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Short Communication

A Rapid and Simple Method for Inoculation with *Fusarium oxysporum* f. sp. *pisi* by Using Hydroponic Culture

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Abstract: Fusarium wilt of pea caused by *Fusarium oxysporum* f. sp. *pisi* (FOP) is categorized as a seed-borne pathogen with plant quarantine significance in Japan. In order to develop a rapid inoculation test procedure to identify the disease, the possibility of utilization of a hydroponic method employing a rotary shaker (Roberts and Kraft, 1971) was studied and evaluated by comparing it with the root-dip inoculation test. The hydroponic method was found not only more efficient but also more sensitive in determining the virulence of FOP than the root-dip method. The study also revealed that the hydroponic method was much faster than the root-dip method, taking 10–13 days from inoculation to show severe symptoms, which was at least 8 days shorter than the root-dip method. As a seed testing protocol of FOP, the hydroponic inoculation method paired with detecting techniques such as the blotter method and morphological observation is recommended.

Key words: *Fusarium oxysporum* f. sp. *pisi*, Hydroponic method, Inoculation

Introduction

Wilt of pea (*Pisum sativum* L.) caused by *Fusarium oxysporum* f. sp. *pisi* (FOP) often causes severe crop losses depending on the population density of the pathogen in the field soil or susceptibility of pea cultivars (Haglund and Kraft, 2001). In 1996, Japan categorized FOP as a seed-borne pathogen with plant quarantine significance. Since 1998, Japanese authorities have been requesting countries of export to carry out "field inspections" of parental plants of pea seeds, because it is difficult to find infested seeds with this fungus at the point of import. Although field inspections are the most effective way to prevent an invasion of FOP, seed inspection at the point of import is also important to secure the reliability of field inspections. In addition, a rapid inoculation test procedure is necessary to differentiate the FOP from non-pathogenic *F. oxysporum* isolated from the seeds. Roberts and Kraft (1971) developed a rapid inoculation test technique using a rotary shaker for FOP, which was reviewed as a "hydroponic method" by Dhingra and Sinclair (1995) and might be useful for our purpose. To our knowledge, there has been no other report on the technique that can be practically applied. Therefore, we evaluated the possibility of application of the hydroponic method for the following three issues in this study: (1) Specificity to FOP (reliability). (2) Disease development with time (rapidness). (3) Working efficiency (simpleness).

Material and Methods

Fungal isolates

The fungal isolates used in this study are shown in Table 1. *F. oxysporum* f. sp. *pisi* (FOP) 1-1-M were used for all tests, and the others were used for the specificity test.

Preparation of Inocula

Two or three mycelial disks prepared from the colony on SNA medium (Nirenberg, 1976) were placed into 100 ml of Kerr's medium (Kerr, 1962), which was modified by adding $FeSO_4 \cdot 7H_2O$ instead of $FeSO_4$ and excluding both agar and antibiotics ($NaNO_3$ 2.0g, KH_2PO_4 1.0g, KCL 0.5g, $MgSO_4 \cdot 7H_2O$ 0.5g, $FeSO_4 \cdot 7H_2O$ 0.01g, sucrose 30g, yeast extract 0.5g, distilled water to make 1,000ml), in 300 ml flasks, and incubated on a reciprocal shaker at 100 rpm

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Table 1. List of *Fusarium oxysporum* f. sp. *pisi* and other fungus used in this study, and reaction of pea seedlings (cv. Akabana-suzunatrisatou) inoculated with each isolate by two kinds of inoculation methods.

Isolates	isolate No.	Original host	Inoculation method	
			Hydroponic	Root-dip
Fusarium oxysporum f. sp.				
pisi (race: unidentified)	1-1-M[*1]	Pea	4/4[*5]	4/4
pisi race2[*2]	−	Pea	4/4	2/4
pisi race5[*2]	−	Pea	4/4	4/4
lycopersici	MAFF 103036	Tomato	0/4	0/4
radicis-lycopersici	MAFF 103007	Tomato	0/4	0/4
adzukicola	NBRC 31629	Azuki bean	0/4	0/4
adzukicola	NBRC 31630	Azuki bean	0/4	0/4
phaseoli	NBRC 9970	Azuki bean	0/4	0/4
F. oxysporum (f. sp.: unidentified)	MAFF 235711	Azuki bean	0/4	0/4
	MAFF 305111	Broad bean	0/4	0/4
	MAFF 305112	Broad bean	0/4	0/4
	MAFF 238041	Mung bean	0/4	0/4
F. oxysporum (Non pathogenic) [*3]	K3-3	Pea (seed)	0/4	0/4
	K4-3	Pea (seed)	0/4	0/4
	K5-2	Pea (seed)	0/4	0/4
F. solani f. sp. *pisi*	2-1-5[*1]	Pea	4/4	0/4
Sterile distilled water (SDW)[*4]	−	−	0/4	0/4

*1. The isolates were originated from diseased pea plants in Aich, Japan in 2002 (Sakoda *et al* ., 2004).

*2. The isolates were kindly provided by Dr. Paola Nipoti (Università degli studi di bologna). Import permit number issued by Ministry of Agriculture, Forestry and Fisheries is 15Y332.

*3. The isolates were isolated from pea seeds (cv. Misasa) by the blotter method, which were non-pathogenic to the original cultivar also by root-dip inoculation test.

*4. Negative control.

*5. The number of diseased seedlings / inoculated seedlings.
Each seedling with a disease severity rate of higher than 50% was evaluated as a diseased plant.

for 5 days at 25 °C under continuous fluorescent light. The culture were filtered through 2 layers of cheesecloth to remove the mycelia, centrifuged twice at 1,095 ×*g* for 15 minutes, conidia collected were re-suspended in sterilized distilled water (SDW) at 1×10^6 cells/ml using a hemacytometer, and used as an inoculum.

Pea cultivars and growing condition

The four commercial cultivars of pea, which have differences in resistance to Fop were used. Cultivars (cvs.) Akabana-suzunarisatou (Ak) and Misasa (Mi) were susceptible, while cvs. Kinukomachi (Ki) and Narikoma-sanjyunichi (Na) were resistant to FOP 1-1-M, which was determined by the root-dip inoculation method. The four cultivars were used to evaluate disease development with time and cv. AK was used for specificity tests also. Each seed was washed with distilled water (DW) in the beaker three times using magnetic stirrer to remove coated agrochemicals before use, surface-sterilized for 10–20 seconds in 70% (v/v) ethanol and in 1% (v/v) sodium hypochlorite for 2 minutes, and washed in sterilized distilled water. The seeds were sown in sterilized vermiculite in stainless trays (30×40×10cm deep; 50 seeds / tray), and were stimulated to germinate and grow in an isolation greenhouse or chamber (22–28°C) for 11 days. The seedlings (4–5 node stage) were carefully uprooted from each tray, and the root system was washed with tap water to remove the vermiculite.

Inoculation

The roots of the seedlings were immersed in the 180 ml of inoculum in a 200 ml wide-mouse glass bottle (milk-bottle) for 15–30 minutes. The inoculated seedlings were individually transplanted into the sterilized vermiculite in the clay pots (No.5:φ15×14cm) with a pole for supporting plants (4 seedlings / pot). Each pot with inoculated plants was placed in aluminum trays, and watered from the bottom (root-dip method; Fig. 1-A). For the hydroponic method (Fig. 1-B), the roots were immersed in the inoculum in the milk-bottle (4 seedlings / bottle). The slit plug (Roberts and Kraft., 1971) was not used to prevent damage to the seedlings. This modification also increased the number of seedlings tested per bottle from 2 to 4. When 2 cultivars (Ki and Na) were tested, a pole was attached in each bottle with polypropylene (PP) tape to support plants because plant-growth was faster than others (Ak and Mi). Each bottle with seedlings

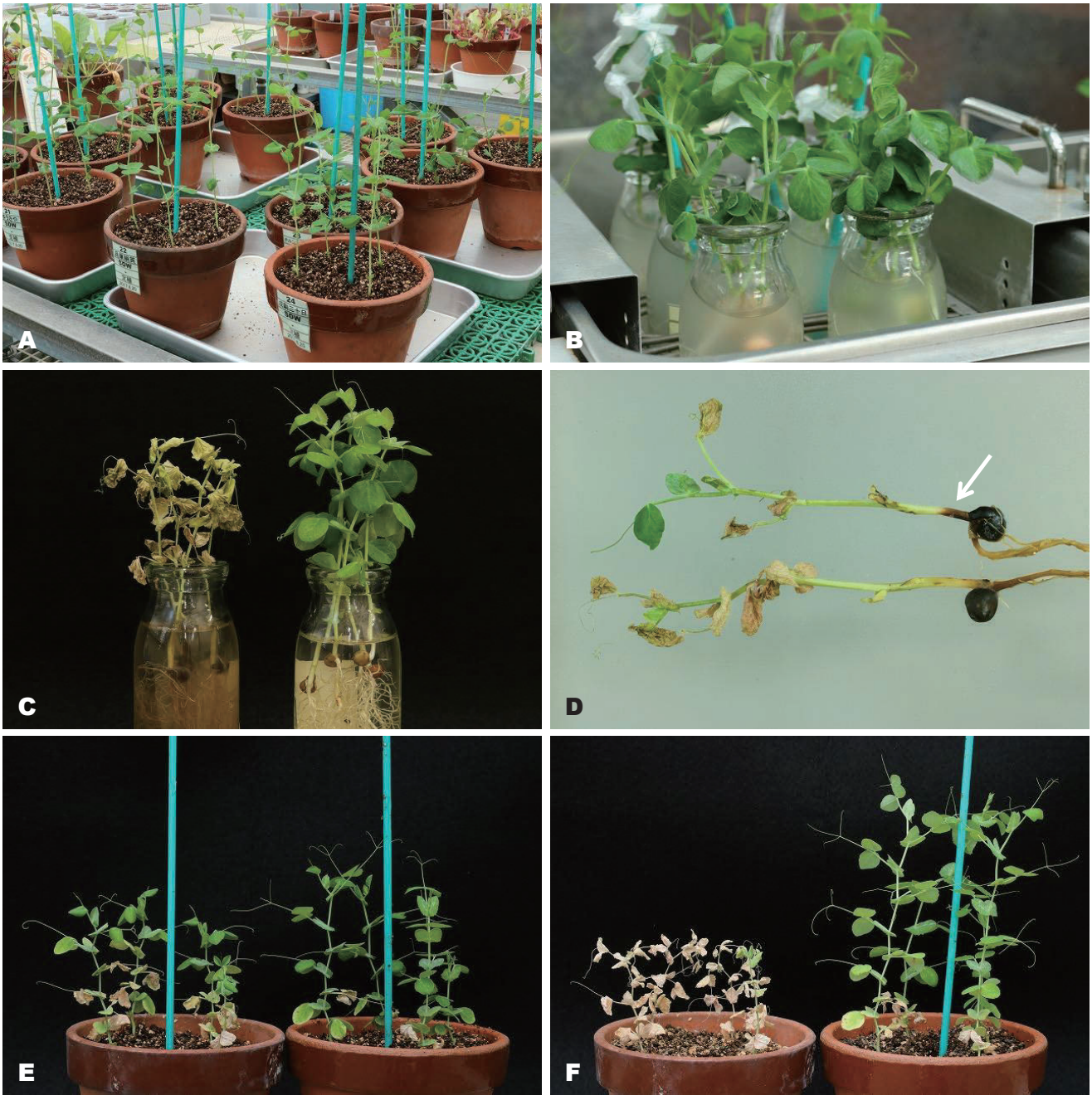

Fig. 1. Comparison of symptoms produced by a hydroponic inoculation method with those of a root-dip inoculation method.
A: Root-dip method, B:Hydroponic method, C: The symptoms (left) 9 days after inoculation with *F. oxysporum* f. sp. *pisi* (FOP) 1-1-M by using hydroponic method (right: control), D: The presence (arrow) and absence of symptoms at the basal stem after inoculation with *F. solani* f. sp. *pisi* 2-1-5 (upper) and FOP 1-1-M (bottom) with hydroponic method. E, F: The symptom (left) development of 14 (E) and 24 (F) days after inoculation with FOP 1-1-M, respectively by using root-dip method (right: control). C-F: Cultivar Akabana-suzunarisatou.

was fixed to the reciprocal shakers with flexible springs (FS-202DN, Fine Co., Ltd., Japan. and NTS-4000A, EYELA Co., Ltd, Japan), and incubated at 115 rpm to ventilate. To make up loss of volume of the inoculum in the bottle by evaporation, SDW was occasionally added to keep the cotyledon immersed in the inoculum. Both experiments were performed in the isolation greenhouse (22–28 °C) under natural light conditions. SDW was served as the negative control.

Disease assessment

Development of symptoms on each plant was visually observed for 14 days in the hydroponic method and for 30 days in the root-dip method after inoculation with each fungus. Disease severity was evaluated as the percentage of both leaves and stipules with the 2nd and upper node which present partial or whole death. A set of stipules encircled at each node were counted as a leaf, while yellow-green to yellow discolored leaves at the beginning stage of disease

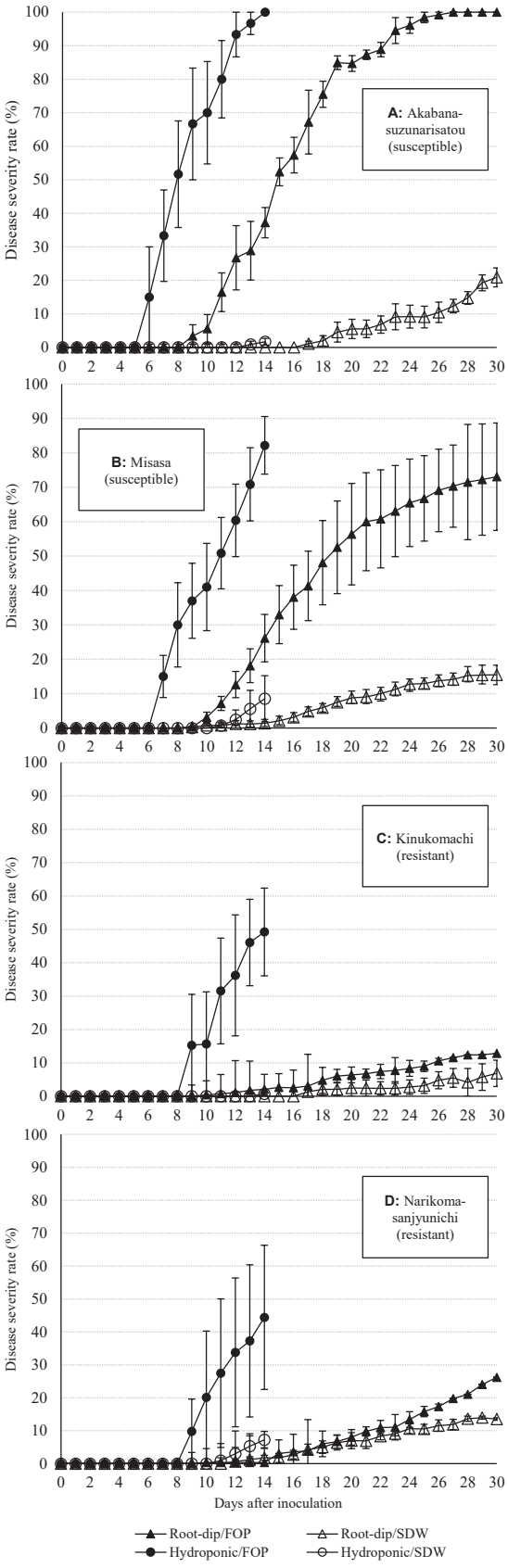

Fig. 2. Symptom development in each pea cultivar (cv.) after inoculation with *F. oxysporum* f. sp. *pisi* (FOP) 1-1-M and sterile distilled water (SDW) used as controls by using hydroponic method and root-dip method. Averages and standard errors (bar) of percentage of dead leaves and stipules on inoculated seedlings (n=12) are indicated.

development were excluded from counting. The experiments with two methods were replicated three times with 4 seedlings / treatment, respectively. In the specificity test, each seedling with a disease severity rate of higher than 50% was evaluated as a diseased plant (4 seedlings / treatment). At the end of the experiments, each inoculated plant was removed, and the presence of internal symptoms within the basal stem (0–1 node) was visually observed by longitudinally cutting with a razor blade, and re-isolation of inoculated fungus was conducted from the stem at the 2–5 node, respectively.

Results and Discussion

Disease symptom

In the hydroponic method, the symptoms caused by inoculation with FOP 1-1-M initially appeared as yellow-green to yellow discoloration from the lower leaves and stipules, and progressed to the upper leaves. Eventually, each of them showed a greyish brown to rice-straw color because of partial or whole death (Fig. 1-C). In addition, the roots of the diseased pea discolored into pale brown and their surfaces were covered with a liquid gel-like material. The red-brown discoloration of the vascular system was observed in plants showing severe wilt, and the fungus isolated from 2–5 node was identical to the fungus used as an inoculum, but not from negative healthy controls. The external symptoms produced by this method were similar to those by the root-dip inoculation method except for that of underground portions because the latter method did not affect roots.

Specificity test

Morphologically similar fungi including *F. solani* with FOP were inoculated to the pea seedlings (cv. Ak) by the two methods described above (Table 1). In the hydroponic inoculation method, there was no isolate except for the root rot pathogen of Pea *F. solani* f. sp. *pisi* 2-1-5 (FSP) showed similar symptoms, such as wilt, like those presented by FOP. The wilt caused by FSP could be distinguished from that caused by FOP based on the symptoms at the basal stem, which discolored into black in FSP but not in FOP (Fig. 1-D).

Disease development with time on susceptible cultivars

The disease severity rate on two susceptible cultivars (Ak and Mi) daily after inoculation with FOP 1-1-M is given in Fig. 2-A and B. In the hydroponic inoculation method, dead leaves or stipules initially appeared on each cultivar about 6–7 days after inoculation. Thereafter, the disease severity rate on each cultivar increased rapidly and reached 70% in 10 days (Ak) and 13 days (Mi). By the root-dip inoculation method, disease severity rates were 37% (Ak) and 26% (Mi) 14 days after inoculation (Fig. 1-E). Thereafter, the rate increased slowly, and reached 70% in 18 days (Ak) and in 27 days (Mi) (Fig. 1-F). The results showed that the hydroponic inoculation method was 8–14 days faster than the root-dip inoculation method to

reach a disease severity rate of 70%.

Varietal difference between methods

The disease severity rate on two resistant cultivars (Ki and Na) are given in Fig. 2-C and D. In the root-dip inoculation method, both cultivars demonstrated their resistance well, with disease severity rates remaining at a low level of less than 30% when the disease severity rates on the susceptible cultivars were 100% (AK) and 73% (Mi) 30 days after inoculation. Similar results were shown in the hydroponic method, and the disease severity rates on the resistant cultivars were 49% (Ki) and 44% (Na) when the disease severity rates on the susceptible cultivars were 100% (AK) and 82% (Mi) 14 days after inoculation. The results showing a difference between methods especially on the resistant cultivars indicate that the hydroponic inoculation method was more sensitive than the root-dip inoculation method, and the sensitivity of the hydroponic inoculation method was rather useful to determine the virulence of FOP isolates whose host was unspecified (e.g. from unknown cultivar or field soil) but not always suitable to differentiate the race of FOP, because varietal differences might be unclear.

In this study, some of the working procedures based on Roberts and Kraft (1971) were modified to improve working efficiency as described in Material and Methods. The inoculum was purified with centrifugation and its concentration was changed from 10^4 to 10^6 cells/ml, which allowed the promotion of disease development (data not shown). By comparing with the root-dip inoculation method, which has been ordinarily used (Bani *et al*., 2012., Bani *et al*., 2014. Haglund, 1989., Merzoug *et al*., 2014), the usability of the hydroponic inoculation method was demonstrated in that it was more rapid and simple than the root-dip inoculation method and equally reliable. As a seed testing protocol of FOP, (1) a detecting technique such as the blotter method (Mathur and Kongsdal, 2003) followed by (2) morphological observation and (3) an inoculation test by using the hydroponic method is recommended.

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和 文 摘 要

水耕栽培によるエンドウ萎凋病菌の迅速・簡易な接種方法

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エンドウ萎凋病菌（*Fusarium oxysporum* f. sp. *pisi*: FOP）の迅速・簡易な接種法を開発する目的で、水耕接種法（Robert & Kraft, 1971 を一部改変）を検証した。まず、抵抗性の異なるエンドウ 4 品種（感受性 2 品種、抵抗性 2 品種）に本菌を接種し、病徴（枯死葉率）の経時的変化を旧来の浸根接種法と比較し、発病に要する日数、諸特性を調査した。浸根接種法では播種 11 日目の幼苗の根部を水洗後、本菌の胞子懸濁液（10^6cell/ml）180ml に浸根接種し、バーミキュライトを詰めた素焼鉢に移植した。水耕接種法では同様に浸根接種し、胞子懸濁液の入った容器ごと振盪培養器に固定し振盪栽培（110rpm）した。その結果、感受性 2 品種において 70% 以上の葉が枯死したのは、浸根接種法では各々 18 日、27 日後であったが、水耕接種法では 10 日、13 日後と迅速であった。また、浸根接種法と同様に維管束の褐変と接種菌の高位節からの再分離が確認された。抵抗性品種の場合、浸根接種法では発病しないが、水耕接種法ではある程度の発病が認められた。つぎに、水耕接種法により近縁の *F. oxysporum* 及び *F. solani* をエンドウに接種した結果、*F. solani* f. sp. *pisi* のみが葉に疑似症状を示したが、地際部が褐変したことから、FOP と識別できた。以上から、FOP の病原性を確認する方法として、水耕接種法は浸根接種法と同等に利用でき、迅速・簡易な点で有用と考えられた。

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