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Metabolism of radioactively labeled quinic acid and shikimic acid in healthy and Fusarium-infected tomato plants

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Abstract

Radioactivity from quinic acid-U-^{14}C was readily incorporated into chlorogenic acid and shikimic acid in healthy and *Fusarium*-infected tomato plants of two varieties, 'Bonner Beste' (susceptible) and 'Moneymaker' (resistant); radioactively labeled shikimic acid, on the other hand, was converted neither to quinic acid nor to chlorogenic acid.
Infection led to increased incorporation of ^{14}C into *n*-butanol extractives, and alcohol-soluble and insoluble esters, except in the resistant variety after feeding of shikimic acid-U-^{14}C. After infection incorporation into the non-hydrolyzable fraction – which a.o. contains lignin – decreased in the susceptible variety, but it increased in the resistant variety, particularly after administration of shikimic acid-U-^{14}C.

Introduction

In studies on the physiology and biochemistry of plant-pathogen interactions it has frequently been established that aromatic compounds are involved in resistant and hypersensitive reactions (see Rohringer and Samborski, 1967); among these aromatics, phenolic acids, coumarins, flavonoids and isoflavones (as well as their alcohol-soluble and insoluble derivatives), and also lignin deserve special mentioning.
Tomato wilt, caused by *Fusarium oxysporum* f. *lycopersici*, is well known for impeded lignification, vascular discolouration, and dysfunction of xylem tissues (cf. Dimond, 1959; Chambers and Corden, 1963). Apparently, the fungus interferes, among others, with the metabolic pathway leading to the formation of lignin.
Fig. 1, which is a schematic representation of the so-called shikimate pathway of aromatic biosynthesis, shows quinic acid and shikimic acid as precursors of aromatic compounds.
Starting from these facts and observations, tomato wilt has been chosen by us as the subject of an investigation on the metabolism of quinic acid and shikimic acid in relation to resistance against and susceptibility to this disease in tomato plants. The experiments are analogous to those carried out by Rohringer et al. (1967) and Fuchs et al. (1967) on the metabolism of aromatic compounds in healthy and rust-infected primary leaves of wheat. This paper gives a preliminary account of our experiments, detailed results of which will be published elsewhere.

Fig. 1. Probable major biosynthetic pathways leading to main phenolic compounds (modified after Neish, 1964, with data derived from El-Basyouni et al., 1964, Zenk and Müller, 1964, El-Basyouni and Neish, 1966, and Zenk, 1966)

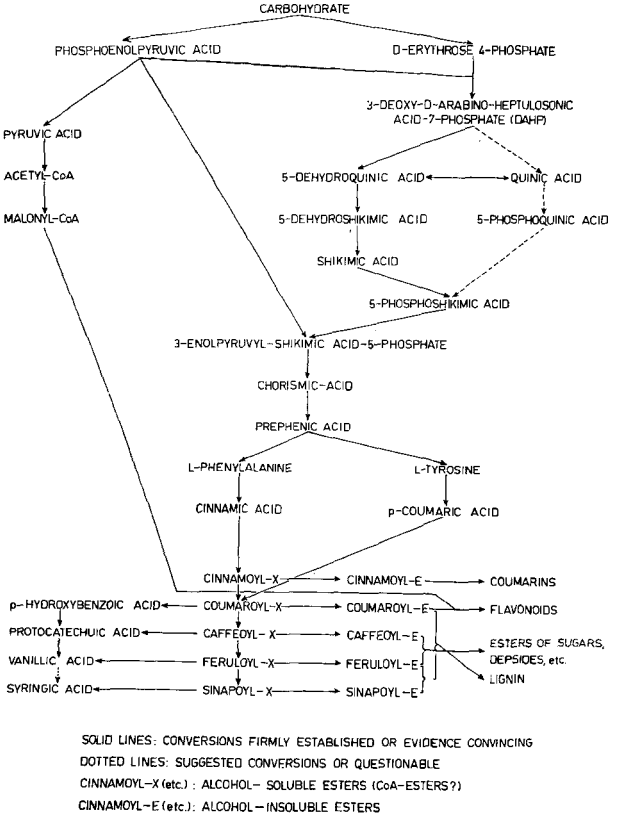

Fig. 1. Stofwisselingswegen, die leiden tot de vorming van de voornaamste fenolische verbindingen (gewijzigd naar Neish, 1964, met gegevens van El-Basyouni et al., 1964, Zenk en Müller, 1964, El-Basyouni en Neish, 1966, en Zenk, 1966)

Materials and methods

Tomato plants of the varieties 'Bonner Beste' (susceptible) and 'Moneymaker' (resistant) were grown under greenhouse conditions, and half of them were inoculated at the four-leaf stage with a highly pathogenic strain of *Fusarium oxysporum* f. *lycopersici**. Inoculation took place by dipping the roots in 800 ml of a suspension of homogenized fungal mycelium, grown in stationary culture in a pectin medium for 28 days. From the time of inoculation all plants, inoculated as well as uninoculated ones, were grown separately in sand culture, each plant receiving 50 ml of Hoagland solution (normal strength) three times a week. To allow optimal fungal growth, the greenhouse temperature was raised to 26 °C within 3 days after inoculation.

* Generously supplied by Professor Dr F. Grossmann from the Institut für Phytopathologie, Justus Liebig Universität, Giessen, Germany.

The plants were cut off at soil level 31 days after inoculation and the shoots were allowed to take up 500 µl of aqueous solutions of either quinic acid-U-^{14}C or shikimic acid-U-^{14}C, followed by at least three rinses with 500 µl of distilled water. Each shoot received 0.2 µmole quinic acid or 0.298 µmole shikimic acid, representing an activity of 1 µC. After uptake of the isotope solutions, the shoots were placed in a dilute Hoagland solution in a perspex photosynthetic chamber for 24 h, receiving artificial light and a 9 h dark period overnight. To minimize reincorporation of respiratory $^{14}CO_2$, the apparatus was flushed continuously with air.

At the end of the metabolic period the shoots were frozen immediately in liquid nitrogen, and ground in mortars with solid CO_2. The powders obtained were then freeze-dried in a Weinkauf L2 lyophilizing apparatus, and stored under N_2 at +2 °C until extraction.

The freeze-dried material was extracted in 25 ml of 90% aqueous methanol at +2 °C, 10 ml of 90% aqueous methanol and 10 ml of 80% aqueous ethanol at room temperature, and 10 ml of 80% aqueous ethanol at 78°C, respectively. Subsequently, the material was extracted with acetone until colourless; then, all extracts were combined and made up to 100 ml. This material is indicated as "solvent soluble material", whereas the remaining is named "solvent insoluble material". The solvent soluble material was freed from chlorophyll and lipids by filtering it through Celite (cf. Rohringer et al., 1967); recovery of radioactivity at this step was always nearly 100%, indicating that chlorophyll and lipids were not labeled.

Fractionation of soluble and insoluble material was carried out using methods essentially similar to those described by Rohringer et al. (1967). The fractions dealt with in this communication were obtained as follows.

(a) Twenty five ml aliquots of the soluble material were adjusted to pH 7 and extracted five times with 15 ml of *n*-butanol; the *n*-butanol extract was dried in vacuo, and the *n*-butanol extractives were taken up in 2 ml of water for further analysis (*n*-butanol extractives).

(b) Ten ml aliquots were made 2N in respect to NaOH, hydrolyzed at room temperature for 6 h and, after having been adjusted to pH 2 with HCl, extracted with peroxide-free ether in a Soxhlet apparatus for 24 h. After evaporation of the ether, the residue was dissolved in 1 ml of 90% methanol for further analysis (soluble esters).

(c) Aliquots of the insoluble material were suspended in 10 ml of 2N NaOH, hydrolyzed, and extracted as described above. The residues were dissolved in 90% methanol for further analysis (insoluble esters).

(d) The insoluble residue, remaining after mild alkaline hydrolysis and ether extraction of the insoluble material, was filtered off and digested in hydrogen peroxide and perchloric acid, according to Mahin and Lofberg (1966) (non-hydrolyzable material).

Paper chromatography (cf. Rohringer et al., 1967), spectrophotometry, and autoradiography served as additional analytical methods, to be described in detail elsewhere.

Radioactivity was measured using a liquid scintillation system (Nuclear Chicago 720 series), and all data were calculated as disintegrations per min. Quinic acid-U-^{14}C and shikimic acid-U-^{14}C were obtained from New England Nuclear Corporation (Boston, Mass., U.S.A.).

Results

In Table 1 data on the distribution of radioactivity in healthy and *Fusarium*-infected tomato plants after feeding of quinic acid-U-^{14}C and shikimic acid-U-^{14}C have been summarized. The data, which have been expressed as % of total activity present in the freeze-dried material, have not been corrected for the effect of "trapping" of radioactivity in the initial precursor pools (cf. Rohringer et al., 1967). These corrections have not been made, because paper chromatographic analysis showed quinic acid, apart from being present in the free form, to be readily converted to chlorogenic acid, which, thus, should be considered an additional "quinic acid" pool. Whereas quinic acid was found to be readily converted to shikimic acid, the reverse proved not to be true; neither was shikimic acid converted to chlorogenic acid to an appreciable degree (details to be published elsewhere).

From Table 1 it is evident, that a much higher percentage of radioactivity was incorporated into the fractions discussed when shikimic acid-U-^{14}C was used as precursor than when quinic acid-U-^{14}C was fed. This difference can be readily explained by the fact, that in the case of quinic acid-U-^{14}C feeding radioactivity leaving the initial precursor pool was partly trapped in chlorogenic acid and shikimic acid before being incorporated into aromatic compounds. However, after administration of shikimic acid-U-^{14}C all radioactivity leaving the initial precursor pool was directly incorporated into the aromatics.

Further it can be seen, that infection led to an increase in % of activity retained in

Table 1. Distribution of radioactivity in some fractions, obtained from healthy and *Fusarium*-infected tomato plants after feeding of quinic acid-U-^{14}C and shikimic acid-U-^{14}C (data expressed as % of total activity recovered)

Administered precursor and plant material	*n-Butanol extractives*	*Soluble esters*	*Insoluble esters*	*Non-hydrolyzable material*
Quinic acid-U-^{14}C fed to:				
susceptible, healthy plants (BB)[1]	6.4*	2.1	0.3	20.0
susceptible, inoculated plants (BB)	13.1	6.2	0.8	19.0
resistant, healthy plants (MM)[2]	4.7	1.3	0.2	8.0
resistant, inoculated plants (MM)	7.1	2.1	0.3	9.6
Shikimic acid-U-^{14}C fed to:				
susceptible, healthy plants (BB)	12.9	3.6	0.7	39.8
susceptible, inoculated plants (BB)	20.0	9.0	1.3	23.6
resistant, healthy plants (MM)	9.0	3.7	0.7	43.8
resistant, inoculated plants (MM)	8.1	3.0	0.6	50.8

* Because the data of only part of the fractions obtained are given in this communication, the numerical values do not add up to 100 % – *Omdat in dit artikel de gegevens van slechts een deel der verkregen fracties besproken worden, is de som der gegeven getallen niet gelijk aan 100.*

[1] BB = 'Bonner Beste'

[2] MM = 'Moneymaker'

Tabel 1. Verdeling van radioactiviteit in sommige fracties, verkregen uit gezonde en met Fusarium geïnfecteerde tomateplanten, na toediening van uniform radioactief gemerkt kinazuur en shikimizuur (gegevens uitgedrukt als percentage van de totale activiteit)

n-butanol extractives, and soluble and insoluble esters, except in plants of the resistant variety 'Moneymaker' after administration of shikimic acid-U-^{14}C, where infection led to a negligible decrease in % of activity. So far, these fractions have not been examined in detail; yet, it might be supposed that quite considerable portions of the activity in the *n*-butanol extractives are present as phenylalanine and tyrosine, which belong to the main products of aromatic biosynthesis in tomato plants (Weinstein et al., 1961), and which are readily extracted by *n*-butanol (Rohringer et al., 1967). In the soluble ester fractions one of the prevailing components was tentatively identified as caffeic acid, the presence of which might be expected in view of the fact, that it is a constituent of chlorogenic acid.

Especially striking is the difference in behaviour of quinic acid-U-^{14}C and shikimic acid-U-^{14}C as to the incorporation of activity into the non-hydrolyzable material. It also should be emphasized that in this fraction infection led to a decreased incorporation of radioactivity in the susceptible variety, whereas the reverse was true in the resistant variety. In the case of *Fusarium*-infected plants of the resistant variety the % of activity incorporated after administration of shikimic acid-U-^{14}C even exceeded 50%.

Discussion

Present views on the so-called shikimate pathway of aromatic biosynthesis, as depicted schematically in Fig. 1, rely heavily upon experimental data obtained in studies with micro-organisms (for literature see Neish, 1964), although this pathway has also firmly been established to operate in mung bean cell suspension cultures (Gamborg, 1966a, 1966b, 1967a). Investigations on the aromatic biosynthesis in other higher plant species, however, have raised doubt as to the general validity of this pathway (cf. Weinstein et al., 1959, 1961, 1962; Neish, 1964; Rohringer et al., 1967). Controversial views have especially been expressed regarding the metabolic sequences which lead from 3-deoxy-D-arabino-heptulosonic acid-7-phosphate (DAHP) to 5-phosphoshikimic acid. Whereas in bacteria and mung beans DAHP is converted to the latter via 5-dehydroquinic, 5-dehydroshikimic, and shikimic acid (Fig. 1, solid lines), in other higher plant species an alternative pathway has been suggested to operate (Neish, 1964) (Fig. 1, broken lines). However, differences do also exist between species of higher plants since conversion of quinic acid to shikimic acid proved to be reversible in wheat plants (Rohringer et al., 1967), but irreversible in bean (Weinstein et al., 1962) and tomato plants (this publication).

Regarding the effects of infection with *Fusarium oxysporum* f. *lycopersici* on the metabolism of quinic acid and shikimic acid in tomato plants, the most conspicuous one seems to be that after administration of shikimic acid-U-^{14}C incorporation of radioactivity into the non-hydrolyzable material decreased in the susceptible variety, whereas it increased in the resistant variety. Most probably the larger incorporation in the latter instance is mainly due to increased incorporation of ^{14}C into lignin. Incorporation of radioactivity into lignin should be consistent with the findings of Gamborg (1967b), who found ^{14}C from various aromatic compounds to be readily incorporated into chlorogenic acid as well as into lignin in potato cell cultures.

The increased incorporation of activity into the *n*-butanol extractives, and the soluble and insoluble esters after infection of the susceptible variety might be attributed to an

accumulation of ^{14}C in these fractions due to impaired lignification. The soluble and insoluble esters are considered to be the direct precursors of lignin and, therefore, impairment of lignification might be supposed to lead to increased retention of activity in these fractions. Supporting evidence for this view can be found in the observation, that a distinct increase of the chlorogenic acid pool was observed after inoculation of the susceptible variety, whereas a decrease in chlorogenic acid concentration was found after inoculation of the resistant variety (unpublished results). Chlorogenic acid has been shown to be a competitive inhibitor of indoleacetic acid oxidase (cf. Hare, 1964). Therefore, an increased chlorogenic acid pool could conceivably be supposed to lead to diminished indoleacetic acid oxidase activity, and thus to an increased indoleacetic acid concentration, which, on the one hand, was found to suppress peroxidase-mediated lignification (cf. Brown, 1964, 1966) and, on the other hand, was shown to induce vascular collapse in tomato plants (Chambers and Corden, 1963). In a comparable way, decreased chlorogenic acid concentrations could be assumed to – indirectly – promote lignin formation, which is actually observed in inoculated plants of the resistant variety. However, more detailed investigations have to be carried out, before this hypothesis should be accepted.

Samenvatting

De stofwisseling van radioactief kinazuur en shikimizuur in gezonde en met Fusarium oxysporum f. lycopersici geïnfecteerde tomateplanten

Radioactief kinazuur werd in gezonde en met *Fusarium* geïnfecteerde tomateplanten behorende tot twee variëteiten, 'Bonner Beste' (vatbaar) en 'Moneymaker' (resistent), in chlorogeenzuur en shikimizuur omgezet; laatstgenoemde verbinding werd echter noch in kinazuur, noch in chlorogeenzuur omgezet.
Infectie leidde tot een toegenomen incorporatie van ^{14}C in met *n*-butanol extraheerbare verbindingen, en in alcohol-oplosbare en onoplosbare esters, behalve na toediening van radioactief shikimizuur in de resistente variëteit. Incorporatie in de zg. niet-hydrolyseerbare fractie, die o.a. lignine bevat, bleek in de vatbare variëteit na infectie geringer te zijn, maar in de resistente variëteit na infectie toe te nemen, vooral na toediening van shikimizuur.

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