First report of *Fusarium oxysporum* f.sp. *niveum* in the Lao PDR

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Abstract *Fusarium oxysporum* f.sp. *niveum* is reported for the first time in the Lao PDR. It was isolated from watermelons (*Citrullus lanatus*) in Songkhon district, Savannakhet province following a limited ad hoc survey during January and February of 2015. Infected plants showed symptoms of wilt, vascular discolouration, necrosis in the collar region, lower stem base and upper taproot regions, and whole plant death. Identification of the pathogen was confirmed through phylogenetic analysis of the *EF1-α* locus and a pathogenicity test satisfying Koch's postulates.

Keywords *Fusarium* wilt · *Citrullus lanatus* · Watermelon

Fusarium oxysporum f.sp. *niveum* (Fon) is the most economically significant pathogen of watermelons worldwide. Fon is host specific on watermelon and identification relies on

a polyphasic approach that typically includes morphological and molecular characteristics, but most importantly, pathogenicity trials to satisfy Koch's postulates (Martyn 2014).

Fusarium oxysporum f. sp. *niveum* colonises the root cortex of watermelon plants and moves into the xylem resulting in an initial loss of turgor pressure, wilting and whole plant death. The pathogen can survive in soil for several years as chlamydospores (Martyn 2014), or through the colonisation of non-host plants (Hendrix and Nielson 1958). If watermelons are grown in the same field over successive seasons, soil inoculum will likely increase, resulting in potential yield losses of up to 100 %. The long-term survival of chlamydospores and their formation in non-host plants is a major factor contributing to the difficulty of managing this pathogen.

A limited ad hoc survey of watermelon farms in Savannakhet province in southern Lao PDR was undertaken during January and February 2015, to investigate the cause of reported wilting, plant death and extensive yield loss. In the southern provinces, watermelons are grown during the “cool dry” period (November–February). At the farms surveyed, watermelons were either grown in pits (approximately 30 cm diameter, 20 cm deep) or on raised beds. Soils tended to be compacted with a high clay content. Watermelons were sown in dormant rice paddies that are normally flooded from late June to late August each year during the wet season when rice is grown. In some cases, watermelons had been cropped in the same field for up to seven seasons, with some fields having up to 90 % or greater yield loss. Irrigation water was sourced from small farm dams and pumped through hand-held hoses in most fields surveyed. It is likely that some water overflows from infested paddies and drains into the farm dams.

Diseased watermelon plants showed typical wilt symptoms and vascular discolouration, especially in the collar region,

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lower stem base and upper taproot (Fig. 1b). Necrosis accompanied vascular discolouration in some diseased plants. Wilt occurred suddenly and was quickly followed by plant death (Fig. 1a). Fruit distortion was also observed in some vines that collapsed gradually. In some cases, white fluffy mycelia could be seen growing within the pith cavity in the collar region of severely infected plants. These symptoms and signs led to the putative identification of *Fon* as the causal agent of the disease.

Stem base samples from symptomatic plants were collected and taken for isolation and identification of the pathogen. Stem sections ~2 cm in length were gently scraped to remove the outer layer of tissue, then washed in sterile water, immersed briefly (5 s) in 70 % ethyl alcohol (ETOH), rinsed immediately in sterile water and patted damp-dry on sterile tissue. Transverse oblique sections of the stem (1–2 mm thick) were aseptically cut from the border of diseased and symptomless tissue. The pieces were pressed firmly, cut-side down onto quarter strength potato sucrose agar (1/4 PSA) amended with 0.2 g/L penicillin and 1 g/L streptomycin sulfate. Plates were incubated at room temperature under a 12 : 12 h light : dark cycle for 2 days before preliminary morphological identification took place. Putative colonies of *F. oxysporum* that emerged from the stem segments were subcultured onto 1/4 PSA and then purified by single sporing onto carnation leaf agar (CLA) (Burgess et al. 2008). The pure cultures were identified putatively as *F. oxysporum* on the basis of morphological characteristics on CLA. The macroconidia were straight to slightly curved, medium length, septate (predominantly 3) (Fig. 2a) and formed in sporodochia on carnation leaf pieces. The micronidia formed on short monophialides in false-heads, and had no septation and were predominantly elliptical in shape (Fig. 2c, d). Typical chlamydospores (Fig. 2b) were also present.

Two pure cultures were deposited in the International Collection of Microorganisms from Plants (ICMP),

Landcare Research, Auckland, New Zealand (<https://scd.landcareresearch.co.nz/>) for accession. The two cultures, accessioned as ICMP 20835 and ICMP 20827, were from two farms at Ban Nong Hai (16°15'31.79"N 105°10'15.82"E) and Ban Nong Khan Yuu (16° 8'12.45"N 105°20'11.72"E) respectively, in the Songkhon district, Savannakhet province.

Genomic DNA extraction required 100 mg of mycelium from each isolate dried in a SpeedVac concentrator for 2 h at 40 °C (Thermo Scientific, USA) and ground to a fine powder using a TissueLyser (Qiagen, USA) with a 2 mm diameter stainless steel ball at a speed of 25Hz for 30 s. DNA was then extracted from powder using a DNeasy Plant Mini kit and QIAcube, robotic workstation (Qiagen, USA).

The translation elongation factor 1 α (*EF1- α*) locus was amplified using primers EF1 and EF2 with conditions described in Carbone and Kohn (1999). Resulting PCR amplicons were sequenced using an ABI PRISM_3700DNA Analyser (Applied Biosystems Inc., Foster City, California, USA). Sequences were subject to phylogenetic analysis with reference *F. oxysporum* ff. spp. obtained from GenBank. Parsimony analysis was conducted in PAUP 4.0b10 (Swofford 2002) using the heuristic search option with 1000 random addition sequences and tree bisection reconnection branch swapping. Gaps were treated as missing data. The Consistency Index (CI) and Retention Index (RI) were calculated to indicate the amount of homoplasy present. Clade stability was assessed in PAUP 4.0b10 using 1000 heuristic search bootstrap replications with random sequence addition (MPBS). Bayesian inference was used to generate posterior probabilities (PP) using MRBAYES (Version 2.0.3) plug-in (Huelsenbeck 2001) in the software Geneious (Version 5.3.6) (Drummond AJ et al. 2011). The Monte Carlo Markov Chain was run with 1,000,000 generations using the appropriate substitution evolution model determined by jModelTest (Posada 2008). The *EF1- α* dataset consisted of 51 taxa with 656 nucleotides, of which 83 were parsimony

Fig. 1 **a** Watermelon with typical wilt symptoms and dead plant, in pit production system. **b** Necrotic lesion on stem of wilted watermelon together with vascular discolouration. **c** Pathogenicity trial of isolates of *F. oxysporum* negative control (left); inoculated watermelons (right)

Fig. 2 Morphological characteristics of cultures of *F. oxysporum* isolated from wilted watermelon. **a** Macroconidia. **b** Chlamydospore. **c** False heads of microconidia formed on short monophialides. **d** Microconidia

informative. The phylogenetic analysis resulted in 76 most parsimonious trees (CI=0.9, RI=0.9). The isolates ICMP 20827 and ICMP 20835 are clustered with members of *F. oxysporum* ff.spp. in a highly supported clade (MBPS, PP), identifying these isolates as *F. oxysporum* (Fig. 3). Although other *Fon* isolates were used in the analysis, isolates ICMP 20827 and 20835 did not cluster with any of the reference *Fon*, however, it has been shown (Laurence et al. 2014) that *F. oxysporum* ff.spp. are polyphyletic and pathogenicity trials were conducted to identify these isolates definitively.

Isolates were initially tested for pathogenicity involving a root dip inoculation technique (Freeman and Rodriguez 1993). Early wilting symptoms appeared in both of the inoculated treatments within 3 days and all of the seedlings had died by day 9, apart from those in the negative control. The stem base region of wilted seedlings was plated out onto 1/4 PSA and *F. oxysporum* was re-isolated.

A more rigorous test was then performed using a soil inoculation method (Dau et al. 2008). A medium consisting of rice hulls and millet seed (1:2 by volume) described by Burgess et al. (2008) was sterilized twice in four 500 ml screw-cap bottles. Two of the bottles were inoculated with ICMP 20827, and the other two bottles with ICMP 20835 using 15 1x1 cm mycelial plugs, per bottle. The bottles were incubated for 2 weeks at room temperature (~25°C), and shaken intermittently to encourage colonisation of the substrate. Following this, 1 bottle of ICMP 20835 and 1 bottle of ICMP 20827 was sterilised in the autoclave for 30 min for use as controls. The four mixtures were air dried for 2 days prior to mixing thoroughly in field soil at 3 %/vol. to give four treatments.

Five replicate pots (10-cm-diameter) each with five seeds (cv. Arree) were used for each of the four treatments (20 pots in all). The pots were arranged randomly on a table and grown outdoors in natural light. The pots were watered daily to

maintain approximate field capacity. The daily air temperature varied from 24 to 34 °C. Typical wilt symptoms developed in at least one seedling in each of the 10 inoculated pots by day 11. All of the seedlings inoculated by ICMP 20827 had wilted and died by day 14 and all of those inoculated by ICMP 20835 had wilted and died by day 18. The control seedlings did not develop wilt symptoms. *F. oxysporum* was re-isolated from the inoculated seedlings, thus fulfilling Koch's postulates, identifying the pathogen as *F. oxysporum* f.sp. *niveum* (Fig. 1c).

The incidence of Fusarium wilt on watermelons grown in the rice paddy soils suggests that *Fon* can survive flooding for 3 months during the wet season each year, as chlamydospores or perhaps through the colonisation of non-host plants (Hendrix and Nielson 1958), such as rice (*Oryza sativa*). Further studies on the mode of survival in these farming systems are needed as a basis for refining integrated disease management (IDM) strategies.

As most of the surveyed farms grew watermelons in the same field every year, crop rotation is an important recommendation in minimising the occurrence of *Fon* in southern Lao PDR. Given the recommended rotation cycle is 5–7 years (Martyn 2014) this management strategy may not be feasible in some small-holder farms where the watermelons must be grown in proximity to the farm dam. Some watermelon farmers in this area are relocating their watermelon production to other areas of their farm where water is also available following our advice. Grafting watermelon onto a resistant rootstock of some other members of the Cucurbitaceae is an area worthy of investigation. It will be necessary to test grafted seedlings in infested paddies both in the 'pit' method of production and on raised beds. A potential issue, particularly for the pit method of growth, is that the graft junction may be inundated with water (suspension of infested soil) during

◀ **Fig. 3** One of 76 most-parsimonious trees inferred from the *EF1- α* dataset, indicating the relationship of isolates ICMP 20827 and ICMP 20835 (*highlighted*) with other *F. oxysporum* f.sp. *niveum* and other formae speciales. Tip labels indicate GenBank accession numbers and species name. Branches with Bootstrap intervals (1000 replications) $\geq 70\%$ and Bayesian posterior probabilities > 0.9 are indicated in bold

watering. This may favour infection of the watermelon scion at the graft junction. Additionally, increased production costs and concerns over potential alterations to the horticultural traits of the plants may act as disincentives as they do elsewhere in the world (Martyn 2014; Lü et al. 2014). Further surveys and trials will be undertaken in order to determine the race(s) of *Fon* present in southern Lao PDR. This information will facilitate the choice of resistant varieties as an economically viable and technically feasible IDM strategy for managing *Fon* in this region of the Lao PDR.

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