UNIVERSIDADE ESTADUAL DE CAMPINAS

INSTITUTO DE BIOLOGIA

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"CARACTERIZAÇÃO DOS EPÍTOPOS E DA ESTRUTURA SECUNDÁRIA DA PROTEÍNA DO CAPSÍDEO DO *Citrus tristeza virus* E UM DIAGNÓSTICO IMUNOMOLECULAR PARA *Xylella fastidiosa*"

Tese apresentada ao Instituto de Biologia para obtenção do Título de Doutor em Genética e Biologia Molecular na área de Imunologia.

Orientador: Profa. Dra. Dagmar Ruth Stach-Machado

Co-Orientador: Prof. Dr. Marcos Antonio Machado

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Aos meus pais Maria Lúcia e Osmar e

ao meu irmão Eduardo

pelo apoio, amor e confiança!

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ÍNDICE

APTA	Centro Avançado de Pesquisa Tecnológica do Agronegócio
BCIP	5-brommo-4chloro-3indoyl phosphate
BLAST	Basic Local Alignment Search Tool
CAT1	Catalase isoforma 1
CAT2	Catalase isoforma 2
CB	Complexo Capão Bonito
CD	Espectroscopia por Dicroísmo Circular
CP	Proteína do capsídeo de 25 kDa (p25) (*coat protein*)
CPd	Proteína de 27 kDa (p27)
CTV	*Citrus tristeza virus*
CVC	Clorose Variegada dos Citros
D.O.	Densidade Óptica
DIBA	*Dot immunobinding assay*
DNA	Ácido desoxirribonucléico
dsRNA	RNA fita dupla
DTT	Dithiothreitol
E. coli	*Escherichia coli*
ELISA	Ensaio imunoenzimático (*Enzyme linked immunosorbent assay*)
EPS	Polissacarídeos extracelulares

EST	Seqüências expressas de RNAm (*expressed sequence tag*)
GPX	Glutationa peroxidase
GST	Glutationa S-transferase
HEL	Helicase
HSP70	Proteína homóloga a “heat shock protein” 70
IAC	Instituto Agronômico de Campinas
IC-PCR	Ensaio de Imunocaptura-PCR
ID-SDS	Imunodifusão na presença de dodecil sulfato de sódio
Ig	Imunoglobulina
IgG1	Imunoglobulina G1
IgG2b	Imunoglobulina G2b
I-PCR	Ensaio de Imuno-PCR
IPTG	Isopropylthiol-β-D-galactoside
ISEM	Microscopia eletrônica imunoespecífica
K_d	Constante de dissociação
kDa	kilo Daltons
LB	Meio Luria-Bertani
M	Molar
mA/cm^2	MiliAmper por centímetro quadrado
MAb	Anticorpo Monoclonal

MALDI-TOF *Matrix-assisted Laser Desorption and Ionization-Time of Flight*

ME Microscopia eletrônica

MT Metiltransferase

MT2 Metalotioneína isoforma 2

NBT Nitroblue tetrazólium

NCBI Centro Nacional de Informação Biotecnológica

Ni-NTA Coluna de afinidade a níquel

ORF Matriz de leitura aberta (*open reading frame*)

P Protease semelhante à papaína

p20 Proteína de 20 kDa.

PAb Soro policlonal

PBS Tampão fosfato salino

PCR Reação em cadeia da polimerase

PEG Polietilenoglicol

Phe Aminoácido fenilalanina

pNPP para-Nitrofenil fosfato

PVP Polivinilpirrolidona

qPCR Reação em cadeia da polimerase quantitativa

RdRp RNA polimerase RNA-dependente

RFLP Análise de Polimorfismo de fragmentos de restrição

RNA	Ácido Ribonucléico
RNAm	RNA mensageiro
ROS	Espécies reativas de oxigênio
rpm	Rotações por minuto
RT-PCR	Reação em cadeia da polimerase com transcrição reversa
SAXS	Difração de Raios X a Baixo ângulo (*Small-Angle X-ray Scattering*)
SDS-PAGE	Eletroforese em gel de poliacrilamida na presença de dodecil sulfato de sódio
SOD Cu/Zn	Superóxido dismutase dependente de cobre e zinco
SSCP	Análise de polimorfismo conformacional de fita simples de DNA
ssRNA	RNA fita simples
TBS	Tampão Tris Salino
Tyr	Aminoácido tirosina
X. fastidiosa	*Xylella fastidiosa*

1. RESUMO

Este trabalho visou efetuar a identificação dos epitopos das proteínas recombinantes CB-22 e CB-104 do capsídeo do *Citrus tristeza vírus* (CTV), reconhecidos pelos anticorpos monoclonais (MAb) produzidos por Stach-Machado e colaboradores (2000). Assim, os epitopos reconhecidos pelos MAbs 30.G.02 e 37.G.11, correspondem às seqüências de aminoácidos NLHIDPTLI e TQQNAALNRDLF, localizadas nas posições 32 a 40 e 50 a 61 das proteínas recombinantes CB-22 e CB-104. O epitopo reconhecido pelo MAb 39.07 que apresenta especificidade apenas para a CB-104, homóloga aos isolados severos do CTV corresponde a seqüência TDVVFNSKGIGN, localizados na posição 120 a 131 da proteína.

Estes dados corroboram com os ensaios de ELISA, confirmando o MAb 30.G.02 como um anticorpo universal, atuando quer como anticorpo de captura ou de detecção em ELISA Sandwich, uma vez que, seu epitopo é conservado em 87.2% dos isolados de CTV. O MAb 37.G.11 deve ser adotado apenas como anticorpo de detecção pois reconhece uma seqüência encontrada em 62,6% dos isolados, permitindo uma identificação ampla, sem efetuar a diferenciação de estirpes. O MAb 39.07 foi classificado como “forte”, pois seu epitopo está presente em apenas 19,7% das seqüências, sendo estas provenientes de isolados fortes do CTV de todo o mundo. A utilização de peptídeos lineares não permitiu a identificação e caracterização do epitopo do MAb IC.04-12, confirmando dados preliminares de nosso laboratório que permitiram inferir que o mesmo reconhece um epítopo conformacional.

O conhecimento das características estruturais da proteína do capsídeo viral possibilita obtenção de informações relevantes quanto às suas propriedades biológicas, como a participação na organização e montagem da partícula viral, proteção do material genético, interação com o inseto-vetor e com a planta hospedeira e também, pela “movimentação” da partícula viral no processo de infecção na planta. Considerando que até a presente data não existem dados disponíveis no Protein Data Bank (PDB) sobre a estrutura tridimensional da proteína do capsídeo do CTV, efetuamos o estudo estrutural da CB-22, utilizando Dicroísmo Circular (CD) e Difração de Raios X a Baixo Ângulo (SAXS).

A análise dos dados de CD mostrou um espectro característico de proteínas cuja estrutura secundária é predominantemente formada por α-hélices, sendo este resultado coerente com a estrutura secundária predita pelo software PSIPRED. Os estudos de SAXS revelaram uma proteína altamente oligomerizada com estruturas formadas por subunidades, que variam de acordo com a concentração da mesma em solução. Este resultado, embora tenha impossibilitado a resolução e modelagem da estrutura secundária da CB-22, demonstrou que esta proteína recombinante mantém a função da proteína nativa, responsável pela formação do capsídeo viral. Assim sendo, podemos postular a sua utilização como uma molécula carreadora, podendo ser aplicada em protocolos de vacinação ou no transporte dirigido de ácidos nucléicos ou proteínas.

Por fim, este trabalho estabeleceu diagnósticos imunomoleculares como Imunocaptura-PCR (IC-PCR) e Imuno-PCR (I-PCR) para a detecção da bactéria gram-negativa *Xylella fastidiosa*, agente etiológico da CVC. Os ensaios imunomoleculares apresentaram o limite de detecção muito superior aos diagnósticos utilizados na rotina como ELISA e PCR, sendo o limite de detecção do IC-PCR e do I-PCR de 10^3 a 10^1 células, respectivamente. Portanto, estes métodos são uma alternativa viável para efetuar o diagnóstico da CVC e também em estudos epidemiológicos, com a vantagem de eliminar as etapas de extração e concentração do material genético bacteriano, permitindo um diagnóstico acurado dessa doença.

2. ABSTRACT

This work aims to carry out the identification of epitopes of the *Citrus tristeza virus* (CTV) recombinant coat protein (CB-22 and CB-104) which are recognized by four monoclonal antibodies (MAb) produced by Stach-Machado *et al.* (2000). MAb IC.04-12 recognizes CB-22 protein, homologous to CTV isolates that induce weak symptoms in indicators plants; while MAb 39.07 reacts to CB-104 protein, homologous to severe CTV isolates and, MAb_s 30.G.02 and 37.G.11 recognize both proteins. MAb 30.G.02 recognized the sequence formed by amino acids NLHIDPTLI, located at positions 32 to 40, while MAb 37.G.11 recognized the amino acids from 50 to 61, whose sequence is TQQNAALNRDLF. On the other hand, the MAb 39.07 recognized the sequence TDVVFNSKGIGN, located at positions 120 to 131 in CB-104 protein.

MAb 30.G.02 was considered a Universal antibody, once its epitope is conserved in 87.2% of CTV isolates, and can be applied as a coating antibody or even as a detection antibody in ELISA Sandwich assays. The epitope of MAb 37.G.11 is conserved in 62.6% of sequences and, can be applied as detection antibody, allowing a quick and wide range screening but without strains differentiation. Finally, MAb 39.07 was classified as “Severe” antibody, once its epitope is present at only 19.7% of CTV sequences, which were derived from severe worldwide CTV isolates. The technique of linear peptides did not allow the epitope determination of MAb IC.04-12, since this antibody may recognize a conformational epitope in the CB-22 tridimensional structure, according to the early data from our laboratory showing that this MAb did not recognize the CB-22 under denaturating conditions, confirming the conformational nature of its eptiope.

The knowledge about structural features of viral coat protein enables acquiring relevant information about biological properties of this protein. The coat protein plays an essential role in the organization and assembly of viral particle, acts in genetic material protection and, probably is involved in the interaction with insect-vector and with host plant, being responsible for the movement of viral particle in the infection process. However, there are not any data in Protein Data Bank (PDB) about the secondary and tertiary structures of CTV coat protein, and so, this work carried out a structural study

about CB-22 by Circular Dichroism Spectroscopy (CD) and Small-Angle X-ray Scattering (SAXS).

The CD data analysis demonstrated a spectrum characteristic of proteins whose secondary structure is predominantly formed by α-helixes, and these results are coherent of predicted structure by PSIPRED software. The SAXS data revealed a highly oligomeric protein comprising many subunits whose quantities are dependent and vary according to the protein concentration in solution. These data, although had impaired the modeling and resolving of CB-22 secondary structure, demonstrated that this recombinant protein maintain the function of native protein, responsible for the assembly of CTV capsid, and this structure without bacterial DNA is also called *virus-like*, which allows to postulate its usefulness as nucleic acid carrier in vaccination protocols.

Finally, the third objective of this work was the establishment of immunomolecular diagnosis to gram-negative bacterium, *Xylella fastidiosa*, which causes Citrus Variegated Chlorosis in Brazil. We carried out two immunomolecular assays, like ImmmunoCapture-PCR and Immuno-PCR for direct detection of *X. fastidiosa* without DNA isolation. Whereas the reactivity of ELISA and PCR ranged from 10^6 to 10^4 bacterial cells, the IC-PCR sensitivity was up to 10^3 and the detection limit of I-PCR was up to 10^1 bacterial cells. Therefore, IC-PCR and I-PCR assays provide an alternative for quick and very sensitive methods to screening *X. fastidiosa*, with the advantage of not requiring any concentration or DNA purification steps while still allowing an accurate diagnosis of CVC.

3. INTRODUÇÃO

3.1. A TRISTEZA DOS CITROS

A tristeza dos citros é considerada uma das mais importantes doenças que afetaram a citricultura mundial e brasileira nos últimos 60 anos. Aparentemente originada na Ásia (WALLACE *et al*., 1956), a tristeza dos citros chegou ao Brasil por volta de 1930 (ROBERTS *et al*., 2001), onde causou a morte de 12 milhões de árvores (OLSEN *et al.*,2000). A natureza infecciosa da tristeza foi confirmada por MENEGHINE *et al*. (1946) que mostrou a transmissibilidade dela através do pulgão preto dos citros. Posteriormente também foi transmitida através de enxertia de borbulhas, confirmando que era causada por um agente patogênico (BAR-JOSEPH *et al.,* 1989). Anteriormente, inúmeras outras causas haviam sido propostas, dentre elas: incompatibilidade fisiológica entre a copa e o porta-enxerto, acidez e alto teor de umidade do solo, prolongados períodos de seca.

As partículas do *Citrus tristeza vírus* (CTV) foram observadas pela primeira vez no Brasil por KITAJIMA *et al*. (1963), através de técnicas de microscopia eletrônica (ME), onde observou partículas longas e flexíveis em preparações de plantas infectadas, não sendo observadas em plantas sadias, bem como em espécies e variedades de citros conhecidas por sua imunidade ou resistência ao CTV, como *Poncirus trifoliata, Severinia buxifolia* ou *Citrange Troyer* (*C. sinensis* x *P. trifoliata*) (KITAJIMA *et al.*, 1963).

A citricultura representa uma das principais atividades agroindustriais do mundo e, atualmente, os pomares mais produtivos resultantes de uma citricultura estruturada estão localizados nas regiões de clima tropical e subtropical, destacando-se o Brasil, Estados Unidos, México, China e África do Sul. O Brasil tornou-se, na década de 80, o maior produtor mundial, com mais de 1 milhão de hectares de plantas cítricas em seu território. A maior parte da produção brasileira destina-se a indústria de suco e está concentrada no Estado de São Paulo, responsável por 70% das laranjas e 98% do suco produzido no país, uma produção que supera 350 milhões de caixas (FUNDECITRUS, 2007; IEA, 2007).

A tristeza foi responsável pelo quase aniquilamento da citricultura paulista na década de quarenta, quando foi detectada atacando todas as combinações onde a laranja azeda (*Citrus aurantium* L.) era utilizada como porta-enxerto. Entretanto, a substituição

por porta-enxertos tolerantes, como limão Cravo (*Citrus limonia* Osb.), minimizaram os prejuízos causados pela doença e permitiram a implantação de pomares mais vigorosos e produtivos, resultando na retomada da exploração comercial de citros no país (MÜLLER e COSTA, 1993; PERSAD *et al.*, 2003). Associado a esse fato, está o estabelecimento do programa de proteção cruzada ou pré-imunização, obtida através da utilização de estirpes fracas do CTV que conferem certa imunidade à planta hospedeira, protegendo-as da ação de estirpes fortes (BAR-JOSEPH *et al*., 1979).

O CTV, além de induzir a morte da planta infectada induz duas doenças graves denominadas de *stem pitting* e *seedling yellows* (GARNSEY, 2002). O *stem pitting* induz um crescimento vascular anormal (SATYANARAYANA *et al*., 2001), fazendo com que o tronco da árvore apresente profundas depressões em algumas áreas da sua casca e também nas dos galhos e ramos, que leva a uma redução do tamanho, número e a qualidade dos frutos, afetando principalmente as mudas susceptíveis (laranja doce e grapefruit) independente da raiz em que as mesmas estão enxertadas (DJELOUAH e D´ONGHIA, 2001). Enquanto o *seedling yellows* induz clorose e interrupção do crescimento e desenvolvimento da planta, mas ocorre apenas em plantas inoculadas experimentalmente com alguns isolados de CTV, não sendo comumente encontrada no campo (DODDS et *al*., 1987).

O vírus da tristeza dos citros

O *Citrus triteza virus* (CTV) pertence à família *Closteroviridae,* cujos membros se caracterizam por possuírem nucleocapsídeos filamentosos e flexíveis, simetricamente helicoidais, com tamanho variando entre 700 a 2.000 nm e diâmetro entre 10 e 13 nm, sendo as partículas virais limitadas ao floema (KITAJIMA, 1963; BAR-JOSEPH *et al.*, 1979; DOLJA *et al.*, 1994) (**Figura 1**).

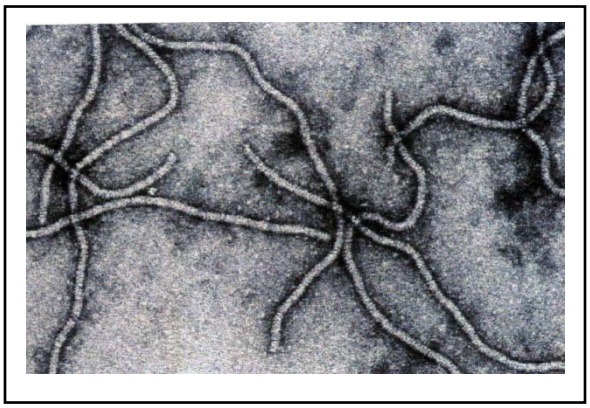

Figura 1. Fotomicrografia eletrônica de transmissão do CTV (Cortesia de G. W. Müller).

O genoma do CTV (**Figura 2**) é constituído por uma molécula de RNA fita simples (ssRNA), polaridade positiva, não segmentado, sem cauda poli-A na extremidade 3', com peso molecular aproximado de 6,5 x 10^3 kDa ou 20 kb, sendo considerado o maior genoma detectado entre os vírus de plantas (SEKIYA *et al.*, 1991).

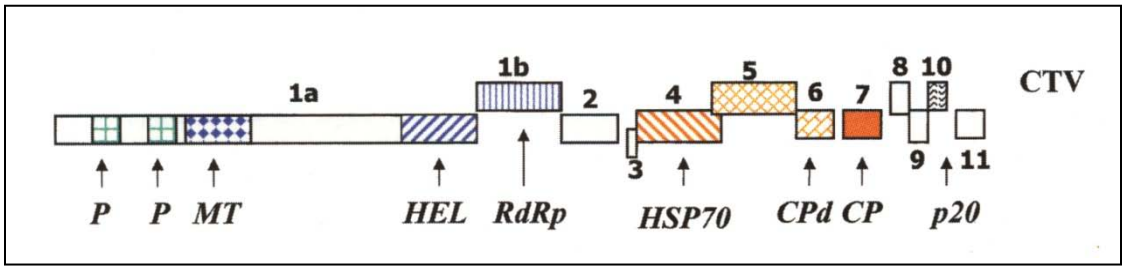

Figura 2. Diagrama esquemático do genoma do CTV adaptado de KARASEV *et al.* (1995). Os retângulos representam as matrizes de leitura aberta (ORF), indentificadas pelo número e respectiva seta: (P) Protease semelhante à papaína; (MT) Metiltransferase; (HEL) Helicase; (RdRp) RNA polimerase RNA-dependente; (HSP70) Proteína homóloga a 70 "heat shock protein"; (CPd) Proteína de 27 kDa (p27); (CP) Proteína do capsídeo de 25 kDa (p25) e (p20) Proteína de 20 kDa.

Os primeiros trabalhos de clonagem e seqüenciamento de nucleotídeos de CTV foram desenvolvidos por SEKIYA *et al.* (1991) com o gene da proteína do capsídeo viral do isolado de CTV T36, proveniente da Flórida. A análise da seqüência revelou a presença de 669 nucleotídeos flanqueados por vários códons de terminação.

Posteriormente, PAPPU *et al.* (1994) e KARASEV *et al.* (1995) seqüenciaram o genoma deste isolado e verificaram que os 19.296 nucleotídeos codificam 12 matrizes de leitura aberta ("open reading frames - ORFs"), que por sua vez, codificam 17 produtos protéicos (PAPPU *et al.,* 1994; KARASEV *et al.,* 2000). Estas ORFs podem ser divididas em dois blocos gênicos: o primeiro inclui os genes responsáveis pela replicação do vírus e pela síntese das proteínas, sendo conservado em outras famílias relacionadas, e o segundo bloco compreende as ORFs de 3 a 7, que estão relacionadas com a síntese das proteínas estruturais, como as do capsídeo viral.

As partículas virais de CTV contêm aproximadamente 4.000 moléculas do capsídeo viral (CP), formado pelas proteínas p27 e p25, com pesos moleculares de 27 kDa e 25 kDa (DOLJA *et al*., 1994; KARASEV *et al.*, 1995), respectivamente sendo codificadas pelas ORFs 6 e 7. Os virions são heterodímeros, sendo que a p27, descrita como uma cópia divergente da p25 (FEBRES *et al.,* 1994 e 1996), compõe apenas uma das extremidades do virion, correspondendo a uma porção de 75 a 85 ηm de comprimento, enquanto a p25 forma o restante da partícula viral, conferindo ao capsídeo uma estrutura polar, que pode estar relacionada com a movimentação do vírus entre as células.

Estudos realizados por ECALE ZHOU *et al.* (2002) confirmaram o reconhecimento específico das proteínas p25 e p27, constituintes do capsídeo do CTV, utilizando os anticorpos monoclonais CTV 908-6 e CTV 1212-3, específico para a p25 e outro, previamente descrito por FEBRES *et al.* (1994), específico para a p27. (**Figura 3**).

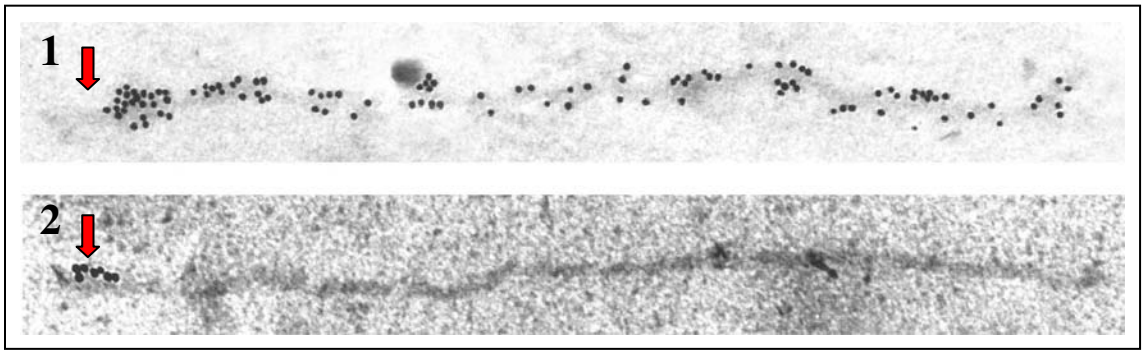

Figura 3. Fotomicrografias eletrônicas de transmissão demonstrando o reconhecimento específico das proteínas do capsídeo do CTV por anticorpos monoclonais (MAb) distintos marcados com partículas de ouro. As setas indicam a porção da partícula viral que possui a proteína p27. No painel **1** foram utilizados anticorpos específicos para a proteína p25, distribuída ao longo de quase toda a partícula viral. No painel **2** foi utilizado anticorpo específico para a proteína p27, localizada apenas em uma das extremidades do vírus (ECALE ZHOU *et al.*, 2002).

Diversidade Biológica

O CTV é constituído por uma mistura complexa de estirpes que diferem nas suas propriedades biológicas, principalmente na severidade induzida em diferentes hospedeiros e na transmissibilidade por afídeos (BAR-JOSEPH *et al.*, 1989). Diferentes estirpes do CTV têm sido encontradas nas diversas regiões de crescimento de citros no mundo causando os mais variados sintomas, desde leve clareamento das nervuras (**Figura 4**) a severas caneluras do lenho, acompanhadas de rápido declínio no crescimento e necrose do floema (GARNSEY *et al.,* 1991; ROCHA-PEÑA *et al.,* 1995).

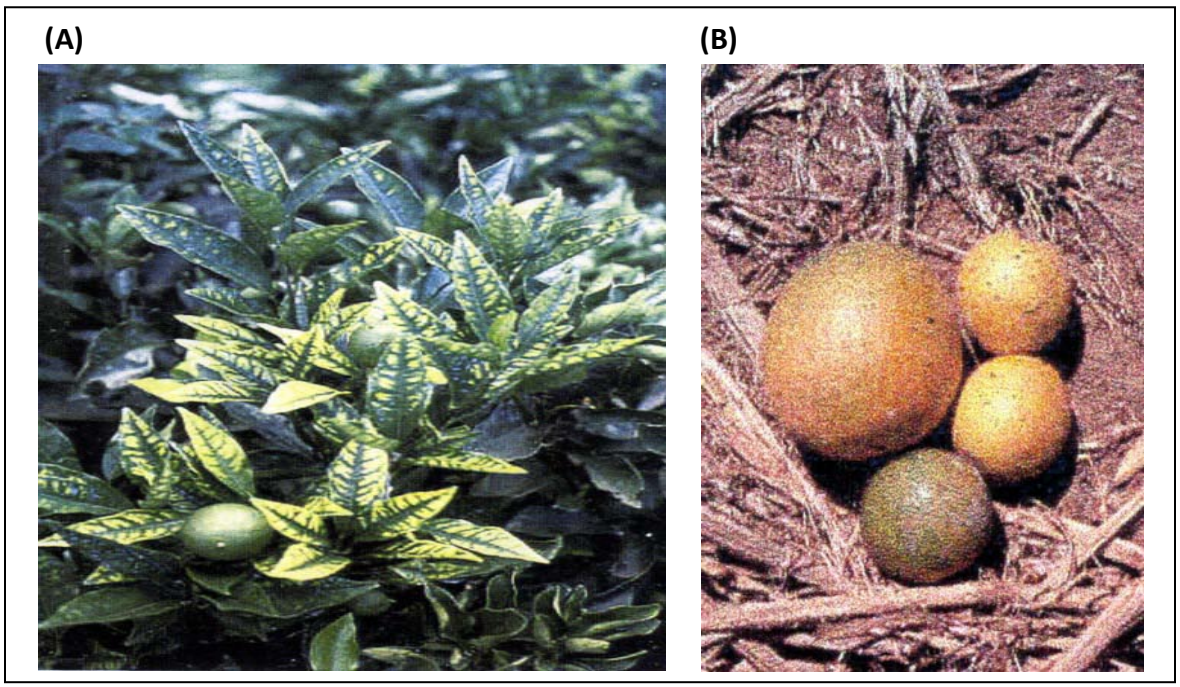

Figura 4. (A) Folhas com sintomas da infecção com CTV, mostrando o clareamento das nervuras; **(B)** Frutos infectados com CTV, apresentando um sabor azedo e tamanho reduzido quando comparado com o fruto sadio (Cortesia do Dr. Marcos A. Machado).

O vírus infecta as células parenquimáticas do tecido floemático das plantas hospedeiras, adjacentes aos elementos de tubo crivado (SEKIYA *et al.*, 1991), afetando essencialmente as plantas do gênero *citrus* e seus parentes próximos. Com uma única exceção, o CTV infecta membros da família *Rutacea,* como a *Passiflora gracilis* (KNORR, 1956). Sua transmissão se dá de duas formas: através da ação humana, como na prática da enxertia e da propagação vegetativa e pela ação de insetos vetores (DJELOUAH e D´ONGHIA, 2001), como os afídeos, insetos pertencentes à ordem Homoptera e a família Aphididae (ROBERTS *et al.*, 2001; PERSAD e HOY, 2003), que variam enormemente quanto a sua eficiência. Dentre eles, o *Toxoptera citricidus* é o mais importante e eficiente vetor do CTV (MEHTA *et al*., 1997), embora outros como *Aphis craccivora, Aphis gossypii, A. spiraecola, M. persicae, T. aurantii e Dactynotus jacea* (BAR-JOSEPH *et al*., 1979) também atuem como vetores do CTV.

O *T. citricidus*, conhecido também como pulgão preto, provavelmente teve sua origem no sudeste asiático, sendo comum em toda Ásia (MICHAUD, 1998) e apresenta um ciclo de vida não-holocíclico (sem gerações com reprodução assexuada) em regiões tropicais e subtropicais, podendo apresentar ciclo holocíclico em condições temperadas (KOMAZAKI, 1993). Seu comportamento alimentar é único e tecido-específico (o animal se alimenta apenas da seiva elaborada que flui pelos elementos de tubo crivado). Prefere se alimentar em estruturas recém-formadas, como folhas jovens e botões florais de citros e seus parentes (TSAI *et al*., 1999), causando danos à planta. No entanto, é considerado uma ameaça, não pelos poucos danos que causa ao se alimentar, mas por transmitir eficientemente o CTV, de maneira semi-persistente e não circulativa (FEBRES *et al.,* 1996; MEHTA *et al.*, 1997), podendo até matar a planta hospedeira (HALBERT E BROWN, 1996).

Os diferentes isolados de CTV são agrupados e caracterizados de acordo com a severidade dos sintomas induzidos na planta hospedeira:

- **Rápido Declínio** (*Quick decline*): Sintoma que afeta várias combinações de porta-enxerto, causador de sérios danos às células do floema, impedindo o fornecimento de seiva para a raiz, e a extinção das reservas de amido com conseqüente morte da raiz.
- **Canelura** (*Stem pitting*): Sintoma caracterizado pelo desenvolvimento de fendas longitudinais no lenho. A planta apresenta perda do vigor e produção de pequenos frutos com sabor azedo.
- **Amarelecimento** (*Seedling yellows*): Sintoma caracterizado pelo amarelamento, clorose e nanismo em mudas novas.
- **Clareamento das nervuras** (*Vein clearing*): Sintoma caracterizado pelo clareamento das nervuras.

O Complexo Capão Bonito

Apesar de no Brasil, o vírus da tristeza ser endêmico, a citricultura brasileira incluindo a paulista, têm convivido razoavelmente com o CTV. No entanto, o complexo Capão Bonito (CB) contém as estirpes mais agressivas. Este complexo, que induz rápido declínio da planta afetada, foi observado inicialmente por MÜLLER *et al.* (1968) na Estação Experimental de Capão Bonito, pertencente ao Instituto Agronômico localizado na Cidade de Capão Bonito, no Estado de São Paulo. As plantas infectadas apresentam baixa taxa de crescimento, brotos axilares curtos, galhos quebradiços, folhas menores que as sadias, com sintomas de deficiência de nutrientes e frutos com diâmetro reduzido, secos e com sementes mal formadas (**Figura 5**). Além disso, as plantas infectadas exibem numerosas caneluras, curtas e superficiais, podendo ser observadas pontuações escuras no lenho, produzidas por impregnações de goma, quando os ramos com caneluras eram cortados no sentido longitudinal (MÜLLER *et al.*, 1968). A principal característica que diferencia esta estirpe é o forte declínio afetando todas as variedades de laranjas doces e a indução de caneluras no porta-enxerto de limão cravo, características não observadas em plantas infectadas por outras estirpes de CTV (MÜLLER *et al.*, 1977).

Estima-se que 90% do parque citrícola do Estado de São Paulo (IEA, 2007) utilizam o porta-enxerto limão Cravo (*Citrus limonia* Osb.) (MÜLLER *et al.*, 1968) e, embora esse porta-enxerto seja tolerante a ação da maioria das estirpes do CTV que ocorrem no país, danos econômicos significativos podem ser observados, principalmente na combinação com laranja Pêra (*Citrus sinensis* L. Osb.) infectadas por estirpes fortes como as do complexo Capão Bonito. Neste caso, a adoção de medidas de controle eficiente é necessária para impedir um prejuízo na plantação e, para tanto, torna-se primordial a rápida detecção e identificação dos isolados, uma vez que esta estirpe representa um risco real e de enorme potencial à citricultura paulista e brasileira.

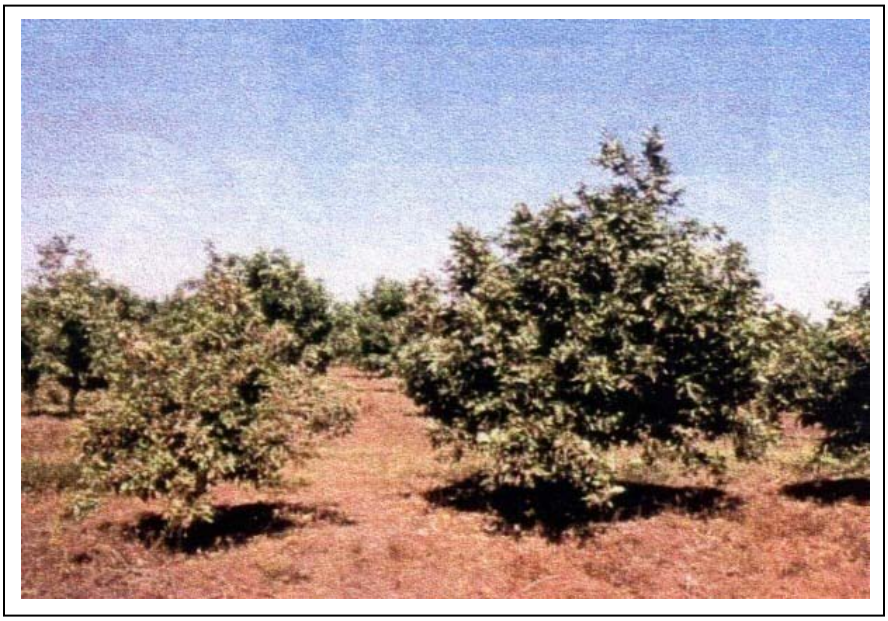

Figura 5. Planta infectada com CTV do Complexo Capão Bonito, apresentando tamanho reduzido (nanismo) e brotos axilares curtos, à esquerda, e uma planta sadia, à direita (Cortesia Dr. Marcos A. Machado).

Detecção de Isolados do CTV

Existem no mundo diversas estirpes do CTV que variam quanto a sua agressividade e indução de sintomas na planta hospedeira, formando um complexo viral de difícil separação e identificação dos seus constituintes. As propriedades biológicas do vírus, como severidade dos sintomas induzidos em plantas no campo ou em plantas indicadoras, principalmente na severidade induzida em diferentes hospedeiros e na transmissibilidade por afídeos (BAR-JOSEPH *et al*, 1989), ou a habilidade de proteção cruzada (MÜLLER E COSTA, 1972), foram inicialmente utilizadas para caracterizar as diferentes estirpes existentes, no programa de indexação biológica. Desse modo, baseado nos sintomas causados pelo CTV em um único hospedeiro, as estirpes foram classificadas em fracas, médias e fortes.

Inicialmente, o principal método de diagnóstico do vírus nos programas de certificação de matrizes e inspeção de pomares era o uso de plantas indicadoras

(indexação biológica), que além de confirmar a presença do vírus também permitia a classificação das estirpes em função da severidade da sintomatologia desenvolvida. Este método tradicional, embora eficiente e de grande utilização até os dias de hoje, é um procedimento lento, uma vez que são necessários de 12 a 15 meses para efetuar a detecção, caro, levando-se em consideração o espaço necessário e as despesas de manutenção, e nem sempre permite a identificação correta dos isolados de CTV.

As técnicas sorológicas só puderam ser utilizadas com sucesso na identificação de isolados de CTV após o estabelecimento de protocolos de purificação das partículas virais, o que possibilitou a produção de soros policlonais e anticorpos monoclonais específicos para os diferentes isolados de CTV (ROCHA-PEÑA e LEE, 1991). A disponibilidade destes anticorpos contra a proteína do capsídeo permitiu o estabelecimento de inúmeros testes imunológicos (CLARK *et al.*, 1986; HOOKER *et al.,* 1993; SHALITIN *et al.*, 1994).

A aplicação de ELISA no diagnóstico de vírus de plantas introduzido por CLARK e ADAMS (1977) foi um marco na nos estudos de vírus de plantas, permitindo um diagnóstico rápido, sensível e seguro para as mais diversas patologias de plantas. No Brasil, a utilização de ELISA para o diagnóstico do vírus era baseada, até bem pouco tempo, no uso de anticorpos monoclonais produzidos contra as estirpes do exterior, como as da Espanha e Flórida (CAMBRA *et. al.,* 1988; PERMAR *et al.*, 1990).

O MAb MCA-13, produzido contra as estirpes do CTV da Flórida, é um dos anticorpos mais utilizado e reage com isolados que induzem declínio de produtividade severo, amarelecimento e caneluras no lenho em plantas de citros provenientes da Flórida, Califórnia e Espanha. Entretanto, o MCA-13 não reage com isolados de severidade intermediária, como os que induzem sintomas primariamente em Lima do México. Além disso, ensaios de imuno eletromicroscopia sugeriram que MCA-13 reconhece um epitopo críptico (PERMAR *et al.*, 1990). Por outro lado, o MAb 3DF1, que vem sendo utilizado na detecção do CTV em vários laboratórios da Flórida e Espanha, reage com inúmeros isolados, independentemente dos sintomas apresentados pela planta hospedeira, com o amarelecimento sendo o sintoma comum a todas as plantas afetadas (VELA *et al.*, 1986).

Os epítopos reconhecidos pelos MAb_s MCA-13 e 3DF1 são lineares. O 3DF1 reage apenas com a proteína do capsídeo viral intacta e não é capaz de reconhecer a proteína do capsídeo após clivagem proteolítica. A reatividade do MCA-13 foi determinada através de mutagênese sítio-dirigida e é controlada pela alteração de um único nucleotídeo, adenina para timina, na posição 371 do gene do capsídeo viral (PAPPU *et al.*, 1993). Esta alteração provoca a modificação de Fenilalanina para Tirosina (Phe $\rightarrow$ Tyr) na posição 124 da capa proteína de isolados severos do CTV, ao passo que a mutação no mesmo sítio em isolados intermediários não-reativos confere reatividade para o MCA-13 (PAPPU *et al.*, 1995).

No entanto, esses anticorpos monoclonais, que reagem em maior ou menor grau com as diferentes estirpes brasileiras importantes do CTV, não permitem a diferenciação dessas estirpes no Brasil (VEGA *et al.,* 1993), limitando, desta forma a sua aplicabilidade no reconhecimento de estirpes severas do CTV como às do Complexo Capão Bonito.

Paralelamente as metodologias quantitativas, o diagnóstico do CTV é também realizado a partir de microscopia eletrônica de transmissão, possibilitando a detecção de partículas virais em amostras de plantas infectadas utilizando uma técnica, denominada *ISEM (Immunosorbent Electron Microscopy)*. Dentre as características da *ISEM* encontram-se sua sensibilidade acurada e a possibilidade de observação direta das partículas virais (HSU *et al.*, 1992), bem como da interação do vírus com outras moléculas, como anticorpos conjugados com partículas eletro-densas, específicos para constituintes da superfície do capsídeo viral, possibilitando evidenciar um padrão de reconhecimento e afinidade entre um anticorpo e seu antígeno específico (KATOCH *et al.*, 2003).

Os primeiros trabalhos de identificação do CTV com técnicas de biologia molecular foram: mapeamento da proteína do capsídeo viral (LEE e CALVERT, 1987; LEE *et al.*, 1988), análise de dsRNA de plantas infectadas (DODDS *et al*., 1987; MORENO *et al.,* 1990; BAR-JOSEPH *et al*., 1989), clonagem e seqüenciamento do genoma viral (PAPPU *et al.,* 1993), análise do polimorfismo de fragmentos de restrição do gene da capa protéica (RFLP) (GILLINGS *et al*. 1993) e análise do polimorfismo conformacional da fita simples de DNA (SSCP) (FEBRES *et al.,*1995). Nos últimos anos, inúmeras outras metodologias foram

estabelecidas como Imunocaptura-PCR e Imuno-PCR, técnicas que associam a imunocaptura de antígenos e a reação em cadeia da polimerase (PCR) (NOLASCO *et al.*, 2002; HEMA e*t al.*, 2003; LIANG *et al.*, 2003), além do RT-PCR (Transcrição reversa-PCR) (HUNG *et al.*, 2000; HUANG *et al.*, 2004), RT-PCR-ELOSA (CHANDELIER *et al.*, 2001), juntamente com a PCR quantitativa (qPCR) em Tempo Real, que estão sendo empregados nas clínicas de certificações (RUIZ-RUIZ *et al.*, 2007; SAPONARI, *et al.*, 2008) e contribuem para a eficiente detecção e caracterização das estirpes brasileiras do CTV.

Proteínas Recombinantes e a produção de anticorpos monoclonais

A detecção do vírus da tristeza através de técnicas sorológicas como ELISA, utilizando anticorpos monoclonais e policlonais produzidos contra a proteína do capsídeo viral ou contra proteínas virais intactas, vem sendo realizada rotineiramente, já que é uma metodologia de alta sensibilidade, fácil manuseio e que pode ser dimensionada para a análise de um grande número de amostras. No entanto, devido ao fato do CTV estar estritamente associado ao floema, a purificação de suas partículas apresenta algumas limitações, dentre elas, grande quantidade de tecido infectado necessária para a purificação do vírus, baixo rendimento e contaminação com proteínas da própria planta hospedeira. Estas limitações, na maioria das vezes, comprometem a qualidade dos anticorpos obtidos (MANJUNATH *et al.,* 1994; NIBLETT *et al.,* 2000) e, apenas com o desenvolvimento da biologia molecular, foi possível a produção e purificação de proteínas recombinantes do capsídeo do CTV, facilitando a sua utilização como fonte de antígeno para a produção de anticorpos para diagnóstico, uma vez que permitem a produção de grande quantidade de antígeno, livres de contaminantes da planta hospedeira (MANJUNATH *et al.,* 1994). Os primeiros trabalhos de clonagem do gene da capa protéica foram realizados por NIKOLAEVA *et al.*, (1995) a partir do isolado SY568 da Califórnia, no vetor pMAL-c2, que promove a expressão de uma proteína de fusão, compreendendo a proteína de interesse fusionada à proteína ligante de maltose (MBP) em *E. coli*.

No Brasil, TARGON (1997 e 2000) e pesquisadores do Laboratório de Biotecnologia em Citros do Centro de APTA Citros 'Sylvio Moreira' (IAC, Cordeirópolis, SP) clonaram o gene da capa protéica do CTV presente em Pêra 135/Cravo 507, que apresentava o complexo severo de Capão Bonito, produzindo duas proteínas recombinantes do capsídeo viral, denominadas CB-22 (homóloga aos isolados que induzem sintomas fracos em plantas indicadoras) e CB-104 (homóloga aos isolados que induzem sintomas severos em plantas indicadoras). Estas proteínas correspondentes a duas seqüências gênicas distintas, com homologia de 91,3% na sua seqüência de ácidos nucléicos e 93,3% na sua seqüência de aminoácidos foram utilizadas em esquemas de imunização em camundongos e coelhos, possibilitando a produção de quatro anticorpos monoclonais, agrupados em três grupos distintos (STACH-MACHADO *et al.,*2000 e 2002; DIAS *et al.,* 2001).

O primeiro grupo denominado IC-04.12, pertencente à subclasse IgG2b/κ, apresenta especificidade para a proteína CB-22, que possui homologia com a proteína do capsídeo viral de isolados que induzem sintomas fracos em plantas indicadoras; o segundo grupo, denominado 39.07, de subclasse IgG1/κ, apresenta reatividade apenas para a proteína CB-104, que tem alta homologia com a proteína do capsídeo de isolados severos de CTV. O terceiro grupo de anticorpos monoclonais denominados de 30.G.02 e 37.G.11, de subclasse IgG1/κ, reconhece as duas proteínas CB-22 e CB-104. Nenhum desses anticorpos monoclonais apresentou reação cruzada com proteínas da planta hospedeira, e foram caracterizados quanto a sua especificidade, sensibilidade e título (DIAS, 2001).

A caracterização inicial dos anticorpos monoclonais demonstrou que eles possuem um título elevado, chegando até a ordem de 1:40.000 e alta sensibilidade, sendo capazes de detectar as proteínas recombinantes purificadas em concentrações variando de 70 a 300 ηg por ensaio (DIAS, 2001). A diferença nas características individuais dos monoclonais produzidos pode ser atribuída às variações experimentais como a idade, sexo e linhagem dos camundongos utilizados; condições de fusão e cultura celular; manipulação dos animais durante a produção de ascite, além da própria resposta imunológica de cada animal frente aos antígenos.

A capacidade de diagnóstico desses MAb_s foi também avaliada em ensaios de ELISA Sandwich, utilizando amostras de plantas provenientes da coleção do Banco Ativo de Germoplasma e plantas Matrizes do Centro de Citricultura Sylvio Moreira (IAC) de Cordeirópolis (SP) e amostras de plantas coletadas em condições de campo da Estação Experimental de Capão Bonito (STACH-MACHADO *et al.*, 2002). Os resultados obtidos mostram um padrão de reatividade distinto entre as amostras de campo de Capão Bonito e das amostras do Centro de Citricultura Sylvio Moreira, que não apresentam o complexo Capão Bonito do CTV, validando deste modo a utilização destes anticorpos monoclonais em imunodiagnósticos para a identificação diferenciação do complexo de Capão Bonito, uma vez que apresentam um padrão de reatividade diferente daquele obtido com plantas infectadas com CTV, mas livres do complexo Capão Bonito.

Mapeamento de Epitopos

Um imunodiagnóstico eficiente e seguro requer a caracterização e identificação das regiões envolvidas na interação com o anticorpo monoclonal, isto é, as regiões funcionais do antígeno, os epítopos. O conhecimento detalhado da estrutura de um epítopo é importante na identificação de reatividade cruzada com proteínas do capsídeo de vírus semelhantes ou mesmo de outras famílias e, também, para estudos de relação entre estrutura e função (MACHT *et al.*, 1996).

Os epítopos de uma partícula viral correspondem às regiões do capsídeo ou às proteínas do envelope viral que são especificamente reconhecidas pelos sítios de ligação das moléculas de anticorpos (VAN REGENMORTEL, 1990). Essas regiões funcionais possuem de 6 a 12 resíduos de aminoácidos que se ligam aos sítios específicos das moléculas de anticorpos, podendo ser constituídas por vários resíduos de aminoácidos (epítopos lineares ou contínuos) ou então por vários resíduos de aminoácidos não contínuos na estrutura primária, mas próximos na estrutura terciária da cadeia polipeptídica (epítopos conformacionais ou descontínuos) (BARLOW *et al.*, 1986; PELLEQUER *et al.*, 1991; CASON, 1994).

Os resultados obtidos através de Western Blot em nosso laboratório têm mostrado que os MAb_s 30.G.02, 37.G.11 e 39.07 reconhecem epítopos lineares enquanto o anticorpo IC.04-12, provavelmente, reconhece epítopo conformacional, já que foi incapaz de reagir com as partículas virais provenientes de tecido vegetal infectado, preparado sob condições desnaturantes (STACH-MACHADO *et al.*, 2002).

Deste modo, o estudo dos epítopos reconhecidos por esses anticorpos monoclonais é de grande interesse para o desenvolvimento e padronização de diagnósticos capazes de identificar as diferentes estirpes do CTV presentes no Brasil.

3.2. A CLOROSE VARIEGADA DOS CITROS

A clorose variegada dos citros (CVC) ou "amarelinho" é hoje a doença bacteriana mais grave que ameaça a citricultura brasileira. Foi inicialmente reconhecida como uma nova doença no final da década de 80, na fronteira citrícola entre São Paulo e Minas Gerais (LEE *et al.*, 1991; ROSSETTI *et al.*, 1991). Estima-se que atualmente cerca de 90% dos pomares do Estado de São Paulo apresentem plantas com sintomas e que as perdas anuais girem em torno de 90 milhões de dólares. Essa assustadora disseminação pode ser atribuída à existência de fontes de inóculos em grande número, a eficiência do vetor (cigarrinhas da família Cicadelidae), abundante em nossos pomares, e a disseminação por mudas contaminadas.

O desenvolvimento dos sintomas está associado à bactéria *Xylella fastidiosa.* São bactérias gram-negativas, apresentam a forma de bastonete, não flageladas, sem pigmentação e são estritamente aeróbicas Essas bactérias, embora tenham sido definitivamente associada à CVC em 1993, possuem uma ampla gama de hospedeiros, incluindo membros de pelo menos 28 famílias de plantas mono e dicotiledôneas (HOPKINS e ADLERZ, 1988), como ameixa, amêndoa, pêssego, café, uva (CHANG *et al.*, 1993; LEE *et al.*, 1993; PURCELL e HOPKINS, 1996; LIMA *et al.*, 1998), além de plantas ornamentais (BARNARD *et al.*, 1998). Entre as culturas de importante expressão comercial, destacamos a *Pierce disease*, presente em vinhedo dos Estados Unidos e da América do Sul; a *Phony disease* em pêssego dos Estados Unidos, a escaldadura da folha da ameixeira, constatada na Argentina, Estados Unidos, Brasil e Paraguai, e também está presente em plantas de café, causando a requeima das folhas do cafeeiro (HOPKINS e MOLLENHAUER, 1973; FRENCH e KITAJIMA, 1978; DAVIS *et al.*, 1983).

No Brasil, *X. fatidiosa* é encontrada associada a escaldadura da folha da ameixeira, clorose variegada dos citros (ROSSETI *et al.*, 1990; CHANG *et al.*, 1993), e mais recentemente a requeima foliar do cafeeiro (PARADELA FILHO *et al.*, 1997). Nos citros, a bactéria coloniza principalmente plantas de laranja doce (*Citrus sinensis* Osbeck), cujos sintomas, inicialmente restrito a folha, evoluem para o fruto inviabilizando-o

economicamente, devido a uma drástica redução de tamanho (**Figura 6**). Os prejuízos decorrentes de presença de *X. fastidiosa* na citricultura paulista chegam a ordem de U$ 150 milhões e a um aumento de 40% no custo de implantação de 1 ha de laranjeira.

Figura 6. Sintomas de CVC em plantas de laranja doce. (A) Manchas cloróticas na parte dorsal das folhas correspondendo a pontos marrons com aspecto de goma na superfície ventral. (B) Aspecto de super brotamento de ramos. (C) diferença entre um fruto sadio (esquerda) e outro com CVC (direita) apresentando tamanho reduzido, endurecidos e com maturação precoce (Figuras A e C, FUNDECITRUS e Figura B cortesia do Dr. Marcos A. Machado).

Devido a isso, foi criado um consórcio brasileiro para o seqüenciamento do genoma da linhagem 9a5c de *X. fastidiosa*, sendo o primeiro fitopatógeno a ter seu genoma completamente seqüenciado (SIMPSON *et al.*, 2000). Posteriormente, o seqüenciamento do genoma dos citros também foi realizado e a análise das seqüências expressas de RNAm (ESTs) permitiu a comparação do perfil de expressão gênica entre folhas saudáveis e folhas infectadas com *X. fastidiosa* em diferentes estágios (PERONI *et al.*, 2007).

Sob condições de estresse pela presença do patógeno, as folhas infectadas, nos primeiros dias de infecção, superexpressam a isoforma da superóxido dismutase dependente de Cobre e Zinco (SOD Cu/Zn), que provavelmente aumenta a capacidade de defesa das folhas infectadas (Foyer *et al.*, 2001). Além disso, passam a expressar Catalase 1 e 2 (CAT1 e CAT2) em níveis similares, enquanto Metalotioneína 2 (MT2) é superexpressa quando comparada com as folhas saudáveis. Em plantas, CAT representa umas das primeiras defesas enzimáticas contra o estresse oxidativo induzido por senescência, desidratação, estresse osmóticos, injúria, ozônio e metais pesados, quebrando rapidamente o peróxido de hidrogênio produzido em resposta a estes processos (SINGH e TEWARI, 2003).

Entretanto, o perfil de ESTs das folhas com 30 dias de infecção por *X. fastidiosa* mostra uma mudança significativa no padrão de expressão das enzimas envolvidas nos mecanismos antioxidantes enzimáticos e não-enzimáticos (PERONI *et al.*, 2007). Estas folhas não expressam mais a SOD, sugerindo que os cloroplastos se tornam incapazes de remover os radicais O_2^- gerados na cadeia transportadora de elétrons na fotossíntese. Em contrapartida, as folhas infectadas superexpressam o gene da Glutationa S-transferase (GST), MT1 e MT2. A GST tem atividade Glutationa peroxidase (GPX), protegendo as células da injúria oxidativa causada por peróxidos orgânicos produzidos nas plantas durante certos processos, como a fotossíntese e a infecção por patógenos. Assim, com o progresso da infecção, as células infectadas acumulam espécies reativas de oxigênio (ROS), enquanto a produção de substâncias "removedoras" (*scavenger*) está diminuída, ou até mesmo, ausente.

Entre os citros hospedeiros de *X. fastidiosa* destacam-se as laranjas doces, principalmente as da variedade Pêra. A *X. fastidiosa* é uma bactéria limitada ao xilema (BOVÉ e GARNIER, 2002) e, embora ainda não exista um consenso sobre seu mecanismo de patogenicidade, a oclusão vascular devido aos agregados bacterianos e a produção de polissacarídeos extracelulares (EPS), conduzindo a planta ao estresse hídrico, é a teoria mais aceita para a causa dessa doença (MACHADO *et al.*, 2001). A bactéria se multiplica e se dissemina dentro dos vasos do xilema da planta hospedeira, desenvolvendo uma

doença sistêmica devido à obstrução dos vasos por pectinas, tiloses e gomas produzidas pela planta em resposta à presença da bactéria.

A obstrução dos vasos causa deficiência hídrica e nutricional e, conseqüentemente, temos o aparecimento de plantas murchas, quase sempre de coloração amarelada, com crescimento irregular da copa, maturação precoce dos frutos, que apresentam tamanho reduzido e sem valor comercial. Os sintomas da CVC em laranjas doce são manchas cloróticas de bordos irregulares na face superior das folhas, com correspondente formação de pústulas de coloração marrom na face inferior, desenvolvimento de frutos pequenos e duros, com maturação precoce nos ramos afetados (LEE *et al.*, 1991; NEGRI e GARCIA JR., 1993).

O lento período de expressão dos sintomas e a possibilidade de transmissão através do uso de borbulhas contaminadas têm requerido técnicas de diagnóstico sensíveis, que possibilitem o monitoramento efetivo do material de propagação (borbulhas) e de mudas livres do patógeno. A técnica da PCR tem possibilitado um diagnóstico seguro para a *X. fastidiosa* e tem sido utilizada rotineiramente nos laboratórios de análise.

O DNA alvo para a PCR vem sendo obtido através de extrações de DNA total do pecíolo da folha, seguindo a metodologia descrita por COLETTA-FILHO *et al*. (1996), ou pelo método de perfusão, ou seja, passagem e coleta de um líquido através do pecíolo da folha, com posterior extração de DNA deste líquido através de resina (LEMOS *et al.*, 1997). Ambos os procedimentos tem resultados confiáveis para diagnóstico da bactéria *X. fastidiosa*, com vantagens e desvantagens inerentes a cada procedimento. Na realização destes diagnósticos é imprescindível o uso de controle positivo (normalmente pecíolo de folha com sintoma de CVC) e negativo (pecíolo de folha de planta mantida sob proteção do vetor).

O DIBA (D*ot Immunobinding* A*ssay),* um ensaio sorológico em suporte de nitrocelulose para o diagnóstico de CVC, assim como a produção de soros policlonais utilizando diferentes isolados de *X. fastidiosa* têm sido descritos na literatura (DAVIS *et al.*, 1983; RAJU *et al.*, 1986; LEE *et al.*, 1993). Todos imunodiagnósticos se baseiam na

interação específica de antígeno e anticorpo. O teste do DIBA apresenta inúmeras vantagens como baixo custo, fácil execução, análise de muitas amostras. No entanto, apresenta também inúmeras desvantagens, pois é uma reação qualitativa com baixa sensibilidade (somente as plantas doentes que já apresentam sintomas são positivas), apresentando freqüentemente reações inespecíficas e, além disso, não permite a quantificação da reação. Por outro lado, o ensaio de ELISA apresenta alta sensibilidade e permite a quantificação da infecção e a análise de grande número de amostras de forma rápida e eficiente.

Embora as reações de PCR constituam método sensível e seguro para o diagnóstico de *X. fastidiosa*, a extração de DNA bacteriano utilizando fenol/clorofórmio, Trizol®, ou similares, causa preocupação devido ao risco de sua manipulação, toxicidade e contaminação ambiental. Assim, visando o estabelecimento de um método capaz de detectar amostras de *X. fastidiosa* sem a necessidade do uso desses reagentes e, ainda, com um aumento de sua sensibilidade e especificidade, foram propostas metodologias como o sistema de Imunocaptura-PCR e Imuno-PCR.

No método de Imunocaptura-PCR, as bactérias íntegras são capturadas por soro policlonal específico, previamente adsorvido em microplacas de 96 poços. Posteriormente, a detecção é feita por PCR, utilizando iniciadores (*primers*) específicos para o DNA bacteriano. Por outro lado, o Imuno-PCR, desenvolvido por SANO *et al.* (1992), compreende a sensibilização da microplaca de 96 poços com uma suspensão de antígenos bacterianos e estes, por sua vez, são detectados pelo anticorpos policlonais biotinilados. Posteriormente, uma molécula "linker", como a streptoavidina é aplicada e o DNA-*repórter*, também biotinilado, é adicionado, formando um complexo. Finalmente, a PCR é realizada utilizando *primers* desenhados especificamente para o DNA-*reporter*. Esta técnica mostrou-se muito sensível, sendo até 10.000 vezes mais sensível do que o ELISA convencional (JOERGER *et al.,* 1995), e tem sido amplamente utilizado na detecção de oncogenes (ZHOU *et al.*, 1993), microrganismos e toxinas (LIANG *et al.*, 2003; CHAO *et al.*, 2004; ALLEN *et al.*, 2006; LUBELLI *et al.*, 2006; MASON *et al.*, 2006; FISCHER *et al.*, 2007), citocinas e hormônios (SANNA *et al.*, 1995; SIMS *et al.*, 2000; KAMATSU *et al.*, 2001),

patógenos humanos e vegetais (LUO *et al.*, 2002; CHYE *et al.*, 2004; ADLER, 2005) e neurotoxina do *Clostridium botulinum* (WU *et al.,* 2001).

Deste modo, a produção de soro policlonal e o desenvolvimento das técnicas de IC-PCR e I-PCR para a detecção de *X. fastidiosa* a partir de amostras de tecido vegetal infectado seriam de grande interesse para estabelecer um diagnóstico preciso, rápido e menos dispendioso do que a técnica de PCR tradicional, na qual é necessária a extração de DNA total da *X. fastidiosa* a partir do tecido vegetal infectado.

4. OBJETIVOS

OBJETIVOS

- Identificar e caracterizar os epítopos das proteínas recombinantes CB-22 e CB-104 do capsídeo do *Citrus tristeza virus* (CTV) do Complexo Capão Bonito, reconhecidos pelos anticorpos monoclonais 30.G.02, 37.G.11, 39.07 e IC.04-12.

- Desenvolver e padronizar ensaios imunomoleculares para o diagnóstico da Clorose Variegada dos Citros.

ESTRATÉGIAS

Capítulo 1

1. Expressar e purificar as proteínas recombinantes CB-22 e CB-104;
2. Expandir e purificar os anticorpos monoclonais 30.G.02, 37.G.11, 39.07 e IC.04-12;
3. Determinar o padrão de reatividade dos anticorpos monoclonais;
4. Desenhar os *primers* para clonagem em pET32/XaLIC, os peptídeos das proteínas CB-22 e CB-104;
5. Identificar e caracterizar os epítopos das proteínas recombinantes.

Capítulo 2

1. Seqüenciar as proteínas recombinantes CB-22 e CB-104;
2. Analisar *in silico* as proteínas recombinantes;
3. Expressar em *E. coli* BL21(DE3) e purificar a proteína recombinante CB-22;
4. Determinar a estrutura secundária da CB-22

Capítulo 3

1. Produzir e caracterizar o soro policlonal para *Xylella fastidiosa*;
2. Desenvolver e padronizar o ensaio de Imunocaptura-PCR;
3. Desenvolver e padronizar o ensaio de Imuno-PCR;
4. Determinar a especificidade e sensibilidade de detecção de *X. fastidiosa*.

5. CAPÍTULOS

Production and Characterization of Monoclonal Antibodies Against Different Epitopes of the CTV Recombinant Major Coat Protein.

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ABSTRACT

Citrus tristeza virus (CTV) complex comprises a number of isolates producing several economically important disease syndromes, and among Brazilian CTV strains, the genotype Capão Bonito (CB) is the most harmful. Therefore, two recombinant proteins CB-22 and CB-104, encoded by ORF 7 for the coat protein (CP) were used to obtain monoclonal antibodies (MAb). In an attempt to develop a serological tool to rapidly differentiate CB isolates, we characterized and mapped the epitopes to identify the antigenic domains onto CB strains. The mAbs reactivity was primarily screened by the recombinant coat proteins, which together contain the complete CP sequence. In ELISA and Western blot assays, the MAb_s 30.G.02 reacted with fragment 1 and 2 (amino acids 2-73) while MAb 37.G.11 reacted only with fragment 2 (amino acids 32-73) of CB-22 and CB-104 and, finally, MAb 39.07 reacted only with fragment 5 (amino acids 114-138) of CB-104. However, the MAb IC.04-12 recognized none of tested fragments. This means the epitopes recognized by MAb_s 30.G.02, 37.G.11 and 39.07 were linear and mainly localized in two regions: amino acids 2-73 and 114-138, while the MAb IC.4-12 epitope may be conformational. Therefore, in this study, four monoclonal antibodies were generated and three antigenic epitopes were identified using these mAbs.

Keywords: *Citrus tristeza virus,* Capão bonito complex, monoclonal antibodies, epitope mapping

INTRODUCTION

Tristeza causes one of the most economically important viral diseases of citrus industry which Brazilian agribusiness together with USA, respond by over 50% of fruit and 80% of juice world production (FAO, 2006). It is caused by *Citrus tristeza virus* (CTV) transmitted by aphids in a semi persistent manner and by grafting during citrus propagation (Bar-Joseph *et al.*, 1989).

CTV belongs to the *Closteroviridae* family, genus *Closterovirus* and is a long flexuous virion with a positive-sense single-stranded RNA (ssRNA) genome, with 12 open reading frames (ORFs) encoding up to 19 protein products (Karasev *et. al.*, 1995). The virus have a unique bipolar architecture incorporating two coat proteins (CP), the ORF 7 encodes a 25 kDa major coat protein which encapsulated approximately 95% of the helical nucleocapsid, whereas the ORF 6 encodes a 27 kDa minor coat protein, which encapsulated a small portion at one end of the capsid (Sekiya, *et.al.*, 1991 and Febres *et al.*, 1996). Genetic and biochemical analyses have shown that the functions of these virion components are distinct. The major coat protein is required primarily for genome protection, whereas the tail represents a specialized device for cell-to-cell movement (López *et al.*, 2000).

There are many worldwide CTV strains which have been initially classified on the basis of their biological symptoms and aphid transmissibility (Bar-Joseph *et al.*, 1989), latter by its serological relationships, peptide mapping of the coat protein, nucleic acid hybridization and nucleotide sequence identity (Lee and Calvert, 1987; Lee *et al.*, 1988; Moreno *et al.,* 1990). Furthermore, a relationship between the CP sequences and the symptoms caused by was also demonstrated (Machado *et al.*, 1996). However, CTV is often referred as a virus complex, since there are many strains and its infections frequently occur as mixtures, which differ in pathogenicity (Bar-Joseph *et al.*, 1989).

The most harmful Brazilian CTV is the “Capão Bonito complex (CB)” that causes important economical losses since its affect mainly sweet oranges (*Citrus sinensis* Osbeck) plants grafted on Rangpur lime (*Citrus limonia* Osbeck) rootstock, the most adopted

variety among Brazilian orchards (Müller *et al.*, 1968). Therefore, a key element in the control strategies of CTV is achieved by eradication programs and by preventive methods such as quarantine, use of disease free budwood or even to find CTV-tolerant rootstocks (Müller e Costa, 1993; Persad *et al.*, 2003). However, the use of the monoclonal antibodies designated as 3DF1 (Vela *et al.*, 1986) and MCA13 (Cambra *et. al.,* 1988; Permar *et al.*, 1990) considered as reference for CTV diagnosis because it react primarily with most severe strains in Florida, could not be used as diagnostic reagent for Brazilian CTV strains such as CB, which sheltered, at least two different strains, a mild and a severe ones (Müller et al., 1968).

So, the CP genes from these CB strains previously cloned and expressed by Targon (2000), were used to obtain high specific monoclonal antibodies (mAbs) that may detect severe and mild strains could be use for differentiating CB strains (Stach-Machado, 2000 and 2002).

This work aims to identify the immune reactive regions of the antigens recognized by monoclonal antibodies, referred as epitope mapping, a critical step in developing of immunoassays, as well as for studying protein-protein interactions and for defining protein topology.

MATERIAL AND METHODS

Materials

Oligonucleotide primers were synthesized at Invitrogen Life Technologies (São Paulo, Brazil). The pET32-Xa/LIC, pET 22 vectors, the BL21(DE3) strains were obtained from Novagen (Madison, WI). The pBluescript KS+ vector was obtained from Stratagene (Cedar Creek, TX). The molecular-mass marker was purchased from Amersham (GE Healthcare, Uppsala, Sweden). All other reagents were of the highest commercially available grade.

Bacterial strains and culture conditions

Escherichia coli strains DH5α and BL21(DE3) were, respectively used for cloning and expression of CP genes. The bacteria were cultured at 37^{o}C in LB broth (10 g tryptone, 5 g yeast extract, 5 g NaCl per 1L) or on agar supplemented with 100μg/mL ampicillin (Sigma Chemical, Saint Louis, MO, USA), when necessary.

Animals

The 6-8 week old female BALB/c mice were purchased from CEMIB (Centro Multidisciplinar para Investigação Biológica, Campinas, SP) and were maintained under specific pathogen free conditions, housed in groups of 5 in propylene cages, fed a standard laboratory diet and given tap water *ad libitum*. All experimental procedures were in accordance with the ethical regulation established by the Brazilian College of Animal Experimentation and approved by the Animal Experimentation Ethics Committee of the State University of Campinas.

CTV coat protein gene cloning and expression

CTV dsRNA was isolated from a 19–year-old Pêra sweet orange tree grafted on Rangpur lime, according to the procedure described by Valverde *et al.* (1990). The first cDNA synthesis and amplification of the coat protein (CP) gene were done as described by Sambrook *et al.* (1989). The CP gene was amplified by PCR using specific primers, containing *EcoRI* and *BglII* restriction sites, and cloned into pBluescript KS+ vector. Two clones, CB-22 and CB-104 were selected, sequenced and expressed in pET 22 (TARGON *et al.,* 2000). The expression of recombinant proteins was induced at an early logarithmic phase by the addition of 100 mM IPTG (Isopropyl-β-D-thiogalactopyranoside) under constant agitation during 5 hours at 37°C. Aliquots (5mL) of this culture were centrifuged at 6,000x*g* for 15 minutes, the pellet was suspended in 300μL sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (0,062M Tris-HCl, pH 6.8; 10% Glycerol; 2% SDS; 5% β-Mercaptoetanol and 0.02% Bromphenol blue) and frozen -20°C until usage.

Purification of CP proteins

The expressed CP proteins were subjected to gel electrophoretic separation in a 12.0% discontinuous SDS-PAGE system as previously described by Laemmli (1970). The proteins were precipitated with 0.25M KCl and 1mM DTT and the recombinants proteins were identified comparing with a negative standard (*E. coli DH 5α* without induction with IPTG). Gel slices containing proteins were transferred to dialysis tube containing 1.0 mL of 0.2M Tris/acetate, pH 7.4, 10% SDS, 100mM dithiothreitol per 0.1 g of wet polyacrylamide gel. The dialysis tubing were crosswise in a horizontal electrophoresis chamber (Pharmacia LKA-GNA 200) containing the running buffer (50mM Tris/Acetate pH 7.4; 0.1% SDS; 0.5mM sodium thioglycolate) and run at 30 volts for 60 minutes. The proteins were concentrated by ultracentrifugation at 4,000 x g during 25 minutes, using Centriprep-10 system (Amicon®, Millipore, Billerica, MA, USA).

Quantification of Protein

The total soluble protein concentration was assayed according to the method presented by Bradford (1976), using bovine serum albumin as standard. In addition, the purified CB-22 protein was also quantified, based on absorbance at 280nm, using a calculated extinction coefficient of 0.945 at 1 mg/mL (Abs 0.1% (1 g/l) = 0.945) [14].

Monoclonal antibodies

Mice were divided in three different groups and immunized intraperitoneally two times with 20 µg the recombinant proteins CB-22, CB-104 or both, respectively on day 0 and 14 using complete and incomplete Freund's adjuvant (Sigma). Further, two times on day 21 and 32 with 10 µg of antigen using PBS pH 7.3. The immune response to the antigen was assessed by measuring the titer of antibody levels in mouse serum by enzyme-linked immunosorbant assay (ELISA) according to Clark *et al.*, (1986), using the purified recombinant proteins as antigens.

Mice from each group with highest titer of antibodies were splenectomized at day 4, after the last antigen injection. The spleen cells were fused with SP2/0-Ag14 myeloma cells at a ratio of 5:1 using 50% (w/v) polyethylene glycol (PEG 3000, Sigma), according to the technique previously described by Köhler and Milstein (1975) and Van Duesen and Whetsstone (1981). Serum blood was saved as a positive control.

The hybridoma cells were suspended in RPMI 1640, supplemented with non essential aminoacid and L-Glutamine, Hypoxanthine Aminopterin Thymidine (HAT), and 20% heat inactivated Fetal Bovine Serum (FBS). The cells were seeded in 24-well tissue culture plates and incubated in a humidified 37°C, 5% CO_2 incubator for 2 weeks. Culture fluids from the hybridomas were screened using ELISA sandwich assays and only positive clones, producing specific antibodies were kept in Hypoxanthine Thymidine (HT) medium for further 2 weeks. After, the positive clones were cloned by limiting dilution (Goding, 1980).

To ensure the monoclonality and stability of the cell line, the positive clones were subcultured twice. The immunoglobulin class was determined using the mouse monoclonal antibody isotyping ELISA kit (ImmunoPure Monoclonal Antibody Isotyping Kit II, Pierce Biotechnology, Rockford, IL, USA), carried out according to the manufacturer instructions. Large quantities of monoclonal antibodies were obtained by intraperitoneal injection of hybridoma cells onto BALB/c mice.

Therefore, ascitic fluid was collected and submitted to low-speed centrifugation for 20 min to remove cellular debris. The IgGs were purified by protein G or A affinity chromatography (Pierce), depending on the immunoglobulin isotype. The IgG1 isotype was purified by protein G column, whereas the IgG2b isotype was purified by Protein A, following the manufacturer's instructions. The protein concentration was estimated by spectrophotometry, assuming an extinction coefficient of 1.40 at A_{280} for 1 mg/mL to mouse IgGs.

Indirect Double Antibody Sandwich Enzyme Linked Immunosorbent Assay (DASI ELISA)

All indirect DAS ELISA assays (Clark and Adams, 1977) were performed in 96-wells microtiter plates (Nunc-Immuno Maxisorb™ plates, Nunc). Briefly, the plates were coated with 100 μL/well of #1006 anti-CTV rabbit polyclonal immunoglobulin (Baptista *et al.* 1996) in 0.05 M sodium carbonate buffer, pH 9.6 and placed at 4°C, overnight. After washing three times in phosphate buffer saline (PBS) containing 0.05% Tween 20 (PBS-T), the free-antigen sites were blocked with 100 μL/well of blocking buffer (2% bovine serum albumin (BSA) in PBS) for 1 h at 37°C.

After washing, 100 μL of purified recombinant protein CB-22 or CB-104 at 2 μg/ml were added, and the plate was incubated for 4 hr at 37°C. After washing three times with PBS-T, 100 μL of culture fluid or the monoclonal antibodies at 2 μg/mL was added to each well and incubated for 2 hr at 37°C. Later, after washing, a 1:30,000 dilution of goat anti-mouse antibody conjugated with alkaline phosphatase (Sigma) was added and incubated for 2 hr at 37°C.

Finally, the plate was washed and the reaction was determined by adding 1 mg/mL of *p*-nitrophenyl phosphate (pNPP) in 10% diethanolamine buffer, pH 9,8 and incubated at room temperature for 1 hr. The optical density (OD) was measured at 405 nm using an Expert Plus Multiplate plate reader (ASYS HITECH, Austria).

Western blot

The reactivity of MAbs were evaluated by Western blot analysis. The recombinant proteins CB-22 and CB-104 were electrophoresed on SDS-PAGE using 12% acrylamide gel (Laemmli, 1970), and transferred onto nitrocellulose membrane (Hybond-C, 0.45 Micron, Amersham), according to Towbin *et al.* (1979), using Mini Trans-Blot Electrophoretic Transfer Cell® (Bio-Rad, Hercules, CA, USA) following the manufacturer's instructions.

Non-specific binding sites onto the membrane were blocked with 5% skim milk in 100 mM Tris-HCl, pH 7.5, 0.9% NaCl, 0.05% Tween 20 (TBS-T) for 2 h at room temperature. Membrane was washed five times with TBS-T, cut into strips and incubated individually with different hybridoma clone supernatants, overnight under constant agitation. After the washing steps, the strips were incubated for 1 h at room temperature with goat anti-mouse immunoglobulin alkaline phosphatase conjugate (Sigma) and finally, after extensive washing with TBS-T, the binding of MAbs to antigen was detected by 0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, 5 mM $MgCl_2$, 80 mg BCIP (5-brommo-4-chloro-3-indoyl phosphate) and 160 mg NBT (nitroblue tetrazolium) for 1 hour at room temperature in darkness.

The reaction was stopped by washing the membrane with distilled water, and dried. In the control experiments, antigen strip was incubated with an irrelevant monoclonal antibody or non immunized mice sera.

Immunogold labelling

Drops of purified CTV preparations were placed onto Formvar-filmed copper grids. The grids were sequentially incubated in a drop of specific Mab (100 μg/mL) for 30 min, in a drop of goat anti-mouse IgG 10nm gold conjugate (Sigma) for 30 min and a drop of 1% ammonium molybdate (Vera and Milne, 1994). The conjugate was diluted 1:30 in PBS-T. All steps were performed at 20^{o}C and the grids were rinsed with 20 drops of PBS after each step. Grids were examined with an electron microscope (LEO 906E, Laboratório de Microscopia Eletrônica, UNICAMP, Brazil).

Peptide design and cloning

The peptides were designed based on hydropathicity analysis and were draw to cover the entire amino acid sequence onto overlapping fragments (**Figure 1**). Therefore, six and eight pairs of primers were designed for CB-104 and CB-22, respectively (**Table 1**). The pairs of PCR primers were designed in order to generate amplicons with vector cohesive overhangs, and, so all forward primers were designed with a complementary sequence to pET32 vector (5'-GGTATTGAGGGTCGC-3') plus the target sequence of peptide, and all reverse primers were designed with another complementary sequence to pET32 vector (5'-AGAGGAGAGTTAGAGCC-3') (**Table 1**).

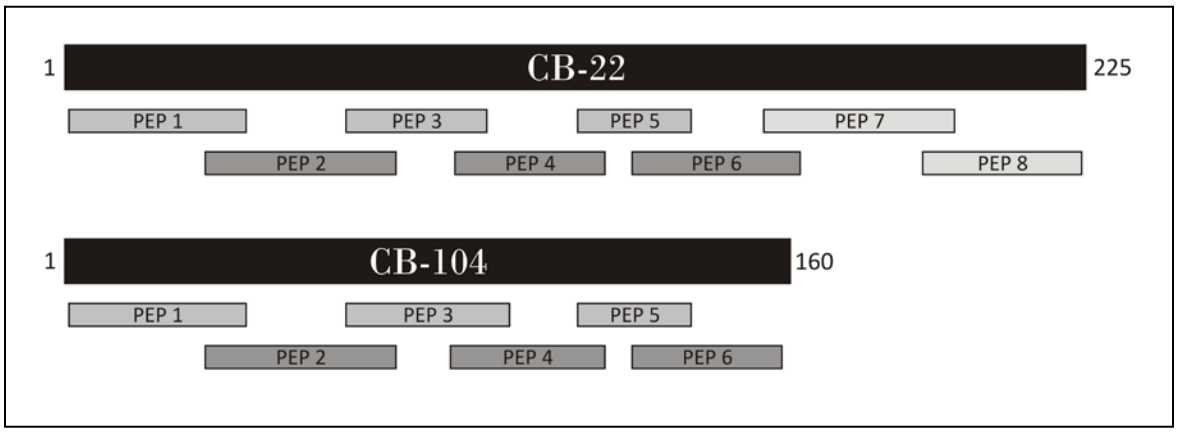


Figure 1. The construction of CTV recombinant peptides. The CB-22 protein was truncated into 8 overlapping peptides and each fragment was expressed as a His-tag fusion protein in *E. coli* BL21(DE3), while the CB-104 protein was truncated into 6 overlapping fragments, expressed in the same way.

Table 1. Synthesized oligonucleotides primers and amino acid locations of the recombinant peptides in CB-22 and CB-104 sequences.

Peptide	Target Protein	Amino acid positions	Pair of primers	Peptide sequences
PEP 01	CB-22	2 - 40	5'-GACGACGAAACAAAGAA-3' 3'-TATCAGAGTCGGATCGAT-5'	DDETKKLKNKNKETKEGDDVVAAESSFGSVNLHIDPTLI
	CB-104			DDETKKLKNKNKETKEGDDVVAAESSFGSLNLHIDPTLI
PEP 02	CB-22	32 - 73	5'-AACTTACACATCGATCCG-3' 3'-GTCAGGCAAGTTAGGATAC-5'	NLHIDPTLITMNDVRQLSTQQNAALNRDLFLALKGKYPNLPD
	CB-104		5'-AACTTACACATCGATCCG-3' 3'-GTCAGGTAAGTTGGGATAC-5'	NLHIDPTLIAMNDVRQLGTQQNAALNRDLFLTLKGKYPNLPD
PEP 03	CB-22	63 - 93	5'-GCTCTGAAAGGGAAGTATC-3' 3'-TGATGAACTCTTAACCGC-5'	ALKGKYPNLPDKDKDFHIAMMLYRLAVKSSS
	CB-104	63 - 98	5'-ACCTTGAAAGGGAAGTATC-3' 3'-ACCGTCGCTTTGTAATG-5'	TLKGKYPNLPDKDKDFHIAMMLYRLAVKSSSLQSDG
PEP 04	CB-22	87 - 119	5'-TTAGCGGTTAAGAGTTCATC-3' 3'-CCAAAGTTTGTCAGACAA-5'	LAVKSSSLQSDDDTTGITYTREGVEVDLSDKLW
	CB-104	86 - 119	5'-CGTTTAGCAGTTAAGAGTTC-3' 5'-CCAAAGTTTGTCAGACAA-3'	RLAVKSSSLQSDGDTTGVTYTREGVEVELSDKLW
PEP 05	CB-22	114 - 138	5'- TTGTCTGACAAACTTTGGAC-3' 3'-GACTCGAAGGGCGTTAGT-5'	LSDKLWTDIVYNSKGVGNRTNALRV
	CB-104			LSDKLWTDVVFNSKGIGNRTNALRV
PEP 06	CB-22	126 - 162	5'-TCTAAGGGTGTTGGTAACCGA-3' 3'-ACGTCCGCCATAACTC-5'	SKGVGNRTNALRVWGRTNDALYLAFCRQNRNLSYGGR
	CB-104	126 - 158	5'-TCTAAGGGTATTGGTAACC-3' 3'-ACTCAAGTTGCGGTTCT-5'	SKGIGNRTNALRVWGRTNDALYLAFCRQNRNLS
PEP 07	CB-22	155 - 196	5'-CGTAATTTGAGTTATGGC-3' 3'-AGCTTGTATGTACACAGCAC-5'	RNLSYGGRPLDAGIPAGYHYLCADFLTGAGLTDLECAVYIQA
PEP 08	CB-22	190 - 224	5'-TGTGCTGTGTACATACAAGC-3' 3'-GAGACGTGTGTTAAATTTCC-5'	CAVYIQAKEPLLKKRGADEVVVTNVRQLGKFNTRL

Legend: 5'→ 3' corresponds to Forward primers and 3'→ 5' corresponds to Reverse ones.

The nucleotide sequences of each peptide was amplified from pET22 vector by polymerase chain reaction (PCR) and cloned onto the pET32Xa/LIC expression vector (Novagen, 2000). The sense primer encodes a Factor Xa recognition site and is in-frame with the open reading frame defined by the vector, while the antisense primer encodes a stop codon. This vector is able to express a heterologous protein fused to the 109 thioredoxin amino acids (to improve solubility), a six amino acid His-tag (to simplify the purification), and 15 amino acid S-tag (for detection and quantification) sequences upstream to the cloning site. The fusion tags together have about 17 kDa and theoretical pI of 5.95, which can be removed by, if is desired, factor Xa cleavage (Xa/LIC Vector Kits Manual, Novagen).

The amplification protocol consisted of a 3 min denaturation at 94°C followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min 30s and extension at 72°C for 2 min, afterwards a final extension at 72°C for 10 min. The blunt PCR products were purified and treated with T4 DNA polymerase in the presence of the dGTP and DTT to generate specific vector cohesive overhangs. The cloning of amplicons in the linearised pET32 vector was carried out according to the manufacturer's protocol (Xa/LIC Vector Kits Manual).

Finally, *Escherichia coli* DH5α competent cells were transformed by recombinant vectors by the standard polyethyleneglycol (PEG) method (Sambrook and Russel, 2001) and plated overnight at 37°C on Luria-Bertani broth (LB) with 100 μg/mL ampicilin. The colonies were selected and individually stored in a permanent LB-glycerol broth and their sequences were verified by PCR and nucleotide sequencing. Afterwards, *E. coli* BL21(DE3) competent cells were transformed with positive CB-22 or CB-104 peptide recombinant vectors by the PEG method and used for protein expression.

Peptide expression

Eschechiria coli BL21(DE3) cells transformed by recombinant vectors were cultured in 30 mL LB broth added to 100 μg/mL ampicilin, grown overnight at 37°C and 300 rpm.

Then, it transferred to 3 L of LB broth with the same antibiotic. The culture was grown at 37°C and 300 rpm until OD_{600} of 0.8, then induced with 5.6 mM lactose for an additional 4 h. Cells were harvested by centrifugation at 2,600xg and 4°C for 10 min. Pelleted bacteria were suspended in 150 mL of 50 mM Tris-HCl buffer, pH 7.5 added to 300 mM NaCl (column buffer) The protease inhibitor 1mM PMSF, as well as, 1 mg/mL of lysozyme were added. The suspension was incubated for 30 min at 4°C and sonicated 8 x 15s at power 20 (70% cycle) in a Sonifer Misonix (Microson Ultrasonic Cell Disruptor XL). The lysed material was clarified by centrifugation at 27,500xg for 10 min at 4°C and the supernatant (soluble fraction) was collected to confirm recombinant protein expression by SDS-PAGE 12%.

The soluble fraction was passed per gravity through an IMAC (Immobilized Metal Affinity Chromarography) column packed with 1.0 mL of nickel-nitrilotriacetic acid (Ni-NTA) resin equilibrated with 10 column volumes (CV) of column buffer. The resin was washed with 8 CV of a 50 mM Tris-HCl buffer (pH 7.5) added to a 1.0 M NaCl. Additional washing was performed with 8 CV from column buffer added to 5 mM of imidazole. Bound proteins were eluted with 5 CV from column buffer added to 200 mM imidazole. Fractions were used to total protein quantification and analyzed in SDS-PAGE 12%.

RESULTS

Expression and purification of recombinant CB-22 and CB-104 proteins

The most common limitation for production of specific MAb to CTV has been the difficulty to obtain a sufficient amount of pure CP due to contamination with plant components because the association of the virus with the phloem. As a result, polyclonal and monoclonal antibodies are often unsatisfactory for an accurate diagnosis. To overcome these drawbacks, the molecular biology had provided the opportunity to produce large amount of proteins expressed in bacterial cells.

Therefore, the CP gene from CB strains had been cloned and the recombinant proteins expressed in *E. coli* BL21 (DE3) after induction by IPTG. The recombinant protein CB-22 has 25 kDa while the CB-104 has 19 kDa, approximately (**Figure 2**). These proteins were purified and yielded, per liter of culture 2.8 mg of CB-22 and 1.68 mg of CB-104 that were used as antigens in all immunoassays, such as ELISA, Westrn blot, immunogold labeling.

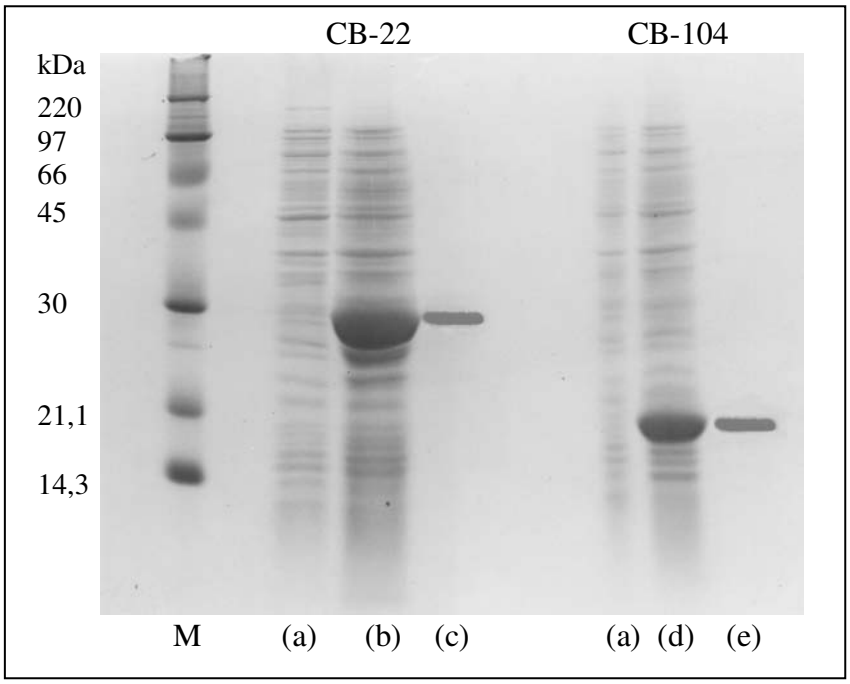


Figure 2. SDS-PAGE 12% analysis of recombinant CB-22 and CB-104 protein expression in (**a**) *E. coli* uninduced (**b**) *E. coli* induced with IPTG, expressing CB-22 protein, (**c**) Purified CB-22 (**d**) *E. coli* induced with IPTG, expressing CB-104 (**e**) Purified CB-104 protein. The sizes of protein molecular weight markers (Amserham) in lane (M) were indicated in kDa.

Monoclonal antibody production and specificity

The four MAbs evaluated in this study were produced from mice immunized with recombinant CB-22 or CB-104 proteins, or with both ones. The MAbs were designated as 30.G.02, 37.G.11, 39.07 and IC.04.12. The first three MAbs belong to subclass IgG1, while the last one belongs to IgG2b isotypes. Furthermore, all monoclonal antibodies contain kappa light chains (**Table 2**).

The specificity and reactivity of the MAb_s against recombinant CP were examined by ELISA and by Western blot assays. The data were very similar demonstrating that MAb 39.07 recognized only the CB-104 protein, while the IC.04-12 reacted only with CB-22, whereas the other two MAbs 30.G.02 and 37.G.11 reacted with both CP recombinant proteins (**Figures 3** and **4**). These results indicated that the binding sites for these MAbs were probably linear.

Table 2. Characterization of monoclonal antibodies raised against recombinant CP proteins, CB-22 and CB-104.

Groups	Immunizing Antigen	Monoclonal antibodies	Isotype		*CB-22*	CB-104
			Heavy Chain	Light Chain		
I	CB-22	IC.04-12	IgG2b	Kappa	+	-
II	CB-104	39.07	IgG1	Kappa	-	+
III	CB-22 and CB-104	30.G.02 37.G.11	IgG1	Kappa	+	+

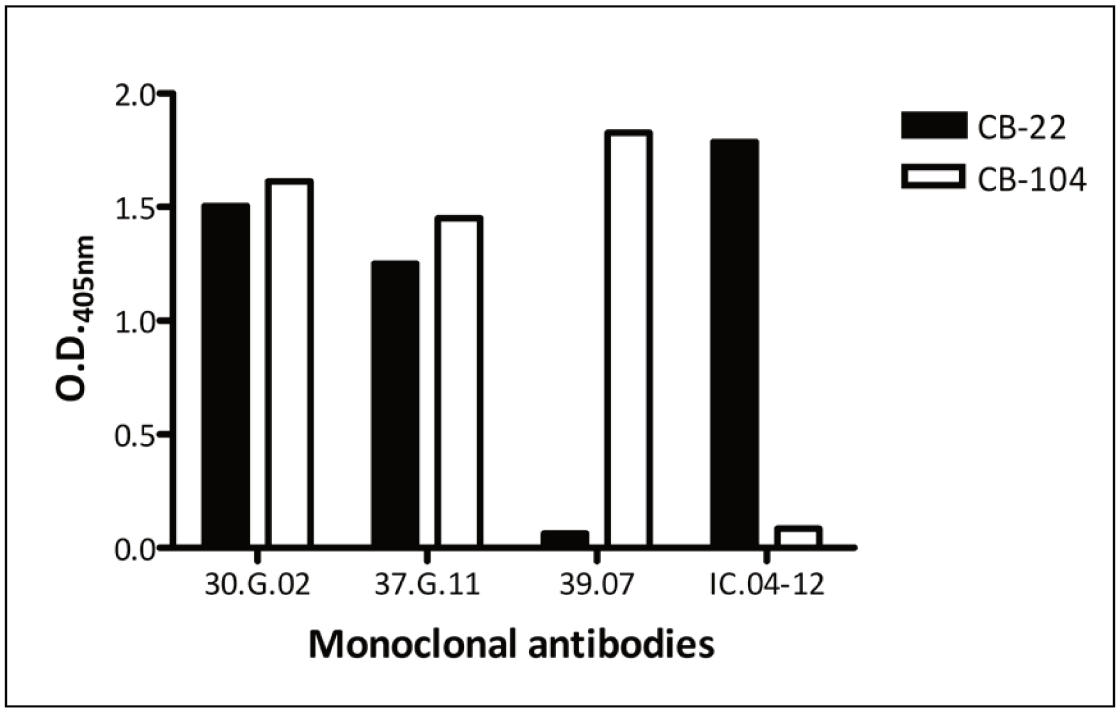


Figure 3. Reactivity of MAbs against recombinant CB-22 and CB-104 proteins determined by indirect DAS-ELISA.

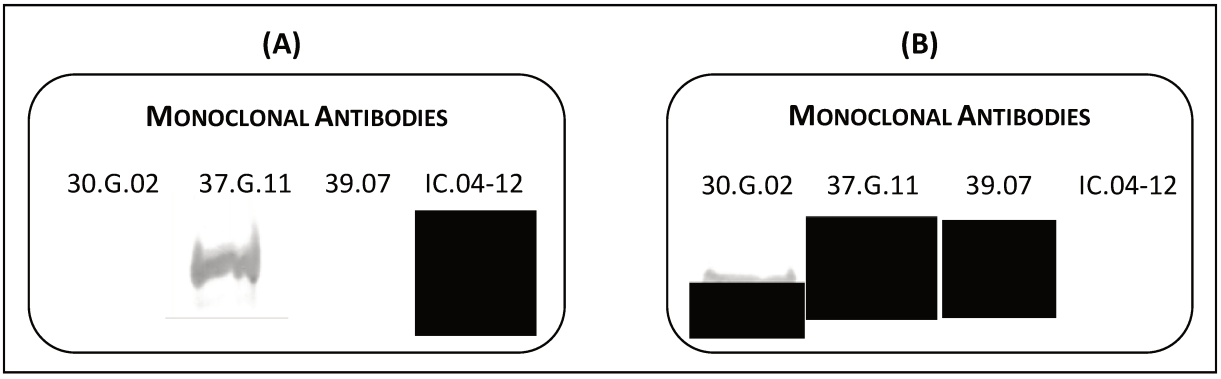


Figure 4. Western blot to analyze the reactivity of CB-22 (**A**) and CB-104 (**B**) using the MAbs 30.G.02, 37.G.11, 39.07 and IC.04-12.

The immunogold-labelled particles of CTV in the electron microscope revealed that the monoclonal antibody Mab 30.G02 was bounded along the entire length of viral particle (**Figure 5**), whereas MAb 37.G.11 marked one of the extremities of the virion (**Figure 6**).

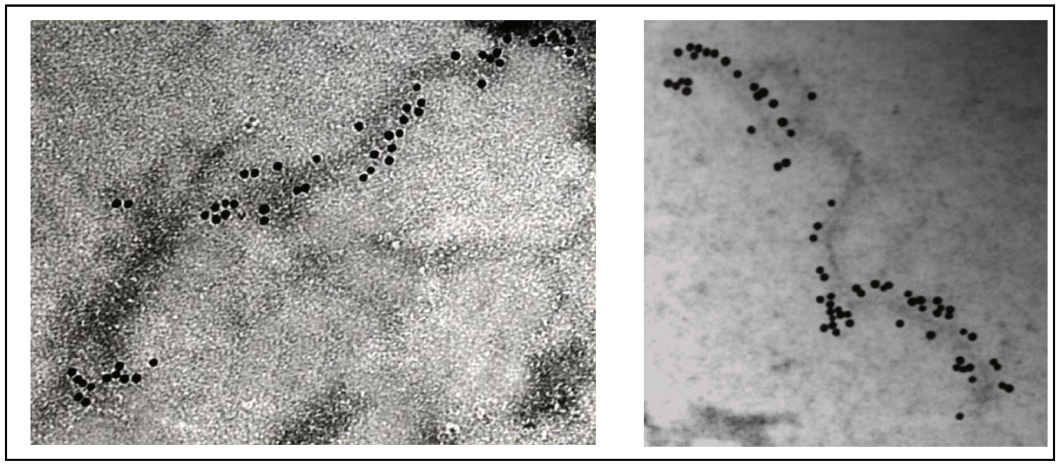


Figure 5. Gold labeling of CTV particles with MAb 30.G.02, showing gold decorations along the entire particle (60,000x).

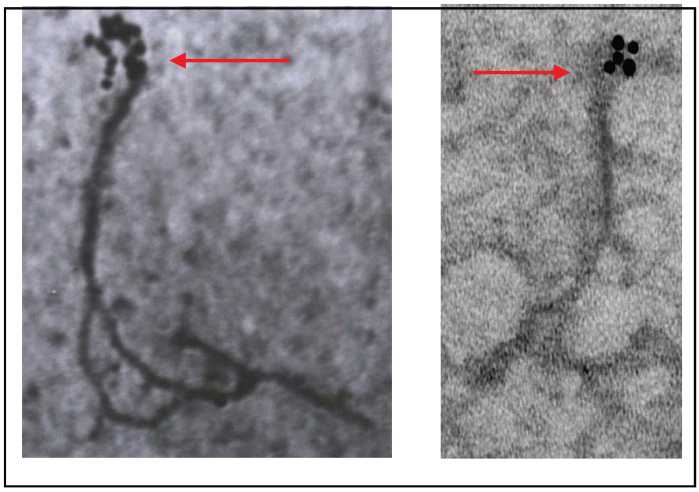


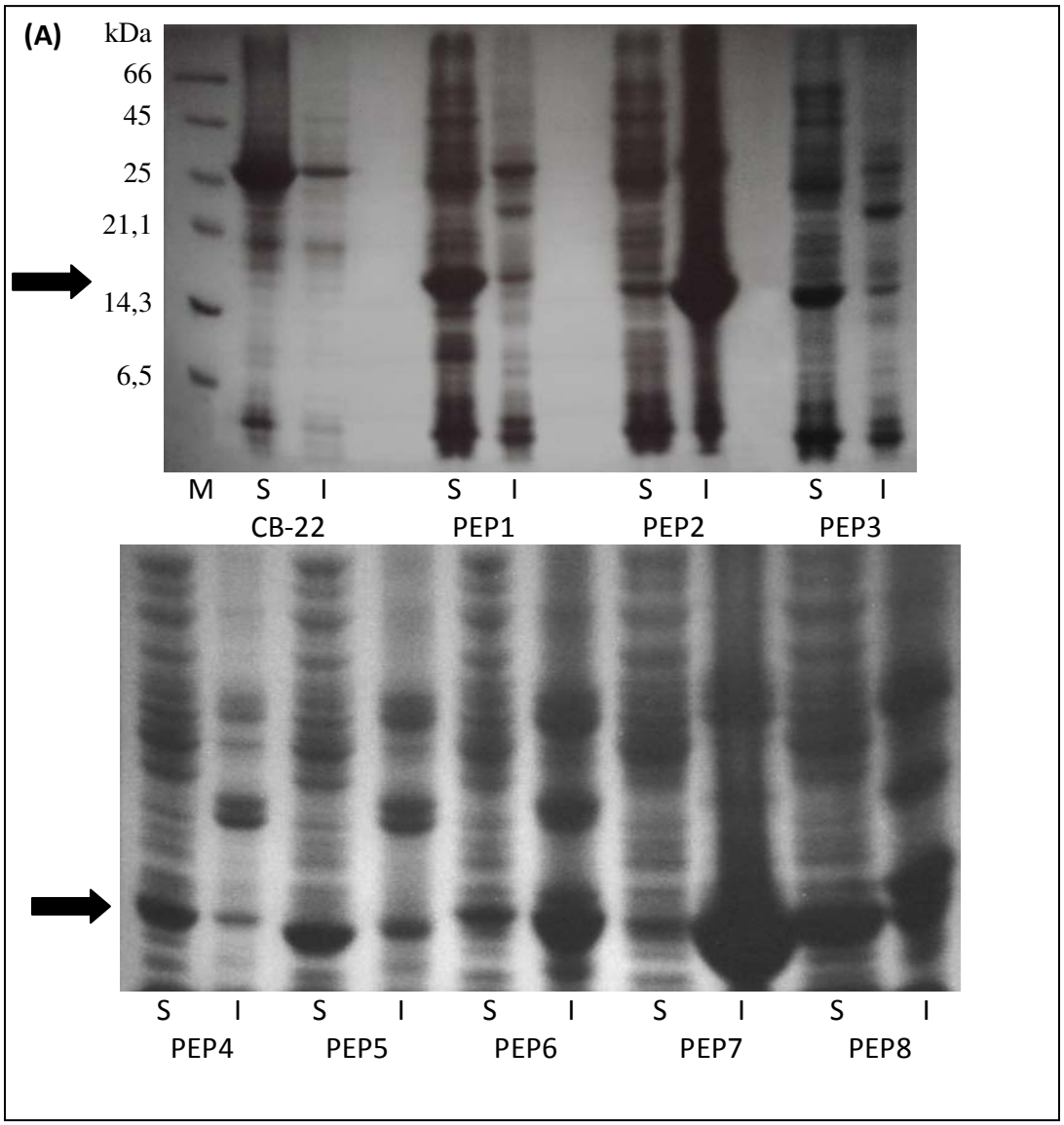
Figure 6. Gold labeling of CTV particles with MAb 37.G.11, showing gold decorations only in one portion of CTV particle (red arrows), restricted to p27 region, as previously observed by Ecale Zhou *et al.* (2002).

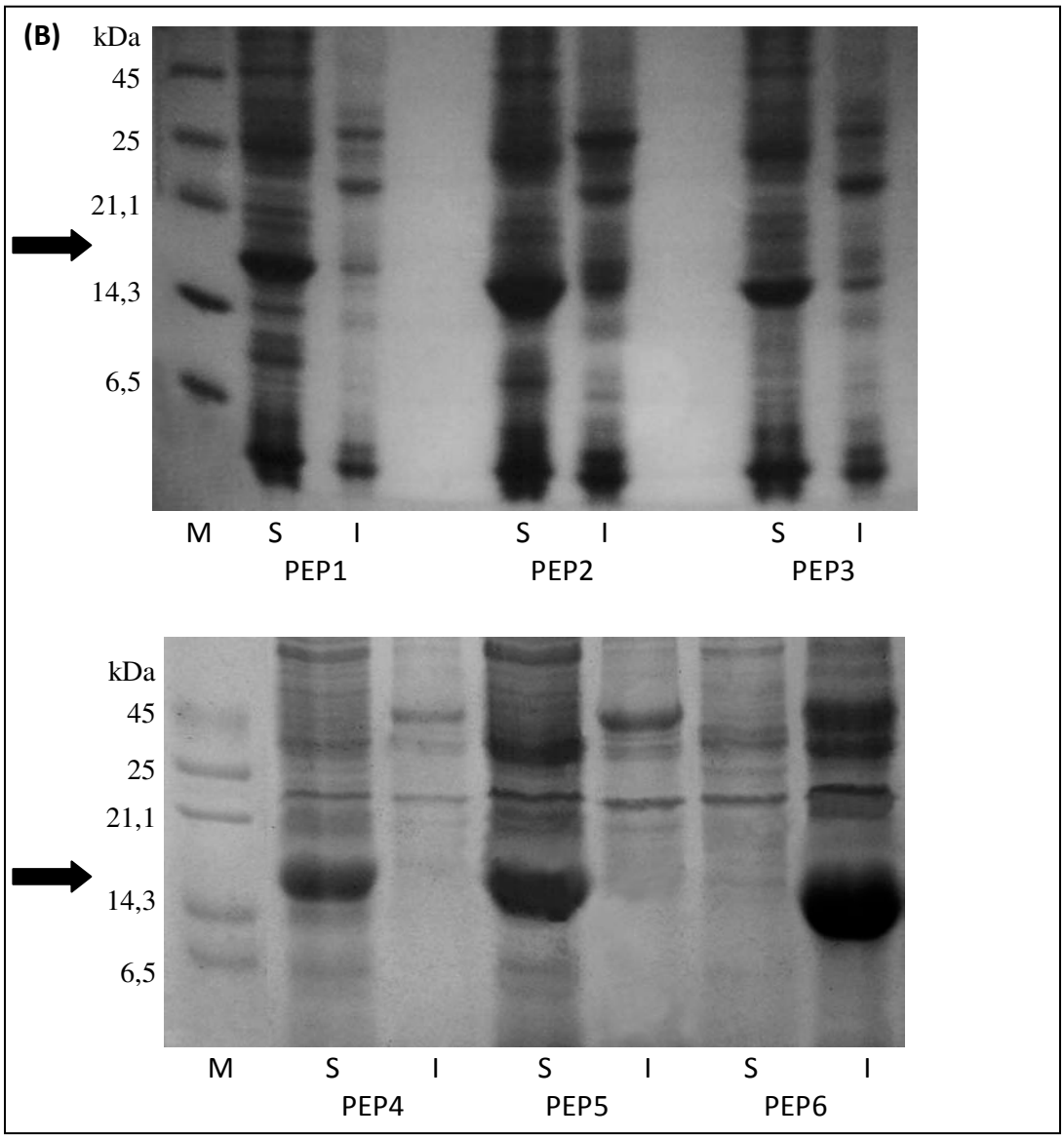
Characterization of MAbs binding epitopes.

To better understand the epitope specificity of MAbs, the CB-22 and CB-104 protein, with 225 and 160 amino acids, respectively, were divided into fragments (Figure 1) based on the hydropathicity analyses according to Kyte and Doolittle (1982) parameters in ProtScale tool (Gasteigeret *et al.*, 2005) on the ExPASy Server.

These recombinant peptides with overlapped fragments were constructed based in CB-22 and CB-104 proteins expressed in *E.coli* using the pET system. The majority of peptides, were expressed predominantly in the supernatant as soluble fraction (**Figure 7, panels A** and **B**), but even peptides expressed predominantly in insoluble fraction have a smaller amount expressed in the soluble fraction, like peptides 2, 6 and 7 of CB-22 (**Figure 7, panel A**) and peptide 6 of CB-104 (**Figure 7, panel B**).

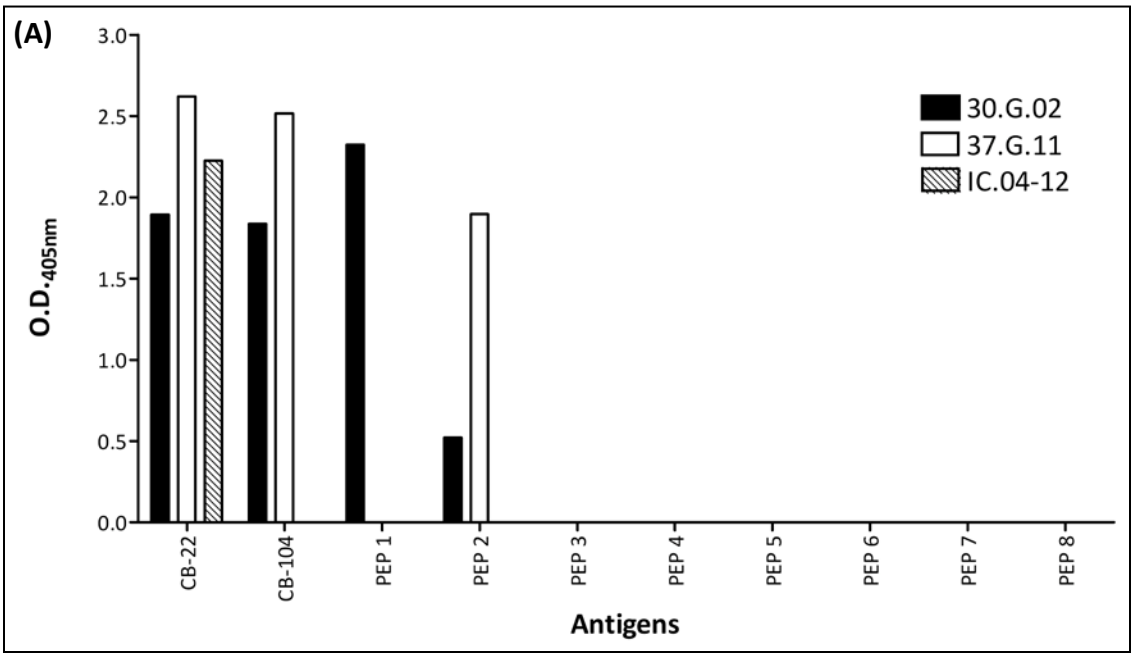
Figure 7. (A) CB-22 and **(B)** CB-104 peptides expression analyzed in SDS-PAGE 12% after induction of 5.6 mM Lactose, and stained with Coomassie Blue R-250. The arrows represent the bands corresponding to expressed peptides. (M) Molecular weight marker, Sigma (S) Soluble fraction (I) Insoluble fraction.




(B)
kDa
45
25
21,1
14,3
6,5
M S I S I S I
PEP1 PEP2 PEP3
kDa
45
25
21,1
14,3
6,5
M S I S I S I
PEP4 PEP5 PEP6

The fusion proteins were purified by Ni-NTA affinity chromatography and used for testing in ELISA (**Figure 8, panels A** and **B**) and Western blot (**Figure 9, panels I, II** and **III**) which showed similar results, demonstrating that the MAb_s 30.G.02 reacted with fragment 1 and 2 (amino acids 2-73) while MAb 37.G.11 reacted only with fragment 2 (amino acids 32-73) of CB-22 and CB-104 and, as expected, MAb 39.07 reacted only with fragment 5 (amino acids 114-138) of CB-104. However, the MAb IC.04-12 recognized none of tested fragments. This means the epitopes recognized by MAb_s 30.G.02, 37.G.11 and 39.07 were linear and mainly localized in two regions: amino acids 2-73 and 114-138, while the MAb IC.4-12 epitope may be conformational.

Figure 8. (A) CB-22 peptides recognition profile by MAb_s 30.G.02, 37.G.11 and IC.04-12 and (B) CB-104 peptides recognition profile by MAb_s 30.G.02, 37.G.11 and 39.07, through DASI-ELISA. The monoclonal antibodies were applied in a saturated concentration and the peptides at 5 μg/ml.



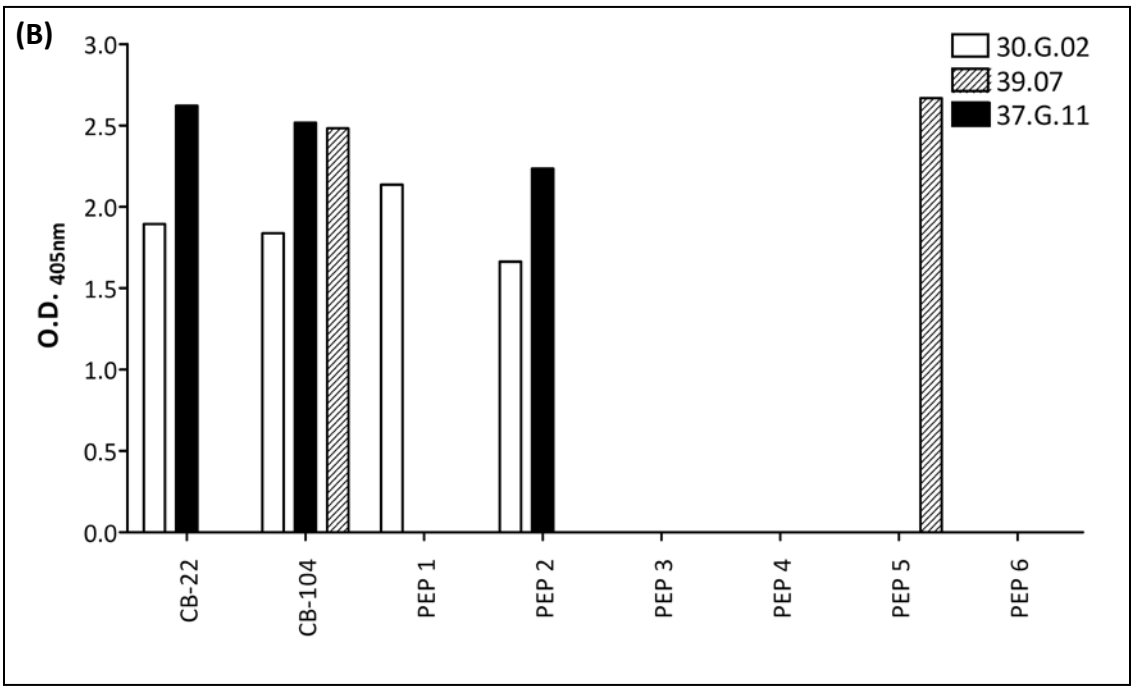
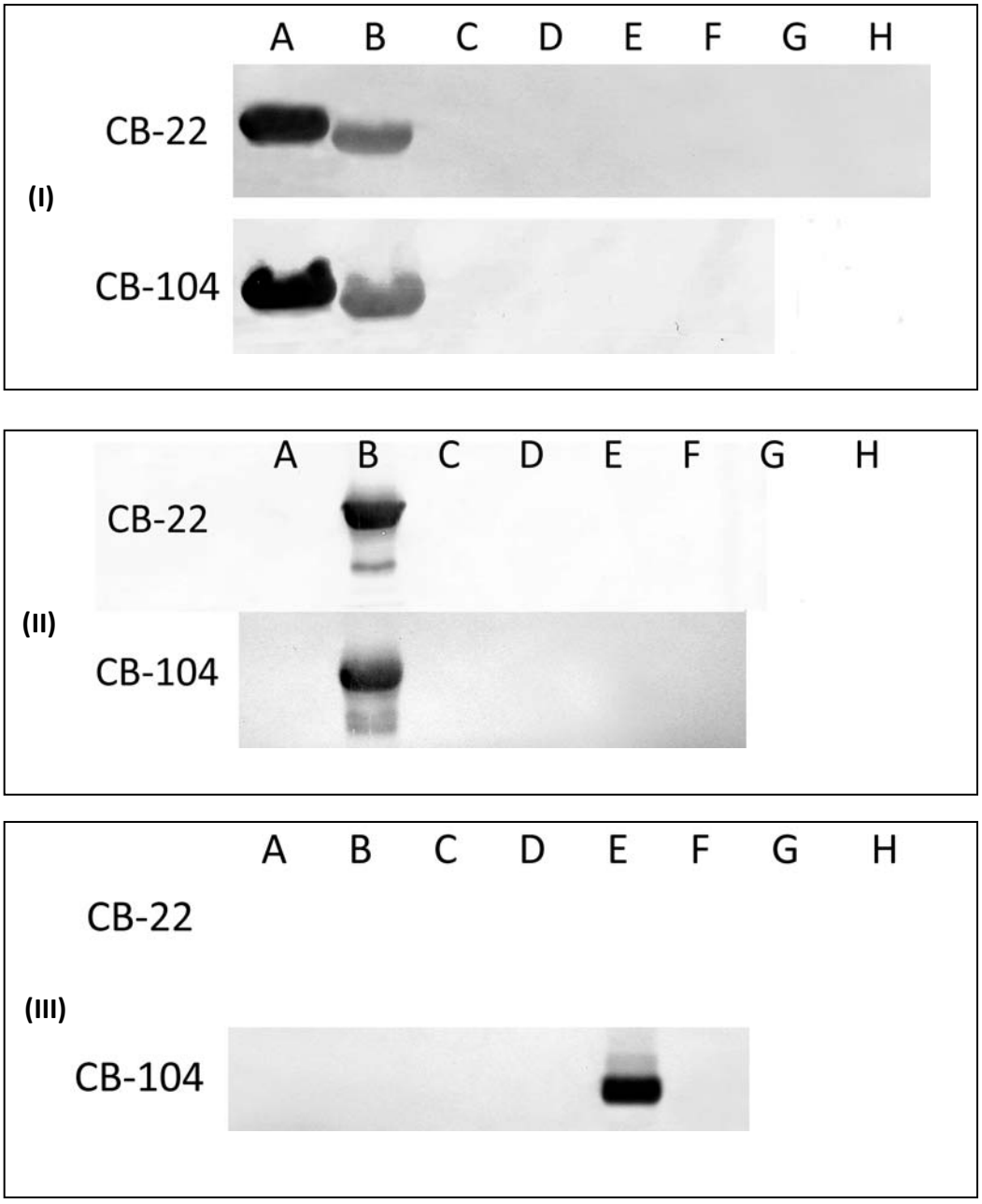


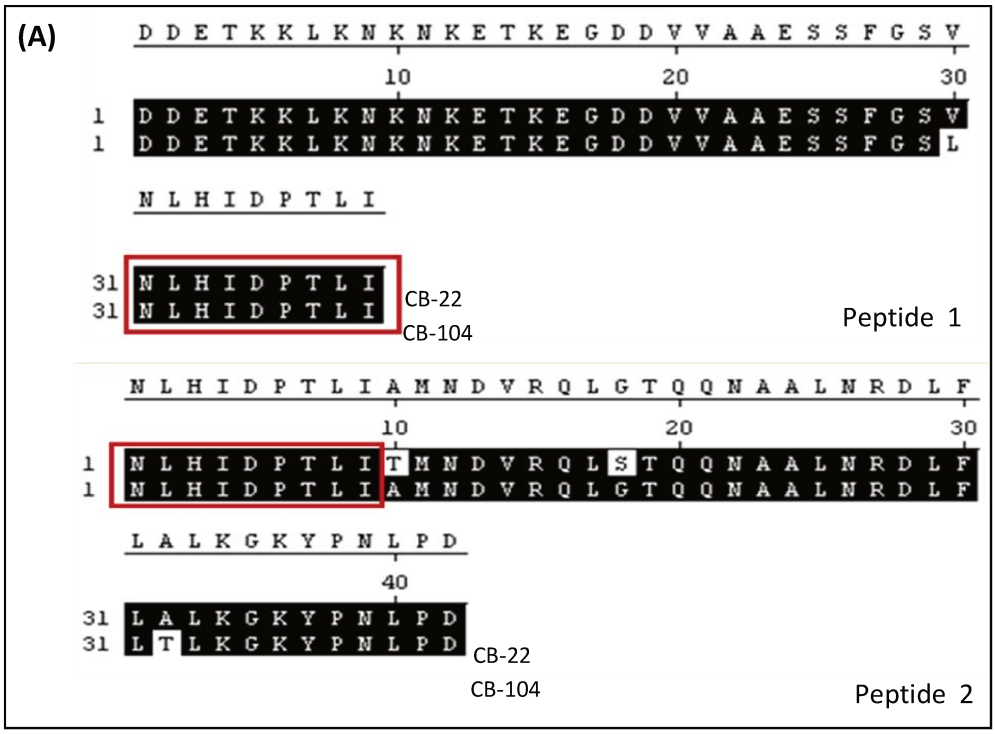
Figure 9. CB-22 and CB-104 peptides recognition pattern by MAb_s **(I)** 30.G.02, **(II)** 37.G.11 and **(III)** 39.07 by Western blot assay. The letters from A to H represent the peptides from 1 to 8 of CB-22 protein and the letters A to F represent the peptides from 1 to 6 of CB-104 one. The monoclonal antibodies were applied in a saturated concentration and the peptides was applied at 100 µg/ml.

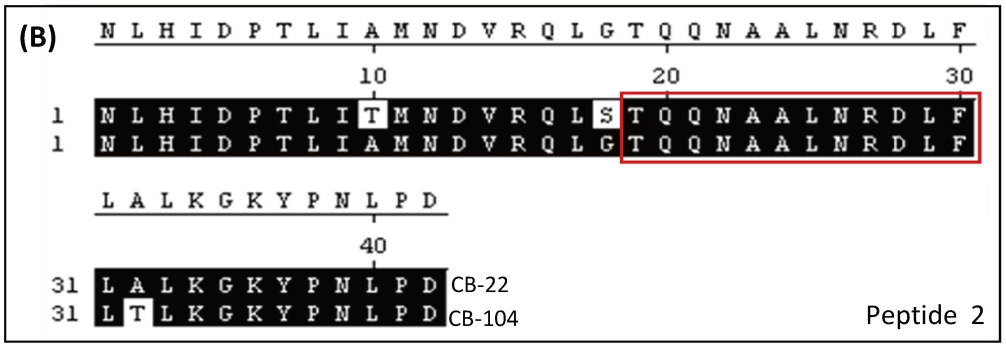


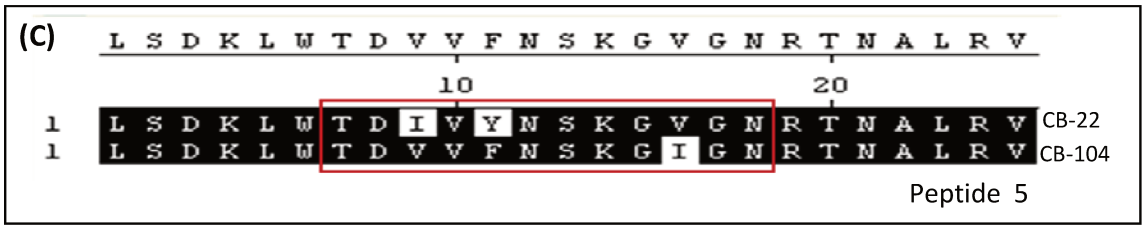
Considering the data from ELISA and Western blot, the positive peptides for each respectively monoclonal antibody, were aligned using Clustal W method in MegaAlign program and the epitope region was determined considering the sequence homologies or divergences, epitope size (from 8 to 14 amino acids) and features like hidrophobicity. To MAb_s 30.G.02 and 37.G.11, the epitopes were localized in an identical region in CB-22 and CB-104 sequences (100% Identities), however, to MAb 39.07, the epitope was determined in a region which has divergences between CB-22 and CB-104.

So, the alignment of the peptides 1 and 2 showed an overlapping region from 32 to 40 amino acid positions (red box in **Figure 10, panel A**), which correspond to sequence [N L H I D P T L I], the specific recognition site for MAb 30.G.02. On the other hand, MAb 37.G.11 recognized the sequence [T Q Q N A A L N R D L F], located at positions 50 to 61 (red box in **Figure 10**, **panel B**), while the MAb 39.07 recognized the sequence [T D V V F N S K G I G N], located from 120 to 131 amino acid positions (red box in **Figure 10, panel C**). This region was determined due to divergences between CB-22 and CB-104 peptides 5 alignment, since MAb 39.07 only recognizes CB-104 protein.

Figure 10. Epitope region for each monoclonal antibody was determined by alignment of ELISA and Western blot positive peptide sequences. Epitope binding site of **(A)** MAb 30.G.02, **(B)** MAb 37.G.11 and **(C)** MAb 39.07.







DISCUSSION

Citrus tristeza virus (CTV) occurs in most citrus producing regions of the world, and it is the most serious viral pathogen of citrus. There are many strains or isolates of CTV and it infections frequently occur as mixtures of strains producing several diseases syndromes in commercial citrus crops. Among Brazilian strains, the most dangerous is the complex denominated Capão Bonito (CB) which affect mainly sweet oranges (*Citrus sinensis* Osbeck) plants grafted on Rangpur lime (*Citrus limonia* Osbeck) rootstock the most adopted variety among Brazilian orchards (Müller *et al.*, 1968). Strategies for minimizing the destructive effect of the CTV include traditional methods, like disease program management and epidemiology studies. However, they are dependent on an appropriate routine diagnosis carried out by ELISA assays based essentially on monoclonal antibodies as MCA 13 and 3DF1 that not differentiate Brazilian CTV strains (Cambra *et al.*, 1998; Permar *et al.*, 1990; Pappu *et al.*, 1995). In all of these assays, the rabbit polyclonal antibodies were used as antigen capture (coating antibody) and MAbs were used as antigen detection.

In attempt to develop a serological tool to rapidly differentiate CB isolates, since it is very convenient method for routine diagnostic once a lot of plant samples must be screened, the coat protein gene of CB strains were cloned and the recombinant expressed protein used for production of monoclonal antibodies (Targon *et al.*, 2000; Stach-Machado *et al.*, 2000). Four stable hybridoma cells lines secreting monoclonal antibodies had been produced in our laboratory which recognition patterns were initially characterized by indirect DAS-ELISA and Western blot. We grouped the MAbs into three groups based on their reactivity with the recombinant proteins CB-22 and CB-104. Therefore, group I recognizes CB-22, group II recognizes CB-104, while group III recognizes both proteins. All of the MAbs recognized the recombinant proteins in Western blot analysis even under reducing conditions, suggesting that the bulk of the antibodies recognize epitopes that are at least partly linear (Stach-Machado *et al.*, 2002).

The MAbs binding epitopes were identified using recombinant peptides**,** which were located in amino acids position 32-40 (N L H I D P T L I) to MAb 30.G.02 and 50-61 (T Q Q N A A L N R D L F) to MAb 37.G.11 in CB-22 and CB-104 proteins. On the other hand, MAb 39.07 reacted to amino acids 120-131 (T D V V F N S K G I G N) in CB-104.

Furthermore, the epitope sequences were blasted in GeneBank (BLASTN 2.2.16, NCBI) to evaluate the worldwide distribution in different CTV isolates (**Table 3**). Therefore, the MAb 30.G.02 can be classified as "Universal" antibody; once recognized an extremely conserved sequence (87.2%) distributed in almost CTV isolates. So, this antibody is optimal as capture (coating) antibody in ELISA Sandwich. Moreover, MAb 37.G.11 recognizes a conserved sequenced in CTV (62.6%), and although it can be used as coating antibody, this monoclonal can also be used as detection antibody to a quick and simple screening. Furthermore, MAb 39.07 was classified as a "Severe" specific antibody, since its epitope was present only in 19.7% of CTV coat protein sequences and these sequences correspond to severe isolates from different countries infected by CTV.

Summarizing, our described monoclonal antibodies had already routinely widespread application and research work in detection of CB Brazilian strains.

Table 3. CTV epitope in worldwide distribution in different CTV isolates. Each epitope was blasted against coat protein from other CTV isolates and classified in severe, mild, or universal epitope.

MAb_s	30.G.02	37.G.11	39.07
Total of sequences	187	187	187
Number of sequences	163 87,2%	117 62,6%	36 19,3%
e-value	$9e^{-6}$	$2e^{-3}$	$2e^{-7}$
CTV epitope	Universal	Mild	Severe

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Purification and secondary structure characterization of the Citrus Tristeza Virus coat protein

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ABSTRACT

The coat protein (CP) is one of the major protein components of the virion, which is primarily required for genome protection. CP may also be involved in translocation into the host cell and for cell-to-cell movement, due to a probable interaction with host cells via specific receptors to start an infection. The capsid of the citrus tristeza virus (CTV) is made up of two types of CPs with 25 and 27 kDa. Therefore, the CTV coat protein from a Capão Bonito complex (São Paulo, Brazil) was produced in *Escherichia coli* BL21 (DE3) and purified by nickel affinity chromatography. The recombinant protein has a predictive molecular mass of 25 950 Da whose identity was confirmed by ELISA and Western blot analyses. The purified CP protein was analyzed for secondary structure through circular dichroism (CD) spectroscopy and analysis showed that CP comprises 44% α-helix, 12% β-sheet and 44% random coil content. In addition, the region between amino acid residues 25 and 100 of CTV CP is predicted to be an antigenic epitope region on the basis of its hydropathicity plot and comparison with the CP sequences of CTV. Our results contribute to structural characterization of CP and may help to understand its biological and physiological functions.

Keywords: Citrus tristeza virus, coat protein, secondary structure, circular dichroism

INTRODUCTION

Citrus tristeza virus (CTV) is the causal agent of one of the most important viral disease of citrus worldwide [1]. CTV belongs to the *Closteroviridae* family, genus *Closterovirus* and is a long filamentous virion with a positive-sense single-stranded RNA (ssRNA) genome, with 12 open reading frames (ORFs) encoding up to 19 protein products [2].

Double stranded RNAs (dsRNA), corresponding to the full length ssRNA replicative form (13.3 $x10^3$ kDa), and several subgenomic RNAs have also been detected in CTV infected plants [3],[4]. The *Closteroviridae* virus have a unique bipolar architecture incorporating at least five proteins that are assembled into a long body of uniform morphology and a short segmented tail, where most of the helical nucleocapsid is encapsulated by the major coat protein (CP) and a small portion, at one end, is encapsulated by the minor coat protein (CPm) [5].

CTV CP (p25) has 25 kDa and encapsulates approximately 95% of the particle length, while the CPm has 27 kDa (p27) and encapsulates one end of it [6]. Genetic and biochemical analyses have shown that the functions of these virion components are distinct. The virion body is required primarily for genome protection, and the tail may represent a specialized device for cell-to-cell movement [7]. It has been reported that recombinant CTV CP from different world regions has been cloned and expressed [5],[8],[9]. However, beyond the primary structure, our knowledge of the molecular and structural aspects of CTV coat protein is very limited and poorly known. To date, there is no information available in the protein data base (PDB), on the secondary structure of any CTV CP protein, and even in literature, this subject is poorly investigated. However, this information is quite important for developing and establishing functional assays to determine the coat protein functions, such as its influence on viral particle movement or to analyze the interaction between coat protein and host plant cells.

In this study, the major capsid protein CP 25 kDa of the Brazilian CTV Capão Bonito isolate (CB-22) was cloned into the prokaryotic expression vector (pET22) to express the

recombinant CB-22 in *E. coli* BL21(DE3). These recombinant protein was also, purified by nickel affinity chromatography a identified by ELISA and Western blot analysis. The secondary structure was determined through Circular Dichroism (CD) spectroscopy.

MATERIAL AND METHODS

E. coli strains, plasmid, enzymes and other materials

The oligonucleotide primers were synthesized at Invitrogen Life Technologies (São Paulo, Brazil). The pET22b vector and the BL21(DE3) strain were obtained from Novagen (Madison, WI, USA). The Ni-NTA affinity resin was obtained from Quiagen (Hilden, Germany) and the molecular mass marker from Fermentas (Burlington, Canada). The Coomassie brilliant blue reagent for total soluble protein determination was purchased from Bio-Rad (Hercules, CA, USA). The protease inhibitor phenylmethylsulfonyl fluoride (PMSF), lysozyme and, bovine serum albumin (BSA) were purchased from Sigma Chemical (Saint Louis, MO, USA). All other chemicals used were of at least reagent grade.

Source of virus

Leaves obtained from a nineteen-year-old 'Pera' sweet orange tree grafted on Rangpur lime, collected in Capão Bonito county (SP, Brazil) were used. This plant had been inoculated with the Capão Bonito complex by grafting at the nursery stage. The samples collected were stored at -70 ºC until use.

Expression of recombinant CB-22

The clone CB-22 containing the full length CTV coat protein (CP) gene in pET22b expression vector (Novagen, Madison, WI, USA) in *Escherichia coli* DH5α was obtained by Targon [10]. The nucleotide sequence of this clone was initially confirmed by automated

sequencing, using universal primers into pET22b vector, and *E. coli* BL21 (DE3) cells were transformed by recombinant vectors by the standard polyethylene glycol (PEG) method [11].

A starter culture of 10 mL LB medium (10 g tryptone, 5 g yeast extract, 5 g NaCl per 1L) supplemented with 100 μg/mL ampicillin was inoculated with single colonies of *E. coli* BL21(DE3) cells transformed by recombinant plasmids and grown overnight at 37ºC and 300 rpm. This inoculum was transferred to a main culture of 1.0 L of LB medium containing ampicillin at the same concentration, and incubated at 37ºC and 300 rpm until reaching $O.D._{600nm}$ = 0.8. Finally, the protein expression was induced with 5.6 mM lactose for an additional 4 h and cooled on ice.

Extraction of CB-22

All extraction and purification steps were performed at 4 – 10ºC. Cells were harvested by centrifugation at 2600 x g for 10 min. Cell pellet was suspended in 44 mL of 50mM Tris–HCl buffer, pH 7.5 containing 300 mM NaCl (adsorption buffer). Protease inhibitor PMSF and lysozyme were added at final concentration of 1.0 mM and 1.0 mg/mL, respectively and incubated for 30 min. The cells were disrupted by sonication (8 x 15 s at 70% of the maximum power in a Misonix Sonifier (Microson Ultrasonic Cell Disruptor XL)). The insoluble fraction of the cell lysate was sedimented at 27,500 x *g* for 15 min and the supernatant was collected. The expression of the recombinant protein was confirmed by SDS–PAGE, according to Laemmli [12].

Immobilized Ni-Affinity chromatography

One milliliter of nickel-nitrilotriacetic acid (Ni-NTA) resin was packed in the 1 cm diameter column and equilibrated with 10 column volumes (CV) of adsorption buffer (50 mM Tris-HCl, pH 7.5, 300 mM NaCl) at room temperature. Six milliliters of the clarified supernatant was applied to the column at 1 mL/min and flow through (FT) was collected for analysis. The column was washed with washing buffer (50 mM Tris–HCl, pH 7.3, 1.0 M

NaCl) to remove non-specific interactions. When the washing buffer reached the baseline, bound proteins were eluted by utilizing imidazole at three different concentrations: 5, 100 and 200 mM in the adsorption buffer. Fractions containing 1.0 CV were collected, assayed for total protein concentration and analyzed by SDS–PAGE. The CB-22-containing fractions were pooled and concentrated by ultrafiltration (Amicon Ultra, 10 kDa cut-off, Millipore, San Diego, CA, USA) and stored at -20ºC.

Quantification of Protein

The total soluble protein concentration was assayed according to the method presented by Bradford [13], using bovine serum albumin as standard. In addition, the purified CB-22 protein was also quantified, based on absorbance at 280nm, using a calculated extinction coefficient of 0.945 at 1 mg/mL (Abs 0.1% (1 g/l) = 0.945) [14].

DASI-ELISA

The indirect sandwich ELISA (DASI-ELISA) was performed according to Clark [15]. The 96-well microplates (Pierce Biotechnology, Rockford, IL, USA) were coated with 100 μL of #1006 anti-CTV rabbit polyclonal antiserum [16] diluted 1:1,000 in 0.2M sodium-carbonate buffer, pH 9.6. After overnight incubation at 4°C, the wells were blocked with 200 μL of 2% bovine serum albumin (BSA) in phosphate buffer saline (PBS) at 37 ºC for 1 h. After three washing with 200 μl PBS, 0.05% Tween 20 (PBS-T), 100 μL of a 3 μg/ml recombinant CB-22 in PBS was allowed to bind to the adsorbed antibodies at 37°C for 2 h. The plates were extensively washed with PBST and loaded with 100 μl of unlabeled monoclonal anti-CB-22 antibody (1:1,000) [17] at 3 μg/ml in PBS.

The antigen-bound monoclonal antibodies were detected using 100 μL goat anti-mouse antibodies conjugated to alkaline phosphatase (Sigma Chemical, Saint Louis, MO, USA), diluted 1:30,000 in PBS. Finally, the wells were loaded with the substrate solution and 1 mg/ml of p-nitrophenyl phosphate (Pierce Biotechnology, Rockford, IL,

USA) and the plates were incubated at room temperature for 1 h. The ELISA reactions were measured using ELISA reader (Asys Expert Plus) at $O.D._{405\ nm}$.

SDS gel electrophoresis

SDS-PAGE according to Laemmli [12] was performed using 12% acrylamide separating and 5% stacking gels containing 0.1% SDS (reducing conditions). Protein bands were stained with Coomassie brilliant blue R-250. Broad-range protein molecular weight marker (Sigma Chemical, Saint Louis, MO, USA) was used for the estimation of protein size.

Western blotting assays

Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA, USA) was used for immunoblotting with Hybond-N (Amersham–Pharmacia Biotech, Uppsala, Sweden) membrane and 85 mm blotting papers according to Towbin [18]. Protein samples (0.5–1.0 µg each) were separated in SDS-PAGE and transferred on to the membrane using transfer buffer (25 mM Tris, 192 mM Glycine, pH 8.3, and 20% methanol) at 200 mA at 4°C for 2 h. Membranes were blocked for 3 h by incubation in 5% (w/v) non-fat dry milk (skim milk) dissolved in 500 mM NaCl, 20 mM Tris–HCl, pH 7.5 (TBS) at room temperature (RT).

Immunoblots were washed three times for 5 min each with TBS containing 0.01% (v/v) Tween 20 (TBST) and subsequently incubated overnight at RT with monoclonal anti-CB-22 antibody (1:1,000) [17] in TBST with 5% skim milk. Membranes were washed three times, incubated with goat anti-mouse alkaline phosphatase conjugated (1:30,000) secondary antibody in TBST at RT for 1 h. Finally, the membranes were washed as described above, and the colorimetric reactions were developed with nitro blue tetrazolium (NBT) (Pierce Biotechnology, Rockford, IL, USA) and 5-bromo-4-chloro-3-indolyl phosphate, p-Toluidine Salt (BCIP) (Pierce Biotechnology, Rockford, IL, USA)

Circular dichroism spectroscopy

The fractions containing the recombinant CB-22 protein were concentrated to 5 mg/mL and dialyzed against 5 mM Tris-HCl buffer, pH 7.5 in preparation for circular dichroism (CD) spectroscopy analysis by a Jasco Spectropolarimeter Model J-810 (Japan Spectroscopic, Tokyo, Japan) to assess the secondary structural integrity and composition. CB-22 dilutions of 1:2, 1:4, 1:5, 1:10 and 1:50 were measured in one of three reference cells (1.0 cm, 1.0 mm and 0.1 mm) at 20°C to determine optimal conditions.

The CD spectra were generated using a 1.0 mm path length cuvette containing 200 μl of the CB-22 protein solution at 0.455 mg/ml in 5 mM Tris–HCl buffer, pH 7.5, at 20°C. The spectrum was presented as an average of four scans recorded from 190 to 250 nm, at a rate of 20 nm/min. Mean residue ellipticity ([ϑ] expressed in deg × cm^2/dmol) was calculated using $[\vartheta] = \vartheta \times 100 \times M_r/(c \times l \times N_A)$, where ϑ is the experimental ellipticity in mdeg, M_r is the protein molecular weight in Daltons, c is protein concentration in mg/mL, l is the cuvette path length in cm and N_A is the number of residues in the protein [19].

Secondary structure was predicted based on the expressed CB-22 amino acid sequence using PSIPRED program [20],[21] according to the method described by Jones [22].

Hydropathicity

The hydropathicity of CB-22 recombinant protein was analyzed according to Kyte and Doolittle [23] parameters using the ProtScale tool [24] on the ExPASy Server (http://www.expasy.org)

RESULTS AND DISCUSSION

Cloning and sequencing of CB-22 recombinant protein

The ORF of the 25 kDa CTV coat protein (CP) gene was successfully amplified by RT-PCR (reverse transcription-polymerase chain reaction) and cloned in pET22 expression vector [10]. It was not difficult to transform the *E. coli* DH5α and BL21 (DE3) strains. The CP gene sequence (CB-22) was confirmed by automated sequencing using universal primers into pET22 vector and compared with other CTV CPs nucleotide sequences in NCBI (National Center for Biotechnology Information) using BLASTN 2.2.16 (Basic Local Alignment Search Tool).

The CB-22 sequence showed high homology (98-95% Identity, e-value 0.0) with many CTV major coat proteins, such as CTV CP from isolate 81P (GenBank accession no. **AY995567**) and 464-2 (GenBank accession no. **AY995566**) from California, USA; isolate B35 (GenBank accession no. **L12175**) and T30 (GenBank accession no. **AF260651**) from Florida, USA; isolate T385 (GenBank accession no. **Y18420**) from Spain; isolate CPCu IJ-12 (GenBank accession no. **AF220503**) from Mexico and isolate TN.2 (GenBank accession no. **AY707460**) from Iran. These data confirmed the identity of CB-22 sequence as a CTV major coat protein.

Expression and purification of CB-22

The CB-22 recombinant protein was expressed in a soluble form and about 11 mL of total protein per liter of culture was obtained. Dose dependence and time course studies of the induction of the recombinant protein expression, analyzed by SDS–PAGE, led to lactose concentration of 5.6 mM and induction time of 4 h at 37°C. Although, no improvement in the expression level was verified when different expression strains, induction temperatures, and growth phase-induction were tested (data not shown).

A high amount of the CB-22 protein was recovered by immobilized metal affinity chromatography. The reduction of non-specific interactions between proteins and the matrix was done by washing the resin with high salt concentration and low imidazole

concentration buffers. The recombinant protein was eluted with 200 mM imidazole (**Figure 1**), a procedure that considerably improved the final protein purity.

The recombinant CB-22 protein remained stable in the solution and the final amount was approximately 2.4 mg/L of initial bacterial broth. The recombinant protein CB-22 was probed by ELISA and Western blot with the monoclonal antibody MAb IC04-12 that specifically recognizes this protein in either natured or denatured conditions [25]. The ELISA revealed a positive reaction when MAb IC04-12 was used, which confirmed the identity of this recombinant protein (**Figure 2**). The western blotting analysis showed a unique band of molecular mass of approximately 26 kDa, this value being close to the theoretical molecular mass calculated from the primary amino acid sequence: 25,950 Da (**Figure 3**).

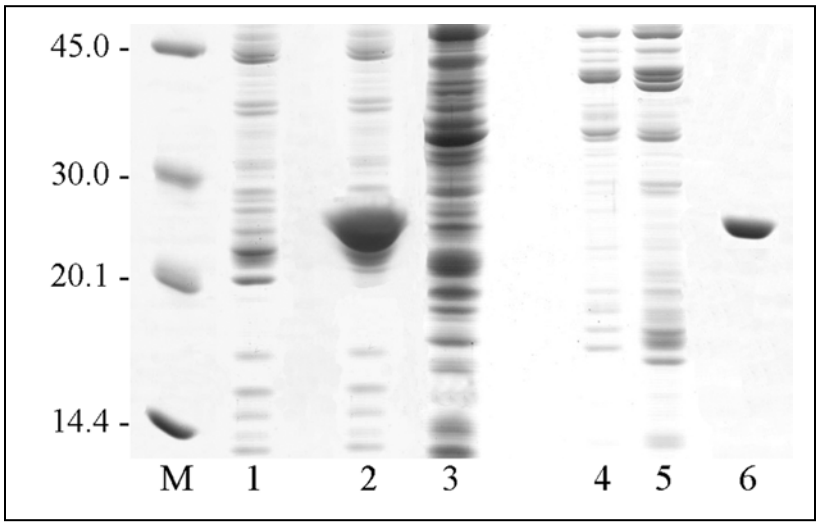

Figure 1. SDS-PAGE gel showing the expression and purification of CB-22 (Coomassie-stained). Lanes: (M) molecular markers, (1) non-induced cells, (2) clarified supernatant after cell induction with 5.6 mM Lactose, (3) flow-through from Ni-NTA column, (4), recombinant CB-22 eluted with 5 mM imidazole, (5) recombinant CB-22 eluted with 100 mM imidazole and (6) recombinant CB-22 with 200 mM imidazole.

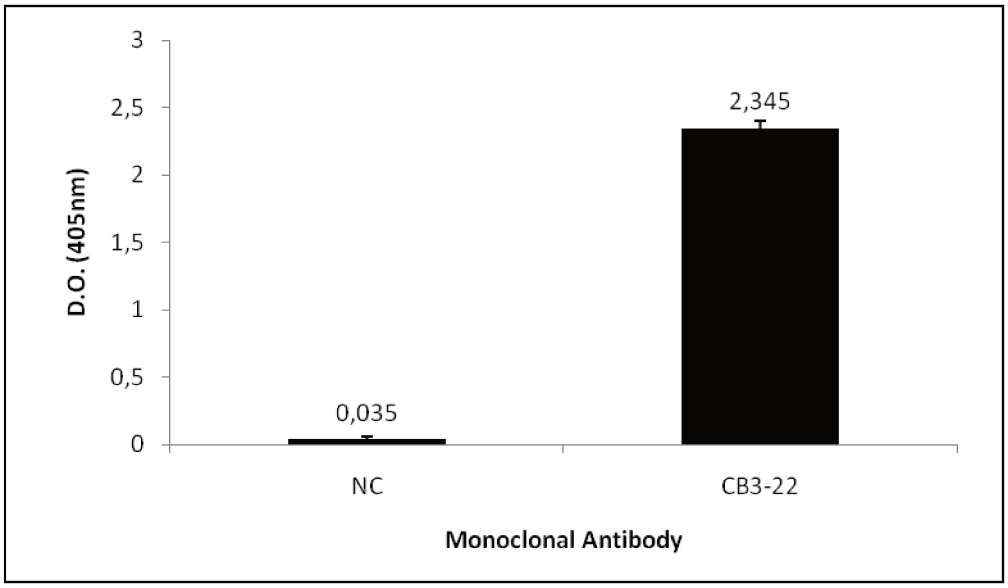

Figure 2. Detection of the recombinant CB-22 protein by ELISA. The protein was probed with monoclonal antibody MAb IC.04.12, which specifically recognizes this protein, while normal serum was applied as negative control (NC).

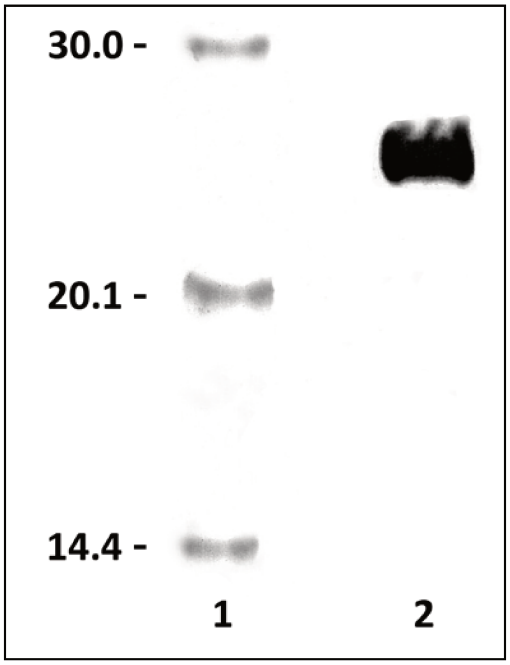

Figure 3. Identification of recombinant CB-22 protein by Western blot. The protein was probed with monoclonal antibody MAb IC.04-12 (lane 2). Lane 1, standard protein markers. The observed molecular weight of this protein was 26 kDa similar to the predicted MW, 25.59 kDa.

Circular dichroism (CD) spectroscopy analysis

The structural integrity and the secondary structure content of the purified recombinant CB-22 were determined by circular dichroism spectroscopy, measured between 190 and 250 ηm (**Figure 4**). The CD spectrum indicated that the recombinant CB-22 contained a substantial amount of secondary structure. The minima at 222 and 209 ηm and the maximum at 192 ηm indicated the presence of α-helical structures in the protein; furthermore, the overall spectrum was characteristic for a protein with mixed α/β-structure [19].

The secondary structure was analyzed by software K2d [26],[27], and the calculated amounts of α-helices, β-strands and random coils were: 45, 15 and 40%, respectively. The experimentally derived composition displayed reasonable agreement with PSIPRED prediction, based on amino acid sequence only (**Figure 5**) which estimated 44% α-helices, 12% β-strands and 44% coils for the CB-22 protein.

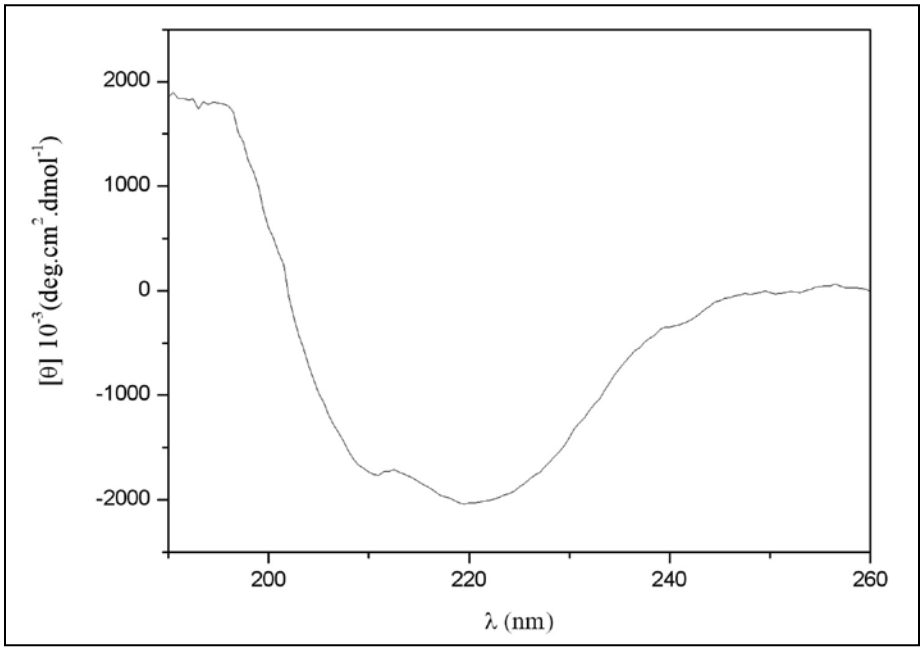

Figure 4. Circular dichroism spectrum of the purified CB-22 protein. The spectrum was generated using CB-22 protein at concentration of 0.455 mg/mL in a 5 mM Tris–HCl buffer (pH 7.5, 20°C). Four scans in a 250–190nm range were performed at a rate of 20 ηm/min and 20°C.

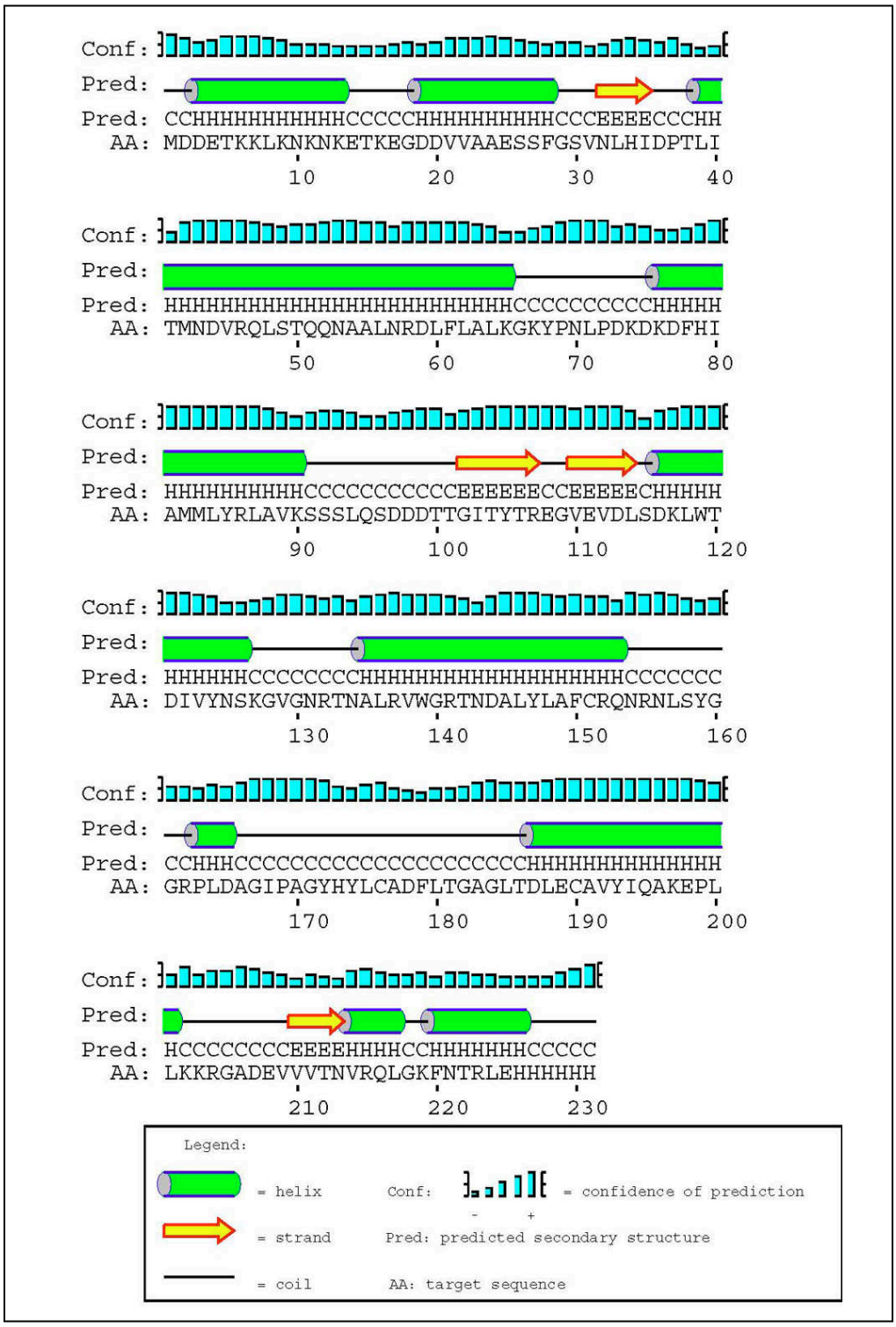

Figure 5. CB-22 secondary structure prediction from the Protein Structure Prediction Server (PSIPRED). The secondary structure analysis revealed a majority of α-helix structures and some β-strands.

The deviation between experimental data and theoretical prediction indicated a higher than expected amount of β-strand, resulting in a less than expected amount of random coil. It should be noted that the program K2d considers *random coil* to include all residues that do not participate in an α-helix or a β-strand, and this term does not imply that such residues lack a defined and stable structure within a given protein. Having more residues in a regular secondary structure conformation probably helps to stabilize the protein, and is an indication of a well folded protein.

The results indicated that the protein is quite stable at room temperature and remained folded throughout the purification process, and the recombinant protein may be suitable for crystallization studies, with the aim of three dimensional structure determinations.

CONCLUSION

The *Closteroviridae* have a unique bipolar architecture incorporating two coat proteins, where most of the helical nucleocapsid is encapsulated by the major coat protein (CP) and a small portion, at one end, is encapsulated by the minor coat protein (CPm). Genetic and biochemical analyses have shown that the functions of these virion components are distinct, the virion body is required primarily for genome protection, and the tail represents a specialized device for cell-to-cell movement.

This report illustrated the expression, purification, and partial characterization of a CTV recombinant coat protein CB-22. The CD analysis of the CB-22 indicated a secondary structure composed mainly of α-helices, although some short β-strands also seemed to be present. No previous studies of the secondary structure of recombinant CP proteins have been conducted, however, the sequence analysis of CPs from several CTV isolates is well documented. Many divergent points in the primary sequences of CP for many biologically and geographically diverse strains of the virus have been found and the main amino acid divergences are found in the C-terminal region. Our central goal was to elucidate the structure of CP with the ultimate hope of using this knowledge to obtain insights into the fundamental basis of molecular recognition of the virus capsid and its interactions with the host plant. This work presents new information for the first time, about the structural features of the CTV coat protein, whose biological role may be related to CTV pathogenicity.

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Assessment of the diagnostic potential of Immmunocapture-PCR and Immuno-PCR for Citrus Variegated Chlorosis

Diagnostic potential of IC-PCR and I-PCR for CVC

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ABSTRACT

Xylella fastidiosa causes significant losses in many economically important crops. An efficient pathogen detection system is critical for epidemiology studies, particularly when large sample size is involved. In this study we report the development of immunomolecular assays like Immmunocapture-PCR and Immuno-PCR for direct detection of *X. fastidiosa* without DNA isolation. Whereas the reactivity of ELISA and PCR ranged from 10^6 to 10^4 bacterial cells, the IC-PCR sensitivity was up to 10^3 and the detection limit of I-PCR was up to 10^1 bacterial cells. These methods can use either plant sample extracts or cultivated media, and show no cross reaction for any other endophytic citrus-bacteria. Therefore, IC-PCR and I-PCR assays provide an alternative for quick and very sensitive methods to screening *X. fastidiosa*, with the advantage of not requiring any concentration or DNA purification steps while still allowing an accurate diagnosis of CVC.

1. Introduction

Xylella fastidiosa subsp. *pauca* (Schaad et al., 2004) is a xylem-limited Gram-negative bacterium (Hopkins, 1989) of a broad range of perennial plants and is responsible for significant losses in many economically important crops. In Brazil, this bacterium is associated with plum leaf scald (Rosseti et al., 1990), Citrus Variegated Chlorosis (CVC) (Chang et al., 1993) and coffee leaf scorch (Beretta et al., 1996). These different strains are transmitted during xylem sap feeding by insect vectors, such as common sharpshooters leafhoppers (Hemiptera, *Cicadelidae*) (Purcell and Hopkins, 1996), and currently, there are no adequate control measures for these diseases.

In citrus, *X. fastidiosa* infects mainly sweet orange trees (*Citrus sinensis* [L.] Osbeck cv. pera), and the chlorosis symptoms are initially restricted to leaf, but later progress to the fruits which make them useless economically, due to the drastic size reduction. The economic losses to São Paulo citriculture are estimated to range from US$ 286–322million annually (FUNDECITRUS).

At the present time, the diagnosis of *X. fastidiosa* is routinely carried out by standard polymerase chain reaction (PCR) using specific primer sets whereas its quantification is performed by agarose gel electrophoresis (Henson and French, 1993; Pooler and Hartung, 1995). The primer set usually used includes the RST31/33 (Minsavage et al., 1994), derived from the RNA polymerase genomic locus, 272-1-int and 272-2-int or those derived from the 16S rRNA gene (Chen et al., 2005). However all PCR procedures involve sample extraction to generate template DNA which can be obtained through petiole perfusion from fresh leaf petioles (Coletta-Filho et al., 1996) since the CVC bacterium forms a strong biofilm within the xylem vessels which render these extraction processes difficult to carry out, making these purification methods very tedious and labor intensive when conducted on a large scale. Furthermore, samples can also be obtained by grounding, but many times uncharacterized components of plant tissues may interfere and even inhibit the PCR (Wilson, 1997).

Therefore, to circumvent these purification problems and the drawback of the initial step of PCR that requires chloroform/phenol manipulation for the nucleic acid extraction, which is a potential environmental pollution risk, we established Immunocapture-PCR (IC-PCR) and Immuno-PCR (I-PCR) for CVC detection. These immunomolecular methods are highly sensitive and specific for detecting CVC, since they unite immunological and molecular detection technologies, which combine the specificity of enzyme-linked immunosorbent assay (ELISA) with the amplification power and sensitivity of the PCR (Sano et al., 1992; Niemeyer et al., 2005).

These methodologies are claimed to be several orders of magnitude more sensitive than conventional ELISA or PCR, thereby enhancing dramatically sensitivity and enabling for a broad range of applications in diagnostics. Recently, IC-PCR and I-PCR have been used to detect a variety of very low antigen concentrations, such as tumor markers (Zhou et al., 1993), microorganisms and toxins (Liang et al., 2003; Chao et al., 2004; Allen et al., 2006; Lubelli et al., 2006; Mason

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et al., 2006; Fischer et al., 2007), cytokines and hormones (Sanna et al., 1995; Sims et al., 2000; Kamatsu et al., 2001), and human and plant pathogens (Luo et al., 2002; Chye et al., 2004; Adler, 2005).

2. Materials and methods

2.1. Bacterial strain and culture

The 9a5c bacterial strain of *X. fastidiosa* (Schaad et al., 2004) was used in this study. This strain was the same one used for genome sequencing and obtained from the Institut National de La Recherche Agronomique (INRA, Bordeuax, France). Bacterial cells were grown for 9days either in solid or liquid periwinkle wilt (PW) media (Davis et al., 1981), at 29 °C and with or without an orbital shaker at 130 to 150rpm. The cells were harvested from liquid and solid medium cultures, pelleted by centrifugation. The suspension of bacteria was standardized to an optical density of 0.25 (10^7 to 10^8CFU/mL) at A_{600}nm with a spectrophotometer (Minsavage et al., 1994).

2.2. Plant samples

The leaves were obtained from healthy, CVC-symptomatic and -asymptomatic citrus sweet orange trees (*C. sinensis* cv. pera), from the crop of the Centro APTA 'Sylvio Moreira', Instituto Agronômico, Cordeirópolis, SP, Brazil. Samples were obtained by grinding 0.5g petioles in 5mL of grinding buffer (2% polyvinylpyrrolidone 40 in PBS-T (136 mM NaCl, 1.4 mM KH_2PO_4, 2.6 mM KCl, 8 mM Na_2HPO_4, 0.05% Tween 20, pH 7.4), using a manual bead grinder. One milliliter of plant extract was transferred to microfuge tubes and clarified by centrifugation at room temperature for 2 min at 21,000×*g*.

2.3. Viability of X. fastidiosa in infected plants

The CVC-infected plant sterilized samples (150 mg/mL) previously milled in sterile PBS, were diluted to 10-fold serial dilutions in sterile PBS (10^{-1} to 10^{-9}). So, 100µL of each solution was placed on three sterile plates with PW medium (0.4% w/v phytone peptone, 0.1% w/v trypticase peptone, 7.35 mM KH2PO4, 6.89 mM K2HPO4, 1.62 mM MgSO4, 0.001% w/v hemin chloride, 0.002% phenol red, 0.6% w/v BSA, 0.4% w/v glutamine, pH 6.8) and one sterile plate with BCYE medium (1% w/w yeast extract, 0.04% w/v, L-cysteine-HCl, 0.025% w/v ferric pyrophosphate, 30 mM KOH, and 54.9 mM ACES; pH 6.85). The growth profile was estimated by counting the colony forming units (CFU) after growing at 25 °C for 10–12days. Finally, the CFU per mg of infected tissue was calculated and all assays (ELISA, IC-PCR and I-PCR) were carried out with 100mg of infected tissue.

2.4. Endophytic bacterial strains

The endophytic bacterial strains were obtained from the collection of the Laboratório de Genética de Microrganismos (Departamento de Genética, ESALQ/USP, Piracicaba, Brazil). The growth conditions were as recommended in the Handbook of Microbiological Media. The bacteria were cultured under aerobic conditions and harvested at the end of the exponential growth phase, centrifuged, washed with PBS and stored at 4 °C.

2.5. Polyclonal antiserum

Female New Zealand white rabbits weighing 2kg were endovenously inoculated with standardized bacterial suspension at 1×10^6 *X. fastidiosa* cells/mL in PBS, and this procedure was repeated three times at 14day intervals. Ten days after the last injection, the blood was collected through cardiac puncture and the serum titer and specificity were determined by indirect enzyme-linked immunosorbent assay (ELISA) (Clark et al., 1986) and preimmune serum was used as a negative control.

2.6. Biotinylation of antibody

The immunoglobulin G (IgG) fraction previously purified from the antisera by protein G affinity column chromatography (Sigma Chemical, Saint Louis, MO, USA), according to manufacturer's instructions using low-salt conditions and eluting with 0.1M glycine, pH 3.0, was biotinylated using the photobiotin method according to Heggeness and Ash (1977). Briefly, the purified antibodies (1 mg/mL) were dialyzed in 0.2M borate buffer, pH 8.5 and mixed with a fresh solution of biotinyl-*n*-hydroxy-succinimide ester (Sigma) in DMSO (1 mg/mL), at the molar ratio of 1:20 in a final volume of 50µL. The conjugation was developed at room temperature for 4 h, dialyzed overnight at 4 °C against PBS pH 7.2, and finally mixed with glycerin 1:1 (v/v) and stored at − 20 °C.

2.7. Synthesis of biotinylated-reporter DNA

The biotinylated-reporter DNA was prepared following the method described by Liang et al. (2003). The *X. fastidiosa usp*A1 (XF1516) gene that encodes a surface-exposed outer membrane protein was previously cloned into vector pGEM-T (Promega Corporation, Madison, WI, USA). This construction was used as the template to produce the reporter 1.122kb DNA sequence. One microgram of the uspA1-R-Biotin primer was used as reporter. The labeling was done using 1× terminal transferase buffer, 25 nmol of Biotin-16 ddUTP (Boehringer Mannheim, Germany) and 60units of terminal transferase (Promega). The reaction was carried out for 30 min at 37 °C. The labeling was confirmed by dot blot using 200ng of uspA1 gene from *X. fastidiosa* as template and labeled primer as probe.

PCR amplifications were performed using the following primers: forward, uspA1-F: 5'5′-AACTCGAGAGCAGGCCGCCGGTGATAG-CAGTA-3′ and reverse, uspA1-R-Biotin: 5′-GACGCTCGAGCCCCCGCCG-CAAGAT-3′. Each 25 µL PCR reaction contained Milli-Q water, 10× buffer (Invitrogen Corporation, Carlsbad, CA, USA), 1.25 mM $MgCl_2$, 0.25 mM dNTP, 0.1 mM of each primer, 0.25U *Taq* DNA polymerase (Invitrogen) and approximately 1ng/µL template plasmid. PCR amplifications were performed at an initial denaturation step at 96 °C for 30 s, followed by an annealing step at 55 °C for 30s and extension step at 72 °C for 1 min and 30s for 35 cycles. The final extension was at 72 °C for 10 min. The PCR products were quantified using gel electrophoresis with a 1.0% agarose gel in 1× TAE (45 mM Tris–acetate, 1 mM EDTA, pH 8.0) followed by ethidium bromide (1 mg/mL) staining and visualization under UV light.

2.8. Enzyme linked immunosorbent assay

Polystyrene microtiter plates (Pierce Biotechnology, Rockford, IL, USA) were coated with 100 µL/well of standardized *X. fastidiosa* cells or plant extract samples diluted serially (10-fold) with carbonate coating buffer (16 mM sodium carbonate, 34 mM sodium bicarbonate, pH 9.6), and incubated overnight at 4 °C. The plates were washed three times with 200 µL of washing buffer (PBS-T), and blocked with PBS containing 2% BSA for 1 h at 37 °C. After washing, the plates were incubated for 3 h at 37 °C with 100 µL/well of anti-*X. fastidiosa* diluted to 1:1000 in PBS. For detection of the antigen–antibody reaction, 100 µL/well of goat anti-rabbit IgG conjugated with alkaline

Table 1
Viability of *X. fastidiosa* in infected plant tissue

Dilution	Mean of colonies	CFU/mg
10^{-4}	40	1.33×10^3
10^{-5}	10	3.33×10^3
10^{-6}	3.3	1.11×10^4
CFU/mg		5.25×10^3

After growing at 25 °C for 10–12 days, the colony forming units (CFU) were counted in three different dilutions and the CFU/mg was calculated.

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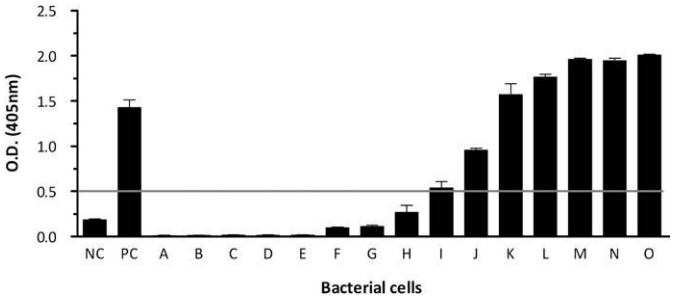

Fig. 1. Detection limit of CVC by Indirect ELISA. (NC) Negative control (without *X. fastidiosa*); (PC) positive control (1×10^6 *X. fastidiosa* cells in PW medium); 1×10^6 bacterial cells of (A) *Methylobacterium mesophilicum*, (B) *Methylobacterium extorquens*, (C) *Curtobacterium flaccumfaciens*, (D) *Bacillus sp*, (E) *Pantoea agglomerans*; (lanes F to O) 10-fold serial dilutions of *X. fastidiosa* ranging from 10^{10} to 10^1 cells. Therefore, the ELISA sensitivity ranges between 10^4 and 10^6 bacterial cells, similar to PCR sensitivity that can amplifiy around 10^6 cells per sample.

phosphatase (Sigma) diluted to 1:30,000 in PBS was added, and incubated for 2 h at 37 °C. After the washing procedures, the colorimetric reaction was developed using 100 μL/well of pNPP (*p*-nitro phenyl phosphate, Pierce) at 1 mg/mL diluted in diethanolamine substrate buffer (Pierce), and stored for 1 h at room temperature in the dark. The absorbance values were read at 405nm in microplate reader Asys Multireader Expert Plus (ASYS Hitech, Austria). The results are means of triplicate sample values of the intensity reaction measured colorimetrically at 405nm. Samples presenting absorbance of three times that of the negative control (O.D. > 0.5) were considered positive (Clark et al., 1986).

2.9. Immunocapture-PCR (IC-PCR)

Polystyrene microtiter plates (Reacti-bind™ Pierce) were coated overnight at 4 °C with 200 μL/well of anti-*X. fastidiosa* polyclonal antibody diluted at 1:1000 in coating buffer, then washed three times with 100 μL/well of washing buffer, and blocked with PBS containing 2% BSA for 1 h at 37 °C. The plates were washed three times with PBS-T, and a 10-fold serial dilution of *X. fastidiosa* cells or plant samples was added to each well. After incubation for 2 h at 37 °C, the plates were washed five times, and 25 μL Milli-Q water was added per well. Finally, bound biotinylated-reported DNA was detached from the plate by denaturing at 100 °C for 5 min, and 10μL of each sample was mixed with Milli-Q water, 10× buffer (Invitrogen), 1.25 mM $MgCl_2$, 0.25 mM dNTP, 0.1 mM of each primer CVC-1: 5′5′-AGATGAAAACAATCATGCAA-A-3′ and 272-2-int: 5′-GCCGCTTCGGAGAGCATTCCT-3′ (Pooler and Hartung, 1995), and 1U *Taq* DNA polymerase (Invitrogen) to perform the PCR in a 25 μL final volume reaction. PCR cycle conditions were an initial denaturation step at 94 °C for 3 min, then 35 cycles (94 °C for 60 s, 55 °C for 60s and 72 °C for 90s) followed by a final elongation step of 10 min at 72 °C. Ten microliters of PCR products were analyzed by standard electrophoresis on a 1.0% agarose gel in 1× TAE followed by ethidium bromide (1 mg/mL) staining and visualization under UV light.

2.10. Immuno-PCR (I-PCR)

Standardized bacterial suspension (10-fold dilution) and plant sample extracts were diluted in the carbonate coating buffer and added at 100 μL/well to polystyrene microtiter plate (Pierce). After incubation for 3 h at 37 °C, the plate was washed three times with 200 μL/well of the washing buffer (PBS-T), and blocked with PBS containing 2% BSA for 1 h at 37 °C. After washing procedures, to each well was added 100 μL of biotinylated polyclonal antibody diluted 1:100 in PBS and incubated for 2 h at 37 °C. Afterwards the plate was washed three times as described above and streptavidin molecules were added at 100 μg/mL in PBS for 1 h at 37 °C. After washing, the plate was incubated with the biotinylated-reporter DNA (UspA1 gene) at 50 μg/mL under the same conditions as above.

Finally, the plates were washed five times with PBS-T, and 25 μL Milli-Q water was added to each well. The plate was steamed at 100 °C for 5 min to denature the biotin–streptavidin complexes and release the biotinylated-reported DNA, and finally 10μL of each sample was mixed with Milli-Q water, 10× buffer (Invitrogen), 1.25 mM $MgCl_2$, 0.25 mM dNTP, 0.1 mM of uspA1-F and uspA1-R-biotin primers, and 1U *Taq* DNA polymerase (Invitrogen) to perform the PCR in a 25 μL final volume reaction. PCR cycle conditions were carried out by an initial denaturation step at 94 °C for 3 min, then 35 cycles (94 °C for 60s, 55 °C for 60s and 72 °C for 90s) followed by a final elongation step of 10 min at 72 °C. Ten microliters of PCR products were analyzed by standard electrophoresis on 1.0% agarose gel in 1× TAE, followed by ethidium bromide (1 mg/mL) staining and visualization under UV light.

3. Results

3.1. Viability of X. fastidiosa

The viability of *X. fastidiosa* was estimated in a serial dilution ranged from 10^{-1} to 10^{-9} and calculated in CFU/mg of infected tissue (Table 1). So, ELISA, IC-PCR and I-PCR assays were carried out with 100mg of infected tissue, therefore, 5.25×10^5 bacterial cells.

3.2. Specificity of indirect ELISA assay

The reactivity of the *X. fastidiosa* antiserum was tested by indirect ELISA, and the average of the results was expressed as the optical density at 405nm as a function of the concentration of antigens

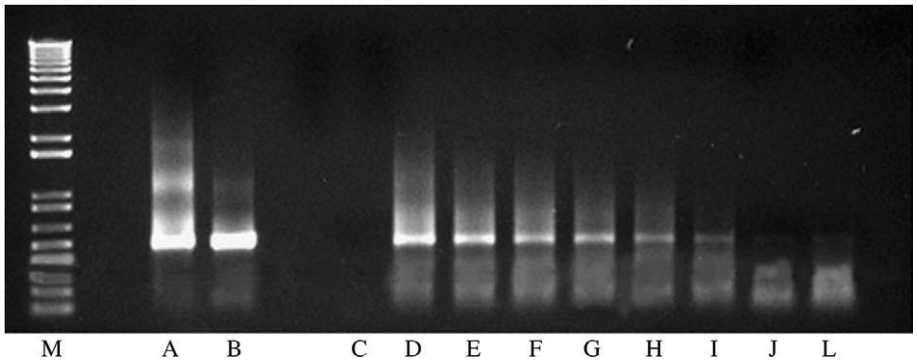

Fig. 2. Detection of *X. fastidiosa* cells cultivated in PW medium by Immunocapture-PCR. Lane M, Ladder 1 kb DNA Plus (Invitrogen); Lanes A and B, Positive controls of PCR (150 ng purified DNA from *X. fastidiosa*); Lane C, Negative control (1×10^6 *Methylobacterium mesophilicum* bacterial cells); Lanes D to L, 10-fold serial dilutions of *X. fastidiosa* ranging from 10^8 to 10^1 cells per lane. Positive samples showed an amplicon of 500 pb and the detection limit of IC-PCR was 10^3 cells (Lane I).

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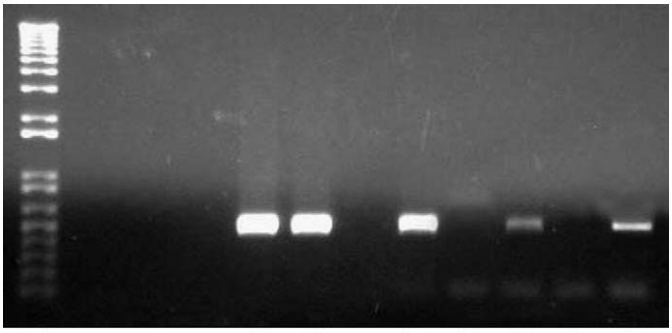

Fig. 3. Detection of *X. fastidiosa* from field citrus samples by Immunocapture-PCR. Lane M, Ladder 1 kb DNA Plus (Invitrogen); Lane A, Negative control (PCR mix without DNA); Lanes B and C, Positive controls of PCR (150 ng purified DNA from *X. fastidiosa*); Lane D, Positive control of IC-PCR (1×10^6 *X. fastidiosa* cells); Lanes E and F, Healthy and CVC-symptomatic citrus plants respectively, milled in water; Lanes G and H, Healthy and CVC-symptomatic citrus plants, respectively, milled in PBS.

(Fig. 1). The antiserum had a robust reactivity and its detection ranged from 10^6 to 10^4 bacterial cells, and detected *X. fastidiosa* with similar magnitude in sample plant extracts.

These polyclonal antibodies are highly specific for *X. fastidiosa*, since it has no cross-reactivity in ELISA with other endophytic citrus-bacteria such as *Methylobacterium mesophilicum*, *Methylobacterium extorquens*, *Curtobacterium flaccumfaciens*, *Bacillus* sp. and *Pantoea agglomerans* (Fig. 1). The cross-reactivity can interfere in the specificity, making the diagnosis useless, due to false positive results even in the absence of the *X. fastidiosa*.

Based on the detection and discrimination activity, these polyclonal antibodies could be useful in diagnosis in all serological tests; however, conventional immunoassays have detection limits, therefore these antibodies could be useful for conducting antibody-based PCR tests like IC-PCR and I-PCR, which combine the power of nucleic acid amplification techniques like PCR with the versatility of immunoassays like ELISA to detect a broad range of antigens.

3.3. Immunocapture-PCR (IC-PCR)

IC-PCR, like ELISA requires the capture of the bacterial cells by coating the wells with specific polyclonal antibody as an initial step. Therefore in preliminary tests, different temperatures and incubation times were evaluated in order to establish the best conditions for IC-PCR, since the coating conditions may influence the accuracy of pathogen detection. The overnight incubation at 4 °C was enough for coating the antibodies onto the plate, and this condition was adopted in all further experiments.

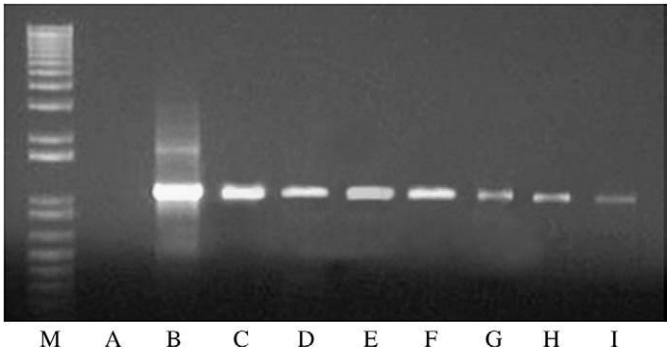

Fig. 4. Detection of *X. fastidiosa* cells cultivated in PW medium by Immuno-PCR. Lane M, Ladder 1 kb DNA Plus (Invitrogen); Lane A, Negative control (150 ng purified DNA of *X. fastidiosa*); Lane B, Positive control (1×10^6 *X. fastidiosa* cells); Lanes C to I, 10-fold serial dilutions of *X. fastidiosa* ranging from 10^7 to 10^1 cells per lane. Positive samples showed an amplicon of 1100 pb and the detection limit was 10^1 cells (Lane I).

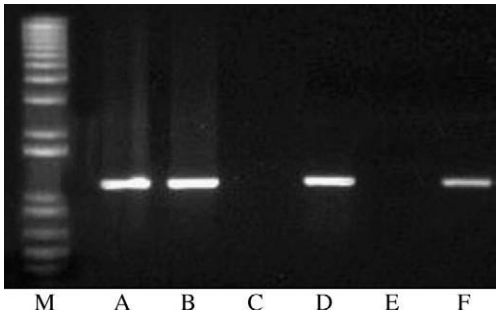

Fig. 5. Detection of *X. fastidiosa* from field citrus samples by Immuno-PCR. Lane M, Ladder 1 kb DNA Plus (Invitrogen); Lane A, Positive control of PCR (150 ng purified DNA of *X. fastidiosa*); Lane B, Positive control of I-PCR (1×10^6 *X. fastidiosa* cells); Lanes C and D, Healthy and CVC-symptomatic citrus plants respectively, milled in water, Lanes E and F, Healthy and CVC-symptomatic citrus plants, respectively, milled in PBS.

However, unlike the standard PCR methodology, IC-PCR did not require any further purification step, since the PCR reaction is carried out by direct DNA amplification with specific designed primers. The expected amplicon of 500bp was coherent with the amplificons described for CVC-1 and 272-2-int primer set used (Pooler and Hartung, 1995), and currently recommended for the diagnosis of CVC.

For the standard curve, serial dilutions of PW-cultivated bacteria were done (Fig. 2), and IC-PCR sensitivity was measured to be up to 10^3 bacterial cells (Fig. 2, lane I), which means that it consistently detected populations of *X. fastidiosa* 10-fold lower than those detected by ELISA and similar results were obtained from infected and symptomatic citrus plant samples (Fig. 3, lanes F and H). IC-PCR was carried out with 100mg of infected tissue which had approximately 5.25×10^5 CFU. Non-infected tissue samples or endophytic bacteria (negative control) did not reveal any false positive results in our assays (Fig. 3, lane C). Preparation procedures of the samples, either in buffer or water, did not interfere in the assay, since similar results were achieved (Fig. 3, lanes F and H).

3.4. Immuno-PCR (I-PCR)

The Immuno-Polymerase Chain Reaction is based on the chimeric conjugation of specific antibodies and nucleic acid (reporter DNA), which is used for the signal generation and amplification step. Therefore, the antibody and the reporter DNA were biotinylated and efficiency was determined by ELISA and PCR (data not shown). In preliminary tests, the optimal concentration of 50µg/mL for biotinylated-reporter DNA was determined, since false–positive reactions may occur as a result of the non-specific binding of reagents, and oligomeric conjugates can be formed by self-assembly. This concentration was adopted in all subsequent I-PCR assays.

The excepted amplicons of 1100kb were obtained and are coherent with the use of *usp*A1 (XF1516) gene as the reporter DNA. The detection limit of I-PCR was up to 10^1 bacterial cells (Fig. 4, lane I), 100-fold lower than those obtained by IC-PCR, and 1000-fold lower than ELISA. The Immuno-PCR assay was successfully detected, with same efficiency for *X. fastidiosa* from CVC-symptomatic plant samples, whether milled in water or PBS (Fig. 5, lanes D and F). These plant samples were prepared in the same manner as IC-PCR, which means, 100mg of infected tissue had approximately 5.25×10^5CFU. No amplification products were observed for any other endophytic citrus-bacteria tested (data not shown).

4. Discussion

X. fastidiosa causes several economically important diseases in North, Central and South America. In citrus, it causes the Citrus Variegated Chlorosis or CVC, a destructive disease of sweet orange, *C. sinensis* (L) Osbeck. CVC is present in almost half of the citrus growing areas in Brazil (FUNDECITRUS) and currently there are no adequate

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control measures. Therefore, it is essential and necessary to maintain an efficient monitoring program that can aid in disease management. This program depends on a detection protocol that must be rapid, reproducible and scalable to large sample sizes; however, it must be sensitive enough to detect low amounts of pathogen, since orange scions are propagated clonally from budwood, and even asymptomatic or healthy plants must be screened.

Today, CVC diagnosis is routinely carried out by PCR and more recently also by Real Time PCR, based on its ability to amplify specific DNA segments, using specific primers (Pooler and Hartung, 1995), whose sensitivity is about 1×10^6 bacterial cells. These techniques have already been used for the identification of many other phytopathogens (Henson and French, 1993; Schneider et al., 1993). Moreover, those methods are not very suitable for analyzing a large number of samples since they require nucleic acid extraction by using chloroform/phenol steps, which present a potential risk to the environment. However, in recent years, many DNA extraction kits have been developed and commercialized to improve the performance and minimize the risks of phenol manipulation. However, the need to improve the sensitivity and specificity for CVC diagnosis and even to circumvent these purification steps still exists. Thus, we developed an Immunocapture-PCR and an Immuno-PCR assay using microtiter plates which successfully detected CVC even from asymptomatic citrus plants without any further purification steps.

These immunomolecular methods unite the immunological with the molecular detection technologies which, in turn, improved the diagnostic capacity, although, the sensitivity of both methods is highly dependent on the quality of the antibodies produced, measured by its affinity and specificity to the target antigen (Barletta, 2006). The high specificity of the antibody is essential for an adequate diagnosis, since non-specific reactions increase the background of the reaction and decrease the sensitivity of the immunoassays.

As our results showed, the produced serum, anti-*X. fastidiosa* was highly specific and had no cross reaction with other endophytic bacteria of citrus, which may colonize similar ecological niches like *X. fastidiosa* (Fig. 1). This serum can therefore be used for establishment of both assays. IC-PCR and I-PCR did not require the DNA isolation step, since in IC-PCR, the bacterial cells were captured directly by the coating polyclonal antibody, and the *X. fastidiosa* DNA sequence is directly amplified by specific primers. The integrity of the DNA template is maintained throughout the whole procedure and loss of DNA, due to degradation during purification protocols, can be avoided. The advantages of the IC-PCR technique allow detection of smaller concentrations of bacteria when compared to PCR, since it combines the antibody specificity of recognizing and capturing the bacteria with the specific DNA amplification by PCR. Moreover, I-PCR was the most sensitive assay and the complex biotinylated polyclonal antibody–reporter DNA allows a direct detection of coated antigens onto microtiter plates and a quick and simple amplification by PCR.

These described methods were highly sensitive and specific for detecting CVC, whose detection limit ranged from 10^3 to 10^1 bacterial cells for IC-PCR and I-PCR respectively, which were clearly more sensitive than standard PCR, whose sensitivity is 10^6 bacterial cells (Minsavage et al., 1994), even for the Real Time PCR designed by Li et al. (2003), which detects 50 cells in infected tissue, and did not require any DNA extraction procedures like the nested-PCR assay described by Ciapina et al. (2004). These methods can be used for routine diagnosis of CVC and may aid in the study of *X. fastidiosa* epidemiology, the sensitivity of the I-PCR makes it possible to determine the amount of the bacteria even in a single insect, which could improve the management of the CVC disease. Moreover, Almeida et al. (2001), using a different technique, found population of *X. fastidiosa* from symptomatic citrus leaves ranging from 10^6 to 10^8 10^8CFU/100mg of tissue. On the other hand, Souza et al. (2003) showed that asymptomatic citrus plant after 180days of infection had approximately 2.6×10^4 copy number of *X. fastidiosa* amplicon/100mg of tissue. Consequently, considering the sensitivity of IC-PCR and I-PCR, these methods can be used to screening citrus asymptomatic plants to determine the infection by *X. fastidiosa.*

Finally, our current version of these assays could be improved by the use of monoclonal antibody and the adoption of real time-I-PCR, which would allow direct quantification of *X. fastidiosa*.

However, one of the drawbacks for even *X. fastidiosa* diagnostic methods from citrus plants is the requirement of extensive perfusion or grinding of the sample since the bacteria form a strong biofilm within the xylem vessels.

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6. DISCUSSÃO

O vírus da tristeza dos citros (CTV) ocorre nas principais regiões produtoras de citros do mundo e é considerado um dos fitopatógenos virais mais prejudiciais para a citricultura. O CTV apresenta diversas linhagens e estirpes e a infecção freqüentemente ocorre por uma mistura dessas estirpes, com diferentes níveis de severidade. No Brasil, o CTV é endêmico devido à alta eficiência do pulgão transmissor *Toxoptera citricidus* (Kirk), sendo que a infecção ocorre de maneira semipersistente e não circulativa, com um período de aquisição de 30 minutos a 24 horas (FEBRES *et al.,* 1996; MEHTA *et al.*, 1997).

No entanto, graças à utilização de porta-enxertos tolerantes, como limão Cravo (*Citrus limonia* Osb.), implantação de genótipos resistentes ou tolerantes ao CTV e, ainda programas de pré-imunização utilizando isolados protetivos, como Pêra IAC (MÜLLER e COSTA, 1993), a citricultura brasileira conseguiu implantar pomares mais vigorosos e produtivos. Entretanto, os pomares infectados pelas estirpes do complexo Capão Bonito sofrem danos econômicos consideráveis, uma vez que estas medidas profiláticas não proporcionaram o efeito de proteção cruzada contra estas estirpes. Desse modo e considerando que nos dias atuais, há leis que exigem a certificação de mudas destinadas à implantação de novos pomares, bem como a inspeção dos já existentes, torna-se necessária a realização de um monitoramento eficiente sobre a produção de cítricos no país.

Desse modo, o monitoramento vem sendo efetuado através de técnicas biológicas, imunológicas e moleculares. O principal método biológico de diagnóstico do CTV nos programas de certificação de matrizes e inspeção de pomares era através do uso de plantas indicadoras, que confirmam a presença do vírus através da manifestação e observação dos sintomas, e também agrupa as diferentes estirpes em função da severidade da sintomatologia desenvolvida (MACHADO *et al.*, 1996). Entretanto, é um método lento, caro e dispendioso, já que necessita de uma grande área plantada para uma adequada observação dos sintomas em plantas indicadoras, o que limita a sua aplicabilidade na análise de um grande número de amostras. Dentre os métodos imunológicos, destaca-se a técnica de ELISA como a de maior importância no diagnóstico do CTV, sendo os primeiros protocolos introduzidos por CLARK e ADAMS (1977).

A técnica de ELISA é amplamente utilizada devido à sua facilidade de aplicação, rapidez, baixo custo e alta sensibilidade, além de possibilitar a quantificação do CTV em um grande número de amostras. Posteriormente, outros testes foram desenvolvidos como a imunodifusão na presença de dodecil sulfato de sódio (GARNSEY *et al.,* 1979), imunofluorescência (TSUCHIZAKI *et al.,* 1978; BRLANSKY *et al.*, 1984), microscopia eletrônica imunoespecífica (GARNSEY *et al.*, 1980) e *Western blot* (LEE *et al.*, 1987).

Nos últimos anos, as técnicas moleculares começaram a ser estabelecidas e dentre elas, destacam-se: mapeamento de peptídeos da proteína do capsídeo viral (LEE e CALVERT, 1987), análise de dsRNA de plantas infectadas (DODDS *et al*.,1987; MORENO *et al.,* 1990; BAR-JOSEPH *et al*., 1989), clonagem e seqüenciamento do genoma viral (PAPPU *et al.,* 1993), RFLP (GILLINGS *et al*. 1993) e SSCP (FEBRES *et al.,*1995). Além disso, ensaios de imunocaptura de antígenos por anticorpos associados a técnicas moleculares de PCR e RT-PCR (NOLASCO *et al.*, 2002; HEMA *et al.*, 2003; LIANG *et al.,* 2003) foram estabelecidos, permitindo um diagnóstico mais sensível e específico do CTV (HUNG *et al.*, 2000; HUANG *et al.*, 2004).

Assim, o diagnóstico do CTV vem sendo realizado através do uso de anticorpos monoclonais (MAb) amplamente descritos na literatura, como o MCA13, que reage com isolados causadores de declínio e/ou caneluras mas não reage com isolados fracos provenientes da Califórnia, Flórida e Espanha (PERMAR *et al.*, 1990) e o 3DF1, que reage com um grande número de isolados da Flórida e Espanha, independentemente dos sintomas apresentados pela planta hospedeira (CAMBRA *et. al.*, 1988; PERMAR *et. al.*, 1990). No entanto, estes anticorpos apresentam uma aplicabilidade limitada na detecção das estirpes importantes do CTV encontradas no Brasil, uma vez que não são capazes de diferenciar entre os isolados fracos, moderados e severos, como os encontrados no complexo Capão Bonito (CB).

Por outro lado, a produção de anticorpos monoclonais requer a extração de antígenos das plantas infectadas, através de métodos de purificação extensos e complexos, uma vez que o CTV está limitado ao floema da planta hospedeira. Assim, a purificação de suas partículas com grau de pureza e quantidade adequadas apresenta

algumas dificuldades, como: grande quantidade de tecido infectado necessário para a purificação do vírus; baixo rendimento do vírus e contaminações com proteínas da planta hospedeira, o que podem comprometer a qualidade do anticorpo produzido (NIKOLAEVA *et al.,* 1995).

Estas dificuldades foram minimizadas com o desenvolvimento da biologia molecular no âmbito nacional, permitindo a produção de proteínas recombinantes e sua utilização como fonte alternativa de antígenos, uma vez que podem ser obtidas em grandes concentrações e estão livres de contaminantes da planta hospedeira (MANJUNATH *et al.,*1994).

No Brasil, TARGON (1997 e 2000) clonou gene da capa protéica do CTV presente em Pêra 135/Cravo 507 que apresentava o complexo severo de Capão Bonito, produzindo duas proteínas recombinantes do capsídeo viral, denominadas CB-22 (homóloga aos isolados que induzem sintomas fracos em plantas indicadoras) e CB-104 (homóloga aos isolados que induzem sintomas severos em plantas indicadoras). Estas proteínas foram utilizadas em esquemas de imunização em camundongos e coelhos, possibilitando a produção de quatro anticorpos monoclonais, agrupados em três grupos distintos (STACH-MACHADO *et al.,*2000 e DIAS *et al.,* 2001).

O primeiro grupo denominado IC-04.12, pertencente à subclasse IgG2b/κ, apresenta especificidade para a proteína CB-22, enquanto o segundo grupo, denominado 39.07, de subclasse IgG1/κ, apresenta reatividade apenas para a proteína CB-104. O terceiro grupo de anticorpos monoclonais denominados de 30.G.02 e 37.G.11, de subclasse IgG1/κ, reconhece as duas proteínas CB-22 e CB-104 (DIAS, 2001).

A caracterização inicial dos anticorpos monoclonais demonstrou que eles possuem um título elevado, chegando até a ordem de 1:40.000 e alta sensibilidade, sendo capazes de detectar as proteínas recombinantes purificadas em concentrações variando de 70 a 300 ηg por ensaio (DIAS, 2001).

No entanto, além das características descritas, outra de importância fundamental para a validação do potencial de diagnóstico dos MAbs é a identificação e caracterização dos epitopos, que fornecem informações relevantes à aplicação desses $MAbs_s$ em

imunoensaios, de modo a identificar e diferenciar as estirpes distintas do CTV presentes no Brasil, e até mesmo em todo o mundo, em função do padrão de reatividade obtido nos ensaios de ELISA.

Inicialmente tentamos efetuar a identificação destes epitopos através da produção de peptídeos obtidos pela clivagem enzimática com enzimas como Tripsina, Glu-c, Lys-c e Arg-c, das proteínas recombinantes CB-22 e CB-104, cuja interação com os anticorpos monoclonais foi avaliada por espectrometria de massa (MALDI-TOF, *Matrix-assisted Laser Desorption and Ionization – Time of Flight*). Alguns trabalhos realizados por SUCKAU *et al.* (1990), MACHT *et al.* (1996), PARKER *et al.* (1996) e van de WATER *et al.* (1997), demonstraram a formulação de ensaios baseados em espectrometria de massa para a determinação de epítopos reconhecidos por anticorpos monoclonais específicos. Após a identificação do peptídeo que interagiu com um dos MAb_s, a sua massa é comparada com um banco de dados formado pelos dados de massas referentes aos peptídeos clivados em uma digestão proteolítica teórica pelo programa PEPTIDE MASS (http://us.expasy.org/tools/peptide-mass.html).

No entanto, os ensaios em espectrometria de massa não apresentaram resultados satisfatórios. Entre as possíveis causas, podemos citar a produção de peptídeos devido a uma clivagem enzimática inespecífica e a supressão de sinal. Esta supressão é devido ao fato de que, dentro de uma amostra complexa de peptídeos, alguns possuem uma maior capacidade de se ionizarem, suprimindo os sinais daqueles com baixa capacidade de ionização. Assim, muitos dos peptídeos gerados na clivagem enzimática não são detectados em uma análise de espectrometria de massa, de modo que identificação dos peptídeos reconhecidos pelos anticorpos monoclonais pode ser prejudicada.

Alternativamente, adotamos a metodologia de construção de bibliotecas de mutantes em uma linhagem especial *Escherichia coli,* denominada *Epicurian Coli* XL1-Red (Stratagene, La Jolla, Califórnia) de acordo com o protocolo descrito por WENGELNIK *et al.* (1999) e SLEATOR *et al.* (2001). Esta linhagem possui uma DNA polimerase alterada, de modo que durante a fase de crescimento e replicação, ocorrem mutações no DNA plasmidial contendo o inserto. Posteriormente, os mutantes são selecionados e as

proteínas recombinantes são expressas por ação de IPTG e avaliadas em ELISA Sandwich (CLARK *et al.*, 1986). Desse modo, as proteínas com mutações na região envolvida no reconhecimento antigênico (epítopo) do anticorpo monoclonal resultam em uma reação negativa no ELISA. Os clones negativos são seqüenciados possibilitando a identificação dos epitopos.

No entanto, o desenvolvimento dessa técnica encontrou problemas na importação e no cultivo da linhagem de *Epicurian coli* e, além disso, como as mutações ocorrem de maneira aleatória ao longo do DNA plasmidial, seria necessária a avaliação de um grande número de mutantes para que fosse possível a identificação do epitopo. Desse modo, optamos por avaliar o reconhecimento antigênico dos MAb_s utilizando peptídeos clonados a partir da seqüência gênica das proteínas CB-22 e CB-104.

Com esse propósito, inicialmente as proteínas CB-22 e CB-104 foram avaliadas quanto ao seu padrão de hidrofobicidade (**Anexo 8.1**) e, com base nas regiões hidrofílicas, foram desenhados 8 pares de primers para a CB-22 e 6 pares de primers para a CB-104 para clonagem e expressão de peptídeos recombinantes com tamanho médio de 30 aminoácidos e com sobreposição entre eles de 7 a 13 aminoácidos. Estes peptídeos foram desenhados de modo a cobrir a seqüência de aminoácidos completa das proteínas.

Assim, a seqüência dos epitopos identificados através do reconhecimento pelos MAbs foram comparadas com as seqüências do capsídeo do CTV disponíveis no GenBank (NCBI) através da ferramenta BLAST N 2.2.16, visando avaliar a distribuição e conservação desses epitopos nos diferentes isolados do CTV. Nossos resultados demonstraram que o MAb 30.G.02 é um anticorpo "Universal", uma vez que, o epitopo é conservado entre a maioria dos isolados de CTV (87,2%), sendo este um anticorpo ideal tanto para atuar como anticorpo de captura ou de detecção em ensaio de ELISA Sandwich. O MAb 37.G.11 reconhece um epitopo conservado em 62,6% das seqüências e, embora teoricamente possa ser usado como anticorpo de captura e de detecção, resultados práticos mostraram que deve ser preferencialmente utilizado como anticorpo de detecção. O MAb 39.07 reconhece um epitopo presente em apenas 19,7% das seqüências, sendo estas obtidas de

isolados severos do CTV, o que permite a detecção e diferenciação dos isolados de CTV presentes em plantas infectadas.

Embora o MAb IC.04-12 reconheça a proteína CB-22, que possui homologia com os isolados fracos do CTV, não foi possível efetuar a identificação do epitopo utilizando peptídeos lineares, permitindo pressupor que este MAb reconhece um epitopo conformacional formado pela estrutura secundária e terciária da proteína do capsídeo viral. Dados preliminares obtidos no laboratório confirmaram estes resultados, uma vez que os MAb_s 30.G.02, 37.G.11 e 39.07 reconhecem epitopos lineares, enquanto o MAb IC.04-12 não é capaz de reconhecer o CTV sob condições desnaturantes, como *Western blot.*

Para avaliar o padrão de reconhecimento dos anticorpos monoclonais na partícula viral do CTV, foram realizados ensaios de imunomarcação com anticorpos conjugados com partículas de ouro coloidal (Capítulo 1). Esta técnica é de grande utilidade em aplicações que busquem evidenciar padrões de reconhecimento e até mesmo de afinidade entre um anticorpo e seu antígeno específico. Trabalhos realizados por ECALE ZHOU *et al.* (2002), utilizando anticorpos policlonais demonstraram que um dos anticorpos reconhecia a proteína p25, que está distribuída ao longo de toda a partícula viral, enquanto outro era específico para a proteína p27, que se limita somente à extremidade do vírus.

Nesse sentido, p27 é de especial interesse, pois é caracterizada como uma cópia divergente da proteína p25, a principal do capsídeo (PAPPU *et al.,* 1994), e por estar presente em apenas uma das extremidades da partícula viral, forma uma cauda e confere polaridade a mesma. Uma estrutura similar foi relatada para o BYV, onde a cópia divergente da proteína principal do capsídeo (p22), denominada p24, reveste uma ponta da partícula viral, dando a mesma um aspecto de *rattlesnakes* (FEBRES *et al*., 1996). Apesar de sua função ser desconhecida, p27 pode estar envolvida na interação entre o afídeo e o vírus, como postulado para a p24 do *Beet yellows vírus* (BYV), o qual também pertence à família Closteroviridae (FEBRES et al., 1996). Outra função estipulada seria a movimentação célula a célula do vírus, uma vez que a mesma conserva grande parte da estrutura da p25 e, portanto, deve reter a capacidade de se ligar ao RNA, além de estar

presente em grande quantidade na parede celular de células infectadas (FEBRES *et al.*, 1994 e PAPPU *et al*., 1994). Além disso, uma função na montagem da partícula viral através da associação com outras proteínas virais, incluindo a p25 também é sugerida (FEBRES *et al.*, 1996).

A proteína p25 é a principal proteína (*major coat protein*) do capsídeo do CTV com 25 kDa. No entanto, os genes das proteínas CB-22 e CB-104, clonados por TARGON (1997) utilizando o vetor de expressão pET22, embora apresentassem o mesmo número de nucleotídeos, quando expressos em células competentes de *E. coli* BL21 (DE3) sob ação de IPTG, as proteínas recombinantes apresentavam pesos moleculares distintos de 25 e 18 KDa, respectivamente quando analisadas em SDS-PAGE. Para explicar esta incongruência, formulamos duas hipóteses. Primeiramente, poderia ter ocorrido algum problema no cultivo ou na indução da expressão destas proteínas por IPTG nas células de *E. coli.* Enquanto que na segunda hipótese, poderia haver um "stop códon" na seqüência de nucleotídeos da proteína CB-104, o que resultaria na expressão de uma proteína menor.

Posteriormente, realizamos um novo seqüenciamento e alinhamento destas proteínas para determinação da seqüência consenso, de modo que os resultados revelaram um erro no seqüenciamento, realizado manualmente por TARGON (1997). A seqüência da proteína CB-104 apresentou um nucleotídeo modificado na posição 481 (G $\rightarrow$ T), de modo a provocar a mudança do aminoácido glicina, codificado pelo códon GGA, para um "stop códon", codificado por TGA, na posição 161 da proteína (**Anexo 8.2**). Este resultado explica a diferença de peso molecular entre as proteínas CB-22 e CB-104, confirmando o peso molecular de 18 KDa da CB-104. De maneira semelhante à CB-104, o seqüenciamento da CB-22 revelou a modificação de alguns nucleotídeos bem como a inclusão de mais aminoácidos na seqüência protéica, totalizando 225 aminoácidos (**Anexo 8.3**). As características físico-químicas das proteínas CB-22 e CB-104 foram avaliadas *in silico* através do programa PROTPARAM disponível na suíte de aplicativos do EXPASY (http://www.expasy.org/tools/protparam.html) quanto a sua estrutura primária, considerando o número de aminoácidos, peso molecular, pI teórico e número total de resíduos carregados positiva e negativamente (**Anexo 8.4**).

A proteína do capsídeo é fundamental na organização e montagem da partícula viral, atua na proteção do material genético viral, e está provavelmente envolvida na interação com inseto-vetor e com a planta hospedeira. Considerando que a estrutura antigênica do vírus é derivada da estrutura protéica do capsídeo viral, a caracterização e o estudo da estrutura secundária dessa proteína podem fornecer informações sobre as regiões ativas envolvidas na infecção viral e movimentação do vírus na célula hospedeira, além de permitir o mapeamento e localização dos epitopos dos anticorpos monoclonais ao longo da estrutura da proteína do capsídeo. Deste modo, efetuamos estudos estruturais com a proteína CB-22, descritos no capitulo 2 deste trabalho.

A identidade da proteína recombinante purificada CB-22 foi confirmada em ensaios de ELISA e *Western blot* e pela comparação pelo BLASTN 2.2.16, revelando que a CB-22 possui alto grau de homologia (Identidade 98% com e-value 0.0) com outras proteína do capsídeo do CTV, como a do isolado 81P (GenBank, acesso n° AY995567) e 464-2 (GenBank, acesso n° AY995566) da Califórnia, EUA; isolado B35 (GenBank acesso n° L12175) e T30 (GenBank, acesso n° AF260651) da Flórida, EUA; isolado T385 (GenBank, acesso n° Y18420) da Espanha; isolado CPCu IJ-12 (GenBank, acesso n° AF220503) do México e isolado TN.2 (GenBank, acesso n° AY707460) do Irã.

A integridade estrutural e a estrutura secundária foram avaliadas por Dicroísmo Circular (CD), medido entre 190 e 250 ηm. O espectro do CD com picos negativos a 222 e 209 ηm e um pico positivo a 192 ηm, demonstra que estruturas em α-hélices são predominantes na estrutura secundária desta proteína e, de uma maneira geral, é um espectro característico de proteínas com estruturas mistas, apresentando estruturas em α-hélices e folhas β. A porcentagem dessas estruturas foi estimada pelo programa K2D (ANDRADE *et al.*, 1993; MERELO *et al.*, 1994), compreendendo 45% de α-hélices, 15% de folhas β e 40% de *coils*, que está de acordo com a predição realizada pelo PSIPRED, baseada apenas na seqüência primária de aminoácidos.

Os dados indicam que a proteína CB-22 é estável a temperatura ambiente e permanece adequadamente enovelada durante todo o processo de expressão e purificação. Considerando estes resultados, a CB-22 foi submetida a estudos de Difração

de Raios X a Baixo ângulo (SAXS) para a resolução e modelagem de sua estrutura secundária. A SAXS revelou que esta proteína quando em solução forma oligômeros com número de unidades variável, mas dependente da concentração (dados não mostrados). Esta condição impediu a resolução e modelagem da estrutura tridimensional, no entanto, evidencia a função nativa da proteína do capídeo viral, ou seja, a capacidade de auto oligomerizar, uma vez que é responsável pela montagem da partícula viral para proteção e armazenamento do material genético viral.

Este resultado, embora tenha impossibilitado a resolução e modelagem da estrutura secundária da CB-22, demonstrou que esta proteína recombinante mantém a função da proteína nativa, responsável pela formação do capsídeo do CTV, de modo que esta estrutura sem a presença do material genético viral é denominada de *vírus-like*, permitindo postular a sua utilização como carreadora de ácidos nucléicos em protocolos de vacinação.

Paralelamente aos estudos do CTV, foi desenvolvido o estabelecimento de diagnósticos imunomoleculares para a bactéria *Xylella fastidiosa*, responsável, no Brasil, pela Clorose Variegada dos citros (CVC). Esta doença causa prejuízos anuais de aproximadamente 150 milhões de dólares e necessita de medidas de controle e manejo que permitam o estabelecimento e manutenção de um programa de monitoramente eficiente. Esse programa depende de um método de diagnóstico rápido, reprodutível e escalonável para milhares de amostras. Deve ser sensível o suficiente para detectar pequenas quantidades do patógeno.

Rotineiramente o diagnóstico da CVC é realizado através da reação em cadeia da polimerase (PCR), com base em iniciadores (*primers*) amplamente descritos na literatura (POOLER e HARTUNG, 1995). A PCR apresenta uma sensibilidade da ordem de $1x10^6$ células bacterianas, no entanto, exige a extração de ácidos nucléicos utilizando fenol e clorofórmio, substâncias com alto risco de contaminação ambiental e de manipulação. Embora atualmente existam disponíveis no mercado, diversos kits de extração que minimizam esses riscos, existe a necessidade do desenvolvimento de diagnósticos mais sensíveis. Deste modo, estabelecemos os protocolos para as técnicas de Imunocaptura-

PCR (**Figura 10**) e Imuno-PCR (**Figura 11**) descritas no capítulo 3, como metodologias alternativas e promissoras para a detecção de *Xylella fastidiosa* (PERONI *et al.*, 2008).

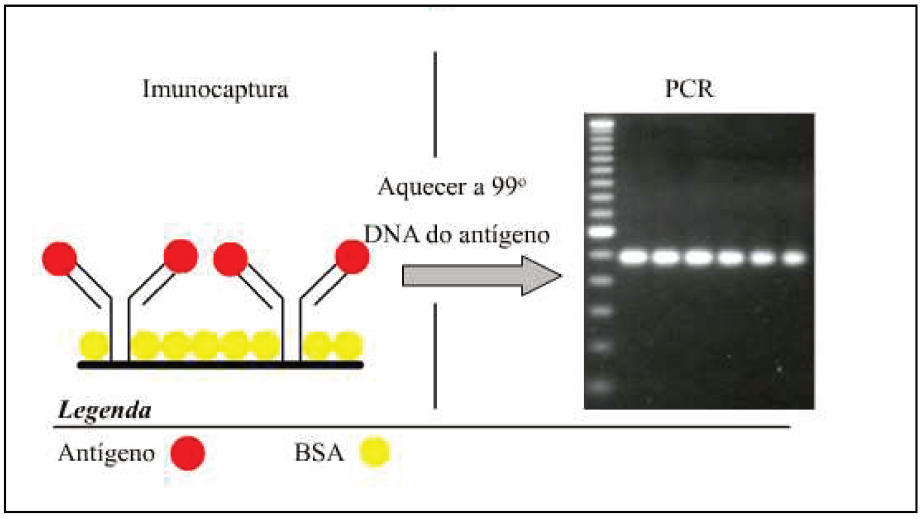

Figura 10. Ensaio de Imunocaptura-PCR. Os antígenos são capturados pelo anticorpo policlonal previamente adsorvido em um suporte sólido (microplaca) e detectados através da PCR utilizando primers específicos para o DNA de *X. fastidiosa.*

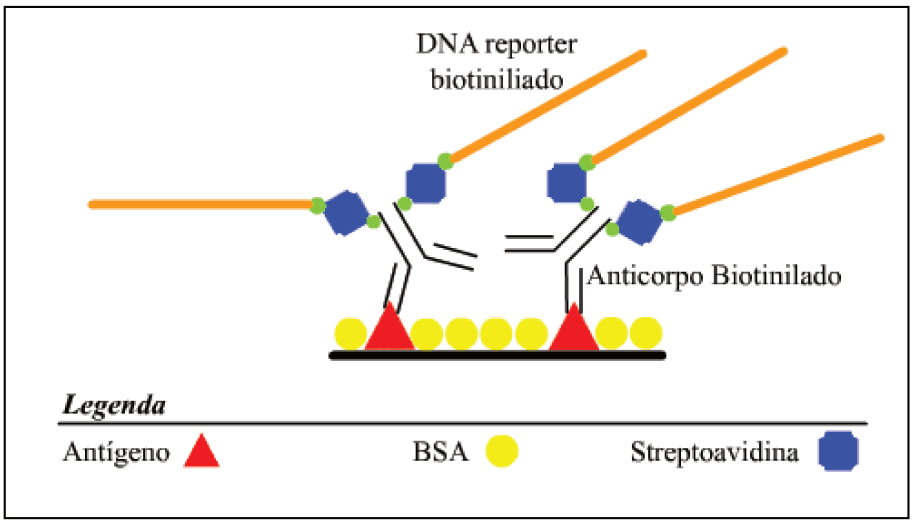

Figura 11. Ensaio de Imuno PCR. Os antígenos são adsorvidos em um suporte sólido (placas de poliestireno) e detectados por um anticorpo específico biotinilado.

Posteriormente, o anticorpo é complexado com streptoavidina, que permite a ligação do DNA-*reporter* biotinilado, sendo este detectado por PCR.

A técnica de Imunocaptura-PCR (IC-PCR) possibilita a captura das bactérias diretamente pelos anticorpos policlonais na microplaca de ELISA e a sua posterior detecção através de PCR, utilizando o par de primers, CVC-1 e 272-int (POOLER e HARTUNG, 1995). Esta metodologia não requer os passos de extração de DNA, no entanto, exige a manutenção da integridade da parede celular da bactéria durante todo o processo de captura, pois a amplificação na PCR é realizada a partir do DNA bacteriano. Esta característica prejudicou o desempenho e a reprodutibilidade da IC-PCR, embora tenha apresentado um limite de detecção da ordem de $1x10^3$ células bacterianas. Assim, o estabelecimento da Imuno-PCR (I-PCR) eliminou esta dificuldade, além de proporcionar um aumento na sensibilidade do imunodiagnóstico.

Na I-PCR, as amostras de tecido vegetal são incubadas em microplacas de ELISA e posteriormente, o soro policlonal previamente biotinilado é adicionado, formando o complexo antígeno-anticorpo. Posteriormente, é adicionada uma molécula ligadora (linker) como a estreptoavidina e em seguida, um DNA-*reporter* biotinilado (gene *UspA1*) é ligado ao complexo. A PCR é baseada em *primers* desenhados especificamente para o DNA repórter, de modo que, nesta técnica as bactérias não precisam necessariamente serem extraídas integras durante o processo de extração, o que torna o teste mais reprodutível. Além disso, utilizando bactérias provenientes de meio de cultura a sensibilidade da I-PCR foi de $1x10^1$ células, isto é, 100 vezes maior do que a IC-PCR, 1.000 vezes maior do que o ELISA e 100.000 vezes maior do que o PCR. No entanto, ainda assim, todos os métodos descritos necessitam de uma intensa perfusão ou maceração das amostras vegetais já que a bactéria forma um forte biofilme nos vasos do xilema da planta hospedeira.

A versão atual desse imunoensaio pode ser melhorada com o desenvolvimento de anticorpos monoclonais e a padronização de ensaios de I-PCR em Tempo Real permitindo uma quantificação direta da *X. fastidiosa*.

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8. ANEXOS

8.1. Padrão de Hidrofobicidade da CB-22 e CB-104

As proteínas também foram avaliadas quanto a sua hidrofobicidade no programa PROTSCALE (http://www.expasy.org/tools/protscale.html) usando os parâmetros de KYTE e DOOLITTLE (1982). Esta análise (**Figura 12**) foi utilizada para auxiliar na determinação da posição e tamanho dos peptídeos a serem clonados em vetor de expressão pET32 Xa/LIC (NOVAGEN, 2000).

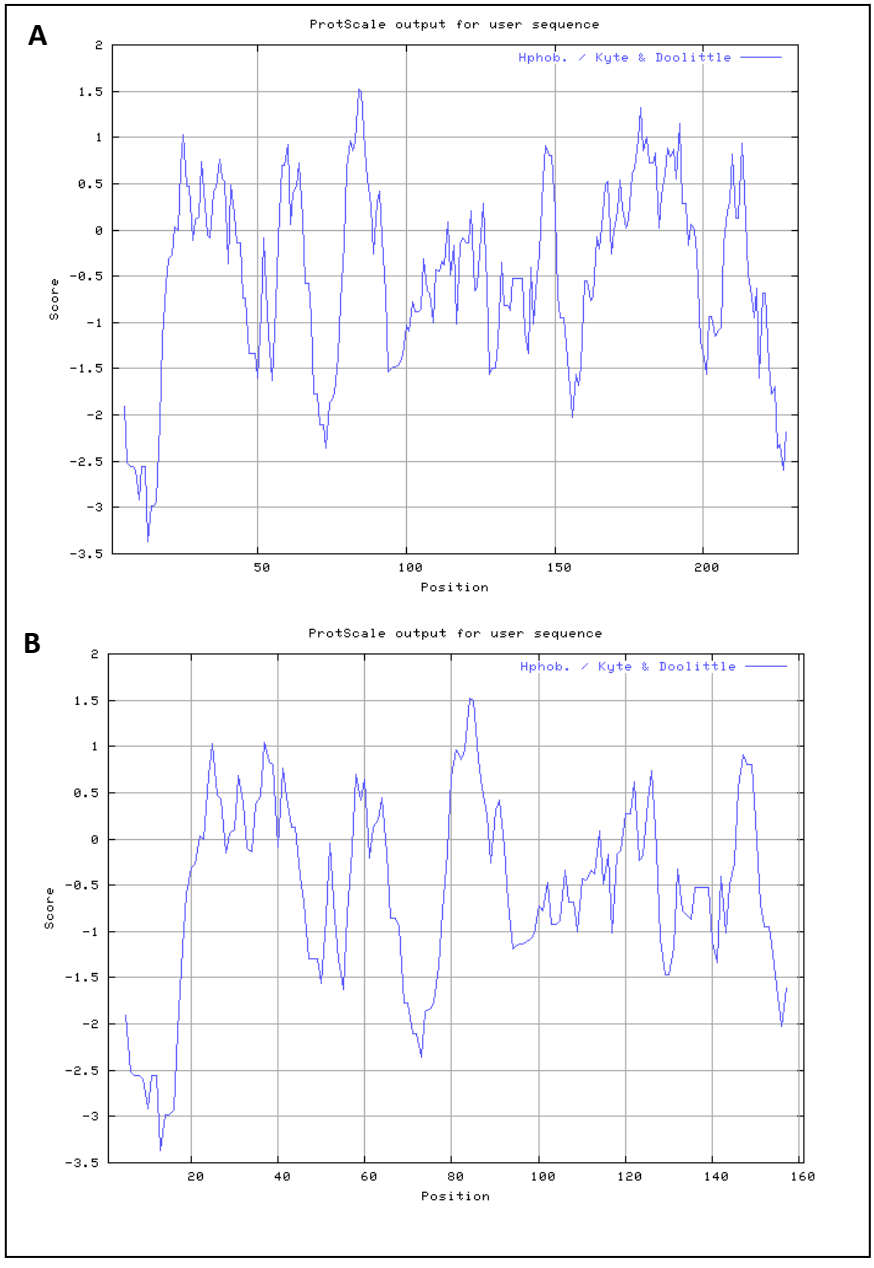

Figura 12. Análise da proteína CB-22 (**A**) e CB-104 (**B**) quanto a sua hidrofobicidade no programa PROTPARAM.

8.2. Seqüenciamento *de novo* da CB-22 e da CB-104

A análise das seqüências da proteína CB-104 apresentou um nucleotídeo modificado na posição 481 (G → T). No entanto, esta alteração ocasionou a mudança do aminoácido glicina (GGA) para um "*stop codon*" (TGA) na posição 161 da proteína (**Figura 13**). Este resultado explica a diferença de peso molecular entre as proteínas CB-22 e CB-104 e determina o peso molecular de 18 KDa para proteína CB-104.

A

469 T T G A G T T A T G G T **G** G A C G T C C G T T A CB-104 (TARGON,1997)

469 T T G A G T T A T G G T **T** G A C G T C C G T T A CB-104 (PERONI,2005)

B

157 L S Y G **G** R P L D A G CB-104 (TARGON, 1997)

157 L S Y G **S** R P L D A G CB-104 (PERONI, 2005)

Figura 13. Seqüência parcial de nucleotídeos **(A)** e de aminoácidos **(B)** da proteína CB-104. O alinhamento mostrou uma alteração no nucleotídeo 481 (G → T), modificando o aminoácido Glicina (G) para um *stop codon* (S).

8.3. Características físico-químicas da CB-22 e CB-104

A comparação das seqüências para a proteína CB-22 mostrou a troca de 4 nucleotídeos (posições nº 149, 150, 152 e 596) e a presença de 6 novos nucleotídeos na porção 3' da cadeia nucleotídica (posições nº 670 a 675). Essas alterações provocaram a modificação de três aminoácidos nas posições 50, 51 e 199 e a adição de dois novos aminoácidos à cadeia polipeptídica nas posições 224 e 225. (**Tabela 1**).

Proteína CB-22			
Posição	**Nucleotídeos**	**Posição**	**Aminoácidos**
149	**A → C**	**50**	**Asn → Thr**
150	**C → T**		
152	**C → A**	**51**	**Pro → Gln**
596	**A → C**	**199**	**Gln → Pro**
670	**- → C**		
671	**- → T**	**224**	**Leu**
672	**- → C**		
673	**- → G**		
674	**- → A**	**225**	**Glu**
675	**- → G**		

Tabela 1. Diferenças de nucleotídeos e aminoácidos entre as seqüências da proteína CB-22 obtidas por TARGON (1997) e pelo novo seqüenciamento.

Dessa forma, os resultados confirmam os pesos moleculares de 25 e 18 KDa, respectivamente para as proteínas CB-22 e CB-104, que são coincidentes com os pesos moleculares aproximados observados em gel desnaturante de poliacrilamida a 12%.

8.4. Análise *in silico* das proteínas CB-22 e CB-104

As seqüências de aminoácidos das proteínas CB-22 e CB-104 foram analisadas no programa PROTPARAM disponível na suíte de programas do EXPASY (http://www.expasy.org) quanto a sua estrutura primária, considerando o número de aminoácidos, peso molecular, pI teórico e número total de resíduos carregados positiva e negativamente (**Tabela 2**).

Para a proteína CB-22, a principal diferença observada foi na quantidade total de resíduos carregados, conferindo uma carga residual negativa ao contrário do que era observado quando do seqüenciamento efetuado por TARGON (1997). Entretanto, em relação à proteína CB-104, os dados mostraram uma diferença significativa no seu ponto isoelétrico e a alteração na carga residual positiva para uma carga neutra, além do tamanho da seqüência como descrito anteriormente (**Tabela 2**).

Características	**Proteína CB-22**		**Proteína CB-104**	
	TARGON (1997)	PERONI (2005)	TARGON (1997)	PERONI (2005)
Número de aminoácidos	223	225	223	160
Pelo Molecular (KDa)	25	26	24.9	18
pI Teórico	6.83	6.75	7.67	6.73
Total de resíduos carregados negativamente (Asp + Glu)	30	31	29	22
Total de resíduos carregados positivamente (Arg + Lys)	30	30	30	22

Tabela 2. Análises e comparação das características estruturais primárias das proteínas CB-22 e CB-104 no programa PROTPARAM.

8.5. Material e Métodos

Proteínas recombinantes (CB-22 e CB-104):

As proteínas recombinantes (CB-22 e CB-104) foram obtidas por TARGON *et al.* (1997) através da clonagem do gene da capa protéica de isolados do complexo CB no vetor pET22. Alíquotas de *E. coli* BL21 DE3 transformadas foram incubadas a 37°C por 12 horas sob agitação constante (300 rpm) em 200 mL de meio LB. A expressão das proteínas recombinantes foi induzida pela adição de 1 mM IPTG (*isopropylthiol-β-D-galactoside*), sob constante agitação por 4 horas.

As proteínas foram purificadas através de coluna de afinidade a níquel (Ni-NTA) e eluídas com Imidazol a 5, 20, 50, 100, 200 e 500 mM. A purificação foi monitorada através de espectrofotometria a 280 ηm, e as alíquotas que apresentaram absorbâncias acima de 0,5 foram reunidas, concentradas em Centriprep 10 (Millipore) e analisadas em SDS-PAGE 12%, segundo LAEMMLI (1970).

Anticorpos monoclonais:

Os hibridomas 30.G.02, 37.G.11, 39.07 e IC.04-12 foram obtidos através da fusão de células esplênicas de camundongos BALB/c imunizados com as proteínas recombinantes, com células de mielona Sp2, segundo protocolo de FASEKAS e SCHEIDEGGER (1980). Os hibridomas foram cultivados inicialmente em placas de 24 poços, contendo "feeder layer" de macrófagos preparados com pelo menos três dias de antecedência em meio RPMI 1640 (Sigma) e incubados em estufa a 5% de CO_2 a 37^{o}C.

Após cerca de 5 a 7 dias, as células foram transferidas para frascos de cultura com capacidade de 25 ml, e destes posteriormente para frascos de cultura de maior capacidade (75 ml). Entre cada transferência, foi realizado ensaios de ELISA para avaliar os sobrenadantes das culturas em relação à presença dos anticorpos de interesse, uma vez que as linhagens de hibridomas podem ser instáveis e deixar de secretar as

imunoglobulinas. Posteriormente, 1.0 x 10^6 células de cada um dos clones de hibridomas foram inoculadas, via intraperiotoneal, em camundongos Balb/c separadamente, previamente estimulados com 0.5 ml de óleo mineral (Nujol®), para produção de ascite.

O fluído ascítico foi coletado por punção peritoneal, centrifugado a 200xg por 10 minutos a 4^{o}C e o sobrenadante coletado e purificado por cromatografia de afinidade. Os anticorpos monoclonais 30.G.02, 37.G.11 e 39.07, da classe IgG1, foram purificados em coluna de Sepharose com proteína G, e o anticorpo monoclonal IC.04-12, da classe IgG2b, foi purificado em coluna de Sepharose com proteína A, segundo os protocolos descritos por WILCHEK *et al.* (1984).

Purificação de anticorpos monoclonais:

Cromatografia em Sepharose de Proteína G:

- Pesar 1.5 g de Sepharose de Proteína G e dissolver em 10 ml de tampão fosfato de sódio 0,1 M, pH 8.5 (tampão de coluna)

- Preparar a amostra a partir da diluição do fluído ascítico em tampão de coluna, até que o pH da solução final seja 8.5

- Homogeneizar a amostra com a Sepharose, com agitação ocasional durante 30 minutos.

- Lavar a coluna com tampão de coluna

- Eluir as imunoglobulinas com tampão 0,1 M Glicina- HCl, pH 2.8

- Coletar em banho de gelo, frações de 5 ml em tubos contendo tampão 1 M Tris-HCl, pH 8.5 para efetuar a neutralização.

Cromatografia em Sepharose de Proteína A:

- Pesar 1.5 g de Sepharose de Proteína A e dissolver em 10 ml de PBS (136 mM NaCl, 1.4 mM KH_2PO_4, 2.6 mM KCl, 8 mM Na_2HPO_4, pH 7.4). Manter durante 60 minutos a temperatura ambiente

- Montar a coluna e equilibrar com tampão fosfato de sódio 0,1M, pH 8.0

- Efetuar a aplicação da amostra, previamente diluída em tampão de coluna

- Lavar a coluna com de tampão de coluna

- Eluir as IgG com tampão citrato 0.1 M, pH 3.0

- Coletar frações de 4,0 ml em tubos contendo tampão carbonato 0,1M, pH 9.0

Dosagem de Proteínas:

As concentrações de proteínas foram determinadas pelo micrométodo de Bradford (BRADFORD, 1976), utilizando BSA como proteína padrão. Este método é rápido e capaz de detectar e quantificar proteínas em concentrações entre 50 μg e 500 μg/ml e se baseia na ligação da proteína ao Corante Coomassie Blue G-250.

ELISA Sandwich:

A avaliação da reatividade dos anticorpos monoclonais contra as proteínas recombinantes do capsídeo viral do CTV foi realizada através de ensaios de ELISA Sandwich, segundo a metodologia descrita por CLARK *et al.,* (1986).

Placas de poliestireno foram sensibilizadas com 100 μl/poço de soro policlonal de coelho #1006 (BAPTISTA *et al.*, 1996) específico para o CTV, diluído 1:1.000 em tampão carbonato 0.2 M, pH 9.6 e incubadas a 4^oC por um período de 12 horas.

As placas foram bloqueadas com 100 μl/poço de PBS com 2 % de leite em pó desnatado (Molico®) e incubadas por 1 hora a 37 ^oC. As placas foram lavadas 3 vezes com 2000 μl/poço de PBS + 0.05 % Tween 20 (PBS-T). Em seguida, foram adicionados 100

μl/poço das proteínas recombinantes purificadas, CB-22 e CB-104 (3 μg/mL em PBS). Após incubação por 2 horas a 37°C, as placas foram lavadas 3 vezes com 150 μl/poço de PBS-T.

Posteriormente, foram adicionados 100 μl/poço do anticorpo monoclonal na diluição desejada, e as placas foram incubadas por 2 horas a 37°C ou por 16 horas a 4°C.

Após a lavagem das placas como descrito acima, foram acrescentados 100 μl/poço do anticorpo anti-IgG de camundongo conjugado com fosfatase alcalina (1:30.000 em PBS).

As placas foram incubadas a 37°C por 1 hora, e lavadas novamente com PBS-T. As reações foram reveladas com 100 μl/poço do substrato pNPP (para-nitrofenil fosfato) na concentração de 1 mg/ml diluído em tampão substrato dietanolamina.

Após incubação no escuro por 1 hora a temperatura ambiente, a absorbância foi quantificada a 405 ηm, em leitor de ELISA (Multireader Expert Plus, ASYS HITECH, Áustria).

Eletroforese SDS-PAGE 12%:

A eletroforese foi realizada em gel descontínuo de poliacrilamida na presença de SDS (dodecil sulfato de sódio), de acordo com a metodologia descrita por LAEMMLI (1970). O gel de resolução foi de 12%, enquanto o gel de empacotamento foi de 5%.

Os géis foram preparados usando o Sistema Mini Protean II® da BioRad, seguindo o protocolo abaixo:

Componentes do Gel SDS – PAGE		
	Gel de Resolução (12 %)	**Gel de concentração (5 %)**
Acrilamida 30%	12 ml	1,25 ml
3 M Tris-HCl, pH 8.8	5,6 ml	-
0,5 M Tris- HCl, pH 6.8	-	2,50 ml
SDS 10 %	270 μl	100 μl
H_2O MilliQ	11,78 ml	5,85 ml
Persulfato de amônia 3%	340 μl	250 μl
Temed	10 μl	7,5 μl
Volume Total	**30 ml**	**10 ml**

Western blot

As proteínas submetidas à eletroforese SDS-PAGE 12% foram imediatamente transferidas para membranas de nitrocelulose (Hybond C® extra, Amersham), segundo a técnica descrita por TOWBIN *et al.* (1979) e as especificações determinadas pela BioRad (Mini Trans-Blot® Electrophoretic Transfer Cell) durante 3 horas a 0,8 mA/cm^2.

Após a transferência, as membranas foram bloqueadas durante 3 horas sob agitação constante em tampão TBS (0.1M Tris-HCl, pH 7.5 e 0,9% NaCl) contendo 5% de leite Molico®. Após lavagem com TBS 0,1% Tween 20 (TBS-T), foram incubadas durante 12 horas a 4°C sob agitação constante em TBS-T contendo 5% de Molico® e os anticorpos monoclonais na diluição desejada.

Posteriormente, as membranas foram lavadas 3 vezes durante 10 minutos com TBS-T e incubadas durante 1 hora em TBS-T com 5% de Molico® e o anticorpo anti-IgG de camundongo conjugado com fosfatase alcalina (Sigma), diluído 1:30.000.

As membranas foram lavadas como descrito acima e incubadas com o tampão de revelação (0.1 M Tris-HCl, pH 9.5; 0.1 M NaCl, 5 mM $MgCl_2$) contendo 8 mg de BCIP (5-brommo-4chloro-3indoyl phosphate) e 16 mg de NBT (nitroblue tetrazolium). Quando a coloração desejada foi obtida, as membranas foram transferidas para água destilada a fim de paralisar a reação e secas a temperatura ambiente.

Imunodetecção por Microscopia Eletrônica de Transmissão (ISEM)

Para avaliar a interação entre os anticorpos monoclonais obtidos por STACH-MACHADO *et al.* (2000) e as proteínas virais, foi realizado um ensaio modificado de DERRICK e BRLANSKY (1976). As grades de microscopia eletrônica, previamente recobertas com parlodium e carbono, foram tratadas com o soro policlonal #1006 (BAPTISTA *et al.*, 1996), diluído em tampão fosfato de potássio 0,06 M, pH 7.2, por 1 hora a temperatura ambiente.

Posteriormente, as grades foram lavadas com o mesmo tampão, tratadas com um extrato de plantas infectadas com o CTV, coletadas no Centro Apta Citros Sylvio Moreira, Cordeirópolis-SP (secções transversais do pecíolo das folhas picotadas em tampão fosfato de potássio 0,06 M, pH 7,2) e mantidas em câmara úmida por 2 horas a temperatura ambiente. As grades foram lavadas em água destilada e incubadas com cada um dos anticorpos monoclonais diluídos em tampão fosfato de potássio 0,06 M, pH 7,2, por 2 horas a temperatura ambiente.

Em seguida, foram lavadas em água destilada, incubadas com o anticorpo anti-IgG de camundongo conjugado com partículas de ouro coliodal de 10 ηm e examinadas ao microscópio eletrônico de transmissão do Laboratório de Microscopia Eletrônica do Departamento de Biologia Celular, da Universidade Estadual de Campinas.

Sequenciamento das proteínas recombinantes CB-22 e CB-104

Inicialmente, os clones de *E. coli* BL21 (DE3) contendo os genes das proteínas CB-22 e CB-104 no vetor de expressão pET22 foram cultivados em meio LB (1% de NaCl, 1% de Bacto triptona, 0,5% de Extrato de levedura, pH 7,0 contendo 100 μg/ml de ampicilina) por 16 horas a 37°C sob agitação constante (300 rpm) e, em seguida, os plasmídeos foram purificados segundo metodologia modificada de SAMBROOK *et al.* (1989 e 2001). A qualidade da extração plasmidial foi avaliada em gel de agarose 0,8%.

Posteriormente, a transformação com polietilenoglicol (PEG) foi realizada utilizando células de *E. coli* DH5α, que foram escolhidas em detrimento da BL21(DE3), já que não possuem enzimas como recombinases ou invertases capazes de provocar alterações no DNA cromossômico ou plasmidial.

Os clones transformantes foram plaqueados em meio de cultura LB sólido contendo 50 μg/ml de ampicilina, utilizando um volume de cultura de 50, 100 e 200 μl. Após crescimento por 16 horas a 37°C, as placas foram observadas e cinco clones isolados (colônias brancas que expressam o gene de resistência à ampicilina) para cada uma das

proteínas recombinantes foram selecionados. Culturas permanentes foram preparadas e armazenadas em freezer -80°C.

Para avaliar e confirmar a transformação das células de *E. coli* DH5α com os plasmídeos pET22 foi realizado uma PCR das colônias selecionadas, utilizando os *primers T7 promoter* e *T7 terminator* que são universais para os vetores de expressão da família pET. A reação de amplificação foi: 94°C por 3 min; 30 ciclos de 94°C por 1 min, 55°C por 1:30 min e 72°C por 2 min com uma etapa final de elongação a 72°C por 7 min. Os produtos de PCR foram analisados em gel de agarose 0,8%, corados com brometo de etídeo.

O seqüenciamento dos plasmídeos foi efetuado com 3 clones de cada uma das proteínas. Para isso, cada clone foi amplificado em uma PCR de colônias como descrito anteriormente, mas com um volume final de reação de 100µl (4 alíquotas de 25µl). Os produtos da PCR foram purificados com o kit *GFX PCR DNA and Gel band purification* (GE-Amersham Bioscience), avaliados em gel de agarose 0,8% e aplicados na reação de sequenciamento.

O termociclador foi programado com 40 ciclos de: 96°C por 10 seg; 55°C por 5 seg e 60°C por 4 min. Os produtos foram precipitados com isopropanol 75% e etanol 70% e o sequenciamento foi efetuado no Seqüenciador Automático ABI PRISM 377. Os cromatogramas foram analisados e as seqüências dos três clones foram alinhadas no programa MEGALIGN da plataforma LASERGENE (DNASTAR Inc.), determinando uma seqüência consenso de nucleotídeos para as proteínas CB-22 e CB-104.

Análise in silico das proteínas CB-22 e CB-104

As seqüências de aminoácidos das proteínas CB-22 e CB-104 foram analisadas no programa PROTPARAM (http://www.expasy.org/tools/protparam.html) quanto a sua estrutura primária, considerando o número de aminoácidos, peso molecular, pI teórico e número total de resíduos carregados positiva e negativamente. As proteínas também foram avaliadas quanto a sua hidrofobicidade no programa PROTSCALE

(http://www.expasy.org/tools/protscale.html) usando os parâmetros de KYTE e DOOLITTLE (1982). Esta análise foi utilizada para auxiliar na determinação da posição e tamanho dos peptídeos a serem clonados em vetor de expressão pET32 Xa/LIC.

Desenho dos primers para síntese dos peptídeos das proteínas recombinantes

Os *primers* dos peptídeos de interesse foram desenhados no programa GENERUNNER v. 3.05 e no PRIMERSELECT da LASERGENE (DNASTAR Inc.). Foram desenhados 14 pares de *primers*, dos quais 8 foram usados para amplificação de peptídeos da proteína CB-22 e 6 pares para os peptídeos da CB-104, uma vez que alguns destes *primers* estão localizados em regiões homólogas das proteínas CB-22 e CB-104.

Cada primer direto (forward) é composto por uma seqüência comum de 15 nucleotídeos (GGT ATT GAG GGT CGC) e uma seqüência que corresponde à região de interesse a ser amplificada, totalizando aproximadamente 35 nucleotídeos. Enquanto que os *primers* reversos (reverse) possuem a seqüência (AGA GGA GAG TTA GAG CC) seguida da seqüência de interesse. Estas seqüências comuns são necessárias para possibilitar a clonagem direta no vetor de expressão pET32 Xa/LIC sem a necessidade do tratamento com enzimas de restrição (**Figura 14**).

Sítio LIC para clonagem

start codon **inserto** **stop codon**

5' GGTATTGAGGGTCGCATG.....sense.....TAAGGCTCTAACTCTCCTCT **3'**

3' CCATAACTCCCAGCGTAC.....antisense.....ATTCCGAGATTGAGAGGAGA **5'**

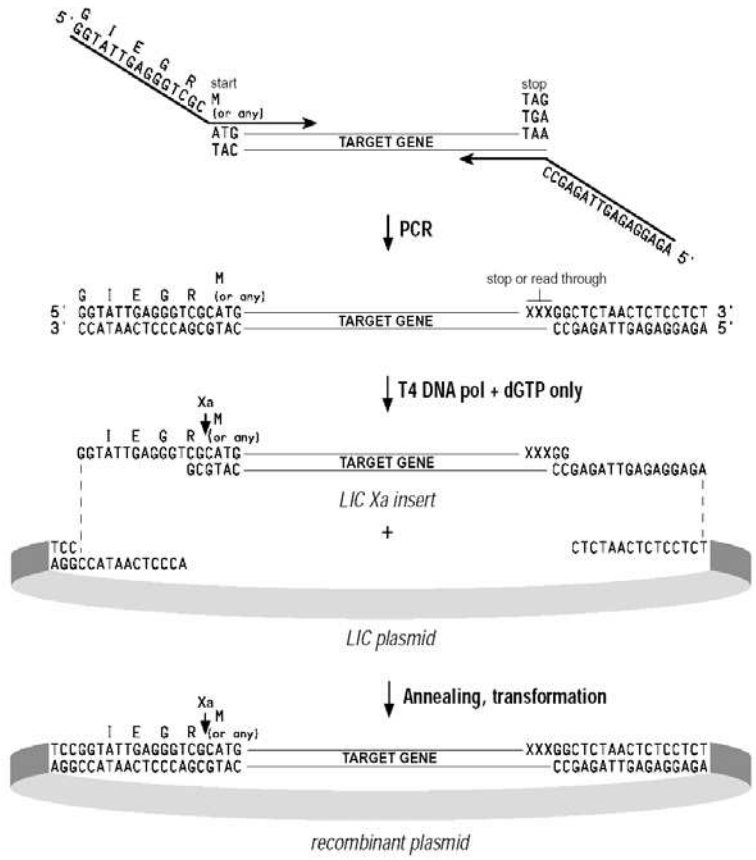

Figura 14. (A) Representação das seqüências adesivas para clonagem do produto de PCR purificado de cada seqüência-alvo no vetor pET32Xa/LIC, **(B)** Esquema da clonagem do produto de PCR purificado no vetor pET32Xa/LIC.

Clonagem em vetor de expressão pET 32 Xa/LIC

Inicialmente foi realizada uma PCR Gradiente para estabelecer as temperaturas ideais de anelamento dos *primers*. Para isso, uma pré-cultura para cada clone das proteínas CB-22 e CB-104 foi realizada e os plasmídeos foram extraídos e diluídos em tampão TE na proporção 1:10 (v/v). Cada um dos pares de *primers* foi testado em seis temperaturas diferentes: 40,0; 41,7; 45,5; 51,7; 56,8 e 59,6°C. Os produtos de PCR foram avaliados em gel de agarose 1% corado com brometo de etídeo.

Após análise da eficiência de amplificação, foram escolhidas as temperaturas na qual o amplificado foi de intensidade forte e de boa qualidade. Uma nova PCR com volume final de reação de 100 µl (4 alíquotas de 25µl) foi re alizada com cada um dos pares de *primers* e as duas proteínas recombinantes, resultando em 14 amplificons. Os produtos da PCR foram purificados, quantificados em gel de agarose 1%, tratados com T4 DNA polimerase e ligados ao vetor de expressão pET32 Xac/LIC. Posteriormente, células competentes de *E. coli* DH 5α foram transformadas com o inserto-vetor e plaqueadas em meio LB contendo 100 µg/ml de ampicilina. Os clones transformantes foram selecionados e o sequenciamento para confirmação da clonagem foi realizado de acordo com a metodologia utilizada no Laboratório de Análises Genéticas e Moleculares, sob responsabilidade da Profa. Dra. Anete Pereira de Souza.

A clonagem permitiu a expressão de peptídeos de 20 a 45 aminoácidos que foram analisados em ELISA e Western blot para o mapeamento dos epitopos reconhecidos pelos quatro anticorpos monoclonais específicos para o CTV.

Minipreparação de DNA plasmidial

A minipreparação do DNA dos clones foi feita de acordo com a metodologia descrita por SAMBROOK *et al.* (1989).

Produção do antisoro policlonal anti-Xylella fastidiosa

Amostras de Xy*lella fastidiosa*, isoladas de plantas de laranja Pêra sintomáticas para CVC (Clorose Variegada dos citros) foram fornecidas pelo Centro de APTA Citros Sylvio Moreira (IAC, Cordeirópolis, SP). As bactérias foram cultivadas em meio de cultura líquido PW (DAVIS *et al.*, 1981) sob agitação constante a 28°C até a fase estacionária de crescimento, em seguida, centrifugadas a 12.000 rpm por 3 minutos e a concentração foi ajustada para $1x10^9$ bactérias/mL em PBS estéril.

As bactérias foram inativadas com 1% de formol por 24 horas, a temperatura ambiente e, lavadas quatro vezes em PBS estéril e inoculadas na veia marginal da orelha de três coelhos da linhagem Branca da Nova Zelândia de acordo com o esquema de imunização de cinco doses (volumes de 0,5; 1; 2; 3 e 4 mL), com intervalos de cinco dias. O sangue foi coletado, centrifugado e o soro inativado a 56°C por 45 minutos.

O título, especificidade e sensibilidade do antisoro foram determinados por ELISA Indireto segundo a metodologia descrita por CLARK *et al.* (1986).

Imunocaptura-PCR

Microplacas de poliestireno foram sensibilizadas com o soro policlonal de coelho anti-*Xyllela fastidiosa* diluído 1:1.000 em tampão carbonato de sódio 0,2 M, pH 9.6 e incubadas a 4°C por 16 horas. Posteriormente, as placas foram bloqueadas com PBS contendo 2% de Molico® com incubação de 1 hora a 37°C.

Em seguida, as placas foram lavadas quatro vezes com PBS-T e em seguida, foram adicionadas as bactérias *Xyllela fastidiosa* nas concentrações desejadas. Após incubação de 1 hora a 37°C, as placas foram lavadas com PBS-T e foram adicionados 25 μl por poço de água Milli-Q estéril.

Posteriormente, as placas foram aquecidas a 99°C por 5 minutos e transferidas imediatamente para banho de gelo. Este procedimento lisa as bactérias liberando o DNA que será utilizado como molde na PCR.

As reações foram realizadas usando Tampão 10X Invitrogen®; 2,5 mM Mg^{++}; 2,5 mM dNTP; 25 ηg dos *primers* CVC-1 (5'-AGATGAAAACAATCATGCAAA-3') e 272-2int (5'-GCCGCTTCGGAGAGC ATTCCT-3'); 1,5 U Taq DNA polimerase e água Milli-Q estéril para um volume final de 25 μL de reação. Os *primers* CVC-1 e 272-2int amplificam uma região conservada de aproximadamente 500 pares de bases em *Xyllela fastidiosa* (POOLER e HARTUNG, 1995).

O termociclador foi programado com: 94°C por 3 minutos; 35 ciclos de 94°C por 1 min, 55°C por 1 minuto e 72°C por 1:30 min e uma etapa final de elongação a 72°C por 10 minutos. Os produtos da PCR foram analisados em gel de agarose 1% corados com brometo de etídeo.

Imuno-PCR

Microplacas de poliestireno foram sensibilizadas com *Xylella fastidiosa* nas concentrações desejadas, diluídas em tampão carbonato 0,2M, pH 96 e incubadas por 3 horas a 37°C. Posteriormente, as placas foram bloqueadas com PBS contendo 2% de Molico® com incubação de 1 hora a 37°C.

Em seguida, as placas foram lavadas quatro vezes com PBS-T e em seguida, foram adicionados o soro policlonal anti-*X. fastidiosa* biotinilado, diluído 1:100 em PBS. Após incubação por 2 horas a 37°C, as placas foram lavadas com PBS-T.

Posteriormente, foi adicionada streptoavidina a 100 ug/ml com incubação por 1 hora a 37°C. As placas foram novamente lavadas com PBS-T e foi adicionado o DNA repórter UspA1 biotinilado na concentração de 50 ηg/μl. Após incubação por 1 hora a 37°C, foram adicionados 25 μl de H_2O MilliQ estéril e as placas foram aquecidas a 99°C por 5 minutos e transferidas imediatamente para banho de gelo.

As reações foram realizadas usando Tampão 10X Invitrogen®; 2,5 mM Mg^{++}; 2,5 mM dNTP; 25 ηg dos *primers* UspA1 Direto (5'-AACTCGAGAGCAGGCCGCCGGTGATAGCAGTA-3') e UspA1 reverso (UspA1-R-Biotin: 5'-GACGCTCGAGCCCCCGCCGCAAGAT-3'.); 1,5 U Taq DNA polimerase e água Milli-Q estéril

para um volume final de 25 μL de reação. O par de *primers* UspA1 amplificam o gene UspA1 de *X. fastidiosa* com um tamanho aproximado de 1.100 pb.

O termociclador foi programado com: 94°C por 3 minutos; 35 ciclos de 94°C por 1 min, 60°C por 1 minuto e 72°C por 1:30 min e uma etapa final de elongação a 72°C por 10 minutos. Os produtos da PCR foram analisados em gel de agarose 1% corados com brometo de etídeo.

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Research Article

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Expression profile of oxidative and antioxidative stress enzymes based on ESTs approach of citrus

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Abstract

Plants not only evolve but also reduce oxygen in photosynthesis. An inevitable consequence of this normal process is the production of reactive oxygen species (ROS). Plants are adequately protected by the presence of multiple antioxidative enzymes in the cytosol and also in the different cell organelles such as chloroplasts, mitochondria, and peroxisomes. Traditionally, ROS were considered to be only a toxic byproduct of aerobic metabolism. However, recently it has become apparent that plants actively produce these molecules which may control many different physiological processes such as abiotic and biotic stress response, pathogen defense and systemic signaling. The search results using the Citrus Genome Program in Brazil (CitEST) for oxidative stress and the antioxidant enzyme system in *Citrus Sinensis* variety 'Pera IAC' indicated that the multiple ROS-scavenging enzymes were expressed throughout all citrus tissues. The analyses demonstrated the ubiquitous expression of metallothioneins, probably indicating a constitutive expression pattern. Oxalate oxidase has been identified as the most abundant expressed gene in developing fruits, which suggests a specific function in the ripening of citrus fruit. Moreover, infected leaves with *Xylella fastidiosa* and *Leprosis citri* showed a massive change in their ROS gene expression profile which may indicate that the suppression of ROS detoxifying mechanisms may be involved in the induction of the diseases.

Key words: citrus, genome, EST, reactive oxygen species, oxidative stress enzyme.

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Introduction

The appearance of oxygen in the atmosphere enabled respiratory metabolism and efficient energy generation systems which use molecular oxygen (O_2) as final electron acceptor, which led to the formation of reactive oxygen species (ROS) in cells (Temple *et al.*, 2005). Although, atmospheric oxygen is relatively non-reactive, it can give rise to reactive oxygen intermediates which include superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$), and singlet oxygen ($^1O_2$) (Scandalios, 2005).

In plants, ROS are produced continuously as byproduct of various physiological metabolic pathways, such as photosynthesis, photorespiration and CO_2 assimilation. Furthermore, ROS production is increased by several environmental factors of stress, such as exposition to high levels of light, drought, heavy metals, salt concentrations, temperature extremes, air pollution, UV radiation, herbicides and pathogen attacks. Whether ROS will act as damaging, protective or signaling factors depends on the delicate equilibrium between ROS production and scavenging at the proper site and time (Gratão *et al.*, 2005).

The enzymatic ROS scavenging mechanisms in plants include: superoxide dismutase (SOD), the water-water cycle (WWC), the ascorbate-glutathione cycle (AGC), the glutathione peroxidase cycle (GPXC), and catalase (CAT) (Apel and Hirt, 2004) (Figure 1).

SOD catalyzes the dismutation of superoxide radical in a broad range of organisms, including plants. The dismutation of superoxide into hydrogen peroxide and oxygen constitute the first line of cellular defense to prevent undesirable biological oxidation by oxygen radical generated during cellular metabolism. Based on the metal co-factor used by the enzyme, SODs are classified into three groups: iron SOD (FeSOD), manganese SOD

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(MnSOD), and copper-zinc SOD (Cu/ZnSOD) (Alscher *et al.*, 2002).

In plant cells, one of the most important detoxification systems is the WWC which operates together with SOD as a mechanism of hydrogen peroxide scavenging in intact chloroplasts (Edreva, 2005; Asada, 2006). The most important function of this cycle is a rapid, immediate scavenging of O_2^- and H_2O_2 at the site of its generation prior to their interaction with the target molecules. Ascorbate peroxidase (APX) uses two molecules of ascorbate to reduce H_2O_2 to water, with the concomitant generation of two molecules of monodehydroascorbate (MDHA). MDHA is a radical with a short lifetime, which is reduced directly to ascorbate within the chloroplast at the thylakoid membrane (Figure 1 A).

Hydrogen peroxide can also be converted into water by the AGC which involves successive oxidations and reductions of ascorbate, glutathione and NADPH by enzymes: APX, glutathione reductase (GR); dehydroascorbate reductase (DHAR); and monodehydroascorbate reductase (MDHAR) (Figure 1 B). The reducing agent in the first reaction catalyzed by APX is ascorbate, which is oxidized into MDHA that can be regenerated by MDHAR using NAD(P)H as a reducing equivalent. MDHA can spontaneously dismutase into dehydroascorbate (DHA). The ascorbate regeneration is mediated by DHAR driven by oxidation of glutathione (GSH) to glutathione disulphide (GSSG). Finally, the cycle closes with GR converting GSSG back into GSH using NAD(P)H as a reducing agent.

The GPXC (Figure 1 C) also detoxifies hydrogen peroxide to water but uses glutathione directly as a reducing agent. The oxidized GSSG is converted into GSH by GR using NAD(P)H.

Catalase (CAT) is responsible to dismutation of hydrogen peroxide into oxygen and water in the peroxisomes, protecting the cell from the deleterious effects of hydrogen peroxide accumulation (Figure 1 D). Multiple isoenzymes of CAT have been studied in higher plants, and in maize, three main CAT isoforms have been characterized (Scandalios, 2005).

Besides all enzymatic scavenging-pathways, plant cells have numerous non-enzymatic antioxidant molecules, which are also involved in protection against oxidative stress and damage caused by ROS. The main non-enzymatic antioxidant molecules are ascorbate and glutathione, which are integrated in the cycles above, flavonoids, alkaloids, phenolic compounds, α-tocopherol and carotenoids, which help in scavenging of ROS (Foyer and Noctor, 2005). Furthermore, the metal chelators, such as metallothioneins (MT) and ferritins (FT), due to their metal-binding activity play an important role in metal metabolism and detoxification (Briat *et al.*, 1999).

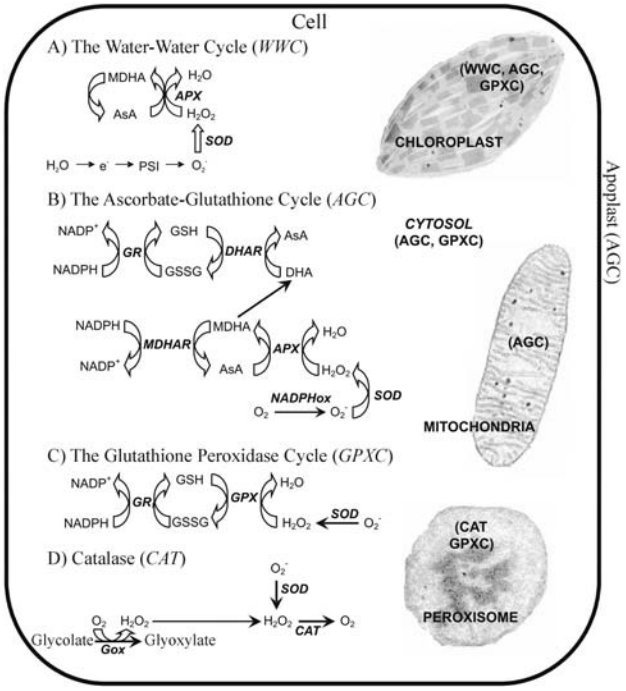

Figure 1 - Localization of reactive oxygen species (ROS) scavenging pathways in plant cells. (A) The water-water cycle (WWC); (B) The ascorbate-glutathione cycle (AGC); (C) The glutathione peroxidase cycle (GPXC); (D) Catalase (CAT). APX, ascorbate peroxidase; AsA, ascorbate; CAT, catalase; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; Gox, glycolate oxidase; GPX, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; GSSG, oxidized glutathione; MDHA, monodehydroascorbate; MDHAR, monodehydroascorbate reductase; NADPHox, NADPH oxidase; PSI, photosystem I and SOD, superoxide dismutase.

In spite of the presence of an efficient antioxidant system, oxidative damage still occur in plant cells either due to uncontrolled production or inefficient scavenging of ROS. Plant tissue senescence and fruit ripening is generally accompanied by higher production of ROS and gradual loss in the ability of scavenging enzymes to neutralize the free radicals (Palma *et al.*, 2006).

In this work, the expression of oxidative and antioxidative stress enzymes in *Citrus sinensis* variety 'Pera IAC' was analyzed. For this purpose, a battery of ROS scavenging enzymes and non-enzymatic antioxidants were searched in young and adult leaves, bark and flower, fruit in different maturation stages, and leaves infected with *Xylella fastidiosa* and *Leprosis citri* using the EST sequencing project.

Material and Methods

cDNA libraries from different citrus tissues such as leaf, bark, fruit and flower were constructed by reverse transcription using mRNA as a model. Traditionally, one problem of Expressed Sequence Tags (EST) library construction is the overabundance of short and truncated EST

fragments due to incomplete reverse-transcription reaction and the ligation bias toward small inserts. This can result in a disproportionately high number of false undiscovered 'novel' sequences due to insufficient coding sequence to establish identity accurately by homology to existing coding sequences. Then, to improve the proportion of full-length and large EST fragments and minimize the overlap of very short inserts in the libraries, the amplified cDNA were size-selected and those with less than approximately 650 bp were discarded.

Considering the entire CitEST database, in this work only *Citrus sinensis* 'Pera IAC' ESTs were considered in the analysis of reads involved in the oxidative stress response, and these ESTs were divided into different groups according to standard CitEST reads nomenclature.

So, each read code refers to leaf (C1), bark (C2), fruit (C3) and flower (C5), as well as, different conditions, such as non-infected tissue (100), tissue infected by *X. fastidiosa*, stage 1 (101), 30 days after infection by *X. fastidiosa* (102), infected by Citrus leprosis virus (401), young tissue (650), healthy plants growing in green house (003), development stage 1 (700), development stage 2 (701), development stage 3 (702), development stage 4 (703), development stage 5 (704), and development stage 6 (705) (Table 1).

Citrus EST database was mined by use of program GeneProject v.1. Sequences related to plant oxidative stress enzymatic and non-enzymatic responses were found by keyword search, and reads presenting a BLASTX match for annotation with an E-value lower than 10^{-15} were selected (Altschul *et al.*, 1990). This E-value was chosen to ensure that the annotation was based only on genes with a high degree of similarity to citrus cDNA clones.

ESTs with identical or extremely similar putative annotations were counted and their frequency was normalized per thousand in relation to the total number of ESTs in each corresponding library. Normalized frequencies were used for expression pattern hierarchical clustering using Hierarchical Clustering Server in GEPAS online tools (Herrero *et al.*, 2003, 2004), separating high and low expressed sequences in each analysis.

Table 1 - EST libraries in *Citrus sinensis* variety 'Pera IAC' showing specific tissues, several treatments and number of reads.

Library	Type	Tissue	Treatment	Number of reads
CS 00 C1 100	cDNA	Leaf	Non-infected	7185
CS 00 C1 650	cDNA	Leaf	Young tissue	2865
CS 00 C2 003	cDNA	Bark	Plants growing in green house	5451
CS 00 C5 003	cDNA	Flower	Plants growing in green house	4330
CS 00 C3 700	cDNA	Fruit	Stage 1 (1.0 cm)	8454
CS 00 C3 701	cDNA	Fruit	Stage 2 (2.5 cm)	7052
CS 00 C3 702	cDNA	Fruit	Stage 3 (5.0 cm)	7909
CS 00 C3 703	cDNA	Fruit	Stage 4 (7.0 cm)	6387
CS 00 C3 704	cDNA	Fruit	Stage 5 (8.0 cm)	6242
CS 00 C3 705	cDNA	Fruit	Stage 6 (9.0 cm)	6712
CS 00 C1 101	cDNA	Leaf	Infected by *Xylella fastidiosa* stage 1	5899
CS 00 C1 102	cDNA	Leaf	Infected by *X. fastidiosa* after 30 days	7231
CS 00 C1 401	cDNA	Leaf	Infected by *Citrus leprosis* stage 1.	945

Results and Discussion

Antioxidant system in *Citrus* EST database

A complex enzymatic system is responsible for the control of the ROS, which are produced by many different processes in plants, and these enzymes can be divided into two groups. The first group is involved in the oxidative stress which produces ROS and comprises the enzymes such as glycolate oxidase (GOX), NADPH oxidase (NADPHox) and oxalate oxidase (OXO); whereas, the second one, the antioxidative stress system, is responsible for ROS scavenging which comprises the enzymes, such as SOD, APX, CAT, glutathione peroxidase (GPX), and α-tochopherol.

The ESTs from *Citrus Sinensis* variety 'Pera IAC' related to the oxidative and antioxidative stress systems comprise 38 isoforms, which 4 were related to ROS production, while 14 isoforms were first described as direct ROS scavenging enzymes and 20 isoforms were related to indirect ROS scavenging system due to their participation in production of antioxidant products such as glutathione and ascorbate (Table 2).

Then, for each EST isoform, the total relative abundance was calculated considering the 13 different libraries (Table 1), the E-value from BLASTX server (Altschul *et al.*, 1990), as well as the amino acid identity according to GenBank non-redundant database from National Center for Biotechnological Information (NCBI).

All EST clones were putatively annotated by similarity to coding sequences in the GenBank non-redundant database with approximately 42% of all reads sharing 80% or higher similarity with existing sequences. The same percentage was observed to reads that had similarity of between 60%-80% and, more than 15% shared less than 60% similarity to known coding sequences (Table 2).

The OXO from *A. thaliana* (E-value = $7e^{-78}$) had the highest relative abundance index (83.5×10^{-4}), followed by Cu/Zn SOD isoform 1 from *M. crystallinum* (12.0×10^{-4}). The other three isoforms (catalase 1 - CAT1; Glycolate

Table 2 - Overview of the oxidative and antioxidative stress systems in *Citrus sinensis* variety 'Pera IAC' showing their similarities with other organisms, relative abundance ($x10^{-4}$) and amino acid identities.

Enzyme	Organism	Total number of ESTs	Relative abundance ($x10^{-4}$)	E-value	Amino acid identity
Production					
GOX	*S. oleracea*	4	1.85	e^{-179}	318 / 368 (86%)
GOX2	*A. thaliana*	52	9.31	0.0	333 / 368 (90%)
NADPH Oxidase	*N. tabacum*	16	5.20	0.0	576 / 720 (80%)
OXO	*A. thaliana*	305	83.5	$7e^{-78}$	145 / 222 (65%)
Direct scavenging					
APX1	*A. thaliana*	15	4.25	e^{-122}	205 / 249 (82%)
APX2	*G. max*	8	2.87	e^{-124}	212 / 248 (85%)
APX3	*A. thaliana*	19	3.85	e^{-165}	293 / 369 (79%)
APX4	*L. esculentum*	3	1.92	e^{-137}	251 / 347 (72%)
CAT1	*G. hirsutum*	75	9.9	0	453 / 492 (92%)
CAT2	*A. thaliana*	37	6.27	0	406 / 493 (82%)
GPX	*P. fluorescens*	5	8.0	$2e^{-77}$	140 / 157 (89%)
GPX1	*P. sativum*	20	4.0	$2e^{-90}$	174 / 246 (70%)
GPX2	*A. thaliana*	9	2.71	$8e^{-72}$	126 / 162 (77%)
GPX3	*A. thaliana*	5	1.75	$2e^{-67}$	120 / 169 (71%)
GPX4	*C. sinensis*	23	4.24	$3e^{-92}$	166 / 167 (99%)
SOD Cu/Zn 1	*M. crystallinum*	40;	12.0	$2e^{-75}$	127 / 152 (83%)
SOD Fe 2	*G. max*	21	4.90	e^{-102}	175 / 231 (75%)
SOD Mn	*H. brasiliensis*	16	3.77	e^{-106}	186 / 231 (80%)
Indirect scavenging					
FT	*P. aeruginosa*	8	3.13	$2e^{-96}$	173 / 213 (81%)
FT1	*S. tuberosum*	8	4.76	e^{-91}	170 / 256 (66%)
FT3	*G. max*	15	3.30	e^{-110}	205 / 262 (78%)
GGCS	*L. esculentum*	8	2.39	0	436 / 526 (82%)
GR1	*P. Sativum*	8	2.26	0	405 / 480 (84%)
GR2	*N. tabacum*	3	3.55	$4e^{-70}$	69 / 82 (84%)
GS	*B. juncea*	7	2.64	e^{-106}	183 / 237 (77%)
GSTF	*H. muticus*	6	2.70	$2e^{-50}$	94 / 212 (44%)
GSTF3	*A. thaliana*	28	5.25	$3e^{-78}$	133 / 206 (64%)
GSTU6	*O. sativa*	24	3.56	$1e^{-62}$	118 / 228 (51%)
GSTX2	*N. tabacum*	25	5.9	$1e^{-61}$	117 / 222 (52%)
GSTX4	*N. tabacum*	7	1.89	$7e^{-98}$	166 / 221 (75%)
GSTX6	*G. Max*	20	4.13	$1e^{-51}$	99 / 215 (46%)
GSTXA	*A. thaliana*	3	3.79	$9e^{-60}$	111 / 211 (53%)
HGGT	*V. vinifera*	9	2.46	e^{-134}	245 / 364 (67%)
MDHAR1	*A. thaliana*	4	2.56	$3e^{-98}$	163 / 216 (74%)
MDHAR4	*C. sativus*	33	5.91	0.0	358 / 434 (82%)
MT2	*A. chinensis*	60	7.83	$4e^{-28}$	54 / 79 (68%)
MT3	*C. papaya*	49	9.23	e^{-23}	46 / 66 (69%)
TPT1	*A. thaliana*	15	3.54	$8e^{-43}$	64 / 136 (47%)

oxidase 2 - GOX2 and Metallothionein 3 - MT3) had approximately 9.48 x 10^{-4} to their relative abundance index. The remaining enzymes represent a mean value approximately of 3.6 x 10^{-4}.

Expression profile of oxidative and antioxidative enzymes in leaf, bark and flower

The production of ROS in plant cells is a normal and continuously occurring phenomenon and in leaf cells, there is an intricate balance between H_2O_2 and O_2^- production and the activities of the ROS-scavenging enzymes. In *Arabidopsis thaliana*, a network of at least 152 genes controls ROS metabolism (Mittler *et al.*, 2004). The network is thought to regulate the rates of ROS production and ROS scavenging in the different cellular compartments and to modulate the steady-state level of ROS.

The ESTs from citrus leaves (Figure 2 Row 1) showed that all major enzymes involved in ROS-scavenging mechanisms were expressed, including SOD, CAT and the majority of AGC enzymes, like APX and GR. In healthy leaves, the chloroplastic antioxidative enzymes FeSOD and Cu/ZnSOD were found and the main peroxisomal enzyme catalase isoform 2 (CAT2) was overexpressed.

In addition to enzymatic detoxification of ROS, the control of the concentration of free metals is an important complementary way to prevent oxidative damage in plant cells. Healthy leaves, also overexpressed metallothionein isoform 2 (MT2), which are low-molecular weight and cysteine-rich proteins are thought to be involved in heavy metal storage, detoxification and homeostasis, due to extensively metal release inherent to their development process in plants (Cobbett and Goldsbrough, 2002). However, MTs are not only involved in maintaining homeostasis of essential metals and metal detoxification, but are also implicated in a range of physiological processes, including scavenging ROS, regulating cell growth, and proliferation.

In young leaves, bark and flower tissues (Figure 2 Row 2, 3 and 4, respectively) several of the well-documented oxidative and antioxidative stress enzymes were not identified in the present study. This is probably because ESTs are only a snapshot of gene expression in a particular

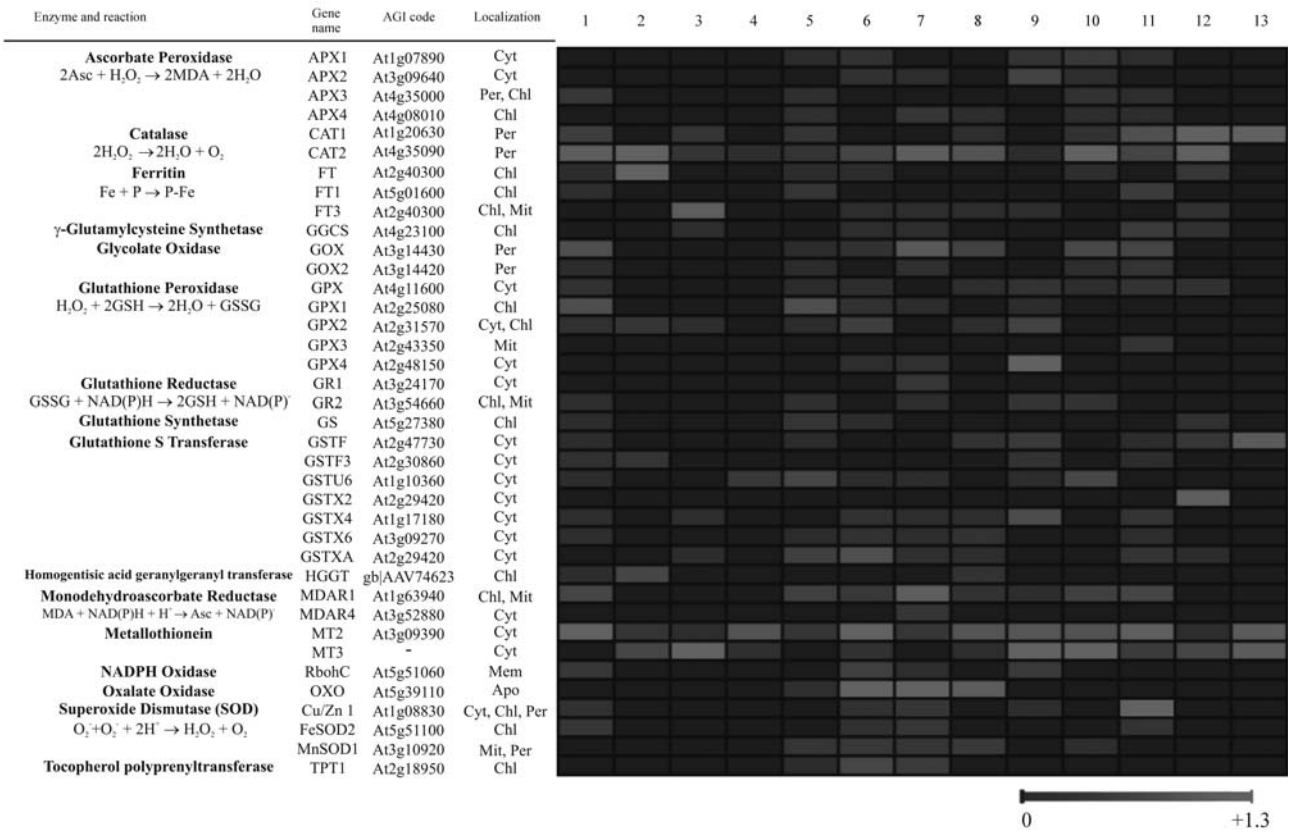

Figure 2 - Expression profile of enzymes and polypeptides of antioxidant system of *Citrus sinensis* variety 'Pera IAC' submitted to several treatments (1) Leaf cDNA from non-infected tissue; (2) Leaf cDNA from young tissue; (3) Bark cDNA of healthy plants growing in green house; (4) Flower cDNA of healthy plants growing in green house; (5) Fruit cDNA of development stage 1; (6) Fruit cDNA of development stage 2; (7) Fruit cDNA of development stage 3; (8) Fruit cDNA of development stage 4; (9) Fruit cDNA of development stage 5; (10) Fruit cDNA of development stage 6; (11) Leaf cDNA from tissue infected by *Xylella fastidiosa* stage 1; (12) Leaf cDNA with 30 days after infection by *X. fastidiosa*; and (13) Leaf cDNA from tissue infected by Citrus leprosis stage 1. All data were multiplied by 10^3 to GEPAS online tool analysis.

tissue and stage of development, and these tissues may have minor photosynthetic activity when compared with adult leaf tissue.

Young leaves overexpressed CAT2 and the enzymes involved in cellular iron homeostasis like FT, MT2 and MT3 (Becana *et al.*, 1998). However, all tissues expressed MT2 and MT3, which prevent the formation of the highly toxic hydroxyl radical via the metal-dependent Haber-Weiss reaction (Fenton reaction) (Van Breusegem *et al.*, 2001).

Expression pattern of oxidative and antioxidative stress enzymes within growing citrus fruits

Fruit development and ripening are complex processes involving major changes in fruit metabolism. Fruit development is a genetically regulated process, and it takes 6-8 months to reach maturity in citrus fruit. Orange fruit needs to be matured on the plant. If harvested prematurely, the fruit does not continue to ripen or sweeten, as there are no starch reserves in the fruit to be converted to sugar.

Fruit ripening has been described as an oxidative phenomenon, characterized by oxidative stress with chlorophyll and protein breakdown. Plant cells produce ROS, particularly superoxide and hydrogen peroxide, which have been implicated as a second messenger in many processes associated with plant growth and development. In general, fruits are divided into two large groups, climacteric and non-climacteric, based upon the presence or absence of an autocatalytic ethylene burst during ripening. In climacteric fruits, such as tomato, apple and banana (Clendennen and May, 1997; Jimenez *et al.*, 2002; Mondal *et al.*, 2004), the burst of ethylene biosynthesis plays a crucial role in the control of the ripening process by regulating the transcriptions of a large number of genes. However, in non-climacteric fruits, such as strawberry and citrus, oxidative stress-related genes were upregulated in ripening (Aharoni and O'Connell, 2002).

The gene expression pattern of oxidative and antioxidative stress enzymes from CitEST libraries using fruits of different size are shown in Figure 2, Rows 5 to 10. All oxidative and antioxidative stress enzymes were present throughout the different fruit size stages. By comparing different fruit stages, it was possible to observe changes in the intensity profile of the expression pattern of enzymes isoforms during fruit development. The largest number of enzymes and their isoforms were found within orange fruits in stage 1, with 1.0 cm of diameter, and the smallest number in fruits stage 6.

CAT represents one of the primary enzymatic defenses against oxidative stress induced by senescence (Zimmermann *et al.* 2006). Although this enzyme was expressed in all fruit stages, it was overexpressed in fruits stages 3 and 4. Furthermore, increases in catalase activity during ripening also have been reported in many fruits (Sala and Lafuente, 1999).

MT genes were the most highly expressed in citrus fruits and were overexpressed in fruits stages 5 and 6. MTs are thought to be involved in both heavy metal detoxification and cellular homeostasis of essential trace metals, such as zinc and cooper. Because of the nature of its metal-binding activity and induction by heavy metal ions, MT genes are strongly believed to play a role in metal ion metabolism and metal tolerance mechanisms.

The data available regarding the expression of MT genes from a variety of plant species indicate that each MT gene type exhibits characteristic temporal and tissue specific expression pattern (Chen *et al.*, 2003). During fruit development, it has been reported that MT has an upregulated gene expression in climacteric as well as in non-climacteric fruits. Citrus and pineapple, non-climacteric fruits, express two MT genes that exhibit similar expression patterns across ripening (Moriguchi *et al.*, 1998; Moyle *et al.*, 2005). Both MT2 and MT3 were detected in developing fruit, but were differentially expressed. MT2 was expressed during the entire ripening process of citrus fruit, whereas MT3 had an expression peak in the later stages of fruit development. The expression of MT is confined to specific stages of fruit development. This differential expression of MT genes strongly suggests that each MT isoform may have specialized functions in different tissues. Some of the functions proposed for plant MTs include a role during development, in senescence and in protection against oxidative stress.

The analysis of expression pattern from developing citrus fruit showed that OXO or germin-like protein (GLP) is the most abundant gene product. OXO is one of the enzymes that produces H_2O_2 in plants. OXO is a glycosylated protein localized in the apoplast and is a known marker protein in the germination of wheat seeds (Custers *et al.*, 2004). Various studies have revealed that GLP may play an important role in plant development and shows responsiveness to biotic and abiotic stresses. These enzymes are highly expressed during germination of wheat and barley and in response of mature leaves to pathogen (Lane, 2002). However the exact biological significance of the H_2O_2 production by OXO in plants remains unknown.

Expression pattern of oxidative stress enzymes during infection with *Xylella fastidiosa* and *Leprosis citri*

Plants have developed a complex defense system to combat invading pathogens, which includes pre-formed and induced components. Plants respond to pathogens by transient increase in the production of ROS and ion fluxes (Dat *et al.*, 2000). This is the so-called "oxidative burst" a

hallmark of successful recognition of plant pathogens (Lamb and Dixon, 1997; De Gara *et al.*, 2003).

Oxidative burst means a massive, rapid and transient activation of oxidative metabolism with the generation of ROS such as superoxide anions and hydrogen peroxide, after exposure to certain abiotic and biotic stress factors (Torres and Dangl, 2005; Torres *et al.*, 2006). This oxidative burst triggered by an imbalance in the production and metabolism of ROS was described by Doke (1983) in plant cells exposed to pathogens. It has been shown that ROS have a direct antimicrobial effect on the pathogen. They are involved in the cross-linking of cell walls around the site of infection and also, activate both local programmed cell death and systemic increase in stress-induced pathogen resistance (Mahalingam and Fedoroff, 2003; Van Breusegem and Dat, 2006).

In incompatible interactions the oxidative burst is a biphasic response that comprises a primary peak 1-2 h after infection, followed by a secondary peak of greater magnitude after 3-6 h. However in compatible interactions, the peak is monophasic (Melillo *et al.*, 2006).

X. fastidiosa is a gram negative bacterium and lives exclusively within xylem vessels (Bové and Garnier, 2002). The bacterium multiplies and spreads within the xylem developing a systemic disease by plugging the xylem vessels with pectins, tyloses, and gums produced by the plant in response to bacteria, and causing the chlorosis variegated disease in citrus. *Citrus leprosis* (CiLV) is non-systemic virus disease that occurs on sweet orange (Rodrigues *et al.*, 2003). The disease is characterized by localized lesions on leaves, twigs and fruits. The early damage on fruit consists of areas with chlorotic yellow spots, and late damage includes necrotic brown lesions which recall the so-called hypersensitive reaction (HR) (Levine *et al.*, 1994).

The comparison of the gene expression profiles between healthy leaves (Figure 2 Row 1) and *X. fastidiosa* infected leaves stage 1 (Figure 2 Row 11) revealed that these two tissues have very similar expression profiles of oxidative enzymes with only slight differences. Infected leaves overexpressed Cu/Zn SOD isoform, which probably enhances the protection of leaves against specific stresses (Foyer *et al.*, 2001). Infected leaves expressed CAT1 and CAT2 at similar levels and higher levels of APX isoforms, while overexpressing the MT2 gene when compared with healthy leaves.

However, the ESTs profile from leaves with 30 days of infection by *X. fastidiosa* or CiLV showed a massive change in the gene expression of enzymatic and non-enzymatic antioxidative mechanisms (Figure 2 Row 12 and 13). Leaves infected with *X. fastidiosa* or CiLV had no expression of SOD, which suggests that chloroplasts from infected leaves are unable to remove the O_2^- radicals generated in the photosynthetic electron transport chain. The Mehler-peroxidase cycle or WWC in chloroplast performs an essential protective function in preventing the accumulation of superoxide and hydrogen peroxide (Foyer and Noctor, 2000; Noctor, 2006).

Infected leaves overexpressed the H_2O_2 scavenging enzymes such as CAT. *X. fastidiosa* infected leaves overexpressed both isoforms CAT1 and CAT2 whereas CiLV infected leaves overexpressed CAT1 only, which has a major role in H_2O_2 scavenging during photorespiration by preventing hydrogen peroxide accumulation. In plants, CAT represents one of the primary enzymatic defenses against oxidative stress induced by senescence, chilling, dehydration, osmotic stress, wounding, paraquat, ozone and heavy metals which rapidly breaks down hydrogen peroxide (Singh and Tewari, 2003).

Infection with *X. fastidiosa* and CiLV induced overexpression of the glutathione S-transferase (GST) gene. GST has GPX activity, thereby protecting cells from oxidative injury by detoxifying organic peroxides produced in plants during processes such as photosynthesis and pathogen attack. Moreover, GST is upregulated in many plants in response to a range of stress conditions (Dean *et al.*, *2005*). *X. fastidiosa* infected leaves expressed MT1 and MT2, while CiLV infected leaves overexpressed both genes.

Our results from the comparison between infected and healthy leaves clearly demonstrated that cells from infected leaves will accumulate ROS while their scavenging capacities are decreased or even absent. The suppression of ROS detoxifying mechanisms can be involved in the induction of these diseases.

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Internet Resources

CitEST GeneProject v.1.0, http://biotecnologia.centrodecitricultura.br.

GEPAS, Gene Expression Pattern Analysis Suite v. 1.1, http://gepas.bioinfo.cnio.es/cgi-bin/cluster.

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Characterization of monoclonal antibodies for identification of the severe strains of 'Capão Bonito' *Citrus tristeza virus*

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ABSTRACT. The severe 'Capão Bonito' (CB) isolates of *Citrus tristeza virus* (CTV) have limited distribution in Brazil, but affect both sweet orange and rootstocks varieties, especially Pera sweet orange grafted on Rangpur lime. The CB complex has at least two different CTV types expressing different coat proteins, whose genes were cloned and expressed in *Escherichia coli*. These proteins, CB-104 and CB-22, were used as antigens, allowing us to obtain three groups of monoclonal antibodies (MAb). All CTV isolates from CB reacted strongly in DASI-ELISA with MAb 30 which seems to react with a conserved epitope. Whereas MAbs 30 and IC.04 react with conformational epitopes, MAbs 37 and 39 recognize linear epitopes on the viral coat protein. It is possible that MAbs 37, 39 and IC.04 can be used diagnostically, and may discriminate severe CB isolates.

Citrus tristeza virus (CTV) is endemic in Brazil due to its introduction many years ago, the presence of the highly efficient vector, the brown citrus aphid, and the use of CTV-tolerant rootstock species that maintain the virus as permanent reservoirs. For the more susceptible varieties such as Pera sweet orange, Mexican lime, and grapefruit, control has been achieved by cross protection using mild protective isolates of the virus. Amongst all Brazilian CTV isolates, the complex known as 'Capão Bonito' (CB) has been considered the most severe due the economic damage caused especially in sweet orange varieties grafted on Rangpur lime (6). Both scion and rootstock varieties infected with the CB complex develop severe stem pitting, and all other tristeza symptoms, except decline (7).

Biological diagnosis of CB isolates has been conducted using Mexican lime and Pera sweet orange as indicators, but there is no significant difference in response compared with other severe CTV complexes. Polyclonal antisera and monoclonal antibodies such as MCA 13 (11), which react with Florida severe isolates, or 3DF1 and 3CA5 (15) that react to a wide range of CTV isolates with differing symptoms, were unable to discriminate CB isolates (4). The occurrence of mixtures in the CB isolates, with at least two CTV types both with similar CP gene expression rate (16) allowed us to use both recombinant coat proteins, called CB-22 and CB-104, as antigens for producing monoclonal antibodies (MAbs) (13). MAb IC.04 has specificity for the protein CB-22, which has high homology with the isolates that induce weak CTV symptoms in Mexican lime. MAb 39 has specificity to the protein CB-104, which has high homology with severe isolates. The third group of monoclonal antibodies, MAbs 30 and 37, recognize both proteins. In this paper, we report the characterization of these MAbs that can probably be used for the identification and differentiation of severe strains of CTV present in the CB complex.

MATERIALS AND METHODS

Polyclonal antiserum 1006 was prepared in rabbits against whole purified virus particles from Mexican lime (1). Monoclonal antibodies against recombinant coat proteins CB-22 and CB-104 were obtained by Stach-Machado et al. (13). Samples were collected from trees with severe

TABLE 1
ABSORBANCE AT 405 NM (OD_{405}) OBTAINED BY DASI-ELISA USING *CITRUS TRISTEZA VIRUS* POLYCLONAL ANTIBODY 1006 ANTISERUM (2 µG/ML) AS COATING ANTIBODY, AND THE MONOCLONAL ANTIBODIES (2 µG/ML) AS DETECTION ANTIBODIES (30.G. 02, 37.G. 11, 39.08 AND IC04-08).

Sample	30.G.02	37.G.11	39.08	IC04-08
Pera I	++++	+	+	—
Pera II	+++	+	+	—
Valencia I	+++	+	—	—
Valencia II	+++	+	+	—
Calderon I	+++	+	—	—
Calderon II	+++	+	+	—
CB-22	++++	+++	—	++
CB-104	+++	++	++	—
Negative control	—	—	—	—

The reactions were scored as +++++ for measurements greater than 2.0; ++++ for measurements of 1.5 to 2.0; +++ for measurements of 1.0 to 1.5; ++ for measurements of 0.5 to 1.0; + for measurements of 0.5 to 0.1; — for measurements less than 0.1.

symptoms of CB tristeza at the Núcleo de Agronomia do Sudeste, Capão Bonito. Different varieties of sweet orange, including nucellar lines of Pera, were also collected from the mother plant blocks at the Centro APTA Citros Sylvio Moreira, Cordeirópolis. These plants did not show tristeza symptoms, but all of them were infected with the mild and protective isolates in the cross protection program. *In vitro* shoot-tip grafted Pera-IAC sweet orange was used as negative control. Samples of 1 mg of lyophilized bark tissue were powdered in liquid nitrogen, and homogenized in extraction buffer (PBS, 0.05% Tween 20-PBS, 2% PVP) at a 1:10 (w/v) dilution. All ELISA tests were performed using double antibody sandwich indirect (DASI)-ELISA, according Garnsey and Cambra (3). Western blots were carried out under denaturing conditions (SDS-PAGE in 12.5% acrylamide gels). The proteins were electrophoretically transferred onto Hybond-C membrane (Amersham) using semi-dry equipment (Biorad), as described by Towbin et al. (17).

TABLE 2
ABSORBANCE AT 405 NM OBTAINED BY DASI-ELISA USING DIFFERENT *CITRUS TRISTEZA VIRUS* MONOCLONAL ANTIBODIES (2 µG/ML) FOR COATING AND POLYCLONAL ANTIBODIES FROM ANTISERUM 1006 (2 µG/ML) AS INTERMEDIATE ANTIBODIES.

Sample	30.G.02	37.G.11	39.08	IC04-08
Pera I	+++++	++	++	++
Pera II	++++	—	—	—
Valencia I	+++++	++	++	++
Valencia II	+++++	++	++	++
Calderon I	+++++	—	+	+
Calderon II	+++++	—	—	—
CB-22	+++++	+++++	—	+++++
CB-104	+++++	+++++	+++++	—
Negative control	—	—	—	—

The reactions were scored as +++++ for measurements greater than 2.0; ++++ for measurements of 1.5 to 2.0; +++ for measurements of 1.0 to 1.5; ++ for measurements of 0.5 to 1.0; + for measurements of 0.5 to 0.1; — for measurements less than 0.1.

The dot-immunobinding assay (DIBA) was performed according Rocha-Peña et al. (12).

RESULTS AND DISCUSSION

Several monoclonal antibodies have been developed against CTV that display varying degrees of reactivity to various CTV isolates (11, 15), but none of them is able to discriminate the CB strains of CTV. The coat protein gene sequences of several geographically and biologically distinct isolates have been cloned (5, 10, 14) and recombinant proteins expressed in *Escherichia coli* have been used as antigens for production of polyclonal antisera (2, 8, 9).

Recombinant coat proteins, named CB-22 and CB-104, were used as antigens for producing MAbs, and four of them from three different fusions were selected for immunore-

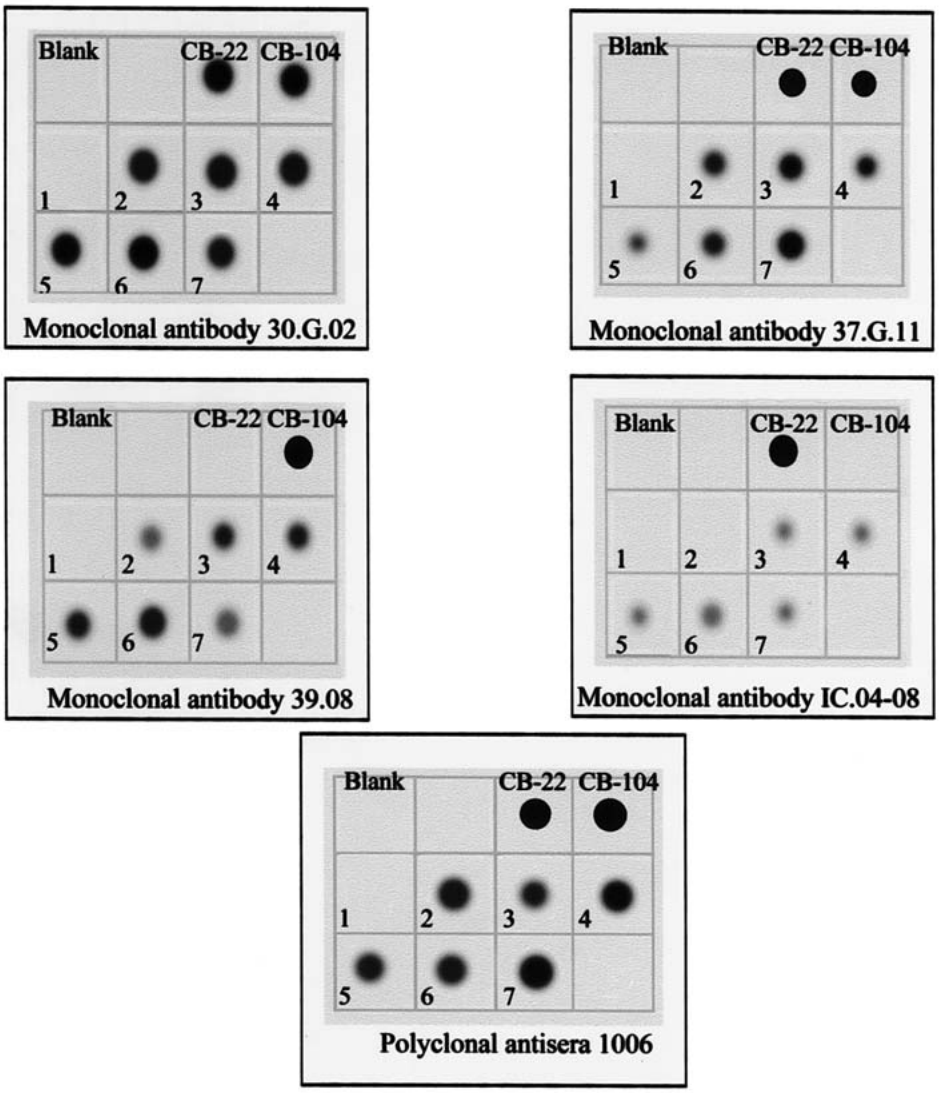

Fig. 1. DIBA carried out with samples of sweet orange varieties infected with *Citrus tristeza virus* complex CB: 1. Negative control; 2. Calderon I; 3. Calderon II; 4. Valencia I; 5. Valencia II; 6. Pera I; 7. Pera II.

activity by DASI-ELISA with recombinant CB-CTV proteins (13). Sweet orange trees with severe symptoms of CB tristeza maintained under field conditions for over 20 yr were used to determine the range of reactivity of the MAbs. The results obtained in DASI-ELISA using polyclonal anti-CTV as coating antibody and the MAbs as detection antibodies are presented in Table 1. All samples of sweet orange showed positive reaction against MAb 30.G.02. Weakly positive reactions were obtained with MAb 37.G.11 and 39.08, whereas IC.04-08 failed to react. When the monoclonal antibodies were used as coating antibodies (Table 2), MAb 30.G.02 still reacted positively with all extracts, but the reaction pattern of the other MAbs changed significantly.

The MAbs were tested using Western blot analysis (Fig. 2). MAb 30.G.02 reacted with *E. coli*-expressed CP protein CB-22 and CB-104, but failed to recognize the protein in the extract of CTV-infected citrus bark tissue. Whereas MAb 37.G.11 which reacted with *E. coli*-expressed CP protein CB-22 and CB-104, was able to detect two bands with molecular weights of approximately 27 and 26 kDa, corresponding to CP1 and CP2, respectively. The explanation for this is probably that the epitope recognized by Mab 30.G.02 is susceptible to denatur-

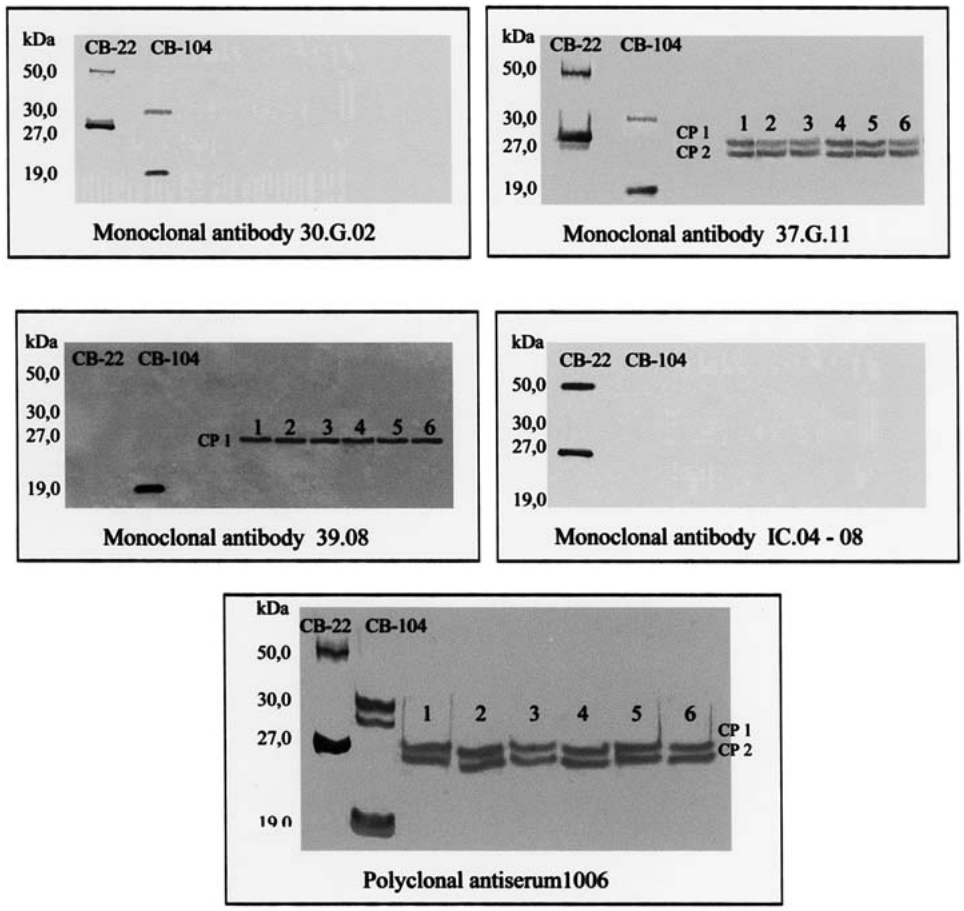

Fig. 2. Immunoblotting of total proteins from the sweet orange bark tissue after electrophoretic analysis in 12% SDS-PAGE. (1) Calderon; (2) Calderon II; (3) Valência I; (4) Valência II; (5) Pêra I e; (6) Pêra II.

ation. MAbs 39.08 and IC.04-08 reacted strongly with the *E. coli*-expressed protein CB-104 or CB-22 respectively, only MAb 39.08 reacted with one protein band of 27 kDa.

The dot-immunobinding assay (DIBA) was used to compare the results obtained by DASI-ELISA and Western blot. The results (Fig. 1) showed that all monoclonal antibodies and polyclonal antiserum react positively with all samples of plants, although the intensity of the reaction varies among the antibodies tested. The intensity of reaction between MAb 30.G.02 and polyclonal antiserum 1006 was similar, and the reactivity of MAbs 39.08 and 37.G.11 were comparable each other. MAb IC.04-08 showed the lowest reaction when compared with the other antibodies.

The pattern of reactivity of monoclonal antibody was compared using samples of Pera sweet orange (old and new clones), infected with CTV, but symptomless. All 16 samples (Table 3) showed strong positive reaction with MAbs 30.G.02, 37.G.11 and 39.08, and only six samples give weak positive reactions with MAb IC.04-08 using polyclonal antiserum 1006 as coated antibody. In contrast, when the monoclonal antibodies were used as coating antibodies (Table 4), only MAb 30.G.02 reacted positively with all plant extracts, and the other monoclonal antibodies were negative.

These results indicate that these monoclonal antibodies obtained against recombinant proteins CB-104 and CB-22 react differentially to the CTV tested. Although direct compe-

TABLE 3
ABSORBANCE AT 405 NM (OD_{405}) OBTAINED BY DASI-ELISA USING THE POLYCLONAL 1006 ANTI-*CITRUS TRISTEZA VIRUS* ANTISERUM (2 MG/ML) AS COATING ANTIBODY, AND THE MONOCLONAL ANTIBODIES (2 MG/ML) AS DETECTION ANTIBODIES.

Sample	30.G.02	37.G.11	39.08	IC .04-08
Sweet oranges				
Pera IAC	+++++	+++	++++	—
Pera Olímpia	+++++	+++	+++	—
Pera EEL	+++++	++	+++	—
Pera Bianchi	+++++	+++	+++	—
Hamlin	+++++	++	++++	++
Rubi	+++++	+++	+++	++
Natal	+++++	++	+++	—
Valencia	++++	++	+++	—
Navel Cabula	++	++	+++	+
Navel Baianinha	++++	+++	+++	+
Mandarins				
do Rio	+++++	+++	++++	—
Ponkan	++++	++	++	—
Cravo	+++++	+++	+++	—
Tangor Murcott	++++	++	++	—
Eureka lemon	+++	++	++	—
Taiti lime	+++++	+++	++++	—
Control				
CB-104	+++++	+++	+++	—
CB-22	+++++	+++	—	+++
Negative	—	—	—	—

The reactions were scored as +++++ for measurements greater than 2.0; ++++ for measurements of 1.5 to 2.0; +++ for measurements of 1.0 to 1.5; ++ for measurements of 0.5 to 1.0; + for measurements of 0.5 to 0.1; — for measurements less than 0.1.

TABLE 4
ABSORBANCE AT 405 NM OBTAINED BY DASI-ELISA USING DIFFERENT *CITRUS TRISTEZA VIRUS* MONOCLONAL ANTIBODIES (2 MG/ML) FOR COATING AND POLYCLONAL ANTIBODIES FROM ANTISERUM 1006 (2 MG/ML) AS INTERMEDIATE ANTIBODIES

Sample	30.G.02	37.G.11	39.08	IC.04-08
Sweet oranges				
Pera IAC	++++	+	—	+
Pera Olímpia	+++	—	—	+
Pera EEL	+++	+	—	+
Pera Bianchi	+++	—	—	—
Hamlin	+++	+	—	—
Rubi	+++	—	—	—
Natal	+++	—	—	—
Valencia	+++	—	—	—
Navel Cabula	++	—	—	—
Navel Baianinha	++	—	—	+
Mandarins				
do Rio	+++	—	—	—
Ponkan	+++	—	—	—
Cravo	+++	—	—	—
Tangor Murcott	+++	—	—	—
Eureka lemon	+++	—	—	—
Taiti lime	+++	—	—	—
Control				
CB-104	+++	++	+++	
CB-22	+++	++	—	+++
Negative	—	—	—	—

The reactions were scored as +++++ for measurements greater than 2.0; ++++ for measurements of 1.5 to 2.0; +++ for measurements of 1.0 to 1.5; ++ for measurements of 0.5 to 1.0; + for measurements of 0.5 to 0.1; — for measurements less than 0.1.

tition assays between these four MAbs were not performed, the differential reactions of each MAbs observed in DASI-ELISA, Western blot and DIBA suggested that they recognize different epitopes. MAb 30.G.02 can be used as universal antibody against the Brazilian isolates in large scale screening like DASI-ELISA.

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Participação e colaboração em projetos:

1. Estudos da bactéria Candidatus liberibacter spp. Agente causal do huanglongbing (ex-greenin) do citros - Diagnóstico, biologia e manejo. (2005-2009). Projeto Temático (FAPESP), Coordenador: Marcos A. Machado

2. Anticorpos poli e monocloanais no diagnóstico de doenças de citros e batata (2006-2008). Financiadora de Estudos e Projetos (FINEP), 2006-2008

3. Identificação dos epítopos de proteínas recombinantes do capsídeo do vírus da tristeza dos citros. (2004). Fundo de Amparo Ao Ensino e a Pesquisa (FAEP), UNICAMP.

4. Imunomarcação com ouro coloidal de X. fastidiosa em diferentes condições de biolfime no projeto "Características biológicas de *Xylella fastidiosa* em biofilme: importância dos genes de adesão e adaptação na patogênese", (2005-2008), FAPESP, Jovem Pesquisador, sob a coordenação da Dra. Alessandra Alves de Souza.

5. Produção de antisoros policlonais para as proteínas de *Leishmania sp.* Em dois projetos da Profa. Dra. Maria Isabel Nogueira Cano (2006)

5.1. Identificação e isolamento de proteínas que se ligam aos telômeros de *Leishmania (L.) amazonensis* utilizando seleção genética em levedura (sistema "one-hybrid"). Projeto de doutorado de Cristina Braga de Brito Lira.

5.2. Estudos funcionais (in vivo) e estruturais das proteínas LaRbp38 e LaRpa-1 que se associa in vitro com a fita telomérica rica em G em *L. amazonensis*. Projeto de doutorado de Jair Lage de Siqueira Neto.

5.3. Caracterização bioquímica e molecular do componente transcriptase reversa da telomerase de *Leishmania spp*. Projeto de doutorado de Miriam Aparecida Giardini.

6. Integração e Melhoramento Genético, Genoma Funcional e Comparativo de Citros (Institutos do Milênio, CNPq) (2004-2006). Coordenador Dr. Marcos Antonio Machado. Participação no processo de anotação e avaliação dos ESTs de citros.

7. Projeto Genoma *Xanthomonas axonopodis pv. citri* na categoria de Anotador das ORFs seqüenciadas (2002).

Outras Atividades

1. Auxiliou na condução de dois projetos de Iniciação Científica:

Fabio Haach Téo (PIBIC/CNPq). *Estudo da interação molecular entre o vírus da tristeza do citros (CTV) e seu principal afídeo-vetor (Toxoptera citricidus)*.

Ângela Luzia Drezza (PIBIC/SAE). *Avaliação de microscopia eletrônica no reconhecimento das proteínas do capsídeo do CTV por anticorpos monoclonais e comparação com outros imunodiagnósticos.* Menção honrosa no XII Congresso Interno de Iniciação Científica da Unicamp, 2004.

2. Programa de Estágio Docente I (PED I): disciplina BS 415, Microbiologia, Imunologia e Parasitologia II, sob a coordenação do Prof. Dr. Fábio T. Costa.

3. Programa de Estágio Docente II (PED II): disciplina BS 410, Módulo Relação Parasita-Hospedeiro, sob a coordenação da Profa. Dra. Dagmar Ruth Stach-Machado.

4. Projeto Social Voluntário "Sonhar Acordado" - Campinas .

5. Projeto Social Voluntário "Cursinho Pré Vestibular Consciência".

8.9. Protocolo de Ética

CEEA-IB-UNICAMP

Comissão de Ética na Experimentação Animal
CEEA-IB-UNICAMP

CERTIFICADO

Certificamos que o Protocolo nº **1212-1**, sobre **"Caracterização dos epitopos e da estrutura secundária do capsídeo do virus da tristeza dos citros e um diagnóstico imunomolecular para Xylella fastidiosa"**, sob a responsabilidade de **Profa. Dra. Dagmar Ruth Stach-Machado / Luís Antonio Peroni**, está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética na Experimentação Animal (CEEA)-IB-UNICAMP em 27 de fevereiro de 2007.

CERTIFICATE

We certify that the protocol nº **1212-1**, entitled **"Characterization of epitopes and secundary structure of citrus tristeza virus coat protein and a immunomolecular diagnostic to Xylella fastidiosa"**, is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). This project was approved by the institutional Committee for Ethics in Animal Research (State University of Campinas - UNICAMP) on February 27, 2007.

Campinas, 27 de fevereiro de 2007.

Profa. Dra. Ana Maria A. Guaraldo
Presidente

Fátima Alonso
Secretária Executiva

CEEA/IB – Unicamp
Caixa Postal 6109
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http://www.ib.unicamp.br/institucional/ceea/index.htm

DECLARAÇÃO

Declaro para os devidos fins que o conteúdo de minha dissertação/ tese de mestrado/doutorado intitulada "*Caracterização dos epítopos e da estrutura secundária da proteína do capsídeo do Citrus tristeza virus e um diagnóstico imunomolecular para Xylella fastidiosa*"

( ) não se enquadra no Artigo 1°, § 3° da Informação CCPG 002/06, referente a bioética e biossegurança.

( ) está inserido no Projeto CIBio (Protocolo n°_____), intitulado _____.

(x) tem autorização da Comissão de Ética em Experimentação Animal (Protocolo n° 1212-1).

( ) tem autorização do Comitê de Ética para Pesquisa com Seres Humanos (Protocolo n°_____).

Aluno Luís Antonio Peroni

Orientadora Dagmar Ruth Stach-Machado

Para uso da Comissão ou Comitê pertinente:

(X) Deferido ( ) Indeferido

Nome:
Função: Profa. Dra. ANA MARIA A. GUARALDO
Presidente
Comissão de Ètica na Experimentação Animal
CEEA/IB - UNICAMP