

カンキツ葉細胞間汁液中のアミノ酸によるXanthomonas campestris pv. citriの増殖抑制とその解除

誌名	日本植物病理學會報 = Annals of the Phytopathological Society of Japan
ISSN	00319473
著者名	後藤,正夫 山中,勝司
発行元	日本植物病理學會
巻/号	47巻5号
巻号補足	
掲載ページ	p. 618-626
発行年月	1981年12月

農林水産省 農林水産技術会議事務局筑波産学連携支援センター
Tsukuba Business-Academia Cooperation Support Center, Agriculture, Forestry and Fisheries Research Council
Secretariat



Growth Inhibition of *Xanthomonas campestris* pv. *citri* and Its Reversal by Amino Acids Found in the Intercellular Fluids of Citrus Leaves

Masao GOTO* and Katsushi YAMANAKA*

後藤正夫*・山中勝司*: カンキツ葉細胞間汁液中のアミノ酸による *Xanthomonas*
campestris pv. *citri* の増殖抑制とその解除

Abstract

Twenty-one amino acids were identified in the intercellular fluids taken from healthy citrus leaves. Quantitative analysis was made on the changes in the concentration of these amino acids after inoculation of *Xanthomonas campestris* pv. *citri*. The amino acids detectable in a significant amount in citrus leaf tissues were tested for their role in bacterial growth. Met was an essential factor needed for growth of the bacterium and the optimum concentration was in the range of 0.05 to 0.1 μ mol per ml which corresponded to the amount detected in the intercellular fluids. In the presence of met, several amino acids such as asp, asn, glu, val, leu, and pro were utilized by the bacterium as nutrient. Pro was particularly important because it was the major amino acid found in the intercellular fluid of citrus leaves, and it increased rapidly after the infection of citrus trees with the bacterium. The concentration several days after inoculation became 10 times higher than that of non-inoculated healthy leaves. On the other hand, ser and hyls were also important because of their ability to inhibit the growth of the bacterium. This activity was, however, prevented by the presence of two amino acids, met and pro, at concentrations equivalent to those in the intercellular fluids. Sensitivity to ser and hyls was higher in the virulent isolate than that in the avirulent isolate. Pro prevented more effectively the growth inhibition of the virulent isolate than that of the avirulent isolate. The activity of pro synergistically increased by the coexistence of ala, although ala itself did not have such an effect. The concentration of pro in the leaves of *Fortunella japonica*, resistant to citrus canker, was lower than that of susceptible *Citrus natsudaidai* leaves, but the reverse phenomenon was observed with ser. The higher ratio of ser: pro may be responsible for induction of the strong resistance of this plant to citrus canker.

(Received May 9, 1981)

Introduction

Citrus canker epidemics occur continually in a severe form on younger trees which produce abundant autumn or angular shoots. The disease becomes less severe or even disappears when the trees mature and start to bear fruits. Decrease in disease incidence is pronounced where functional windbreaks are provided to protect citrus trees from storms. However, the disease has not yet been eradicated and sporadic epidemics occur every ten or fifteen years. Moderate resistance of Satsuma orange is easily broken down under these epidemics. These sudden outbreaks of citrus canker have been attributed to

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the increase of inoculum in fields established under warm climate from late autumn to early spring. Warm winter is assumed to affect the physiology of citrus leaves in being conducive to the development of the disease, although nothing has so far been elucidated from physiological point of view. This is partly due to the scarcity of information on host-parasite interaction in citrus canker.

We have already demonstrated that various amino acids and electrolytes are released into the intercellular spaces when susceptible citrus leaves are inoculated and the rates of leakage differ depending on the kind of amino acids, pro being most predominant.²⁾ It is interesting to note that bacterial infection induces such selective accumulation of amino acids in the citrus leaves. Some phytopathogenic bacteria have been reported to be inhibited in various degrees by the presence of certain amino acids, but the inhibition was reversed by the addition of some other amino acids. Therefore, there may be some important implications suggesting that the differential diffusion of amino acids plays a role in the host-parasite interactions of citrus canker.

This paper deals with the results of a study on interactions among various amino acids released by diseased citrus leaves in terms of growth inhibition of *Xanthomonas campestris* pv. *citri* (Hasse) Dye and its reversal.

Materials and Methods

Bacteria. Two strains, U9-1 and U37, of *X. campestris* pv. *citri* used in the study were originally isolated from diseased leaves of Unshu (*Citrus unshu* Marcov.). Strain U9-1 was strongly virulent producing typical canker lesions on leaves of various *Citrus* spp., whereas strain U37 was avirulent. Virulence of the latter was lost during subculturing on potato-peptone-sucrose agar at 4 C for some 20 years. This strain has, however, retained differential characteristics such as the inability to utilize mannitol and susceptibility to the host specific phage Cp2 and the temperate phages of *X. campestris* pv. *citri*, which were useful for the identification of this bacterium.

Media. All chemicals were obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan. All media were adjusted with N NaOH or N HCl to pH 6.8. The composition of the media is as follows. Peptone-sucrose broth: peptone 10 g, sucrose 10 g, deionized water 1,000 ml. Peptone-sucrose agar: peptone-sucrose broth with agar 15 g per 1,000 ml. Synthetic basal medium: (NH₄)₂SO₄, 2.5 g; KH₂PO₄, 1.0 g; MgSO₄·7H₂O, 0.5 g; CaCl₂·2H₂O, 0.1 g; NaCl, 0.1 g; glucose, 10 g; distilled water, 1,000 ml. Synthetic methionine medium: methionine was added to the synthetic basal medium at the concentration of 0.01 μmol per ml which corresponded to the concentration in the intercellular fluid of citrus leaves.

Plants. Two-year-old seedlings of Natsudaidai (*Citrus natsudaidai* Hayata) were grown in a greenhouse and their fully expanded leaves were used for inoculation. The leaves of Kinkan (*Fortunella japonica* Swingle) for amino acid analysis were provided by Dr. A. Yamaguchi, Okitsu Branch, Fruit Tree Research Station, Shimizu, Shizuoka Prefecture.

Determination of bacterial growth in liquid media. The bacterium was grown in liquid media shaken on a shaker (Iwashiya Co. Ltd. Tokyo) with 7 cm amplitude at the frequency of 117 strokes per min at 28 C for 2 days. Bacterial growth was determined by the turbidity measured with a Hirama photoelectric colorimeter at the wavelength of 660 nm.

Growth inhibition of *X. campestris* pv. *citri* by amino acids at different concentrations. Amino acid to be tested was added at various ratios to the synthetic basal medium to which met and pro were supplemented at the concentrations of 0.01 and 1.0 μmol per ml, respectively, which corresponded to those in the intercellular fluid of diseased citrus leaves.

Paper disk tests. Sterilized paper disks (8 mm in diameter and 2 mm thick, Toyo Roshi Co. Ltd., Tokyo) were dipped in each amino acid solution and dried in vacuum. They were placed on the surface of agar plates seeded with *X. campestris* pv. *citri* and incubated at 28 C until growth inhibition zones became visible. The concentration of amino acids was adjusted so that the disks contained 10 μmol amino acid. To test the reversal activity of growth inhibition, the disks with the challenging amino acids were placed on agar plates close to those with the amino acids responsible for growth inhibition so that the former were located within the inhibition zone due to the latter. When growth occurred around the challenge disks in the inhibition zone, the amino acids were estimated to be effective for preventing the inhibition.

Amino acid analysis of the intercellular fluids. The methods for sampling the intercellular fluids from citrus leaves and for assaying the amino acids were the same as those described in the previous papers^{1,2)}.

Results

Analysis of amino acids in the intercellular fluids taken from citrus leaves

Amino acid concentration was determined with the intercellular fluids taken from citrus leaves 3 and 5 days after inoculation, and healthy leaves as the control, respectively. The results are shown in Fig. 1. Among the 21 amino acids identified in the intercellular fluids, the major amino acid was pro as previously reported²⁾. Although amino acid concentration increased with the time after inoculation, the rate of increase differed depending on the kind of amino acids. Significant increase was observed in ser, asn, pro, gly, tyr, GABA, and hyls. The accumulation of pro was particularly pronounced, reaching about 10 times the concentration of healthy leaves. The concentration

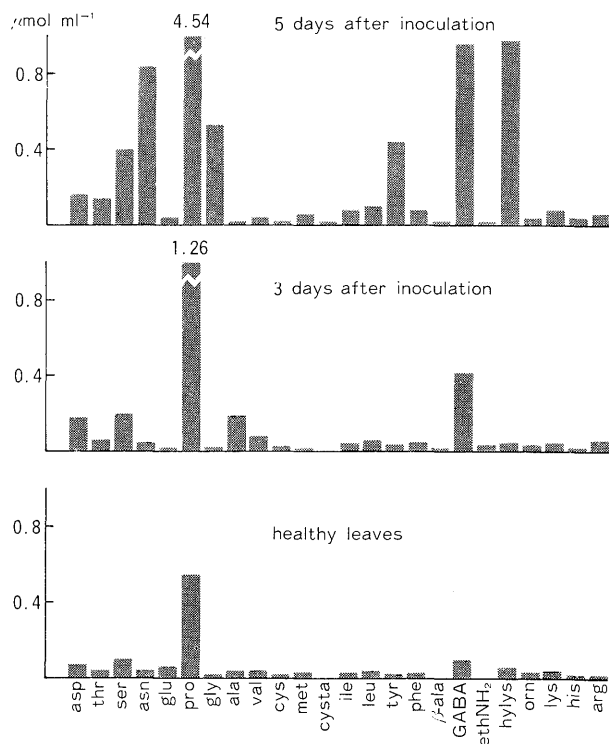


Fig. 1. Amount of free amino acids ($\mu\text{mol ml}^{-1}$) in intercellular fluid of Natsudaikai leaves inoculated with *Xanthomonas campestris* pv. *citri* at different time interval after inoculation

of the remaining amino acids unchanged or only slightly increased.

Effect of amino acids on growth of *X. campestris* pv. *citri*

The amino acids detected in the intercellular fluid of diseased citrus leaves were individually added to the synthetic methionine medium at the concentration of 1 mg per ml. The results are shown in Fig. 2. Distinct growth inhibition was induced by thr, ser, gly, β -ala, and hylys. Conversely, growth increase was achieved by the addition of amino acids such as asp, asn, glu, pro, val, orn, lys, his, leu and arg. Although the rate of growth enhancement of pro was not considerable, this amino acid was thought to play a major role in the multi-

plication of the pathogen in the citrus leaves because it showed the highest concentration in the intercellular fluids taken from diseased citrus leaves.

The results were similar to those obtained with paper disks. Bacterial growth was induced only by methionine. With the synthetic methionine medium, however, growth was intensified around the disks carrying amino acids such as asp, asn, glu, ala, leu, and his. Growth inhibition zones were formed around the disks containing ser, gly, hylys, but not thr and β -ala. Pro, the major amino acid found in citrus leaves, did not induce bacterial growth when it was added singly or together with met to the synthetic medium at this concentration. Although the mixture of 21 amino acids induced bacterial growth on the synthetic basal medium, growth did not occur when met was omitted from the mixture. This fact indicates that this amino acid is essential for the growth of *X. campestris* pv. *citri*.

Effect of methionine on the growth of *X. campestris* pv. *citri*

Growth of *X. campestris* pv. *citri* was studied in the synthetic basal medium to which met had been added at various concentrations (Fig. 3). The maximum growth of the virulent isolate U 9-1 was obtained at the methionine concentration of 0.05 to 0.1 μ mol per ml which was two times lower than that of the avirulent isolate U 37. Growth was gradually decreased as with the increase in the concentration of met. Thus, the virulent isolate grew well in the presence of a lower concentration of met compared with the avirulent one.

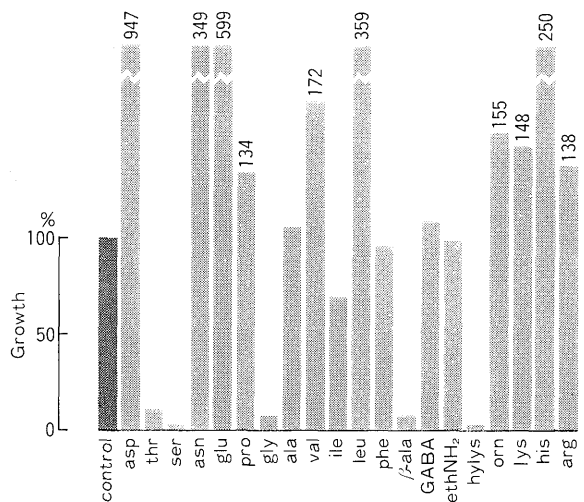


Fig. 2. Growth rate of *X. campestris* pv. *citri* in the synthetic methionine medium added with the individual amino acid at the ratio of 1 mg ml⁻¹. Turbidity measured 48 hr after inoculation was the mean value of 5 repetition which was expressed by the percent turbidity of the control.

Growth inhibition of X. campestris pv. citri by serine

Ser was added at various concentrations to the synthetic basal medium to which met and pro had been supplemented and its effect on growth inhibition was examined (Fig. 4). Growth of the virulent isolate U9-1 was greatly depressed at a concentration of $0.1 \mu\text{mol}$ per ml and became negligible at a concentration of $0.5 \mu\text{mol}$ per ml. However, the growth inhibition of the avirulent isolate U37 took place at half the concentration in the isolate U9-1. Thus, the growth inhibition by ser was more effectively demonstrated in the avirulent strain than in the virulent one.

Survey of amino acids effective in reversing growth inhibition due to serine by using paper disk method

Twenty amino acids were individually examined for their biological effectiveness to prevent growth inhibition by ser. Reversion was observed only in pro, although the growth was enhanced when it was mixed with met and ala. This fact was also confirmed by deletion tests. Bacterial growth could not be induced only when pro was removed from the mixture of 21 amino acids.

Effectiveness of proline in preventing growth inhibition by serine

Pro was added at various concentrations to the synthetic medium containing met and

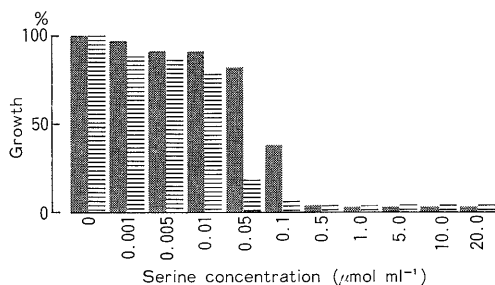


Fig. 4. Growth inhibition of *X. campestris* pv. *citri* by serine added at various ratio to the synthetic basal medium containing methionine ($0.01 \mu\text{mol ml}^{-1}$) and proline ($1.0 \mu\text{mol ml}^{-1}$), respectively. Growth shown by turbidity was the mean value of 3 repetition.

■ virulent isolate U9-1
 ≡ avirulent isolate U37

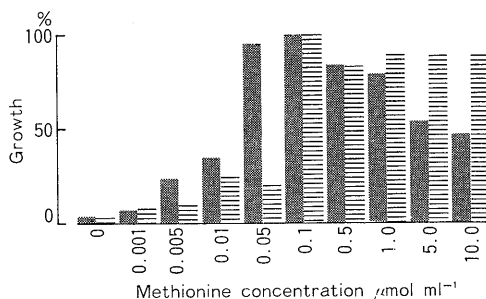


Fig. 3. Effect of methionine added to the synthetic basal medium at various concentration on growth of *X. campestris* pv. *citri*.

Turbidity taken 48 hr after inoculation was the mean value of 3 repetition which was expressed by the percent turbidity of the maximum obtained with the virulent and avirulent isolates, respectively.

■ virulent U9-1 isolate
 ≡ avirulent U37 isolate

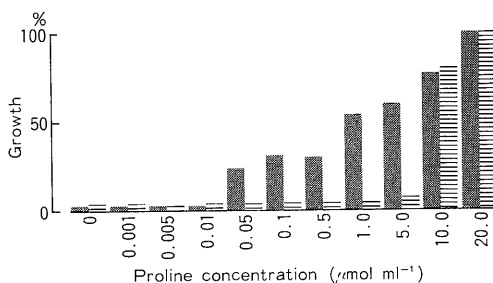


Fig. 5. Effect of proline on the reversal of growth inhibition of *X. campestris* pv. *citri* due to serine. The synthetic basal medium contained methionine ($0.01 \mu\text{mol ml}^{-1}$) and serine ($0.1 \mu\text{mol ml}^{-1}$), respectively. The tests were repeated 3 times.

■ virulent isolate U9-1
 ≡ avirulent isolate U37

ser at concentrations of 0.01 and 0.1 μmol per ml, respectively (Fig. 5). Growth of the virulent isolate U 9-1 occurred at the pro concentration of 0.05 μmol per ml and progressively increased as pro content rose.

In the avirulent isolate U 37, however, growth was obtained at the concentration of 10 μmol per ml, which was 200 times higher than that in the virulent isolate. Thus, pro appears to be more effective in blocking growth inhibition in the virulent isolate compared with the avirulent one.

Growth inhibition of *X. campestris* pv. *citri* by hydroxylysine and its reversal by proline

Hyls was added at various concentrations to the synthetic medium containing met and pro at the concentrations of 0.01 and 1.0 μmol per ml, respectively (Fig. 6). A similar trend of growth inhibition was observed between the virulent U 9-1 and avirulent isolate U 37. Growth was remarkably depressed at concentrations ranging between 0.05 and 0.1 μmol per ml, becoming insignificant at a concentration higher than 0.5 μmol per ml. Reversal of the inhibition was tested by adding pro at various concentrations to the synthetic medium containing met and hyls at the concentrations of 0.01 and 0.05 μmol per ml, respectively (Fig. 7). Growth of the virulent isolate U 9-1 was sharply increased with the addition of pro at concentrations higher than 0.1 to 0.5 μmol per ml, whereas the reversal in the avirulent isolate U 37 was poor. Growth in the isolate U 37 corresponded to that of U 9-1 in the medium with about a concentration of pro 50 times higher.

Growth inhibition by the mixture of threonine, glycine and β -alanine, and its reversal by proline

The mixture of three amino acids was added to the synthetic basal medium containing met and pro (Fig. 8). Growth of the virulent isolate U 9-1 was inhibited at a concentration of 5 μmol per ml although a 30% depression was observed at concentrations ranging from 0.005 to 1.0 μmol per ml. The growth of the avirulent isolate U 37 decreased at

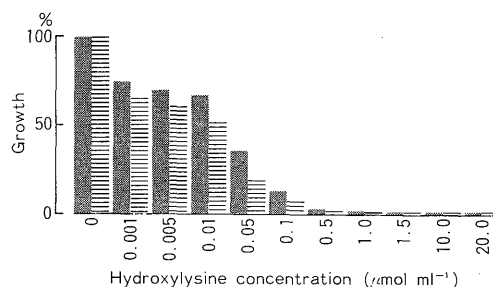


Fig. 6. Growth inhibition of *X. campestris* pv. *citri* by hydroxylysine added at various ratio to the synthetic basal medium containing methionine (0.01 $\mu\text{mol ml}^{-1}$) and proline (1.0 $\mu\text{mol ml}^{-1}$), respectively. The tests were repeated 3 times.

■ virulent isolate U9-1
 ▨ avirulent isolate U37

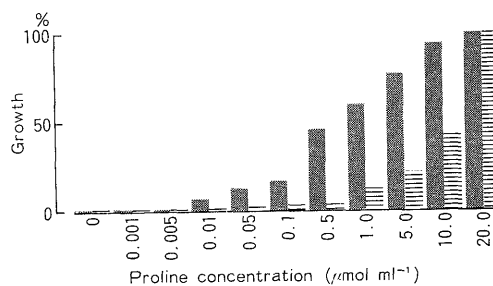


Fig. 7. Effect of proline on the reversal of growth inhibition of *X. campestris* pv. *citri* due to hydroxylysine. The synthetic basal medium contained methionine (0.01 $\mu\text{mol ml}^{-1}$) and hydroxylysine (0.05 $\mu\text{mol ml}^{-1}$), respectively. The tests were repeated 3 times.

■ virulent isolate U9-1
 ▨ avirulent isolate U37

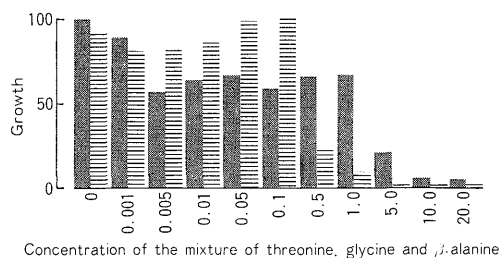


Fig. 8. Growth inhibition by the mixture of threonine, glycine and β -alanine added at various ratio to the synthetic basal medium containing methionine ($0.01 \mu\text{mol ml}^{-1}$) and proline ($1.0 \mu\text{mol ml}^{-1}$), respectively. The tests were repeated 3 times.

■ virulent isolate U 9-1
 ≡ avirulent isolate U 37

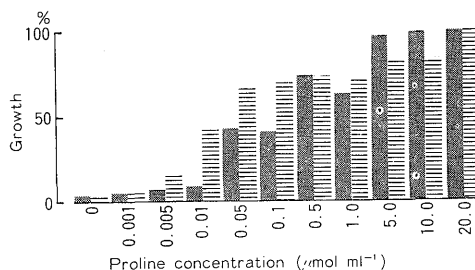


Fig. 9. Effect of proline on the reversal of growth inhibition of *X. campestris* pv. *citri* due to the mixture of threonine, glycine and β -alanine. The medium contained ($0.01 \mu\text{mol ml}^{-1}$) and the mixture of three amino acids ($0.1 \mu\text{mol ml}^{-1}$), respectively. The tests were repeated 3 times.

■ virulent isolate U 9-1
 ≡ avirulent isolate U 37

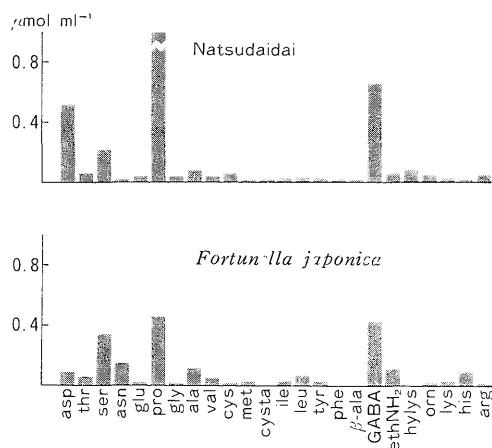


Fig. 10. Amount of free amino acids ($\mu\text{mol ml}^{-1}$) in the supernatant of leaf homogenate of Natsudaaidai and *Fortunella japonica*

Fortunella japonica which are susceptible and resistant to citrus canker, respectively (Fig. 10). *F. japonica* had the higher ser and lower pro content compared with *C. natsudaaidai* resulting in the higher ratio of ser: pro in the former. This fact indicates that the growth inhibition due to ser may be induced more strongly in *F. japonica* than in *C. natsudaaidai* because the content of pro in the latter is sufficient enough to reverse the growth suppression by ser.

Discussion

It has already been reported that homoserine strongly inhibits phytopathogenic pseudo-

concentrations higher than $0.5 \mu\text{mol}$ per ml. The inhibition due to the mixed amino acids was reversed by the addition of pro at the ratio of $0.01 \mu\text{mol}$ per ml or above (Fig. 9). Thus, the inhibition by the mixture of three amino acids occurred on considerably higher concentrations and was reversed on relatively lower concentrations of pro compared with the inhibition due to ser and hylys.

Assay of amino acids in leaf homogenates of susceptible *C. natsudaaidai* and resistant *Fortunella japonica*

Amino acid concentrations in the supernatants of citrus leaf homogenates were determined with *C. natsudaaidai* and

monads of the *P. syringae* and *P. cichorii* group, but not the saprophytic pseudomonads, whereas cysteine uniformly inhibits all the pseudomonads. Certain strains of the *P. syringae* group were also inhibited by thr, val, gly, ala, and a mixture of phe and tyr⁵⁾. The inhibition was prevented by the presence of met, thr, and lys which are the biosynthetic pathway's end products of the inhibiting amino acids. Growth inhibition of the pathogens due to amino acids present in the plant tissues was thought to be responsible for the induction of host resistance^{3,5,6,7)}.

The interaction between *X. campestris* pv. *citri* and host tissues induces the release of electrolytes as well as amino acids which are needed for bacterial multiplication. The selective accumulation of certain amino acids was assumed to reflect the altered amino acid biosynthesis in host cells because the amino acid patterns of the intercellular fluid were similar to those of the supernatant of the leaf homogenate^{1,2)}.

Amino acids, which had a biological activity in relation to the host-parasite interaction of citrus canker, included met, ser, hyls, thr, gly, β -ala and pro. The biological activity of these amino acids found in the intercellular fluid was divided into three categories, i. e., growth stimulation, growth inhibition and reversal of growth inhibition. Although the concentrations of GABA, asn and tyr were also significantly increased in the infected citrus leaves, the role of these amino acids in the host-bacterium interaction remains to be elucidated. Since GABA plays a significant role in the pathogenesis of crown gall⁴⁾, it may be involved in the hormone metabolism during the course of disease development of citrus canker. This subject will be taken up in future studies.

Met was required by the bacterium as an essential growth factor. As in the case of *X. campestris* pv. *oryzae*⁸⁾, the citrus canker organism seemed to lack the enzyme systems for synthesizing methionine from inorganic sulphate. The increase in the release of growth stimulating amino acids such as asp, asn, glu, pro, val, and leu was accompanied by the increase in growth inhibiting amino acids such as ser, hyls, or gly. The concentrations of these inhibiting amino acids were high enough to depress bacterial multiplication.

The causal bacterium induces the synthesis of several amino acids in the host tissues and releases them into the intercellular spaces to prevent the growth inhibition by the above amino acids. Pro as the major amino acid of the intercellular fluid of healthy citrus leaves was particularly important in this respect because its natural concentration was effective enough to prevent inhibition. Since the biosynthesis of pro quickly responded to the infection of *X. campestris* pv. *citri*, the interaction between ser and pro may play an important role in the susceptibility of citrus leaves to canker disease. This was also substantiated by the fact that the avirulent isolate was more sensitive to ser than the virulent one, requiring higher concentrations of pro to reverse the inhibition. Among the five growth inhibitory amino acids, the most important ones were ser and hyls because the concentrations of these two amino acids in the intercellular fluids were high enough to inhibit bacterial growth even in healthy leaves. Ser was particularly important because it quickly increased after inoculation, reaching concentrations two to three times higher than the minimum concentration required to effectively inhibit bacterial growth. Thr, gly, and β -ala seemed to be less important because the concentrations of these amino acids even 5 days after inoculation were less than 1/10 of minimum concentrations required for growth inhibition. The reason why pro reverses the growth inhibition by ser has not yet been determined.

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和文摘要

カンキツ葉細胞間汁液中のアミノ酸による *Xanthomonas campestris*
pv. *citri* の増殖抑制とその解除

後藤正夫・山中勝司

健全なナツミカン葉細胞間汁液(IF)中に見出される21種のアミノ酸について、かいよう病菌 *X. campestris* pv. *citri* の感染に伴う濃度変化を定量的に調べ、高濃度の主要アミノ酸について、病菌増殖との関係を調べた。本病菌は増殖にメチオニンを必須成分として要求した。その最適濃度は $0.05 \sim 1.0 \mu\text{mol/ml}$ で、IF はこれに匹敵するメチオニンを含有した。メチオニンの存在下でアスパラギン酸、アスパラギン、グルタミン酸、バリン、ロイシン、プロリン等が IF 濃度で有効に増殖に利用された。特にプロリンは IF に多量に含まれ、しかも接種後数日で約10倍量に増加する点で、重要な栄養源と考えられた。一方セリンおよびヒドロキシリジンは IF 内濃度でかいよう病菌の増殖を顕著に抑制した。病原性菌株は非病原性菌株に比較して低濃度で増殖抑制を受けた。この抑制作用は IF 内濃度に相当するメチオニンとプロリンの存在下で解除された。プロリンによるこの増殖抑制の解除は、アラニンの共存下で顕著になり、しかも非病原性菌株よりも病原性菌株に対して効果的に現われた。かいよう病抵抗性のキンカン葉磨碎汁は、ナツミカンに比べてプロリン濃度が低く、セリン濃度は高い数値を示したことから、両アミノ酸の濃度比の相違が、両カンキツのかいよう病抵抗性の差の一因をなしているものと考えられた。