Immunological and molecular detection of *Colletotrichum falcatum* Went causing red rot disease in sugarcane

R. Velazhahan, K. Nithya, Khalid A.I.M. Bukhari,
V. Valluvaparidasan and V. Paranidharan

Department of Plant Pathology
Tamil Nadu Agricultural University
Coimbatore, India

Sugarcane Red rot

- Red rot disease of sugarcane caused by the fungus ***Colletotrichum falcatum*** Went (Perfect state: *Glomerella tucumanensis* (Speg) Arx and Muller), is the most destructive disease of sugarcane worldwide
- Red rot epidemics cause up to **100% loss of sugarcane yields** under favourable conditions for disease development
- Several epiphytotics of red rot resulted in failure of important Indian commercial sugarcane varieties (Co 312, Co 997, Co419, Bo3, Bo11, Bo17, CoJ82, CoS245, CoS510, CoS770, Co 1148, CoJ64, CoJ84, Coc90061,Co68027, CoC 671, Co419, CoC 86062 and CoC 92061)

Sugarcane Red rot

- Use of red rot-resistant sugarcane cultivars can provide some degree of control of this disease, however the occurrence and **development of new pathogenic races is a continuing problem**
- If a disease resistant variety is released for cultivation, it becomes susceptible to red-rot disease within 8-10 years, because of development of new more virulent races of the pathogen (Yadav, 2006).
- **Eleven races of the pathogen** have been described in India based on host differentials (Satyavir, 2003).

Sugarcane Red rot

- **In the early stages of infection it is difficult to recognize the presence of the disease in the field** (reddening of the internal tissues with interrupted red and white patches, the characteristic symptoms of the disease, develops on stem only at later stages).
- **Latent infection occurs frequently**, making visual diagnosis impossible. Consequently, planting of the infected sugarcane setts can spread the disease.
- **Use of pathogen-free seed canes** in commercial production is one of the most efficient avenues for prevention of red rot disease.
- Detection of the pathogen in seed canes prior to planting can prevent introduction of infected materials into fields, and could be an effective management practice.
- **Sensitive and reliable methods for detection of *C. falcatum* are needed in certification programs.**

Disease diagnosis

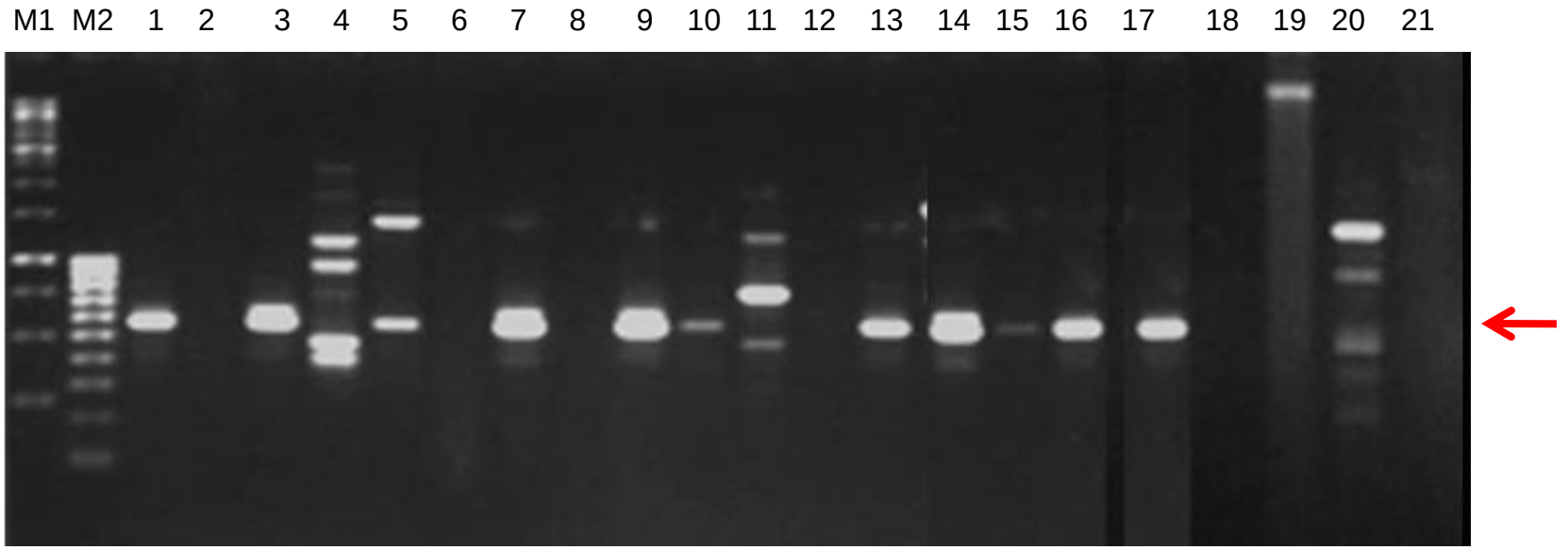
- Disease diagnosis and pathogen identification by conventional methods involve isolating the pathogen and characterizing it by inoculation tests (labour-intensive and time-consuming).
- Organisms need not be cultured prior to detection by PCR and large numbers of samples can be processed in a short time by these methods
- Immunological and molecular diagnostic methods have increasingly received attention over the past few decades as an alternative or complement to conventional methods (Schaad et al. 2003).
- Serological methods (Enzyme-linked immunosorbent assay) are sensitive, specific, and simultaneous analysis of many samples in a single microplate.
- Hiremath and Naik (2004) developed a protocol for rapid diagnosis of sugarcane red rot infection in the planting materials by using Dot-immunobinding assay (DIBA) technique.
- Viswanathan et al. (1998) developed polyclonal antisera against a 101- kDa protein of *C. falcatum* and standardized the ELISA, dot-immunobinding assay and western blot methods for detection of *C. falcatum* in sugarcane

Objectives

To develop Immunological and molecular methods for detection of *Colletotrichum falcatum* in planting materials

Development of SCAR markers for sensitive detection of *C. falcatum* in sugarcane setts

Agarose gel electrophoresis of PCR-amplified products from genomic DNA of *C. falcatum* using the RAPD primer OPE 01



Lane M1: 1kb marker
Lane M2: 100bp marker
Lane 1: Cf 86032
Lane 2: Cf 90063
Lane 3: Cf 93v297a
Lane 4: Cf 95020
Lane 5: Cf 671a
Lane 6: Cf 671b
Lane 7: Cf 671c
Lane 8: Cf 8368

Lane 9: Cf95045
Lane 10: Cf 98061
Lane 11: Cf 93v297b
Lane 12: Cf 93009a
Lane 13: Cf 01
Lane 14: Cf 03
Lane 15: Cf04
Lane 16: Cf 05
Lane 17: Cf 06
Lane 18: Cf 09

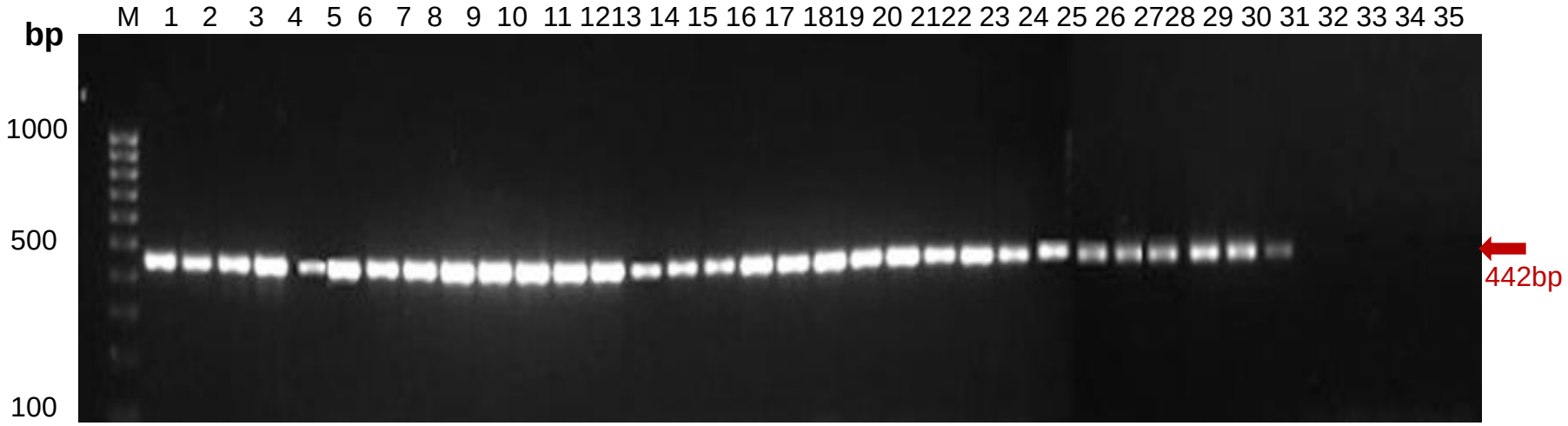
Lane 19: *C.lindemuthianum*
Lane 20: *C.capsici*
Lane 21: *C.gleosporioides*

Nucleotide sequence of the PCR-amplified product obtained from genomic DNA of *C.falcatum* late using the RAPD primer OPE 01

CF 1 (Co 86032) 566 bp insert

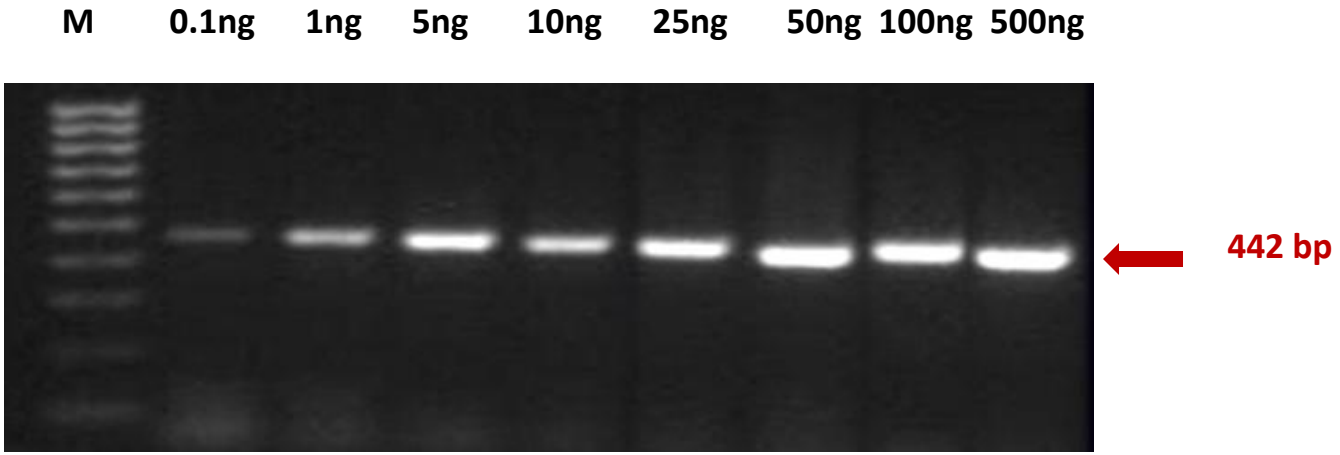
CCCAAGGTCCTCAGAGATGGAGGATGAGGGGATGAGGGGAAGAAGAG
TGTGTTGTTTCGAGCCAGACATGGAACGGCACATAAACCA**CCTACCCA**
ACCGAGTATCGAGTACGTGCCTGTTTACTTTCGTAGCACTCGTGGCTC
AAGAGGACATGGGGTCGGCGGGAGCATTCTGATTGGCTTGGAGAGGG
AGAGCATGGCAAGGAGAGGGTTGACGTTGTCAGAGGGCGGTGAAGGT
ACGGCAGCACACACACACACACACACACACAAGGCGGCCACACTCGTCA
CTCTAACCCCGGAGAAAGAGTACGATGAGAGACAGAGGCACTGCAACT
AAGACATGGGAAAGAAGGAGGACCCTTTGGATTGTGTGGGGCAGGAG
ACAGCGCATGGTCGACATGGCCAGCACGGATCATCAACAGACCACTG
CTGGAGGAGTGGGGCGGGGGACAGTTTCCACCTTTTGTAGTACTTGC
GGAGTACGGATACTGAGGCACGGATTCATACACAC**GCTCTTGAGAGCA**
AGCTGCGCTGCTTCTGCTGCGGCTTGCCGGCCTTGGACCTTGGGA

Molecular detection of *C. falcatum* using SCAR marker

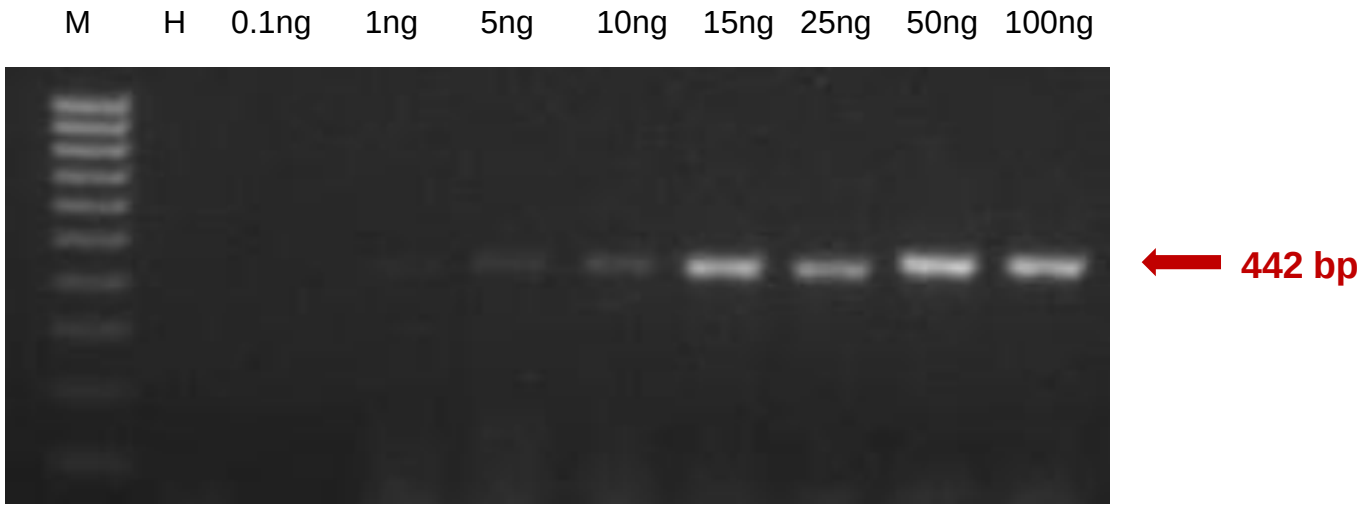


Lane M2: 100bp marker
Lane 1: Cf 86032
Lane 2: Cf 90063
Lane 3: Cf 93v297a
Lane 4: Cf 95020
Lane 5: Cf 671a
Lane 6: Cf 671b
Lane 7: Cf 671c
Lane 8: Cf 8368
Lane 9: Cf95045
Lane 10: Cf 98061
Lane 11: Cf 93v297b
Lane 12: Cf 93009a
Lane 13: Cf 93009b
Lane 14:Cf 94025
Lane 15: Cf 87012a
Lane 16: Cf 87012b
Lane 17: Cf 94045a
Lane 18: Cf 8371
Lane 19: Cf 910171
Lane 20: Cf 6304
Lane 21: Cf 94045
Lane 22: Cf 93009c
Lane 23: Cf 93009d
Lane 24: Cf98061
Lane 25: Cf 94045b
Lane 26: Cf 01
Lane 27: Cf 03
Lane 28: Cf 04
Lane 29: Cf 05
Lane 30: Cf 06
Lane 31: Cf 09
Lane 32: *C.lindimethianum*
Lane 33: *C.gleosporoides*
Lane 34: *Fusarium sp*
Lane 35: *Alternaria sp*

Sensitivity of SCAR marker in detection of *C.falcatum*

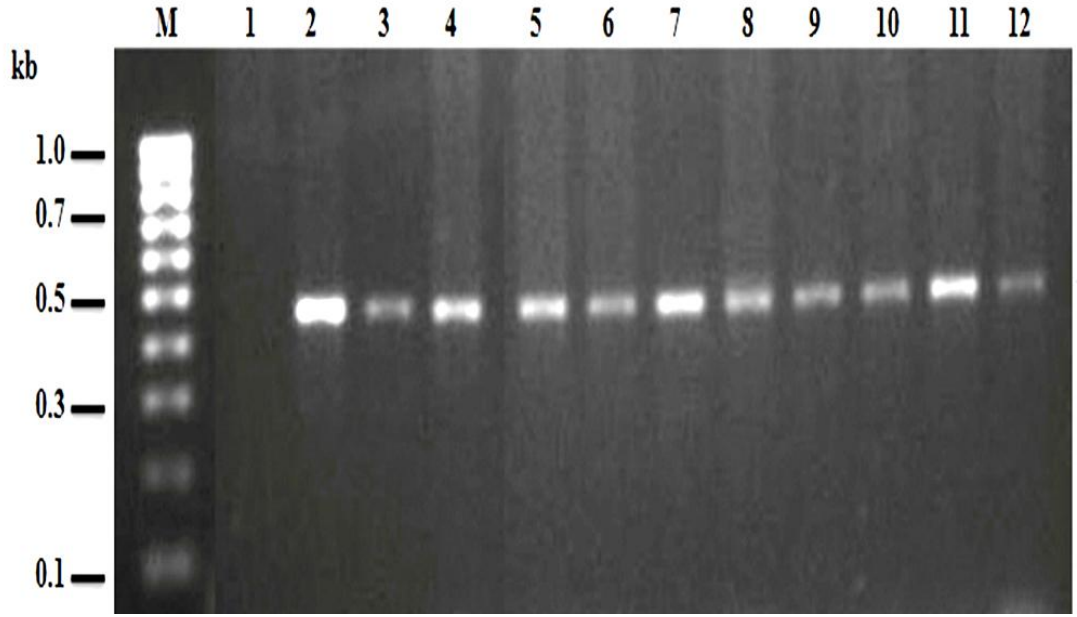


Sensitivity of SCAR marker in detection of *C. falcatum* in infected Sugarcane setts



H- Healthy Sugarcane sett DNA

Detection of *C. falcatum* in artificially inoculated sugarcane tissues by PCR using SCAR primers

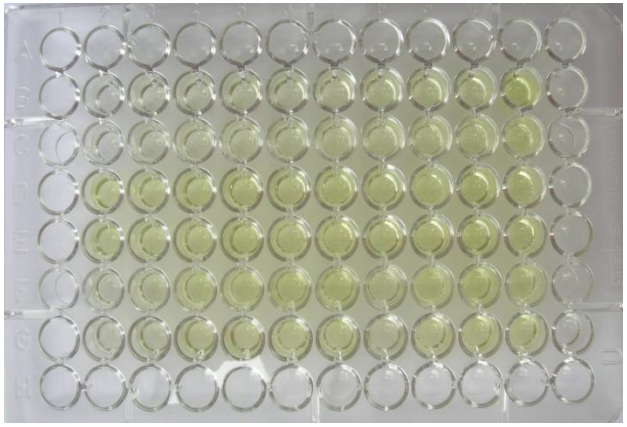


Lanes 1, Healthy sugarcane tissue DNA; Lanes 2, Cf 671a; Lane 3, Cf 98061; Lane 4, Cf 671b; Lane 5, Cf 671c; Lane 6, Cf 93009a; Lane 7, Cf 93v297; Lane 8, Cf 90063; Lane 9, Cf 95020; Lane 10, Cf 87012; Lane 11, Cf 93009b; Lane 12, Cf 91017.

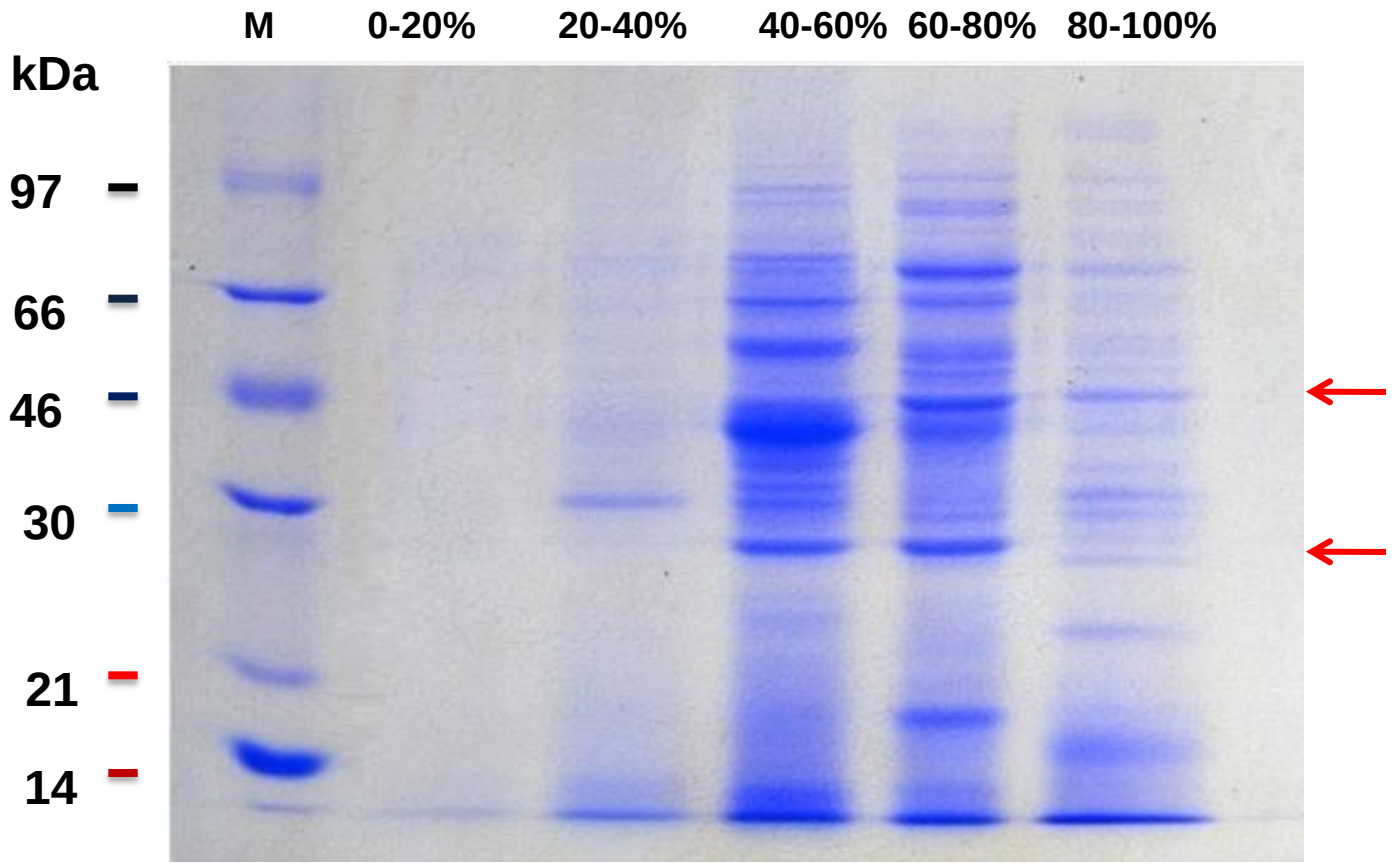
Development of immunological method of detection of *C. falcatum*

C. falcatum **protein**

ELISA

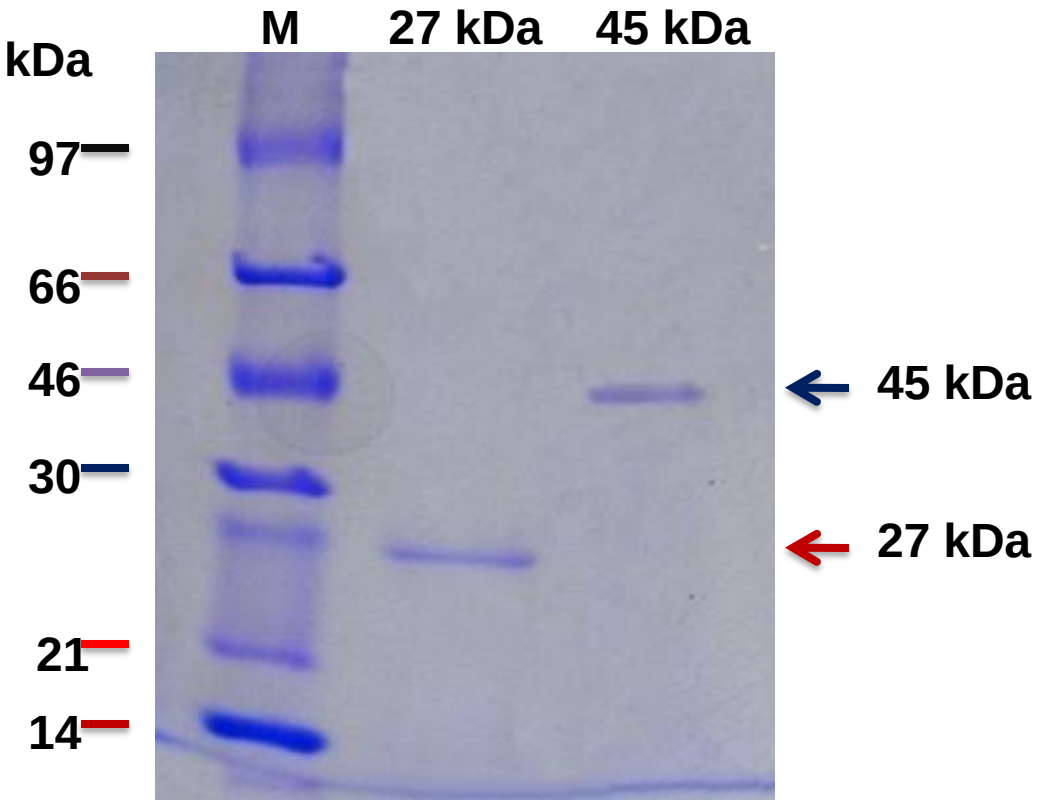


Ammonium sulphate fractions of mycelial proteins of *C. falcatum* race Cf 05



Proteins separated by 12% SDS-PAGE were stained with Coomassie Brilliant blue. The molecular weight of marker proteins is indicated on the left

SDS-PAGE of purified 27kDa and 45kDa proteins from the mycelium of *C. falcatum* race *C*f 05



The 27 kDa and 45-kDa proteins eluted from the gel were subjected to 12% SDS-PAGE followed by Coomassie blue staining. The molecular weight of marker proteins (Lane M) is indicated on the left.

Sensitivity of the antibodies in detection of *C. falcatum* by enzyme-linked immunosorbent assay

Dilution of antibody	Optical density at 405 nm	
	Antiserum against 27 kDa protein	Antiserum against 45 kDa protein
1:500	2.269	2.449
1:1000	1.947	2.048
1:10000	0.943	0.844
1:20000	0.575	0.517
1:30000	0.429	0.317
1:50000	0.330	0.283
Control (buffer)	0.062	0.076

Total proteins extracted from *C. falcatum* race Cf 05 (380 µg/ml) were used as antigen. Each measurement is an average of the readings from three wells

Sensitivity of enzyme-linked immunosorbent assay in detection of *C. falcatum*

Dilution of antigen	Optical density at 405 nm	
	Antiserum against 27 kDa protein	Antiserum against 45 kDa protein
No dilution	0.900	0.906
1:10	0.721	0.750
1:100	0.475	0.475
1:250	0.452	0.437
1:500	0.319	0.387
1:1000	0.227	0.284
1:5000	0.216	0.276
1:10000	0.158	0.180
Control (buffer)	0.089	0.081

The ELISA test was performed using the antibodies raised against the 27 and 45-kDa mycelial protein of *C. falcatum* diluted to 1:10000. Each measurement is an average of the readings from three wells

Reactivity of polyclonal antisera towards *C. falcatum* and other *Colletotrichum* spp. as determined by ELISA

Fungus	Optical density at 405 nm	
	Antiserum against 27 kDa protein	Antiserum against 45 kDa protein
C. falcatum race Cf 01	0.718	0.802
C. falcatum race Cf 05	0.789	0.790
C. falcatum isolate Cf 671	0.862	0.791
C. falcatum isolate Cf 8371	0.716	0.814
C. capsici	0.288	0.221
C. gloeosporioides	0.243	0.204
C. musae	0.257	0.212
C. lindemuthianum	0.213	0.173
Control (buffer)	0.098	0.077

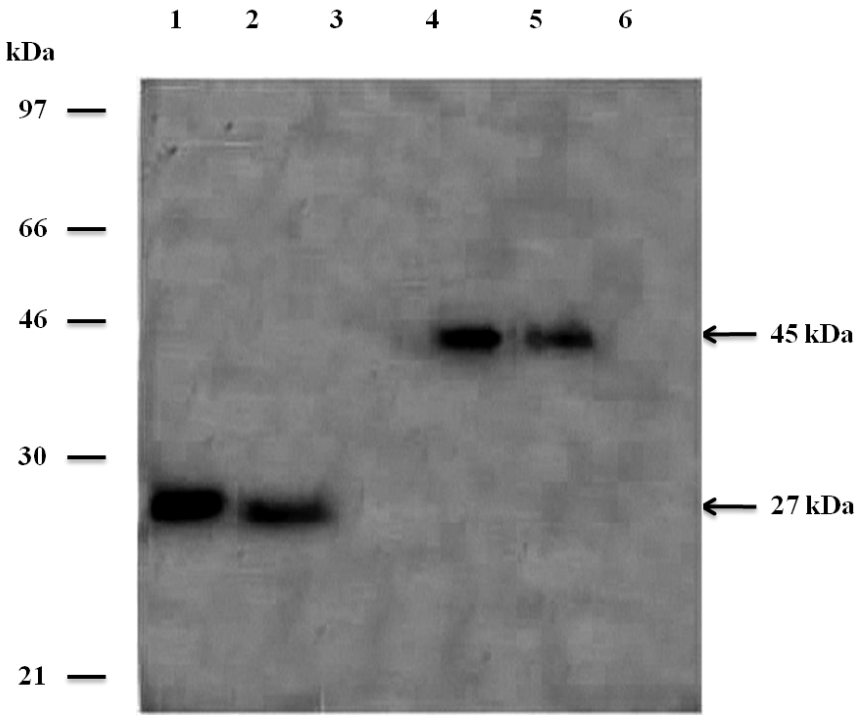
The ELISA test was performed using the antibodies raised against the 27 and 45-kDa mycelial protein of *C. falcatum* diluted to 1:10000.

Detection of *C. falcatum* in red rot-infected sugarcane extracts by ELISA

Sample	Optical density at 405 nm	
	Antiserum against 27 kDa protein	Antiserum against 45 kDa protein
Protein extracts from red rot-infected sugarcane tissues	0.667	0.736
Healthy sugarcane tissues	0.179	0.198
Control (buffer)	0.096	0.069

The ELISA test was performed using the antibodies at a dilution of 1:10000. Each measurement is an average of the readings from three wells

Immunoblot analysis of the total proteins extracted from red rot- infected or healthy sugarcane plants



Lanes 1 & 4, 100 µg of mycelial proteins from *C. falcatum* race Cf 05; Lanes 2 & 5, protein extract from infected sugarcane tissues; Lanes 3 & 6, protein extract from healthy sugarcane tissues

Conclusions

- The SCAR primers are highly specific to *C. falcatum*
- The molecular detection sensitivity of *C. falcatum* is 0.1 ng of purified *C. falcatum* DNA template and 15 ng of DNA from red rot infected sugarcane tissue.
- The developed antibody is very sensitive and could detect *C. falcatum* proteins even at a dilution of 1:50000
- The antibodies are specific to *C. falcatum* and all the isolates/races of *C. falcatum* reacted strongly with the antibodies in the ELISA
- The PCR-based assay and ELISA-based detection methods may be highly useful in detection of *C. falcatum* in seed canes

Acknowledgements

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Thank you