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Effect of chemotherapeutically active amino acids on pathogenicity and growth of a wild type strain and a nutritional mutant of Cladosporium cucumerinum Ell. et Arth.

O. M. VAN ANDEL

Laboratory of Phytopathology, Agricultural University, Wageningen, The Netherlands*

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Abstract

The pathogenicity of a wild type strain and a nutritional mutant of *Cladosporium cucumerinum* was not changed after prolonged culturing on media containing D-serine, L-threo-β-phenylserine or DL-β-aminobutyric acid. Growth of the wild type strain was inhibited by D-serine, whereas the mutant was found able to utilize this compound. Both strains were able to grow in a medium containing L-threo-β-phenylserine as sole source of nitrogen. These results support the hypothesis that the increase in resistance to both strains of cucumber seedlings treated with the compounds just-mentioned is not due to a direct inhibition of the development of the pathogen.

Introduction

According to several authors (Hrushovetz, 1957; Lowther, 1964; Williams, 1965) culturing pathogenic fungi on media containing certain amino acids changed their virulence. Williams (1965) pointed out that such a phenomenon, resulting perhaps from a change in enzyme systems, would be a valuable tool in the study of the physiology of host–pathogen relationships. It was tried to gain an impression of the effect of some amino acids with chemotherapeutic activity against cucumber scab on the pathogenic behaviour of *Cladosporium cucumerinum* by investigating the pathogenicity of the fungus grown in the presence of the compounds just mentioned. D-serine, L-threo-β-phenylserine and DL-β-aminobutyric acid were used as chemotherapeutically active compounds (van Andel, 1958, 1962); for comparison L-serine and glycine, being inactive in this respect, were chosen.

Materials and methods

Cladosporium cucumerinum Ell. et Arth. was grown at 23 °C on cherry agar to which amino acids were added to a final concentration of 0.01 M. Cultures were transferred weekly to fresh media of the same composition. Controls were grown on plain cherry agar.

Pathogenicity tests were carried out by inoculating 9 day old cucumber seedlings cv 'Lange gele Tros' in the way described before (van Andel, 1958).

Spore germination was determined in drops of suspensions of 2×10^6 spores/ml sterile

* Present address: Institute for Biological and Chemical Research on Field Crops and Herbage, Wageningen, the Netherlands

tap water on microscopic slides, after 16–24 h incubation in a moist chamber at 23 °C. Linear growth of mycelium was measured on cherry agar plates kept at 23 °C. Growth as dry weight production was determined from shake cultures, consisting of 25 ml nutrient solution in 100 ml erlenmeyers inoculated with 1 or 2 × 10^6 spores after 3, 5, 8 and sometimes 11 days of incubation at approximately 25 °C. Four replicates were used for each determination. The nutrient solution contained 2% glucose; 0.5% KH_2PO_4; 0.1% $(NH_4)_2SO_4$; 0.05% $MgSO_4$ 7 aq; 0.05% NaCl in tap water. Amino acid solutions were separately sterilized by filtration and added to autoclaved cherry agar or nutrient solution.

The amino acids used were obtained from Nutritional Biochemical Corporation, Cleveland, Ohio, U.S.A., except L-threo-β-phenylserine, which was kindly provided by S.A.F. Hoffmann-La Roche and Co., Basel, Switzerland.

Results

Cultural characteristics

Cultures of *C. cucumerinum* maintained on plain cherry agar and on cherry agar containing 0.01 M L-threo-β-phenylserine, DL-β-aminobutyric acid, L-serine or glycine showed a similar appearance even after 9 weekly transfers: darkish green mycelium sporulating abundantly. On a D-serine containing medium the colour was pale grey and sporulation scarce already at the first transfer. At the 7th transfer a darker area was noticed on one such culture. The strain isolated from this part of the culture, henceforth indicated as BR, grew and sporulated very well on D-serine media and could not be distinguished visually from the original strain growing on a D-serine free substrate. Linear growth of mycelium obtained after 9 transfers from the cultures mentioned above was measured: (a) on plain cherry agar, and (b) on cherry agar containing 0.01M of the corresponding amino acid. No differences in growth rate were found in either case for cultures previously grown in the presence of L-threo-β-phenylserine, DL-β-aminobutyric acid, L-serine or glycine, or without amino acids. Mycelium obtained from a culture on D-serine showed on plain cherry agar about the same growth rate as the control, but the rate was decreased to some extent in the presence of D-serine.

Table 1. Effect of amino acids incorporated in the culture media on the pathogenicity of a wild type strain and a mutant of *C. cucumerinum* at three inoculum levels

	Disease index		
inoculum level (spores/ml)	*200,000*	*50,000*	*12,500*
Wild type strain, grown on			
cherry agar	3.7	2.4	2.6
cherry agar with D-serine	3.3	2.3	2.8
cherry agar with L-threo-β-phenylserine	3.4	2.3	2.0
cherry agar with L-serine	3.5	2.3	2.5
cherry agar with glycine	3.5	2.2	2.6
mutant BR, grown on cherry agar with D-serine	3.4	2.7	3.0
Maximum disease index 4			

Tabel 1. Pathogeniteit van een "wild type" stam en een mutant van C. cucumerinum na kweken op media met verschillende aminozuren

Table 2. Effect of treatment with D-serine and L-threo-β-phenylserine on the susceptibility of cucumber seedlings to infection by a wild type strain and a mutant of *C. cucumerinum* grown on media containing 0.01 M of these compounds

Culture medium of pathogen and strain	*Treatment of seedlings:*	*Disease index*		
		control	*D-serine 0.05 M*	*L-threo-β-phenylserine 0.01 M*
control, wild type strain		3.0	1.5	1.3
D-serine, wild type strain		3.4	1.6	1.8
L-threo-β-phenylserine, wild type strain		3.4	1.6	1.0
L-serine, wild type strain		3.0	1.4	1.0
D-serine, mutant BR		3.8	1.4	1.3

Tabel 2. Effect van behandeling van komkommerkiemplanten met D-serine en L-threo-β-phenylserine op de vatbaarheid voor twee stammen van C. cucumerinum, gekweekt op media met 0.01 M van de betrokken verbindingen

Strain BR grew more rapidly than the control on cherry agar even after addition of 0.01 or 0.05 M D-serine.
Spores obtained from the cultures on the various media after 8 transfers germinated to about 80%, those produced by strain BR to 89%.
Although D-serine inhibited mycelium growth and sporulation of C. *cucumerinum* to some extent, as had been found before (van Andel, 1958), growing the fungus in the presence of this or any other amino acids did not induce any lasting changes in its cultural characteristics.

Pathogenicity
Six to seven days after each transfer spores from cultures on the six media mentioned before were used to inoculate cucumber seedlings. No significant differences in the severity of the disease were found even after 9 transfers, when the same spore concentrations were used (Table 1). Treatment of the seedlings with D-serine or L-threo-β-phenylserine via the roots before inoculation reduced disease indices to a similar extent independently of the origin of the spores used for inoculation (Table 2).

Effects of amino acids on growth of C. cucumerinum
As was to be expected from the results just mentioned dry weight production of the wild type strain of the fungus in shake culture was decreased in a complete nutrient solution containing D-serine. Strain BR, however, showed an increase in growth rate in these conditions, which suggested that D-serine might be used as a nutrient. Table 3 shows considerable growth of strain BR in a solution containing D-serine as the only source of nitrogen. Even the wild type strain, however, was able to grow slightly. It seems plausible that the decreased sensitivity of strain BR to D-serine is due to its ability to metabolize the compound. It should be added that strain BR grew faster than the wild type strain in the control medium as had been observed on cherry agar.
Of the other compounds investigated L-threo-β-phenylserine proved to cause an increase in dry weight production in shake cultures of strain BR when added to a complete medium, but had no effect on that of the wild type strain. In a medium lack-

Table 3. Growth response of a wild type strain and a mutant of *C. cucumerinum* to D-serine in the presence and absence of an inorganic nitrogen source. Growth measured as dry weight production after 8 days incubation and expressed as percentage of the dry weight produced in a nutrient solution containing $(NH_4)_2SO_4$.

	Medium containing 0.0375 M $(NH_4)_2SO_4$		*Medium without* $(NH_4)_2SO_4$	
		0.075 M D-serine added		*0.075 M D-serine added*
Wild type strain	100	55.8	6.1	15.4
Mutant BR	100	123.8	2.0	90.5

Tabel 3. Invloed van D-serine op de groei van een "wild type" stam en een mutant van C. cucumerinum in aanwezigheid en afwezigheid van een anorganische stikstofbron. De groei is bepaald als hoeveelheid drooggewicht gevormd in 8 dagen en uitgedrukt als percentage van het drooggewicht in een medium met $(NH_4)_2SO_4$.

ing other sources of nitrogen than L-threo-β-phenylserine about the same dry weight was found for strain BR as in the control containing an equivalent amount of nitrogen as $(NH_4)_2SO_4$, whereas for the wild type strain $(NH_4)_2SO_4$ could apparently only be partially replaced by the amino acid. L-serine proved to be an excellent source of nitrogen for both strains. Of the other D-amino acids investigated none were found to inhibit growth of either strain in a complete medium. Replacement of inorganic nitrogen by equivalent amounts of nitrogen as D-threonine, D-alanine, D-methionine, D-leucine, D-isoleucine or D-phenylalanine resulted in a distinctly slower growth of BR as compared with the control; D-histidine proved a very unsuitable source of nitrogen.

Discussion

In previous publications the chemotherapeutic effect of a number of amino acid analogues on cucumber scab has been ascribed to an increase of the resistance of the host rather than to an effect on the pathogen. This hypothesis was based, among other things, on the fact that in preliminary experiments the compounds did not inhibit growth of *C. cucumerinum* in vitro or were inhibitory only at relatively high concentrations (van Andel, 1958, 1962). However, the possibility of inhibition of fungal growth as the result of local accumulation of large amounts of, for instance, D-serine, in host tissue could not be excluded on the one hand; on the other hand pathogenic development of the fungus might be affected in a different way than by growth inhibition.
The experiments described above do not provide any evidence for the latter possibility, which may be due, for one thing, to the fact that not the infecting mycelium but only the spores it originated from had been in contact with the amino acids. No lasting effect was found on growth rate or spore viability, although D-serine was able to reduce growth of the wild type strain considerably in a rich as well as in a poorer medium Spore germination and in particular growth of the germination hyphae was inhibited by D-serine and L-threo-β-phenylserine only in water (van Andel, 1958; van Zaaijen, unpublished). A nutritional mutant, however, strain BR, was not inhibited by D-serine, but able to even utilize the compound, whereas both strains utilized L-threo-β-phenylserine to some extent. Treatment of cucumber seedlings with D-serine and

L-threo-β-phenylserine, however, resulted in an increase in resistance to both strains of the pathogen to a similar extent. These results may be considered to support the hypothesis that no direct effect on the development of the fungus is involved in the increase in resistance.

There are few data on the breakdown of D-amino acids by fungi. D-amino acid oxidase whose presence was demonstrated in several species and strains of fungi (Horowitz, 1944; Emerson et al., 1950), did not oxidize D-serine or D-threonine, whereas D-methionine, D-leucine and D-phenylalanine were rapidly broken down, compounds which proved less suitable sources of nitrogen in our experiments, so it seems doubtful that a similar enzyme is involved in the utilization of D-serine. Emerson et al. (1950) claim that there is no correlation between the ability of a fungus to utilize a D-amino acid as a source of nitrogen and the presence of D-amino oxidase, which might be in agreement with our results, but in his experiments DL-serine was used, which might confuse the issue. Zenk and Scherf (1964) demonstrated conversion of a number of D-amino acids to N-acetyl-D-amino acids in several fungi, but these compounds are not known to be broken down, whereas again D-serine was among the amino acids least easily converted.

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Samenvatting

Het effect van chemotherapeutisch werkzame aminozuren op de pathogeniteit en groei van een "wild type" stam en een mutant van Cladosporium cucumerinum Ell. et Arth.

De pathogeniteit van een "wild type" stam en een mutant van *Cladosporium cucumerinum* werd niet beïnvloed door kweken op media, die D-serine, L-threo-β-phenylserine of Dl-β-aminoboterzuur bevatten. De groei van de "wild type" stam werd door D-serine geremd, terwijl de mutant deze verbinding als stikstofbron kon gebruiken. Beide stammen waren in staat te groeien in een medium dat alleen L-threo-β-phenylserine als stikstofbron bevatte. Deze resultaten steunen de hypothese dat de toename in resistentie van komkommerkiemplanten tegen beide stammen, veroorzaakt door behandeling met de genoemde verbindingen, niet het gevolg is van een directe remming van de ontwikkeling van het pathogeen.

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