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Full Length Research Paper

Molecular characterisation of *Mycovellosiella koepki*, the causal agent of yellow spot disease of sugarcane

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Yellow spot of sugarcane is a fungal disease caused by *Mycovellosiella koepki*. Yellow spot symptoms are variable and differ in several sugarcane varieties and the existence of more than one strain of the fungus has been evoked. 29 isolates of the fungus were characterised morphologically and no significant differences were observed on the type, colour and size of the spores. Following restriction digestion of the ITS region, no significant differences were observed among isolates of the fungus and DNA sequencing of this region of the ribosomal DNA confirmed the absence of polymorphisms among the fungal strains.

Key words: Yellow spot, PCR, sequence analysis.

INTRODUCTION

Yellow spot is considered to be the most important fungal disease which affects sugarcane leaves and is known to reduce yield and sucrose (Autrey and Saumtally, 1986). Yellow spot disease has been reported in different sugarcane growing countries including Australia, India, Madagascar, Reunion and the Philippines. In Mauritius, yellow spot has been reported since 1965 (Antoine, 1965) and it is prevalent in the humid and superhumid zones, where it is considered to be a disease of prime importance in profusely flowering sugarcane varieties. From 1976 to 1980, losses due to this disease were estimated to be 20,000 tonnes in Mauritius in the highly susceptible variety Saipan 17. Saipan 17 is no longer grown commercially and the main commercial varieties of sugarcane which are affected by yellow spot disease are M134/32 and R570. These varieties are not highly recommended for planting in the humid and superhumid zones.

The causal agent of yellow spot disease is the imperfect fungus *Mycovellosiella koepki* (Kruger) Deighton for which the perfect stage has not been reported. Hughes and Ocfemia (1961) cultured *M. koepki* on artificial media with some difficulty while Autrey and Saumtally (1986) cultured the fungus on various nutritive media and found that growth and sporulation were enhanced on cane leaf agar. More than one strain of the fungus *M. koepki* is thought to have occurred since its first report in 1890. Two or three strains of *M. koepki* probably existed in Australia as Egan (1970) observed a difference in the varietal reaction to yellow spot. In Mauritius, varieties which were initially considered to be resistant showed considerable susceptibility to yellow spot after a few years (Ricaud et al., 1978) and possible existence of different strains of the fungus was evoked. The existence of more than one strain of the fungus could be possible because the disease symptoms of yellow spot vary in different sugarcane varieties. On M1658/78, M52/78, R570, S17 the spots are yellow while they are red on B33/37 and M134/32. (Figure 1). The possibility that more than one strain of the fungus exist is not only supported by the fact that the foliar infection varies between sugarcane varieties but the disease symptoms can sometimes even vary within and between geographical regions. However, no definite proof of strain variation to this disease have yet been obtained. In this study, strain variation within *Mycovellosiella koepki* was assessed using morphological description, inoculation experiment and molecular characterization.

MATERIALS AND METHODS

Collection and morphological characterization of *M. koepki* isolates

Isolates of *M. koepki* were collected from different sugarcane varieties in the humid and superhumid zones from April, the period

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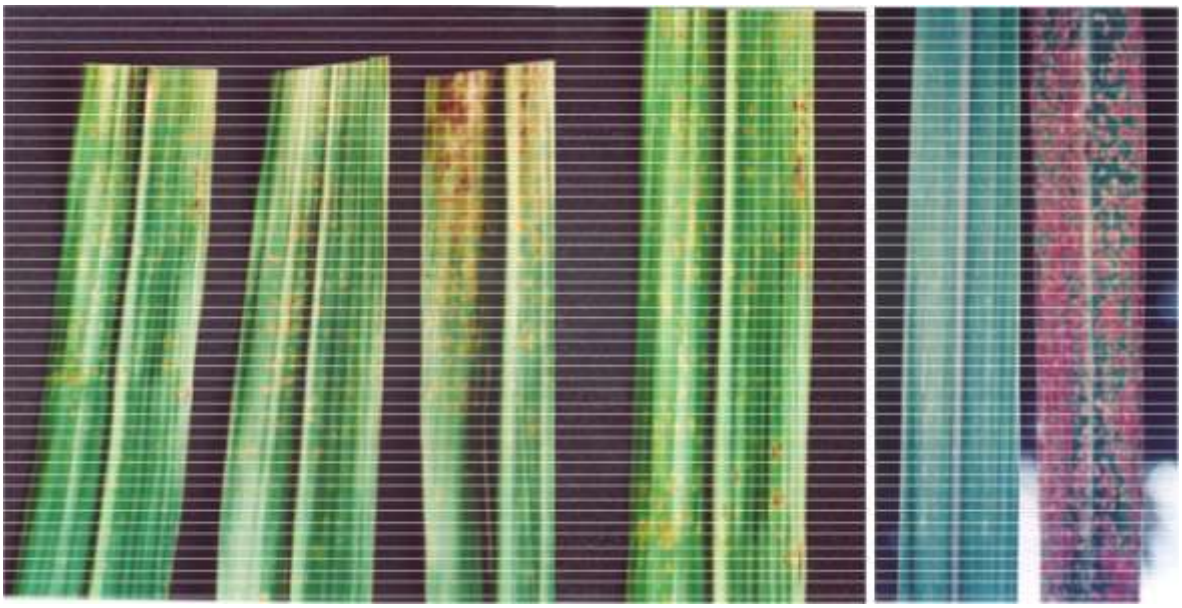

Figure 1. Symptoms of yellow spot in varieties R570, S17, M1658/78 and B33/37

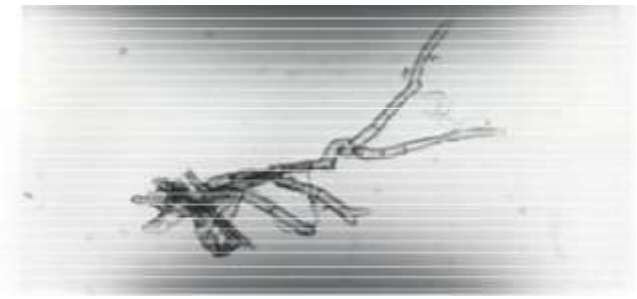

Figure 2. Spores of *Mycovellosiella koepki*.

during which usually high humidity prevails and the first infections start to be visible on leaves. In order to establish whether there are more than one strain of the fungus, leaf samples from different sugarcane varieties infected with yellow spot were collected. Variations in the disease symptoms (e.g. colour of lesions) on these leaves were noted along with the morphological features of the fungus (e.g. colour, septation, size) which would serve as a first indication of the presence of more than one strain of the fungus.

Inoculation of sugarcane plantlets with spores of yellow spot

An initial inoculation experiment was set up on young sugarcane (S17) plantlets in the greenhouse; plantlets of variety S17 derived from tissue culture were inoculated with mycelium and spores from cultures of *M. koepki* and spores collected from infected leaves of variety B33/37 in the field. (Figure 2).

Another experiment was set up by artificially inoculating the fungus on sugarcane varieties of variable resistance to the disease. Sugarcane cuttings from varieties M596/78 (resistant), M134/75 (slightly susceptible), B33/37, R570 and S17 were grown in the greenhouse. After two months they were inoculated with spores of yellow spots from infected leaves of variety R570 and B33/37 collected in the fields.

DNA extraction and RAPD-DNA fingerprinting

DNA extraction was carried out using a modified Doyle and Doyle (1987) method (Ranghoo et al., 1999). Isolates were initially screened by random amplified polymorphic DNA (RAPD) to find variability among the different strains. Each 50 µl of reaction consisted of 2 U of *Taq* polymerase, 50 mM KCl, 10 mM Tris-HCl pH 8.8, 4 mM $MgCl_2$, 100 µM dNTP, 0.22 µM primer and approximately 50 ng (≈10 µl) of template DNA. Forty amplification cycles were performed on a MJ Research minicycler. Each cycle consisted of denaturation at 94°C for 3 min, annealing at 36°C for 1 min, followed by a 2 min rise to 72°C for primer elongation. The final primer elongation segment of the run was extended to 7 min. Reaction products were resolved in a 2% agarose gel at 4 V/cm for 2 h. PCR product were visualised by UV-fluorescent staining with ethidium bromide**.** Forty primers were screened to identify those which produced maximum polymorphisms.

Amplification, digestion and sequencing of the ITS region

The ITS regions was amplified for the *M. koepki* isolates using primer pairs ITS 4 and ITS 5. Each 50 µl of reaction consisted of 2 U of *Taq* polymerase, 50 mM KCl, 10 mM Tris-HCl pH 8.8, 5 mM $MgCl_2$, 20 µM dNTP, 0.4 µM primer and approximately 10 ng (≈2 µl) of template DNA. Thirty amplification cycles were performed on a MJ Research minicycler. An initial denaturation step was performed at 94°C for 3 min followed by a denaturation step at 94°C for 1 min, then annealing at 52°C for 1 min and finally 72°C for primer extension. The final primer elongation segment of the run was extended to 10 min at 72°C. Amplified products were digested with restriction enzymes according to the manufacturer's instructions (Boehringer Mannheim). Digested DNA fragments were resolved in a 2% agarose gel at 4 V/cm for 2 h, stained with ethidium bromide and photographed under UV light. The amplified product was diges-

Table 1. List of sugarcane varieties from which fungal isolates were obtained.

Sugarcane variety	Locations	Climatic zones
S17	Union Park	Very humid
R570	Belle Rive	Very humid
R579	Belle Rive	Very humid
M695/69	Le Val	Very humid
R570	Rose Belle	Very humid
M52/78	Rose Belle	Very humid
B33/37	Union Park	Very humid
R570	Le Val	Very humid
M52/78	Cluny	Very humid
M52/78	Beau Bois	Humid
M1658/78	Riche Bois	Dry
M134/32	Union Park	Very humid
M337	Union Park	Very humid
M52/78	Valetta	Very humid
R570	Riche Bois	Dry
R570	Henrietta	Humid
R570	La Nicoliere	Very humid
R570	Reduit	Humid
M1658/78	Union Park	Very humid
M1658/78	Mare Tabac	Humid
M292/70	Montagne Blanche	Very humid
M377/56	Union Park	Very humid
R570	Deep River Beau Champ	Dry
M596	Union Park	Very humid
M695/69	Rose Belle	Very humid
R570	Union Park	Very humid
M695/69	Mare Tabac	Humid
R570	Mare Tabac	Humid
S17	Britannia	Dry

ted using restriction enzymes *EcoR* I, *Hae* III, *Rsa* I, *Taq* I, *Cfo* I, *Dde* I, *Hinf* I, *Hpa* II and *Msp* I.

Sequencing method used

The ITS DNA fragment from 4 isolates B33/37, S17, R570 and M695/69 were successfully cloned in pPCR-Script Amp SK+ Cloning vector (Stratagene). The fragment was then sequenced using universal primers KS, M13-20, M13/R, T3 and T7 as well as with the internal primers ITS 4 and ITS5 using the ABI 310 DNA analyzer. 300 - 400 ng of the cloned, purified pPCR-Script Amp SK+ Cloning vector (Stratagene) were mixed with 8.0 μ l of terminator reaction mix (BigDye Terminator Cycle Sequencing Kit) along with 3.2 pmol of the respective sequencing primers. 25 cycles of rapid thermal ramp to 96°C, then 96°C for 10 s, rapid thermal ramp to 50°C, 50°C for 50 s, rapid thermal ramp to 60°C, 60°C for 4 min, followed by a rapid thermal ramp to 4°C were carried out. The extension reaction product was then mixed with 2.0 μl of 3 M Sodium acetate and 50 μl of 95% ethanol. The mixture was then vortexed and left at room temperature for 1 h for precipitation. The pellet was then washed and then left to dry. 15 μl of Template Suppression Reagent was then added to the DNA pellet, then denatured, and then loaded on the ABI 310 Genetic analyzer.

RESULTS

Collection and morphological characterization of *M. koepki* isolates

29 isolates (Table 1) of the fungus were collected from 11 varieties of sugarcane and the size, colour and number of septations of the spores were noted. No variations were noted on the size, shape and colour of the spores. The spores were hyaline, and ranged from 20 to 50 μm length and from 5 to 8 μm in width (Figure 2) . Single spore isolations were made on corn meal agar supplemented with mycological peptone (Figure 3). Colonies were irregular in shape, grey in colour with fluffy, cottony and grey mycelium.

Inoculation of sugarcane plantlets with spores of yellow spot

Infections of yellow spot were observed on varieties

Figure 3. Growth of *Mycovellosiella koepki* on corn meal agar.

Figure 4. Seedlings of S17 infected with yellow spot isolates from B33/37.

B33/37, R570 and S17 after six weeks following inoculation. The infections were more severe on S17. No symptoms were observed on M596/78 and M134/75. Results obtained from this artificial inoculation experiment showed that sugarcane varieties exhibit similar reactions to yellow spot infection in the greenhouse as well as in the fields e.g. B33/37 has red lesions of yellow spot even though the plant was infected with spores collected from R570 while those in S17 are yellow with inoculate from B33/37 (Figure. 4).

DNA extraction and DNA fingerprinting

DNA yield was low but of high quality (10 μg/μl). RAPD amplification was successful with operon primers (series R and J) for the different fungal isolates. Polymorphisms were observed with primer OPR 01, OPR 03, OPR13 and OPR 15. More polymorphisms were observed with primer OPR 01. However the results were not reproducible.

Amplification, digestion and sequencing of the ITS region

The ITS region was amplified for the *M. koepki* isolates and a 600- bp PCR product was obtained following amplification using primer pairs ITS 4 and ITS 5. Restriction digestion with enzymes *EcoR*I, *Hae* III, *Rsa* I, *Taq* I *Cfo* I, *Dde* I, *Hinf* I, *Hpa* II and *Msp* I were also attempted but no polymorphisms were observed between the restriction digestion patterns of the 29 isolates.(Figure 5)

The nucleotide sequences of the ITS region and the 5.8S rDNA are shown in Figure 6. These sequences were aligned with Clustal X but no variation in the sequences was detected among the four isolates the species. Sequence analysis showed that there was no similarity between the sequences.

DISCUSSION

Collections of yellow spot were made from sugarcane varieties ranging from those which are slightly susceptible to those which are highly susceptible. However no distinct morphological variations within the fungus were found among the isolates of yellow spot indicating that only one strain of the fungus exist.

Fungal isolate from the red lesions of B33/37 produced yellow lesions in S17 and R570 suggesting that the same strain exist in all those varieties screened. Similar experiments can be set up to artificially inoculate sugarcane seedlings or plantlets of unknown reaction to yellow spot so as to obtain their relative susceptibility within a few weeks. Hence varieties can be easily and quickly selected for yellow spot resistance in the greenhouse.

DNA fingerprinting using RAPD showed no polymerphism with the 4 Operon primers used but the reactions were not reproducible. The extensive divergence in the ITS region has been the basis for the development of rapid RFLP analysis which are able to distinguish many different fungal species, different races of a fungal isolate as well as intraspecific variation. The DNA sequences that encode ribosomal RNAs have been extensively used to study the taxonomic relationships and genetic variations in fungi (Bruns et al., 1992; White et al., 1990). The ribosomal RNA gene cluster is found in both nuclei and mitochondria and consists of highly conserved and variable regions. The fungal nuclear rRNA genes are arranged as tandem repeats with several hundred copies per genome. The conserved sequences found in the large subunit (LSU) and small subunit (SSU) genes have been exploited to study the relationships among distantly relat-

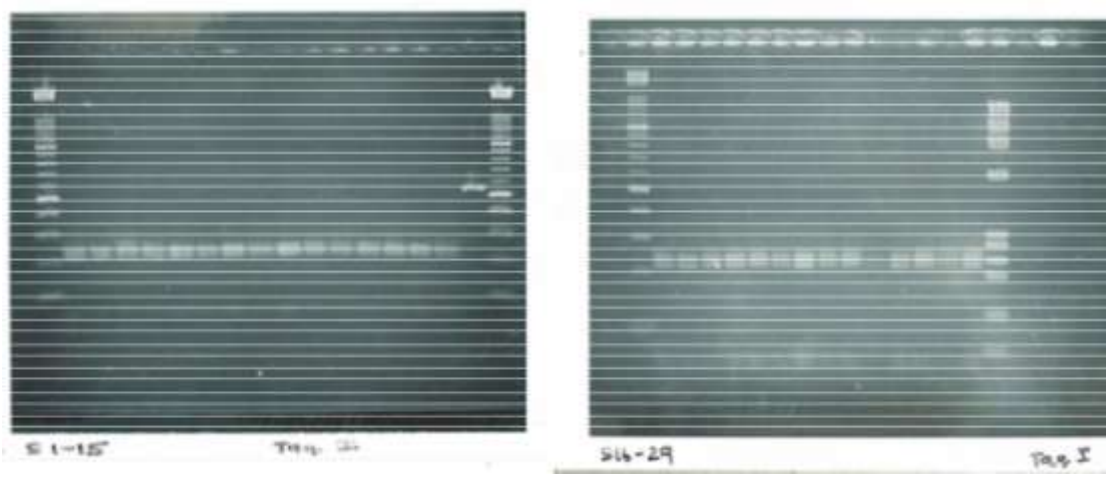

Figure 5. Restriction digestion patterns of the ITS fragments using *Taq* I.

TTTTACGCGAACCGCGCATTATTCCTCACTAAAGGGAACAAAAGCTGGAGCTCC
ACCGCGGTGGCGGCCGCTCTAGCCCGGAAGTAAAAGTCGGAACAAGG**TCTCCGTAGGTGA**
ACCTGCGGAGGGATCATTACCGGAAGCGCAGCCCTCGGGCTTCACCCTTCCCACCCTCTGT
GAACAACTTGTTGCTTTGGGGCGACCCTGCCGTACCCGCAGCGAGCGCCCCCAGAGGAC
CCTCAACACTGCATCCTTGCGTCGGAGCCTGAAGTAAAATGGAAGCAAAACTTTCAACAA
CGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAAT
TGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCGAAG
GGCATGCCTGTTCGAGCGTCATCTCACCACTCAAGCCTCGCTTGGTGTTGGGCGCCGCGGT
AGTGCCCGCGCGCCTCAAAGTCCCCGGCTGGGCGGTCTGCCTCTGAGCGTAGTGACTCCG
CGATCGCTCTAGGGTGTAGGCTGCGGTGGCCGTTAAACCTCCACATGGTTGACCTCGGATC
AGGTAGGGATACCCGCTGAACTTAAGCATATCAAATAAGCGGAGGAGGGCGGATGCTCC
CCGGGCTGCAAGGAATTCGAGTATCCAAGCTTATCGAATACCGTCGACCTCGAGGGAGGC
CCGGTAGCCAAT

Figure 6. ITS Sequence of isolates, ITS4 (blue) and ITS5 (red) are highlighted.

ed fungi (Bruns et al., 1992). The spacer regions between the subunits, called the internal transcribed spacers (ITS) and between the gene clusters, called the intergenic spacers (IGS) are considerably more variable than the subunit sequences and have been widely used in studies on the relationships among species within a single genus or infraspecific populations. The extensive divergence in the ITS region has been the basis for the development of rapid RFLP analysis which are able to distinguish many different fungal species (Chen et al., 1999, Schilling et al., 1996), different races of a fungal isolate (Hamelin et al., 1996) as well as intraspecific variation (Edel et al., 1996).

RFLP of the ITS region of the nuclear ribosomal DNA did not reveal any difference among the different isolates of yellow spot. Sequencing of the ITS fragment of 4 isolates of yellow spot confirmed the absence of divergence within the ITS fragment. This result is a definite proof for the existence of only one strain of *M. koepki* prevailing in Mauritius.

Conclusions

In this study, morphological as well as molecular characterization including RFLP and DNA sequencing have shown that there exist only one strain of the causal agent of yellow spot, *M. koepki.* The presence of only one strain of *M. koepki* will definitely be an advantage as far as disease control is concerned. In addition, the existence of only one strain of the fungus is an advantage in line of the application of marker-assisted selection for yellow spot in the breeding programme of sugarcane.

Differential reaction (i.e. increasing susceptibility to yellow spot) of variety S17 with the passing years as mentioned by Ricaud et al. (1978) can be possibly explained by the build-up of more inoculum in the fields as opposed to the existence of a more virulent strain of the same fungus. Moreover, climatic conditions prevailing at the time of the introduction of S17 in Mauritius may have not favoured the yellow spot infection in S17. With

the passing years, however, inoculum build-up coupled with favourable climatic condition i.e. heavy rainfall and high humidity led up to severe yellow spot infection in the fields. Interestingly enough, there may have been more than one strain of *M. koepki* in 1978. With time and with changing climatic conditions a more virulent strain may have been selected for.

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