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PRODUCTION OF ANTIBIOTICS BY FUNGI. PART II: PRODUCTION BY *FUSARIUM JAVANICUM* AND OTHER *FUSARIA*.

H. R. V. ARNSTEIN, A. H. COOK AND M. S. LACEY.

From Imperial College, London, S.W. 7.

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THE production of antibiotic metabolism solutions by a miscellany of fungi under varying culture conditions was described in a previous paper (Cook and Lacey, 1945). The present paper records the results of a similar study of 19 species and strains of *Fusarium*, and a more detailed examination of the behaviour of *F. javanicum* and the antibiotic pigment, javanicin, produced by it.

EXPERIMENTAL.

Methods.

In general, the methods and media employed for the examination of *Fusarium* culture fluids for antibacterial activity were similar to those described by Cook and Lacey (1945). It was found, however, that the agar plate method of testing did not give a reliable quantitative estimation of the antibacterial titre of javanicin solutions. In an acid medium javanicin diffused freely into the agar, producing clear zones of inhibition, but in neutral or alkaline solutions little or no diffusion occurred. The serial broth dilution method was therefore employed throughout in testing javanicin solutions for antibacterial activity.

Results with Species other than F. javanicum.

(*a*) Negative results were obtained in all tests of cultures of the following: *F. culmorum* (one strain), *F. caeruleum*, *F. oxysporum cubense* and 4 unidentified species. In addition a strain of *F. dianthi* and an unidentified species developed only a trace of activity, and this only irregularly.

(*b*) The culture fluid of a second strain of *F. culmorum* grown on a medium containing 1 per cent Bacto-Tryptone and 4 per cent glucose was inactive against *Staphylococcus aureus*, but was both bacteriostatic and bactericidal towards *Myco. phlei* in 1 : 10 dilution from the 6th to the 15th day; the activity had disappeared by the 19th day.

(*c*) Culture solutions of *F. fructigenum* were always inactive towards *Staph.*

aureus. No activity towards *Myco. phlei* developed in media containing 1 per cent Bacto-Tryptone without glucose, but media containing 1 per cent glucose became bacteriostatic and bactericidal (dilution 1 : 10) towards *Myco. phlei* between the 13th and 17th days ; in 2 or 4 per cent glucose activity was delayed until the 20th day, the solutions being bacteriostatic (1 : 10) but not bactericidal.

(*d*) Three strains, F 71 and F 75 (? *F. lateritium*) and F. 80 (*F. avenaceum*) grown in a medium containing 1 per cent Bacto-Tryptone and 4 per cent glucose caused complete inhibition (1 : 10) of *Staph. aureus* and (1 : 20) of *Myco. phlei* from the 13th day onwards.

(*e*) *F. sambucinum* was examined at intervals over 18 months in a variety of media. In synthetic media containing 0·5–4 per cent glucose and supplemented with varying amounts of yeast extract, feeble activities towards *Staph. aureus* and *Str. pyogenes* and stronger activities towards *Myco. phlei* were developed but growth of *Bact. coli* was unaffected. Results were similar when yeast extract was replaced by 0·5 per cent Bacto-Tryptone. In media containing 1 per cent Bacto-Tryptone, 0, 0·5, 2 or 4 per cent glucose, but no added salts, plate tests showed very feeble activity towards *Staph. aureus* and *Str. pyogenes* (1 : 5–1 : 10), with stronger activity towards *Myco. phlei* (1 : 25) in all cultures alike. Dilutions of 1 : 10 were bactericidal towards *Myco. phlei*. The last titres were reached on the 6th day, and persisted for 6 weeks.

(*f*) An unidentified *Fusarium* (F 6) grown in 1 per cent glucose and 1 per cent Bacto-Tryptone produced activity similar to that developed by *F. fructigenum* (+ towards *Myco. phlei*, — towards *Staph. aureus*). It differed from *F. fructigenum* however in developing activity towards both organisms in Bacto-Tryptone media containing 4 per cent glucose ; 1 : 10 dilutions were bactericidal towards both from the 6th until the 17th day, when the last tests were made. Activity of the last kind was also shown by another unidentified *Fusarium* (F. 72). Still another strain (F. 64) produced complete inhibition of *Staph. aureus* and *Myco. phlei* in 1 : 5 dilution at 12–15 days on a medium containing 1 per cent Bacto-Tryptone and 4 per cent glucose. The solutions were bactericidal towards *Myco. phlei* on the 12th day, but only bacteriostatic by the 19th day. In this instance, but not in others, Bacto-Tryptone could be replaced by Eupepton.

The Production of Antibiotics by F. javanicum.

It was earlier reported (Cook and Lacey, 1945) that of several *Fusarium* cultures *F. javanicum* was the most potent in developing antibiotic solutions. Many of the 18 strains used in the preceding section exhibited pink, orange or yellow mycelia, but the pigments did not diffuse into the medium and appeared to have no connection with the antibacterial activity of the solution (Sideris, 1925). Strains of *F. javanicum* differed, however, from the preceding ones in producing, under selected cultural conditions, a blood-red pigment which diffused into the medium (liquid or solid) and was found to be the active antibacterial principle. The pigment fraction was sometimes substantially homogeneous, but at other times contained two antibacterial pigments. These have been termed javanicin, $C_{15}H_{14}O_6$, the major constituent, and oxyjavanicin, $C_{15}H_{14}O_7$. Their preparation and chemistry is described elsewhere (Arnstein, Cook and Lacey, 1946 ; Arnstein and Cook, in press), and the present account is restricted to considerations of biological interest.

Cultural conditions.

Addition of Bacto-Tryptone or Eupepton to the medium was found essential for production of antibacterial activity so that these nutrients in synthetic medium containing 4 per cent glucose could not be replaced by yeast extract (three preparations were examined), 1 per cent malt extract, 2 per cent concentrated corn-steep liquor, 1 per cent. meat extract (two preparations) or 1 per cent Gurr's peptone.

When glycine (0·05, 0·1, 0·2 and 0·4 per cent) was substituted for Bacto-Tryptone, pigmentation appeared to be equally good, but the activity of the medium was much less (about 1 : 5 after 17 days). The effect of glycine at the various levels was compared by extracting the metabolism solutions with ether, removing inactive pigment with sodium bicarbonate, and taking the residue to dryness. The solid residue was then dissolved in ethanol and the colours compared visually. It was found that 0·5 per cent glycine was the optimum concentration. Although no solid javanicin was isolated from this medium there can be no doubt that the colour was due to javanicin, for the absorption spectrum of this solution was almost identical with that of pure javanicin. Brown and Horne (1926) found that the addition of ammonium chloride increased the amount of yellow pigmentation of certain species of *Fusaria*. When ammonium chloride (0·05, 0·1 and 0·2 per cent) was substituted for Bacto-Tryptone the mycelium of *F. javanicum* was pigmented red, but although growth appeared otherwise normal, there was no diffusible pigment of any kind. The activity of these media was not tested.

Four other preparations were tested as substitutes for Bacto-Tryptone and Eupepton. The effect of these materials is shown in Table I, none being a useful substitute.

TABLE I.—*Effect of Various Preparations on the Production of Antibiotic by* F. javanicum.

	Time of incubation (days).	Antibiotic activity against *Staph. aureus*. (Dilution of culture fluid.)
Glycine (0·05 per cent)	12	1 : 5
	17	1 : 5
Eupepton (0·5 per cent)	12	1 : 50
	14	1 : 100
Vitamin-free acid digest of casein (0·5 per cent)	15	1 : 25
Low ash casein digest (0·5 per cent)	8	1 : 25
	15	1 : 50 to 1 : 100
Meat protein papain digest (0·5 per cent)	8	1 : 10
	15	1 : 25
Baker's peptone (0·5 per cent)	13	1 : 10

Deep red pigmentation diffusing into the medium and an activity titre of 1 : 100 towards *Staph. aureus* and *Myco. phlei* was produced in synthetic media containing 4 per cent glucose and 0·5 per cent Bacto-Tryptone. Smaller

amounts of glucose (1 per cent) caused earlier development of activity and pigment (observable after 3 days), but production soon ceased and the maximum activity titre was lower. Without glucose there was no development of either pigment or activity.

The effect of varying the initial pH was investigated using a Czapek-Dox medium containing 4 per cent glucose and 0·5 per cent Eupepton. At pH 4 the fungus grew very slowly with slow development of pigment and activity. At pH 5 this development was more rapid and was maximum at pH 6. Brownish coloration of the fluid and green mycelia were observed in cultures initially at pH 7, and these effects were enhanced at pH 8. At pH 7–8, however, only a trace of anti-staphylococcal activity developed. The optimum pH was slightly higher (6·8) in Bacto-Tryptone media.

The optimum temperature of incubation was about 25° C. At 22° C. growth and pigment production were slower, though the ultimate maximum titre was the same. At 30° C. rate of growth and antibiotic production as well as the absolute amount of antibacterial activity were diminished, and growth failed at 37° C.

Although the antibacterial effect was most readily observed in the culture fluid, considerable quantities of javanicin were present in the mycelia. Thus a 15-day culture in Bacto-Tryptone-glucose broth (40 ml., anti-staphylococcal titre 1 : 80) was drained and the felt ground with acetone (20 ml.). The extract completely inhibited the growth of *Staph. aureus* at a dilution of 1 : 400–1 : 500 and of *Myco. phlei* at 1 : 800.

Variation of pigment production with strain.

Colonies of *F. javanicum* grown on Bacto-Tryptone or Eupepton glucose agar media became deep red, the pigment being at first observed within the hyphae and gradually diffusing into the medium until the whole plate was stained blood-red. The red pigment was also produced slowly and less strongly on malt extract agar, but on other media such as potato-agar the cultures remained white. On a synthetic medium containing 3 per cent glucose the deep mycelial coloration as noted above in liquid cultures at pH 8 was observed, the solid medium itself being stained yellow. Both green and yellow pigmented extracts were inactive towards *Staph. aureus*.

Ten cultures on Bacto-Tryptone broth which were 4–8 months old and in which the fungal growth had degenerated into a jelly-like mass were subcultured on to a synthetic medium containing 3 per cent glucose and on to a Eupepton-glucose-agar medium ; two cultures produced no pigment under these conditions, but the remaining eight behaved in the original manner ; they produced green pigment on the former medium and red diffusing antibacterial pigment on the latter.

Single spore cultures of *F. javanicum* showed a marked variation in antibiotic production. On Bacto-Tryptone-glucose-agar the antibiotic pigmentation ranged from blood-red, orange-red and orange to almost colourless. Some colonies showed definite sectors of blood-red and white forms, the former being of markedly more restricted growth. By continued selection of deeply pigmented colonies a culture was obtained which produced nearly twice as much javanicin as the original culture.

Antibiotic activity of javanicin.

When *F. javanicum* was grown in conical flasks (150 ml.) containing Bacto-Tryptone-glucose medium the crude culture fluids normally developed a bacteriostatic titre of 1 : 200 towards *Myco. phlei* and 1 : 100 towards *Staph. aureus*, *B. subtilis* and the Gram-positive plant parasite *Bact. fascians*. The culture fluids were less potent towards *Str. pyogenes*, very feebly active towards *Bact. coli* and inactive towards *Ps. pyocyanea*, *Ps. fluorescens liquefaciens* and the Gram-negative plant parasite *Bact. tumefaciens*. After extracting pigments (Arnstein, Cook and Lacey, 1946; Arnstein and Cook, in press) the residual liquid was biologically inactive. The extracted pigments ultimately yielded javanicin and oxyjavanicin, both being antibacterial naphthoquinones. Table II shows the antibacterial activity of javanicin. We have to thank Dr. A. T. Fuller, National Institute of Medical Research, London, for tests on the haemolytic streptococcus and *Clostridium welchii*; these inhibitory effects were observed in broth. In view of the powerful action towards *Myco. phlei* its action towards *Myco. tuberculosis* was examined by Prof. W. H. Tytler, Welsh National School of Medicine, Cardiff, to whom our thanks are also due.

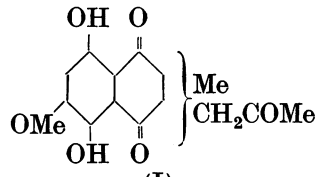
In in-vivo tests mice tolerated a maximum dose of 10 mg. (intraperitoneal injection). The bacteriostatic effect towards *Myco. tuberculosis* was not markedly affected, if at all, by serum though there was no definite evidence of a bactericidal action; however, in further tests in blood media javanicin produced a brown colour (probably methaemoglobin) at 1 : 50,000 and failed to inhibit the growth of the haemolytic streptococcus at 1 : 20,000, so that it seemed to have serious defects as a drug for systemic treatment. Prof. W. H. Tytler kindly carried out tests in which 8 guinea-pigs were inoculated intracutaneously, each on both flanks, with *Myco. tuberculosis*, and one of the resulting ulcers in each animal treated daily for 3 weeks with a 1 : 500 solution of javanicin, in N/50 alkali. On application there was a rapid colour change and precipitation, so that the effective

TABLE II.—*Antibacterial Activity of Javanicin.*

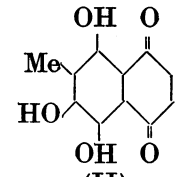
Bacterium.	Effective concentration for—	
	Bacteriostasis.	Bactericidal action.
Staph. aureus	1 : 200,000	1 : 50,000
Myco. phlei (1st strain)	1 : 200,000	1 : 40,000
,, ,, (2nd strain)	1 : 100,000	..
B. subtilis	1 : 200,000	1 : 50,000
Str. pyogenes	1 : 40,000	>1 : 40,000
Bact. fascians	1 : 100,000	..
Bact. coli	>1 : 10,000	..
Ps. pyocyanea	,,	..
B. fluorescens liquefaciens	,,	..
Bact. tumefaciens	,,	..
Myco. tuberculosis (human)	1 : 50,000 to 1 : 100,000	..
Haemolytic streptococcus	1 : 100,000	..
Cl. welchii	1 : 100,000	..

concentration of residual javanicin may have been very low or indeed negligible. There was at most a slight indication that the treated ulcers did not progress as rapidly as the untreated ones, but there was no suggestion of healing.

Javanicin (I) is a naphthoquinone related structurally to hydroxydroserone (II), and appears to owe its antibacterial properties in large measure to its



(I)



(II)

quinone nature. Thus the action of javanicin was inhibited by thiols though with difficulty as the behaviour with penicillamine shows :

Test solution. (mg./ml.) Javanicin + Penicillamine.	Limiting diluting towards *Staph. aureus.*
1·15 mg. ..	1 : 100,000–1 : 200,000
,, 0·5	1 : 100,000–1 : 200,000
,, 1·0	1 : 50,000 –1 : 100,000
,, 2·0	1 : 25,000 –1 : 50,000
,, 4·0	1 : 100,000
,, 8·0	1 : 50,000 –1 : 100,000

The higher figures at greater concentrations of penicillamine reflect the known but small antibacterial action of penicillamine itself.

Structural features other than the quinone grouping appear to have an influence as the smaller activity of hydroxydroserone, especially towards *Myco. phlei*, shows :

	Staph. aureus.	*Mycobact. phlei.*
Javanicin	1 : 200,000	1 : 200,000
Hydroxydroserone . . .	1 : 50,000	1 : 10,000 (44 hrs.) or inactive (3 days).

Oxyjavanicin inhibited the growth of *Staph. aureus* at 1 : 200,000, and a similar degree of activity was shown by certain derivatives of javanicin retaining the quinone system such as anhydrojavanicin and the acetyl derivative thereof.

Antifungal action of F. javanicum.

When malt-Bacto-Tryptone- or Eupepton-agar plates were inoculated with *F. javanicum* and at another point with *Verticillium albo-atrium*, *V. dahliae*, *Trichoderma* sp., *Phoma* sp., or other *Fusarium* sp., *F. javanicum* had no action on the other fungi and was itself slightly inhibited by *Trichoderma*. When, however, *F. javanicum* was first grown on Bacto-Tryptone-agar until javanicin was being freely produced and the plate then inoculated with the above fungi, zones of inhibition limited by the coloured circles of javanicin were observed.

Malt-agar plates were impregnated with a crude culture filtrate of *F. javanicum* (active against *Staph. aureus* at 1 : 50) in 1 : 10 dilution and the plates then sown with *Aspergillus parasiticus*, *Verticillium albo-atrium*, *V. dahliae*, *Trichoderma* sp., *Phoma* sp., *Penicillium notatum*, *Fusarium sambucinum*, *F. lateritium* or *F. javanicum*. The first five fungi were unaffected, but growth of *P. notatum* and the *Fusarium* species was reduced without being entirely suppressed; after 3 days, growth was about 30 per cent of the controls. A weak but specialized effect was thus apparent.

Effect of javanicin on seed germination.

In 1 : 100,000 or 1 : 50,000 concentration javanicin had no effect on germination of turnip or lettuce seeds. At a concentration of 1 : 20,000 germination of lettuce seeds was entirely suppressed, whilst with turnip seeds 60 per cent of the radicles emerged compared with 100 per cent in control solutions, but no further growth occurred. Root inhibition was still more marked in the case of tomato seeds in javanicin at a concentration of 1 : 20,000. Whereas 18 control seeds germinated and developed normally, 7 out of 18 treated seeds failed to germinate, and in the remaining 11 there was delayed emergence and growth of the plumule and complete absence of any root growth.

SUMMARY.

Twelve of 19 species and strains of *Fusarium* developed antibacterial culture solutions under selected conditions. There is evidence for the development of a plurality of antibacterial substances, some of which have been isolated.

The production of two antibacterial pigments, javanicin and oxyjavanicin, by selected strains of *F. javanicum* under a variety of conditions has been examined.

The antibacterial action of javanicin (including its in-vitro and possible in-vivo antitubercular action) and of some nearly related compounds was studied.

Javanicin had a weak specialized antifungal action and a marked effect on the germination of certain seeds.

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