***Aphanomyces euteiches* Zoospore Attractant Isolated from Pea Root ; Prunetin**

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Abstract

Zoospores of *A. euteiches* were attracted to the site immediately behind the root caps of pea roots. The isolation and structural determination of attractant in pea root and its exudates were performed. Isolated attractant was 5,4'-dihydroxy-7-methoxy-isoflavone, prunetin. This compound was detected both root and its exudates, and strongly attracted *A. euteiches* zoospore at the concentration 5×10^{-7} mol. Prunetin had no attractive effect on *A. cochlidioides* zoospore at the concentrations 10^{-5} - 10^{-9} mol.

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Key words : *Aphanomyces euteiches*, pea, attractant (prunetin).

Introduction

There are many reports on the taxis of zoosporic plant pathogens to the underground parts of plants.

Though zoospores are attracted in general regardless of hosts and non-hosts or susceptible and resistant cultivars^{3,5,9,10,14,24,31)}, attraction had important role to increase the chance of contact between hosts and pathogens, and especially encysted zoospore mass on the host root or hypocotyl resulting from the attraction enable the mass infection^{9,18,31)}.

Cunningham and Hagedorn³⁾ showed that zoospores of *Aphanomyces euteiches* attracted to pea and other plant roots and most strongly to the site immediately behind the root cap of pea.

Few studies have been reported on the structural determinations of attractants of zoospore. It was found that zoospores of *Pythium aphanidermatum*²⁹⁾ and *A. raphani*³²⁾ attracted by serine and indole-3-aldehyde, respectively, at reasonable concentrations to elucidate zoospore taxis to their hosts.

The objectives of present study are to examine the site of encysted zoospore mass formation of *A. euteiches* on pea root, to determine the structure of attractant of *A. euteiches* zoospore in pea and to estimate the activity of the determined attractant.

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Materials and Methods

Fungi. Three *A. euteiches* isolates, AE-F3, AE-T7 and AE-031 which were isolated from pea root rot field soils were used. Three *A. cochlioides* isolates, AC-I, AC-K and AC-O which were isolated from sugar beet field soils, were used.

Zoospore formation. Fungal isolates were grown for 3-4 days on corn meal agar plates at 25 C. Each agar plate bearing mycelium was transferred to Petri dish, 15 cm diameter, containing 400 ml of distilled water and it was rinsed with four changes of distilled water within 2 hr to remove nutrient material inhibitory to zoospore formation. The material thus rinsed was then incubated for 12-20 hr at 25 C. Zoospore concentration was adjusted to 5,000/ml with distilled water.

Plant and its cultivation. Pea (*Pisum sativum*) was used as a test plant.

Cultivation of pea for the isolation of the attractant was as follows. Seeds were sterilized by momentary immersion in 75% ethanol and 0.1% mercuric chloride solution for 3 min. Washing with running tap water, the seeds were scattered on the wet quartz sand in stainless steel container (30 cm×30 cm×20 cm height). The container was kept at room temperature for 3 days. In this period, non-germinated and polluted seeds were excluded and healthy germinating seeds were covered with wet quartz sand in depth 1.5 cm. These were grown for 2-3 weeks at room temperature.

Aseptical cultivation of pea was as follows. Sterilization of seeds were made as mentioned above. Then the seeds were washed fully with sterilized water and kept on nutrient agar for 3 days at 25 C to select the contaminated free seeds. Fifty utilizable seeds were planted in sterilized wet quartz sand in one liter Erlenmyer flask and it was stoppered with sterilized cotton plug. After 15-20 days at room temperature, maintenance of sterility within the flask was tested by plating quartz sand on nutrient agar.

Isolation of attractant. As it seemed difficult to obtain enough amount of aseptically grown seedlings for the isolation and the structural determination of attractant, it was, at first, made with non-aseptically grown seedlings as material. Presence of the attractant in aseptically grown seedlings and those exudates were confirmed by comparing those attractive fraction with the attractant determined from non-aseptically grown seedlings, using thin layer chromatography (TLC) and gas chromatography (GC).

The isolation procedures for the attractant was shown in Fig. 1.

Attraction test. The methanolic solution of the test sample was filled in glass capillary tube (2.5 cm long, 0.8 mm outer diameter) and the solvent was thoroughly evaporated spontaneously. Then distilled water was filled in the capillary tube and its one end was sealed with vaseline. Two cm part from unsealing end of the capillary tube was inserted stantingly into zoospore suspension in a small plastic vessel (44 mm long, 22 mm wide and 5 mm high) setting on a microscope stage and behaviour of zoospores to the capillary tube was observed at a magnification of 40×.

Degree of the attraction of samples were rated as follows: non, -; attractive effect perishes within 10 min, +; attractive effect persists 10-30 min, ++; attractive effect persists 30-60 min, +++; attractive effect persists more than 60 min, ++++.

Fig. 1. Isolation procedure of *A. euteiches* zoospore attractant from pea seedling. Drying and concentration were made *in vacuo* at 40 C. Solvents and chromatographies were designated as follows : acetone, A ; benzene, B ; chloroform, C ; ethanol, D ; ether, E ; ethylacetate, F ; hexane, G ; column chromatography on silica gel, C. C ; thin layer chromatography, TLC.

Results

Taxis of zoospore to root of pea seedling

Pea seedling, grown aseptically for 10 days at room temperature, was submerged in zoospore suspension of *A. euteiches* for 30 min. Immediately after this treatment microscopical observation of the sites of encysted zoospore mass formation on the root was made at a magnification of $40\times$. Three replicate tests were performed.

In all seedlings, encysted zoospore mass were observed constantly at the sites of immediately behind the root caps. In a few cases, it was also observed at the differentiating point of lateral root from taproot. Result of one of three tests was illustrated in Fig. 2.

Fig. 2. Illustration of encysted zoospore mass formation of *A. euteiches* on pea seedling root. A seedling was submerged in zoospore suspension for 30 min. Sites of mass were shown as arrows.

Structural determination of attractant

The structure of attractant was determined by infrared (IR), ultraviolet (UV), proton nuclear magnetic resonance ($^1\text{H-NMR}$) spectra and physical properties as collected in Table 1. The molecular formula was determined by high resolution electron impact mass spectrum (HREIMS). This compound showed positive ferric chloride test.

These physical and spectral data suggested that structure of attractant has trisubstituted isoflavone skeleton.

The position of three substituent groups were elucidated that the one of them is

Table 1. Physical and spectral data of attractant

Mol. Formula ; C ₁₆ H ₁₂ O ₅
HREIMS (calcd.) ; 284. 0650 (error -3.3 MU), (284.0684)
mp (synthetic) ; 245-246 (246-248)°C (colorless needles)
IR (KBr) ν ; 3382, 1688 and 1617cm ⁻¹
¹ H-NMR (CDCl ₃) δ ; 3.87 (3H, s), 6.38 (2H, s), 6.89 (2H, d, J=8.8 Hz), 7.41 (2H, d, J=8.8 Hz), 7.85 (1H, s) and 12.80 (1H, s) ppm
¹ H-NMR (methanol-d ₄) δ ; 3.88 (3H, s), 6.36 (1H, d, J=2.3 Hz), 6.54 (1H, d, J=2.3 Hz), 6.84 (2H, d, J=8.8 Hz), 7.38 (2H, d, J=8.8 Hz) and 8.11 (1H, s) ppm
UV (EtOH) $\lambda_{max}(\epsilon)$; 262 (31877) and 325 (3844) nm
UV(EtOH-AlCl ₃) λ_{max} ; 273 and 376 nm
MS(relative intensity, %) ; M ⁺ 284 (100), 166 (10) and 167 (8)

C-5 hydrogen bonding hydroxy group from ¹H-NMR spectrum, and the another one is C-7 methoxy group by fragment ion (m/e 166 and 167) from retro Diels-Alder cleavage of MS as showed in Fig. 3. The other substituent group was C-4' hydroxy group from ¹H-NMR spectrum.

So the structure of attractant was determined 5,4'-dihydroxy-7-methoxy-isoflavone, prunetin (Fig. 4).

This attractant was identified by comparing spectral and physical data with that of synthetic sample, which was synthesized following the route⁷⁾ in Fig. 5.

Fig. 5. Synthetic route of Prunetin.

The presence of prunetin in a crude extraction with ethyl acetate from aseptically grown seedlings and those exudates were identified by direct comparison with synthetic sample using pre-coated silica gel TLC. Spots were detected by UV lamp (254 nm). Extraction from the spot corresponded to synthetic sample was the only spot that attracted *A. euteiches* zoospore.

Moreover, the crude extraction was derived to trimethylsilyl (TMSi) ether by usual method and TMSi derivatives were identified by GC (glass column, 3 mm×1 m, packed with 1.5% OV-1 Chromosorb W, column temperature 200-270 °C, carrier gas N₂ at a flow rate of 60 ml/min, detector FID). The retention time (8.99 min) of both samples agreed with the synthetic sample.

It was confirmed that prunetin, attractant of *A. euteiches* zoospore, existed in pea seedling root and its exudates.

Fig. 3. Fragment ion of attractant.

Fig. 4. Structure of Prunetin.

Judging from the relative abundance of GC peak, prunetin content of exudates sample was 1.3 times as much as root sample.

Activity of prunetin

Effects of prunetin on the zoospore behaviour of *A. euteiches* (isolate AE-031, AE-F3 and AE-T7) and *A. cochlioides* (isolate AC-I, AC-O and AC-K) were tested using the concentrations between 10^{-9} – 10^{-6} mol (Table 2).

Zoospores of all *A. euteiches* swam to mass in few seconds to the end of the test capillary tube at the concentrations between 10^{-8} – 10^{-6} mol, but the extensiveness of zoospore mass and persistence of attractiveness were different in concentrations (Fig. 6).

At the concentration 10^{-6} mol, zoospore masses of three isolates spread wide along the flow of test solution diffused from the capillary tube and swimming zoospore masses persisted more than 60 min and in some cases continued about 90 min. Zoospores of AE-031 and AE-F3 showed same response as above at the concentration 5×10^{-7} mol,

Table 2. Attractive effect of Prunetin to the zoospores of *Aphanomyces* spp.

Concentration		Degree of Attraction ^{a)}					
		<i>A. euteiches</i>			<i>A. cochlioides</i>		
		AE-031	AE-F3	AE-T7	AC-I	AC-O	AC-K
ppm	mol						
2.84	10^{-6}	+++	+++	+++	—	—	—
1.42	5×10^{-7}	+++	+++	++	—	—	—
2.84×10^{-1}	10^{-7}	++	+	++	—	—	—
2.84×10^{-2}	10^{-8}	+	+	+	—	—	—
2.84×10^{-3}	10^{-9}	—	—	—	—	—	—

a) non, —; attractive effect persists within 10 min, +; attractive effect persists 10–30 min, ++; attractive effect persists 30–60 min, +++; attractive effect persists more than 60 min, +++.

Fig. 6. Massing of *A. euteiches* zoospore to the capillary tube containing prunetin solution. Concentration of prunetin is 5×10^{-7} mol.

1. Setting after 3 seconds. 2. Setting after 20 seconds. 3. Setting after 40 seconds.

but in AE-T7 persistence of attractiveness was shortened slightly. At the concentration 10^{-7} mol, swimming zoospore masses of AE-031 and AE-T7 persisted 10-30 min and in AE-F3, it faded away within 10 min. At the concentration 10^{-8} mol, swimming zoospore masses of three isolate lost in a few minutes. No effect was observed at the concentration 10^{-9} mol in all isolates.

Three *A. cochlioides* isolates did not show any response at the concentration between 10^{-9} and 10^{-6} mol.

Discussion

As in many zoosporic plant pathogens, it was observed in *Aphanomyces* spp. that zoospores attracted strongly to the specific sites of underground parts of plants and formed encysted zoospore masses. Zoospores of *A. raphani*³¹⁾ and *A. cochlioides*²³⁾ were found to mass at the hypocotyl of cabbage and sugar beet seedlings, respectively.

Zoospore of *A. euteiches*³⁾ was reported to mass strongly at the sites of immediately behind the root caps. We also had a same result in this study. Original disease symptom by *A. euteiches* takes place at the sites encysted zoospore masses formed⁴⁾.

Massing of *A. euteiches* zoospore to pea root seems to have important function in disease occurrence as in that of *A. raphani*³¹⁾ zoospore to the hypocotyl of cabbage seedling.

Works had been made to clarify the mechanisms of massing of zoospore to the plants from view points of substance exuded from roots^{10,23,25,32)} and electrical relationship^{13,17,27,30)} between zoospore and root surface.

But many of explanations concerning with this phenomenon seem to have some difficulties when one compares the response of zoospore to the test model with that to the plant and compares the concentration of substance tested with its actual concentration on the plant surface.

Zoospore of *Pythium aphanidermatum*²⁹⁾ and *A. raphani*³²⁾ were known to be attracted swiftly and strongly to their host metabolites, serine and indole-3-aldehyde, respectively, at considerably low concentration. Prunetin was a same level of attractant of *A. euteiches* zoospore as two compounds mentioned above. At the concentration between 5×10^{-7} and 10^{-6} mol, zoospores of *A. euteiches* were attracted strongly to prunetin. This response of zoospores quite resembles to the response to the pea root, though actual concentration of prunetin on the root surface is obscure.

Prunetin was originally isolated from bark of plant closely allied to *Prunus emarginata*⁸⁾. Subsequently prunetin was isolated as a constituent of some of *Rosaceae*^{19,22)} and *Leguminosae*^{2,16,21)} plants, *Iris millesii*¹⁾, *Oroxylum indicum*¹²⁾ and as a metabolite of *Mycobacterium phlei*¹¹⁾. This compound is known to show estrogenic activity^{20,26)}, hypolipidemic activity¹⁵⁾, antioxidative activity⁶⁾ for edible oil, stimulative effect to indole acetic acid oxidase²⁸⁾.

Zoospore in soil establish themselves in host plant root after taking steps as follows; to reach adjacent the root, to contact with the root, to adhere and encyst on the root, to germinate and invade to the root. In these steps, prunetin has a part to induce zoospore of *A. euteiches* to the root. So the other mechanisms or compounds may need to

explain these all steps.

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和 文 摘 要

横沢菱三・国永史朗・関崎春雄：エンドウから単離される *Aphanomyces euteiches* 遊走子の誘引物質；プルネチン

A. euteiches の遊走子はエンドウの根冠上部に特に強く誘引され、ひき続き塊状となり、被のう遊走子塊を形成する。エンドウの根、根の分泌物中には *A. euteiches* 遊走子を強く誘引する物質が含まれているので、その単離、同定を行った。単離された誘引物質は 5,4'-dihydroxy-7-methoxy-isoflavone (Prunetin) と同定された。*A. euteiches* に対する Prunetin の誘引作用は 10^{-8} mol でわずかに認められ、 5×10^{-7} mol では、きわめて強い誘引作用が見られた。*A. cochlioides* の遊走子に対しては $10^{-9} \sim 10^{-6}$ mol で、誘引作用を示さなかった。エンドウの根への *A. euteiches* 遊走子の走性の主要な原因は Prunetin によると思われた。