STUDIES ON THE TOXINS OF *PYRICULARIA*

1. Detection of Pyriculol in Cultures of *Pyricularia**

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ABSTRACT

Six isolates of *Pyricularia* from cultivated and wild Gramineae and 10 races of *P. oryzae* were studied for production of the phytotoxic compound pyriculol in different media under different cultural conditions. Pyriculol production varied with the isolate, the medium and the cultural condition. The results are discussed in relation to the role of toxins in the blast disease of Gramineae.

INTRODUCTION

Two toxins, *viz.*, α-picolinic acid ($C_6H_5NO_2$) and piricularin ($C_{18}H_{14}N_2O_3$) were implied by Tamari and Kaji (1954) in the blast disease of rice caused by *Pyricularia oryzae* Cavara. Both toxins have been isolated from culture filtrates of the fungus in crystalline form and also detected in extracts from diseased rice plants by paper chromatography. Independently or together, the two toxins have not only been shown to cause a lesion, closely resembling that produced by the parasite, on rice leaves but also induce severe growth inhibition in rice seedlings (Tamari *et al.*, 1965).

Since Tamari and co-workers demonstrated picolinic acid and piricularin as the toxins of *P. oryzae*, there has surprisingly been no other report confirming their observations. Nor is it known if *Pyricularia* occurring on a number of Gramineae other than rice produce the toxins. However, a third phytotoxic compound called pyriculol ($C_{14}H_{16}O_4$) has since been isolated from cultures of *P. oryzae* (Iwasaki *et al.*, 1969 ; Sato and Kozaka, 1970). The compound, classified as a heptaketide (Turner, 1971), causes a dark necrotic spot, resembling the natural blast lesion, on rice leaves and inhibits growth of

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rice seedlings (Sato and Kozaka, 1969). Whether pyriculol is also detectable in cultures of other *Pyricularia* spp. and in leaves of diseased plants does not appear to have been investigated.

As part of a continuing programme of research in this laboratory on the 'blast' disease, repeated attempts were made to detect piricularin and picolinic acid in cultures of *Pyricularia* spp. from cultivated and wild Gramineae (*vide*, Materials and Methods) according to the methods of Tamari and Kaji (1954) and Tamari *et al.* (1965). The cultures were grown in Tamari's media (Tamari *et al.*, 1965), sucrose-nitrate medium (Suryanarayanan, 1958) and Sabouraud's liquid medium (Difco Manual, 1953). Tamari and Kaji (1954) isolated the two toxins from surface cultures incubated for 30 days. Tamari *et al.* (1965) also reported that aerated submerged cultures of *P. oryzae* yielded 0·5–0·8 μg/ml of piricularin after 40 h growth. They, however, indicated that the fungus tended to lose its toxin producing ability through repeated serial cultures. Fresh isolates were, therefore, used in the present work, but the two toxins could not be detected in stationary cultures of any of the *Pyricularia* spp. under report even when incubated upto 40 days. Similarly, the two toxins could not be detected in shake or aerated submerged cultures grown for varying lengths of time. Since piricularin was reported to be bound to a protein (Ogasawara *et al.*, 1957 *a*, *b*) attempts were also made to detect the toxin in culture filtrates desalted with Sephadex G25 (coarse) but without success. Supplements of 3-hydroxyanthranilic acid, a presumed precuror of picolinic acid in animal systems (Mehler and May, 1956), or tryptophane to cultures of *Pyricularia* under study did not promote production of this toxin. Conidia of the blast fungus were reported to contain picolinic acid which was discharged from the spores into water during germination (Ogasawara *et al.*, 1961 ; Tamari *et al.*, 1965). However, this toxin could not be detected in germination fluid of any of the isolates tested. Similarly, both toxins could not be detected in leaves of the six gramineous hosts infected by the respective isolates of *Pyricularia.*

Since we could not detect piricularin and picolinic acid in our studies, attempts were made to detect pyriculol in cultures of *Pyricularia* spp. and races of *P. oryzae* and the results are reported here.

METERIALS AND METHODS

Cultures used.—The following 6 monospore isolates of *Pyricularia* were used : *Pyricularia oryzae* Cavara from *Oryza sativa* L., *P. setariae* Nisikado from *Setaria italica* (L.) P. Beauv., *Pyricularia* spp. from *Eleusine coracana* L., *Panicum repens* (L.) Derf., *Brachiaria mutica* (Forssk.) Stapf and *Leersia*

hexandra Swartz. The virulence of the isolates was tested by inoculating them on their respective hosts from time to time. In addition, 10 races of *P. oryzae*, kindly supplied by The Central Rice Research Institute, Cuttack, and The International Rice Research Institute, The Philippines, were also examined for pyriculol production. The isolates were maintained on potato-sucrose agar (2% sucrose).

Cultural conditions.—The isolates were grown in sucrose–nitrate medium (Suryanarayanan, 1958) with Robbins trace element solution (Robbins, 1939), Tamari's medium (Tamari *et al.*, 1965) and Iwasaki's medium (Personal communication ; Glucose 20·0 g, KH_2PO_4 0·5 g, K_2HPO_4 0·5 g, $MgSO_4$ 0·5 g, $CaCl_2$ 0·01 g, yeast extract 5·0 g, water 1,000 ml) as shake or stationary cultures at room temperature 30 ± 1°C. The media were sterilized at 15 1b/15 min and inoculated with a mycelial agar block, from freshly isolated 10–day old cultures. Shake cultures were grown in 200 ml medium in 500 ml Erlenmeyer flasks. The inoculated flasks were left undisturbed for 60 h and then placed on a rotary shaker. Stationary cultures were grown in 250 ml medium in 1 litre flasks. Cultures were filtered after the required days of incubation, through 2 layers of muslin cloth and under suction through Whatman No. 1 filter-paper.

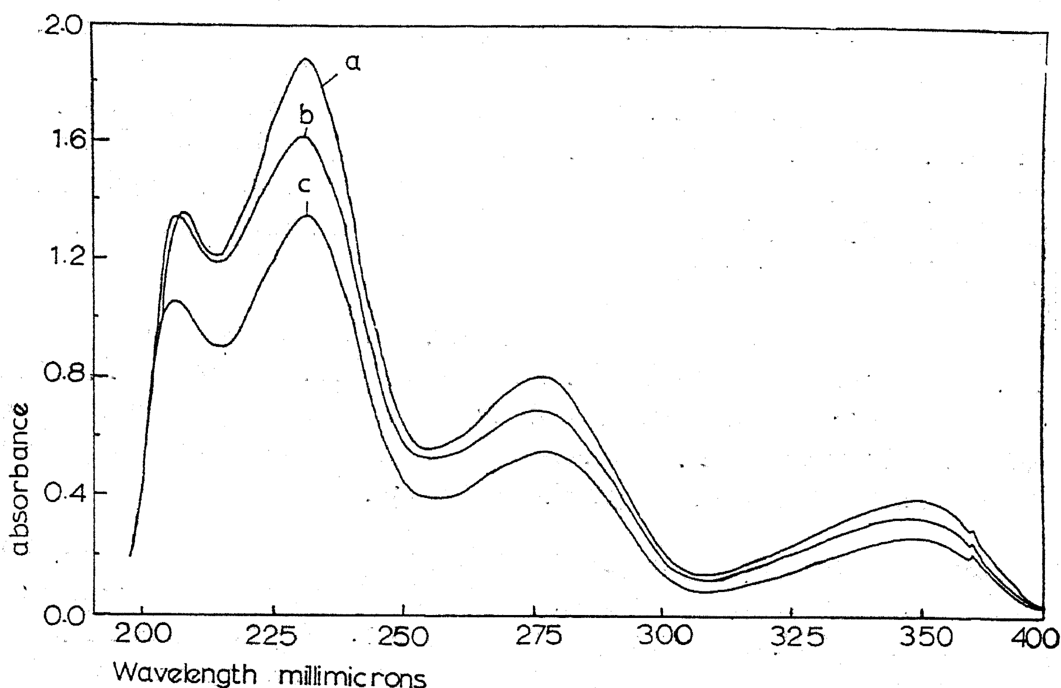
Extraction and detection of pyriculol.—The culture filtrates were extracted thrice in separatory funnels with 1/3 volume of ethyl acetate. The ethyl acetate extracts were concentrated to dryness in a rotary evaporator at 50° C under reduced pressure. The residues were taken in small amounts of ethyl acetate and subjected to thin-layer chromatography.

Thin-layer chromatography (TLC).—Glass plates (16 × 7 cm) with 250 μm thick silica gel G layers, applied by DESAGA (Brinkmann) applicator, were air-dried for 20 h and activated for 30 min at 120°.C prior to use. The test samples in ethyl acetate, equivalent to 200 ml of the filtrates, were streaked on TLC plates and chromatographed with authentic pyriculol in benzene: ethyl acetate (7 : 3) solvent system. The plates were developed to a distance of 100 mm. After development, chromatograms were viewed under U.V. light (254 and 356 nm) and pyriculol could be recognized by its characteristic reddish-brown colour especially at the latter wavelength. The marker pyriculol and areas corresponding to it were scrapped off the plates, extracted twice with twice distilled ethanol and centrifuged at 4,000 r.p.m. for 5 min. The ethanolic samples were scanned in the U.V. region for their absorption spectra with a UNICAM SP 800 Spectrophotometer. Spectra of the samples were compared with that of authentic pyriculol. The samples were dried at

room temperature and further purified by multiple development in benzene, and benzene : ethyl acetate (9: 1, 8: 2 and 7: 3) solvent systems. The U.V. absorption spectra of the purified samples were again studied as before.

EXPERIMENTAL AND RESULTS

TLC of ethyl acetate extracts.—When the ethyl acetate extracts were chromatographed in benzene : ethyl acetate (7: 3) a number of fluorescent/ absorbing zones could be distinguished under U.V. The number of such zones varied with the isolate, medium and cultural conditions but usually 7-10 zones were evident as illustrated in Plate II. When pyriculol was present, it could be distinguished by its characteristic reddish-brown colour under U.V. at an R_f of $0{\cdot}30 \pm 0{\cdot}05$.



TEXT-FIG. 1. U.V. spectra of authentic pyriculol before (*a*) and after TLC (*b*) and spectrum of purified pyriculol (*c*) from zone 5 of the thin-layer chromatogram shown in Plate II.

Text-Figure 1 shows the U.V. absorption spectra of authentic pyriculol and pyriculol isolated from culture filtrate in the present study by TLC. The authentic sample, before and after TLC, showed 3 absorption maxima at 232, 278 and 354 nm which agreed closely with the values of 232·5, 280 and 358 nm reported by Iwasaki *et al.* (1969). In the present studies, pyriculol was always identified by its characteristic U.V. spectrum after purification of the pyriculol zone by multiple development TLC (*vide,* Materials and Methods).

Detection of pyriculol in shake and stationary cultures of Pyricularia *grown in different media.*—Details of the cultures used, media, cultural conditions and the results obtained are given in Table I. Shake cultures were grown for 15 days in sucrose-nitrate medium, 6 days in Iwasaki's medium and 10 days in Tamari's medium. Stationary cultures were grown for 40 days.

TABLE I

Production of pyriculol in shake/stationary cultures of Pyricularia *grown in different media*

Isolate from	MEDIA: Shake cultures			MEDIA: Stationary cultures	
	Sucrose-nitrate	Iwasaki's	Tamari's	Sucrose-nitrate	Iwasaki's
O. sativa ..	—	—	—	—	—
E. coracana ..	+	—	—	+	—
S. italica ..	+	—	—	+	—
P. repens ..	+	+	—	+	—
B. mutica ..	+	+	—	+	—
L. hexandra ..	+	+	—	+	—

— : Not detected. + : Detected.

It is evident from the results that pyriculol could not be detected in cultures of the isolate from *O. sativa* (*P. oryzae*) in shake or stationary cultures in any of the media tested. However, pyriculol was detectable in other isolates in both shake and stationary cultures when grown in sucrose-nitrate medium. On the contrary, pyriculol was not detectable in any of the isolates when grown in Iwasaki's medium as stationary cultures. However, three isolates from the grasses, *P. repens*, *B. mutica*, and *L. hexandra*, produced pyriculol in the same medium when grown as shake cultures. The compound could not be identified in shake cultures of the isolates grown in Tamari's medium.

Detection of pyriculol in shake and stationary cultures of races of P. oryaze.—Since pyriculol was not detectable in cultures of *P. oryzae* used in the earlier

experiment, a number of races of *P. oryzae* were tested for pyriculol production. The races were grown in sucrose-nitrate and Iwasaki's media as shake and stationary cultures as before. The results are presented in Table II.

TABLE II

Production of pyriculol in shake/stationary cultures of races of P. oryzae *grown in two media*

Races	MEDIA			
	Sucrose-nitrate		Iwasaki's	
	Cultural conditions			
	Shake	Stationary	Shake	Stationary
IC1 ..	−	+	+	−
IC17 ..	−	+	−	−
ID1 ..	−	+	+	−
IE1 ..	−	−	+	−
IF1 ..	−	+	+	−
P16 ..	−	−	+	−
P26 ..	−	+	+	+
P92 ..	−	+	+	+
P149 ..	−	−	+	+
P150 ..	−	−	−	−

− : Not detected. + : Detected.

None of the races produced pyriculol when grown as shake cultures in sucrose-nitrate medium but the compound was detectable in 6 of the 10 races when grown as stationary cultures in the same medium. In Iwasaki's medium 8 of the 10 races produced pyriculol in shake cultures but only 3 of these produced it in stationary cultures. Pyriculol was not detected in shake/stationary cultures of the race P 150 in either medium.

Effect of temperature on pyriculol production.—The above two experiments showed that pyriculol production varied not only with the isolates but also with the media and cultural conditions. The production of this phytotoxic compound by 6 isolates of *Pyricularia* grown as stationary cultures at two temperatures in two media is shown in Table III.

TABLE III

Production of pyriculol in stationary cultures of Pyricularia *grown at two temperatures*

Isolate from	Sucrose-nitrate		Iwasaki's	
	Temperature °C			
	30	25	30	25
O. sativa ..	—	—	—	—
E. coracana ..	+	—	—	—
S. italica ..	+	+	—	—
P. repens ..	+	—	—	—
B. mutica ..	+	—	—	—
L. hexandra ..	+	—	—	—

— : Not detected. + : Detected.

Regardless of the temperature at which the cultures were grown, pyriculol was not detectable in any of the 6 isolates when grown in Iwasaki's medium. On the other hand, in sucrose-nitrate medium all isolates except that from *O. sativa* produced pyriculol when grown at 30° C, but in cultures grown at 25° C, pyriculol was detectable in the same medium only in the case of the isolate from *S. italica*.

DISCUSSION

Our best efforts to identify the earlier reported toxins, *viz.*, piricularin and picolinic acid (Tamari and Kaji, 1954) in cultures of *Pyricularia* from culti-

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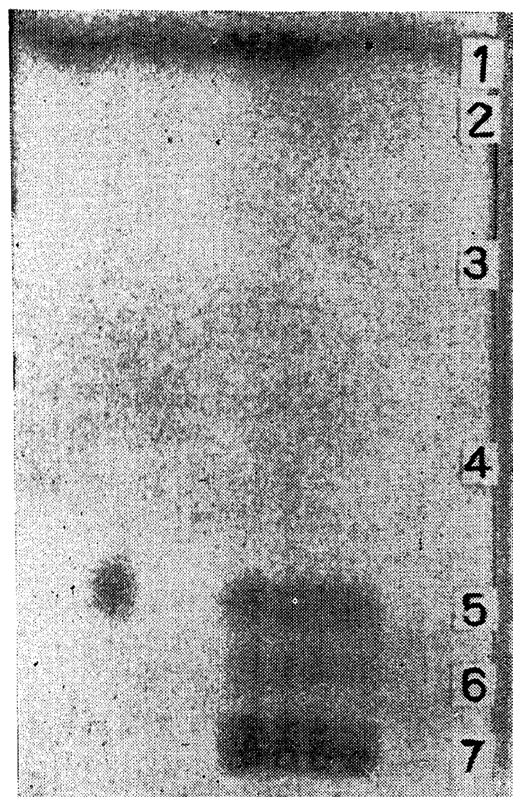

Thin-layer chromatogram of ethyl acetate extract of culture filtrate of *Pyricularia* sp. from *B. mutica* grown in sucrose-nitrate medium as shake culture. The numbers refer to the fluorescent/absorbing zones marked under U.V. The visible bands are due to H_2SO_4 charring. Note zone 5 corresponds to the marker pyriculol.