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Identification of *Fusarium* spp. causing dry rot of seed potato tubers in northern, Thailand

Falert, S. [1,2] **and Akarapisan, A.** [1,2*]

[1]Division of Plant Pathology, Department of Entomology and Plant Pathology, Faculty of Agriculture, Chiang Mai University, Chiang Mai 50200, Thailand; [2]Center of Excellence on Agricultural Biotechnology: (AG-BIO/PERDO-CHE), Bangkok 10900, Thailand.

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Abstract Fusarium dry rot (FDR) is an important potato disease causing post-harvest tuber rot and seed piece decay worldwide. The causative agent of potato dry rot was identified in northern Thailand, based on the characterization and pathogenicity of the isolated pathogen. Infected potato tuber samples from different areas in northern Thailand showed potato dry rot symptom. *Fusarium* species were identified, based on morphology and the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA (rDNA) and pathogenicity. The effect of wounding on infection by *Fusarium* species in potato tubers was studied, four injury levels were evaluated to determine the influence of wound severity on infection by each *Fusarium* species. *F. graminearum* and *F. solani* were isolated from diseased potato tubers in Chiang Mai and Tak Province in Thailand, respectively. *F. graminearum* was the most aggressive and exhibited typical external dry rot lesions expressed as brown to black flecks on the tuber surface. This is the first report to evaluate the importance of *F. graminearum* as potato pathogen in Thailand.

Keywords: Fusarium dry rot (FDR), *Fusarium graminearum*, *Fusarium solani*, ITS regions, Potato disease

Introduction

Potato (*Solanum tuberosum* L.) is the world's third most important food after rice and wheat. Many studies have been conducted to increase the quality and quantity of potato production in the world (Aydin *et al*., 2016). Australia, Netherlands, Scotland, and Canada are important countries for potato seed production. The world production of potato is 321 million tons (Muthoni and Nyamongo, 2009). In Thailand three provinces are the main potato production areas - Tak Provinces, with 40.25 % of total production, and Chiang Mai (28.34 %), and Chiang Rai (14.79 %). Many diseases, including several seed borne, foliar and soil borne diseases affect the

* **Corresponding Author:** Akarapisan, A. ; **Email**: angsana.aka@gmail.com

production of the potato crop in Thailand (Mirhendi *et al*., 2010).

Fusarium dry rot (FDR) or potato dry rot is an important potato disease worldwide and is caused by 13 species such as *Fusarium solani*, *F. sambucinum*, *F. avenaceum*, *F. graminearum*, and *F. oxysporum*, and causes post-harvest tuber rot and seed piece decay after planting. This disease can reduce crop establishment by affecting the developing potato sprouts and causing crop losses estimated to be up to 25%, while more than 60% of tubers can be infected during storage (Singha *et al*., 2016). These pathogens are soil borne and survive as resistant spores in soil or within decayed plant tissues. Small, brown lesions appear at wound sites 3–4 weeks after harvest and continue to enlarge during storage (Hooker, 2001). In 2004–2005, a survey of potatoes from stores in the north-central potato-producing region of the USA showed that *F. graminearum* and *F. sambucinum* were the important causes of the disease (Alison *et al.*, 2005; Gashgari and Gherbawy, 2013). Moreover, *F. graminearum*, *F. solani*, *F. oxysporum*, and *F. sambucinum* were the most predominant causative agents of FDR in Tunisia (Aydin *et al*., 2016; Al-Mughrabi, 2010). In 1997 and 2000 in Scotland, *F. avenaceum* was the most predominant cause of FDR (Choiseul *et al*., 2007), while *F. solani* var. *coeruleum* dominated in four regions of Great Britain, including eastern Scotland, in 2000–2002 (Aktaruzzaman *et al*., 2014).

There is no literature concerning FDR in Thailand and study the severity of potato dry rot disease. Therefore, the present study aims to characterize morphologically and molecularly by polymerase chain reaction-internal transcribed spacer (ITS) *Fusarium* spp. isolates recovered from infected potato tuber and assess their pathogenicity.

Materials and Methods

Fungal isolates and pathogen determination

Pathogen isolates were obtained from potato tuber cv. Atlantic collected from Chai Prakan District in Chiang Mai Province and Phop Phra District in Tak Province in 2017-2018. Infected tuber samples were cut (ca. 5 × 5 mm) with a sterile blade and sterilized by dipping in 10% (w/v) sodium hypochlorite solution (NaOCl) for 1 min and washed two times with sterile water. Next, the potato pieces were placed on the surface of potato dextrose agar medium (PDA) and incubated at 28 ℃ for 4 days (Booth, 1971).

Potato tubers of cv. Spunta were surface sterilized with 70% ethanol and air-dried overnight. Potato tubers were inoculated with a 0.5 cm disc of

mycelium of *Fusarium* isolates grown on PDA for 5 days. The control consisted of a PDA disc on potato tubers. The fungal pathogens were re-isolated from the disease lesions of the inoculated tubers and the re-isolated pathogens exhibited the same morphological characteristics as those of the original isolates (Booth, 1971).

Morphological identification

Cultural characteristics of each *Fusarium* isolate were then determined after 10 days on PDA, acidified potato dextrose agar (APDA), yeast malt agar (YMA) and synthetic nutrient deficient agar (SNA). Microscopic features of the size and shape of conidia, chlamydospores, and colony pigment according to published descriptions were determined. Fifty macroconidia were observed randomly, and the width and length were measured (Alison *et al.*, 2005; Singha *et al*., 2016; Gardes and Bruns, 1993).

Molecular identification based on ITS

Fusarium isolates were identified by sequencing of the ITS region of the nuclear ribosomal DNA (rDNA). The ITS gene was amplified by PCR with the primers ITS1 (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (Gardes and Bruns, 1993; Estrada *et al*., 2010). PCR amplifications were performed in a final volume of 50 µl by mixing 2 µl of DNA with 1.6 µM of each primer, 25 µl of PCR Master Mix and 22.2 µl of sterile water. Amplification was conducted in a thermal cycler with an initial denaturation of 3 min at 94 ℃, followed by 35 cycles of 1 min at 94 ℃, 1 min at 58 ℃, 1 min at 72 ℃, and a final extension of 10 min at 72 ℃. Aliquots of PCR products were checked by electrophoresis on a 1.5% agarose gel revealed with RED Taq® DNA Polymerase Taq for routine PCR with inert dye. The ITS nucleotide sequences for each isolate were compared to those in the public domain databases of National Center for Biotechnology Information (NCBI) (www.ncbi.nih.gov) using Basic Local Alignment Search Tool for Nucleotide sequences. Alignment of *Fusarium* sequences was done using the Clustal W program and confidence of the branching was estimated by bootstrap analysis (Gardes and Bruns, 1993).

Effect of wounding on infection by Fusarium isolates in potato tubers

In order to establish pathogenicity of the *Fusarium* isolates four inoculation methods were assessed for two *Fusarium* isolates, FCP01 and

FPP03. The inoculated tubers (five tubers per treatment) were placed in plastic bags to maintain a high humidity and then incubated for 3 weeks at room temperature (28 ±2 ℃).

Non-wounded: Tubers were inoculated by placing a 5-mm mycelial plug from 5-day-old actively growing *Fusarium* cultures directly onto potato tubers (non-wounded).

Bruise injury: Bruising was created using a mortar smash for black spot bruise testing of potato cultivars using previously described methods (Estrada *et al*., 2010). A 5-mm-diameter mycelial plug from a 5-day-old actively growing *Fusarium* culture was placed directly on the bruised area.

Skinning injury: A peridermal area of tubers approximately 10 × 10 mm was removed using a plastic scrub. Next, *Fusarium* isolates were inoculated by placing a 5-mm mycelial plug from 5-day-old actively growing *Fusarium* cultures directly onto the abraded area.

Plug injury: Potato tubers were inoculated by removing a plug of tissue 5 mm in diameter by 5 mm deep using a sterile cork borer, and replacing it with a 5-mm-diameter mycelial plug from a 5-day-old actively growing *Fusarium* culture.

After the incubation period, tubers were cut along the longitudinal axis across the inoculation sites. For disease assessment, symptomatic lesions were measured both externally and internally. Disease severity was calculated following previously described methods (Boutheina *et al*., 2015).

Statistical analyses

A factorial analysis of variance (One-Way ANOVA) was performed to determine the significance of the main factors and their interactions using a completely randomized factorial design with two factors i.e., fungal isolates and method of inoculation. Mean separations were performed by a completely randomized design (at $P < 0.05$) and the data were subjected to statistical analysis using the Least Significant Different (LSD).

Results

Fungal isolates and pathogen determination

The fungal pathogens were isolated from potato cv. Atlantic in Chiang Mai and Tak, Thailand in 2017-2018, with isolates FCP01, FCP02 and FCP03 isolated from Chai Prakan District in Chiang Mai Province and isolates FPP01, FPP02, FPP03 and FPP04 isolated from Phop Phra District in Tak Province (Table 1). These isolates were isolated from potato tubers

exhibiting large lesions and internal light to dark brown or black rot. According to the pathogenic test done on potato tuber, 2 out of 7 isolates of *Fusarium* spp. were found to be pathogenic and the others were weakly or not pathogenic. The FCP01 and FPP03 isolates were tested pathogenicity on potato tubers, three weeks after inoculation with FCP01, potato tubers showed large lesions around the wound site, and internal symptoms characterized by necrotic areas. Pathogenicity tests proved that these *Fusarium* isolates caused FDR. FPP03 was generally less aggressive in causing dry rot disease than FCP01.

Morphological identification

Fusarium isolates FCP01 and FPP03 were identified from FDR tubers based on the morphology of macroconidia, microconidia, and chlamydospores. FCP01 possessed a distinctive banana-shaped macroconidium. These conidia contained multiple septa and often had a foot cell. Aerial mycelium of FCP01 was variable in color and texture: pink, starting out as light orange, or orange on PDA (Figure 1). The size of macroconidia was 23.5-30.9 x 5.6-14.5 μm and FCP01 on PDA, and it demonstrated formation of chlamydospores at 25 ^{o}C. The macroconidia produced by FPP03 were slightly curved, hyaline, often aggregating in fascicles, with a size of 12.5-25.6 x 2.6-6.7 μm. Colonies of FPP03 were faster growing than FCP01, variable in color and texture, purple, or lavender, but may have started out as white on PDA (Figure 1) and FPP03 showed low chlamydospore formation on PDA under prolonged 25 ^{o}C after a 10-day incubation. The size of macroconidia on APDA of isolate FCP01 was 21.0-29.2 x 6.2-11.8 μm and FPP03 was 12.4-24.5 x 3.5-4.2 μm. The size of macroconidia on YMA of FCP01 was 18.6-30.6 x 5.4-12.8 μm and FPP03 was 15.3-20.7 x 3.8-6.7 μm. The size of macroconidia on SNA of FCP01 was 19.0-28.2 x 5.1-12.7 μm and FPP03 was 19.7-31.8 x 3.3-4.7 μm. Colonies of FCP01 showed pale yellow pigmentation of the culture medium and FPP03 showed purple pigmentation on APDA, but showed white pigmentation on SNA and YMA (Figure 1).

Table 1. Isolates of *Fusarium* species isolated from potato tubers collected from potato-growing areas in northern, Thailand showing dry rot symptoms

Province origin	No. of tested isolates	Isolate	Pathogenic
Chai Prakan District, Chiang Mai Province	3	FCP01, FCP02, FCP03	FCP01
Phop Phra District, Tak Province	4	FPP01, FPP02, FPP03, FPP04	FPP03

Molecular identification based on ITS

The PCR results confirmed that isolates FCP01 and FPP03 from potato tubers were *Fusarium* species consistent with the morphological identification. About 522 bp fragments were amplified from the genomic DNA of the *Fusarium* species isolates. *Fusarium* sequences obtained in table 2 from amplification of the conserved ribosomal ITS region were compared with sequences from the NCBI database using BLAST 2.0 (https://blast.ncbi.nlm.nih.gov /Blast.cgi). These sequences were identified and deposited in NCBI Genbank; FCP01 clustered close to *F. graminearum* and FPP03 clustered close to *F. solani*. However, both species had significant subleasing (Figure 2).

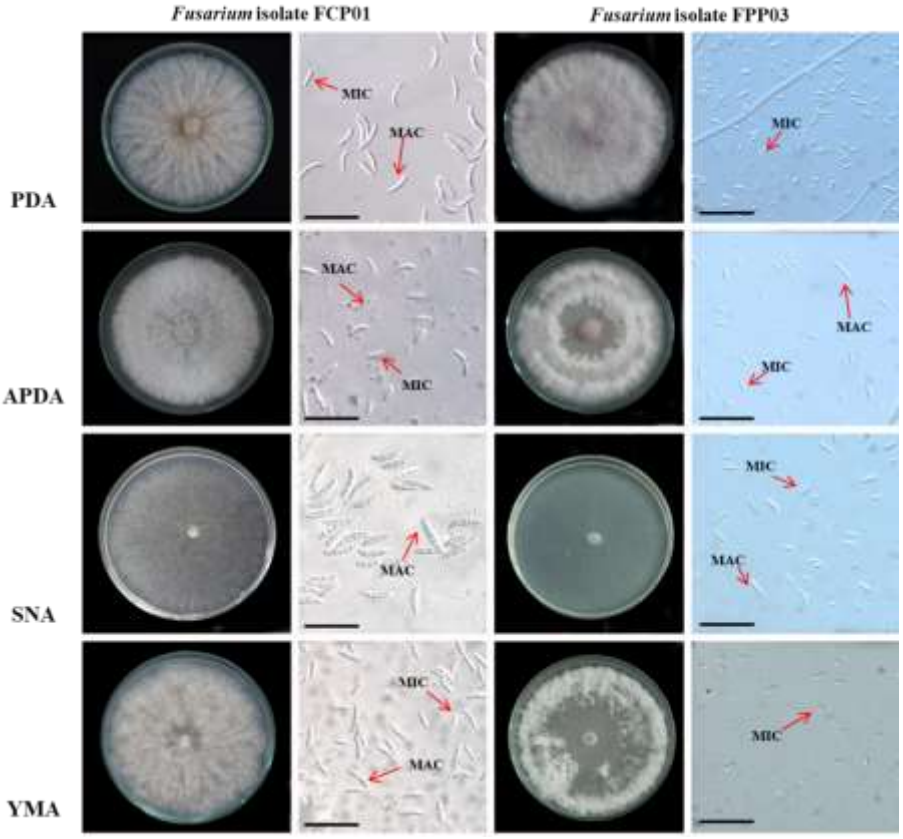

Figure 1. The fungal colonies and macroconidia of *Fusarium* isolates FCP01 and FPP03 grown on potato dextrose agar (PDA), acidified potato dextrose agar (APDA), yeast malt agar (YMA), synthetic nutrient deficient agar (SNA) incubated at 25 ^{o}C for 10 days. Bar = 20 µm. MAC = Macroconidia, MIC = Microconidia

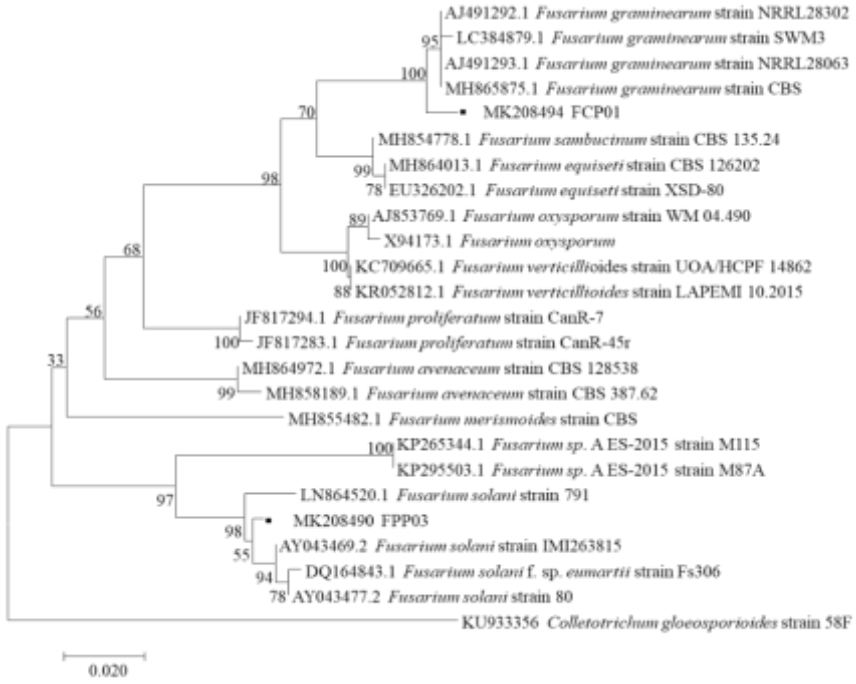

Figure 2. Neighbor-joining phylogenetic trees of *Fusarium* isolates FCP01 and FPP03 and related species were identified from GenBank based on ITS gene sequences. Numbers at the nodes indicate bootstrap values from a test of 1,000 replications. The scale bar indicates the number of nucleotide substitutions. Evolutionary analyses were conducted by using the MEGA7 program

Effect of wounding on infection by Fusarium isolates in potato tubers

Both *Fusarium* isolates were pathogenic to potato tuber cv. Spunta. Significant differences in disease severity were observed among isolates in the four treatments. In treatment 3 skin injured tubers inoculated with *Fusarium* isolates FCP01 and FPP03 developed lesions which were 4.750 (LA) mm, and 4.050 mm (LA), respectively. In treatment 4 plug-injured tubers inoculated with *Fusarium* isolates FCP01 and FPP03, lesion sizes were 15.025 mm (HA) and 5.350 mm (LA), respectively. Significant differences among the main effects of tuber injury ($P<0.05$) and between *Fusarium* isolates ($P<0.05$) were also observed. Tuber injury treatment (none, skinning, bruising or plugging) significantly affected the disease severity caused by both *Fusarium* isolates (FCP01 and FPP03). A significant difference was observed in disease severity between *Fusarium* isolates FCP01 and FPP03 in treatment 4, but treatments 1, 2 and 3 showed no significant difference (Figure 3, 4).

Table 2. GenBank accessions nos. of ITS sequences used in the phylogenetic analysis

No.	ITS identification	Host	Origin	Strain	GenBank Accession No.
1	*F. graminearum*	Potato	Thailand	FCP01	MK208494
2	*F. graminearum*	Potato	Thailand	FPP03	MK208490
3	*F. graminearum*	Wheat head	Japan	NRRL28302	AJ491292
4	*F. graminearum*	Corn Seed	Iraq	SWM3	LC384879
5	*F. graminearum*	Maize	USA	NRRL28063	AJ491293
6	*F. graminearum*		Netherlands	CBS	MH865875
7	*F. sambucinum*		Netherlands	CBS135	MH854778
8	*F. equiseti*		Netherlands	CBS126202	MH864013
9	*F. equiseti*		China	XSD-80	EU326202
10	*F. oxysporum*		Australia	WM04	AJ853769
11	*F. oxysporum*	Elegans	Netherlands		X94173
12	*F. verticillioides*		Greece	UAO/HCPF14862	KC709665
13	*F. verticillioides*		Brazil	LAPEMI10.2015	KR052812
14	*F. proliferatum*	Oilseed	China	CanR-7	JF817294
15	*F. proliferatum*	Oilseed	China	CanR-45r	JF817283
16	*F. avenaceum*		Netherlands	CBS128538	MH864972
17	*F. avenaceum*		Netherlands	CBS387.62	MH858189
18	*F. merismoides*		Netherlands	CBS	MH855482
19	*Fusarium* sp.	Potato	Poland	M115	KP265344
20	*Fusarium* sp.	Potato	Poland	M87A	KP295503
21	*F. solani*		Pakistan	791	LN864520
22	*F. solani*	Potato	Brazil	IMI263815	AY043469
23	*F. solani* f.sp. *eumartii*	Potato	California	Fs306	DQ164843
24	*F. solani*	Potato	Brazil	80	AY043477
25	*C. gloeospoioides*	Carambola	lndia	58F	KU933356

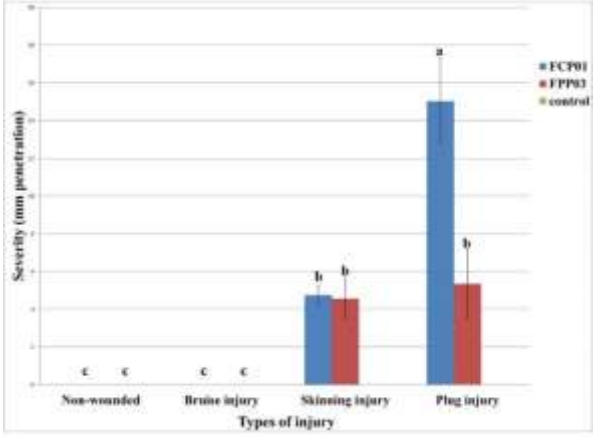

Figure 3. Disease severity of dry rot in potato tubers inoculated with either *Fusarium* isolates FCP01 or FPP03 and subjected to tree types of injury

Figure 4. Effect of wounding on infection by *Fusarium* isolates in potato tubers cv. Spunta. After the incubation period, tubers were cut along the longitudinal axis across the inoculation sites. (A) non-wounded, (B) bruise injury, (C) skinning injury, (D) plugging injury

Discussion

This was the first study of the FDR disease in northern, Thailand, and reports on *F. graminearum* novel isolate FCP01 of potato tuber rot and a highly aggressive pathogen. An isolate of *F. solani* was also demonstrated to cause FDR but a key issue is tuber damage; dry rot would not develop without an initial wound. The *F. graminearum* grown on PDA produced light orange aerial mycelia with pale yellow pigments and white fluffy mycelium on APDA discernible by Alison *et al*. (2005). SNA was also found to be favorable for the growth of the *Fusarium* isolate as also seen previously (Steven, 2005). The macroconidia ranged from 18.5-30.6 x 5.4-12.8 µm in size and the slender macroconidia typically possessed 3-5 cells demarcated by septa and two apical cells possessing obvious asymmetry, as each one gradually tapered toward a rounded end as also previously reported of Steven (2005). The *F. solani* produced yellowish purple aerial mycelia with purple pigments. This isolate's macroconidia ranged from 12.4–25.5 x 2.6–6.5 µm and the number of septa in macroconidia and microconidia were 3-5 and 0-1, respectively, similar to that previously reported (Padvi *et al*., 2018; Islam and Datta, 2017). The *F. graminearum* isolate was significantly more pathogenic than the other isolate that belonged to these species (Stefańczyk *et al*., 2016). However, a similar lesion size was observed to

that caused by *Fusarium* species causing dry rot in China (Du *et al*., 2012). *Fusarium* species were unable to infect non-wounded potato tubers. This corresponds to the previous report of the potato disease (Singha *et al*., 2016) indicating that progeny tubers were not usually infected until harvest because dry rot pathogens were unable to cause infection unless they penetrated the skin, and the potato skin was rarely injured during the growing season. Growth cracks, however, provide an entry-point for infection by *Fusarium* species. Significant differences in disease severity were observed between species in the four wound-inoculated treatments. In treatment 3, *Fusarium* isolates FCP01 and FPP03 inoculated into the abraded area of potato tubers infected and caused small lesions. In treatment 4, *F. graminearum* isolate FCP01 inoculated into plug-injured potato tubers produced large lesions whereas *F. solani* isolate FPP03 produced small lesions like those occurring in the abraded treatment. These results support the highly dynamic nature of different *Fusarium* species as the causal agents of potato dry rot; *F. graminearum* were highly pathogenic species and also the most dominant species in this survey, suggesting that the predominant distribution of *Fusarium* species is likely associated with its pathogenicity (Du *et al*., 2012). These result is in contrast to Gachango *et al.* (2012) pathogenicity of *Fusarium* isolates obtained from Michigan potato seed pieces with dry rot symptoms and inoculated onto seed potato tubers to founded *F. graminearum* were non virulence. Further work should include mycotoxin biosynthesis, factor of pathogenicity and genetic comparisons between *F. graminearum* and *F. solani*. Moreover, rebuilding the plant microbiome both with endophytes and rhizosphere microorganisms by using beneficial microbes when available has proven to improve the plantlets performance against *Fusarium* spp. in the field.

A novel isolate of *F. graminearum* from a diseased potato tuber was found to be the causative agent of FDR, and represents a new record in Thailand. Isolates of *Fusarium* spp. isolated from potato tubers with dry rot were tested for aggressiveness to potato tubers. The disease severity was highly variable among the 7 isolates, this is the first work that shows that *F. graminearum* most aggressive agent causing potato dry rot disease in Thailand. An isolate of *F. solani* was also demonstrated to cause FDR.

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