Fusarium verticillioides from Sugarcane, Vegetative Compatibility Groups and Pathogenicity

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Abstract

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Sugarcane plant samples affected by the disease pokkah boeng were collected from the major sugarcane producing areas in Iran. Fifty isolates of *Fusarium verticillioides* were recovered from diseased sugarcanes. Results of pathogenicity tests revealed that all isolates were pathogenic to sugarcane. Four hundred and eighty-five nitrate non-utilising mutants were generated from *F. verticillioides* isolates. Media used for nit mutant generation included potato dextrose agar, minimal medium and Czapeck agar amended with 1.8%, 2%, 2.5%, and 3% potassium chlorate. Nit mutants were divided into three phenotypic (nit1, nit3, and nitM) classes based on their growth on different nitrogen sources in the culture media. Among the isolates tested, 280, 115 and 90 nit1, nit3, and nit M were generated, respectively. Nit mutants were used to force the heterokaryon formation to determine the distribution of VCGs and their relation to pathogenicity and geographic origin. Forty-eight VCGs of *F. verticillioides* were isolated from sugarcane. Forty-six of the VCGs were represented by a single isolate, whereas the remaining two comprised two isolates. None of the VCGs was common.

Keywords: pokkah boeng; *Fusarium;* mutant; nit

Sugarcane is cropped in many countries such as India, Cuba, USA, and South Africa (Purseglove 1979; Whittle & Irawan 2000). The disease 'pokkah boeng' was originally described in Java and the name is a Javanese term denoting a malformed or distorted top. It is caused by the fungus *F. verticillioides*. McRae (1932) isolated *F. verticillioides* from infected sugarcane. The disease is present in most, if not all, sugarcane-producing areas of the world (Whittle & Irawan 2000). Pokkah boeng rarely causes serious yield losses in commercial plantings. Reported outbreaks of the disease, while looking spectacular, have caused small economic losses (Whittle & Irawan 2000). In Iran, sugarcane is cropped in Khuzestan province and the incidence of pokkah boeng has been reported in Iranian sugarcane fields (Taherkhani *et al.* 1998). The genetic diversity of *F. verticillioides* populations has been investigated in other species such as maize and cereals by VCGs (Sunder & Satyavir 1998; Li *et al.* 2000). The formation of heterokaryons between different strains is an important and common component of the life cycle of many filamentous fungi. Lineages that are capable of fusing (anastomosis) and forming stable and functional heterokaryons are known as sexually or vegetatively compatible, the former being frequently described as members of the same group of vegetative compatibility or vegetative compatibility group – VCG (Leslie 1993). VCG analysis will provide an identification tool and a way to assess genetic variability in the *Fusarium* population. In addition, it increases our understanding of the population biology of the genus. Data on the morphological characteristics,

pathogenicity test on healthy sugarcane and VCG's will form integrate information to correctly identify the *Fusarium* species causing the pokkah boeng disease. Studies of the genetic diversity of *F. verticillioides* from sugarcane have recently been conducted and this paper provides the first research report on the diversity of *F. verticillioides* in sugarcane fields using the vegetative compatibility group technique.

MATERIAL AND METHODS

Plants showing symptoms of pokkah boeng were randomly sampled from sugarcane fields in Iran over several years. Each sample was surface sterilised in a 5.5% solution of NaOCl for 1 min, rinsed three times in sterilised water and air-dried on sterile paper towel. Each disinfected sample was plated with Nash and Snyder medium (Taherkhani *et al.* 1998; Jo *et al.* 2008). Nash and Snyder is a selective medium for *Fusarium* species and facilitates the formation of large, easily recognisable colonies (Chen *et al.* 2007). Fusarium colonies were observed microscopically, those colonies identified as *F. verticillioides* were transferred to carnation-leaf agar (CLA) and potato dextrose agar (PDA). Single-spore isolates were made from each colony and isolates were identified morphologically to the species level based on the characteristics of macroconidia, phialides, microconidia, chlamydospores and colony growth traits (Leslie & Summerell 2006).

Pathogenicity test. A stem wound was made using a sterilised cork borer, a PDA agar block from a 5-days old culture of *F. verticillioides* was placed in the wound and the inoculated portion was wrapped with parafilm. An uninfested PDA block was used with control plants. The wrapping material was removed from the stems 2 weeks after inoculation (Figure 1E). Plants were monitored for the development of disease symptoms and isolations were attempted to confirm pathogenicity in inoculated stems (Taherkhani *et al.* 1998).

Vegetative compatibility. VCGs were determined using the complementation of nitrate non-utilising (nit) mutants as a visual indicator of heterokaryon formation (Jo *et al.* 2008). Nit mutants were generated from each of the 50 *F. verticillioides* isolates on PDA, minimal medium (MM) and Czapeck agar amended with 1.8, 2, 2.5, and 3% potassium chlorate, respectively (Puhalla 1985; Klittich & Leslie 1988). The fast-growing, chlorate-resistant sectors originating from the initially restricted colony, which grew thinly but expansively on Puhalla's minimal medium (Puhalla 1985), were considered nit mutants. Nit mutants were phenotypically classified by their growth on basal medium (MM without $NaNO_3$) amended with one of several nitrogen sources (Pasquali *et al.* 2005). Several nit1, nit3 and nitM mutants from all isolates were stored in sterile distilled water at 4°C. Before complementation tests among isolates, vegetative self-incompatibility of each isolate was examined (Pasquali *et al.* 2005). Nit1 and nitM mutants of all *F. verticillioides* isolates were then paired in all possible combinations on minimal medium and the plates were incubated at 25°C in the dark (Figure 1D). Vegetatively compatible isolates were recognised by the robust growth at the interface of the two colonies after 10 days (Klittich & Leslie 1988).

RESULTS

Seventy isolates in *Fusarium* section *Liseola* were recovered from sugarcane stalks infected with pokkah boeng disease including 50 isolates of *F. verticillioides*. Previous studies showed that *F. verticillioides* predominates in sugarcane (Taherkhani *et al.* 1998). Results proved that all *F. verticillioides* isolates were pathogenic to sugarcane. Inoculated stalks in the pathogenicity test showed discoloration from red to brown (internal and external discoloration); all isolates caused discoloration of the sugarcane stalks while no changes were observed in the control treatment (Figures 1E–F) .

Table 1. Frequency and phenotypes of *nit* mutants recovered from *Fusarium verticillioides* in Iran

Phenotype of nit mutants	Growth on nitrogen sources				Total nit
	nitrate	nitrite	hypoxanthine	ammonium tartrate	
Nit1		+	+	+	280 (57.74%)
Nit3		–	+	+	115 (23.71%)
NitM		+	–	+	90 (18.55%)

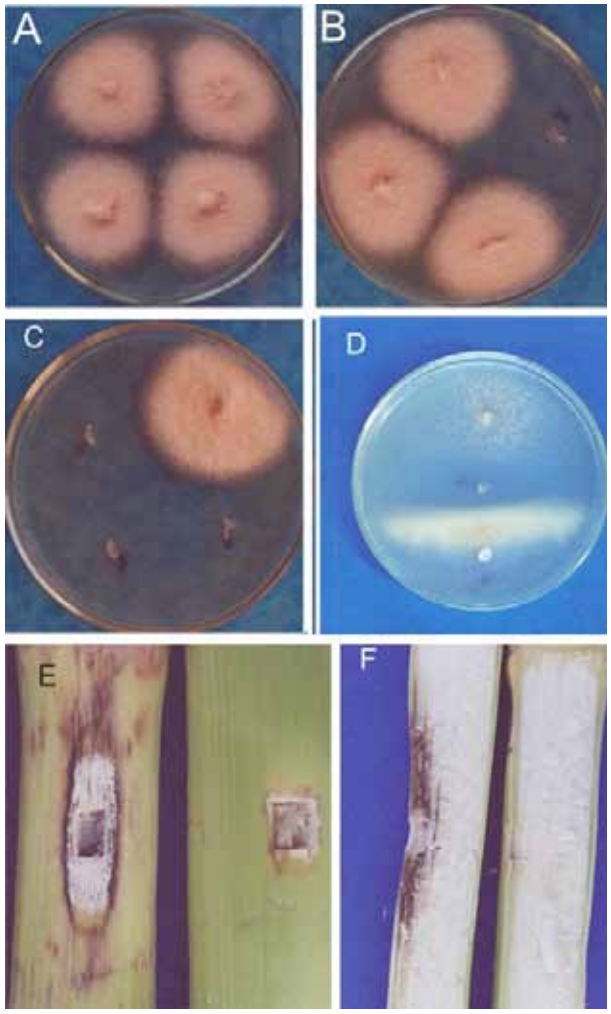

Figure 1. Nit mutants on a nitrogen source (A–B), complementation among nit mutants (C), pathogenicity test (E–F)

F. verticillioides isolates produced chlorate-resistant sectors on media complemented with chlorate. Large differences in sectoring frequency occurred between isolates. The majority of the chlorate-resistant isolates recovered was unable to utilise nitrate as the sole nitrogen source and consequently grew as thin expansive colonies without aerial mycelium on MM. However, a few chlorate-resistant sectors were able to utilise nitrate. The frequency of resistant sectoring was different when different concentrations of chlorate were supplemented. The 3.0% concentration of chlorate produced the highest frequency of sectoring in this study. Sectoring frequency of *F. verticillioides* has been shown to be heritable and to vary among isolates. No chlorate-resistant sector was observed in PDA complemented with chlorate. The phenotypes of 485 nit mutants were determined by their colony morphology on media containing nitrate, nitrite, hypoxanthine, uric acid, or ammonium tartrate as a sole nitrogen source (Figures 1A–C).

The nit mutants were divided into three classes; nit1 (a mutation of the nitrate reductase structural locus), nit3 (a mutation of the nitrate-assimilation pathway specific locus), and nitM (mutations that affect the assembly of a molybdenum-containing cofactor necessary for nitrate reductase activity). Among the tested isolates, 280 (57/74%), 115 (23/71%) and 90 (18/55%), nit1, nit3 and nitM were generated, respectively (Tables 1 and 2). The majority of nit mutants were nit1 followed by nit3 and nitM (Table 2).

Physiological complementation among nit mutants with different mutations was indicated by the development of dense aerial mycelia where the mycelia of the nit mutants came in contact and anastomosed to form a heterokaryon (Figure 1D). Forty-eight VCGs were determined in the tested isolates, two groups with two isolates and 46 VCGs with one isolate. There was no correlation between isolate characterisation and geographical distribution, pathogenicity and VCG groups.

DISCUSSION

In the present study *Fusarium* species were isolated from infected sugarcane. Results showed that *F. verticillioides* was the most frequently isolated species (70% of isolates). This data is

Table 2. Frequency and phenotypes of *nit* mutants recovered from three media

Phenotype of nit mutants	Media and percentage of chlorate							Total
	PDA		MMC			Czapeck		
	1.5	1.8	1.8	2.0	2.5	2.5	3.0	
Nit1	0	90	0	30	45	0	115	280
Nit3	0	14	0	15	40	0	46	115
NitM	0	10	0	6	15	0	59	90
Total	0	114	0	51	100	0	220	485

Table 3. Isolates tested and their VCGs

Isolate	Location	VCG	Isolate	Location	VCG
K1	Karun agro industry	1	S7	Emam Khomaini agro industry	25
K2		2	S10		26
K5		3	S13		27
K6		4	S14		28
K7		5	S17		29
K9		6	S18		30
K11		7	S19		31
K12		8	S21		32
K14		9	S23		33
K16		10	S26		34
K17		11	A1	Amirkabir agro industry	35
K18		12	A2		36
K19		13	A3		37
K20		14	A4		38
K21		15	A5		39
K24		16	A6		40
H1	Haft Taeh agro industry	17	A7		41
H2		17	A8		38
H7		18	A9		42
H8		19	A10		43
H10h		20	A11		44
H12		21	A12		45
H14		22	A15		46
S1	Emam Khomaini agro industry	23	A19		47
S6		24	A20		48

supported by previous studies showing that *F. verticillioides* predominates in infected sugarcane (Taherkhani *et al.* 1998) and more than 73% of the isolated fusarium population was from maize (Gohari *et al.* 2008).

Nit mutants in three phenotypic classes were recovered from each of the fifty isolates of *F. verticillioides* tested. The majority (57.74%) of nit mutants recovered were nit1 mutants. With all fifty isolates, nit3 and nitM mutants were recovered at a much higher frequency from minimal agar medium amended with chlorate than from PDA and Czapeck agar amended with chlorate. The wide range of sectoring frequency in plant pathogenic fungi at different concentrations of chlorate has been suggested as a selective advantage for rapid adaptation to environmental stresses such as fungicide toxicity and host resistance (Klittich & Leslie 1988). However, a similar frequency of sectoring was observed in other studies where chlorate was added to both PDA and MM in studies with other *Fusarium* spp. (Correll *et al.* 1987). According to Bowden and Leslie (1992) and Puhalla (1985), the frequency of nit1 mutants is higher than the frequency of the other types of nit mutants. Generation and phenotype assignment of *nit* mutants in this study showed that the ratio of nitM phenotype was relatively high. Similar results were also described in *F. graminearum* (Puhalla 1985; Bowden & Leslie 1992) and in the coelomycete fungus *Colletotrichum orbiculare* (Gohari *et al*. 2008*)*.

It is presumed that the isolates in the same VCG are clones even if the isolates are geographically isolated. This could be so because the loci and alleles of VCG are selectively neutral with respect to traits such as pathogenicity and vegetative vi-

ability (Huang *et al.* 1997). The segregation of *F. verticillioides* isolates obtained from sugarcane into VCGs in Iran is reported for the first time. We identified 48 VCGs among 50 isolates, accounting for a genetic diversity (number of VCGs/number of isolates) of 0.96. Forty-eight VCGs had only one member, and the remaining four belonged to two VCGs. The analysed population of *F. verticillioides* is genotypically diverse. These results are in agreement with results from previous studies on *F. verticillioides*, demonstrating that this fungus is genotypically highly diverse (Corell *et al.* 1989; Danielsen *et al.* 1998; Chulze *et al.* 2000; Gohari *et al.* 2008). No specific relationship was observed between VCGs and geographic origin of the isolates in this study.

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