Plant Pathol. J. 38(4) : 403-409 (2022)
https://doi.org/10.5423/PPJ.NT.04.2022.0051
eISSN 2093-9280

Note Open Access

The Plant Pathology Journal

Isolation and Identification of Ice Nucleation Active *Fusarium* Strains from Rapid Apple Declined Trees in Korea

Diane Avalos-Ruiz[1], Leonid N. Ten[1], Chang-Kil Kim[2], Seung-Yeol Lee [1,3]*, and Hee-Young Jung[1,3]

[1]*School of Applied Biosciences, Kyungpook National University, Daegu 41566, Korea*
[2]*Department of Horticultural Science, Kyungpook National University, Daegu 41566, Korea*
[3]*Institute of Plant Medicine, Kyungpook National University, Daegu 41566, Korea*

(Received on April 5, 2022; Revised on May 25, 2022; Accepted on June 2, 2022)

In biological particles such as *Fusarium* species, ice nucleation activity (INA) has been observed. *Fusarium* strains isolated from apple declined trees in Korea were identified with a multilocus sequence analysis using the *tef1* and *rpb1* genes. Droplet-freezing and tube-freezing assays were used to determine the INA of the strains, using *Pseudomonas syringae* pv. *syringae* KACC 21200 as a positive control and resulting in seven INA+ fungal strains that were identified as *F. tricinctum* (KNUF-21-F17, KNUF-21-F18, KNUF-21-F29, KNUF-21-F32, KNUF-21-F38, KNUF-21-F43, and KNUF-21-F44). The effect of *Fusarium* INA+ KNUF-21-F29 was compared to that of INA– strains on *Chrysanthemum morifolium* cv. Shinma explants. A higher callus formation and no-shoot formation were observed, suggesting that fungal INA could play a role in cold injuries and be a factor to consider in rapid apple decline. To the best of our knowledge, this is the first report of INA fungal strains isolated in Korea.

Keywords **:** cold injury, *Fusarium*, ice nucleation activity

*Corresponding author.
Phone) +82-53-950-5761, FAX) +82-53-950-6758
E-mail) leesy1123@knu.ac.kr
ORCID
Seung-Yeol Lee
https://orcid.org/0000-0003-1676-0330

Handling Editor : Yong Hoon Lee

Plants are constantly exposed to a variety of environmental stress conditions, including pathogen infections and pest attacks. Under some of these circumstances, plant growth can be severely impacted (Suzuki et al., 2014). Rapid apple decline (RAD) or sudden apple decline has been studied extensively in the last decade, but its causative agent is still unknown (Peter, 2020; Singh et al., 2019). Its common symptoms include the presence of dead tissue at the graft union, in addition to cankers or cracks (Rosenberger, 2017; Stokstad, 2019). RAD has been linked to several factors, leading researchers to believe that this phenomenon is due to more than one of them (Stokstad, 2019). Herbicides, boring insects, pathogen infections, cultivar-rootstock incompatibility, and environmental stress are all factors that have been associated with RAD (Rosenberger, 2017; Singh et al., 2019). Weather-related stress, which includes cold injuries, is one of the hypotheses circulating around the cause of RAD (Rosenberger, 2017; Singh et al., 2019; Suzuki et al., 2014). The affected graft union tissue is the last to go dormant in the tree, making trees become vulnerable to unfavorable weather conditions like late-season freezes, and hence making them susceptible to opportunistic pathogen invasion or pest attacks (Ali et al., 2019; Singh et al., 2019; Stokstad, 2019; Suzuki et al., 2014). *Fusarium* is a genus of cosmopolitan, mycotoxigenic plant pathogens that is also the first discovered and best-studied ice-nucleating fungus (Kunert et al., 2019). Ice nucleation activity (INA) is the ability of foreign particles to catalyse water freezing at temperatures above ~ –38°C, water's freezing temperature on its ultrapure state (Failor et al., 2021; Kunert et al., 2019). Biological particles such as bacteria and fungi have been shown to act as important ice nuclei, causing ice to form at temperatures ranging from −15°C to 0°C (Kunert et al., 2019; Lagzian et al., 2014; Pouleur et al., 1992). In the case of *Fusarium*, INA has been reported above −12°C

but the frequency, distribution, and mechanisms of this INA within the genus are still not well known (Kunert et al., 2019). In Korea, only *Pseudomonas syringae* pv. *syringae* has been reported as an INA-positive microorganism (Kim et al., 1987; Lim et al., 2019), however, no fungal strains have been identified as INA-positive. Therefore, it is important to evaluate this characteristic in fungal isolates and further study its potential ecological role. This research aims to evaluate INA in fungal strains related to RAD in hopes of further understanding the role of this characteristic in RAD, cold injuries, and plant growth.

In 2021, during a survey of RAD trees in apple orchards in Chungbuk, Jeonbuk, Gyeongbuk, and Gyeonggi provinces, Korea, fungal strains were isolated from branches of RAD trees (*Malus domestica*) and cultured in potato dextrose agar (PDA; Difco, Detroit, MI, USA) at 25°C. *Fusarium* isolates were chosen based on cultural characteristics and further used in this study, in addition to collected pathogens such as *Botrysphaeria* sp., *Diaporthe* sp., *Rosellinia* sp., and *Valsa* sp. that were used as control strains for the experiment. The HiGene Genomic DNA Prep Kit (BIOFACT, Daejeon, Korea) was used to extract total genomic DNA from the strains according to the manufacturer's instructions. A polymerase chain reaction (PCR) was used to amplify the internal transcribed spacer (ITS) region using the ITS1F/ITS4 (Gardes and Bruns, 1993; White et al., 1990) primer pair. The amplified PCR ITS region products were purified and sequenced (Bioneer Co., Daejeon, Korea) and a total of 29 fungal strains; 21 *Fusarium* spp., 3 *Diaporthe* spp., 3 *Botryosphaeria* spp., 1 *Rosellinia* sp., and 1 *Valsa* sp. were identified at the genus level using the National Center for Biotechnology Information's (NCBI) Basic Local Alignment Tool (BLAST) (data not shown).

Based on previous research, a droplet-freezing assay was used to assess the strains' INA (Lindow et al., 1978; Pouleur et al., 1992). All the isolated fungal strains were grown on PDA and potato dextrose broth (PDB; Difco) at 25°C for 7 days. PDA-grown mycelium was collected and suspended in 500 µl of sterile double distilled water (DDW) and vigorously homogenized. Five 10-µl droplets per sample were placed on a working surface maintained at −8°C to −7°C in a styrofoam container with an ethanol-ice mixture. The working surface was prepared by coating aluminum foil with mineral oil and folding it into a tray shape. Frozen droplets were observed visually, and a strain was considered as INA positive if 3 to 5 droplets froze within 1 min. DDW was used as a negative control and *Pseudomonas syringae* pv. *syringae* strain KACC 21200 was used as a positive control. Other RAD tree isolated strains; *Botrysphaeria* spp., *Diaporthe* spp., *Rosellinia* sp., and *Valsa* sp. were also tested. As a result of the mycelial suspension droplet-freezing assay, 7 *Fusarium* sp. isolates out of 21 revealed high INA (5/5 frozen droplets), the same outcome as *P. syringae* pv. *syringae* KACC 21200. The remaining 14 *Fusarium* sp. isolates, *Botryosphaeria* spp., *Diaporthe* spp., *Valsa* sp., and *Rosellinia* sp. showed negative INA (0/5-2/5 frozen droplets) (Table 1). PDB liquid cultures were filtered with AVANTEC 0.45 µm disposable membrane filters to remove the mycelium, and the same process was repeated with these mycelium-free suspensions. *P. syringae* pv. *syringae* KACC 21200 was also cultured on liquid medium (Luria-Bertani broth, Difco) and filtrated using ADVANTEC 0.22 µm disposable membrane filters. As a result, 4 of the previously 7 *Fusarium* sp. INA-positive isolates; KNUF-21-F29, KNUF-21-F32, KNUF-21-F38, and KNUF-21-F44 continued to show positive INA (3/5-5/5 frozen droplets) (Table 1). INA was not observed in the control culture filtrate of *P. syringae* pv. *syringae* KACC 21200. INA was confirmed again through a tube-freezing assay, in which 50 µl of culture filtrate were added in 0.2 ml tubes and the tubes were placed for 2 min on a refrigerated circulator bath (JEIO Tech Co., Daejeon, Korea) maintained at −8°C, obtaining the same results as the droplet-freezing assay (Supplementary Fig. 1). Although *Fusarium* is one of the best studied INA fungi, little is known about its INA mechanism (Kunert et al., 2019). Ice-nucleating proteins anchored to the outer cell membrane of INA+ bacteria have been reported to induce the formation of ice (Pandey et al., 2016), which could explain why INA was lost in the cell-free filtrate of *P. syringae* pv. *syringae* KACC 21200. In the case of fungi, INA may also be due to proteinaceous compounds produced by them (Failor et al., 2021). High activity has been reported in ice nucleation particles found in *F. avenaceum*. Additionally, it was discovered that these proteinaceous particles function independently from the fungal cells and are nanometers in scale (O'Sullivan et al., 2015). Fungal ice-nucleating proteins, unlike bacterial ones, are not anchored to the cells and therefore can be separated from the fungus. Because of these characteristics, even after filtration, the four above-mentioned *Fusarium* strains still showed INA. The absence of INA after filtration for KNUF-21-F17, KNUF-21-F18, and KNUF-21-F43 indicates the presence of fungal structures or ice-nucleating macromolecules with a more complex nature. Many *Fusarium* species produce a wide range of metabolites that belong to several classes of compounds; however, the biological activity of all these compounds has not been researched (Vesonder and Golinski, 2014). INA in *Fusarium* strains is not due to one single

Table 1. Sample collecting location, strain identification and ice nucleation activity results

Strain code	Location	Species complex	Species	Ice nucleation activity				
				From solid culture		0.45 μm filtrate		Final classification
				Result	Classification	Result	Classification	
KNUF-21-F15	Boeun-gun, Chungbuk province	FTSC	*Fusarium tricinctum*	0/5	–	0/5	–	Negative
KNUF-21-F16	Boeun-gun, Chungbuk province	FTSC	*F. tricinctum*	0/5	–	0/5	–	Negative
KNUF-21-F17	Boeun-gun, Chungbuk province	FTSC	*F. tricinctum*	5/5	+	0/5	–	Positive
KNUF-21-F18	Boeun-gun, Chungbuk province	FTSC	*F. tricinctum*	5/5	+	1/5	–	Positive
KNUF-21-F20	Goesan-gun, Chunbuk province	FOSC	*F. oxysporum*	1/5	–	0/5	–	Negative
KNUF-21-F23	Boeun-gun, Chungbuk province	FNSC	*F. commune*	2/5	–	0/5	–	Negative
KNUF-21-F24	Boeun-gun, Chungbuk province	FOSC	*F. oxysporum*	0/5	–	0/5	–	Negative
KNUF-21-F25	Boeun-gun, Chungbuk province	FSSC	*F. solani*	0/5	–	0/5	–	Negative
KNUF-21-F26	Boeun-gun, Chungbuk province	FSSC	*F. solani*	0/5	–	0/5	–	Negative
KNUF-21-F27	Boeun-gun, Chungbuk province	FSSC	*F. solani*	0/5	–	0/5	–	Negative
KNUF-21-F28	Boeun-gun, Chungbuk province	FNSC	*F. commune*	0/5	–	0/5	–	Negative
KNUF-21-F29	Gunwi-gun, Gyeongbuk province	FTSC	*F. tricinctum*	5/5	+	4/5	+	Positive
KNUF-21-F32	Muju-gun, Jeonbuk province	FTSC	*F. tricinctum*	5/5	+	4/5	+	Positive
KNUF-21-F33	Cheongsong-gun, Gyeongbuk province	FOSC	*F. oxysporum*	0/5	–	0/5	–	Negative
KNUF-21-F34	Muju-gun, Jeonbuk province	FTSC	*F. tricinctum*	0/5	–	0/5	–	Negative
KNUF-21-F36	Cheongsong-gun, Gyeongbuk province	FTSC	*F. acuminatum*	2/5	–	0/5	–	Negative
KNUF-21-F37	Cheongsong-gun, Gyeongbuk province	FTSC	*F. acuminatum*	2/5	–	0/5	–	Negative
KNUF-21-F38	Boeun-gun, Chungbuk province	FTSC	*F. tricinctum*	5/5	+	5/5	+	Positive
KNUF-21-F42	Yeoju-si, Gyeonggi province	FTSC	*F. acuminatum*	1/5	–	0/5	–	Negative
KNUF-21-F43	Muju-gun, Jeonbuk province	FTSC	*F. tricinctum*	5/5	+	0/5	–	Positive
KNUF-21-F44	Muju-gun, Jeonbuk province	FTSC	*F. tricinctum*	5/5	+	3/5	+	Positive
KNUF-21-D1	Boeun-gun, Chungbuk province	n/a	*Diaporthe* sp.	2/5	–	0/5	–	Negative
KNUF-21-D2	Gunwi-gun, Gyeongbuk province	n/a	*Diaporthe* sp.	2/5	–	0/5	–	Negative
KNUF-21-D3	Muju-gun, Jeonbuk province	n/a	*Diaporthe* sp.	1/5	–	0/5	–	Negative
KNUF-21-Ros	Boeun-gun, Chungbuk province	n/a	*Rosellinia* sp.	0/5	–	0/5	–	Negative
KNUF-21-B1	Boeun-gun, Chungbuk province	n/a	*Botrysphaeria* sp.	0/5	–	0/5	–	Negative
KNUF-21-B2	Boeun-gun, Chungbuk province	n/a	*Botrysphaeria* sp.	0/5	–	0/5	–	Negative
KNUF-21-B3	Uiseong-gun, Gyeongbuk province	n/a	*Botrysphaeria* sp.	0/5	–	0/5	–	Negative
KNUF-21-Val	Muju-gun, Jeonbuk province	n/a	*Valsa* sp.	0/5	–	0/5	–	Negative

FTSC, *Fusarium tricinctum* species complex; FOSC, *Fusarium oxysporum* species complex; FNSC, *Fusarium nisikadoi* species complex; FSSC, *Fusarium solani* species complex; +, (positive) presents ice nucleation activity; –, (negative) does not present ice nucleation activity; n/a, does not apply.

compound; therefore, further systematic studies are needed to better understand the INA phenomenon at a molecular level. INA-positive *Fusarium* sp. KNUF-21-F29, INA-negative *Fusarium* sp. KNUF-21-F37, and INA-negative *Botryosphaeria* sp. KNUF-21-B1 were selected to test the effect of *Fusarium* sp. INA on plant growth. *Chrysanthemum morifolium* cv. Shinma leaves were cut into 0.5-1.0 cm long segments and cultured on Murashige and Skoog

(MS) medium containing 3 g/l of Gelrite and supplemented with 6-benzyladenine and α-naphthaleneacetic acid. Explants were cultured with 16 h photoperiod (Naing et al., 2016). Furthermore, 1.5 ml tubes were prepared with 1 ml of 0.45 µm filtrates of the previously mentioned strains and MS liquid medium as control. In sterile conditions, three to four explants were placed per treatment tube and tubes were placed on a refrigerated circulator bath (JEIO Tech Co.) set at −8°C for 1, 2, or 3 min. Explants were removed from the tubes after the cold treatment and excess treatment solution was dried out with sterile tissue paper. The explants were placed again in the plates containing MS medium and cultured as previously mentioned. After 7, 14, 24, and 30 days, the plates were photographed to assess explant development, shoot regeneration percentage, and the number of shoots per explant. Additional explants were left without any intervention. When comparing the treatments to the controls after 30 days, there was no significant difference in the regeneration percentage and the number of shoots per explant (data not shown). However, three types of tissue were overall observed in the *Chrysanthemum* explants: callus, shoot buds, and shoots (Fig. 1). For the explants with no intervention, shoots were mostly present

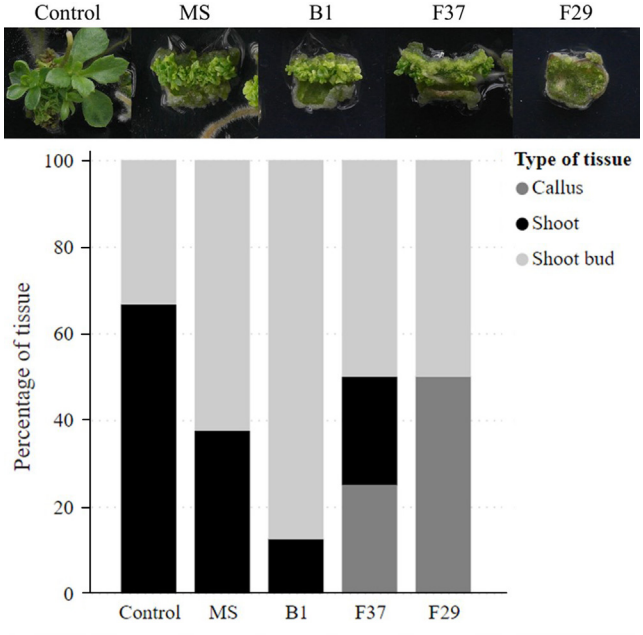

Fig. 1. Effect of different treatments and fungal ice nucleation activity (INA) on *Chrysanthemum morifolium* cv. Shinma explants and regeneration percentage after 30 days. Control, no intervention; MS, Murashige and Skoog medium; B1, *Botrysphaeria* KNUF-21-B1 (INA–); F37, *F. acuminatum* KNUF-21-F37 (INA–); F29, *F. tricinctum* KNUF-21-F29 (INA+). Data belongs to the 2-min treatment.

(over 60%), while for the MS control and the INA negative strains KNUF-21-F37 and KNUF-21-B1, shoot buds were predominant. This shows that even without an INA effect, the explants suffered from cold stress. Both *Fusarium* strains KNUF-21-F37 and KNUF-21-F29 presented callus formation, with the INA-positive strain KNUF-21-F29 having the highest percentage of callus formation among treatments (50%). Callus consists of unspecialized and unorganized cells that can undergo differentiation when in the right conditions (Bhatia, 2015), and, in this case, the presence of this tissue can be related to the effect of the strain's INA on the explants.

To identify the isolated *Fusarium* species, EF1/EF2 and RPB1-Fa/RPB1-R8 (or RPB1-F5/RPB1-G2r) were used to amplify the translation elongation factor 1-α (*tef1*) and the RNA polymerase largest subunit (*rpb1*), respectively (O'Donnell et al., 1998, 2010). Sequences from 21 samples for *tef1* and *rpb1* were obtained, registered in NCBI (*tef1*: LC702293-LC702313, *rpb1*: LC701711-LC701731), and identified through NCBI's BLAST. A neighbor-joining phylogenetic tree constructed using MEGA version X (Kumar et al., 2016) based on the *tef1* partial sequences showed that the fungal strains clustered together with their expected species complex (Supplementary Fig. 2), including species from *F. tricinctum* species complex (FTSC), *F. oxysporum* species complex, *F. nisikadoi* species complex, and *F. solani* species complex. In particular, INA-positive *Fusarium* strains were identified as members of the FTSC. A further phylogenetic analysis was done to get a better resolution on the phylogeny of the FTSC strains, combining *tef1* and *rpb1* partial sequences. The INA-positive strains KNUF-21-F17, KNUF-21-F18, KNUF-21-F29, KNUF-21-F32, KNUF-21-F38, KNUF-21-F43, and KNUF-21-F44 clustered together with *F. tricinctum* isolate 24E with high bootstrap values (Fig. 2).

Fungal pathogens such as *Fusarium* have been associated with apple decline syndrome (Villani, 2018). This genus comprises several species with reported INA, such as *Fusarium acuminatum*, *F. avenaceum*, *F. tricinctum* (FTSC), *F. armenium*, *F. langsethiae* (*F. sambucinum* species complex), *F. begoniae*, *F. concentricum*, and *F. langsethiae* (*F. fujikuroi* species complex) (Crous et al., 2021; Kunert et al., 2019). As mentioned above, the seven positive INA strains belonged to the FTSC and were identified as *F. tricinctum*, matching previously reported data. It is important to highlight that other INA negative strains such as KNUF-21-F16 and KNUF-21-F34 were also clustered together within the FTSC with *F. tricinctum* 24E (Supplementary Fig. 2). These findings are in good agreement with what was suggested by Kunert et al. (2019), indicating that not all strains

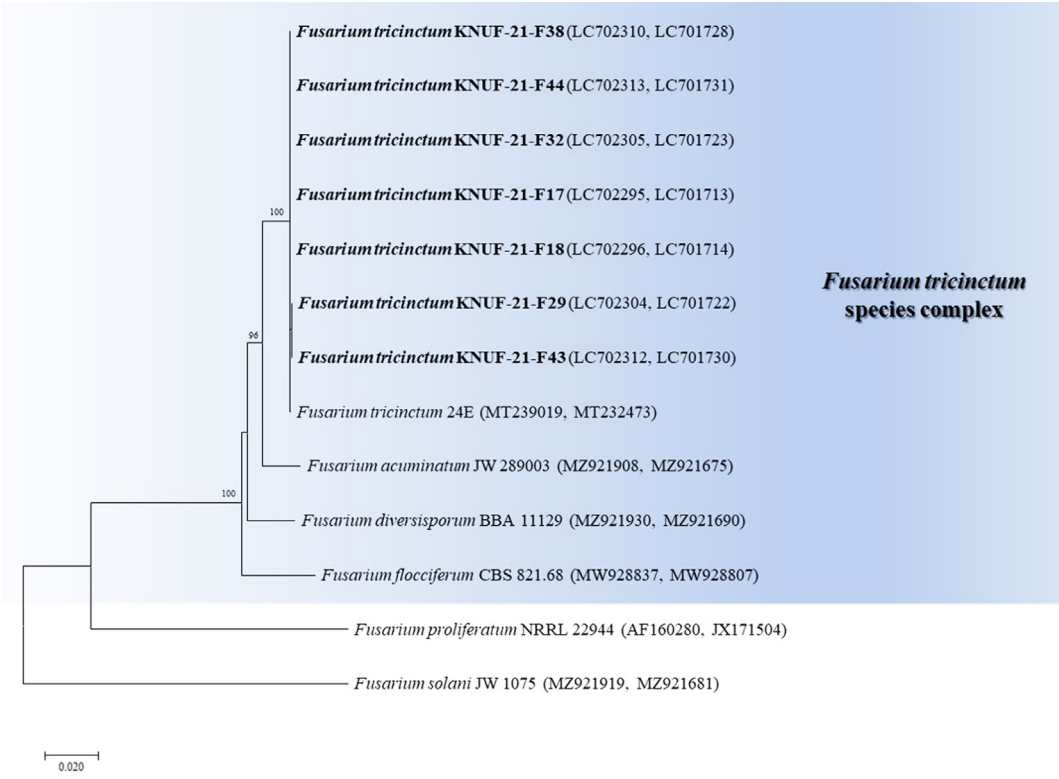

Fig. 2. Neighbor-joining phylogenetic tree analysis based on *Fusarium tricinctum* species complex combined translation elongation factor (*tef1*) and RNA polymerase largest subunit (*rpb1*) partial sequences. *Fusarium solani* JW 1075 was used as an outgroup. The strains isolated in this study are bolded and values below 90 are not shown. Bootstrap values are based on 1,000 replications. Bar, 0.02 substitutions per nucleotide position. Sequence accession numbers are shown in parentheses.

within one *Fusarium* species exhibit ice nucleation activity. The production of ice nucleation proteins requires energy; hence, it could be a trait that won't be expressed all the time. Environmental conditions are thought to have an effect on the gene expression of this characteristic; however, the specific trigger is still not identified. Moreover, after a series of subcultures, some *Fusarium* strains can show a reduction of their INA, with some of them even losing this ability (Kunert et al., 2019).

In bacteria, INA has been extensively studied, and it has been suggested that bacterial ice nuclei play an important role in cold damage in many cold or frost-sensitive plants (Kishimoto et al., 2014; Lindow, 1983; Lindow et al., 1978). Furthermore, cold injury has been proposed to be a predisposing factor in terms of plant infection, making the infection more severe if the pathogen is INA-positive (Kennelly et al., 2007; Lindow, 1983). Cold extremes are expected to decrease because of climate change (IPCC, 2021). With temperatures getting continuously warmer and ice-nucleating microorganisms present in apple orchards, cold injuries can become more frequent and so does other related problems like RAD. With the present study, we look forward to contributing to a better understanding of the ecological role of ice nuclei active fungi. However, more research on fungal INA, its mechanisms, and its effect on different crops, like apple, both *in vitro* and under on-field conditions is necessary to eventually identify the role of INA fungi on cold injury and RAD.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

Acknowledgments

This work was carried out with the support of the Cooperative Research Program for Agriculture, Science and Technology Development (Project No. PJ014998022022), Rural Development Administration, Republic of Korea.

Electronic Supplementary Material

Supplementary materials are available at The Plant Pathol-

ogy Journal website (http://www.ppjonline.org/).

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