INHIBITORY ACTION OF PISATIN, A PHYTOALEXIN OF *PISUM SATIVUM*, FOR SPORE GERMINATION OF PLANT FUNGI

Siti Muslimah Widyastuti*, Fukuji Nonaka**, Nobumichi Sako**, and Kinji Tanaka**

Abstrak

Pisatin, fitoaleksin dari kacang kapri (*Pisum sativum* L.), diekstraksi dari polong kapri dengan menggunakan perangsang $HgCl_2$ dan $AgNO_3$. Konsentrasi optimum $HgCl_2$ dan $AgNO_3$ untuk merangsang pembentukan pisatin adalah 10^{-4} M. Spora *Ascochyta pisi*, patogen kacang kapri, ternyata lebih tahan terhadap pisatin dibanding dengan *Botrytis cinerea* (patogen polyfagus kacang kapri) dan *Pestalotia funerea* (bukan patogen kacang kapri).

Introduction

Phytoalexin was first introduced by Muller and Borger in 1940 to describe fungistatic or fungitoxic compound produced by the hypersensitive response of potato tubers to incompatible races of *Phytophthora infestans* (Montagne) de Bary. Since that time it has been shown that many other plant species produced phytoalexins in response to infection, and a number of these have been successfully isolated and characterized. According to the definition widely accepted, phytoalexins are low molecular weight antimicrobial compounds that are both synthesized and accumulated in plants after their exposure to microorganisms (Bailey and Mansfield, 1982). The synthesis or accumulation of phytoalexin is not specially induced only by pathogens, but chemical and mechanical injuries are also able to induce the appearence in some cases (Schwochau and Hadwiger, 1968; Nonaka and Hara, 1975; Darvill and Albersheim, 1984).

*Alumnus of Faculty of Agriculture, Gadjah Mada University, Yogyakarta, and the present status is graduate student at Faculty of Agriculture, Saga University, Saga 840, Japan.

**Faculty of Agriculture, Saga University, Saga 840, Japan.

The accumulation of pisatin has long been hypothesized as one mechanism of active disease resistance in pea. Though pisatin has been successfully cristalized and chemically characterized for 25 years ago, the further studies on its role on the disease resistance, metabolism and toxicity, potential as therapeutic agents against diseases plants and animals, and its possible dangers to human and animal health, have not be completely done. The difficulty to obtain a large quantities of pisatin is probably one of the constraint in doing these studies. Thus it is necessary to determine the conditions which yield greatest amount of pisatin.

The purpose of this present study was to determine the best concentration of abiotic elicitors for producing pisatin in *Pisum sativum* L. The activity of the pisatin to inhibit the spore germination of several fungi was also described.

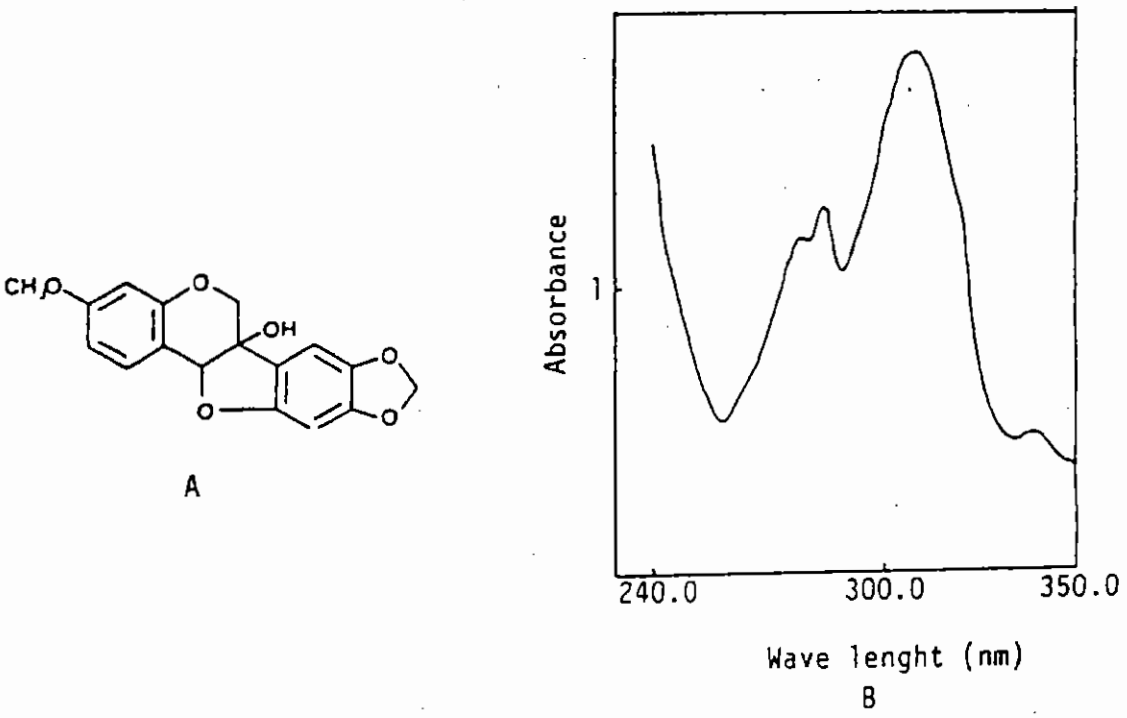

Fig. 1. The structure of pisatin (A) and its ultraviolet absorbtion spectrum (B)

Materials and Methods

Fungi.-- 13 fungi used in this experiment were isolated from their host plants, cultured on potato sucrose agar(PSA) at 25°C, and transferred periodically to fresh medium (Table 1).

Pisatin.-- Pisatin was produced by using 'drop diffusate' technique which was originally proposed by Muller (1956) and adapted on large scale of operation by Chruickshank and Perrin (1961) (Fig. 2). Pea pods were cut longitudinally and the seeds removed. The endocarp lining the seed cavities of pods was 'inoculated' with a drop (0.2) of $AgNO_3$ or $HgCl_2$ as abiotic elicitors. The concentrations of elicitors were 10^{-3} M, 10^{-4} M, and 10^{-5} M. Sterilized distilled water was used for control. After 24 h incubation at room temperature in moist chamber, the 'inoculation drops' were collected with capillary pipet, and pisatin was extracted from it by using petroleum ether. After the *in vacuo* concentration and addition of 100% ethanol, the extract was fractioned by silica gel thin layer chromatography (TLC). Pisatin could be easily identified under ultraviolet (UV) light. Quantitative measure of pisatin was done by using spectrophotometer (Fig. 1B).The extract of pisatin could also be obtained from the pea pod by dipping it in 80% ethanol for overnight, followed by filtration using filter paper. The extract of pisatin was obtained from the filtrat by concentrating it *in vacuo* procedure, and it was further processed by using procedure as described above.

Spore germination test.-- 1-2 weeks old cultures were suspended in 0.05 M phosphate buffer (pH 6) and centrifuged (800 g, 10 min). The pellet was resuspended in the same buffer and adjusted so that suspension contained 5×10^5 spores/mililiter. An appropriate amount pisatin in alcohol was then added to the spore suspension to give a final concentration of 2% (v/v) of alcohol, and then the suspension was incubated in water bath at 25°C hours. The spore germination was counted under light microscope. The spores were considered as germination when germ tube lenght exeeded the lenght of the spore.

Result and Discussion

Yield of pisatin.-- Many chemicals (abiotic elicitors) have been employed to induce the accumulation of phytoalexin from different hosts (Bailey and Mansfield, 1982). In this experiment we could also succeed to produce pisatin from pea pod using $HgCl_2$ and $AgNO_3$. The optimum concentration of both elicitors was 10^{-4} M (Fig. 3). The finding confirms the previous report (Perrin and Chruickshank,1965). The lower yield obtained by higher concentration of elicitor was probably caused by damage on cells or tissues by the chemicals.It was reported that only physiologically active tissues could produce high concentration of phytoalexin(Bailey and Mansfield, 1982).

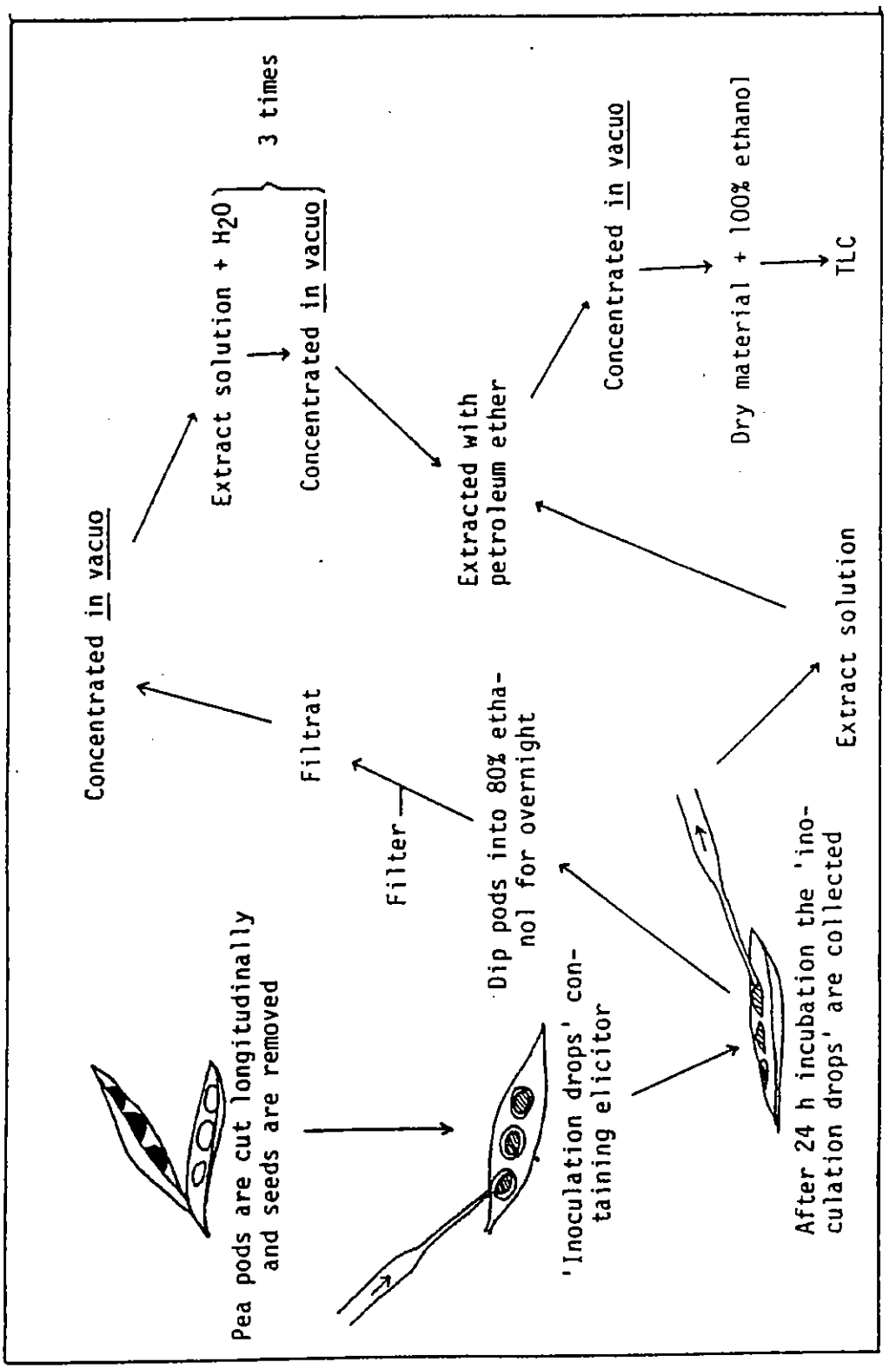

Fig. 2. Preparation of pisatin

The ultraviolet absorbance profile of the pisatin was taken with a spectrophotometer Shimadzu 265 using solvent of 100% alcohol. Both crude (before subjected to TLC) and pure (after subjected to TLC) pisatin gave almost same pattern of ultraviolet absorbance with minimum and maximum 258 - 259 nm and 308-310 nm, respectively (Fig. 1B). The crude pisatin obtained in this experiment was 56 ug/g of fresh weight of tissues whereas 10 ug/g of fresh weight of tissues for purified pisatin.

Inhibition of spores germination.-- It was found that the 13 fungi tested were sensitive to pisatin (Table 1). However, they differed greatly from sensitivity to pisatin. Namely, pisatin could strongly inhibit the spore germination of the fungi that are not pathogenic on pea (Nonaka, 1967). The low sensitivity to pisatin was obtained with *Ascochyta pisi*, a monophagus pathogen to pea. Whereas, intermediate sensitivity to pisatin was obtain with a fungi that polyphagus pathogen to pea (Nonaka *et al.*, 1977; 1978).

It suggested that in resistant host, the production of phytoalexin may cause the failure of spore to germinate , before penetration (Bailey and Mansfield, 1982). In the further study, similar result was found that *Botrytis cinerea* and *Pestalotia funerea* seemed to be higher sensitive to pisatin than *Ascochyta pisi* (Fig. 4). ED_{50} for *B. cinerea*, *P. funerea* and *A. pisi* was 68.3 ppm, 72 ppm, and 120 ppm, respectively.

The phenomenon was already observed in the previous study by using bio assay of mycelium growth on agar surface (Nonaka *et al.*, 1977; 1978). However, the time of pisatin application influenced the sensitivity of *A. pisi* to pisatin during germination period (Widyastuti *et al.*, 1987).

Pisatin is only one example of phytoalexin that can be isolated from leguminoceae. In addition of pisatin, many kind of phytoalexin such as phaseollin, phaseollidin, phaseollinoisofalvan, kievitone, and glyceollin, have been successfully isolated from leguminoceae (Bailey and Mansfield, 1982).

In agreement with the previous study, our present data suggested that pisatin has antifungal activity to pathogenic fungi. Thus, it would be expected that pisatin has contribution to the mechanism of disease-resistance in the pea. Further studies to manipulate phytoalexin on the disease management expecially in leguminoceae are still needed.

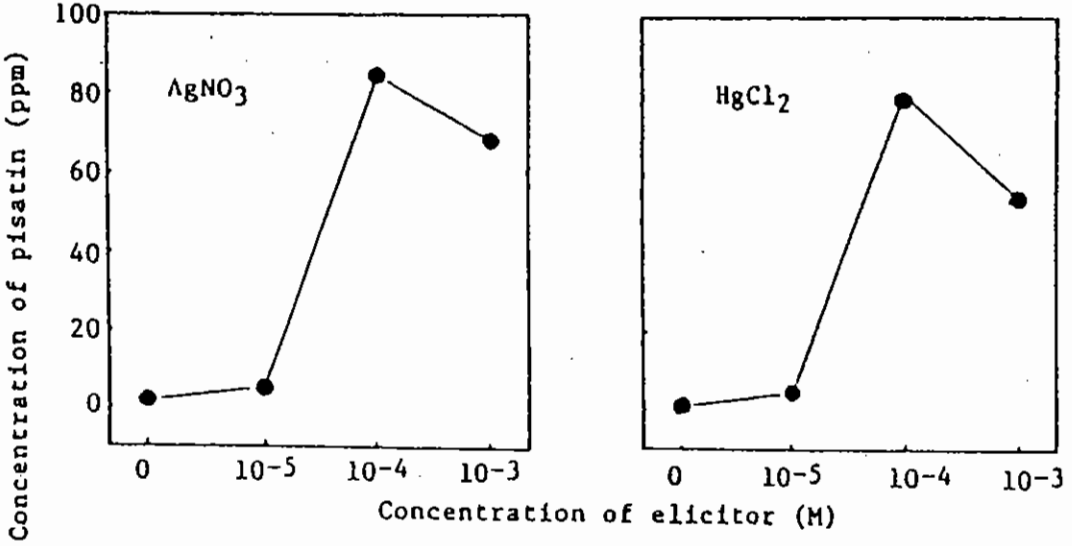

Fig. 3. Accumulation of pisatin in pod of Pisum sativum by treatment of different concentrations of elicitors

Table 1. The effect of 50 ppm pisatin treatment on the spore germination of the 13 fungi

Fungi	Host plants	Germination rate(%) Control	Treatment
Ascochyta pisi Libert	Pisum sativum L. (Pea)	90	74
Botrytis cinerea Persoon	Fragaria ananassa Duch (Strawberry)	91	62
Sclerotinia sclerotiorum (Libert) de Barry	Pisum sativum L. (Pea)	88	72
Fusarium solani (Martius) Appel et Wollenweber f. sp. pisi (F.R. Jones) Snyder et Hansen	Pisum sativum L. (Pea)	86	66
Colletotrichum gloeosporioides Penzig	Eriobotrya japonica Lindley (Loquat)	84	68
Pestalotia funerea (Desmazieres) Steyaert	Eriobotrya japonica Lindley (Loquat)	90	60
Glomerella cingulata (Stonemon) Spaulding et Schrenk	Vitis spp. (Grapes)	91	72
Dendrophoma obscurans (Ellis et Everhart) H.W.Anderson	Fragaria ananassa Duch (Strawberry)	80	61
Mycosphaerella melonis (Passerini) Chiu et Walker	Cucumis sativus L. (Cucumber)	94	63
Botrytis alli Munn	Allium cepa L. (Onion)	80	55
Cochliobolus miyabeanus (S. Ito et Kuribayashi) Dreschsler ex Dastur	Oryza sativae L. (Rice)	90	69
Corynospora melonis (Cooke) Lindaii	Cucumis sativus L. (Cucumber)	90	70
Cladosporum fulfum (Cooke)	Lycopersicon esculentum Mill. (Tomato)	92	54

Conclusion

1. The best concentration of 10^{-4} $HgCl_2$ and $AgNO_3$ for producing pisatin using pea was 10^{-4} M.
2. *A. pisi*, a non polyphagus and pathogen of pea, was lower sensitive to pisatin than polyphagus or non-pathogenic fungi.

Acknowledment

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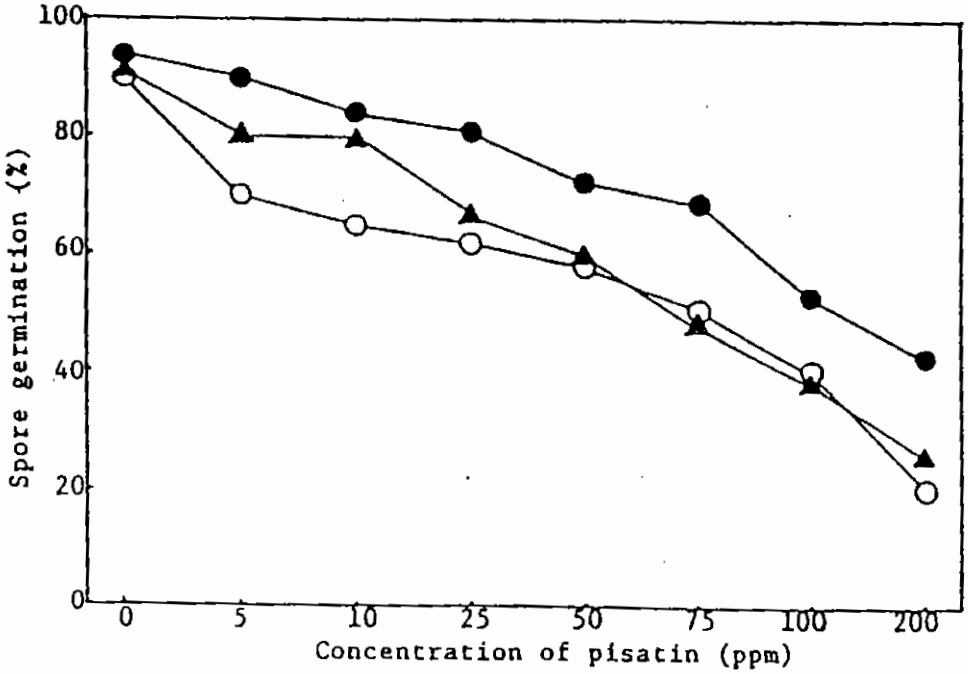

Fig. 4. The effect of pisatin at different concentrations on the spore germination of three fungi. (●) Ascochyta pisi, (▲) Botrytis cinerea, (○) Pestalotia funerea. Data represent means of three replications in each experiment and 100 spores per replication observed.

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