Phytoalexin Production by Flowers of Garden Pea *(Pisum sativum)*

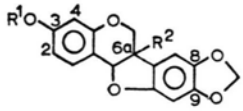
John L. Ingham

Phytochemical Unit, Department of Botany, University of Reading, Reading RG6 2AS, England

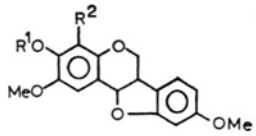
Z. Naturforsch. **34 c**, 296–298 (1979);
received December 4, 1978

Leguminosae, *Pisum*, Isoflavonoids, Pterocarpans, Phytoalexin

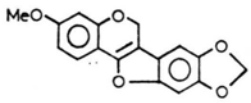
The isoflavonoid phytoalexin pisatin has been isolated from fungus-inoculated petals of 16 *Pisum sativum* cultivars. Pisatin was usually accompanied by trace quantities of maackiain. No other fungitoxic compounds were detected.



1: R^1=Me; R^2=OH
2: R^1=R^2=H



3: R^1=Me; R^2=H
4: R^1=R^2=H
5: R^1=Me; R^2=OH



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Pods of the garden pea, *Pisum sativum* L. (Leguminosae-Papilionoideae; tribe Vicieae) rapidly produce the isoflavonoid phytoalexin [1] pisatin (**1**) (3-methoxy-6a-hydroxy-8,9-methylenedioxypterocarpan) following inoculation with a wide range of micro-organisms including both obligate and facultative fungi [2]. The same compound also accumulates in the fungus-inoculated leaflets [3–5], epicotyls [6] and stipules [4] of *P. sativum* and is similarly produced by roots growing under non-sterile conditions [7]. Because pisatin is fungitoxic [8], its formation is widely regarded as a mechanism whereby microbial invasion and/or colonisation of *P. sativum* can be prevented or greatly reduced.

In addition to pisatin, trace quantities of the chemically related phytoalexin maackiain (**2**) (3-hydroxy-8,9-methylenedioxypterocarpan) have recently been isolated from the fungus-inoculated pods, leaflets and stipules of *P. sativum* [4, 5, 9]. Three other pterocarpan derivatives (2,3,9-trimethoxy-(**3**); 2,9-dimethoxy-3-hydroxy-(**4**); and 2,3,9-trimethoxy-4-hydroxy-(**5**)) also co-occur with pisatin in pea epicotyls infected by *Fusarium solani* f. sp. *pisi* [6] although as yet, compounds **3**–**5** have not been associated with any other fungus-*P. sativum* interaction. This paper describes the isolation of pisatin and maackiain from petals of *P. sativum* and is the first report of phytoalexin production by the floral structures of any plant species.

Sixteen commercially-available *P. sativum* cultivars (see Table I for details of seed sources) were grown to flowering in sheltered, outdoor plots; the standard (all cultivars), wing and keel (cultivar Pilot only) petals were then excised and inoculated as previously described [10, 11] with a conidial suspension of the fungus *Helminthosporium carbonum* Ullstrup. After 48 h incubation, the resulting diffusates [10] (1–4 ml) were collected, diluted to 10 ml with absolute EtOH and immediately reduced to dryness *(in vacuo*, 40 °C). TLC (Merck Si gel 60, F254 [12]) of the residues ($CHCl_3$: MeOH, 50 : 1) gave, for each cultivar, a single fluorescence-quenching band (R_F 0.71) corresponding to a marker of authentic pisatin. This zone was eluted (EtOH) and the pisatin firmly identified by UV and TLC (3 or 4 solvent systems) comparison with authentic material and (after spectrophotometric determination) by acidic (conc HCl) conversion to the spectroscopically distinct anhydro-derivative (**6**) (3-methoxy-8,9-methylenedioxypterocarp-6a-ene) [13]. Only one antifungal zone – attributable to pisatin – was observed on TLC plates when fungus-induced diffusates from the standard petals of Pilot and Meteor were bioassayed [11] against spore germination of *Cladosporium herbarum* Fr.

As shown in Table I, diffusates from the *H. carbonum*-inoculated petals of each *P. sativum* cultivar contained pisatin at concentrations ranging from 19–45 μg/ml. Comparable, or slightly higher, pisatin levels have also been associated with diffusates from the excised, fungus-inoculated stipules of Pilot (41 μg/ml) [4] and leaflets of Meteor (35 μg/ml), Myzar (30 μg/ml), Pilot (54 μg/ml) [4] and the Hungarian variety Apollo II (approx. 55 μg/ml) [5]. Overall, the standard petals of early, second-early and late/maincrop varieties produced pisatin in roughly equivalent amounts (Table I). Although some striking varietal differences were ap-

Reprint requests to Dr. J. L. Ingham.

0341-0382 / 79 / 0300-0296 $ 01.00/0

Table I. Pisatin concentration (μg/ml) in diffusates (48 h) from the *Helminthosporium carbonum*-inoculated petals of 16 *Pisum sativum* varieties.

Variety	Seed [a] Source	Petal [b] Type	Pisatin [c] Concentration	Mean [d]
A. Early Varieties				
Feltham First	TM	S	37	
Hurst Beagle	TM	S	45	
Kelvedon Wonder	S	S	34	
Meteor	S	S	32	
Myzar	S	S	29	
Pilot	S	S	24	31
		W	26	
		K	22	
Pioneer	S	S	19	
Progress No. 9	S	S	31	
B. Second Early Varieties				
Achievement	S	S	38	
Kelvedon Monarch	SD	S	36	
Lincoln	SD	S	28	34
Onward	TM	S	35	
C. Late/Maincrop Varieties				
Alderman	SD	S	22	
Giant Stride	SD	S	34	
Lord Chancellor	S	S	32	33
Senator	S	S	44	

[a] TM, Thompson and Morgan Ltd., Ipswich, Suffolk, England; S, Suttons Seeds Ltd., Torquay, Devon, England; SD, Samuel Dobie Ltd., Llangollen, Clwyd, Wales.

[b] S, W and K denote standard, wing and keel petals respectively.

[c] Pisatin concentrations were determined spectrophotometrically using the extinction coefficient, $\varepsilon = 7{,}244$ at 309 nm[13].

[d] Mean values (to nearest whole number) are for standard petals only.

parent – notably between the early cultivars, Pioneer and Hurst Beagle and the late/maincrop cultivars, Alderman and Senator – these may reflect the influence of variable parameters such as inoculum density and incubation conditions or, more probably, losses incurred during TLC purification and subsequent recovery of the phytoalexin. Pisatin was never obtained from petals treated with droplets of de-ionised H_2O.

Maackiain, previously reported as a minor phytoalexin of *P. sativum* [9], was isolated in trace amounts from the diffusates of every cultivar except Alderman, Giant Stride, Lord Chancellor, Pioneer and Progress No. 9. For each variety a band of Si gel opposite the maackiain marker (R_F 0.56) was eluted (EtOH), the eluant removed *in vacuo* and the residue then re-chromatographed as a discrete spot. After development ($CHCl_3$: MeOH, 50: 1 or *n*-pentane: Et_2O: HOAc, 75: 25: 3), the chromatogram was sprayed with diazotised *p*-nitroaniline; samples of authentic and *Pisum*-derived maackiain were immediately visible as bright yellow spots. Maackiain, which might conceivably function as a biosynthetic precursor of pisatin, was never obtained from diffusates in quantities sufficient for spectrophotometric measurement.

There was no evidence to suggest that petal diffusates contained any of the *F. solani* f. sp. *phaseoli*-induced pea phytoalexins **3** – **5** reported by Pueppke and VanEtten [6]; markers of all three compounds were located above pisatin (**3**, R_F 0.92; **4**, R_F 0.82; and **5**, R_F 0.80) on chromatograms developed in $CHCl_3$: MeOH (50 : 1).

As yet, the chemical defense mechanisms of flowers have received scant attention although Schönbeck and Schroeder [14] recently reported that resistance of tulip *(Tulipa gesneriana;* Liliaceae) pistils to colonisation by *Botrytis cinerea* (grey mould) was related to the post-infectional formation of two fungitoxic lactones termed tulipalin A and B: however, both compounds apparently arise *via* enzymic hydrolysis of pre-existing but inactive glucosides and must therefore be regarded as post-inhibitins [15] rather than as phytoalexins. In contrast, the present study has clearly shown that petals of *P. sativum* readily accumulate the isoflavonoid phytoalexin pisatin in response to fungal inoculation. Pisatin has also been isolated from both the petals (standard; 127 μg/g fresh tissue) and sepals (159 μg/g) of *P. sativum* cv. Pilot following irradiation with short-wavelength (254 nm) UV light [4], a widely used abiotic phytoalexin inducer. A variety of sugars (*e. g.* fructose, glucose and sucrose) occur in nectar, the sweet, insect-attractive, fluid produced by basipetalous nectaries [16]. This liquid is presumably a suitable medium for the growth of many fungi and bacteria including plant pathogens, and it is conceivable, therefore, that phytoalexin production by petals of *P. sativum* – and possibly other plant species – may represent a mechanism by which growth of these organisms can be inhibited and floral colonisation thereby prevented or greatly reduced.

The author thanks H. D. VanEtten for authentic samples of compounds **1** and **3** – **5** and the Science Research Council for financial support.

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