# 新しい免疫学的手法, gel penetrate-blotted immuno-binding assay による土壌中の Fusarium spp. の検出


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Original Article

# Gel Penetrate-blotted Immunobinding Assay, a Novel Method for Serological Detection of *Fusarium* spp. in Soil

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A new serological method for the detection of *Fusarium* spp. in soil was established. On a sheet of nitrocellulose membrane, about 1 mm thick layer of agar gel was overlaid, and soil sample suspension was spread over the gel. After 10 to 20 hr of incubation, fungi in the sample grew through the gel-layer and reached the membrane leaving the soil particles on the gel surface. The membrane blotted with the fungi was recovered, and was treated with a monoclonal antibody API9-2 against *Fusarium* spp., secondary antibody labeled with alkaline phosphatase, and finally the substrate solution. The samples of *Fusarium*-infested soil successfully showed positive reaction on the membrane by this immunobinding assay (IBA). We named the method as gel penetrate-blotted immunobinding assay (GP-IBA). As the series of assay procedures can be completed within 24 hr and the detection limit is estimated to be about $10^3$ conidia/g soil, this method is considered to be suitable for detection of *Fusarium* spp. in soil.

## INTRODUCTION

Soilborne diseases, especially those due to *Fusarium* spp., cause serious problems on cultivating crops. To control the diseases, use of soil fumigants such as chloropicrin, D-D (a mixture of 1,3-dichloropropene, 1,2-dichloropropane, and related hydrocarbons), or methyl bromide has been in practice. Because of the risks, involved in using these chemicals, such as unfavorable impact on the environment and soil becoming vulnerable to pathogens, one should be careful about the timing of the treatment and the dose of the fungicide.[1)]

Several ways of biological control of soilborne diseases using non-pathogenic fungi or anti-fungal microorganisms have been reported.[1-4)] As the disadvantage in biological control of diseases is its insecurity, integrated pest management (IPM) with chemical and/or biological methods is necessary depending upon the actual pathogen level in the soil as well as in the seedlings.

For the detection of soilborne pathogenic fungi in soil, few methods have been reported other than selective media or those with susceptible indicator plants.[5-7)] But they are sometimes time-consuming and not practical for rapid and accurate assay. Therefore, development of some good detection methods is called for.

Immunological methods are now widely used not only in medical examination but also in plant diagnosis because of their high reaction specificity, rapidity and reliability.[8-10)] Polyclonal antibodies or monoclonal antibodies (MAbs) are reported to be useful to detect plant pathogenic fungi including soilborne pathogens such as *Phytophthora* sp.,[11,12)] *Pythium* sp.,[13,14)] *Plasmodiophora brassicae*,[15-17)] *Fusarium* sp.,[18)] and *Rhizoctonia solani*[9)] in plant tissues.

Previously we reported methods for the detection of *Fusarium* spp. in plant tissues by immunofluorescence assay (IFA)[18)] and direct-tissue blotted-immunobinding assay (DT-IBA)[18)] with MAb API9-2,[19)] which is one of MAbs reacting specifically with the genus *Fusarium*. Using the MAb, immunoassays such as ELISA or DIBA with soil suspension were assessed in preliminary experiments; however, they were successful only when a high number of propagula ($10^{8-9}$ cfu/g soil) of pathogen exists in the soil sample. Since such a high population of *Fusarium* spp. is not a common occurrence, it is required to develop a new serological method for soil diagnosis. The failure in detection of the less propagula might be due to adsorption of immunoprotein on soil particles. To prevent this non-specific reaction between antibody and soil particles, separation of fungi from soil particles is necessary for applying serological diagnostic tests to soil. However, it is very difficult to isolate fungi from soil with ordinary physical methods, such as density gradient or sieving.

This paper deals with a new method to separate fungi from soil particles with a layer of agar gel in contact with a piece of membrane, and a serological way to specifically detect *Fusarium* spp. on the membrane.

## MATERIALS AND METHODS

### 1. *Monoclonal Antibody (MAb)*

Hybridoma clone API9-2 was used as a producer of a *Fusarium* spp.-specific MAb.[19] Supernatant fluid containing the MAb was prepared by the method already established[18] and was used after diluting with FTBS (TBS, 10 mM Tris-HCl [pH 7.4] and 140 mM NaCl, containing 10% [v/v] fetal calf serum). The binding site of MAb API9-2 was identified to be on the surface of mycelia of *Fusarium* spp.[18]

### 2. *Fungal Isolates and Preparation*

For artificial infestation of soil, *F. oxysporum* Schlechtend.: Fr. f. sp. *lycopersici* (Sacc.) Snyd. & Hans. race 2 (880621a-1), a causal pathogen of tomato wilt and *Verticillium dahliae* Kleb. (910312a-1), a pathogen of Verticillium wilt of eggplant and tomato, were used. These isolates were cultured on potato dextrose broth (PDB) medium, which consists of 0.7 g of freeze-dried mashed-potato (Snow Brand, Sapporo) and 0.5 g of dextrose in 100 ml of tap water, in a 300 ml of Erlenmeyer flask on a rotary shaker (Bio-Shaker, BR-3000, Taitec, Saitama) at 120 rpm, 27°C for about 4 days. After incubation, the culture fluid was centrifuged ($3000\times g$, 15 min) to collect conidia, and inoculum was prepared at a concentration of about $10^7$ cfu/ml with 0.1 M phosphate buffered saline (PBS ; pH 7.5).

### 3. *Soils*

Commercial gardening soil (Kureha-soil, Kureha Chemical Industry, Tokyo) was sterilized by autoclaving (121°C, 40 min) and was artificially infested with the pathogens by pouring the desired amount of inocula to pots with the soil.

We also examined soil samples from tomato field of Imaichi, Tochigi, which were severely infested with *F. oxysporum* f. sp. *lycopersici*. These infested soils and sterilized soil were mixed in different ratios (1 : 4 ; 1 : 49 ; 1 : 499 ; 1 : 4999 ; 1 : 49,999 ; 1 : 499,999 ; 1 : 4,999,999) to prepare a series of soils with decreasing population of the pathogen. As a control, non-infested soil sampled from a healthy tomato field in Imaichi was used without sterilization.

For GP-IBA, 1 g of each test soil was suspended in 5 ml of PBS and mixed thoroughly by voltexing, followed by 1 hr of incubation at room temperature.

### 4. *Gel Penetrate-blotting System (GP-system)*

A piece of gel layer ($3\times4$ cm and ca. 1 mm in thickness) of potato dextrose agar (PDA, PDB containing 1.5% [w/v] agar) was cut from a plate medium and was laid over a piece of 0.45 $\mu$m pore size nitrocellulose membrane ($3\times4$ cm, Trans-Blot Transfer Medium, Bio-Rad Laboratories, Richmond, CA, U.S.A.) with care to avoid air bubbles between them (Fig. 1). Then this GP-system was kept in a plastic petri dish under high-humidity conditions with a sheet of filter paper immersed in distilled water.

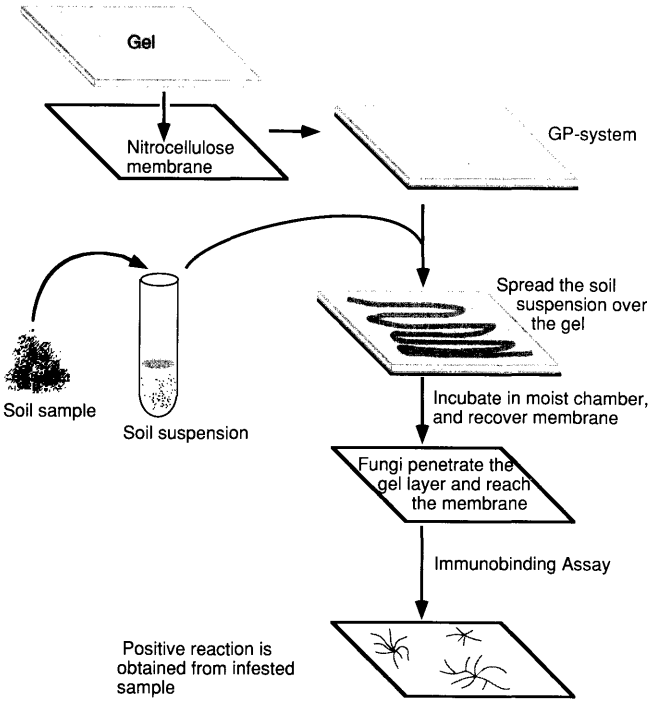


Fig. 1 Pictorial explanation of gel penetrate-blotted immunobinding assay (GP-IBA).

### 5. *Gel Penetrate-blotted Immunobinding Assay (GP-IBA)*

Soil suspension was prepared as mentioned above, and 100 $\mu$l of each sample was spread over the gel layer of GP-system. After 10 to 20 hr of incubation at 27°C under humidified conditions, the membrane was separated from the gel and blocked with FTBS containing 1% [w/v] bovine serum albumin (BSA), for 1 hr at room temperature. After blocking, it was incubated with MAb API9-2, diluted with TBS, at room temperature for 1 hr. Following three washes with TBST (TBS containing 0.1% [v/v] Tween 20), the blot was incubated with 2000 times dilution of the secondary antibody (goat anti mouse IgM, Zymed Co., So. San Francisco, CA., U.S.A.) conjugated with alkaline phosphatase for 1 hr at room temperature. Then, the membrane was washed three times, 5 min each, with 200 ml of PBST, and was immersed in AP-buffer (100 mM Tris, 100 mM NaCl, and 5 mM $MgCl_2$) containing 5-bromo-4-chloro-3-indolyl phosphate (BCIP color development substrate, Promega, Madison, WI, U.S.A.) and nitro blue tetrazolium (NBT

color development substrate, Promega) for color development. Presence of pathogen was indicated by the development of blue color on the membrane.

*6. Conventional Diagnosis Methods*

In order to compare credibility of GP-IBA method with the conventional methods, *in planta* assay and the isolation test with a selective medium were performed.

*6.1 In planta test with indicator plants*

For the indirect assay of Fusarium-infestation of the field soil, *in planta* assay[6] was carried out with cv. Momotaro, a susceptible cultivar of tomato (*Lycopersicon esculentum* Mill., Takii Seeds, Kyoto). Each soil with various population of the pathogen was taken in plastic pots, and seeds were sown. The pots were maintained in a phytotron that is controlled at 26–29°C, 50% humidity, 12 hr light (10,000–30,000 lx) and 12 hr dark conditions. Forty-five days after seeding, the disease severity (disease index: 0–3) of each plant was assessed by symptoms developed on the plants.

*6.2 Estimation of population of Fusarium spp. with a selective medium*

To estimate the population of *Fusarium* spp. in soil, a selective medium, Komada's medium,[5–7] made of 0.1% $K_2HPO_4$, 0.05% KCl, 0.05% $MgSO_4 \cdot 7H_2O$, 0.001% Fe(III)-EDTA, 0.2% L-asparagine monohydrate, 2% D-galactose, a.i. 0.075%[6] pentachrolonitrobenzene (PCNB, 1000 times diluted Earthcide 75WP; Nissan Chemical Industries, Tokyo), and 0.05% sodium cholate (Tokyo Kasei, Kita, Tokyo), was used. Soil suspension was spread over the medium and was incubated at 27°C for 9 days. The number of the colonies that appear like *Fusarium* spp.[5] was counted and the population in soil was estimated.[20]

## RESULTS AND DISCUSSION

After spreading the soil-suspension onto the gel layer, 10 to 20 hr incubation was enough to allow the growing fungal mycelia to penetrate the gel and reach the membrane under it. However, the resting form of *F. oxysporum*, chlamydospore, requires about 10 hr just to germinate, so, 20-hr incubation is better to get more accurate results with the field's soil sample. Many species of fungal propagula possibly present in the soil might also be blotted on the membrane, but as MAb API9-2 reacts specifically with the genus *Fusarium*, only those belonging to this genus blotted on the membrane were detected after the immunobinding assay (IBA) using the MAb API9-2.

As shown in Fig. 2, when *F. oxysporum* f. sp. *lycopersici*-infested soils were tested, the colonies of *Fusarium* spp. became visible on the membrane by immunostaining, showing a mycelial pattern of the blot (Fig. 2-5, indicated by an arrow). The more propagula of *Fusarium* spp. in the soil established the stronger reactions was observed depending on the mycelial density (Fig. 2-1, 2, and 3). The samples without infestation (Fig. 2-8) and infested with *V. dahliae* showed no reaction.

With this blotting system, the fungi in soil were sieved from soil particles through a gel layer due to the intrinsic nature of most of the fungi; While the soil particles stayed on the surface of the gel layer, the fungi could grow to penetrate the gel with mycelial elongation. So, we named this method as gel penetrate-blotted immunobinding assay (GP-IBA).

GP-IBA requires only 15 to 24 hr from preparing soil suspension to achieve the final results. Conventionally, it has been difficult to isolate *Fusarium* spp. in soil for detection except with some *Fusarium*-selective media, but GP-IBA with MAb API9-2 offers us an easy way to isolate and detect *Fusarium* spp. in soil distinctly from the other genera of fungi. Further, this method takes much less time to get the results than any other method so far reported, such as isolation with *Fusarium*-selective media or *in planta* assay with the indicator plants.

Recently, another novel method for the separation of *Phytophthora infestans* from soil was reported.[12] This method also exploits the pathogen's biological feature, *i.e.*, when the sample soil is soaked with water, zoospores

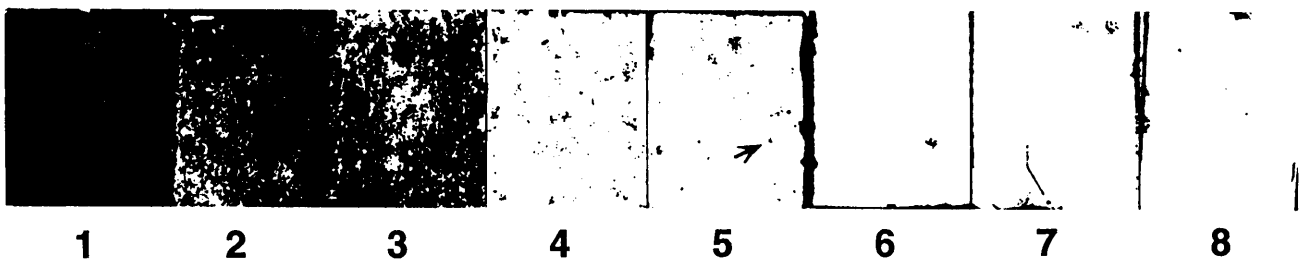


Fig. 2 GP-IBA of artificially *Fusarium*-infested soil.

**1**, $10^7$ conidia/g soil; **2**, $10^6$ conidia/g soil; **3**, $10^5$ conidia/g soil; **4**, $10^4$ conidia/g soil; **5**, $10^3$ conidia/g soil; **6**, $10^2$ conidia/g soil; **7**, $10^1$ conidia/g soil; **8**, soil without infestation. Ten hour incubation after spreading the soil suspension onto the gel layer of GP-system, following IBA with *Fusarium*-specific monoclonal antibody API9-2. The color spot showing positive reaction consists of mycelial blot (ex. **5**, indicated by an arrow). More than five replicates were used and one of the results is shown.

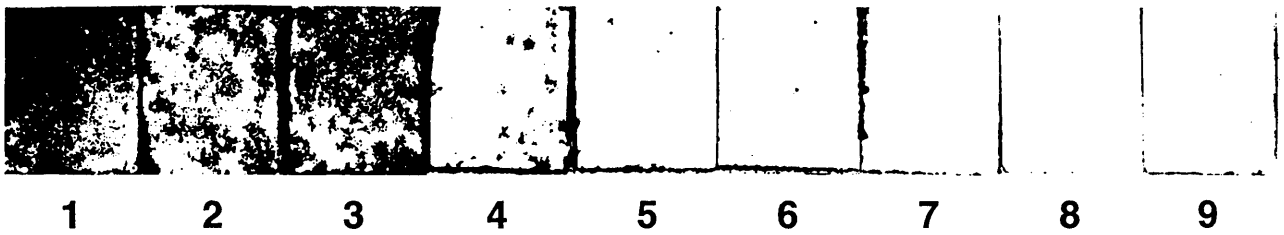


Fig. 3 GP-IBA of *Fusarium*-infested tomato field's soil.

**1**, original field's soil ; **2**, the field's soil and sterilized soil was mixed at the ratio of 1 : 4 ; **3**, 1 : 49 ; **4**, 1 : 499 ; **5**, 1 : 4999 ; **6**, 1 : 49,999 ; **7**, 1 : 499,999 ; **8**, 1 : 4,999,999 ; **9**, healthy field's soil. Twenty hour incubation after applying the soil suspension on to GP-system. More than five replicates were used and one of the results is shown.

Table 1 Detection of *Fusarium* spp. in soil by methods with selective medium and indicator plant.

| Assay method | Soil sample[a] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** |
| Selective medium[b] ($log_{10}$ CFU/g soil) | 5.3 | 4.3 | 3.3 | 3.1 | 0.0[d] | 0.0[d] | 0.0[d] | 0.0[d] | 0.0[d] |
| Indicator plant[c] | 3.0±0.0 | 3.0±0.0 | 2.8±0.1 | 1.2±1.0 | 0.6±0.5 | 0.4±0.6 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 |

[a] The number given to soil samples is corresponding to that of samples in Fig. 3 ; **1**, original field's soil ; **2**, the field's soil and sterilized soil were mixed at the ratio of 1 : 4 ; **3**, 1 : 49 ; **4**, 1 : 499 ; **5**, 1 : 4999 ; **6**, 1 : 49,999 ; **7**, 1 : 499,999 ; **8**, 1 : 4,999,999 ; **9**, healthy field's soil.

[b] The population density of *Fusarium* spp, in soil was estimated by using Komada's medium.

[c] Disease severity of each plant was evaluated from 0 to 3 with the symptoms such as yellowings, wilt and browning of vessels. 0 : no symptom, 1 : yellowing of lower leaf, 2 : wilt of lower to higher leaf, 3 : severe wilt or dead. *In planta* assay was done with at least nine plants for each set and the standard deviation is shown with the average value.

[d] Below the detectable limit.

released into the water phase are trapped by a slip of membrane immersed in the water to be used for immunodetection. It suggests that basic biological features are invaluable for developing new methods of this kind.

Since $10^3$ to $10^5$ conidia of pathogenic *F. oxysporum* in a gram of soil are needed to cause wilt disease in tomato plants,[7] the inferior limit of GP-IBA, estimated at about $10^3$ conidia/g soil as shown in Fig. 2, appears sensitive enough for the soil diagnosis. To test the applicability of GP-IBA for the field diagnosis, then we performed the assay on a severely *Fusarium*-infested tomato field's soil using MAb API9-2. The soil showed strong positive reaction even when it was mixed with sterilized-soil at the ratio of 1 : 49 (Fig. 3-1, 2, and 3). Moderate reaction was observed in the sets of 1 : 499, 1 : 4999, and 1 : 49,999 mixtures (Fig. 3-4, 5, and 6). The population of *Fusarium* spp. in the original field's soil was estimated to be around $10^7$ conidia/g soil by comparing the strength of color development with that of the artificially infested soil (Fig. 2). When the field soil was assessed by *in planta* assay with indicator plants, test plants showed severe symptoms in the soil mixed with the sterilized-soil at 1 : 4, 1 : 49 ; moderate symptoms in the 1 : 499, 1 : 4999, and, 1 : 49,999 ; no symptom in further mixture (Table 1). The results obtained by GP-IBA were more corresponding to *in planta* test than that of Komada's medium method (Table 1).

This GP-IBA method to detect *Fusarium* spp. in soil may be applicable to field diagnosis. However, since MAb API9-2 used in this report reacts with every species or isolates of *Fusarium* spp.,[19] we might have detected all the strains of *Fusarium* spp. including some pathotypes (formae or races) and/or non-pathogenic strains in the field's soil. But again, the fact that healthy field's soil showed no positive reaction by GP-IBA and selective medium method (Table 1 and Fig. 3-9) suggests that bulk of *Fusarium* spp. in soil is mostly comprised of pathogenic forms in this study.

The purpose of the field diagnosis is to affirm whether disease will occur when plants are cultivated in that field. Therefore, the diagnosis with MAb API9-2 is sometimes not conclusive. To make the immunoassay of *Fusarium* spp. more specific, the production of MAb that reacts specifically to each pathogenic form (f. sp.) or each race of the f. sp. is desired. The GP-IBA method that we offer in this paper would be more advantageous if more specific antibodies could be obtained.

In order to estimate the population of *Fusarium* spp. in soil, we tried to see the strength of the developed color in figures by using a colorimeter (NR-3000, Nippon Denshoku Co., Sengoku, Bunkyo, Tokyo). Although in the case of a large *Fusarium*-population, color density and population were correlated, the method did not work well in the case of a smaller *Fusarium*-population (data

not shown), possibly due to the unequal distribution of the colonies on the membrane as can be seen in Fig. 3-4.

Soilborne *Fusarium* diseases are still a major threat for vegetable farming. For the IPM of *Fusarium* diseases, diagnosis of *Fusarium* infestation of the field plays an important role, and sensitive and quicker method like GP-IBA are suited for this purpose.

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## 要　　約

### 新しい免疫学的手法，gel penetrate-blotted immunobinding assay による土壌中の *Fusarium* spp. の検出

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土壌中の *Fusarium* spp. の免疫学的検出法を新たに確立した．ニトロセルロースメンブレン上に厚さ約 1 mm の寒天ゲルを重ね，その寒天表面に土壌試料懸濁液を塗布する．およそ 10～20 時間のインキュベーションの間に，土壌サンプルに含まれる糸状菌は土壌粒子と分離される．そのメカニズムは，糸状菌は菌糸を伸長し，寒天ゲル層を貫通してメンブレンに達するが，一方で土壌粒子は寒天表面に残存することによる．こうして糸状菌がブロットされたメンブレンを，*Fusarium* 属菌に対して特異的に反応するモノクローナル抗体 API9-2，二次抗体，基質の順で免疫学的に発色させることで，ブロットされた糸状菌のうち，*Fusarium* spp. のみを検出できた．この方法を gel penetrate-blotted immunobinding assay（GP-IBA）と呼ぶこととした．本法によれば，24 時間以内に *Fusarium* 汚染土壌を識別することが可能であり，また，その検出可能な最小密度は $10^3$ 個分生子/g 土壌であり，実際の最小発病単位と近似するため，本方法が *Fusarium* spp. の検出に適していると考えられた．