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Monoclonal Antibody-Based Indirect-ELISA for Early Detection and Diagnosis of Epiphytic *Didymella bryoniae* in Cucurbits

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Gummy stem blight caused by *Didymella bryoniae* occurs exclusively in cucurbits. This fungus has been known not to produce its pycnidium *in vitro* unless irradiated. In this study, cultural conditions for the mass-production of pycnidiospore by Metal Halide (MH) lamp irradiation were maximized. The mycelia were cultured at 26°C on PDA for 2 days under dark condition, and then the plate was illuminated with MH lamp continuously for 3-4 days at 26°C. Results show that a great number of pycnidia were simultaneously formed. The pycnidiospores produced were then used as immunogen. Fusions of myeloma cell (v-653) with splenocytes from immunized mice were carried out. Two hybridoma cell lines that recognized the immunogen *D. bryoniae* were obtained. One monoclonal antibody (MAb), Db1, recognized the supernatant while another MAb, Db15, recognized the spore. Two clones were selected which were used to produce ascite fluid of the two MAb, Db1 and Db15, the immunotypes of which were identified as IgG1 and IgG2b, respectively. Titers of MAb Db1 and MAb Db15 were measured and the absorbance exceeded 0.5 even at a 10^{-5} dilution. The MAbs reacted positively with *D. bryoniae* but none reacted with other viral isolates, *Cucumber mosaic virus* and *Cucumber green mottle mosaic virus*. Sensitivity of MAb was precise enough to detect spore concentration as low as 10^3/well by indirect ELISA. Characterization of the MAbs Db1, Db15 antigen by heat and protease treatments, which suggests that the epitope recognized by these two MAbs was glycoprotein.

Keywords **:** *Didymella bryoniae,* gummy stem blight, indirect ELISA, monoclonal antibody.

Gummy stem blight occurs exclusively in cucurbits, showing a variety of symptoms such as leaf spot, stem canker, vine wilt, and black rot of fruit. Detection of specific fungi in phyllosphere or in plant tissues in the presence of other fungi is difficult. Classical methods such as isolation on selective media are useful but subject to limitations, because many pathogens are masked by overgrowth of faster growing fungi (Meyer and Dewey, 2000). The damage caused by the occurrence of gummy stem blight is enormous, and the disease is difficult to control. Therefore, rapid and accurate diagnostic tools are required for its detection and identification.

Clark (1981) emphasized the potential of enzyme-linked immunosorbent assay (ELISA) for detecting and identifying plant pathogen in much lower concentrations than is possible by classical methods. Majority of assays and experiments have been done with plant viruses, and the same techniques have been attempted to detect for bacterial and fungal plant pathogens.

Sundaram et al. (1991) reported that polyclonal antibody (PAb) based indirect ELISA, prepared against purified mycelial proteins from *Verticillium dahliae*, reacted positively with 11 of 12 isolates from potato, cotton, and soil, but negatively with one isolate from tomato and also with non-target pathogens such as *Fusarium oxysporum, Colletotrichum lindemuthianum, Rhizoctonia solani, Verticillium nigrescens,* and *Verticillium tricorpus*. Their antiserum reacted strongly with *V. dahliae* and intensely with *Verticillium albo-atrum.* Velicheti et al. (1993), using PAb based DAS-ELISA, attempted to develop a technique for the early detection of *Phomopsis phaseoli* and *Phomopsis longicolla* of soybean. Unfortunately, the assay system yielded cross-reaction with all *Phomopsis* sp. and *Colletotrichum truncatum* although it discriminated *Phytophthora sojae, Rhizopus* sp., *Rhizoctonia solani, Septoria glycines,* and *Cercospora kikuchii*. Holtz et al. (1994) used soluble protein extracts of mycelium of *Thielaviopsis basicola* black root rot pathogen of cotton to raise polyclonal mouse ascites antibodies. The IgG based ELISA detected both brown and gray cultural types and had negligible cross-reactivity with other soil-borne fungi commonly found in field soils.

Problems encountered with these PAb based ELISA were lack of specificity of antisera. Therefore, monoclonal antibody technique was subsequently introduced.

Marjolein et al. (1995) developed highly specific monoclonal antibody (MAb) based ELISA assay system

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that could detect *V. dahliae* and *V. albo-atrum* in infected roses and chrysanthemums with no reaction to sap of healthy plant and without cross-reactions to six other fungi from rose.

A Mab of immunoglobulin class M (IgM) against surface antigens from *Gaeumannomyces graminis* var. *tritici* by ELISA assay recognized isolates of *Gaeumannomyces graminis* var. *tritici*, *Gaeumannomyces graminis* var. *avenae*, and *Gaeumannomyces graminis* var. *graminis* (Thornton et al., 1997).

As an alternative to serological assays, polymerase chain reaction (PCR) tests were developed using specific primer to pathogens. The PCR assay proved to be a highly sensitive method for detecting very low titers of pathogens (Somai and Keinath, 2002). However, ELISA is often preferred over PCR because PCR test is costly and time-consuming for testing large number of samples (Singh et al., 2000).

ELISA method is simple to operate, specific, and highly sensitive in detecting and identifying the target pathogen. This study developed an ELISA system that can specifically detect *D. bryoniae* and discriminate other epiphytic microbes on the host plants prior to symptom development.

Materials and Methods

Antigen preparation. *D. bryoniae* were isolated from cucurbits in Gyeongnam province, Korea (Table 1). Sample slices were placed on water agar followed by incubation for 2 days at 26°C. Growing mycelial tips from the samples were then cut and transferred into potato dextrose agar (PDA, Difco) for further studies. Other viral isolates, CMV and CGMMV, were used for screening the cross-reactivity of MAb.

Mycelial colony on the PDA plate was cultured at 26°C for 2 days under dark condition, and then the plate was irradiated under Metal Halide (MH) lamp (Hanyoung Electric Co., Seoul, Korea) for 3-5 days at 26°C. Large quantities of mass-produced pycnidiospores on PDA were collected by washing the surface of PDA plate with 2 mL of ultra pure water and filtering with cheesecloth. The spore suspension was centrifuged for 5 minutes at 8,000 rpm, and re-suspended to about 1×10^8/mL in physiological salt solution (0.9% NaCl). The suspension used as immunogen was aliquot and was stored at -20°C until use.

Antibody production. Mice were injected three times at 2-week intervals with spore suspension. Three days after the second injection, the titer of antisera was determined; those that yield higher OD_{490} 0.2 over that of negative control were used for cell fusion. Three days after the last injection, the mice splenocytes were fused with myeloma cells (P3X63Ag8.V653) using PEG 1500 (Lee, 2003). Hybridomas were selectively grown in Dulbecco's modified Eagles Medium supplemented with HAT (Gibco), and hybridoma cell-culture supernatant were screened for the presence of specific antibodies by ELISA as described below. Hybridoma clones that produced antibodies that reacted only with *D. bryoniae* antigens were selected. Each clone was subcloned before injected into the mice for the production of ascites.

Table 1. Isolates of *Didymella bryoniae*, 9 fungi and 2 viruses tested in this study

Species	Isolate[a]	Host	Location
Didymella bryoniae	GS01-01	Pumpkin	Namhea
D. bryoniae	GS01-02	Cucumber	Myeongseok
D. bryoniae	GS01-03	Pumpkin	Uiryeong
D. bryoniae	GS01-04	Pumpkin	Uiryeong
D. bryoniae	GS01-05	Watermelon	Hadong
D. bryoniae	GS01-06	Watermelon	Hadong
D. bryoniae	GS01-07	Watermelon	Hamyang
D. bryoniae	GS01-08	Melon	Hamyang
D. bryoniae	GS01-09	Melon	Hamyang
D. bryoniae	GS01-10	Watermelon	Hamyang
D. bryoniae	GS01-11	Watermelon	Jinju
D. bryoniae	GS01-12	Cucumber	Jinju
D. bryoniae	GS01-13	Cucumber	Jinju
D. bryoniae	GS01-14	Cucumber	Jinju
D. bryoniae	GS01-15	Cucumber	Jinju
D. bryoniae	GS01-16	Watermelon	Jinju
D. bryoniae	DW96-88[b]	Watermelon	CNU[c]
D. bryoniae	DW96-123[b]	Watermelon	CNU[c]
Pythium ultimum[d]		Watermelon	Jinju
Rhizoctonia solani		Watermelon	Jinju
Phytophthora capsici		Watermelon	Jinju
Colletotrichum orbiculare		Watermelon	Jinju
Alternaria cucumerina		Watermelon	Jinju
Cladosporium cucumerinum		Watermelon	Jinju
Fusarium oxysporum		Watermelon	Jinju
Sclerotinia sclerotiorum		Watermelon	Jinju
Botrytis cinerea		Watermelon	Jinju
CMV		Watermelon	Jinju
CGMMV		Watermelon	Jinju

[a] Potato dextrose agar at 26°C.
[b] Isolates kindly provided by Prof. K. C. Kim (Chonnam National University, Korea).
[c] CNU: Chonnam National University, Korea.
[d] Pathogens concomitantly isolated from watermelon.

ELISA method. Indirect ELISA was performed for screening of hybridoma supernatants. Spore was dissolved in carbonate coating buffer (10^7 spore mL^{-1}), and was dispensed 100 μL per well at 4°C overnight. Coated wells were washed three times with PBST, and blocked (1% skim milk) for 30 minutes at room temperature. After washing three times, wells were incubated at 37°C for 1 hour with hybridoma supernatants 50 μL per well, washed three times with PBST, then incubated at 37°C for 1 hour with secondary antibody (Sigma A0412, goat anti-mouse polyvalent IgG, IgA, IgM, peroxidase conjugate diluted 1:10,000 in 1%BSA). After washing five times, 50 μL substrate solution (O-phenylenediamine, 4 mg/5 mL; PCB 0.1 M phosphate-citrate,

10 μL H_2O_2) was added per well, and the reaction was stopped by adding 50 μL of 2 N H_2SO_4 for 15 minutes, followed by OD reading at 490 nm.

Determination of Ig subclass. The Ig subclass of MAbs from selected cell line was determined with a commercial mouse MAb isotyping kit according to the manufacturers instruction (Sigma, ISO-1).

Antigen characterization. To characterize the Db1, Db15 antigen, two types of degradation method were applied to the spore and supernatant: enzymatic and heat.

In the enzymatic treatments, spore and spore washings immobilized in micro titer wells (50 μL) were incubated with pronase (0.25 units per well sigma P5147) and proteinase K (0.15 units per well sigma P2308) or trypsin (1 mg mL^{-1} Sigma T4424) solution at 37°C for 10 minutes, and then washed three times with PBST. Wells incubated with trypsin were treated for 10 minutes with a 0.1 mg mL^{-1} solution of trypsin inhibitor and washed three times with PBST. Washed wells were blocked with 1% skim milk. Positive control wells received PBST without pronase and proteinase K or trypsin and inhibitor but were otherwise treated similarly.

Heat treatments were performed to determine thermostability of the Db1, Db15 antigen. Spore and spore washings were placed in microcentrifuge tubes, boiled in a water bath for 10-70 minutes, and cooled to 25°C. Both samples were coated in micro titer plate and reactivity with Db1 MAb, Db15 MAb was determined by indirect ELISA. Both treatments were carried out with duplicate samples.

Results

Production of immunogen. The effect of light irradiation on the production of pycnidia *in vitro* was evaluated. Metal Halide (MH) illumination was best for immunogen (pycnidiospore) production, followed by UV treatment, which was reported by Kwon et al. (1997) to be effective for inducing conidiation of this fungus. They proposed what is called a standard procedure for mass-production of pycnidiospore as inoculum. Their method was compared with this studys improved cultural technique for the production of pycnidia *in vitro*.

Two days incubation in dark condition followed by 24 hours/day MH light illumination resulted in substantially higher number of pycidia in 2 days, which increased to about three times by further incubation up to 4 days after light treatment (Table 2). Consequently, this study optimized cultural conditions that provide fresh and mature pycidiospores representing epiphytic inoculum on the host phyllosophere: 2 days incubation in dark condition with subsequent 4 days MH light illumination (Table 3). Fresh and mature pycnidiospores produced were used as immunogen for monoclonal antibody production.

Antigen determination. To determine the antigen, the plate was coated with washed pycnidiospore *vs.* mucous pycnidiospore surface washing. Then, Db1 MAb and Db15 MAb were introduced and visualized in indirect ELISA.

Table 2. Improved cultural technique for the production of pycnidia *in vitro*

Irradiation[a]	No. of Pycnidia/cm^2 [b]			
	1 DAT[c]	2 DAT	3 DAT	4 DAT
2 days in dark incubation 12/12 hr UV light/dark[d]	–	–	132 ± 34	
2 days in dark incubation 24 hr/day MH[e]	–	634 ± 83	1524 ± 108	1755 ± 78
Continuous Fluorescent light 24 hr/day	–	–	–	–
Dark 24 hr/day	–	–	–	–

[a] All treatments were done at 26°C.
[b] Mean values of three replicates.
– = no visible pycnidia.
[c] DAT: days after treatment.
[d] Standard method of Kwon et al. (1997).
[e] MH: Metal Halide lamp (250-450 nm).

Table 3. Effect of MH illumination on the maturity of pycnidium as determined by germinability of pycnidiospores

Isolate	MH treatment 2 days after incubation in the dark[a]	Germination (%) of pycnidiospore in corresponding hours[b]			
		24	48	72	96
GS01-16	3	12	36	41	45
	4	28	68	72	76
	5	31	70	75	77
DW96-123	3	18	29	36	42
	4	32	64	69	70
	5	35	68	71	75

[a] All treatments were done at 26°C.
[b] Mean values of three replicates.

This study found interesting results showing that Db1 MAb binds with the supernatant fraction (mucous spore surface washing), whereas, Db15 MAb does not bind with washed pycnidiospore. Two chosen antibodies, Db1 and Db15, were immunotyped and identified as IgG1 and IgG2b respectively.

Specificity and sensitivity of MAbs. In titration experiments, both Db1 MAb and Db15 MAb had the highest level of reactivity to *D. bryoniae.* Absorbance readings exceeded 0.5 even at a 10^{-5} dilution. To determine the possibility of nonspecific cross reaction with other fungi and virus from cucurbits, the MAbs were tested against isolates of *D. bryoniae.* Absorbance values exceeded 1.0, but no reaction was detected with other isolates (Fig. 1). Sensitivity of MAb was precise enough to detect spore concentration as

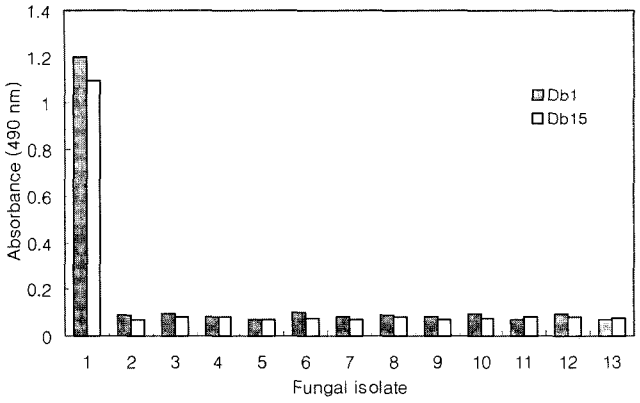

Fig. 1. Test of monoclonal antibody Db1, Db15 for cross-reaction with pathogenic isolate from cucurbits. Plates were coated with fungal homogenates (100 μL per well) and antiserum was diluted to 1:1,000. 1: *Didymella bryoniae*, 2: CMV, 3: CGMMV, 4: *Pythium ultimum*, 5: *Rhizoctonia solani*, 6: *Phytophthora capsici*, 7: *Colletotrichum orbiculare*, 8: *Alternaria cucumerina*, 9: *Cladosporium cucumerinum*, 10: *Fusarium oxysporum*, 11: *Sclerotinia sclerotiorum*, 12: *Botrytis cinerea* 13: Negative Control.

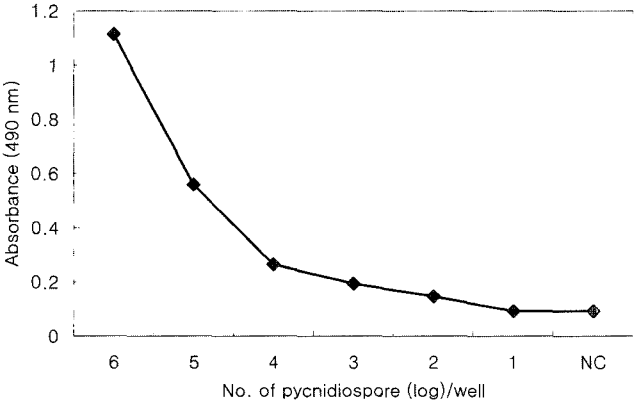

Fig. 2. Sensitivity of Db15 MAb to the threshold pycnidiospore concentration of *D. bryoniae* GS01-14 by indirect ELISA. Plates were coated with pycnidiospore and antiserum was diluted to 1:1,000. NC: Negative Control.

low as 10^3/well by indirect ELISA (Fig. 2).

Characterization of antigen. The epitope recognized by these MAbs were characterized by boiling and protease treatment. Supernatant antigen recognized by the Db1 MAb was found to be heat resistant (data not shown). When the supernatant antigens were treated with pronase and proteinase K, the absorbance values for MAb Db1 were reduced. Trypsin treatment did not result in reduction of absorbance value (Fig. 3A). Spore antigens of *D. bryoniae* recognized by the Db15 MAb were heat labile. There was a significant reduction in absorbance value, as determined by indirect ELISA, when spore antigen was boiled for 10-70 minutes (Fig. 3B). Spore antigen was resistant to proteinase

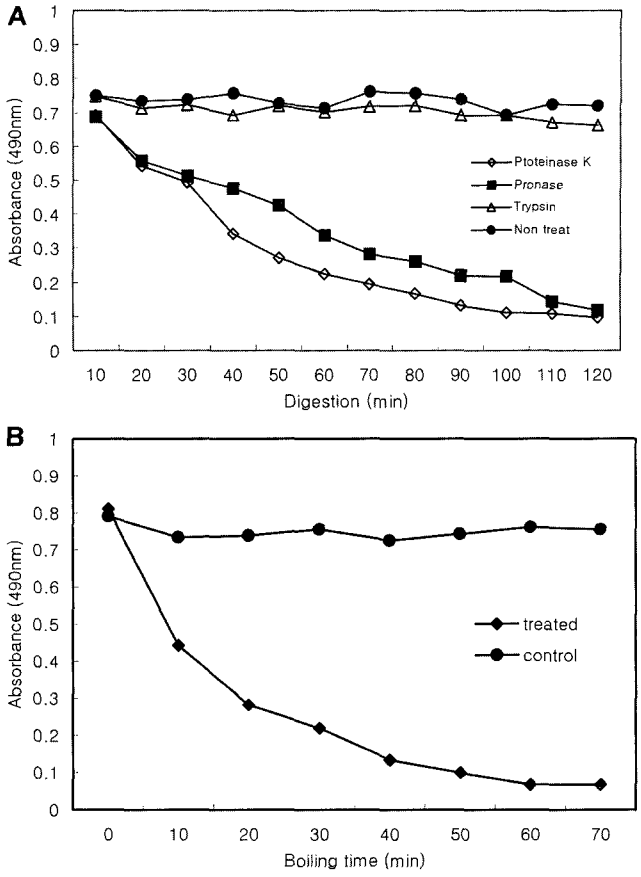

Fig. 3. (**A**) Effect of enzyme treatment on binding of the Db1 MAb to immobilized spore washings of *D. bryoniae* GS01-14. (**B**) Effect of boiling on binding of the Db15 MAb to immobilized pycnidiospore of *D. bryoniae* GS01-14. Plates were coated with pycnidiospore 10^6/well and antiserum was diluted to 1:1,000. Each point represents the mean of duplicate samples.

K, pronase, and trypsin (data not shown).

Discussion

D. bryoniae causes gummy stem blight (GSB) exclusively on cucurbits. It is the most destructive disease of melon and watermelon. Unfortunately, it is very difficult to monitor the inoculum accurately and rapidly on plant surface before the onset of GSB through plant pathological methods so far available. One of the approaches to achieve this goal would be to develop a monoclonal antibody based ELISA kit that can specifically detect *D. bryoniae* and discriminate other epiphytic microbes on the host plants prior to symptom development. In order to determine appropriate antigens for their specific antigenicity, BALB/c mice were immunized with pycnidiospore, mycelium, secreted protein during broth culture, and proteins extracted from mycelium. The antiserum obtained from the spore was promising for its low cross reactivity and the spore is a major source of

primary inoculum for disease cycle of GSB. So far, pycnidiospores have been massively produced *in vitro* by UV irradiation and were used as artificial inoculum to screen the resistance of watermelon cultivars to GSB (Kwon et al., 1997). This study evaluated various light sources for their effects on pycnidia production and sporulation *in vitro*. Results indicated that MH lamp illumination was better for pycnidiospore production than Ultra Violet light illumination, while sporulation was not induced by LED-RED, LED-Far/RED, and LED-BLUE (data not shown). MH is known to emit wavelengths that are closest to those solar lights among commercial lights in the market in Korea.

Wavelengths of light sources were 253.7 nm for UV lamp, 250-450 nm for MH lamp, 550 nm for LED-BLUE, 660 nm for LED-RED, 720 nm for LED-Far/RED, and 280-320 nm for Fluorescent light (Lee, 2003). Considering the wavelength ranges of treated light sources, the most effective wavelength for pycnidia formation and sporulation were at 250-280 nm from lower limit wavelengths for effective MH to below that for non-effective Fluorescent light. Effect of light treatment on sporulation was carried out by initial incubation at 26°C for 2 days under darkness, followed by continuous illumination with MH lamp under the same temperature for 3-5 days. Number of pycnidia induced by above scheme was compared with the mass-production technique of Kwon et al. (1997), which involved 2 days dark incubation and UV treatment of 12/12 L/D for 2 days followed by subsequent dark incubation for 4-5 day to allow pycnidiospores to mature (Table 2).

MH lamp irradiation for 4 days resulted in numerous pycnidia with fully matured pycnidiospores developed uniformly throughout the colony surface, whereas, UV irradiation induced pycnidia development in rather uneven and patchy pattern on the colony surface. UV irradiation might have been harmful to mycelial growth. Thus, this study proposed that a standard procedure for fresh and mature pycnidiospore production be used as antigen for MAb production, i.e., initial incubation of PDA cultures in dark condition at 26°C for 2 days, and subsequent MH lamp illumination (24 hours/day) for 4 days (Table 3).

In this study, pycnidiospores were used as immunogen for MAb production. Splenocytes from immunized mice were fused with myeloma cell and five-hybridoma cell lines were obtained. Among them, two clones were selected for further testing on the basis of absorbance value and designated Db1 MAb and Db15 MAb, respectively. Both Db1 MAb and Db15 MAb recognized pycnidiospores only but not mycelia of GSB pathogen and others associated with cucurbits and viruses. This indicates that the two MAbs could be used to develop early diagnostic kits.

Db1 cell line recognized the mucous conidia surface washing of GSB pathogen, while Db15 did recognize pycnidiospore surface washing. This indicates that the antigen of the two cell lines may be different from each other.

Db1 and Db15 cell line were immunotyped and identified as IgG1 and IgG2b, respectively. Ascites of both MAb revealed high specificity to *D. bryoniae*, the titer of which was as high as 10^{-5} dilution. Generally, the threshold level of conidial concentration to penetrate and infect host tissue should be higher than 10^6 spores per mL. The sensitivity of MAb was determined by ELISA system by coating serial dilution of pycnidiospore concentrations. Absorbance value was 0.2 or higher at 10^3 concentrations compared with 0.05 at negative control. Furthermore, actual number of spores coated to ELISA plate should be way below 10^3 concentrations. If the coating efficiency could be improved, it is probable that the detection limit could be as low as 10^2 spores (Fig. 2).

Db1 and Db15 antigens were characterized by various protease and heat treatment. When Db1 antigen was treated with trypsin, proteinase K, and pronase, it was resistant to trypsin, but treatment of proteinase K and pronase resulted in reduction of absorbance value as a function of time (Fig. 3A). On the other hand, Db15 antigen was resistant to trypsin, proteinase K, and pronase (data not shown). This result suggests that Db1 MAb binds to a protein epitope that does not contain residue of the amino acids, serine and arginine. Heat treatment did not affect the absorbance value of spore supernatant antigen of Db1 (data not shown), but reduced that of Db15 (Fig. 3B). Therefore, the epitopes were presumed to be glycoprotein. Db1 antigen is heat resistant to glycoprotein, while Db15 is heat labile (Avila et al., 1995; Natalia et al., 1998; Thornton et al., 1997).

In conclusion, IgG1 MAb Db1 and IgG2b MAb Db15 are proven to be very sensitive and highly specific to target pathogen *D. bryoniae*, apparently discriminating other unrelated pathogen, viruses, or epiphytes. This kit fulfills the requirements for detecting inoculum before infection and onset of GSB, which would provide highly accurate information compared with the PCR method or classical identification method (Singh et al., 2000). Moreover, PCR methods, so far reported, have relied on DNA from mycelium and hence, may be disadvantageous in the detection of inoculum as conidia, because the current technique to isolate DNA directly from conidia has not been fully established yet.

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