LUÍS GUSTAVO DE LÁZARO RAMPAZO

AVALIAÇÃO DE AGENTES BIOLÓGICOS E SEUS PRODUTOS NA INCIDÊNCIA DE LESÕES FOLIARES DO CANCRO CÍTRICO

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Dissertação apresentada ao Programa de Pós-Graduação em Microbiologia da Universidade Estadual de Londrina como parte dos requisitos para a obtenção do título de Mestre em Microbiologia.

Orientador: Prof. Dr. Galdino Andrade

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DEDICATÓRIA

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RESUMO

O cancro cítrico, causado por *Xanthomonas axonopodis* pv. *citri* (Xac), é um grave problema para a citricultura, pois o principal método de controle é a erradicação das plantas contaminadas. O controle biológico com microrganismos representa uma alternativa no controle da doença. Foram obtidos quatro isolados bacterianos, de lesões de cancro cítrico em folhas (LV, LN), fruto (FRC) e água residual de leiteria (EC), que demonstraram antagonismo contra Xac. Mudas de laranja (*Citrus sinensis* cv. Valencia) foram pulverizadas com suspensões de bactérias e sobrenadantes de culturas dos isolados antagonistas, associados com pré ou pós-aplicação com suspensão de Xac. Os resultados mostraram redução na média de lesões de cancro cítrico por folha com as suspensões dos quatro isolados. Com os sobrenadantes, EC e FRC reduziram o número total de lesões, a média de lesões por folha e, assim como LV, a porcentagem de folhas lesionadas. Foi observado um aumento do número de lesões de cancro com o sobrenadante de LN e do total de folhas lesionadas com a suspensão de células de LV. Estes resultados indicam que a antibiose é um importante mecanismo de interação dos isolados no filoplano e que o antagonismo entre bactérias pode ser uma excelente ferramenta para o controle do cancro cítrico.

Palavras-chave: Cancro cítrico; Agentes no controle biológico de pragas.

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ABSTRACT

Citrus canker, caused by *Xanthomonas axonopodis* pv. *citri* (Xac), is a serious problem to the citriculture, because the main method of control is the eradication of the contaminated plants. Biological control with microorganisms represents an alternative in the disease control. Four bacterial isolates were obtained from citrus canker lesions in leaves (LV, LN), in a fruit (FRC) and from drainage water from a milk plant (EC), which demonstrate over Xac. Orange (*Citrus sinensis* cv. Valencia) plantlets were sprayed with bacterial suspensions and supernatants of liquid cultures of the antagonist isolates, in association with pre- and post-application with Xac suspension. The results showed reduction in the mean number of citrus canker lesions per leaf with bacterial suspensions of the four isolates. With the supernatants, EC and FRC reduced the total number of lesions, the mean number of lesions per leaf and, like the LV isolate, the percentage of lesioned leaves. An increase in the total number of lesions was observed with LN supernatant and an increase in the total number of lesioned leaves was observed with LV bacterial suspension. These results indicate that antibiosis is an important mechanism of bacterial interaction on the phylloplane and that antagonism among bacteria could be an excellent tool for citrus canker control.

Key-words: Citrus bacterial canker discase; Citrus canker; Biological agents for pest control.

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1 REVISÃO BIBLIOGRÁFICA

O agente causal do cancro cítrico é a bactéria *Xanthomonas axonopodis* pv. *citri* (Xac), que foi primeiramente isolado por Hasse (1915). De acordo com Bebendo (1995) e Brunings e Gabriel (2003), é um bastonete Gram-negativo, estritamente aeróbio, dotado de um único flagelo polar e com colônia de formato côncavo e pigmentação amarela e com aspecto bastante viscoso devido à produção de exopolissacarídeos.

As condições ideais para o seu crescimento são umidade elevada e temperatura entre 20 e 39ºC (Rossetti, 2001), com a capacidade de infecção otimizada entre 20 e 30ºC (Koizumi, 1985). Em relação à sobrevivência no ambiente, Xac pode permanecer viável por até 6 meses em órgãos infectados, mesmo após a queda destes (Rao e Hingorani, 1963a; Rao e Hingorani, 1963b), além de se estabelecer como “epífita” no filoplano de plantas não hospedeiras (Goto, 1972; Goto *et al,* 1975a; Goto *et al.*, 1975b; Carvalho *et al.*, 1984). Leite e Mohan (1984) relatam a sobrevivência por volta de 15 dias em ambiente aberto (em solo esterilizado ou não) e por até 62 dias tanto no filoplano quanto no rizoplano de gramíneas como *Paspalum* sp., *Trichachne insularis* e *Panicum maximum*. Na presença de solo argiloso, a sobrevivência pode chegar a 56 dias. Os exopolissacarídeos produzidos por Xac também são importantes na preservação do inóculo em condições ambientais (Koizumi, 1979; Goto e Hyodo, 1985).

A disseminação da bactéria a curtas distâncias se dá principalmente em estações do ano com elevadas temperaturas e chuvas acompanhadas de ventos fortes, como inferem Peltier e Frederich (1926), Stall (1988), Rossetti (2001) e Schubert e Sun (2003). Quando associados a chuvas, ventos com velocidade

superior a 8 m/s (28,8 Km/h) são suficientes para causar um estado de congestão nas folhas (Gottwald *et al.*, 2002b; Schubert *et al.*, 2001; Serizawa *et al.*, 1969; Serizawa e Inoue, 1975). Neste evento, um filme de água é formado desde o filoplano até as células do mesófilo, passando por entre as células estomáticas. Há casos onde apenas uma célula do patógeno é suficiente para causar infecção (Gottwald e Graham, 1992; Graham *et al.*, 1992). Apesar disso, o cancro cítrico caracteriza-se por não ser uma infecção sistêmica (Whiteside, 1988; Schubert *et al*., 2001).

Para longas distâncias, Gottwald *et al.* (1988), Gottwald e Timmer (1995) e Rossetti (2001) afirmam que a disseminação fica a cargo de mudas e frutos infectados, ferramentas, roupas, caixas de colheita, restos de cultura, e veículos provenientes de áreas endêmicas.

Gottwald *et al.* (1997a), Gottwald *et al.* (1997b) e Gottwald *et al.* (2001) relatam que as tempestades tropicais, características da região da Flórida, também colaboram com o avanço do cancro cítrico a longas distâncias. Em 1996, tais tempestades foram responsáveis pela dispersão da bactéria por mais de 11 quilômetros. Além disso, existem relatos que correlacionam alguns sistemas de irrigação com a disseminação da bactéria, principalmente os sistemas baseados na aspersão de água sobre as plantas (Gottwald *et al*., 1989).

A partir do contato de Xac com os tecidos jovens de uma planta sadia, Koizumi e Grierson (1979), Leite (1990), Amorin (1995) e Schubert e Sun (2003), descrevem que a invasão da bactéria se dá pelas aberturas naturais de suas folhas, como estômatos, ou por escoriações e ferimentos provocados por espinhos, insetos ou partículas de solo carregadas pelo vento. De acordo com Gottwald e Graham (1992) e Graham *et al.*, (1992), quase todas as infecções ocorrem em folhas

com até 40 dias após o seu surgimento, com 50 a 80% de expansão, e em frutos com até 90 dias contados a partir da queda das pétalas. Koizumi (1976) relata que, ao se pulverizar tecidos jovens de plantas cítricas com uma suspensão com 10^8 UFC/ml de Xac, lesões características de cancro cítrico surgem em um período de 17 a 21 dias. A colonização do tecido é notavelmente rápida. Em uma a duas semanas depois do aparecimento da primeira lesão, geralmente comportando 10^6 bactérias, estarão formadas 10 lesões (com 10^7 bactérias) e, em outras duas semanas, podem existir em torno de 1000 lesões, comportando cerca de 10^9 bactérias no total (Franco, 2001).

Feichtenberger (1998), Gravena (1998) e Rossetti (2001) chamam a atenção para a introdução involuntária, em 1996, do inseto *Phyllocnistis citrella* no Brasil. Aqui, ele recebeu o nome comum de "larva minadora dos citros" por construir, em sua fase larval, galerias sob a cutícula das folhas novas de plantas cítricas. Isto pode ajudar a infecção de duas maneiras: por deixar as células do mesófilo expostas ou pela própria contaminação do corpo da larva com Xac que esteja no ambiente, transportando-a diretamente para estas células (Koizumi, 1985; Gottwald *et al.*, 2002b; Schubert e Sun, 2003). Segundo Gotto (1992), para o estabelecimento da infecção na presença da larva minadora é necessário um inóculo de 10^2 UFC/ml de Xac, enquanto que dependendo apenas da capacidade da bactéria ingressar pelas aberturas estomáticas, a quantidade necessária aumenta para 10^5 UFC/ml. No mesmo ano de sua introdução no Brasil, Feichtenberger (1998) destaca que 55% dos focos da doença nas principais áreas produtoras foram devidos à ação da minadora dos citros. Esta associação chegou a 95% em 1997 e, já no primeiro semestre do ano seguinte, 100% dos 253 focos detectados de cancro cítrico nestas áreas estavam associados à referida praga.

De acordo com Gottwald *et al.* (2002b), todas as plantas cítricas são suscetíveis ao cancro cítrico, pelo menos nos estágios de desenvolvimento em que possuem tecidos jovens. Rosseti (2001), Gottwald *et al.* (2002b) e Schubert e Sun (2003) classificam os cultivares cítricos de acordo com a resistência varietal da seguinte maneira, com alguns exemplos:

a) **Altamente suscetíveis**: limão (*C. limon*) Siciliano, lima ácida Galego, tangerina (*C. reticulata*) Lee e grapefruit (*C. paradisi*);

b) **Suscetíveis**: laranjas doces (*C. sinensis*) Bahia, Ruby, Seleta e Hamlin;

c) **Moderadamente suscetíveis**: laranja doce (*C. sinensis*) Natal e tangerinas (*C. reticulata*) Cravo, King, Ortanique e Romana;

d) **Moderadamente resistentes**: laranjas doces (*C. sinensis*) Pera, Valencia, Lima e Navelina e tangerina (*C. reticulata*) Dancy;

e) **Resistentes**: laranjas doces (*C. sinensis*) Folha Murcha e Moro e tangerinas (*C. reticulata*) Ponkan, Tankan e Satsuma;

f) **Altamente resistentes**: Calamondin (*C. mitus*) e *Fortunella* spp.

Segundo Leite (1990) e Rossetti (2001), são conhecidos cinco tipos de cancro cítrico, os quais podem ser distinguidos de acordo com a diferença em patogenicidade e sintomas em espécies de *Citrus* e outros gêneros afins. São eles:

a) **Cancro cítrico asiático ou "cancrose A"**: causado pela estirpe "A" de Xac na grande maioria dos cultivares cítricos, é a forma mais importante da doença, com grande disseminação na Ásia, África e Américas;

b) **Cancro cítrico B ou "cancrose B"**: atribuído à estirpe "B" de *X. axonopodis* pv. *aurantifolii*, com ocorrência restrita à Argentina, Paraguai e Uruguai, onde afeta limões verdadeiros (*Citrus limon*);

c) **Cancrose do limoeiro Galego ou "cancrose C"**: causada pela estirpe "C" de *X. axonopodis* pv. *aurantifolii* e está restrita ao Estado de São Paulo, onde afeta somente o limão Galego;

d) **Cancrose "D"**: provoca lesões no limão Galego somente no México. No entanto, esta doença ainda não foi muito bem caracterizada;

e) **Mancha bacteriana dos citrus**: causada a *X. axonopodis* pv. *citrumelo*, ataca variedades de citrumelo em viveiros na Flórida;

Schubert *et al.* (2001), Gottwald *et al.* (2002b) e Schubert & Sun (2003) afirmam que uma planta pode apresentar lesões em todos os órgãos da parte aérea e que geralmente são acompanhadas de um halo clorótico nas folhas e frutos. Tais lesões reduzem drasticamente a produção devido à queda prematura de frutos e à severa desfolhação. Com isso, além da produção de frutos menores, os poucos que são produzidos acabam com um valor comercial bastante prejudicado, quase sempre economicamente inviável (Civerolo, 1984; Leite , 1990; Romeiro, 1995).

Devido a estes prejuízos, o cancro cítrico sempre constituiu uma séria e constante ameaça à citricultura mundial (Civerolo, 1984; Whiteside, 1988; Vernière *et al*., 1998; Mohammadi *et al*., 2001; Rossetti, 2001; Gottwald *et al.*, 2002b). As primeiras indicações de sua existência datam dos anos entre 1827 e 1831, na Índia, em folhas de *Citrus medica* (Romeiro, 1995; Schubert *et al.*, 2001). A partir daí, esta doença tem se disseminado pelo mundo inteiro, principalmente via órgãos vegetais contaminados, estabelecendo-se endemicamente ao longo dos

países e ilhas banhados pelo Oceano Índico, em países da Ásia Oriental, Oriente Médio, África, América do Sul e do Norte (Koizumi, 1985; Leite , 1990; Romeiro, 1995; Bergamin Filho e Kimati, 1995; Feichtenberger, 1998). Em países como Austrália e Nova Zelândia, depois de estabelecido, o cancro cítrico pôde ser erradicado (Koizumi, 1985). No Brasil, sua primeira detecção ocorreu na região de Presidente Prudente, Estado de São Paulo (Bitancourt, 1957). Provavelmente, a introdução deste patógeno ocorreu com a utilização de material propagativo contaminado.

A maneira mais eficaz de se prevenir a contaminação com o cancro cítrico sempre foi evitar que o patógeno entre em contato com a planta cítrica. Dentre as principais medidas de prevenção, Koller (1994), Rossetti (2001) e Gottwald *et al.* (2002b) indicam:

a) instalação de viveiros somente em áreas comprovadamente livres de cancro cítrico por, pelo menos, 1 ano;

b) utilização de mudas provenientes de viveiros rigorosamente inspecionados;

c) descontaminação de todo tipo de equipamento de colheita proveniente de áreas suspeitas com o uso de produtos como a amônia quaternária;

d) uso de quebra-ventos arbóreos protetores, construídos com sansão-do-campo, grevilha, jambolão ou pinus;

e) controle (biológico ou químico) da larva minadora dos citros;

f) inspeção constante dos pomares;

g) e, principalmente, o uso de variedades resistentes à doença.

No caso de plantas já infectadas, a medida mais aceita e eficiente de controle da doença é a redução do inóculo pela remoção e destruição das plantas infectadas e expostas ao patógeno (Stall *et al.*, 1987; Leite e Mohan, 1990; Romeiro, 1995; Rossetti, 2001). Em certos casos, esta prática pode até eliminar totalmente a doença, porém não há garantia de que voltarão cancro cítrico não ocorra novamente, caso as medidas de prevenção sejam "amenizadas". No exemplo da Flórida, após sua introdução por material propagativo contaminado por volta de 1910, o cancro cítrico foi erradicado após mais de 30 anos de combate, o que incluiu a eliminação de 250 mil árvores e a destruição de 3 milhões de mudas, em um gasto acumulado de dezenas de milhões de dólares (Schubert *et al*., 2001). Em 1984, uma nova onda de detecção da doença levou à eliminação de 20 milhões de plantas cítricas e a um prejuízo acumulado de US$ 94 milhões. Hoje, a Flórida possui novos focos da doença, principalmente devido à disseminação da bactéria em pomares residenciais (Gottwald *et al.*, 1997b; Gottwald *et al.*, 2001; Gottwald *et al.*, 2002a).

No Brasil, vários fatores devem ser levados em consideração quando se trata da política de erradicação do cancro cítrico. Tenta-se erradicar esta doença desde sua primeira constatação, na década de 50. Conforme cita Romeiro (1995), a principal iniciativa governamental para este fim foi a criação da Campanha Nacional de Erradicação do Cancro Cítrico (CANECC), conforme Decreto nº 70.601 de 09/12/1974. Inicialmente, esse autor descreve que o processo de erradicação envolvia o mapeamento e avaliação de todas as plantas cítricas das zonas urbana e rural. Havendo a constatação da doença, a propriedade podia sofrer interdição total ou parcial, de acordo com a análise dos engenheiros agrônomos da CANECC. A erradicação em si se dava pela eliminação das plantas infectadas e suspeitas em um raio de 50 metros a partir do foco inicial da doença. Para viveiros de mudas, todas

deveriam ser eliminadas, fossem doentes ou sadias, além das mudas de outros viveiros em um raio de até 200 metros. Há casos onde quaisquer plantas cítricas foram eliminadas em um raio de até 1000 metros de uma planta infectada (Rossetti, 1977; Leite e Mohan, 1990) ou até mesmo em um município inteiro. Para a eliminação das plantas, podia se usar herbicidas, porém a melhor alternativa indicada sempre foi a queima do material *in loco*, depois de terem sido arrancadas inclusive as raízes, impedindo que o patógeno se albergue em qualquer resto de tecido do hospedeiro e tenha chance de infectar brotações novas que possam surgir (Carvalho, 1984; Leite e Mohan, 1984).

A severidade da CANECC revestiu-a de polêmica, notadamente nos seus primeiros anos de implantação, pois tais medidas prejudicaram principalmente os pequenos produtores. Não era difícil encontrar produtores que perderam todo pomar por causa de uma única planta infectada, muitas vezes tendo poucas folhas afetadas. Polêmica semelhante acontece nos Estados Unidos, pois o raio de erradicação de 125 pés (~38 metros), estabelecido com base em estudos de dispersão de inóculo realizados na Argentina (Stall *et al.*, 1980), passou a ser insuficiente na tentativa de se conter o avanço da doença no final da década de 80. Assim, ficou estabelecido um novo raio de erradicação de 1900 pés (~579 metros) a partir de qualquer planta cítrica contaminada (Gottwald *et al.*, 1997a; Gottwald *et al.*, 2001; Gottwald *et al.*, 2002a). Esta medida tem por finalidade eliminar até 95% dos possíveis novos focos de infecção dentro de um período de 30 dias a partir da descoberta de uma nova ocorrência.

No Brasil, a discussão entre produtores, cientistas e entidades governamentais fez com que os limites impostos pela CANECC fossem reduzindo com o passar dos anos. Hoje, principalmente no Estado de São Paulo, as inspeções

são feitas, ao longo de um talhão, no esquema 3x1: vistoria-se uma planta, pulam-se 3 e volta-se a analisar a quarta. Caso o talhão tenha menos de 0,5% de suas plantas infectadas, cada uma delas deve ser eliminada, junto com quaisquer outras em um raio de 30 metros ao seu redor. Caso o talhão tenha mais de 0,5% de plantas afetadas, todo ele deve ser eliminado. Além disso, um período de quarentena de no mínimo um ano deve ser respeitado, no qual nenhuma planta cítrica deve ser plantada nos locais onde ocorreram as erradicações (Leite e Mohan, 1990).

Ocontrole biológico de fitopatógenos com o uso de microrganismos antagonistas apresenta-se como uma estratégia promissora para a proteção de plantas em geral (Kloepper *et al.*, 1999). Cook e Baker (1983) definem o controle biológico como sendo a redução da soma de inóculo ou das atividades determinantes da doença provocada por um patógeno, realizada por um ou mais organismos que não o homem. Ainda segundo estes autores, toda doença depende da interação entre o patógeno e o hospedeiro, além de outros não-patógenos que habitam o local de infecção e que podem limitar a atividade do patógeno ou aumentar a resistência do hospedeiro, sempre sob a influência do ambiente.

No contexto do controle biológico, Baker (1985), Bettiol e Ghini (1995) definem que os mecanismos das interações entre os microrganismos patogênicos e antagônicos podem ser divididos em:

a) **antibiose**: interação onde um ou mais metabólitos produzidos por um organismo têm ação danosa sobre outro;

b) **competição**: ocorre quando dois ou mais organismos concorrem entre si, principalmente por nutrientes, oxigênio e espaço;

c) **parasitismo**: quando um microrganismo vive sobre e/ou alimenta-se de outro (ex.: parasitas que atacam hifas e estruturas de

reprodução e sobrevivência de patógenos, reduzindo seu inóculo);

d) **predação**: situação onde um microrganismo obtém seus nutrientes a partir do patógeno (ex.: predação por amebas e *Bdellovibrio bacteriovorum*);

e) **hipovirulência**: diz respeito à introdução de uma linhagem menos agressiva ou não patogênica do patógeno, a qual pode, ainda que raramente, transmitir esta característica às linhagens patogênicas;

f) **indução de defesa do hospedeiro**: ação direcionada ao hospedeiro por meio dos próprios organismos ou seus metabólitos.

Para o presente trabalho, destacamos a antibiose como um dos mecanismos de interação entre o patógeno e os antagonistas a serem estudados. Como exemplos de ocorrência de antibiose em interações bactéria-bactéria, podemos citar os trabalhos de Aldwinckle e Beer (1979), Paulin e Lachaud (1978), Völksch *et al*. (1996), May *et al*. (1997), Kearns e Mahanty (1998) e Völksch e May (2001), que demonstram o uso de *Pantoea agglomerans* e isolados avirulentos de *Pseudomonas savastanoi* e *E. amylovora* no controle da queima tanto da soja, causada por *P. savastanoi* pv. *glycinea*, quanto da pêra, causada por *E. amylovora*. Kerr (1980) descreve o uso de *Agrobacterium radiobacter* K-84 no controle da galha da coroa causada por *A. tumefasciens*. Gremy *et al.* (1992) utilizaram tanto de cultivos líquidos de *P. fluorescens* quanto de substâncias extraídas deles contra patógenos do algodão. Apesar de existirem trabalhos sobre o uso de microrganismos antagonistas no controle biológico de bactérias do gênero *Xanthomonas* (Fukui *et al.*, 1999; Wulff *et al.*, 2002; Stromberg *et al.*, 2004), ainda não se tem informação desta aplicação especificamente para Xac no caso do cancro cítrico.

De acordo com Cook (1993), são duas as principais razões para o uso pouco difundido de agentes biológicos no controle de doenças em plantas: as dificuldades técnicas de utilização, principalmente devido à falta de conhecimento de sua biologia e ecologia, e os custos de desenvolvimento e obtenção de aprovação regulatória, o que não é facilmente justificável por causa de suas condições restritas de aplicação.

Somadas a estas dificuldades, o ambiente específico do filoplano sempre representou um grande obstáculo para uso de microrganismos no controle biológico. Segundo Bettiol e Ghini (1995) e Mercier e Lindow (2000), a baixa disponibilidade de nutrientes, como exsudatos foliares e resíduos orgânicos, é um fator bastante limitante para este ambiente. A superfície foliar também apresenta grandes diferenças em comparação ao solo, caracterizando-se pela ocorrência de variações maiores e mais rápidas, principalmente em relação à temperatura e umidade, além da exposição à radiação ultravioleta (Blakeman, 1985; Chen *et al.*, 1995). No caso da laranja, a cera que as folhas possuem na superfície é um fator agravante, pois as torna bastante impermeáveis (Kenerley e Andrews, 1990). Problemas como estes, no caso do cancro cítrico, fazem com que projetos sobre controle biológico não recebam tanta atenção nos esforços conjuntos para o controle desta doença (Dixon *et al.*, 2000).

Ainda assim, a possibilidade do menor uso de pesticidas químicos é um dos aspectos positivos que mais incentiva as tentativas de implementação do controle biológico. Hoje, existe uma preocupação crescente com relação ao uso destas substâncias, principalmente devido aos resíduos que se acumulam tanto nos alimentos quanto no meio ambiente (Baker e Cook, 1974; Cook e Baker, 1983; Baker e Dunn, 1989; Safiyazov *et al.*, 1995; Walsh *et al.*, 2001; Quimby *et al.*, 2002;

Gan-Mor e Matthews, 2003). Por causa disso, as indústrias se vêem forçadas a desenvolver novas e efetivas estratégias para o controle de doenças e o controle biológico de fitopatógenos, utilizando bactérias antagonistas ou seus metabólitos, mostra-se como uma estratégia promissora para a proteção de plantas (Baker e Dunn, 1989; Kloepper *et al.*, 1999; Földes *et al.*, 2000). De fato, alguns países possuem metas de substituição gradual dos pesticidas químicos por práticas de manejo integrado de pragas, o que inclui o controle biológico (Hall e Barry, 1995).

Uma vantagem dos agentes biológicos é a possibilidade de adaptação às pressões ambientais e às alterações na população do patógeno, o que não acontece com os agentes químicos (Holt e Hochberg, 1997). Outro ponto favorável aos agentes biológicos é que eles são mais seletivos que os químicos e não são tóxicos contra organismos não-alvo (Mendelsohn *et al.*, 1995; Capalbo e Nardo, 2000). No entanto, os agentes biológicos podem não persistir no ambiente por um tempo suficiente, como é o caso dos agentes químicos (Cornish *et al.*, 1993; Smits, 1996).

Um aspecto bastante negativo dos agentes químicos é o impacto que eles podem ter sobre o chamado “controle biológico natural”, situação em que um patógeno não consegue se estabelecer ou causar doença em um ambiente favorável a ele devido à existência de antagonistas naturais (Baker e Cook, 1974). Este equilíbrio pode ser alterado pela ação antrópica (Wilson, 1998), com a aplicação de pesticidas químicos, o que pode levar à instalação de patógenos oportunistas que inicialmente possuíam importância secundária, causando as chamadas “doenças iatrogênicas” (Griffiths, 1981). De fato, é defendido que o controle biológico pode ser utilizado pelo aprimoramento seletivo do crescimento e

da atividade dos componentes antagonistas da microbiota natural das plantas (Garret, 1982).

Muitas doenças têm sido controladas com aplicações de compostos cúpricos, inclusive o cancro cítrico (Koizumi e Grierson, 1979; Stall e Seymour, 1983; Leite *et al.*, 1987; Leite, 1990), embora a um custo que chega a US$ 56 por acre (Schubert *et al*., 2001) em épocas de surgimento de novas brotações. Porém, muitos casos de resistência a este e outros compostos têm sido relatados (Cooksey, 1990). Em isolados de *X. axonopodis* pv. *vesicatoria*, a resistência ao cobre está ligada a genes plasmidiais (Stall *et al.*, 1986; Bender *et al.*, 1990; Cooksey *et al.*, 1990). Rinaldi (1998) relata a resistência a sulfato de cobre (50 ppm) em 45,5% dos isolados de Xac provenientes de pomares comerciais que recebem pulverizações com compostos cúpricos, comparando-se com 13,4% de isolados resistentes provenientes de pomares isentos de pulverizações. Interessantemente, bactérias resistentes ao cobre e que apresentam atividade antimicrobiana têm sido isoladas (Casida, 1992).

Uma característica que pode indicar se um agente antagonista terá sucesso ou não no controle biológico de doenças é a sua capacidade de multiplicar-se e colonizar este ambiente, ou seja, ocupar o mesmo nicho ecológico do patógeno que irá combater (Baker e Cook, 1974; Bettiol e Ghini, 1995; Paulitz e Bélanger, 2001; Völksch e May, 2001; Gau *et al*., 2002). Além disso, Baker e Cook (1974) e Cook (1985) afirmam que é muito importante procurar por tais agentes em locais onde, apesar da presença de hospedeiros suscetíveis, a doença não esteja presente. É o caso, por exemplo, de algumas poucas plantas cítricas sadias no meio de um pomar gravemente afetado pelo cancro cítrico. Este fato é explicado pela ocorrência de controle biológico natural (discutido anteriormente), o que indica a

possível presença de organismos que mantêm a população do patógeno em uma densidade inferior à necessária para a instalação da doença.. Um exemplo ainda mais extremo seria o de Baker *et al*. (1983; 1985) que relatam o uso bem sucedido de *Bacillus subtilis*, isolado do solo, na redução de pústulas de ferrugem causadas por *Uromyces appendiculatus* no feijoeiro.

Entretanto, merece destaque o fato de que a maioria dos agentes selecionados para controle biológico, apesar de apresentarem antagonismo em etapas *in vitro*, não obtêm sucesso em condições *in vivo* ou de campo (Blakeman e Fokkema, 1982; Garret, 1982; Lindow, 1987; Canaday, 1991; May *et al.*, 1997; Aysan *et al.*, 2003). Assim, o antagonismo *in vitro* não deve ser usado como único critério para a seleção de potenciais agentes de controle biológico (Tani *et al.*,1990). Contribui para isso o fato de a maioria dos experimentos em casa de vegetação freqüentemente envolver um único antagonista contra um patógeno de cada vez, o que não permite levar em conta a influência que os isolados em estudo recebem da comunidade microbiana associada ao ambiente natural da doença (Schottel *et al.*, 2001).

Uma justificativa para a diferença *in vitro-in vivo* é que o sucesso de tais agentes depende de condições ambientais controladas, como uma casa de vegetação ou viveiro de mudas, para favorecer tanto sua viabilidade quanto sua eficiência (Paulitz e Bélanger, 2001). Outro fator importante para isso pode ser a pouca eficácia da formulação e/ou a falta de um equipamento adequado para a aplicação do agente (Steinke e Giles, 1995; Gan-Mor *et al*., 1996; Navon, 2000). Para o caso do filoplano, outro detalhe que pode revelar a superestimação de um evento de antibiose é que a superfície de um meio de cultura sólido não reflete as condições químicas e físicas reais deste ambiente, de modo a não reproduzir

fielmente as condições que influenciam as atividades microbianas. De acordo com McCormack *et al.* (1994) uma diferença fundamental entre um meio artificial e o ambiente natural do filoplano é que a folha possui duas dimensões, de tal forma que ocorre muito pouca difusão vertical de soluto. Em contraste, um meio sólido permite um elevado grau de difusão de nutriente. Outro ponto importante a destacar é que um teste realizado em meio sólido pode favorecer a ação do antagonista (Baker e Cook, 1974).

2 OBJETIVOS

A partir de isolados bacterianos obtidos a partir de amostras ambientais e de folhas e frutos com e sem lesões de cancro cítrico pretende-se:

2.1 Objetivo Geral:

Selecionar bactérias que sirvam de agentes para o controle de lesões foliares do cancro cítrico, utilizando o isolado 306 de Xac (Xac306).

2.2 Objetivos Específicos:

a) utilizar inóculos em *pour plate* de Xac306 para avaliar a capacidade de antagonismo tanto das colônias crescidas quanto dos filtrados de culturas líquidas dos isolados em estudo;

b) empregar tanto a suspensão bacteriana quanto o sobrenadante de culturas dos isolados em condições *in planta* de pré- e pós-tratamento (24 horas) para se avaliar o efeito destes na redução da formação de lesões provocadas por Xac306;

c) caso haja resultados positivos, avaliar qual é a melhor aplicação e o tratamento mais eficiente no controle do cancro cítrico induzido em casa de vegetação; do contrário, tentar elucidar seus motivos.

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EVALUATION OF THE EFFECT OF BIOLOGICAL AGENTS AND THEIR PRODUCTS ON THE INCIDENCE OF CITRUS CANKER FOLIAR LESIONS

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SUMMARY

- Four antagonist bacterial isolates were used in attempts to control foliar lesions induced by *Xanthomonas axonopodis* pv. *citri* (Xac), the causal agent of citrus canker, an important problem for citriculture all over the world.

- Orange (*Citrus sinensis* cv. Valencia) plantlets were sprayed with bacterial suspensions and supernatants of antagonist isolate cultures, in combination with pre- or post-application with a Xac suspension.

- The results showed a reduction in the mean number of leaf citrus canker lesions with bacterial suspensions of the four isolates. The supernatant of the EC and FRC isolates reduced the total number of canker lesions, the mean number of lesions per

leaf and, like the LV isolate, the percentage of diseased leaves. An increase in the total number of lesions was observed with the LN supernatant and an increase in the total number of diseased leaves was observed with the LV cell suspension.

- These results indicate that antibiosis is an important mechanism of bacterial interaction on the phylloplane and that antagonism among bacteria could be an excellent tool for citrus canker control.

Key words: citrus canker, *Xanthomonas axonopodis* pv. *citri*, antagonist bacteria, biological control, phylloplane.

INTRODUCTION

Citrus canker, caused by the bacterium *Xanthomonas axonopodis* pv. *citri* (Xac), is a serious problem for world citrus production (Schubert et al., 2001; Gottwald et al., 2002). Lesions produced in the aerial part of the trees cause premature leaf and fruit drop, with a consequent significant loss of production. In addition, eradication is the main alternative of control after establishment of the disease (Civerollo, 1984; Gottwald et al., 2001; Leite, 1990). The losses can reach hundreds of millions of dollars per year. Only in Florida, US$ 200 millions had been spent until 2001, not including the losses with the elimination of millions of contaminated trees and plantlets (Schubert et al., 2001). Furthermore, countries in which the disease occurs can only commercialize the processed juice since they are forbidden to export the fruit *in natura* (Stall & Seymour, 1983).

The application of chemical products, like copper compounds, has helped in the prevention of many bacterial diseases, including citrus canker (Stall & Seymour,

1983), although at a cost reaching US$ 56 per acre (Schubert et al., 2001). However, cases of resistance to these and other compounds have been reported (Cooksey, 1990). Also, there is a growing concern about the residues of these substances, which accumulate both in foodstuffs and in the environment (Cook & Baker, 1983; Cook et al., 1996; Quimby et al., 2002). Chemical pesticides are also known to cause a disturbance in the plant-associated microbiota, favoring some pathogens (Blakeman & Fokkema, 1982; Griffiths, 1981). In fact, some countries intend to gradually replace chemical pesticides with integrated pest management practices, which include biological control (Hall & Berry, 1995).

For these reasons, biological control of phytopathogens with the use of antagonist bacteria or their metabolites has been proposed as a promising strategy for plant protection (Földes et al., 2000). Although several biological control studies involve soil pathogens and, among these, the fungi, recent studies have reported the successful use of antagonist bacteria for biological control of plant pathogenic bacteria. May et al. (1997) and Völksch & May (2001) describe the use of *Pseudomonas syringae*, *P. fluorescens* and *Pantoea agglomerans* strains in the control of soybean blight caused by *Pseudomonas savastanoi* pv. *glycinea*. Mercier & Lindow (2000) report the use of *P. fluorescens* against *Erwinia amylovora*, the causal agent of the fire blight of pear. Gremy et al. (1992) report the use of both liquid cultures of *P. fluorescens* and of the substances extracted from them against cotton pathogens. However, it has been suggested that attempts at biological control should involve both biological as chemical methods. (Baker & Cook, 1974; Stockwell et al., 1996).

Perhaps the main challenge in the development of a microbial biological agent is its adaptation to the same ecological niche of the pathogen that it will fight (Gau et

al., 2002; Völksch & May, 2001). Even so, such agents show advantages over chemicals products, such as greater selectivity against specific pathogens and less toxicity to non-target organisms (Mendelsohn et al., 1995), although, in contrast to chemical agents, often they do not persist for a sufficient period of time in the environment (Smits, 1996). In the case of biological agents for citrus canker control, it is necessary to consider the difficulties due to the specific environment of the phylloplane. Among them, there are the low nutrient availability, the exposition to ultraviolet radiation, the wide variations in temperature and humidity along the day and, mostly, the high impermeability of the surface because of the presence of waxes (Mercier & Lindow, 2000). Because of these problems, projects about the biological control of citrus canker do not receive so much attention in joint efforts against this disease (Dixon et al., 2000).

The purpose of the present study was to isolate and characterize Xac-antagonist bacteria and to assess their potential as biological control agents of foliar citrus canker lesions caused in *Citrus sinensis* cv. Valencia by isolate 306 of Xac (Xac306), whose genome has been recently sequenced (Silva et al., 2002).

MATERIAL AND METHODS

Experimental design

In the first experiment, suspensions of the four antagonist isolates (EC, FRC, LV and LN) were used and the positive control (Xac306) was applied 24 h later. In the second experiment, Xac306 was applied 24 h before the antagonist bacteria. The experimental design of both experiments was: 4 antagonist isolates x 7 replicates and the respective controls. In the third experiment, the cell-free supernatants of the

antagonist isolates EC and FRC were pre- and post-applied, with a 24 h interval in relation to the cell suspension of Xac306. The experimental design was: 2 antagonist isolates x 2 applications (pre- and post) x 7 replicates and the positive controls. The fourth experiment had the same experimental design, but the LV and LN isolates were used. For all experiments, recently pruned Valencia orange plantlets with 30-d-old leaves were used.

The highest and lowest values obtained in the seven *in planta* replicates were excluded because of the high variation in the number of lesions due to the different maturation stages of the leaves. Using the remaining quintuplicate values, we determined the total number of lesions, the total and percentage of diseased leaves and the mean number of lesions per leaf. Data were analyzed statistically by the Fischer LSD test ($P = 0.05$) using the Statistica® for Windows software (Version 5.1).

Isolation of antagonist bacteria

Leaves and fruits samples with and without citrus canker lesions were collected from disease-infested orchards in Astorga, PR, Brazil. Ten grams of each sample were added to tubes containing 20 ml of sterile distilled water and shaken vigorously for 20 min and the CFU number was determined by the decimal serial dilution technique. One-hundred microliters of each suspension were plated in duplicate onto nutrient agar (NA) and incubated at 28ºC for 3 d. Dilutions of 10^{-2} and 10^{-3} were obtained from samples without lesions and dilutions of 10^{-5} and 10^{-6} were obtained from samples with lesions. Morphologically distinct colonies were selected and their antagonistic activity against Xac306 was tested. An isolate (EC) obtained from the residual water of a dairy plant was also tested. This isolate was used in previous studies, in which it showed an antagonist effect against Xac306.

In vitro antagonism tests

The selected isolates were inoculated into a circle of approximately 1 cm in diameter in NA, supplemented or not with copper chloride ($CuCl_2.5H_2O$, 100 mg l^{-1}) due to the fact that many antagonist bacteria are resistant to copper (Casida, 1982), and incubated at 28ºC for 2 d. After this period, 9 ml of semi-solid NA (0.75% agar) melted at 45ºC, to which 1 ml of Xac306 suspension (10^8 CFU ml^{-1}) was added, was poured over the grown colonies of the isolates. After 2 d, the presence/absence of inhibition halos of Xac306 growth was evaluated and, in positive cases, their size was determined.

The bacteria that showed antagonistic activity in the previous step were inoculated into nutrient broth (NB), supplemented or not with copper chloride, and incubated with or without shaking (100 rpm) at 28ºC. Samples were collected at 1, 3, 7 14, 21 and 28 d of culture and filtered through nitrocellulose membranes (with pores of 0.2 μm in diameter) for evaluation of their antagonistic effect against Xac306. To this end, a 150 μl volume of each filtrate was dispensed in wells of 9 mm in diameter cut into semi-solid AN inoculated with Xac306 as described above. After 2 d of incubation at 28ºC, the antagonistic activity of the filtrates was evaluated by measuring the inhibition halos of Xac306 growth around the wells.

Preparation of bacterial suspensions for in planta antagonism tests

The Xac306 bacteria and the antagonists were grown NA (3 d at 28ºC), suspended in phosphate buffer (1.0 g KH_2PO_4, 1.5 g K_2HPO_4, and 1 liter of distilled water) supplemented with Tween 20 (final concentration of 0.25%), and adjusted to the concentration of 10^8 UFC ml^{-1}. Each bacterial suspension was applied with a hand sprayer on both surfaces of young orange plantlet leaves (*C. sinensis* cv.

Valencia), with care taken to avoid runoff as much as possible. After 1 h, the plantlets were covered with moist plastic bags in order to form a moist chamber that would improve the efficiency of Xac306 infection, and that was left in place for approximately 16 h. The experiments were performed in a greenhouse (28/22ºC day/night) and the number of citrus canker lesions was determined after 21 d.

Preparation of bacteria-free supernatants for the in planta antagonism tests

The antagonist isolates were inoculated into 1 liter of NB, with or without copper chloride, and incubated at 28ºC with or without shaking (100 rpm) for 14 d, according to the conditions needed to reach the highest values of inhibition halo diameter in Xac306, verified in the *in vitro* tests (Table 1). After incubation, the cultures were centrifuged (9,000 rpm at 4ºC) and Tween 20 (0.25%) was added to the supernatants for application of the leaves of young orange plantlet. The plantlets were sprayed and maintained as described in the previous step and the number of citrus canker lesions was determined after 21 d.

RESULTS

Isolation and morphological characterization of antagonist bacteria

More than 100 morphologically distinct colonies were detected in the samples of orange leaves and fruits with and without citrus canker lesions. However, in the *in vitro* tests, only three of these bacteria showed high antagonistic activity against Xac306. A previously obtained isolate (EC) was included in the experiments, for a total of four isolates used in the experiments for the study of antagonism against Xac306 under *in vitro* and *in planta* conditions. Accordingly to the morphological

characterization of the bacteria, all the tested isolates were Gram-negative rods. EC and FRC were isolated in culture media lacking copper chloride and LV and LN were isolated in culture media containing copper chloride (Table 1).

In vitro antagonism tests

Regarding the inhibition halos observed in the tests with grown colonies, only EC showed the formation of a halo smaller than 30 mm in diameter. With the filtrates, only LN showed a halo smaller than 20 mm in diameter, and the others did not differ significantly from one another in the same treatment (Table 1). Under different culture conditions, the isolates showed higher antagonism against Xac306 when cultivated at 28ºC under shaking at 100 rpm and the inhibition halos reached a maximum size after 14 d of culture. These culture conditions were chosen for the *in planta* experiments with the supernatants.

In planta antagonism tests with bacterial suspensions

In pre-treatment, significant improvements in relation to the positive control occurred in the percentage of diseased leaves with EC ($P \leq 0.032$), and in the total number ($P \leq 0.005$) and percentage of diseased leaves ($P \leq 0.001$) with LV (Table 2). For post-treatment, there was a significant reduction in mean number of lesions per leaf with all four cell suspensions (EC, $P < 0.001$; FRC, $P \leq 0.003$; LV, $P \leq 0.001$; LN, $P \leq 0.033$) (Table 3). No significant differences were observed in the other parameters evaluated.

In planta antagonism tests with bacteria-free supernatants

In pre-treatment with EC supernatant, significant reductions were observed compared to the positive control for total number of lesions, number of diseased leaves, percentage of diseased leaves and mean number of lesions per leaf ($P \leq 0.003$, $P \leq 0.009$, $P \leq 0.006$ and $P < 0.001$, respectively). With FRC supernatant, the reductions were significant for total number of lesions ($P \leq 0.004$) and mean number of lesions per leaf ($P < 0.001$) (Table 4). For the LN supernatant, significant improvements occurred in total number of lesions, mean number of lesions per leaf and percentage of diseased leaves ($P \leq 0.001$, $P \leq 0.005$ and $P \leq 0.019$, respectively) (Table 5). In post-treatment with EC supernatant, significant reductions in relation to the positive control were observed for total number of lesions ($P \leq 0.028$) and mean number of lesions per leaf ($P \leq 0.017$). With FRC supernatant, the differences were significant for total number of lesions ($P \leq 0.009$), percentage of diseased leaves ($P \leq 0.047$) and mean number of lesions per leaf ($P \leq 0.002$) (Table 4). For LV supernatant, a significant reduction ($P \leq 0.038$) occurred only in percentage of diseased leaves (Table 5).

The reduction in total number and percentage of diseased leaves, which was significant for the EC supernatant in pre-treatment, became non-significant in post-treatment (Table 4). The opposite occurred for percentage of diseased leaves with the FRC supernatant. The same also occurred for percentage of diseased leaves with treatment with the FRC and LV supernatants (Tables 4 and 5).

For LV and LN, the percentage of diseased leaves obtained in post-treatment with cell suspensions (18.417% and 29.081%, respectively) (Table 3) was similar to the values observed for the supernatants of their liquid cultures (19.52% and 29.380%, respectively) (Table 5), even when the experiments were performed at

different times. Interesting trends were also found for these two isolates, as observed with pre-treatment with the supernatants, when LV values tended to be smaller than LN ones, with a significant differences between them in total number and percentage of diseased leaves ($P \leq 0.020$ and $P \leq 0.003$, respectively) (Table 5). The opposite occurred with their cell suspensions, also in pre-treatment, when LV values tended to be higher, with significant differences compared to LN for total number of lesions, mean number of lesions per leaf and number of diseased leaves ($P \leq 0.005$, $P \leq 0.028$ and $P \leq 0.007$, respectively) (Table 2).

DISCUSSION

The bacterial population on the surface of citrus leaves and fruits with and without lesions was evaluated. The results indicated that there was a higher bacterial population in the presence of lesions (data not shown). In the case of healthy phylloplane, this difference is probably due to the presence of hydrophobic waxes, to high solar exposure or to the lack of nutrients (Mercier & Lindow, 2000). In the lesion environment, the bacteria are more protected from solar radiation by colonizing the intracellular spaces and can obtain nutrients from the cellular exudates.

In vitro tests with grown colonies showed the occurrence of antibiosis against Xac306. This was confirmed in tests with liquid culture filtrates, which demonstrated the production of substances with antimicrobial activities against Xac306 by the selected antagonist isolates.

In the pre-treatment with bacterial suspensions, the improvement in the number of diseased leaves after treatment with LV and in the percentage of diseased leaves after treatment with EC and LV may have been related to some interaction or

to the production of some compound that acted synergistically in the induction of lesions by Xac306. This is quite similar to what occurs in the iatrogenic diseases, with some substance altering the balance existing between natural antagonists and pathogens present on the plant surface (Griffiths, 1981). However, more detailed studies should be performed to demonstrate this effect. On the other hand, there was no difference among the other treatments when compared with the positive control, indicating that pre-treatment (24 h) with the isolates and conditions used were not efficient in the prevention of citrus canker. These results differ from those obtained by Lindow & Leveau (2002) and Wilson et al. (2002), who demonstrated the occurrence of preemptive competition exclusion for both nutrients and space.

For the post-treatment with bacterial suspensions there was a significant difference in mean number of lesions per leaf in all treatments, indicating that spraying the antagonist reduced the Xac306 population on the leaves. The success of this application strategy, named "inundative" (Cook et al., 1996), is due to the application of large quantities of the inoculum of the control agent to ensure a maximum and fast suppression of the pathogen. These results are quite interesting if we consider that studies on phylloplane antagonism using viable microorganisms usually report the use of a high antagonist:pathogen ratio, such as 4:1 or 9:1 (May et al., 1997; Völksch & May, 2001), inoculated simultaneously. It is also interesting to note that no reports are available in the literature regarding the use of bacterial suspensions in post-treatment for the application of a pathogen. Thus, post-treatment (24 h) condition demonstrates the potential of these isolates for biological control of citrus canker.

Bacterial suspensions of the two isolates obtained from orange leaves with citrus canker lesions (LV and LN) were the least efficient in reducing foliar lesions

caused by Xac306. On the other hand, the two isolates obtained from environments differing from the orange phylloplane (drainage water from a dairy plant for EC and, probably, soil particles adhered to a diseased fruit collected from the ground for FRC) showed the best results. These data agree with observations made by other authors (Baker & Cook, 1974), who reported that the best antagonists are isolated from environments where the disease is not present.

The efficiency of liquid culture supernatants varied according to isolate and to time of application. EC was more successful in pre-treatment condition, indicating that the antagonistic effect of its supernatant is reduced if Xac306 gets in contact with the phylloplane up to 24 h before its application (post-treatment). This may be explained that, during this period of time, Xac306 might be able to adhere to the phylloplane and/or penetrate the intercellular spaces of the leaves, thus protecting itself from these substances. However, Koizumi (1988) stated that, even after 48 h on the leaf, the bacterium is not fully established. For FRC supernatant, its activity against Xac306 in general did not seem to depend on the time of application because, when reductions in the incidence of citrus canker lesions occurred, they were significant both during pre- and post-treatment. For LV, a higher reduction in the percentage of diseased leaves occurred only with post-treatment, indicating that its antagonist compounds may be sensitive to the environmental conditions if they are exposed on the phylloplane for up to 24 h before contact with Xac306. For LN, even though *in vitro* results indicated antimicrobial activity against Xac306, the same did not occur under greenhouse conditions. The improvement in the number of lesions verified after pre-treatment with this supernatant may also indicate the occurrence of iatrogeny or synergism with Xac306, as observed for EC and LV cell suspensions in pre-treatment. We suggest that, as is the case for LV, the compounds present in this

supernatant are sensitive to these conditions, a fact that leads them to lose their antimicrobial activity. These data confirm reports showing that many antagonists with *in vitro* activity may not be successful under *in vivo* conditions (Blakeman & Fokkema, 1982; May et al., 1997).

The variation found in the results, i.e., the occurrence of leaves showing either many or few (or even none) citrus canker lesions, could be explained, according to Kinkel (1977), by a lack of uniform application of bacterial suspensions or supernatants with the hand sprayer. Jacques et al. (1995) also emphasized the fact that foliar age can have an effect on population size in some systems. This detail is important because, when 30 d were allowed to elapse after pruning, although still young, these leaves differed in age along the branches. Also, broad variations have been reported to occur in phylloplane biological control experiments, even when performed under highly controlled conditions (Stromberg et al., 2004).

It should be emphasized that more studies are necessary on the use of the isolates described in the present study under different conditions of application, as well as the identification and more precise description of both isolates themselves and the substances contained in their supernatants. These measures are of crucial importance because it is necessary to determine the possible impact of these isolates and their products on the environment (Cook et al., 1996).

The reduced incidence of citrus canker foliar lesions demonstrated in the experiments also indicates the possibility of a more rational use of chemical agents against this disease. In fact, Gau et al. (2002) and Quimby et al. (2002) pointed out the use of antagonistic microorganisms in biological control of phytopathogenic bacteria. This may help reduce the intensive application of pesticides and consequently minimize the persistence of their residues in the environment. This is

particularly important when we observe that overall phytopathogens have developed resistance to commercial products (Cooksey, 1990). However, it should be pointed out that, in contrast to chemical control, biological control may not show immediate effects, since the level of control obtained with biological method alone may be below that needed to prevent the occurrence of production losses. In this way, it is necessary to integrate both methods (Baker & Cook, 1974; Cook & Baker, 1983), always searching for the best conditions of application of each one (Stockwell et al., 1996). Thus, an additive or synergistic effect will occur, with biological control acting within a context of biological balance. Otherwise, its chance of success can be lower. In case of citrus canker, we must not forget the important and well established measures of control and prevention commonly used in countries which have problems with this disease (Leite & Mohan, 1990; Schubert et al., 2001).

In general, the present results are quite promising in terms of citrus canker control, mostly under greenhouse conditions. However, further studies are currently underway to determine the nature and efficiency of the substances produced by these bacteria in the control of this important disease.

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Table 1. *In vitro* antagonism of bacterial isolates against Xac306.

Isolate	Source/isolation local	Copper tolerance	Inhibition halo (mm)	
			Colonies	Filtrates
Xac306	IAPAR	n.t.[v]	n.t.	n.t.
EC	Dairy drainage water	-	21[w]	24[y]
FRC	Diseased fruit from the ground	-	34[w]	23[y]
LV	Diseased leaves	+	32[x]	25[z]
LN	Diseased leaves	+	34[x]	19[z]

[v] Not tested.

[w] In nutrient agar (NA).

[x] In NA with copper (NACu).

[y] Culture in nutrient broth (NB), for 14 d at 28ºC under agitation at 100 rpm.

[z] Culture in NB with copper (NBCu) for 14 d at 28ºC under agitation at 100 rpm.

Table 2. Effect of pre-treatment with bacterial suspensions of EC, FRC, LV and LN isolates on citrus canker foliar lesion development in Valencia orange plantlets.

Treatments	Total n of lesions	Total n of diseased leaves	Percentage of diseased leaves	Mean n of lesions per leaf
Control[x]	1858	93	38.4	20.383
EC	2109 (-13.51)[y]	128 (-37.63)[y]	54.7 (-42.45)[y] *	16.850 (17.33)[y]
FRC	1828 (1.61)	111 (-19.35)	41.7 (-8.59)	16.092(21.05)
LV	3852 (-107.32)	152 (-63.44) *	66.4 (-72.92) *	25.910 (-27.11)
LN	2028 (-9.15)	105 (-12.90)	42.4 (-10.42)	19.783 (2.94)

[x] Positive control (Xac306).

[y] Percent reduction compared to the positive control (Xac306). Negative values indicate an increase in relation to the control.

* Significant difference compared to control according to the Fischer LSD test ($P \leq 0.05$).

Table 3. Effect of post-treatment with bacterial suspensions of EC, FRC, LV and LN isolates on citrus canker foliar lesion development in Valencia orange plantlets.

Treatments	Total n of lesions	Total n of diseased leaves	Percentage of diseased leaves	Mean n of lesions per leaf
Control[x]	844	51	25.5	20.775
EC	136 (83.89)[y]	20 (60.78)[y]	16.2 (36.47)[y]	3.734 (82.03)[y] *
FRC	857 (-1.54)	96 (-88.23)	39.2 (-53.72)	8.324 (59.93) *
LV	240 (71.56)	31 (32.21)	18.4 (27.84)	4.606 (77.83) *
LN	859 (-1.78)	53 (-3.92)	29.1 (-14.12)	11.560 (44.36) *

[x] Positive control (Xac306).

[y] Percent reduction compared to the positive control (Xac306). Negative values indicate an increase in relation to the control.

* Significant difference compared to control according to the Fischer LSD test ($P \leq 0.05$).

Table 4. Effect of pre- and post-treatment with bacteria-free supernatants of liquid cultures of EC and FRC isolates on citrus canker foliar lesion development in Valencia orange plantlets.

Treatments	Total n of lesions	Total n of diseased leaves	Percentage of diseased leaves	Mean n of lesions per leaf
Control[x]	6413	79	35.4	59.773
EC (pre-treat.)	30 (99.53)[y] *	8 (89.87)[y] *	3.4 (90.39)[y] *	2.4 (95.98)[y] *
FRC (pre-treat.)	90 (98.60) *	34 (56.96)	14.5 (59.04)	2.273 (96.20) *
EC (post-treat.)	1874 (70.78) *	65 (17.72)	26.0 (26.55)	25.414 (57.48) *
FRC (post-treat.)	901 (85.95) *	31 (60.76)	13.4 (62.15) *	13.272 (77.80) *

[x] Positive control (Xac306).

[y] Percent reduction compared to the positive control (Xac306). Negative values indicate an increase in relation to the control.

* Significant difference compared to control according to the Fischer LSD test ($P \leq 0.05$).

Table 5. Effect of pre- and post-treatment with bacteria-free supernatants of liquid cultures of LV and LN isolates on citrus canker foliar lesion development in Valencia orange plantlets.

Treatments	Total n of lesions	Total n of diseased leaves	Percentage of diseased leaves	Mean n of lesions per leaf
Control[x]	657	62	34.887	8.941
LV (pre-treat.)	829 (-26.18)[y]	68 (-9.68)[y]	31.798 (8.85)[y]	11.675 (-30.58)[y]
LN (pre-treat.)	1492 (-127.10) *	76 (-22.58)	52.580 (-50.71) *	19.885 (-122.40) *
LV (post-treat.)	327 (50.23)	51 (17.74)	19.562 (43.93) *	8.478 (5.18)
LN (post-treat.)	574 (12.63)	65 (-4.84)	29.380 (15.78)	8.540 (4.48)

[x] Positive control (Xac306).

[y] Percent reduction compare to the positive control (Xac306). Negative values indicate an increase in relation to the control.

* Significant difference compared to control according to the Fischer LSD test ($P \leq 0.05$).

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A Absorbance
C3 plant Plant with C3 metabolism (also C4 plant)
Chl Chlorophyll (also Chl*a*, Chl*b*)
d. wt Dry weight
Fig. (Figs) Figure
f. wt Fresh weight
g Acceleration due to gravity (not rpm)
GA, GA1 Gibberellin, gibberellin A1 GA3
Gibberellic acid
Loge Natural logarithm (not Ln)
n Number of replicates
ns Not significant
P Probability
Pr/Pfr Far-red/red light-absorbing form of phytochrome
Pi Inorganic orthophosphate
sp. (spp.) Species
ssp. Subspecies
var. Variety

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