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The isolation and properties of a fungicidal compound present in seedlings of Vicia faba

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Abstract

The isolation of a fungicidal compound in 0.001% yield from healthy broad bean seedlings by extraction with benzene is described. After column chromatography, purification was achieved by low temperature recrystallisation from hexane, using ultra-violet light absorption, inhibition of spore germination of *Alternaria brassicicola* and thin layer chromatography as criteria of purity. The compound has been given the name wyerone. Its in vitro activity against phytopathogens and dermatophytes and its protectant and eradicant activity against certain fungal pathogens of plants are reported.

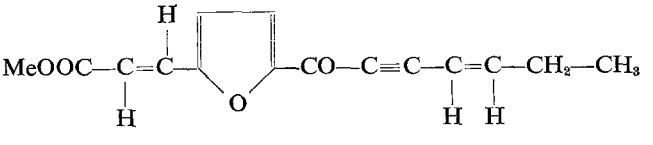
Introduction

Inhibition of fungal growth on nutrient agar arising from the presence of a segment of stem or root tissue of broad bean (*Vicia faba*) was reported by Spencer et al. (1957). Evidence was also presented which indicated that a substance of phenolic nature was responsible for the antibiotic effect observed.

Detailed investigations of the fungitoxic compounds present in *Vicia faba* were described by Wain et al. (1961). Fawcett reported that apart from a stable phenolic constituent, two other unstable antifungal compounds of major importance could be extracted in a crude state. One of these was a hydrophilic organic compound containing sulphur, phosphorus and nitrogen, whilst the other was lipophilic in character.

Numerous unsuccessful attempts were made over several years to isolate stable samples of the lipophilic compound, in order to study its chemical and physiological properties. The loss of antifungal activity due to polymerisation caused by the ultra-violet component of daylight was found to be one important factor in the instability of the compound. A full description of the successful method finally employed is given here.

The recognition of wyerone as a diethylenic keto-acetylenic methyl ester (Fawcett, 1963) was confirmed when it was synthesised by Sir Ewart Jones and his colleagues (Fawcett et al., 1965).



wyerone

Subsequent work which resolved the nature of two other acetylenic compounds related to wyerone which are also present in broad bean seedlings, together with details of syntheses of further analogues not found in bean seedlings have recently been reported (Fawcett et al., 1968). Data on the antifungal activity of wyerone are presented here.

Experimental

Isolation of wyerone from broad bean seedlings

5 kg broad bean seeds (var. 'Green Windsor') were soaked in water for 24 h. After growing for 8 days in the dark at room temperature between wet sacks which had been steam sterilised, the seedlings (~ 10 kg) were placed in plastic bowls and kept in darkness at 2 °C in a refrigerator for 24 h.

The cold seedlings were minced in a Hobart machine and steeped in 10 l redistilled benzene in a glazed earthenware vessel, which had a glass tap and an opaque lid.

After standing at room remperature for 24 h the pale yellow benzene extract was slowly run off by gravity – but the residual tissue was not pressed. This procedure was found by experiment to lead to easier purification in the subsequent stages. The benzene solution was stirred with 100 g dried magnesium sulphate and then decanted on to two large columns externally wrapped with black cloth. Each column (6 cm diam., 60 cm length) which contained acid-washed neutral dry silica-gel, was equilibrated with redistilled benzene and through this the benzene liquors were allowed to percolate slowly. After some 2 hours, when the benzene liquors had run through, the gradient elution sequence was started employing 0.5 % dry ether in benzene; by the time fraction No. 15 was being eluted 5 % dry ether in benzene was being used.

The fractions up to No. 16 were colourless, Nos. 17, 18 and 19 were yellow and those from No. 20 onwards were almost colourless.

Each of the 50 ml fractions collected was stored in darkness until it was examined in the spore germination test using *Alternaria brassicicola*. For this, 0.05 ml of the fraction was slowly pipetted on to a depressed-well glass slide in a draught of air to remove the solvent. The well (1.5 cm diam.) was ringed with a wax pencil and 0.2 ml of a suspension of approximately 30,000 spores per 1 ml of 0.2 % sucrose solution was pipetted into the ring. The slides were incubated in moist chambers for 18 h at 25 °C after which the percentage germination was measured (Fig. 1A). The fractions from No. 15 onwards were also examined by putting 0.3 ml in a 1 mm silica cell and measuring the optical density at 3450 Å, with benzene in the 1 mm reference cell. The results of an experiment are shown in Fig. 1B. The correlation of light absorption with the inhibition of spore germination of *Alternaria brassicicola* can be seen by comparison of Fig. 1A with Fig. 1B.

The benzene/ether solvent was removed from the active fractions obtained from both columns by employing a rotary film evaporator in the dark at 15 °C and water pump vacuum (~ 14 mm). The cream coloured waxy residue from this 10 kg broad bean tissue weighed 117 mg and was stored at -20 °C in the dark.

The residual solid (805 mg) from 70 kg broad bean tissue after recrystallisation six times from spectroscopy grade hexane gave 255 mg pale yellow wyerone m.p. 63.5 °–64.5 °C. Each recrystallisation was carried out in the dark by dissolving the crude wyerone in a minimum quantity of hexane at room temperature (~ 20 °C) and cooling the solution to -20 °C after filtration. When examined by thin layer chromatography,

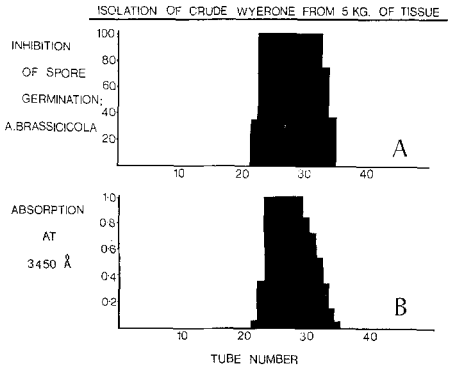


Fig. 1. Material obtained from a benzene extract of broad bean seedlings by column chromatography on silica gel with gradient elution using benzene/ether.
A: Inhibition of spore germination caused by the residue from 0.05 ml of each fraction when tested with 0.2 ml of *A. brassicicola* spore suspension.
B: Optical density at 3450 Å of each fraction in a 1 mm cell.

Fig. 1. Materiaal uit een benzeen extract van tuinboonzaailingen verkregen door kolomchromatografie op silicagel met gradient elutie door middel van benzeen/ether.
A: Remming van de sporekieming door het residu van 0,05 ml van elke fractie, getoetst met 0,2 ml van een sporesuspensie van A. brassicicola.
B: Optische dichtheid bij 3450 Å van elke fractie in een 1 mm cuvet.

using Kieselgel-G as the adsorbent and benzene–ether (9:1) as the solvent, wyerone was detected as a single spot at R_F 0.45 with various chromogenic reagents, including 2,4-dinitrophenylhydrazine and acidified potassium permanganate solutions.
Full details of the chemical analysis of wyerone, together with data relating to its ultra violet, infra red, proton magnetic resonance and mass spectra have been reported (Fawcett et al., 1968).

Antifungal activity in vitro against phytopathogens
The ability of wyerone to inhibit the germination of spores of six phytopathogenic fungi was compared with that of several other fungitoxicants of plant origin (Table 1). A stock solution was prepared at 1,000 μg/ml of each compound in a suitable solvent,

Table 1. Antifungal activity of some naturally occurring compounds from plants. Indication of amount of compound (μg/ml) required to reduce germination of spores to less than 50 per cent of that in controls.

Compound	*Botrytis cinerea*	*Aspergillus niger*	*Uromyces fabae*	*Uromyces appendiculatus*	*Alternaria brassicicola*	*Glomerella cingulata*
Capillin	<1	<5	<5	<10	<1	<25
Juglone	<10	<10	<10	<5	<25	<5
Wyerone	<100	<10	<25	<10	<5	<10
Orchinol	<50	<10	<50	<25	<50	<25
β-Thujaplicin	<250	<100	<500	<250	<500	<250
Chlorogenic acid	<1,000	<1,000	<50	<100	<250	>1,000
Protocatechuic acid	<1,000	<1,000	<100	<500	<100	>1,000
Benzoxazolinone	<500	<500	<500	<500	<500	<1,000
Captan*	<5	<5	<1	<1	<5	<1

* N-trichloromethylthio-4-cyclohexene-1:2-dicarboxyimide

Tabel 1. Schimmelremmende werking van enige natuurlijke verbindingen uit planten. Hoeveelheid (μg/ml) nodig om sporekieming tot minder dan 50% van de controle terug te brengen.

usually acetone. Aliquots of this stock solution were pipetted into the cavities of double depressed-well slides and the solvent was allowed to evaporate under a draught of air at ambient temperature. The amounts of stock solution used were such as to provide a range of concentrations when 0.2 ml spore suspension was run onto the deposit in each cavity. The spore suspension was confined to the area of the slide bearing the chemical deposit by means of a ring drawn on the glass surface with a soft wax pencil. Spore suspensions of *Alternaria brassicicola*, *Aspergillus niger*, *Botrytis cinerea* and *Glomerella cingulata* were prepared by running sterile distilled water on to sporulating cultures 8–10 days old and rubbing the surface of the colony with a glass rod. The suspension was filtered through butter muslin and centrifuged at 2,000 r.p.m. for 30 sec. The supernatant was discarded and the spores resuspended in sterile distilled water were then spun down again. This washing procedure was repeated once more and the spores were finally suspended in 0.2% sucrose solution. The density of spores in the suspension was adjusted to approximately 30,000/ml using a haemacytometer slide, and 0.2 ml of this suspension was pipetted into each of the cavities.

The two *Uromyces* species (Table 1), being obligate parasites, were maintained on growing plants. Young plants of broad bean (for *U. fabae*) and dwarf bean (for *U. appendiculatus*) at the two-leaf stage, were inoculated with an aqueous suspension of uredospores in water. Infection was encouraged by keeping the plants in a humid atmosphere for 48 h immediately after inoculation, after which time they remained in the greenhouse. Leaves bearing uredosori could then be removed from the plants 2 weeks after inoculation. The spores were removed by rubbing the sori with a glass rod under water and they were washed in a manner similar to that used for the spores of the facultative parasites. The spores were then suspended in 0.2% sucrose solution before application to the prepared slides.

Large petri-dishes lined with wet filter paper served as containers in which the slides were incubated for 18 h at 25 °C. They were then examined at the microscope and the percentage of spores which had germinated at each concentration of the test compounds, as well as in 0.2% sucrose solution alone, was determined. When these figures had been corrected for natural mortality they were plotted on log-probability graph paper, log dose against percentage germination, this latter figure being plotted as the probit response. A straight line dosage/response slope was thus obtained from which the concentration which caused spore germination to be reduced by 50% (ED_{50}) could be read. The results obtained are presented in a simplified form in Table 1.

Antifungal activity against dermatophytes

The compound (10 mg) was dissolved in 2 ml absolute alcohol and, from this, a solution of 1,000 μg/ml was prepared in Sabouraud's broth. Two-fold serial dilutions were then made in Sabouraud's broth and inoculated with spore suspensions of the organisms to be tested. The flasks were incubated for 7 days at 27 °C before the minimum inhibitory concentrations given in Table 2 were recorded.

Antifungal activity – protectant

Dwarf bean – Uromyces appendiculatus. Seeds of dwarf bean (*Phaseolus vulgaris*) var. 'Canadian Wonder', were sown in compost in plastic drinking cups, which had been perforated at the base to permit drainage, and kept in the greenhouse where the mini-

Table 2. Antifungal activity of wyerone against dermatophytes

Dermatophyte	*Minimum inhibitory concentration (μg/ml) of wyerone*
Trichophyton rubrum	7.8
T. megnini	2.0
T. soudanense	7.8
T. mentagrophytes 687E	7.8
T. mentagrophytes 858E	4.0
Microsporum canis 764E	4.0
M. canis 834E	7.8

Tabel 2. Activiteit van wyerone ten opzichte van dermatophyten

mum temperature was 15 °C. When the pair of simple leaves were fully expanded the plants were used to test a group of naturally-occurring compounds for their protectant fungicidal activity.

The compounds, at known concentration in 60 % aqueous acetone, were each applied, using a standard spraying procedure to ten dwarf bean seedlings. After drying, the plants were inoculated with *Uromyces appendiculatus* by spraying the leaves to an even cover of discrete droplets with a suspension of approximately 50,000 spores per 1 ml of water.

The plants were immediately transferred to a glass cabinet where the relative humidity was maintained at a level above 98 % in a room kept at 15 °C. After they had been in darkness in the cabinet for 12 hours the plants were illuminated at a light intensity of 6600 lux for 12 hours and they were then removed from the humidity cabinet and returned to the greenhouse for disease development. The level of protectant activity was determined after 2 weeks when the numbers of uredosori on each leaf were counted and compared with the numbers occurring on leaves of plants which had been sprayed before inoculation with aqueous acetone only. The results, expressed as a percentage of the infection in controls, are given in Table 3. Two subsequent experiments gave similar results. The fungicide captan was included as a standard for comparison.

Broad bean – Botrytis fabae

The compounds at known concentration in 60 % aqueous acetone were applied, using a standard spraying procedure (Spencer, 1957) to ten broad bean plants at the four-leaflet stage of growth. When they were dry, the plants were inoculated with the spores of *Botrytis fabae* as described by Fawcett et al. (1958). After 48 h incubation at 98–100 % relative humidity, disease assessment as a percentage of that occurring in controls was recorded (Table 4). Substantially similar results were obtained in two further experiments. Captan was again included as a standard for comparison.

Antifungal activity – eradicant

Dwarf bean – Uromyces appendiculatus. Dwarf bean seedlings grown in compost to the stage at which their simple leaves were fully expanded were inoculated by spraying the underside of the leaves with a suspension containing approximately 50,000 uredospores of the rust organism per 1 ml of water. Infection was allowed to occur, as described

Table 3. Protectant activity of naturally occurring compounds against rust of dwarf bean

Compound	*Concentration* (μg/ml)	*Lesion counts* (*as percentage of controls*)
Wyerone	4	111
	20	110
	100	67**
	500	2***
β-Thujaplicin	500	1***
Juglone	100	22***
	500†	1***
Orchinol	100	18***
Protocatechuic acid	500	44***
Benzoxazolinone	500	57***
	1000	12***
Capillin	100	98
Chlorogenic acid	500	111
Captan	500	4***

† Slight leaf scorch
** Significantly different from control at $P = 0.01$
*** Significantly different from control at $P = 0.001$

Tabel 3. Beschermende werking van natuurlijke verbindingen tegen boneroest

Table 4. Protectant activity of naturally occurring compounds against chocolate spot of broad bean

Compound	*Concentration* (μg/ml)	*Lesion counts* (*as percentage of controls*)
Wyerone	4	96
	20	60**
	100	40***
	500	13***
Orchinol	100	35***
Juglone	100	97
	500	21***
β-Thujaplicin	500	46***
Benzoxazolinone	500	86
	1000	42***
Protocatechuic acid	500	73*
Chlorogenic acid	500	107
Capillin	100	159***
Captan	500	5***

* Significantly different from controls at $P = 0.05$
** Significantly different from controls at $P = 0.01$
*** Significantly different from controls at $P = 0.001$

Tabel 4. Beschermende werking van natuurlijke verbindingen tegen de chocoladevlekkenziekte van tuinboon

Table 5. Eradicant activity of compounds against dwarf bean rust

Treatment	*Uredosori on leaves (as percentage of controls)*
Control (water)	100
Dispersing agent†	99
Wyerone (1,000 μg/ml) + dispersing agent†	81***
N-phenyl-N′-3-sulpholanyl-hydrazine (1,000 μg/ml)	0***
Sulphanilic acid (1,000 μg/ml)	5***
Captan (1,000 μg/ml)	95

† Sucrose laurate (0.1 % aqueous solution)
*** Significantly different from controls at $P = 0.001$

Tabel 5. Curatieve werking van enige verbindingen tegen boneroest

earlier and 48 h after inoculation the undersides of the leaves were again sprayed using the solutions shown in Table 5. The plants were subsequently returned to the greenhouse bench and 12 days later the developing uredosori were plainly visible and could be counted. Eradicant activity was indicated by the reduction in numbers of uredosori. The results obtained are given in Table 5.

Discussion

Acetylenic compounds have to date been obtained from a limited number of plant families. Bu'Lock (1964) mentioned eight and Schulte et al. (1965) have reported the isolation of acetylenic hydrocarbons from Gramineae species. The isolation of wyerone represents the first acetylenic compound obtained from a member of the Papilionaceae. Jones (1966) has suggested the polyacetylenic precursor which may be important in its biosynthesis and the work of Bohlmann (1966) on the biosynthesis of carlina oxide in *Carlina aucalis* provides support for this suggestion.
Wyerone showed high in vitro antifungal activity particularly against *Alternaria brassicicola*, but was some fifty times less active against *Botrytis cinerea* (Table 1). This is of considerable interest since *A. brassicicola* does not infect broad bean plants whereas *Botrytis* spp. are pathogens which cause the well known chocolate spot disease.
In general, the in vitro fungicidal activity of wyerone was found to be comparable with that of capillin (C_6H_5–CO–C≡C–C≡C–CH_3) another naturally occurring acetylenic compound which was obtained from *Artemisia capillaris* by Imai et al. (1956) and Imai (1956). The high activity of capillin was also referred to by Sörensen (1961). The structural basis for the antifungal activity of these two compounds is of interest. In the capillin molecule we have: phenyl-keto-acetylenic-acetylenic-methyl and in wyerone: furyl-keto-acetylenic-ethylenic-ethyl. It appears that in these molecules the sequence of groups which accounts for antifungal activity can be generalised to the R-keto-acetylenic moiety where R is a group possessing aromatic character. This view is supported by the fungistatic activity shown by a series of synthetic R-keto-acetylenic compounds, in which R was furanyl-2- or 5-bromofuryl-2- (Hillers et al., 1966).
A number of compounds with notable antifungal activity have been obtained from plants in recent years (see Spencer, 1962) and wyerone was compared with some of these in tests for antifungal activity both in vitro and in vivo. Juglone (Fischer and

Stander, 1932), capillin (Imai, 1956), orchinol (Gäumann and Kern, 1959), β-thujaplicin (Anderson and Gripenberg, 1948), chlorogenic acid (Johnson and Schaal, 1957), protocatechuic acid (Walker and Stahmann, 1955) and benzoxazolinone (Virtanen and Hietala, 1955) were examined in detail (Table 1, 3 and 4). Several other naturally occurring compounds including caffeic acid, gallic acid, vanillin and phloroglucinol which have been implicated in the disease resistance of plants by various authors were also included in the test but the results obtained have not been included in the tables since they were generally less active than the seven compounds referred to above (Fawcett and Spencer, 1968).

In tests carried out by Dr R. M. Evans of Glaxo Research Ltd. on certain dermatophytic fungi (Table 2) wyerone was found to be toxic, but other tests showed it to be somewhat less active than the commercial crop fungicide captan against strains of these organisms. For example, the minimum inhibitory concentration of captan against *Trichophyton rubrum* D 354 was 10 μg/ml; against *T. mentagrophytes* D 279 it was 7.8 μg/ml and against *Microsporum canis* D 688 0.5 μg/ml.

The similarity of activity in vitro shown by wyerone and capillin was not observed when they were tested for protectant fungicidal activity (Table 3 and 4). When the two compounds at the same concentration (100 μg/ml) were sprayed on to dwarf bean plants wyerone gave significant protection against rust infection (*U. appendiculatus*) whereas that given by capillin was negligible (Table 3). It was noted that juglone (5-hydroxy-1,4-naphthoquinone) also showed high in vitro fungitoxicity (Table 1), but its activity as a protectant was marred by its phytotoxicity (Table 3). Although wyerone showed low activity against broad bean pathogens in vitro, at higher concentrations it provided protection against the chocolate spot organism (*B. fabae*), with no phytotoxicity. In these experiments, however, there was a clear indication that capillin predisposed the broad bean plant to infection by chocolate spot (Table 4).

The antifungal activity of unsaturated keto compounds is thought to arise from their ability to react with essential sulphydryl groups within the fungal cell (Geiger and Conn, 1945; Geiger, 1948; Allen, 1953; Baluja et al., 1964). If wyerone exerts its antifungal action in this manner it is of interest that it does not appear to react similarly with the sulphydryl compounds in the broad bean plant. The reason for this apparent difference has not been investigated but wyerone is not uniformly distributed in broad bean tissues and the compound shows no systemic fungicidal activity in broad bean. The results obtained with wyerone also show that it has slight systemic fungicidal activity against rust of dwarf bean. It was also inactive in comparative tests when sulphanilic acid and lithium sulphate respectively gave good systemic protection against rust and powdery mildew of wheat.

In view of the high in vitro and protectant activity of wyerone, its inactivity in tests for systemic fungicidal activity and its low eradicant activity (Table 5) are of interest. Apart from its high chemical reactivity, which might be expected to lead to its rapid immobilisation within plant tissues, the lipophilic nature of the wyerone molecule would tend to make it non-systemic. Some of the compounds which have proved successful as systemic fungicides, e.g. lithium sulphate (Carter and Wain, 1964), sulphanilic acid (Livingston, 1953), and N-phenyl-N′-3-sulpholanylhydrazine (Evans and Saggers, 1962) are readily soluble in water and can be presented to the plant at relatively high concentrations in aqueous solution. On the other hand a saturated aqueous solution contains only 30 μg/ml of wyerone. It is possible that the introduction of

certain polar groupings into the molecule of wyerone might increase its water solubility without lowering its fungicidal activity and such research could well lead to useful systemic fungicides.

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Samenvatting

De isolatie en de eigenschappen van een fungicide verbinding voorkomend in zaailingen van Vicia faba

Een beschrijving wordt gegeven van de isolatie – door extractie met benzeen – van een fungicide verbinding die in een gehalte van 0,001 % voorkomt in zaailingen van tuinboon. Na toepassing van kolomchromatografie werd de verbinding gezuiverd door herkristallisatie bij lage temperatuur uit hexaan. Als criteria voor zuiverheid werden gebruikt UV absorptie, remming van de sporekieming bij *Alternaria brassicicola* en dunne laag chromatografie. De verbinding werd "wyerone" genoemd. De activiteit in vitro tegen fytopathogenen and dermatophyten en de preventieve en curatieve werking tegen bepaalde fytopathogene schimmels zijn onderzocht.

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