were 7.6, 4.6, 1.4, and 0.7. The numbers of lesions at the second, third, and fourth inoculations were 60, 18, and 9% of the first inoculation, respectively.

Leaves of the grapefruit had significantly more lesions per square centimeter at the first inoculation date than those of the oranges or mandarin. More lesions developed in leaves of Navel orange than in those of Valencia orange, but the difference was not statistically significant. There were no significant differences among the cultivars after the first inoculation.

DISCUSSION

Leaves of plants commonly change in resistance to pathogens with time (18). The change from susceptibility to resistance to cancrosis of citrus is particularly evident. The mechanism of the resistance was thought to be localized at the leaf surface because injury nullified the resistance (12). The stomata of leaves can be considered the first line of defense against bacterial plant pathogens. The amount of inoculum that moves into the mesophyll area may be limited by numbers of stomata or by the size and shape of the stomatal pore (10). The population of *X. campestris* pv. *citri* that occurred naturally in rainwater on leaves (between 10^4 and 10^6 cells per milliliter) resulted in confluent necrosis if infiltrated into leaves (20). Confluent necrosis was not seen in nature, probably because only small quantities of rainwater enter the leaves. The forces that prevent movement of relatively large amounts of water containing bacteria through stomatal pores are not defined.

A second heretofore overlooked line of defense against citrus cancrosis is the resistance in the mesophyll. Injection of inocula into the mesophyll bypassed leaf-surface resistance and did not cause enough injury to nullify mesophyll resistance. Mesophyll resistance was strongest in mature leaves and was demonstrated by the infectivity titration system (1), which compares the relationship of inoculum density with the quantitative response in grapefruit leaves of different ages. The decrease in slope of inoculum density/disease incidence regression lines as leaves aged is consistent with an increase in resistance.

The purpose of this report was not to compare the relative importance of the two lines of defense to cancrosis. However, the mesophyll resistance must play an important role. The close agreement of the time needed for development of resistance in the mesophyll as reported here and the susceptibility period of leaves determined in experiments on timing of sprays for control of cancrosis (19) is evidence for that importance. It also seems highly unlikely that bacteria could be completely excluded from the mesophyll of old leaves by stomatal morphology. Ingress of bacteria through "closed" stomata of corn was demonstrated by

Gitaitis et al (4). Yet, visible lesions of cancrosis do not occur in mature leaves of citrus in nature unless injury occurs with inoculation.

Very little infection of citrus leaves occurred with *X. campestris* pv. *citri* in nature during the first 14 days of leaf development as determined by experiments on timing of sprays for control (19). It is not known whether mesophyll resistance or some other mechanism is responsible for very little infection in leaves that have not fully expanded. Inoculations of citrus leaves by injection could not be made up to 14 days after the beginning of shoot development because of physical limitations. Shoots were about 85% fully expanded by that time (19).

Different numbers of lesions developed after injections with low numbers of cells of *X. campestris* pv. *citri* into leaves of citrus cultivars and species between 14 and 21 days after shoots began growth. A ranking of the citrus types according to number of lesions per square centimeter after injection inoculation was very similar to a ranking of the types for resistance under natural conditions (21), but there were exceptions. Use of several inoculum doses with each age of leaf might have given more accurate rankings. However, important reasons for the exceptions were thought to be errors in selection of shoots of the same age and variation of some environmental factors in the field which affected the rate of maturation of leaves. If more attention is given to uniformity of the host, it may be possible to evaluate cultivar resistance to cancrosis after infiltration of young leaves with low doses of inoculum.

The fact that the number of lesions that developed at each inoculation date was dependent upon the inoculum concentration underscores the importance of determining the number of bacteria in inocula in each test for comparisons of mesophyll resistance among cultivars. Viability of low numbers of bacteria was maintained in autoclaved tap water in our experiments. Populer (18) discussed the importance of reproducibility of quantitative inoculations.

Boelema (1) gave some biological explanations for why only certain bacterial cells can multiply and cause lesions. This work supports the hypothesis that cells of the mesophyll have areas that support growth and areas that do not support growth of cells of *X. campestris* pv. *citri*. These areas may be designated as susceptible sites and resistant sites. The proportion of resistant to susceptible sites increased during maturation of citrus leaves. Some susceptible sites probably existed even on very mature cells because high inoculum doses resulted in necrosis of mature leaves. The chances of a bacterium contacting a rare susceptible site would increase as the number of bacteria per unit volume of inoculum was increased.

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