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Use of RAPD-PCR and Phage display techniques to study variation in *Colletotrichum gloeosporioides* isolated from Papaya (*Carica papaya* L)

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ABSTRACT

ScFv monoclonal antibodies were raised against Colletotrichum gloeosporioides (Penz.) Penz. and Sacc. isolates of papaya using phage display technology. Phages obtained after fourth round of biopanning were used to generate monoclonal antibodies against the pathogen. Four monoclonals were identified having the highest binding affinity to C. gloeosporioides and were selected to differentiate isolates of C. gloeosporioides along with isolates of C. capsici, Fusarium oxysporum f.sp. cubense and Alternaria spp. Cetyle trimethyl ammonium bromide (CTAB) method with some modifications was followed to isolate total genomic DNA in another experiment. Initially, DNA samples were amplified with 20 different OPA random primers and two of them were selected to amplify individual DNA samples from the eight papaya isolates and C. capsici, Fusarium oxysporum f.sp. cubense and Alternaria spp . All monoclones found to have high binding affinity towards different isolates of C. gloeosporioides compared to three other species, indicating their specificity towards C. gloeosporioides. There was a greater variation observed among isolates according to band patterns when amplified with OPA 3 and OPA 14 primers selected. However, with both these primers, there were common bands(1200 bp with OPA 3 and 965 bp with OPA 14) that may be specific to C. gloeosporioides.

Key words: *Colletotrichum gloeosporioides,* Monoclonal antibody, RAPD, Phage display, variation

INTRODUCTION

Species of *Colletotrichum,* commonly known for causing anthracnose disease, attack a large number of important tropical and sub-tropical crop species and cause economically significant diseases of cereals, grain legumes, forage legumes, vegetables, fruit crops and other perennial crops. Currently around 40 species of the Genus *Colletotrichum* reported based on more detailed studies on morphology, cultural characters, and pathogenic abilities (Cannon, *et. al.,* 2000). The variations among *Colletotrichum* species have been described by various authors (Sutton 1980; Sutton 1992) and a variety of molecular approaches have also been used to discriminate various *Colletotrichum* species (Brown *et al.* 1996; Buddie *et al.* 1999; Afanador-Kafuri *et al.,* 2003) or isolates (Denoyes-Rothan *et al.* 2003; Weeds *et al.* 2003). Different forms of nucleic acid analysis currently provides the most reliable framework to build a classification of Genus *Colletotrichum*. However, no attempts have been made to study in detail specifically about differentiation of papaya isolates of *C. gloeosporioides* based on molecular characteristics.

Phage display is a molecular technique by which peptides, proteins or antibody fragments are expressed at the surface of phage particles (Smith 1985). This is an extremely powerful tool for selecting peptides or proteins with specific binding properties from vast numbers of variants. Using phage display, vast numbers of variant nucleotide sequences may be converted into populations of variant peptides and proteins, which may be screened for, desired properties (Willats 2002). Traditionally, antibodies were obtained only from immunized animals; however, recent progress in molecular biology has made it possible to produce monoclonal antibody fragments displayed on the surface of phages as fusion protein with one of the coat protein gene. Antibodies bind antigens, including microorganisms, with high specificity and have been used in immunoassays for the rapid detection of pathogens (Koo *et al*. 1998). The use of antibodies has shorten the time required for microbial enrichment and isolation from a few days to a few hours (Koo *et al.* 1998). Several authors have suggested the potential use of phage display technique in identification of plant pathogens at species level or even beyond that (Griep *et al.* 1998; Koo *et al.* 1998; Gough *et al.* 1999; Griep *et al.* 1999 and Di Carli *et al.* 2003).

The current experiment was carried out to study the variation in *C. gloeosporioides* isolated from papaya using Randomly Amplified Polymorphic DNA (RAPD) technique and monoclonal antibodies developed through phage display technique.
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MATERIALS AND METHODS

All experiments were conducted at the Indian Institute of Horticultural Research (IIHR), Hessaraghatta, Bangalore. Papaya leaves and fruits infected with anthracnose disease were collected from different parts of southern India and pathogen was isolated, purified and maintained on Potato Dextrose Agar (PDA) medium.

Development of monoclonal antibodies

Screening of phage display library was accomplished by an affinity selection (bio-panning) process during which phage populations were exposed to the target (*C. gloeosporioides* IIHR-0 isolate) in order to selectively capture binding phages. Throughout the successive rounds of binding, washing, elution and amplification, the originally very diverse phage population was increasingly enriched with phages with a propensity to bind to the *C. gloeosporioides* in question. M13 K07 bacteriophages constructed by Griffin scFv library (Griffiths *et al.* 1994) were used in this study to generate monoclonal antibodies against *C. gloeosporioides*. The library was obtained from MRC centre for Protein Engineering, Cambridge, UK. The phages carrying antibody specific to *C. gloeosporioides* was selected using immunotubes by a process called biopanning. Selection on immunotubes is the method of several biopanning procedures followed here, to generate antibody against the pathogen.

First Round of Biopanning

C. gloeosporioides (isolate IIHR – 0) grown for seven days on Richard's broth was filtered through Whatman no. 45 filter paper disc, washed with sterile distilled water and air dried. One gram of fungal mycelium was ground with 4ml coating buffer (Na_2CO_3 1.59g, $NaHCO_3$ 2.93g, Distilled water 1000ml, pH9.6). The antigen was coated overnight on to the nunc immunotube (Gibco BRL, Life Technologies Ltd., UK) at room temperature (25^{o}C) at a concentration of 250mg/ml in a final volume of 4ml. The tube was washed thrice in the following day with 1x Phosphate buffer saline (PBS) (8.0g NaCl, 0.2g KCl, 1.44g Na_2HPO_4, 0.24g KH_2PO_4, 1000ml distilled water, pH7.4) followed by blocking with 4ml of 3% BSA made in 1X PBS, for 2 hours at 37^{o}C . The tube was then washed thrice with 1X PBS.

Phages ($1x10^{12}$) previously isolated from ScFv Griffin library were added to 4ml of 3% BSA in 1x PBS and binding of those phages to the antigen coated in the tube was carried out by incubating for 30 minutes at room temperature and rotating continuously on an under-and-over turnable equipment. The tubes were further incubated for 90 minutes at room temperature without shaking then washed 10 times with 1X PBS containing 0.05% tween-20, and 10 times with 1x PBS to remove the detergent.

Each washing step was performed by pouring buffer in and immediately pouring it out. Excess PBS in the tube was shaken out and then the bound phages were eluted by adding 1ml 100mM triethylamine and rotating continuously for 10 minutes. The phages were quickly neutralized by adding 0.5ml of 1M Tris HCl (pH 7.4) to the eluted phage.

In a 50ml tube, 9.25ml of an exponentially growing culture of K12 bacteria was taken and the 0.75ml of the eluted phages (Elute) was added to it to elute tight binders. Four millilitres of the K12 culture was also added to the immunotube (Nunc). Both cultures (Elute and Nunc) were incubated for 30 minutes in a 37^{0}C water bath without shaking. From 10ml and 4ml of the above infected cultures, 100ml cultures were taken separately to make 4 to 5, 100 fold serial dilutions. These dilutions were plated on Tryptone yeast extract (TYE) media containing 100mg/ml Carbenicillin, 2mg/ml Tetracycline and 1% glucose and the plates were incubated at 37^{0}C overnight. Remaining infected K12 culture was spun at 3,300rpm for 10 minutes to pellet the cells and the cell pellet was suspended in 1ml 2xTY. Eluted phages (10ml) and phages in nunc tube (4ml) were used for plating on petriplates separately with TYE containing 100mg/ml Carbenicillin, 2mg/ml Tetracycline and 1% glucose and were incubated overnight at 37^{0}C.

Further rounds of selection (biopanning)

To amplify the scFv phages obtained after the first round of selection from the first biopanning, the following procedure was used. Two to three milliliters of 2xTY, 15% glycol mixture was added to the bacterial colonies growing overnight on the petri dishes in the previous experiment and the cells were loosened with a glass spreader. Hundred micro liters of the above scraped bacteria were then added to 100ml of 2xTY containing 100mg/ml Carbenicillin, 2mg/ml Tetracycline and 1% glucose. The remaining bacteria were stored at –70^{o}C. Bacteria were grown with shaking at 37^{0}C until the OD at 600nm reached 0.5. Ten milliliters of this bacterial culture was taken separately into two clean tubes and infected with M13K07 helper phage by adding helper phage in the ratio of 1:20 (bacterial cells: helper phage). These tubes were then incubated without shaking in 37^{0}C in a water bath for 30minutes. The infected cells were centrifuged at 3,300rpm for 10 minutes at 10^{0}C. The pellet was gently resuspended in 50ml of 2xTY containing 100mg/ml Carbenicillin and 25mg/ml Kanamycin

and incubated shaking at 37^0C overnight. The phages were isolated from the supernatant by PEG/ NaCl method. One-fifth volume of PEG/NaCl (20% polyethylene glycol 6000, 2.5M NaCl) was added to the supernatant containing M13 bacteriophage and incubated for 1h on ice to precipitate phages. The phages were collected by centrifugating at 10,800g for 15minutes. Pellet was resuspended in 2ml of PBS and again added 1/5 volume PEG/NaCl and incubated for 1h on ice. Tube with PBS containing phages was spun at 10,800g for 15minutes. The pellet was dissolved in 4ml of 1x PBS and filter sterilized using 0.45mm filter. One milliliter of this phage was used in the next round of biopanning and remaining 1ml was stored at 4°C.

Second round of biopanning was performed similar to that of first round except that the immunotube was blocked with 3% Boehringer Mannheim (B&M) blocking reagent and by adding the phages obtained from the first round of biopanning instead of library phages. The rest of the steps were same as those of first biopanning. After second round of biopanning, third and fourth rounds of biopanning were performed in the same manner as that of second round. However, nunc tubes were blocked with 2% Marvin instead of B&M blocking reagent in the third round and with 3% BSA in the fourth round and infected with phages obtained from the previous round of biopanning for each round of selection.

Screening of phage particles by ELISA
Polyclonal ELISA
Populations of scFv phage produced at each round of selection were screened for their binding affinity to *C. gloeosporioides* by ELISA to identify polyclonal phage antibodies. Binding of scFv phages to *C. gloeosporioides* in ELISA was detected by anti-M13 conjugate and OPD substrate (Peroxidase substrate sigma fast O-phenylenediamine Dihydrochloride).

The antigen was prepared by finely grinding *C. gloeosporioides* mycelium (IIHR – 0) in carbonate buffer so as to get 100mg/ml concentration. Ten micro gram of the antigen in 100ml final volume was coated per well of an ELISA plate. The control consisted of carbonate buffer without the antigen. Coated ELISA plate was incubated overnight at room temperature. Unbound phages were rinsed 3 times with 1x PBS and the excess liquid was discarded by flipping over the ELISA plates. The wells were blocked with 2% B & M blocking solution (100ml per well) and incubated for 2h at 37°C. The wells were washed thrice with 1xPBS and excess buffer was discarded by flipping over the ELISA plate. Phages from each biopanning at a concentration of $4x10^{12}$ was prepared in 2% B&M in 1x PBS solution and were added as 100ml per well except in controls. The ELISA plate was incubated for 90 minutes at room temperature and was washed 3 times with 0.05% PBS/Tween 20 followed by 3 times with 1x PBS. Anti-M13 conjugated to horse rabbit peroxidase (HRP) diluted in 2% B&M in 1x PBS at 2:10,000 was added at the rate of 100 ml per well. The ELISA plate with the antibody was incubated for 90 minutes at room temperature. The plate was washed 3 times with 0.05% PBS/Tween-20 followed by 3 times with 1x PBS. Hundred milliliters of freshly prepared OPD substrate was added to each well and was incubated in the dark. ELISA readings at 450 nm was done 30 minutes after adding the substrate.

Generation of monoclonal antibodies
Phages obtained after fourth round of biopanning were used to generate monoclonal antibodies against *C. gloeosporioides*. K12 bacterial cells from the glycerol stock was incubated into 5ml 2xTY containing tetracycline 2mg/ml and incubated shaking at 37^0C till OD at 600nm reached 0.6. Those cells were then infected with Eluted phages obtained after 4^{th} biopanning in the ratio of 1:20 (Number of bacterial cells to the number of phages). Infected cells were collected and plated on petri-plates containing TYE with Carbenicillin 100 mg/ml, Tetracycline 2mg/ml and 1% glucose. The plates were incubated overnight at 37°C. Following day, single bacterial colonies were picked and inoculated into 3ml of 2xTY medium containing 100 mg/ml Carbenicillin, 2mg/ml Tetracycline and 1% glucose. Colonies were grown at 37°C under shaking till the OD reached 0.6 at 600 nm. One milliliter of the above culture was infected with M13 K07 helper phage as mentioned before. After infection, the cells were allowed to grow in 5ml of 2xTY containing 25mg/ml Kanamycin, 100mg/ml Carbenicillin and 2mg/ml Tetracycline overnight, under shaking condition at 37^0C

Isolation of ScFv phages from each colony
Since each colony was infected with one particular scFv phage obtained at the end of fourth biopanning, the phages isolated from each of these colonies after M13 K07 helper phage infection represents monoclonal phages against *C. gloeosporioides*. Five milliliters of overnight grown cultures from above, were spun at 10,000rpm for 10 minutes at 4^0C. To the supernatant, $1/5^{th}$ volume PEG/NaCl was added, mixed and incubated on ice for one hour. Tubes containing PEG/NaCl and supernatant were spun at 10,000rpm for 20 minutes at 4°C. Pellet was resuspended in 2ml of 1x PBS and was spun briefly to remove left over bacteria. Again $1/5^{th}$ volume of PEG/NaCl was added to the supernatant and incubated on ice for 1 hour. Supernatant was spun at 10,000rpm and the phage pellet was

resuspended in 200ml 1x PBS and stored at 4^0C for further use. Phages isolated from each colony were spectrophotometrically quantified.

Monoclonal ELISA

After the isolation of monoclonal phages in the above procedure, the scFv phage with highest binding affinity to *C. gloeosporioides* was identified by performing monoclonal ELISA. In this experiment, phages obtained from each of 50 clones were used and the best ones were identified as follows.

In the 96-well ELISA plate, 100ml of *C. gloeosporioides* (10mg/ml carbonate buffer) per well, were coated along with appropriate controls. The plate was incubated overnight at room temperature. Next day morning, the wells were rinsed thrice with PBS and excess buffer was removed by flipping over the ELISA plate. All the wells were blocked with 100 ml of 3% BSA-PBS for 2h at 37^0C. Wells were then rinsed thrice with 1x PBS. To each well, 100ml of $1x10^{12}$ scFv's obtained from different colonies prepared in 3% BSA-PBS were added and the plate was incubated for 90 minutes at room temperature. Wells were washed thrice with 0.05% PBS in tween-20 and then three times with 1x PBS. HRP-anti M13 conjugate was prepared in 3% BSA-PBS in 1:10,000 dilution and added 100ml to each well. ELISA plate was incubated for 90 minutes at room temperature. Wells were washed thrice with 0.05% PBS in Tween 20 and then 3 times with 1x PBS. OPD substrate (10ml) was prepared by dissolving substrate tablets in 10ml of water and 100ml of this substrate was added to each well. ELISA readings were taken 30 minutes after adding the substrate.

Differentiation of C. gloeosporioides isolates using monoclonal antibodies

After doing monoclonal ELISA, four monoclones were identified as having the highest binding affinity to *C. gloeosporioides* and were selected to differentiate 8 isolates of *C. gloeosporioides* along with isolates of *C. capsici, Fusarium oxysporum* f.sp. *cubense* (FOC) and *Alternaria* spp. The same ELISA procedure was used in differentiation of isolates. The antigen concentration used was 100 mg/ml for coating ELISA plate in all isolates. The controls used for in the experiment are as follows:

Each antigen was coated to the wells and all reagents were added except the momoclonal antibodies used in the experiment. This was done so as to detect non-specific binding of anti-M_{13} monoclonals to various antigens used in the experiment. The ELISA readings were obtained 30 minutes after adding the substrate.

Characterization of C. gloeosporioides isolates by RAPD-PCR

Cetyle Trimethyl Ammonium Bromide (CTAB) method (Scott and Arnold 1998) was followed in isolation of total genomic DNA from the fungal mycelium. Nucleic acids from fungal cells were liberated by CTAB and later precipitated with ethanol and further purified with phenol: chloroform to remove proteins and other contaminants. The resulting DNA pellet was dissolved in minimum volume of TE buffer and stored at $4\ ^0$C. The DNA isolated from *C. gloeosporioides* was subjected to polymerase chain reaction (PCR). Initially, the DNA samples were amplified with 20 different OPA random primers. Two of them were more informative and were used to amplify individual DNA samples from the eight isolates.

In small PCR tubes, with 37ml of sterile double distilled water, 2ml (175ng of DNA) of template DNA was added and boiled for 10 minutes and quenched on ice. Five ml of 10x polymerase buffer, 1ml of 1mM dNTP's, 3ml of 25mM $MgCl_2$ was added to the above mixture and mixed gently. One ml of the primer was added to the above and mixed gently. The reaction mixture was boiled for one minute in a water bath and 1ml (5 units) of Taq polymerase enzyme was added. Reaction tube was centrifuged briefly and was loaded in the thermocycler with the following programme. Denaturation of DNA at 94^0C for 5 minutes, 35 cycles of 94^0C for 2 minutes followed by primer annealing of 35^0C for 1 minute and primer extension of 72^0C for 1 minute. Final extension was done at 72^0C for 10 minutes. This PCR product was electrophoresed on 1.2 per cent agarose gel.

Experimental data were analyzed using SAS software (SAS Institute, Cary, NC) and means were separated by Duncan's multiple range test (P=0.05).

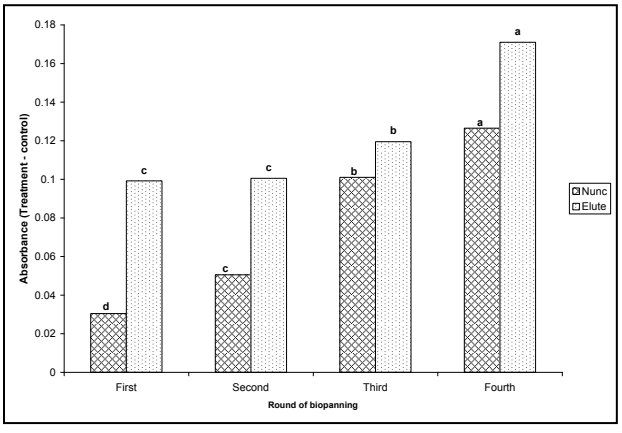

Figure 1: Binding affinity of polyclonal scFv antibodies to *C. gloeosporioides* after each round of selection

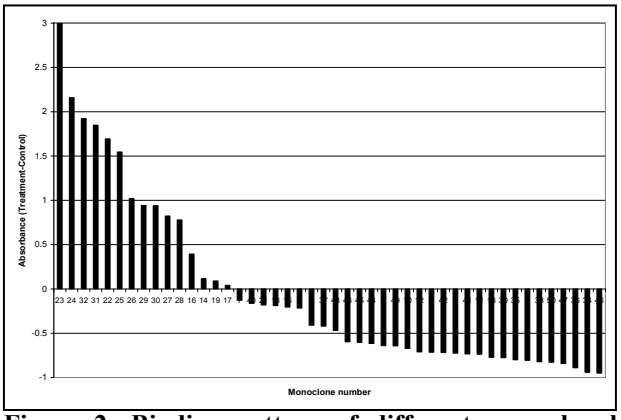

Figure 2: Binding pattern of different monoclonal phage scFvs to *C. gloeosporioides*

RESULTS AND DISCUSSION

Polyclonal phage ELISA

There was a trend of increasing binding affinity of scFv phages to *C. gloeosporioides* in progressive rounds of selection. A high binding affinity to *C. gloeosporioides* was observed when phages produced from the fourth round of biopaning (Figure. 1) and the difference was significant (P=0.05). Since, scFv phages were selected every time in each biopanning, an increasing binding affinity towards the pathogen at each round of selection could be expected. From the results obtained in the polyclonal ELISA, it is clear that phages eluted at the 4th biopanning, had the highest binding affinity to *C. gloeosporioides* than the other phages obtained after each round of selection. Therefore, eluted phages in the 4th round of selection (Elute IV) were selected and further used for generation of monoclonal antibodies against *C. gloeosporioides*.

Monoclonal phage ELISA

There was a variation in binding affinity among 50 monoclonals (colonies) tested against *C. gloeosporioides* (Figure. 2). Fifteen monoclonals were found to have higher binding affinity (positive ELISA readings) to *C. gloeosporioides* compared to control. Among these high binders, the highest binding affinity was observed in monoclone 23, 24, 32 and 31 and these values were more than the mean plus one standard deviation therefore, chosen to differentiate isolates. These monoclones are highly specific and binds strongly with the specific antigen. Although they are raised against the same pathogen, it is not necessarily that specific binding sites of monoclones should always match with the binding sites of the antigen (IIHR-0 in this experiment) in question (Hawkins, *et al.* 1992). This was seen in the results where only fifteen monoclones among 50 tested had higher binding affinity towards *C. gloeosporioides* IIHR-0 isolate.

Differentiation of isolates using monoclonal antibodies

There was a significant difference among isolates and their interaction with different monoclonals. However, there was no significant difference among monoclonals (P=0.05) for their binding affinity to different isolates (Table. 1). Among the isolates, IIHR-0 had the highest ELISA reading (0.122) followed by AP-FB (0.118), AP-FP (0.105) and HD-3 (0.101). However, these four isolates did not differ significantly in their response to scFv antibodies (Table. 1). The binding affinity of *Alternaria* spp, FOC and *C. capsici* was found to be significantly less compared to *C. gloeosporioides* isolates. The least binding affinity towards scFv monoclones was observed in *Alternaria* spp. Among papaya isolates, IIHR-1, CB, NM-F and NM-P had less binding reaction with monoclone phages and differ significantly with IIHR-0 isolate. *C. capsici* isolate had significantly higher binding affinity for monoclonal scFv phages developed against *C. gloeosporioides* compared to two other non *Colletotrichum* species *viz* FOC and *Alternaria* spp. (Table. 1).

The interaction between isolates and scFv monoclones was significant at P=0.05. The highest ELISA reading (0.170) was observed in monoclone 23 with AP-FB isolate while the lowest value (0.028) was recorded in the same monoclone (23) with *Alternaria* isolate (Table. 1). Among *C. gloeosporioides* isolates, the lowest value (0.053) was recorded in IIHR-1 with monoclone 23. According to above results, IIHR-0, the isolate used to raise monoclones, had the highest binding affinity with four different monoclones. All monoclones found to have less binding affinity towards non-*C. gloeo-*

Table 1: ELISA readings of *C. gloeosporioides* isolates with four scFv monoclones 30 minutes after adding the substrate

Isolates	Monoclone				Mean*
	24	23	31	32	
IIHR-0	0.165	0.122	0.099	0.105	0.122^{a}
IIHR-1	0.087	0.053	0.122	0.109	0.092^{c}
CB	0.076	0.083	0.102	0.102	0.090^{c}
HD-3	0.104	0.104	0.100	0.099	0.101^{bc}
AP-FP	0.103	0.131	0.096	0.093	0.105^{abc}
AP-FB	0.089	0.170	0.112	0.104	0.118^{ab}
NM-F	0.082	0.106	0.097	0.101	0.096^{c}
NM-P	0.099	0.103	0.055	0.114	0.092^{c}
FOC	0.034	0.032	0.076	0.034	0.049^{e}
***Alternaria* spp.**	0.042	0.028	0.037	0.048	0.038^{e}
C. capsici	0.047	0.067	0.074	0.105	0.071^{d}
Mean*	0.086^{a}	0.093^{a}	0.091^{a}	0.086^{a}	-

***** Means with same letters within the column and row are not significantly different using Duncan's multiple range test (DMRT) at P=0.05.

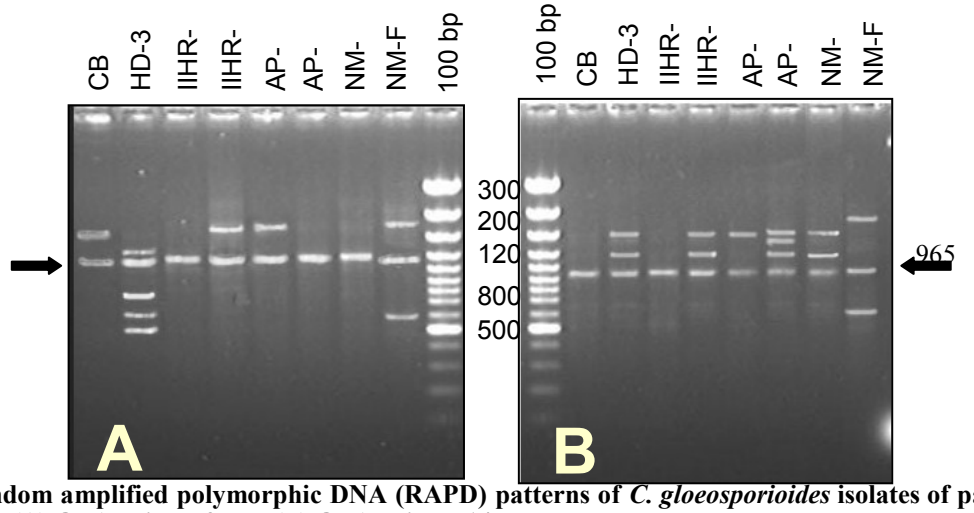

Plate 1: Random amplified polymorphic DNA (RAPD) patterns of *C. gloeosporioides* isolates of papaya obtained with (A) OPA primer 3 and (B) OPA primer 14.

sporioides isolates, indicating their specificity towards *C. gloeosporioides*. It is evident from the results that no considerable variation existed in the four monoclones on their binding affinity to these isolates and can be suggested that any of these clones can be used in differentiation of isolates.

However, among four monoclones tested, monoclone 23 found to be good for differentiation of isolates since it showed greater degree of variability between isolates. For example, the highest ELISA reading (0.170) was observed in the experiment in monoclone 23 with AP-FB isolate while the lowest value (0.028) was also recorded in the same monoclone (23) with *Alternaria* isolate. Based on this, it is clear that the monoclones developed in this research can be used to distinguish *C. gloeosporioides* from other species. Since there were different responses observed in different isolates with monoclonal antibodies, these scFv fragments could represent a valuable tool for inexpensive immunodiagnosis of *C. gloeosporioides* isolates. Similar work has been reported by Koo *et al.* (1998), where they developed small recombinant antibody fragments, which had *Bacillus cereus* spore binding ability and antigen specificity. Gough *et al.,* 1999, also reported scFv antibodies subcloned as fusions at the C-terminus of maltose binding protein (MBP) were bound to *P. infestans* germlings and to mycelial homogenates from various *Phytophthora* species. According to their study, the binding activities of these scFv antibodies to mycelial homogenates of fungal species not belonging to the order *Peronosporales* were substantially lower.

Differentiation of isolates using RAPD-PCR

OPA 3 and OPA 14 primers gave polymorphic PCR products from all isolates of *C. gloeosporioides* (Plate 1). With OPA 3 primer, IIHR-1, AP-FP and CB isolates produced similar band pattern and the band patterns of AP-FB, NM-P and IIHR-0 isolates were also identical (Plate 1A). HD-3 and NM-F isolates both produced different type of band pattern compared to other isolates. It is observed that 1200 bp band was common to all isolates when amplified with OPA 3.

CB and IIHR-0 isolates had similar band pattern with primer OPA 14 (Plate 1B). There was a similarity observed among isolates of HD, IIHR-1 and NM-P with this primer. Isolates AP-FP, AP-FB and NM-F all differed in their band pattern among themselves and also with others. However, 965 bp band found to be common for all isolates when amplified with OPA 14 (Plate 1B).

It is often assumed that DNA fragments of the same molecular size amplified in RAPD assays represent homologous sequences (Jiménez-Gasco and Jiménez-Díaz 2003). Therefore, isolates with monomorphic banding pattern can be considered having similar genetic composition. Grouping isolates based on similarity in band pattern could not be performed in this study due to many variations in their band patterns with these two primers, however, common bands to all isolates with OPA 3 and OPA 14 could be specific to papaya isolates of *C. gloeosporioides*

CONCLUSION

Monoclonal antibodies produced using phage display technique showed variations in the binding affinity of scFv to *C. gloeosporioides*. Those high biding scFv phages could distinguish *C. gloeosporioides* isolates of papaya from three other fungi *viz., C. capsici, Alternaria* spp., and *Fusarium oxysporum* f.sp. *cubense*. There was no literature available to date on the development of monoclonal

antibodies against *C. gloeosporioides* using phage display technique and its application in differentiation of isolates. The current study exhibits the usefulness of this technique in detection and identification of subgroups within the species of *Colletotrichum*. There was a greater variation observed among isolates according to band patterns of the RAPD-PCR experiment. Two common bands observed with OPA 3 and OPA 14 primers can be specific to *C. gloeosporioides* isolates of papaya

REFERENCES

Afanador-Kufuri L, Maymon DMM and Freeman S 2003 Characterization of *Colletotrichum* isolates from tamarillo, passiflora, and mango in Colombia and identification of a unique species from the genus. *Phytopathology,* 93 (5):579-587.

Brown AE, Sreenivasaprsad S and Timmer LW 1996 Molecular characterization of slow-growing orange and key lime anthracnose strains of *Colletotrichum* from citrus as *C. acutatum. Phytopathology,* 86:523-527.

Buddie AG, Martinez-Culebras P, Bridge PD Garcia MD, Querol A, Cannon PF and Monte E 1999 Molecular characterization of *Colletotrichum* strains derived from strawberry. *Mycol. Res.,* 103:385-394.

Cannon PF, Bridge PD and Monte E 2000 Linking the past, present, and future of *Colletotrichum* systematics. pp. 1-20. In. Prusky, D. Freeman, S. and Dickman, M B (eds) Colletotrichum*: Host Specificity, Pathology, and Host-Pathogen Interaction.,* APS Press, St. Paul, Minnesota,

Denoyes-Rothan B, Guerin G, Delye C Smith B, Minz D, Maymon M and Freeman S 2003 Genetic diversity and pathogenic variability amoung isolates of *Colletotrichum* species from strawberry. *Phytopathology,* 93 (2): 219-228.

Di Carli M, Villani ME, Lico C and Benvenuto E 2003 Isolation of single chain Fv antibody fragments against potato virus X (PVX) from phage display libraries. In: *Proceedings of the XLVII Italian society of Agricultural Genetics – SIGA Annual congress Verona, Italy, 24-27 september, 2003,* Poster abstract.

Gough KC, Li Y, Vaughan TJ, Williams AJ, Cockburn W and Whitelam GC 1999 Selection of phage antibodies to surface epitopes of *Phytophthora infestans*, *J. Immunol. Methods.* 228 (1): 97-108.

Griep RA, Twisk van C, Beckhoven van JRCM, Van der Wolf JM and Schots A 1998 Development of specific recombinant monoclonal antibodies against the lipopolysaccharides of *Ralstonia solanacearum* Race 3, *Phytopatholog.,* 88 (8): 795-803.

Griep RA, van Twisk C and Schots A 1999 Selection of beet necrotic yellow vein virus specific single-chain Fv antibodies from a semi-synthetic combinatorial antibody library, *Eur. J. Plant Pathol*., 105: 147-156.

Griffiths AD, Williams SC, Hartley O, Tomlinson, IM, Waterhouse P, Crosby WL, Kontermann R E, Jones PT, Low NM, Allison TJ, Prospero T D, Hoogenboom HR, Nissim A, Cox JPL, Harrison JL, Zaccolo M, Gherardi E and Winter G 1994 Isolation of high affinity human antibodies directly from large synthetic repertoires, *EMBO J.* 13 (14): 3245-3260.

Hawkins ER, Russel SJ and Winter G 1992 Selection of phage antibodies by binding affinity mimicking affinity maturation, *Mol. Biol.* 226: 889-896.

Jiménez-Gasco M del M and Jiménez-Díaz RM 2003 Development of a Specific Polymerase Chain Reaction-Based Assay for the Identification of *Fusarium oxysporum* f. sp. *ciceris* and Its Pathogenic Races 0, 1A, 5, and 6, *Phytopathology,* 93(2): 199-207.

Koo K, Foegeding PM and Swasigood HE 1998 Development of a streptavidin-conjugated single chain antibody that binds *Bacillus cereus* spores, *App. Envt. Microbiol.* 64(7): 2497-2502.

Smith GP 1985 Filamentous fusion phage: Novel expression vectors that display cloned antigens on the virion surface, *Science.* 228: 1315-1317.

Sutton BC 1980 *The Coelomycetes: Fungi imperfecti with Pycnidia, Acervuli, and Stromata*, Commonwealth Mycological Institute, Kew, UK.

.Sutton BC 1992 The genus *Glomerella* and its anamorph *Colletotrichum*. In: *Colletotrichum: Biology, Pathology and Control,* 1–26 pp, (eds.) J.A. Bailey and M.J. Jeger, CAB International, Wallingford, UK.

Willats WGT 2002 Phage display: practicalities and prospects. *Pl. Mol. Biol.,* 50:837-854.