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Carbohydrate composition of apoplastic fluids isolated from tomato leaves inoculated with virulent or avirulent races of Cladosporium fulvum (syn. Fulvia fulva)[1]

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Abstract

Inoculation of tomato (*Lycopersicon esculentum*) with virulent races of *Cladosporium fulvum* (compatible interactions), resulted in substantial changes of the carbohydrate composition of apoplastic fluids isolated from the leaves, during the course of the infection process. In addition to a decrease in the concentration of the translocation sugar sucrose, a transient accumulation of the hexoses glucose and fructose and an accumulation of the polyol mannitol were observed. The latter coincided with a rising level of mannitol dehydrogenase, an enzyme that reduces fructose to mannitol. Only minor changes were detected in the carbohydrate composition of apoplastic fluids isolated from leaves of uninoculated control plants or plants inoculated with avirulent races of *C. fulvum* (incompatible interactions). The fungal metabolite mannitol was not detected in apoplastic fluids isolated from the latter plants.

These results suggest that, upon colonization of the intercellular spaces by virulent races of *C. fulvum*, apoplastic sucrose is hydrolyzed by a host and/or fungal invertase and the resulting hexoses, glucose and fructose, are converted into mannitol by the fungus. In incompatible tomato-*C. fulvum* interactions a functional nutritional relationship between plant and fungus is prevented by plant defense responses, which might explain why in these interactions the carbohydrate composition of apoplastic fluids is similar to that of uninoculated control plants.

Additional keywords: Lycopersicon esculentum, sucrose, glucose, fructose, mannitol, mannitol dehydrogenase, invertase.

Introduction

Cladosporium fulvum (Cooke) (syn. *Fulvia fulva* [Cooke] Cif.), the causal agent of tomato leaf mould, is a biotroph that colonizes the intercellular spaces between mesophyll cells of leaves of tomato (*Lycopersicon esculentum* Mill.), without the formation of haustoria (Lazarovits and Higgins, 1976a, b; De Wit, 1977).

In a compatible tomato-*C. fulvum* interaction, where extensive fungal colonization takes place, the intercellular hyphae are closely appressed to the host cell walls, usually causing slight indentation of the walls. Relatively few ultrastructural changes of the

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host mesophyll cells occur until the fungus starts to sporulate, indicating that initially a stable biotrophic relationship exists between host and parasite. In the incompatible interaction a rapid defense response by host cells (e.g. callose deposition, accumulation of phytoalexins, production of pathogenesis- related (PR) proteins) prevents the establishment of a functional nutritional relationship between host and pathogen (Lazarovits and Higgins, 1976b).

Up to now little is known about the mechanism by which this fungus obtains the carbohydrates necessary for growth and reproduction. A candidate is sucrose, the main product of photosynthesis, which is either exported into the phloem, temporarily stored in the leaf, or metabolized. Although the mechanism by which sucrose is transported from mesophyll cells into the phloem of tomato leaves is unknown, there exists substantial support for a loading of the phloem via the apoplast (Giaquinta, 1983). Apoplastic sucrose could serve as an important carbon source for *C. fulvum*, as this fungus does not produce cell wall degrading enzymes to release carbon-containing compounds from plant cell walls.

Studies by Lewis and Harley (1965) showed that the mycorrhizal fungus of beech is able to absorb carbohydrates from the host and to convert them into mannitol, trehalose and glycogen. As mannitol is the most abundant reserve sugar in this mycorrhizal fungus and the plant is unable to utilize this polyol, the hypothesis was put forward that the fungus absorbs carbohydrates from the host and transforms them into sugars that can only be metabolized by the fungus. In this way a concentration gradient with respect to the carbohydrates of the host is created. As many plants cannot metabolize polyols, Lewis and Harley (1965) suggested that this hypothesis may not only hold for mycorrhizal fungi but also for fungi pathogenic on plants. In their review Smith and coworkers (1969) suggested that biotrophic parasites such as rusts, smuts and powdery mildews obtain carbohydrates from their hosts in a way similar to that of the mutualistic ectotrophic mycorrhizal fungi. Sucrose, the common translocation sugar, was shown to be the main carbohydrate source for these parasitic fungi. It was hydrolyzed to glucose and fructose, while subsequently the latter two compounds were taken up and converted into fungal carbohydrates such as trehalose, mannitol and arabitol. There are several reports on the redirection of the translocation of the assimilatory carbohydrates of the host upon infection by biotrophic or hemibiotrophic fungi (Long and Cooke, 1974; Long et al., 1975; Billett et al., 1977; Mitchell et al., 1978; Clancy and Coffey, 1980; Callow et al., 1980; Brem et al., 1986; Hwang and Heitefuss, 1986; Hwang et al., 1989). In many cases an increased invertase activity (from host and/or fungus) was responsible for sucrose depletion and increases in the concentration of the hexoses glucose and fructose, while many carbohydrates of fungal origin could be detected in the infected tissue.

The present study was undertaken to investigate whether the described mechanism of nutrient uptake described above also holds for *C. fulvum* and to what extent plant defense mechanisms are affecting the nutritional relationship between plant and fungus. In contrast to previous studies, where the sugars present in total leaf extracts were examined, this study focuses on changes in carbohydrates present in the apoplast. The changes in carbohydrate composition of apoplastic fluids in relation to the mode of nutrition of the intercellularly growing fungus are discussed, as well as the possibility of using mannitol concentration or mannitol dehydrogenase activity present in apoplastic fluids as a way of estimating fungal biomass in infected tomato leaves.

Materials and methods

Plant, fungus and inoculation. Cultivation of tomato plants and races of *C. fulvum* and inoculation were carried out as described previously (De Wit, 1977; De Wit and Flach, 1979). Conidial suspensions of races 4 and 5 of *C. fulvum* were used to inoculate two near-isogenic lines of tomato, carrying resistance gene Cf4 (susceptible to race 4 and resistant to race 5) or Cf5 (susceptible to race 5 and resistant to race 4). Uninoculated control plants were kept under the same conditions as the inoculated ones.

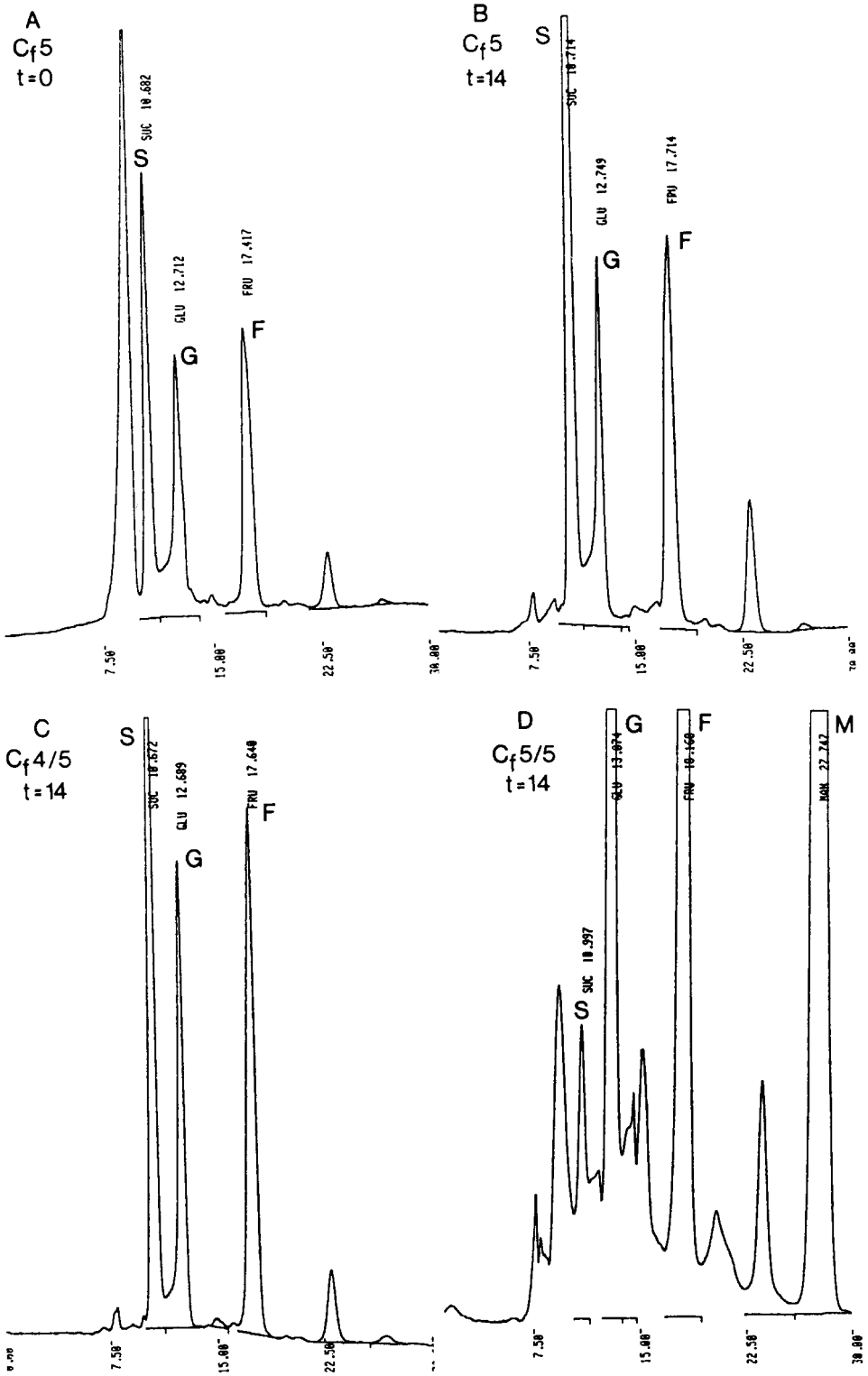
Preparation of apoplastic fluids and analysis of carbohydrate composition. For time-course experiments about 15 to 25 plants were inoculated for every interaction and 10 to 20 leaflets were picked at random at 3, 5, 7, 9, 12, and 14 days after inoculation. The whole leaflets were infiltrated with distilled water *in vacuo* and apoplastic fluid was isolated by centrifugation, according to the method described by De Wit and Spikman (1982).

Proteins present in 300-μl aliquots of apoplastic fluid were precipitated overnight at -20 °C with four volumes of acetone and pelleted by centrifugation at 1500 *g* (5 min). The acetone was evaporated from the supernatant and the remaining aqueous solution was freeze-dried. The residue was dissolved in 100 μl of H_2O, centrifuged at 15 000 *g* (5 min) and analyzed by high pressure liquid chromatography (HPLC). Twenty μl was injected onto an Aminex Carbohydrate HPX-87P column (Bio-Rad, 300 $\times$ 7.5 mm, at 85 °C) fitted into a Waters HPLC system. The column was eluted with H_2O at a flow rate of 0.6 ml min^{-1} and the eluate was monitored using a refractive index detector. The amounts of glucose, fructose, sucrose and mannitol were calculated using the respective sugars as external standards. Other external standards used were xylose, galactose, arabinose and inositol.

Mannitol dehydrogenase (MTLDH) assay. MTLDH activity in apoplastic fluids was assayed by monitoring oxidation of NADPH at 340 nm in the presence of fructose (Morton et al., 1985). In a total volume of 1 ml, a sample of untreated apoplastic fluid (50 to 100 μl) was incubated in 50 mM Tris-HCl buffer, pH 7.5, containing 800 mM fructose and 0.25 mM NADPH. In this assay one unit of activity represents the amount of enzyme which catalyses the oxidation of 1 nmole NADPH min^{-1}.

Results

Sugar composition of apoplastic fluids isolated from uninoculated and inoculated tomato leaves. To investigate the mechanism of nutrient uptake for *C. fulvum* several time-course experiments were carried out. Here we describe the results of a representative experiment using uninoculated Cf5 plants, the incompatible interaction Cf4/race 5 and the compatible interaction Cf5/race 5. The chromatogram presented in Fig. 1A shows that sucrose, glucose and fructose are the main sugars present in apoplastic fluid obtained from uninoculated Cf5 plants that were kept under greenhouse conditions. One ml of apoplastic fluid contained about 150 μg of each of the three sugars. The compound eluting around 23.0 min was identified as inositol and the peak preceeding sucrose contained an unidentified substance. This peak may consist of a mixture of substances (phenols, oligosaccharides) which were not separated on the column.


A
C_f5
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S
SUC 10.682
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C
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GLU 12.689
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◀ Fig. 1. HPLC analysis of the mono- and disaccharides present in apoplastic fluids. A and B show the HPLC traces of apoplastic fluids obtained from leaves of uninoculated Cf5 plants before and after incubation under the same conditions as the inoculated plants for 14 days, respectively. In C and D the analysis of apoplastic fluids (isolated 14 days after inoculation) from the incompatible interaction Cf4/race 5 and the compatible interaction Cf5/race 5 is shown. The peaks representing sucrose, glucose, fructose and mannitol are marked S, G, F and M, respectively.

In the uninoculated Cf5 plants, kept under the same conditions as the inoculated ones for 14 days, the apoplastic sugar concentrations had changed (Fig. 1B). There was an increase in sucrose and fructose levels and the peak of unidentified substances had disappeared after 14 days. Apoplastic fluid isolated from the incompatible Cf4/race 5 interaction showed about the same sugar content as the uninoculated control plants that had been incubated under the same conditions (Fig. 1C). In the compatible Cf5/race 5 interaction substantial changes in apoplastic sugar composition had occurred at 14 days after inoculation (Fig. 1D). At this stage the leaf tissue was heavily colonized by the fungus. Besides a decrease in sucrose concentration, levels of glucose and fructose had increased 4- to 14-fold, respectively. The compound with a retention time around 27.7 min was identified as mannitol and reached a concentration of more than 2000 μg ml^{-1} of apoplastic fluid. There were no changes in the inositol content of the apoplastic fluids isolated from the uninoculated or inoculated plants within 14 days after inoculation. In addition to the five sugars discussed above, several minor peaks were detected but could not be identified with the set of external sugar standards used.

Changes in carbohydrate composition of apoplastic fluids during the infection process. Time-course experiments showed that in apoplastic fluids isolated from leaves of uninoculated Cf5 plants or Cf4 plants inoculated with race 5 of *C. fulvum* (incompatible interaction), an increase in sucrose, glucose and fructose concentration occurred upon incubation under conditions of high relative humidity. However, compared to the apoplastic fluid isolated from the compatible interaction Cf5/race 5, the glucose and fructose levels remained low (Fig. 2A,B). In the compatible interaction, a twofold increase in sucrose content during the first 7 days was followed by a decrease in sucrose content which coincided with a substantial increase in glucose, fructose and mannitol concentrations (Fig. 2C). From 9 days after inoculation onwards the sucrose level decreased slowly, but a sharp decrease of glucose and fructose was observed, while mannitol accumulation slowly reached its maximum in this period.

In the compatible Cf5/race 5 interaction, when using a low inoculum concentration, fungal biomass in the intercellular spaces remained low, even when abundant sporulation was visible at 10 to 12 days after inoculation. There was no visual damage to the leaves up to 18 days after inoculation. Although the amounts of the different sugars remained at a much lower level compared to the Cf5/race 5 interaction where colonization was abundant as a result of using a high inoculum concentration and no sporulation was visible, the same trends in sugar accumulation could be observed (results not shown). A decrease in sucrose content coincided with a fast increase in glucose, fructose and mannitol, followed by decreases in glucose and fructose levels when sucrose concentration was low. At 18 days after inoculation the mannitol content had reached 600 μg ml^{-1} of apoplastic fluid.

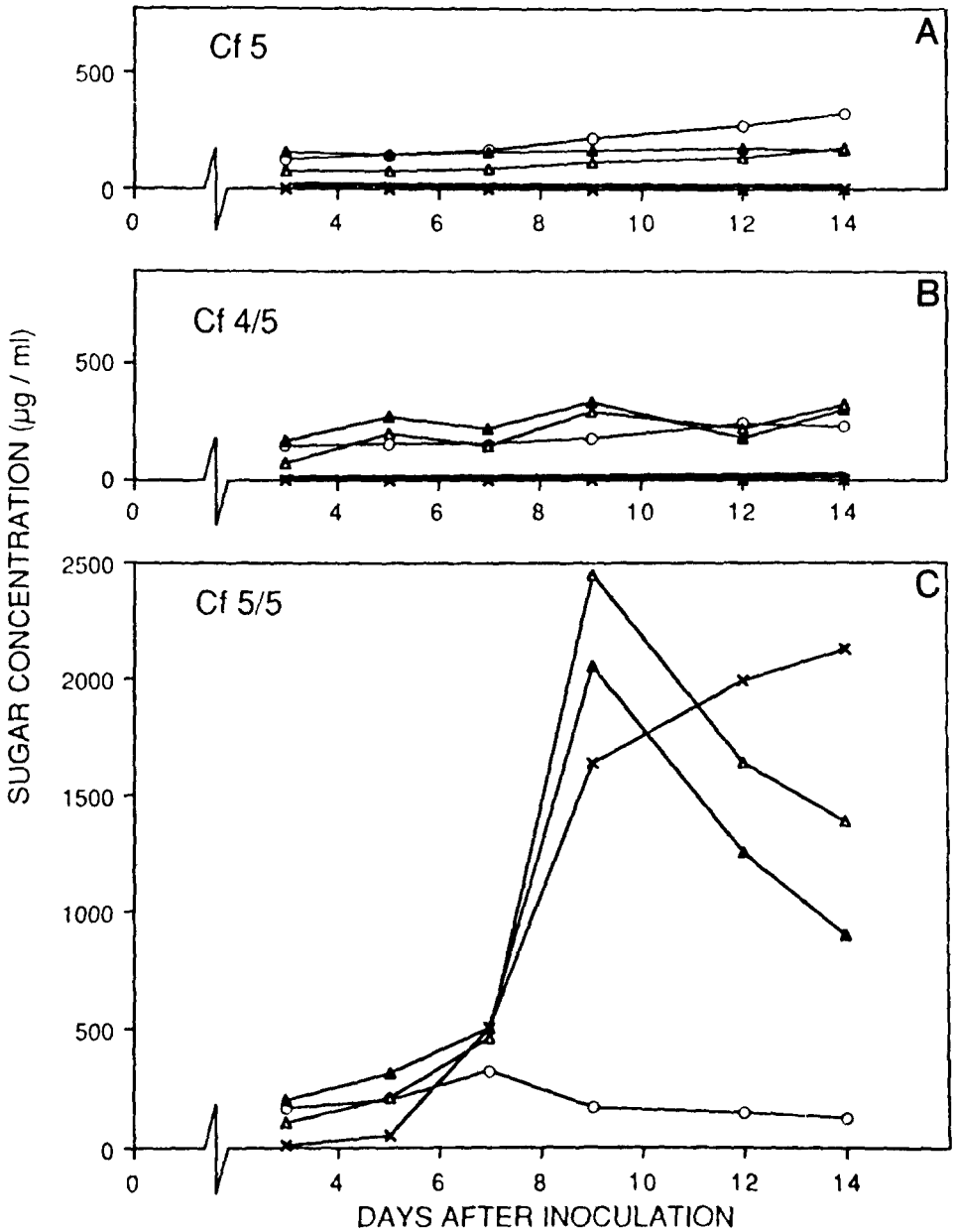

Fig. 2. Time-course of sucrose (o), glucose (▲), fructose (△) and mannitol (×) concentration in apoplastic fluids. Apoplastic fluids were isolated from leaves of uninoculated Cf5 plants (A) and from leaves of tomato cultivars Cf4 (B) and Cf5 (C) inoculated with race 5 of *C. fulvum*. Apoplastic fluids were isolated 3, 5, 7, 9, 12 and 14 days after inoculation and the carbohydrate composition was determined as described in materials and methods.

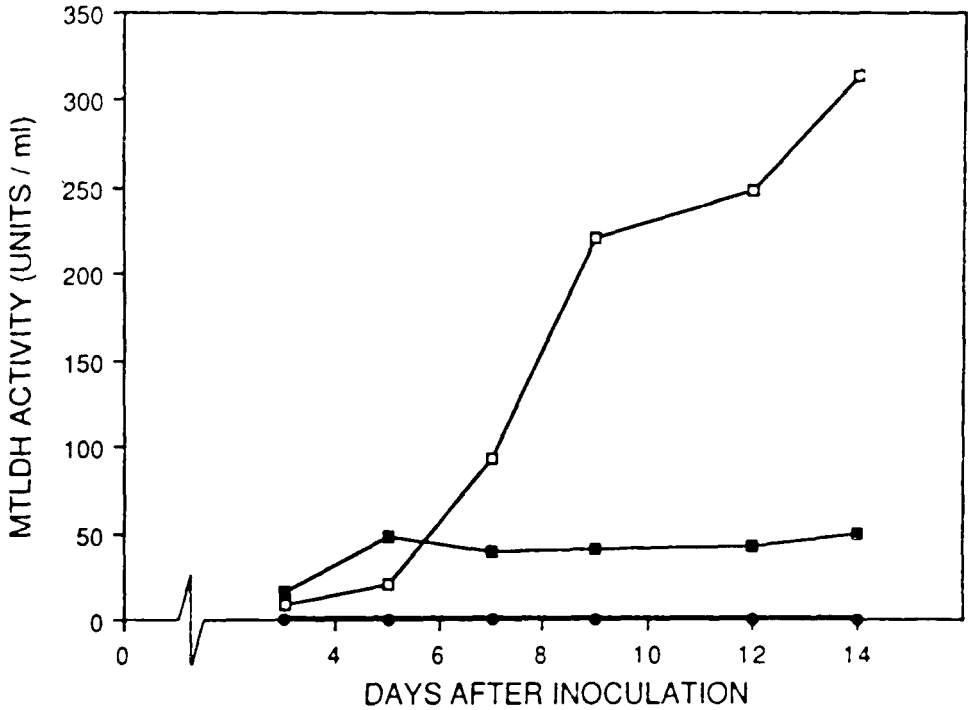

Fig. 3. Time-course of mannitol dehydrogenase (MTLDH) activity in apoplastic fluids. Apoplastic fluids were isolated from leaves of tomato cultivars Cf4 (■) and Cf5 (□) inoculated with race 5 of *C. fulvum* and from leaves of uninoculated Cf5 plants (●). Apoplastic fluids were obtained 3, 5, 7, 9, 12 and 14 days after inoculation and MTLDH activity was determined as described in materials and methods.

Similar results were obtained in time-course experiments with the incompatible interaction Cf5/race 4 and the compatible interaction Cf4/race 4. However, in general race 4 did grow less abundantly than race 5, resulting in a lower rate of accumulation of the various carbohydrates in the Cf4/race 4 interaction (results not shown).

Changes in mannitol dehydrogenase activity present in apoplastic fluids during the infection process. Measurements of apoplastic mannitol dehydrogenase (MTLDH) activity are presented in Fig. 3. MTLDH converts fructose into mannitol, using NADPH as a cofactor. In apoplastic fluid obtained from the leaves of uninoculated Cf5 plants no MTLDH activity could be detected, while in the incompatible Cf4/race 5 interaction a fast increase was observed within the first 5 days. In the compatible Cf5/race 5 interaction, a slower initial increase was followed by a substantial rise in MTLDH activity between day 5 and 9 after inoculation. No NADPH oxidation could be detected in control assays where fructose or apoplastic fluid was omitted.

Measurements on MTLDH activity in apoplastic fluids isolated from the interactions with race 4 gave similar results, but the differences were less pronounced (results not shown).

Discussion

Changes in the metabolism of host carbohydrates (sucrose, glucose and fructose) after infection by biotrophic fungi have been frequently reported (Long and Cooke, 1974; Long et al., 1975; Billett et al., 1977; Mitchell et al., 1978; Clancy and Coffey, 1980; Callow et al., 1980; Brem et al., 1986; Hwang and Heitefuss, 1986; Hwang et al., 1989). In most cases increased invertase activity of host and/or fungus resulted in a depletion of sucrose and the production of high levels of the hexoses glucose and fructose. Besides these quantitative changes in host sugars, mannitol, trehalose and/or erythritol were detected as fungal metabolites.

In *C. fulvum*-infected tomato leaves similar changes in the carbohydrate composition of apoplastic fluids were observed. During the colonization process of the intercellular spaces of tomato leaves a sucrose depletion, transient accumulation of glucose and fructose and accumulation of the polyol mannitol were observed. Possibly the fungus induces leakage of sucrose into the apoplast by changing the permeability of the plasma membrane, as a substantial rise in apoplastic sucrose concentration was observed during the early stages of infection in the compatible Cf5/race 5 interaction. The drop in the amount of sucrose between day 7 and 9 after inoculation was correlated with an increase of glucose and fructose in the apoplast, suggesting that these substances accumulate at the cost of the apoplastic sucrose. At the same time the concentration of mannitol increased substantially. The drop in glucose and fructose levels around 9 days after inoculation coincided with a levelling-off of the mannitol accumulation, suggesting that this accumulation is dependent on glucose and fructose.

These results suggest that apoplastic sucrose is hydrolyzed by host and/or fungal invertases and the resulting hexoses, glucose and fructose, are metabolized by the fungus. Glucose can be converted into fructose, which is subsequently converted into mannitol by MTLDH. Presumably hydrolysis of sucrose occurs in the apoplast and the generated glucose and fructose are absorbed by the fungus and subsequently metabolized into mannitol. Part of the mannitol may either be actively exported or leach passively into the apoplast. In incompatible tomato-*C. fulvum* interactions plant defense responses prevent the establishment of a functional nutritional relationship, resulting in apoplastic sugar compositions similar to those of uninoculated plants.

The relatively high activity of MTLDH measured initially in apoplastic fluid isolated from the incompatible Cf4/race 5 interaction could be the result of a release of MTLDH from the penetrating hyphae caused by deleterious effects of plant defense responses. These effects could be partly caused by the hydrolytic activity of apoplastic 1,3-β-glucanases and chitinases which are rapidly induced in the incompatible combination (Joosten and De Wit, 1989). In the compatible combination the course of MTLDH activity followed that of mannitol accumulation.

When heavy sporulation takes place the amounts of the various sugars detected remain at a lower level. This may be because intercellular growth is less extensive and storage carbohydrates such as mannitol are metabolized to provide energy for spore production or are directly translocated to the spores. Mannitol is the most common polyol in fungi (Lewis and Smith, 1967) and could serve as a carbohydrate reserve or a translocatory compound. It could also have a function in osmoregulation, storage of reducing power and coenzyme regulation (Jennings, 1984). As mannitol could not be detected in uninoculated control plants or incompatible interactions, its presence

in apoplastic fluids can be used as a marker for compatibility. Among the various methods to estimate the amount of fungal biomass present in plant tissue (Matcham et al., 1984) the accumulation of the polyol could be a reliable alternative.

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Samenvatting

Koolhydraatsamenstelling van apoplastische vloeistoffen geïsoleerd uit tomatebladeren geïnoculeerd met virulente of avirulente fysio's van Cladosporium fulvum (syn. Fulvia fulva)

Inoculatie van tomaat (*Lycopersicon esculentum*) met virulente fysio's van *C. fulvum* (compatibele interacties), leidde tot aanzienlijke veranderingen in de koolhydraatsamenstelling van apoplastische vloeistoffen die uit de bladeren werden geïsoleerd in de loop van het infectieproces. Naast een sterke daling van de concentratie van de transportsuiker saccharose, vond er ophoping van de hexoses glucose en fructose en de polyol mannitol plaats. De accumulatie van mannitol ging gepaard met een toename in de activiteit van mannitol dehydrogenase, een enzym dat fructose reduceert tot mannitol. In de koolhydraatsamenstelling van apoplastische vloeistoffen geïsoleerd uit bladeren van niet geïnoculeerde controleplanten, of planten geïnoculeerd met avirulente fysio's van *C. fulvum* (incompatibele interacties), werden slechts kleine veranderingen waargenomen. De schimmelmetaboliet mannitol kon niet worden aangetoond in de apoplastische vloeistoffen die uit deze planten werden geïsoleerd.

Deze resultaten suggereren dat bij de kolonisatie van de intercellulaire ruimtes door virulente fysio's van *C. fulvum*, saccharose uit de apoplast wordt gehydrolyseerd door invertase afkomstig van de plant of de schimmel waarna de ontstane hexoses, glucose en fructose, door de schimmel worden omgezet in mannitol. Bij incompatibele tomaat-*C. fulvum* interacties wordt een functionele voedingsrelatie tussen plant en schimmel voorkomen door het optreden van afweerreacties van de plant, hetgeen kan verklaren waarom in deze interacties de koolhydraatsamenstelling van apoplastische vloeistoffen vergelijkbaar is met die van niet geïnoculeerde controleplanten.

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