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POLYPHENOL OXIDASES AND PHENOLICS IN RELATION TO RESISTANCE AGAINST CUCUMBER SCAB IN *CUCUMIS SATIVUS*

I. FUNGAL AND HOST POLYPHENOL OXIDASES

Polyfenoloxydasen en fenolen en hun verband met resistentie tegen vruchtvuur in komkommers I. De polyfenoloxydasen van schimmel en gastheer

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In culture filtrates of *Cladosporium cucumerinum*, the fungus causing cucumber scab, a constitutive, exocellular catechol oxidase was found; moreover, dihydroxy-phenylalanine and chlorogenic acid oxidases were produced. Catechol oxidase was detected in noticeable activity as soon as the pH of the culture medium had reached a value of 6.0, or if the medium was adjusted to this pH before sterilizing. The Michaelis constant K_m of the fungal catechol oxidase was 62.5×10^{-3} M. In very young non-inoculated green and etiolated cucumber seedlings no catechol oxidase activity was found. In scab diseased seedlings of the susceptible variety 'Lange Gele Tros' high polyphenol oxidase activities were recorded. In seedlings of the resistant variety 'Vios' polyphenol oxidase was also produced upon inoculation, although in much lower activities. In both instances, the Michaelis constant proved to be 15.6×10^{-3} M. This difference in Michaelis constants of catechol oxidases of fungal culture filtrates and of diseased seedlings suggests, that the polyphenol oxidase activity in the diseased plant is of plant origin; however, the presence of the fungus seems to elicit its synthesis or its activation.

INTRODUCTION

Inoculation of etiolated cucumber seedlings of the variety 'Lange Gele Tros' with *Cladosporium cucumerinum* Ell. & Arth. causes the following main symptoms: a. disintegration of parenchyma, especially in the hypocotyls and b. formation of red to reddish-purple spots on cotelydons and streaks on hypocotyls. Although these red discolourations are also found in the resistant variety 'Vios', the degree of discolouration is far less than in the susceptible variety 'Lange Gele Tros'. These red discolourations can now be ascribed to the presence of perylene quinones (OVEREEM *et al.*, in press). Although the biosynthetical pathways leading to the formation of these compounds are not yet known, oxidative enzymes might be assumed to be responsible for their ultimate quinonoid character. Among these, polyphenol oxidases, which have extensively been studied in host-parasite interactions (*cf.* review by FARKAS & KIRÁLY, 1962), deserve special attention.

Another difference in reaction between the two varieties mentioned has been described by HIJWEGEN (1963), who found that as a result of fungal penetration local lignification reactions occurred in etiolated plants of the resistant variety. Such a reaction did, however, not occur in the susceptible variety. Nowadays, *p*-hydroxy-phenylpropanoids, such as ferulic acid and sinapic acid, and their corresponding alcohols are considered to be precursors in lignin formation (*cf.*

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reviews by BRAUNS & BRAUNS (1960), WATKIN *et al.* (1960), BROWN (1961) and FREUDENBERG (1962, 1965)). Therefore, it is likely, that the occurrence and metabolism of phenolic substances might play a part in resistance against cucumber scab in cucumber seedlings.

The above mentioned observations prompted an examination of the polyphenol oxidases present in inoculated and non-inoculated cucumber seedlings of the susceptible and resistant varieties mentioned. Moreover, an investigation of *p*-hydroxy-phenylpropanoid substances and related aromatic compounds in cucumber seedlings was undertaken.

In the first of a series of publications the experiments on fungal and host polyphenol oxidases will be described.

MATERIALS AND METHODS

Green cucumber seedlings

Seeds of the two varieties examined were germinated in steamed river sand at 27 °C in a humid atmosphere for three days; then the seedlings were placed at 20 °C under artificial light (12 hours a day) for another three till four days. After being placed in water, part of the seedlings were inoculated by spraying with a dense spore suspension of *Cladosporium cucumerinum*. The inoculated and uninoculated seedlings were then kept for five days at 18–20 °C under polyethylene sheets to keep a high R.H.

Etiolated cucumber seedlings

Other seeds were germinated on filter papers soaked with Hoagland solution (2/5 strength), the papers laid on wax paper and wrapped up to give so-called rag-dolls. These rag-dolls were placed under polyethylene sheets to keep a high R.H. After having been in the dark at 23 °C for five days, part of the seedlings was inoculated. The inoculated and uninoculated seedlings were rewrapped and replaced in the dark at 17 °C for another five days.

Chemicals

For preparing nutrient media chemicals of common purity or analytical grade were used. The phenols used as substrates for polyphenol oxidase were of analytical grade quality; the firms which supplied the diverse phenolic compounds are mentioned in the text.

Enzyme preparations

Crude enzyme preparations either from fresh plants or from seedlings, which – after cutting off the roots – were kept in the deep-freeze till use, were obtained by grinding the seedlings in a mortar and squeezing the material through cheese cloth. The plant juices were centrifuged at approximately 12000 $\times$ *g* for 20 minutes and the clear supernatant used as such. In the later experiments acetone precipitates were prepared by adding three parts of cooled acetone (–21 °C) to one part of crude plant extract. The acetone insoluble precipitates were spun down at 1600 $\times$ *g* and dissolved in distilled water. This operation was repeated and the final enzyme solution in distilled water used in assays.

Measurement of polyphenol oxidase activity

Enzyme solutions prepared as mentioned above added to buffered solutions of catechol (The British Drug Houses Ltd.), chlorogenic acid (Nutritional Biochemicals Corporation), dihydroxy-phenylalanine (dopa; Hoffman-la Roche) or other di- and polyphenols caused a yellow, light brown or red discolouration, which, depending on enzyme activity, turned dark brown to black after some time, giving insoluble melanins. This rapid change of colour rendered quantitative spectrophotometric measurements of polyphenol oxidase activity virtually impossible. Only catechol oxidase activity could be measured spectrophotometrically by the increase of coloured oxidation products provided measurements were limited to the first three minutes of enzyme action and provided that acetone precipitates were used. The reaction mixtures, usually consisting of 1 ml of enzyme solution plus 2 ml of 1 % catechol in McIlvaine buffer pH 6.0, were incubated at 30°C. Under these experimental conditions, the reaction mixtures showed a maximal absorbance at 400 mμ for at least five minutes. With the reaction time chosen, oxygen tension proved not to be a limiting factor; therefore, the enzyme-substrate solutions were not aerated.

Unfortunately this method, which is essentially the same as that used by MATTA & DIMOND (1963), proved to be unsuitable for the measurement of chlorogenic acid and dopa oxidase activities. Chlorogenic acid oxidase, however, could be estimated by a decrease of substrate concentration according to a method elaborated by VAN KAMMEN & BROUWER (1964).

All spectrophotometric measurements were performed using a Beckman DU spectrophotometer with a 1 cm light path.

RESULTS

Polyphenol oxidases in culture filtrates of Cladosporium cucumerinum

Prior to the examination of the host-parasite complex, the occurrence of polyphenol oxidases in the fungus and in cucumber seedlings was investigated separately. To this end the fungus was grown in two different media, *viz.*

1: G 5 glass-filter sterilized sap of green cucumber seedlings, variety 'Lange Gele Tros', and

2: Richards' solution, containing 1 % KNO_3, 0.5 % KH_2PO_4 and 0.25 % $MgSO_4$ in distilled water, with addition of 2 % glucose and 0.1 % Difco yeast extract.

After three days very heavy growth occurred in both media. Up to five days, only in medium 1 a distinct catechol oxidase activity was found in cell-free culture filtrates; dopa, however, was not oxidized. Only after six days both media showed catechol as well as dopa oxidase activity.

Due to the difficulties encountered in measuring enzyme activities quantitatively, as outlined under MATERIALS and METHODS, the preliminary experiments can not be expressed quantitatively. However, they permit the following conclusions:

1. Comparing the catechol, dopa and gallic acid oxidase activities in different media, it has been found (Table 1), that in medium 2 plus catechol, dopa or gallic acid (Light & Co), catechol and dopa oxidase activities were usually lower than in medium 2 without these phenols added; only concentrations of 0.01 % or 0.001 % catechol or 0.001 % dopa did not alter the catechol oxidase activity. Gallic acid oxidase activity was absent in all cases examined. These observations

TABLE 1. The effect of phenolics on the growth of *Cladosporium cucumerinum* and on the production of some phenol oxidases in medium 2 (see p. 159).

Addition to medium 2	Growth			Catechol oxidase			Dopa oxidase			Gallic acid oxidase	
Number of days	3	4	5	4	5	6	4	5	6	5	6
None	+	+++	+++	++	+++	+++	+	++	—	—	—
Catechol 0.1 %	—	—	—	—	—	—	—	—	—	—	—
Catechol 0.01 %	+	+++	+++	+	+++	+++	+	+	+	—	—
Catechol 0.001 %	+	+++	+++	+	+++	+++	—	++	+	—	—
Dopa 0.1 %	+	+	++	—	—	—	—	—	—	—	—
Dopa 0.01 %	+	++	++	—	—	—	—	—	—	—	—
Dopa 0.001 %	+	+++	+++	+	++	+++	—	+	+	—	—
Gallic acid 0.1 %	—	—	—	—	—	—	—	—	—	—	—
Gallic acid 0.01 %	—	—	—	—	—	—	—	—	—	—	—
Gallic acid 0.001 %	+	+++	+++	—	+	++	—	—	—	—	—

— : no growth, or no enzyme activity
\+ : poor growth, or slight enzyme activity
++ : moderate growth, or moderate enzyme activity
+++ : good growth, or high enzyme activity

are in agreement with those of SANDERSON (1965), who found that many polyphenols, even at low concentrations, inhibited the synthesis of catechol oxidase.

2. After freezing, culture filtrates of medium 1 showed a higher catechol oxidase activity. Apparently, freezing had an enhancing effect on polyphenol oxidase activity (*cf.* KAARS SIJPESTEIJN & PLUIJGERS, 1962).

3. From the above mentioned data, it is obvious, that the polyphenol-oxidizing enzymes of *Cladosporium cucumerinum* are constitutive enzymes. Apparently, they are secreted into the culture medium. Endocellular activity was not found.

The relation reaction velocity *(v)* / substrate concentration *(c)* was examined by measuring absorbances of enzyme-substrate mixtures in relation to time. In Fig. 1, according to LINEWEAVER & BURK (1934), $1/v$ has been plotted against $1/c$ for four different enzyme concentrations. From this graph the Michaelis constant K_m for the fungal catechol oxidase can be calculated to be 62.5×10^{-3} M. The relation reaction velocity / enzyme concentration, plotted from the same results, proved to be a linear one; in Fig. 2 it is given for two out of four different enzyme concentrations.

The production of catechol oxidase in growing cultures of *Cladosporium cucumerinum* was now followed quantitatively (Tables 2 and 3). From these Tables it is obvious, that catechol oxidase activity was not found before the pH in the growing culture had increased to a value of 6.0. The addition of small amounts of cucumber sap to medium 2 caused a more rapid increase in growth and pH; likewise, catechol oxidase activity increased faster in this instance. If the pH of the culture medium was adjusted to 6.0 before sterilizing, catechol oxidase was found to be produced from the first day on. From these observations it might be concluded that the formation of catechol oxidase in medium 2

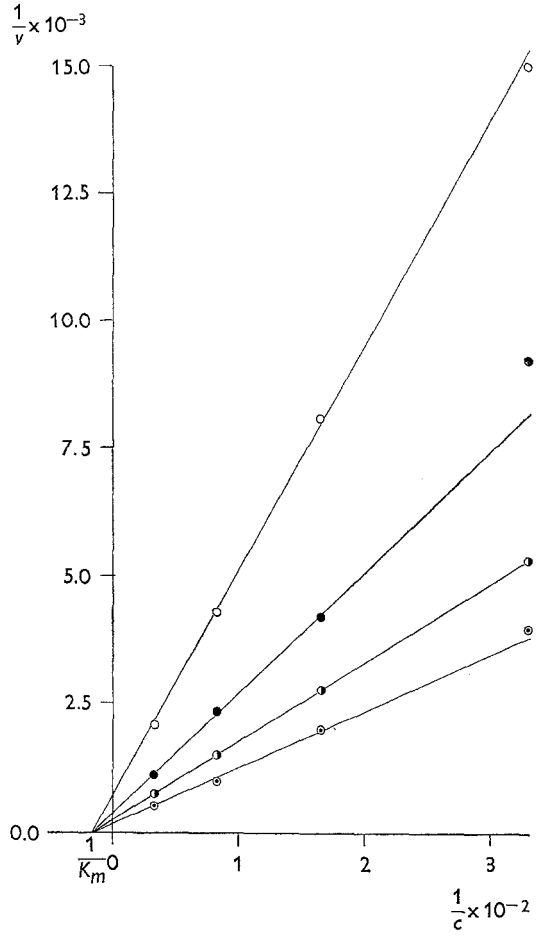


FIG. 1. Relation of reaction velocity (v) and substrate concentration (c), plotted according to LINEWEAVER & BURK (1934), in order to determine the Michaelis constant K_m. c is molar concentration of catechol, v is reaction velocity as ΔA at 400 mμ/sec/3 ml reaction mixture. The four lines represent four different concentrations of an extract of *Cladosporium cucumerinum* (0.20, 0.40, 0.66 and 1.00 ml of enzyme extract/3 ml reaction mixture).

FIG. 2. Relation of reaction velocity ($\times 10^3$) and enzyme concentration, at two different catechol concentrations (0.006 and 0.012 M). Reaction velocity given as ΔA at 400 mμ/sec/3 ml reaction mixture.
Data from Fig. 1.

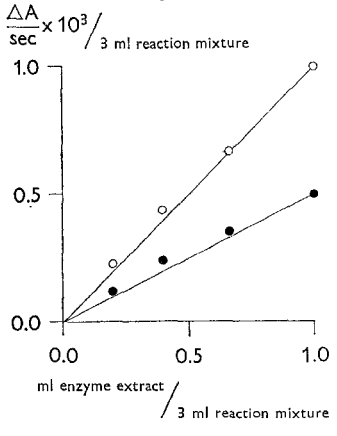


TABLE 2. Production of catechol oxidase in relation to pH in growing cultures of *Cladosporium cucumerinum* (medium 2). Catechol oxidase activity is given as ΔA ($\times 10^3$) at 400 mμ/sec/added ml of crude culture filtrate (2 ml of reaction mixture; final substrate concentration 0.045 M).

Number of days	Catechol oxidase	pH
2	–	4.8
3	–	4.9
4	0.11	5.0
5	0.13	5.7
6	0.48	6.1
9	1.88	6.7

–: activity not measurable

is strongly influenced by its pH, activity becoming detectable only if the pH has reached a value of about 6.0.

TABLE 3. Production of catechol in relation to pH in growing cultures of *Cladosporium cucumerinum* (medium 2), as influenced by the addition of small amounts of cucumber plant sap and by adjusting the pH of the medium to 6.0 before sterilization. Catechol oxidase activity measured as indicated in Table 2.

Number of days	No cucumber sap added; pH not adjusted to 6.0		Cucumber sap added; pH not adjusted to 6.0		No cucumber sap added; pH adjusted to 6.0	
	Catechol oxidase	pH	Catechol oxidase	pH	Catechol oxidase	pH
2					0.03	6.2
3	–	4.8	0.20	5.2	0.47	6.2
4	–	5.8	0.24	5.4		
5	–	4.9	0.35	5.5	0.86	6.4
6	–	5.0	0.68	5.9		
7					1.29	6.4
8	0.12	5.2	1.72	6.2		
10	0.18	6.0	1.96	6.4		

–: activity not measurable

Chlorogenic acid oxidase activities proved to be negligible. Although dopa oxidase activities certainly were very high, neither of the methods used was suitable to measure these enzyme activities quantitatively.

Polyphenol oxidase in non-inoculated cucumber seedlings

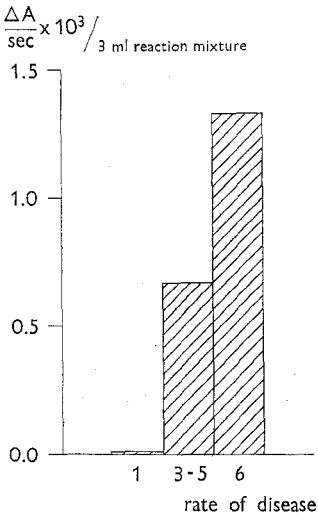
When crude extracts of non-inoculated green and etiolated cucumber seedlings or acetone precipitates were incubated with phenolic substrates, no discolouration took place with any of the substrates tested. Only in extracts of older seedlings (eight till ten days) a very slight activity was found in some cases (*cf.* OPEL *et al.*, 1961).

None of the compounds known to activate latent polyphenol oxidases (Na-dioctyl-sulphosuccinate, pectin and polygalacturonic acid; DEVERALL & WOOD, 1961) resulted in an activation of the enzyme, nor did changing of pH or heat treatment. Freezing at –21 °C (KAARS SIJPESTEIJN & PLUIJGERS, 1962), however, activated the latent polyphenol oxidases in inoculated cucumber seedlings of the susceptible and the resistant variety (see below). With uninoculated seedlings and also with fruit homogenates, on the other hand, cold storage of intact plants, homogenates or plant extracts did not result in activation of a polyphenol-oxidizing enzyme system. Therefore, it might be concluded that polyphenol oxidases are absent in young uninoculated cucumber seedlings of the varieties examined.

Polyphenol oxidases in inoculated cucumber seedlings

High polyphenol oxidase activities have been found in inoculated etiolated and green cucumber seedlings of the susceptible variety, provided the seedlings had been frozen after harvest. Judging from colour changes in enzyme-substrate mixtures, caffeic acid (Nutritional Biochemicals Corporation), catechol, chlorogenic acid, dopa, gallic acid, *p*-hydroxy-phenylpropionic acid (Fluka AG),

FIG. 3. Relation of enzyme activity ($\times 10^3$) and rate of disease (according to an arbitrary scale from 0 (healthy) – 6 (entirely diseased)). Enzyme activity given as ΔA at 400 mμ/sec/3ml reaction mixture. Catechol concentration 0.006 M.



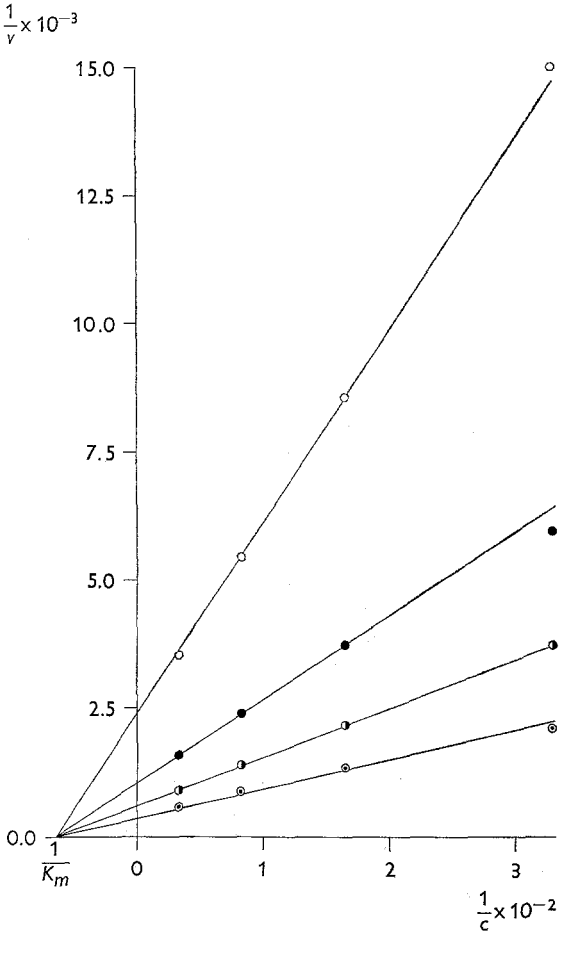


FIG. 4. Relation of reaction velocity (v) and substrate concentration (c), plotted according to LINEWEAVER & BURK (1934), in order to determine the Michaelis constant K_m. c is molar concentration of catechol, v is reaction velocity as ΔA at 400 mμ/sec/3 ml reaction mixture. The four lines represent four different concentrations of an extract of scab diseased cucumber seedlings var. 'Lange Gele Tros' (0.04, 0.08, 0.13 and 0.20 ml of enzyme extract/3 ml reaction mixture).

phloridzin (Fluka AG), phloroglucinol and pyrogallol proved to be substrates for the polyphenol oxidase present.

From these data, at first sight it seems obvious that polyphenol oxidase activity in scab diseased cucumber seedlings originates from the fungus *Cladosporium cucumerinum*. This conclusion seems to be corroborated by the results of an experiment in which catechol oxidase activity has been measured in relation to the rate of disease (Fig. 3). Apparently, the catechol oxidase activity increases readily with disease development, becoming detectable only when symptoms become visible.

Determination of the Michaelis constant K_m of the catechol oxidase from inoculated green seedlings of the susceptible variety 'Lange Gele Tros' revealed a value quite different from the one obtained for the fungal catechol oxidase, *viz.* 15.6×10^{-3} M (Fig. 4). Also in this instance the relation reaction velocity / enzyme concentration, plotted from the same results as used in Fig. 4, proved to be a linear one, as has been shown in Fig. 5 for two out of four different enzyme concentrations.

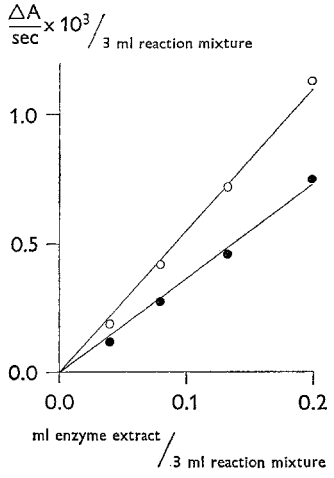


FIG. 5. Relation of reaction velocity ($\times 10^3$) and enzyme concentration, at two different catechol concentrations (0.006 and 0.012 M). Reaction velocity given as ΔA at 400 mμ/sec/3 ml reaction mixture. Data from Fig. 4.

Exactly the same K_m-value was obtained for the catechol oxidase from inoculated green seedlings of the resistant variety 'Vios'. In order to determine this K_m-value the extracts had to be concentrated considerably, the catechol oxidase activity in the inoculated resistant variety being only 3.9% of that in the inoculated susceptible variety, at the same age of seedlings and at a catechol concentration of 0.03 M.

The striking difference in K_m-value of the catechol oxidases of *Cladosporium cucumerinum* and of inoculated green cucumber seedlings (62.5×10^{-3} and 15.6×10^{-3} M, respectively) is evidently in contradiction with the hypothesis outlined above, according to which the catechol oxidase in scab diseased cucumber seedlings should be of fungal origin. Because the Michaelis constants of the catechol oxidases of cucumber seedlings are smaller, not larger, than that of the fungal catechol oxidase, the supposition, that a competitive inhibitor of the fungal catechol oxidase in the inoculated cucumber seedlings should be

responsible for the difference in K_m-values can be ruled out. It seems more likely that the fungus elicits the production or the activation of a catechol oxidase by the plant. In the host-parasite complex the activity of the plant catechol oxidase might by far surpass that of the fungal catechol oxidase.

Information relevant to this hypothesis could be obtained by the use of purified enzymes.

DISCUSSION

As a result of infection, increases in enzyme activities in host-parasite complexes are frequently established. In virus infected plants, in which every increase in enzyme activity must be of plant origin, sharp increases in many different enzyme activities (*e.g.* polyphenol oxidase: OPEL *et al.*, 1961; FARKAS & KIRÁLY, 1962; FARKAS *et al.*, 1963/64; HARPAZ & KLEIN, 1964; VAN KAMMEN & BROUWER, 1964) are reported. With fungal diseases it is less easy to determine, whether the increased enzyme activities are of host or of fungal origin. Evidence, however, is increasing that the host plant can either activate or synthesize enzyme proteins as a result of fungal invasion (*e.g.* polyphenol oxidases (FARKAS & KIRÁLY, 1958; URITANI, 1963, 1965; TOMIYAMA & STAHMANN, 1964; FARKAS, 1965; STAHMANN, 1965). The increased enzyme activity seems to be confined nearly entirely to those plant cells immediately adjacent to the site of infection (STAHMANN, 1965). Before, however, a role in host-parasite interaction should be postulated for these increased enzyme activities, it is necessary to obtain a more precise knowledge on the enzyme substrates, either already present in the plant cells before infection, or synthesized *de novo* after infection.

Therefore, a subsequent publication will deal with the presence and concentration of phenolics in the host-parasite complex, as studied by us; there, the significance of increased polyphenol oxidase activities after infection will be discussed in relation to substrate concentration.

SAMENVATTING

Een onderzoek werd ingesteld naar het voorkomen van polyfenoloxydasen in relatie tot resistentie tegen en vatbaarheid voor vruchtvuur in komkommers. Daartoe werden de activiteiten van dit enzymsysteem (waarbij catecholoxydase als voorbeeld werd gekozen) van een vatbaar ras, 'Lange Gele Tros', en een resistent ras, 'Vios', met elkaar vergeleken, zowel in gezonde als in met *Cladosporium cucumerinum* geïnoculeerde zaailingen. Ook in cultuurfiltraten van de schimmel werd de polyfenoloxydase-activiteit bepaald.

In cultuurfiltraten van de schimmel werd een constitutieve exocellulaire catecholoxydase gevonden; bovendien werden dihydroxy-fenylalanine en chlorogeenzuuroxydasen gevormd. Catecholoxydase werd alleen in aanzienlijke hoeveelheden geproduceerd indien de pH van het cultuurmedium tot 6.0 was gestegen, of indien de pH van te voren op 6.0 werd gebracht. De Michaelis-constante K_m van de schimmelcatecholoxydase bleek 62.5×10^{-3} M te zijn.

In zeer jonge, niet-geïnoculeerde "groene" en geëtioleerde zaailingen werd geen catecholoxydase-activiteit gevonden. Hoge polyfenoloxydase-activiteiten werden daarentegen gemeten in geïnoculeerde zaailingen van het vatbare ras, mits deze – na het oogsten – bevroren werden. Ook in zaailingen van het resistente ras werd na inoculatie polyfenoloxydase gevormd, hoewel met een veel geringere activiteit. In beide gevallen bleek de Michaelis-constante $15.6 \times$

10^{-3} M te zijn. Dit verschil in Michaelis-constanten van de schimmelcatecholoxydase en van die geproduceerd in geïnoculeerde zaailingen suggereert, dat de polyfenoloxydase in het laatste geval niet van de schimmel stamt, doch dat infectie met de schimmel de plant induceert tot *de novo*-produktie van dit enzym, of wel tot activering van een latente polyfenoloxydase.

De betekenis van deze verhoogde polyfenoloxydase-activiteit na infectie voor de gastheer-parasiet-combinatie kan pas begrepen worden na een onderzoek naar het voorkomen van polyfenoloxydase-substraten in de gastheer.

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