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Short communication

Impaired purine biosynthesis affects pathogenicity of *Fusarium oxysporum* f. sp. *melonis*

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Abstract

The vascular wilt pathogen *Fusarium oxysporum* f. sp. *melonis* causes worldwide yield losses of muskmelon. In this study, we characterized a UV-induced non-pathogenic mutant (strain 4/4) of *F. oxysporum* f. sp. *melonis*, previously identified as a potential biological control agent. During comparative analysis of vegetative growth parameters using different carbon sources, mutant strain 4/4 showed a delay in development and secretion of extracellular enzymes, compared to the wild type strain. Amendments of the growth medium with yeast extract, adenine or hypoxanthine, but not guanine, complemented the growth defect of strain 4/4, as well as secretion and partial activity of cellulases and endopolygalacturonases, indicating that the strain is an adenine auxotroph. Incubation of strain 4/4 conidia in adenine solution, prior to inoculation of muskmelon plants, partially restored pathogenicity to the mutant strain.

Fungi belonging to the species *Fusarium oxysporum* are distributed worldwide in soil and organic substrates. The soilborne wilt fungus *F. oxysporum* f. sp. *melonis* (FOM), containing four specialized races 0, 1, 2 and 1,2, causes vascular wilt disease of melon (*Cucumis melo* L.), affecting specific host cuitivars; race 1,2 is the most virulent affecting most cultivars to date (Cohen et al., 1989). One way of identifying pathogenicity factors during pathogen–plant interactions is by analyzing non-pathogenic mutants (Ba1hadère et al., 1999). Freeman et al. (2002) first characterized strain 4/4, which was derived by UV-mutagenesis from FOM, race 1,2, and characterized it as non-pathogenic on melon and watermelon plants. The mutant was capable of penetrating and colonizing stem vessels of the host plants, but did not cause vascular wilt disease symptoms. According to these results and based on the strain characteristics and its ability to confer cross protection they proposed using the 4/4 mutant as a biological control agent.

During vascular wilt disease development, *F. oxysporum* produces a wide array of extracellular hydrolytic enzymes (Di Pietro and Roncero, 1996a, b, c). Even though it was demonstrated by disruption of functional genes that they are not important for pathogenicity, it was proposed that other factors might compensate activity of the disrupted target (Di Pietro and Roncero, 1998). This is likely to reflect functional redundancy because such genes might be members of large gene families. Therefore, initially it was assumed that mutant strain 4/4 might possibly be lacking part of the extracellular enzyme array. The purpose of this study was to determine the nature of impaired pathogenicity of strain 4/4 of FOM on melon, with emphasis on characterization of its ability to

secrete extracellular enzymes, such as cellulases and endopolygalacturonase. This mutant was further used in an attempt to better understand the underlying pathogenicity factor involved in Fusarium wilt of melon seedlings on the one hand and implications for biocontrol on the other.

Biomass accumulation, vegetative growth rate and conidiation of wild type and mutant isolates were not significantly different when cultured on rich media, such as liquid FLC (Freeman et al., 2002) or solid PDA. When the same development parameters of mutant strain 4/4 were examined on synthetic medium (SM) (Di Pietro and Roncero, 1996a), containing 1% of carboxymetylcellulose (CMC), pectin, sodium polygalacturonate (NaPG) or polygalacturonic acid (PGA) (Sigma, St. Louis, MO) as sole carbon sources, a delay in all the tested parameters was observed. Extracellular protein production of mutant strain 4/4 on SM medium supplemented with different sole carbon sources was reduced (to approx. 30 μg ml^{-1} and 19 μg ml^{-1} on media containing CMC and pectin, respectively) in comparison to the wild type strain 1,2 (300 and 485 μg ml^{-1} on the same media respectively). The two predominant extracellular proteins secreted, when the wild type strain 1,2 was cultured in the presence of CMC or GA (Figure 1) were partially purified and identified (using Q2-TOF mass spectrometer) as exocellobiohydralase (54 kD) (Figure 1a) and endopolygalacturonase (endoPG) (38 kD) (Figure 1b), respectively. Although these enzymes were not detected in mutant strain 4/4 culture filtrate by SDS-PAGE, low levels of cellulase activity were detected by the Nelson (1944) and Somogyi (1945) methods. Enzymatic activity was expressed in nanokatals (nkat) – the amount of enzyme that released 1 nmol of monomer equivalent per second under standard conditions. Specific cellulase activity in protein purified from CMC amended medium (Eshel et al., 2000) was 1.38 ± 0.20 and 0.46 ± 0.06 nkat ml^{-1} protein for strains 1,2 and 4/4, respectively. Specific endoPG activity, purified from pectin or NaPG containing medium (Prusky et al., 1989), was 8.49 ± 3.49, 1.73 ± 0.52 and 0.93 ± 0.26, 0.12 ± 0.02 nkat mg^{-1} protein for strains 1,2 and 4/4, respectively, which was significantly different when analyzed by Student's test ($\alpha = 0.05$).

In order to examine the possibility that a mutation leading to nutritional defects might be the cause of differential development of strain 4/4 under the tested conditions, yeast extract was used to supplement the growth medium. Growth of mutant strain 4/4 increased to rates similar to that of the wild type strain 1,2 when minimal medium (FMM) (Corell et al., 1987) was supplemented with different carbon sources and yeast extract. Likewise, addition of yeast extract to SM, supplemented with CMC or D-galacturonic acid (GA) as the sole carbon source, resulted in increased extracellular enzyme production by the mutant strain 4/4. Partial sequence of the two predominant proteins, produced under these conditions

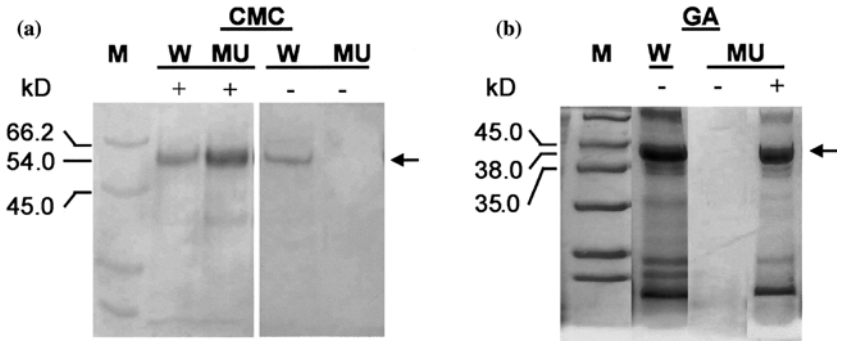

Figure 1. Effect of yeast extract on extracellular enzyme production by wild type, 1,2 (W) and mutant 4/4 (MU) strains of *Fusarium oxysporum* f. sp. *melonis* (FOM). Extracellular enzyme patterns from culture filtrates of the FOM isolates, grown on synthetic medium supplemented with 1% of carboxymethylcellulose – CMC (a) or galacturonic acid – GA (b) with (+) and without (-) yeast extract. Five mg of protein was separated on 12 % SDS-PAGE and stained with Coomassie blue. In the presence of yeast extract, both W or MU secreted proteins with molecular weights of 54 kD in (a) or 38 kD in (b) (marked with arrows), which were sequenced and identified as cellobiohydrolase (a) and endopolygalacturonase (b). M represents protein weight markers in kilo Dalton (kD).

identified them as exocellulase (Figure 1a) and endoPG (Figure 1b), identical to those secreted by wild type strain 1,2. Specific activity of total cellulases in the mutant increased to similar levels of that of the wild type strain 1,2 (1.10 ± 0.08 and 1.03 ± 0.13 nkat mg^{-1} protein for strains 1,2 and 4/4, respectively). Specific activity of endoPG, purified from pectin or NaPG containing medium, of mutant strain 4/4 increased significantly – to 23.06 ± 2.67 and 0.81 ± 0.10 nkat mg^{-1} protein, respectively, when supplemented with yeast extract.

Various combinations of amendments such as amino acids, vitamins and purines or pyrimidines were added to FMM at a final concentration of 100 μg ml^{-1} to determine whether strain 4/4 behaved as an auxotrophic mutant. Growth was partially restored by complementation with two of the three tested purines: adenine, hypoxanthine, but not guanine. When different concentrations of adenine or hypoxanthine (0.01–5 mg ml^{-1}) were tested, no difference in vegetative growth rate was found between strains 4/4 and 1,2. In order to determine the effect of nutritional supplements on growth of mutant strain 4/4 *in planta*, pathogenicity tests were performed. Addition of adenine, but not yeast extract, partially restored pathogenicity of mutant strain 4/4 on melon plants as determined by plant inoculation experiments conducted in glass tubes (Figure 2). Pathogenicity was restored only after pre-incubation of the conidial suspension in the adenine solution for 24 h at 25 °C prior to seedling inoculation, which is in agreement with results described by Namiki et al. (2001). The effect of adenine on pathogenicity was concentration dependent indicating that the mutant is most likely an adenine auxotroph.

The studies of non-pathogenic mutant fungal strains are valuable for progressing our understanding of factors involved in pathogenicity. Furthermore, potential uses of non-pathogenic strains, including *Fusarium* spp., in cross protection phenomena have been demonstrated (Huertas-Gonzalez et al., 1999a). Earlier studies demonstrated that disruption of single genes encoding extracellular enzymes did not affect pathogenicity of *F. oxysporum* (Huertas-Gonzalez et al., 1999b). However, the effect of multiple extracellular encoding gene disruptions in *F. oxysporum* has not yet been analyzed. The general

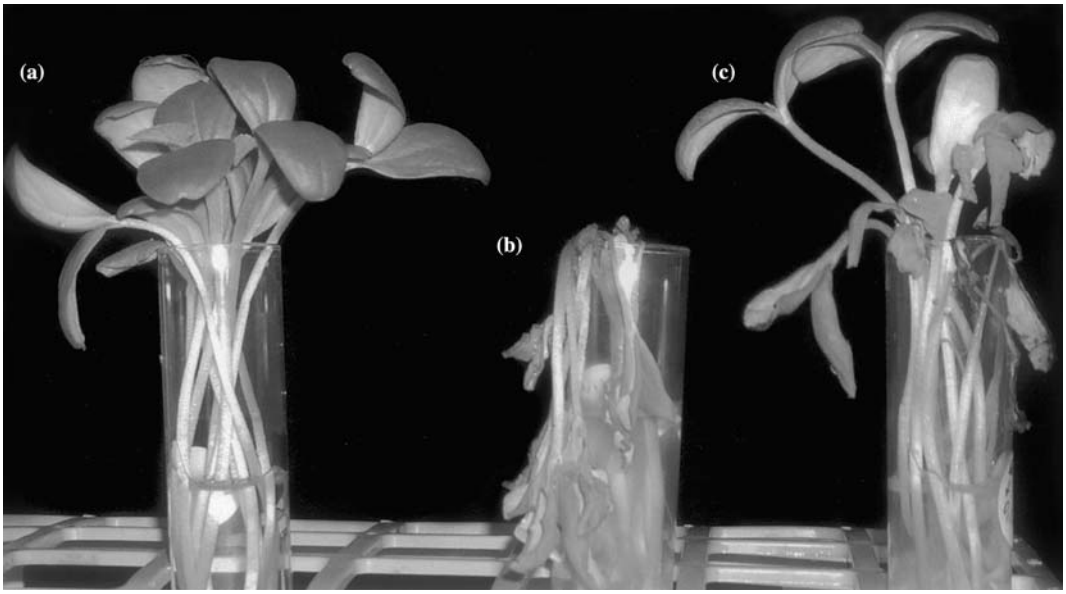

Figure 2. Effect of adenine on pathogenicity of mutant strain 4/4 of *Fusarium oxysporum* f. sp. *melonis*, three days post-inoculation. Melon seedlings were inoculated with (a) conidial suspension of mutant strain 4/4 (1.5×10^6 conidia ml^{-1}) or water controls; (b) mutant 4/4 conidia pre-incubated in a 5 mg $m1^{-1}$ adenine solution for 24 h prior to inoculation, and then transferred to water or 0.1 mg ml^{-1} adenine solution and (c) conidial suspension of wild type strain 1,2 (1.5×10^6 conidia $m1^{-1}$). No seedling mortality was observed when plants were exposed to 0.1 mg ml^{-1} adenine solution, mutant strain 4/4 or water controls.

reduction in extracellular enzyme activity that was observed in mutant strain 4/4 may be one of the reasons contributing to its lack of pathogenicity. The cause of this impairment may be attributed to multiple loci affected by the UV mutagenesis process or by the possibility that an upstream factor was affected that is required for normal production and/or secretion of the enzymes. As BLAST indicated, a high degree of conservation of purine biosynthetic enzymes in filamentous fungi such as *Neurospora crassa* (Galagan et al., 2003), *Magnaporthe grisea*, *Aspergillus nidulans* and *Fusarium graminearum* (www.genome.wi.mit.edu) was found and it is highly conceivable that a similar pathway is present in *F. oxysporum*. Since adding histidine or tryptophan did not have an affect on growth of mutant strain 4/4 the defect is likely located in the purine biosynthetic pathway, between 5-phosphoribosyl pyrophosphate amidotransferase and inosine monophosphate 3 cyclohydrolase. As purine biosynthesis is important for a wide spectrum of processes it is highly likely that a defect in this process can affect different cellular functions, including pathogenicity. We conclude that impaired adenine biosynthesis in mutant strain 4/4 of FOM partially affected growth and development, and appears to be the cause of lack of pathogenicity of this strain in melon plants. Furthermore, caution should be exercised when considering auxotrophic mutants of otherwise pathogenic fungi for biocontrol, as genetic revertants on the one hand and alterations in nutritional sources on the other, may contribute to re-establishment of strain pathogenicity.

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