Alternaria 属菌によるテヌアゾン酸産生とその病理学的評価

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Distribution of Tenuazonic Acid Production in the Genus *Alternaria* and its Pathological Evaluation*

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木下忠孝**・蓮仏由美子**・I. D. KHAN***・甲元啓介**・西村正暘**: *Alternaria* 属菌によるテヌアゾン酸産生とその病理学的評価*

Abstract

Tenuazonic acid which was reported hitherto from *Alternaria tenuis* and *A. longipes* has now also been found in *A. kikuchiana* and *A. mali*, during our investigations on the host-specific toxins in their culture filtrates. However, further investigation on the 19 different isolates of *A. kikuchiana* has revealed that tenuazonic acid was produced not in all isolates used, but in only 6 isolates. A similar investigation on *A. mali* showed that its productivity was in different proportions with the isolates. All virulent isolates of both the *Alternaria* spp. are well-known to produce a host-specific toxic filtrate, in which so-called host-specific toxin is contained. Tenuazonic acid was fairly toxic to young leaves of Japanese pear and apple which are host plants of both the fungi respectively, but not involved in a host specificity of culture filtrates, because both the resistant and susceptible cultivars of each plants were equally sensitive to tenuazonic acid. Among the isolates used, however, tenuazonic acid production was frequently found to be in harmony with that of host-specific toxin in culture filtrate. Screening of 185 isolates, belonging to several species of *Alternaria*, for tenuazonic acid production revealed its widespread occurrence in this genus. Results suggesting tenuazonic acid as a characteristic metabolite and not a pathogen toxin are also discussed in relation to chemotaxonomical interest. (Received February 3, 1972)

Introduction

In some plant diseases, it is well-known[12,13] that pathogens produce host-specific toxic filtrates and a toxin, called host-specific or host-selective toxin (HST), might be involved in disease development. In so far as we know[12], there are nine distinct examples where a toxin produced *in vitro* displays the same host specificity as the pathogen itself. At present, these are limited only to 3 genera of fungi; three species (*A. citri*, *A. kikuchiana* and *A. mali*) of *Alternaria*, five species of *Helminthosporium* and one species of *Periconia*.

During our investigation on the phytotoxic metabolites in *Alternaria kikuchiana* Tanaka and *A. mali* Roberts, causing black spot of Japanese pear and blotch of apple respectively, it was observed that the same acidic fraction which contained HST in the respective culture filtrates gave a strong

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orange-red color with ferric chloride. This was not attributable to any of the compounds described so far. Subsequently, extraction from a large amount of culture filtrates yielded tenuazonic acid (TA) which was identified by direct comparison with an authentic specimen. TA was first isolated by Rosett *et al.* (1957)[16] as a metabolite of *Alternaria tenuis* Nees, and its chemical structure was established by Stickings *et al.* (1959)[18] as 3-acetyl-5-*sec.* butyl-4-hydroxy-3-pyrrolin-2-one. Later, Ramm and Lucas (1963)[15] reported that *A. longipes* (Ell. and Ev.) Mason was also a TA producer. Recent reports by Mikami *et al.* (1970, 1971)[5~7] showed that TA was toxic to tobacco leaves and among several pathogenic isolates of *A. longipes*, differences in pathogenicity correlated with differnces in TA production. Nonpathogenic isolates lacked its production in culture.

In our present fungi, *i.e.*, *A. kikuchiana* and *A. mali*, the ability to produce a toxin specific to only the leaves of susceptible cultivars of Japanese pear and apple, respectively, is prescribed as an unique feature of the virulent isolates only. We can find easily each HST in their culture filtrates. Nevertheless, study of TA production is of value, because the compound is produced adundantly in culture and is fairly toxic to plants without any display of host specificity. The co-occurrence of HST and TA would offer synergistic effect on toxicity, although, at present, there is no evidence that this acid is involved in disease development.

One of the purpose of this communication is to report for the first time the occurrence of TA from *A. kikuchiana* and *A. mali*. Furthermore, data are given on comparative TA production by several isolates of both the pathogens in relation to fungal growth rate and HST productivity in culture. In addition, since we feel that the frequent reports of TA from *Alternaria* are of great pathological or taxonomical interest, a number of *Alternaria* species were surveyed for the detection of TA. A preliminary report of this work has been presented[4].

We wish to thank many plant pathologists for supplying the isolates of *Alternaria* used, and also to Dr. Mikami, Central Research Institute of the Japan Monopoly Corporation, for providing authentic specimen of TA.

Materials and methods

Organisms and culture conditions

Isolates of the genus *Alternaria*, selected from our culture collection and also kindly provided by several plant pathologists in Japan, were maintained on potato-dextrose agar slants. For isolation and identification of TA, isolate No. 19 of *A. kikuchiana*, highly virulent to Nijisseiki cultivar of Japanese pear was selected for the growth in liquid culture.

Cultures for experimental purpose were grown in 300 m*l* Erlenmeyer flasks containing 100 m*l* either of Richards' medium or its modification, with addition of 0.5% peptone. Incubation was done at 28°C for a period of 15 days without agitation.

Identification of TA in culture filtrate

The culture filtrate from *A. kikuchiana*, No. 19 was extracted with benzene by vigorous shaking. Then, the benzene extracts were shaken with 10% sodium bicarbonate solution. The sodium bicarbonate extracts were acidified with 1N-HCl and extracted with petroleum ether. The petroleum ether extracts, after removal *in vacuo*, left an orange-colored gum. This residue showed a remarkable spot of orange-red color with ferric chloride on TLC (MeOH : benzene, 19 : 1, v/v). This compound was separated and purified by the method of Rosett *et al.*[16] to give TA, and was shown by direct comparison (TLC, IR and UV) to be identical with an authentic TA.

Qualitative and quantitative assays for TA from culture filtrates were made spectrophotometrically.

Ten m*l* of the culture filtrate acidified with 1N-HCl was extracted twice with 10 m*l* portions of diethyl ether. The ether phases were combined, and to it anhydrous sodium sulfate was added. The solution was evaporated to dryness. To the residue 10 m*l* of petroleum ether was added and boiled for 10 min. under a reflux. The petroleum ether was filtered and evaporated to dryness under reduced pressure. The residue thus obtained was dissolved in 95% ethanol and measured for its optical density at 277 mμ with a Hitachi 124 spectrophotometer. TA content was determined by comparison with a standard curve prepared with the pure compound. Pure TA shows two maxima in ethanol at 217 and 277 mμ, but in an alkali condition there is no absorption peak at 217 mμ, but only at 239 mμ. However, in an acidic condition, the lower maximum is again shifted to 220 mμ. Such a behavior of TA in UV spectra were useful in qualitative detection of TA from the culture filtrates.

Assays of HSTs and TA of *A. kikuchiana* and *A. mali*

Both the HSTs from the culture filtrates of the fungi were bioassayed by the leaf necrosis method. Detached young leaves of the susceptible plants (cv. Nijisseiki of Japanese pear for *A. kikuchiana* and cv. Indo of apple for *A. mali*), kept on sponge plates in a moist chamber, were slightly cross-wounded with a needle. Such leaves were then inoculated by applying one drop of culture filtrate to each wound with the help of capillary pipet and incubated at 28°C. After a period of 24 hr, the dilution end point for toxic activity of culture filtrates was determined. Resistant cultivars, Chojuro (Japanese pear) for *A. kikuchiana* and Jonathan (apple) for *A. mali* were used as checks. In case of resistant cultivars, even the undiluted culture filtrates did not exhibit any sign of the characteristic necrosis induced only by HSTs.

Results and discussion

TA production in *A. kikuchiana*

Culture filtrates from 19 isolates of *A. kikuchiana*, which varied in their host-specific pathogenicity, were examined for TA production (Table 1). Only six isolates showed TA-producing ability. The remaining 13 were TA-nonproducers even in peptone-containing Richards' medium, which might help in providing amino acids from the peptone to the TA biosynthesis[19]. It is of interest that TA and HST, when present, tend to co-occur frequently. For this reason, a more detailed study on the production of TA in *A. kikuchiana* was conducted with other 69 isolates. Results (Table 3) indicated that 65 isolates were HST producers, and only 17 were TA producers. Hence, most of the virulent isolates of *A. kikuchiana* can't be expected to provide a reliable constant character of TA production. However, the possibility of TA production by some isolates in very small amounts which might be below the limits of detection can't be overlooked.

TA production in *A. mali*

Out of 12 isolates, majority of them (8 isolates), which showed host-specific pathogenicity could also produce TA. The remaining four isolates were avirulent and completely lacked TA production (Table 2). There existed no significant correlation between the amounts of TA and HST produced. The concentrations of TA as well as of HST varied among the isolates.

To confirm the observed harmony between TA and HST productions in *A. mali*, which was suggested by the results obtained above (Table 2), further experiments were conducted with other freshly isolated 41 isolates of the pathogen. It can be observed that 36 isolates produced both TA as well as HST, two produced only HST and two lacked both the metabolites (Table 3).

Table 1. Tenuazonic acid and host-specific toxin production by *A. kikuchiana* in culture

Isolate	Medium[a)]	Growth rate[b)] (g)	TA (mg/100 ml)	Host-specific toxin[c)]
No. 1	R	1.36	0.00	0
	P	2.42	0.00	0
No. 2	R	0.75	0.00	0
	P	0.86	0.00	0
No. 3	R	0.95	0.85	0
	P	1.11	3.90	0
No. 9	R	0.60	0.00	0
	P	0.77	0.00	0
No. 11	R	1.26	0.00	0
	P	1.35	0.00	0
No. 13	R	0.85	0.00	0
	P	1.10	0.00	0
No. 14	R	0.79	0.00	0
	P	1.10	0.00	0
No. 15	R	1.52	Trace	128
	P	1.66	1.43	128
No. 16	R	1.04	0.00	0
	P	1.55	0.00	0
No. 17	R	0.72	0.00	128
	P	0.74	0.00	128
No. 18	R	1.45	0.38	0
	P	1.85	1.55	0
No. 19	R	1.56	4.85	128
	P	1.88	5.80	128
No. 20	R	1.60	0.00	0
	P	1.73	0.00	0
No. 21	R	1.68	0.00	0
	P	1.84	0.00	0
0-15	R	1.37	0.41	32
	P	1.83	4.10	64
0-32	R	1.39	0.38	Trace
	P	1.77	5.50	Trace
0-78	R	0.62	0.00	0
	P	1.02	0.00	0
0-79	R	0.79	0.00	0
	P	0.94	0.00	0
0-97	R	0.94	0.00	0
	P	1.56	0.00	0

a) R and P denote Richards' and 0.5% peptone-containing Richards' medium respectively.

b) Dried weight of mycelial mats after 15-days-culture.

c) Maximum dilution end point of culture filtrates which exhibited black necrosis to young leaves of susceptible Japanese pear. Only the host-specific toxin producing isolates were pathogenic to Nijisseiki pear.

Table 2. Tenuazonic acid and host-specific toxin production by *A. mali* in culture[a)]

Isolate	Medium	Growth rate (g)	TA (mg/100 ml)	Host-specific toxin
0-39	R	0.92	0.35	4
	P	1.25	1.61	1
0-41	R	1.48	0.23	16
	P	1.61	5.05	16
0-69	R	1.96	0.00	0
	P	2.44	0.00	0
0-70	R	0.74	Trace	128
	P	1.50	4.45	128
0-71	R	1.20	0.00	0
	P	1.20	0.00	0
0-72	R	1.63	0.00	0
	P	1.54	0.00	0
0-73	R	1.05	0.00	0
	P	0.71	0.00	0
0-74	R	1.35	3.60	512
	P	1.62	30.00	512
0-75	R	1.29	4.75	128
	P	1.78	11.57	128
0-91	R	1.38	2.65	16
	P	1.53	2.60	1
0-92	R	0.86	0.41	2048
	P	1.58	4.10	2048
0-93	R	0.54	5.40	64
	P	0.43	4.70	512

a) Criterion of growth rate and denotation of medium are given in Table 1. Bioassay method for host-specific toxin was similar to that of *A. kikuchiana*, except that the leaves used were of Indo cultivar of apple.

Table 3. Distribution of tenuazonic acid among the fresh isolates of *A. kikuchiana* and *A. mali*

A. kikuchiana			*A. mali*		
No. of isolates	TA	HST	No. of isolates	TA	HST
17	+	+	36	+	+
48	−	+	3	−	+
4	−	−	2	−	−
Total 69			Total 41		

TA: Tenuazonic acid, HST: Host-specific toxin, +: Producer, −: Nonporducer.

Thus the idea that TA production in *A. mali* may co-occur with that of HST was not always warranted from the present data.

The pathological evaluation of TA showed that it was fairly toxic to young leaves of Japanese pear and apple, which are host plants of both the *Alternaria* species, but are not involved in host specificity of culture filtrates, because both the resistant and susceptible cultivars of each plants were shown to be equally sensitive to TA till 125 ppm.

TA production in the genus *Alternaria*

The above data suggested that TA production, at least, in some isolates of *A. kikuchiana* and *A. mali* can be produced regadless of their ability to produce respective HSTs. Furthermore, TA might be found in other species as well, because of morphological similarities of the species in this genus[9,20]. Hence, the TA producing abilities of a total number of 185 isolates of *Alternaria* belonging to the different species and 2 isolates of *Stemphylium* were determined qualitatively (Table 4). Though the isolates of *Alternaria* used represnted a fairly broad spectrum of the genus as regards to its taxonomical classification and host species from which they were isolated, yet the collection is not highly satisfactory.

Table 4. Tenuazonic acid production by *Alternaria* species

Species	Number of isolates	
	Total	TA producers
A. bataticola[a] (*Macrosporium bataticola* Ikata)	3	0
A. citri Pierce	7	5
A. japonica Yoshii	3	2
A. kikuchiana Tanaka	88[b]	22
A. longipes (Ell. and Ev.) Mason	11	3
A. mali Roberts	53	44
A. oryzae Hara	2	1
A. porri (Ell.) Sawada	3	0
A. solani (Ell. and Mart.) Sorauer	1	0
A. tenuis auct.	4	1
A. spp.[c]	10	5
Stemphylium lycopersici (Enj.) Yamamoto	2	0

a) Tentatively used name for *M. bataticola* Ikata *sensu* Neergaard's basis of classification of *Alternaria* given in his monograph[9], which doesn't describe *A. bataticola*.
b) In *A. kikuchiana* and *A. mali*, the data were shown by the sum total of Table 1, 2 and 3.
c) Isolates that appeared to be insufficiently distinct to permit species designations are included.

It was a surprise to find that, in addition to *A. kikuchiana* and *A. mali*, five out of the seven isolates of *A. citri*, two out of three of *A. japonica*, and one out of two of *A. oryzae* were TA-producers. In the case of *A. longipes*, three isolates out of eleven were TA producers, confirming the findings of Ramm and Lucas[15], and Mikami *et al*[5~7]. In the three species of *Alternaria*, namely, *bataticola*, *porri* and *solani*, which belong to the section of Noncatenatae according to the Neergaard's classification[9,20], all isolates tested to date were completely lacking TA in their culture filtrates. The two isolates of *Stemphylium lycopersici* were also devoid of TA.

As regards to the systematic arrangement of the taxa within the *Alternaria*, we still have a fragmentary knowledge. The present surveys in connection with TA production by some species and their isolates might reveal a certain relationship between chemotaxonomical and morphological classification[9,15]. It appears reasuringly because several morphologically similar species of *Alternaria*, including *A. citri*, *A. kikuchiana*, *A. longipes* and *A. mali*, are regarded as being closely associated on the property of TA production and other biochemical grounds. Parallel situations regarding the selective distribution of fungal metabolites are also known in other genera of plant pathogenic fungi.

For example, among *Fusarium* spp., only *F. oxysporum* and *F. moniliforme* can produce fusaric acid[10,11]; among *Helminthosporium* spp., only *H. victoriae* and certain isolates of *H. carbonum* can produce victoxinine[13]. Therefore, in *Alternaria* spp., superimposed upon the basic pattern of TA, might be other constant metabolites showing selective distribution within the genus. Further work alone can distinguish such a possibility.

Literature on TA indicates that synthetic pathway of TA not only occurs in *Alternaria* but in *Aspergillus* spp. and a number of Sphaeropsidales[3] and an isolate of *Pyricularia oryzae*[2]. This points out that pattern of TA may not show any obvious significance only in *Alternaria*. Recently, TA has also been involved in various fields of study, such as promising chemotherapeutants against human adenocarcinoma-1[3], antiviral agent[8] and mycotoxin[17] in addition to the phytopathological interest.

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和文摘要

Alternaria 属菌によるテヌアゾン酸産生とその病理学的評価

木下忠孝・蓮仏由美子・I. D. KHAN・甲元啓介・西村正暘

Alternaria kikuchiana および *A. mali* の宿主特異的毒素の研究中，それらの培養ろ液中に比較的多量の非特異的な毒性を示す物質の存在を知った。単離，精製の結果，それがテヌアゾン酸 (Tenuazonic acid) であることを確認した。

A. kikuchiana および *A. mali* において，宿主特異的毒素の産生能とテヌアゾン酸産生能の関連性をみるため，前者では88菌株，後者では53菌株を用いて調査した。結果は，両病原菌とも，両物質の同時産生菌株がかなり認められた。テヌアゾン酸は，リンゴあるいはナシ葉に対して，125 ppm 前後まで黒色壊死斑を形成した。しかし，その毒性は宿主特異的ではなかった。

引き続き，*Alternaria* 属菌185菌株を用いて，さらに同属内におけるテヌアゾン酸産生の範囲を検討した。その結果は，上記2種のほかに，*A. citri*, *A. japonica*, *A. longipes*, *A. oryzae* および *A. tenuis* においてもその産生が認められ，*Alternaria* 属内でかなり普遍的に産生されているものと思われた。