ANALYTICAL STUDY OF IPOMEAMARONE & CHLOROGENIC ACID ALTERATIONS IN SWEET POTATO ROOTS INFECTED BY CERATOCYSTIS FIMBRIATA [1, 2]

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Several metabolic alterations have been studied, from biochemical and pathological viewpoints, in sweet potato roots infected by the black rot fungus, *Ceratocystis fimbriata* (2, 11, 13), among which the formation of ipomeamarone is of particular interest. Because this substance is a unique furanoterpenoid produced by host tissues in response to infection, and because it has remarkable biological properties, such as uncoupling and antipathogenic actions, elucidation of its biosynthetic mechanism would be of great value for the further study of terpene formation and of the host-parasite relationship. Furthermore, an increase of polyphenolic substances, e.g., chlorogenic acid, has been commonly observed in diseased plants (6, 10, 14), and some workers have implied that these play an important role in the defense mechanism, although contradictory evidence has been presented (5, 11). We have undertaken an analytical study of ipomeamarone and chlorogenic acid (3). However, recent chromatographic evidence (1) showing a concurrent formation of many ipomeamarone-like terpenoids in injured root tissues indicates the necessity of a more critical analysis of such metabolites in host tissues. The present paper deals with the quantitative analyses of ipomeamarone and chlorogenic acid in the infected root tissues of resistant and susceptible sweet potato varieties, and their possible role in the defense mechanism.

MATERIALS & METHODS

FUNGUS INOCULATION & PREPARATION OF SAMPLE: Sweet potato roots grown at the farm of the National Agricultural Experiment Station, Konosu, Saitama, were sent to us after harvest in October 1959. They were stored at 10° C until used. During this period neither microbial damage nor sprouting was visible. Varieties Norin no. 1 and no. 10 were selected as exemplifying varieties resistant to the black rot fungus and Norin no. 2 and no. 5 as susceptible ones. The basic procedure for fungus inoculation of root tissues and the subsequent handling was as follows. Individual roots were cut into two pieces lengthwise, and both halves cut lengthwise in 1.5 to 2.0 cm thick slices. One half was the uninoculated control sample; the other half of the slices was treated with a dense water suspension of the black rot fungus prepared from slant cultures, and both groups were then incubated for 5 days at 30° C. The relative humidity was kept 100 % throughout this period. The fungus grew and spread quite uniformly on the root slices and gradual penetration occurred. However, there was a significant difference in the symptoms exhibited by the resistant and susceptible varieties. In the former the successful penetration of fungus was prevented concomitantly with the appearance of the typical necrotic browning of the infected tissue after 72 hours. In the latter case, mycelial growth was more extensive and the necrotic reaction was less pronounced. At 24-hour intervals, infected tissue and uninfected tissue, about 0.5 to 1.0 mm thick adjacent to the infected portion, was harvested for chemical analysis of ipomeamarone and chlorogenic acid. For the analysis of chlorogenic acid in the uninoculated sample, sections 1.0 mm in thickness from the inner part of the sliced tissue were also taken. The thickness of the infected tissue increased as the infection developed: about 0.5 mm at 24 hours, 0.5 to 1.0 mm at 48 hours, and 1.0 mm afterwards in the case of the resistant variety. In the case of the susceptible variety, it was about 0.5 mm at 24 hours and 1.0 mm thereafter. It was desirable to avoid contaminating the adjacent tissues in handling infected and uninfected tissues, but a minor inclusion was technically inevitable. At the 24-hour period the former included a negligible portion of the latter since fungus growth was slight. On the contrary, at the later stages of infection, a slight contamination of the necrotized flecks in uninfected tissue was unavoidable. Thus, a careful interpretation of the analytical data obtained was necessary.

CHEMICAL ANALYSIS: To 0.5 g (fr wt) of each sample was added 5 ml of 95 % ethanol, and the tissue homogenized with the Potter-Elvehjem glass homogenizer. The resulting suspension was gently boiled (with the aid of a reflux condenser) in a hot water bath for 30 minutes, and filtered after cooling. A 0.1 ml aliquot was added to a silica gel chromatostrip with a micro-pipette for both the qualitative examination of component substances and the quantitative estima-

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tion of ipomeamarone. Details of the experimental procedure for the chromatostrip technique were given in a previous paper (1, 4). Its application to the quantitative analysis was developed in a manner similar to that of Stanley and Vannier for the analysis of coumarins in citrus oil (9). Since Ehrlich's reaction is rather unstable, a photograph was taken immediately after spraying the color reagent on the strip. In order to determine the magnitude of ipomeamarone synthesis, the portion of the silica gel containing this component was located after the development of strip, eluted with pure methanol, and the carbonyl value determined according to the colorimetric method of Lappin and Clark (7). Its quantity can be calculated from a standard curve obtained from data using pure ipomeamarone. Both pure and crude samples of ipomeamarone used as the reference were prepared according to a previous paper (1).

The ethanol extract was placed on an alumina column and the amount of chlorogenic acid determined by the method of Zucker and Ahrens (15). The calibration curve plotted from data using recrystallized chlorogenic acid and pure isochlorogenic acid was practically the same. The series of analyses of those compounds were carried out using one piece of root per experiment, and quite comparable results were repeatedly obtained. In every experiment analyses were carried out in duplicate and the averaged value presented.

Treatment of Sweet Potato Roots With Poisonous Chemicals: Sliced healthy root tissues were treated with 0.1 % $HgCl_2$ containing 5 % NaCl, or 0.1 % monoiodoacetate following the method of Uritani et al (13). Five per cent NaCl was used as the control reagent. Two additional applications were given 12 and 24 hours later. Forty-eight hours after the first treatment, the tissues, about three to five mm thick, were completely degenerated in the case of the former two chemicals. Both the degenerated and the adjacent tissues were subjected to analysis in the same manner and used above for the infected tissues. NaCl-treated tissues did not show any marked change in appearance.

Results

Chromatographic Study of Ipomeamarone Formation: Figure 1 represents the time-course formation of ipomeamarone and other Ehrlich's reagent-positive substances in infected tissue examined

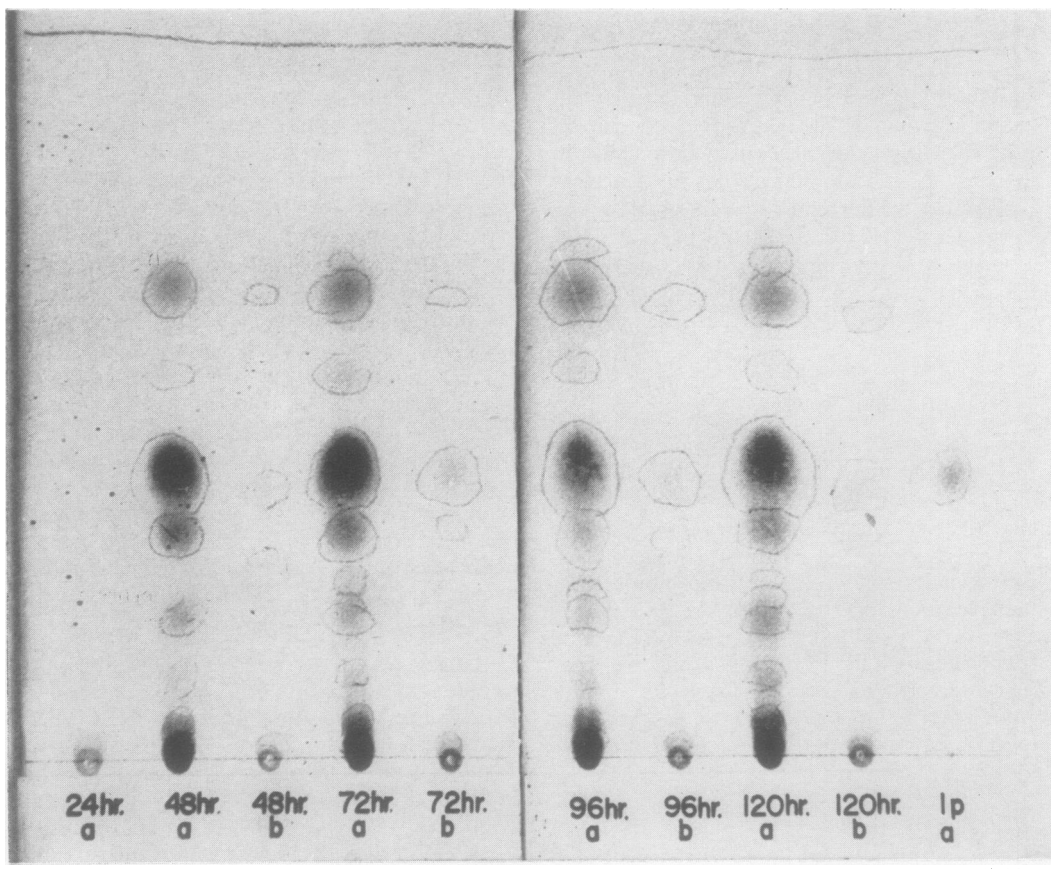

Fig. 1A. Chromatographic examination of the formation of ipomeamarone and other related Ehrlich's reagent-positive substances in sweet potato roots as a function of infection period (hr). (A): Resistant variety Norin no. 1.

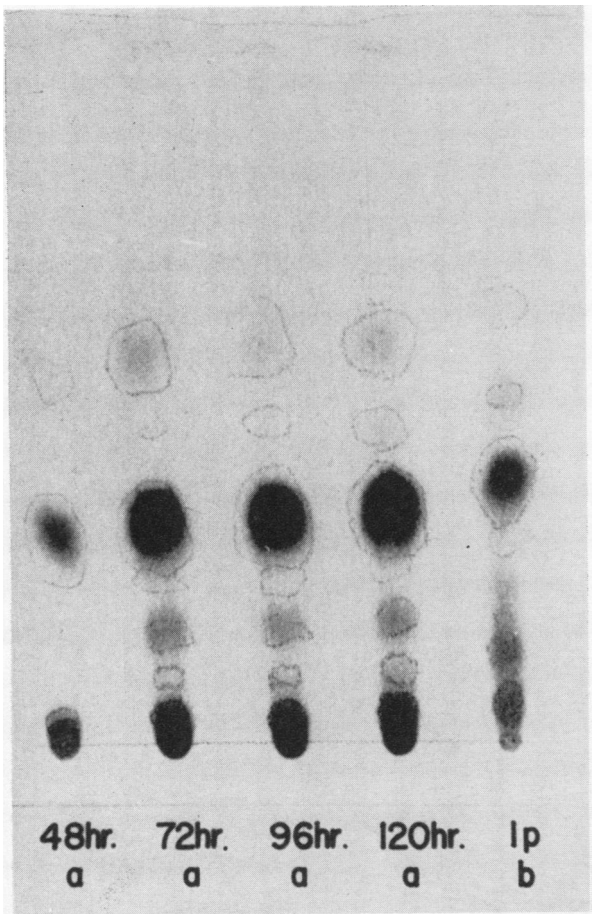

FIG. 1B. Chromatographic examination of the formation of ipomeamarone and other related Ehrlich's reagent-positive substances in sweet potato roots as a function of infection period (hr).

(B): Susceptible variety Norin no. 2. (a) is the infected tissue and (b) the tissue adjacent to this region. Ip. (a) and (b) represent pure and crude ipomeamarone. respectively.

by silica gel chromatostrip. It should be pointed out that, in a qualitative sense, the synthetic pattern of these substances was very similar in both the susceptible and resistant variety. Another important point is that there was almost no visible synthesis of either ipomeamarone or other similar compounds during the initial 24 hours, and a rather striking synthesis in the subsequent 24 hours, thus showing an induction of the drastic metabolic disturbance in host tissues 48 hours after fungus inoculation. After this period little change of the chromatographic picture was seen in either variety. In spite of such similarity in the chromatographic pattern, synthesis of each component in the resistant variety seems to be greater than that in the susceptible one. Formation of Ehrlich's reagent-positive substance in uninfected tissue was very faint and only the results obtained using the resistant variety are shown in figure 1.

QUANTITATIVE DETERMINATION OF IPOMEAMARONE SYNTHESIS: Figure 2 represents a typical result showing the rate of ipomeamarone synthesis in inoculated root tissues. Two points deserve attention. First, in both the resistant and susceptible varieties, there was a lag phase (24 hr) which agrees with the preceding chromatographic evidence. Second, the rate of ipomeamarone synthesis during the two following 24-hour periods was more striking in the resistant than in the susceptible variety. This point is more clearly summarized in table I in which analytical data for four varieties are presented. Synthesis in the highly resistant variety, Norin no. 10,

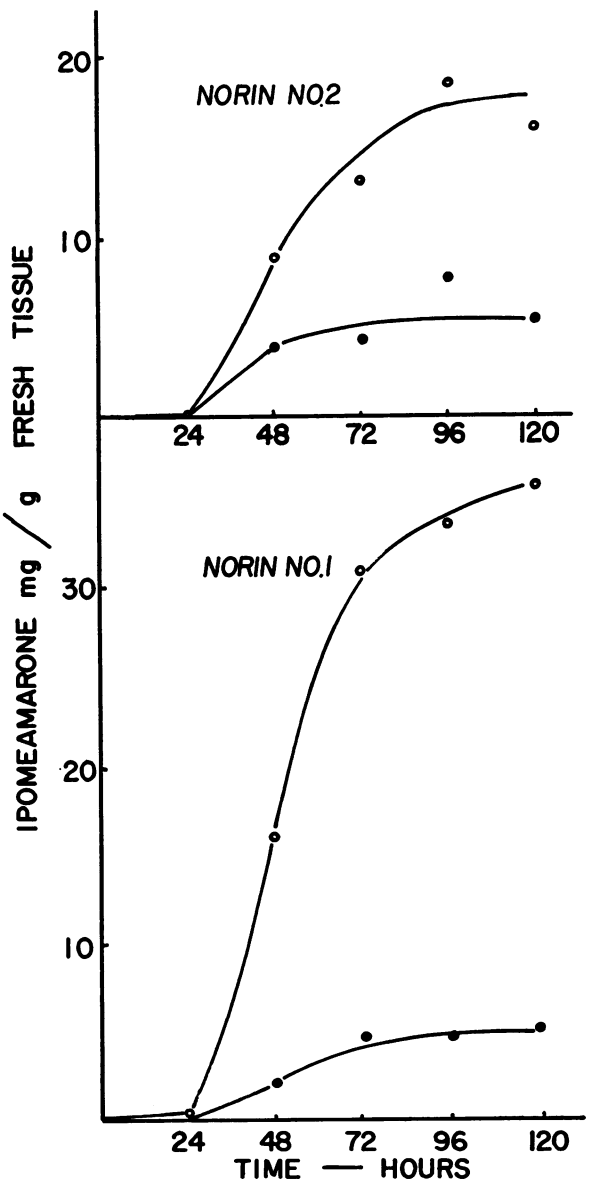

FIG. 2. Synthesis of ipomeamarone in infected tissue (open circle) and adjacent uninfected tissue (closed circle) as a function of infection period (hr).

TABLE I

SYNTHESIS OF IPOMEAMARONE IN INFECTED ROOT TISSUES OF 4 SWEET POTATO VARIETIES AT 2 INTERVALS 24 TO 48 HOURS & 48 TO 72 HOURS

VARIETY	RESISTANCE	IPOMEAMARONE SYNTHESIZED mg/g FR TISSUE	
		24–48 HR	48–72 HR
Norin no. 10	Very high	45.5	14.0
Norin no. 1	High	16.4	14.3
Norin no. 2	Low	8.9	4.3
Norin no. 5	Very low	1.6	1.0

was surprisingly high in the second 24-hour period (from 24 hr – 48 hr), in contrast to an almost negligible synthesis in the least resistant variety, Norin no. 5. The rate of synthesis leveled off somewhat 72 hours after infection, concomitantly with the termination of fungus growth. This event was more or less coincident with the death of the infected root tissues, particularly in the case of the resistant variety, thus the maximum level of ipomeamarone synthesized was significantly higher in the resistant varieties (table I). About three to five milligrams ipomeamarone per gram fresh weight were found in uninfected tissue in both varieties. The ratio uninfected/infected representing the relative amount of ipomeamarone in the adjacent uninfected tissue compared to the infected part, was at the maximum level about one-seventh for the resistant variety and about one-third in the case of the susceptible variety. As discussed above, contamination of infected in uninfected tissue might partially explain such an analytical result, but the formation of ipomeamarone in the healthy tissue in situ would be another possible explanation.

QUANTITATIVE DETERMINATION OF CHLOROGENIC ACID SYNTHESIS: Analytical data for chlorogenic acid synthesis are shown in figure 3. Independent chromatographic experiments have shown that both the chlorogenic acid and isochlorogenic acid content increase in infected roots, in this case the latter compound was found to be the major component (Akazawa, unpubl data). The total amount was expressed as mg chlorogenic acid per g fresh weight in this paper. The concentration of chlorogenic acid in the fresh sweet potato roots was very low, but a significant increase occurred due both to the effect of slicing (wounding) and fungus infection. Three points should be considered. First, throughout the incubation period, the concentration of chlorogenic acid in uninfected tissue was much higher than that in infected, a relationship which was exactly opposite to that observed in the ipomeamarone formation. However, an increase of chlorogenic acid is not a specific reaction of the host tissue induced by the pathogenic infection, because it occurred even in healthy tissues which received a simple mechanical treatment, slicing. Second, unlike ipomeamarone, there was no marked lag phase in the synthesis of chlorogenic acid in uninfected tissue, rather a steady increase was observed. Third, the pattern of chlorogenic acid in the three kinds of tissue samples was quite comparable between two varieties, although the concentration in each tissue was significantly higher in the resistant variety when compared to the susceptible one.

RESPIRATORY INCREASE & IPOMEAMARONE: Ipomeamarone has been found to be a potent uncoupling agent and its role as a respiratory stimulant in in-

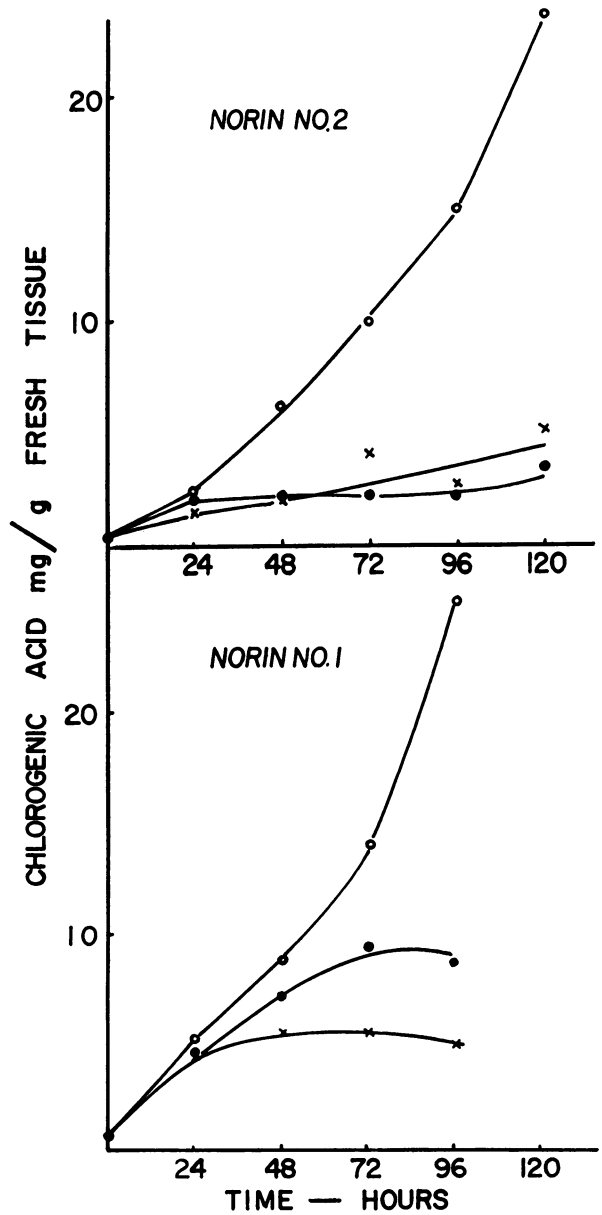

FIG. 3. Synthesis of chlorogenic acid in infected tissue (○——○), tissue adjacent to infected tissue (●——●), and in the sliced healthy tissue (×——×) as a function of infection period (hr).

fected host tissue has been suggested (12). The effect of pure ipomeamarone on the tissue respiration was reinvestigated and an effect similar to that produced by the crude material was found (1). However, the following two points may disprove the hypothesis that ipomeamarone to an appreciable extent is active naturally in infected roots (2, 11); an increase in the respiratory rate is evident in the inner region adjacent to the infected part where no ipomeamarone can be detected, and the respiratory increase begins 24 hours after fungus infection, whereas ipomeamarone synthesis is negligible at this stage even in the infected tissue itself. The analytical results of figure 2 showing the presence of ipomeamarone in the uninfected tissues closely adjacent to the infected tissue do, however, suggest the possible participation of this substance in the respiratory increase in that part. Regarding this point, information obtained in the chemically injured root tissues should be noted. Trends in the respiratory increase similar to that of the fungus-infected tissues were observed in the healthy region of root tissues treated with $HgCl_2$ or monoiodoacetate (13). Chromatographic examination shows a marked synthesis of many substances including ipomeamarone in these tissues (fig 4), and their pattern was practically identical to that of the fungus-infected tissues (cf. fig 1). NaCl treatment, on the other hand, resulted in the formation of a minute amount of Ehrlich's reagent-positive substances which were not clearly detected in the chromatogram. Quantitative estimation of ipomeamarone in the former two cases was not undertaken, but the concentration seemed to exceed that inducing a respiratory increase and was somewhat equivalent to the level exhibiting an inhibitory effect in the in vitro experiment. The question of whether exogenously added ipomeamarone behaves in a similar way to that in living tissues remains open.

Discussion

Uritani and Akazawa (11) have suggested that the pathological disturbance of carbohydrate and fatty acid metabolism in the host may induce the synthesis of ipomeamarone, an abnormal sesquiterpenoid. It has been postulated that its biosynthesis involves the incorporation of mevalonic acid as a precursor (13). Concurrent formation of many substances in the infected tissues is an important finding in this respect and its possible connection to the biosynthesis of ipomeamarone has been discussed (1). It cannot be overemphasized that the present analysis has uncovered a drastic synthesis of ipomeamarone in host tissues only after a lag of 24 hours after fungus infection. The infected region may not be a suitable material for the bio-synthetic study, however, because it is composed of two different systems, host tissues and the pathogenic microorganism. However, chemically-treated sweet potato tissues which form a significant amount of ipomeamarone in their healthy region may offer an alternative way to elucidate this problem. The information gained in this work supports the view that this substance is synthesized by the root tissue per se.

It was found previously that ipomeamarone has several antibiotic properties against the black rot fungus; inhibition of growth, protein synthesis, phosphate metabolism, and respiration among others. Thus its fungitoxic action in host tissues is quite clear (11). Along with this evidence, it should be emphasized that there exists a close correlation between the amount of ipomeamarone synthesized in the host and the degree of host resistance against the pathogen. Müller has proposed a function for phytoalexin in the defense mechanism of host plants based on finding the formation of some organic substance having antipathogenic activity in the inner epiderms of pea pods infected with *Sclerotinia fructicola* and *Phytophthora infestans* (8). In a pathological sense, mode of action of ipomeamarone seems to be quite similar to that of the Müller's phytoalexin, and its physiology warrants further experimentation.

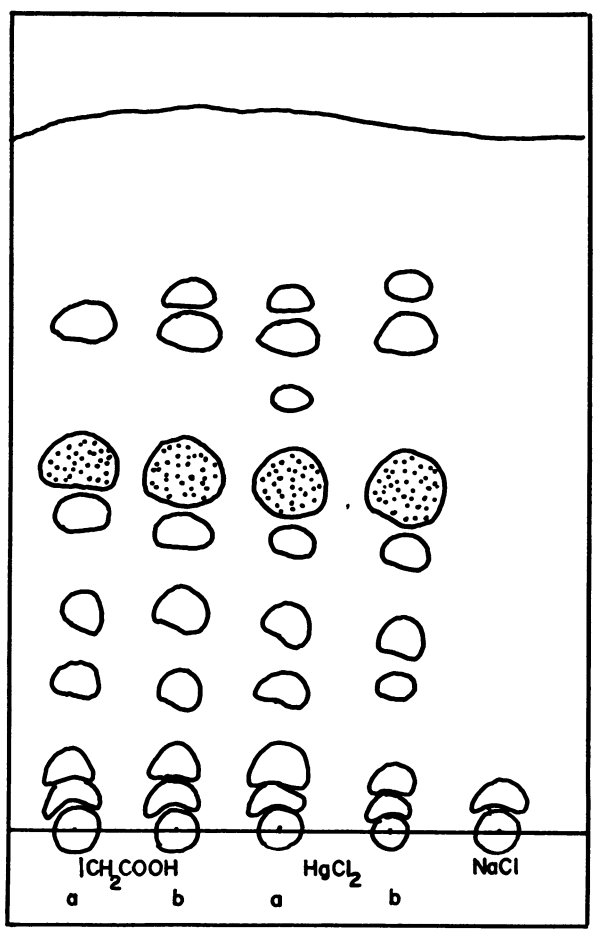

Fig. 4. Chromatographic examination of the formation of ipomeamarone and other related substances in the chemically treated root tissues after a 48-hr period, (a) and (b) represent degenerated and adjacent tissue, respectively. Ipomeamarone was marked by spots.

Turning to the antipathogenic function of polyphenolic substance, conclusive evidence for their participation is lacking so far, although many workers have reported a remarkable synthesis of these compounds in diseased plants (6, 10, 14). Present data does not provide any favorable information whatsoever, but it would be of interest to investigate the nature of chlorogenic acid synthesis as a different approach to the study of metabolic alteration.

Though several hypotheses have been put forward, the crucial mechanism in the respiratory increase in infected plant tissues remains unclear (11). Analytical data shown in figure 2 may indicate a minor participation of ipomeamarone in the respiratory increase of tissue closely adjacent to the infected region, but this is obviously not the case during the very early stages of infection. In fact, independent isotopic work using C^{14}-labeled glucose has given evidence that two different mechanisms are operating in the respiratory system of sweet potato roots with black rot (Akazawa, unpubl data). The respiratory increase in the chemically injured tissues may presumably be attributed to the uncoupling effect of ipomeamarone and the mechanism appears to be different from that of the infected tissues.

Summary

The formation of ipomeamarone and other related substances was compared by silica gel chromatostrip technique in two sweet potato varieties, one resistant the other susceptible to black rot. Chromatographs showing the synthetic pattern were practically the same for both varieties. When ipomeamarone synthesis was quantitatively analyzed, it was found that synthesis occurred quite strikingly after a lag phase of 24 hours and reached a maximum level at about 72 hours after infection. The rate of synthesis and the maximum level in the infected tissues were higher in the resistant than in the susceptible variety, thus the possible function of the substance in the defense mechanism of host tissues was inferred. A steady increase in chlorogenic acid content was found in uninfected tissue adjacent to infected tissues, which was an entirely different picture from that of ipomeamarone. Findings that its increase was induced even by slicing and that no significant difference in the distribution existed between two varieties may refute the primary function of this compound in the defense mechanism of the host. The role of ipomeamarone in the respiratory increase of infected root tissue was discussed from the analytical data of both infected and chemically treated root tissues.

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Literature Cited

1. Akazawa, T. 1960. Chromatographic isolation of pure ipomeamarone & reinvestigation on its chemical properties. Arch. Biochem. Biophys. 90: 82–89.
2. Akazawa, T. & I. Uritani. 1955. Respiratory increase & phosphorus & nitrogen metabolism in sweet potato infected with black rot. Nature 176: 1072–1075.
3. Akazawa, T., I. Uritani, & T. Hirai. 1957. Phytopathological chemistry of black-rotted sweet potato. Part 24. The relation of metabolic activation to resistance in black-rotted sweet potato. J. Agr. Chem. Soc. Japan 32: 634–648.
4. Akazawa, T. & K. Wada. 1960. Quantitative estimation of ipomeamarone by means of chromatostrip technique. Bull. Agr. Chem. Soc. Japan. (in press).
5. Allen, P. J. 1959. Physiology & biochemistry of defense. In: Plant Pathology, J. G. Horsfall & A. E. Dimond, eds. Academic Press, New York. 435–467, Vol. I.
6. Johnson, G. & L. A. Schaal. 1952. Relation of chlorogenic acid to scab resistance in potatoes. Science. 115: 627–629.
7. Lappin, G. R. & L. C. Clark. 1951. Colorimetric method for the determination of traces of carbonyl compounds. Anal. Chem., 23: 541–542.
8. Müller, K. O. 1958. Studies in phytoalexins. I. The formation & the immunological significance of phytoalexin produced by *Phaseolus vulgaris* in response to infections with *Sclerotinia fructicola* & *Phytophthora infestans*. Australian J. Biol. Sci. 11: 275–300.
9. Stanley, W. L. & S. H. Vannier. 1957. Analysis of coumarin compounds in citrus oils by liquid-solid partition. J. Assoc. Offic. Agr. Chem., 40: 582–588.
10. Uritani, I. 1953. Phytopathological chemistry of black rotted sweet potato. Part 5. Physiology of the polyphenols in injured sweet potato. J. Agr. Chem. Soc. Japan. 23: 57–62.
11. Uritani, I. & T. Akazawa. 1959. Alteration of respiratory pattern in infected plants. In: Plant Pathology, J. G. Horsfall & A. E. Dimond, eds. Academic Press, New York. 349–390.
12. Uritani, I., T. Akazawa, & M. Uritani. 1954 Increase of respiratory rate in sweet potato tissue infected with black rot. Nature: 174.
13. Uritani, I., M. Uritani, & H. Yamada. 1960. Similar metabolic alterations induced in sweet potato by poisonous chemicals & by *Ceratostomella fimbriata*. Phytopathology. 50: 30–34.
14. Valle, E. 1957. On antifungal factors in potato leaves. Acta. Chem. Scand. 11: 395–397.
15. Zucker, M. & J. F. Ahrens. 1958 Quantitative assay of chlorogenic acid & its pattern of distribution within tobacco leaves. Plant Physiol. 33: 246–249.