



**ROBERTO LANNA FILHO**

**CONTROLE DA MANCHA (*Xanthomonas vesicatoria*) E  
PINTA (*Pseudomonas syringae* pv. *tomato* GFP-marcada)  
BACTERIANA DO TOMATEIRO POR ISOLADOS  
ENDOFÍTICOS DE *Bacillus* sp.**

**LAVRAS – MG**

**2011**

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Agronomia/Fitopatologia, área de concentração em Fitopatologia, para a obtenção do título de Doutor.

Orientador

Dr. Ricardo Magela de Souza

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APROVADA em 01 de agosto de 2011.

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**2011**

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**OBRIGADO**

## RESUMO

Neste trabalho foi reportado o biocontrole exercido pelas bactérias endofíticas *Bacillus pumilus* e *Bacillus amyloliquefaciens*, nativas de tomateiro, contra as fitobactérias *Pseudomonas syringae* pv. *tomato* (*Pst*) NS4 GFP-marcada e *Xanthomonas vesicatoria* (*Xv*). O isolado NS4 foi obtido por transformação da *Pst* NW (selvagem) pelo plasmídeo integrativo pNKGFP. Em todos os bioensaios o isolado NS4 apresentou características semelhantes ao NW, sendo escolhido como modelo para estudos contra as bactérias endofíticas. Para os experimentos de biocontrole, plantas de tomate foram pulverizadas previamente com as bactérias endofíticas e, após quatro dias, inoculadas com os isolados NW e NS4. Estes isolados tiveram suas populações reduzidas em filoplano e apresentaram poucas lesões da pinta bacteriana, comparados com oxicloreto de cobre. Em microscopia de epifluorescência (ME), poucas células GFP-alvo do NS4 foram observadas colonizando nichos importantes em filoplano, tendo o contrário sido observado com os antagonistas sob microscopia eletrônica de varredura (MEV). No entanto, folíolos não tratados com os antagonistas apresentaram grande número de agregados de células GFP-alvo. O controle da *Xv* ocorreu em plantas previamente pulverizadas com as frações proteicas provenientes das bactérias endofíticas, as quais foram cultivadas em meio pobre até atingirem o ponto de inflexão na fase exponencial de crescimento e, em seguida, centrifugadas. O *pellet* foi descartado e o sobrenadante liofilizado, filtrado (0,22 µm), dialisado (ponto de corte = 12 kDa) e fracionado em coluna contendo Sephacryl S-300 HR equilibrada com tampão fosfato salina (PBS). As frações com maiores concentrações de proteína foram pulverizadas em plantas e, após quatro dias, foi inoculado o patógeno teste. As frações 42 e 75, correspondentes aos picos proteicos de *B. pumilus* (BPP2) e *B. amyloliquefaciens* (BAP2), controlaram eficientemente a fitobactéria, em comparação com o controle (água). Adicionalmente, as frações proteicas promoveram em plântula o aumento da atividade das enzimas peroxidase (POX) e polifenoloxidase (PPO), caracterizando o fenômeno de indução de resistência. Em SDS-PAGE corado com nitrato de prata, as frações proteicas 42 e 75 revelaram bandas simples com massa molecular estimada em 43 e 28 kDa, respectivamente. Os estudos demonstraram que as bactérias endofíticas controlaram *Pst* NS4 expressando o gene *GFP* em filoplano, bem como sintetizaram duas macromoléculas de natureza proteica que atuaram como eliciadoras das respostas de defesa em tomateiro contra *Xv*.

Palavras-chave: Controle biológico. Bactéria endofítica. GFP. Proteínas. Indução de resistência.

## ABSTRACT

In this work we report the biocontrol exercised by the endophytic bacteria *Bacillus pumilus* and *Bacillus amyloliquefaciens*, native tomato, against the plant pathogenic bacterium *Pseudomonas syringae* pv. *tomato* (*Pst*) strain NS4 constitutively expressing the *GFP* gene and *Xanthomonas vesicatoria* (*Xv*). The strain NS4 was obtained from the transformation of *Pst* wild-type strain NW by pNKGFP integrative plasmid. In all of the bioassays the strain NS4 presented characteristics similar to the NW, being chosen as phytobacteria model for studies against the endophytic strains. For the biocontrol experiments, tomato plants were previously sprayed with the endophytic bacteria, and after four days inoculated with the NW and NS4. On the tomato phylloplane the pathogens had their populations reduced, and leaves presented low number of bacterial speck lesions, compared with copper oxychloride. In epifluorescence microscopy (EM), few GFP-tagged cells of the strain NS4 were observed colonizing important niches on phylloplane, the opposite was observed with the antagonists by scanning electron microscopy (SEM). However, leaves not treated with the antagonists presented a large number of GFP-tagged cell aggregates. The control of *Xv* was performed by induced resistance in plants previously sprayed with the proteic fractions from the endophytic bacteria. The endophytic bacteria were cultivated in nutrient poor medium until they reached the inflection point of the exponential growth phase and soon afterwards were precipitated by centrifugalization. The precipitate was discarded and the supernatant lyophilized, filtered (0.22  $\mu$ m), dialyzed (cut-off = 12 kDa) and fractionated in a column containing Sephacryl S-300 HR equilibrated with PBS. Fractions that presented the highest protein concentrations were sprayed on tomato plants, and after four days, the test pathogen was inoculated. The protein peaks BPP2 and BAP2 corresponding to the fractions 42 and 75 controlled the phytobacteria efficiently, in comparison with the control (water). Additionally, the protein peaks promoted the increase of the POX and PPO enzyme activity in the plant, characterizing the phenomenon of resistance induction. In SDS-PAGE stained with silver nitrate, the protein fractions 42 and 75 showed as simple bands with estimated molecular mass at 43 and 28 kDa, respectively. Our results clearly demonstrated that the endophytic bacteria can control on phylloplane tomato the plant pathogenic bacterium *Pst* strain NS4 GFP-marked, and synthesize two macromolecules of proteic nature that act as elicitors of resistance induction in tomato against the *Xv*.

Keywords: Biological control. Endophytic bacteria. GFP. Protein. Induction resistance.

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## PRIMEIRA PARTE

### 1 INTRODUÇÃO

O tomate (*Solanum lycopersicum* L.), segundo Filgueira (2000; 2003), é a segunda hortaliça mais cultivada no mundo, sendo superada apenas pela batata. Na atualidade, o Brasil ocupa a nona posição em produtividade, com participação de 3,36% do total mundialmente produzido (FAOSTAT, 2011). A região sudeste é responsável por 47% da produção nacional, ou seja, a maior produção de tomate está concentrada nos estados de São Paulo, Minas Gerais, Rio de Janeiro e Espírito Santo (ESTADOSAT, 2010). O estado de Minas Gerais ocupa a terceira colocação em produtividade, com participação de 13% do total produzido e, dessa forma, é estado polo na cadeia produtiva desta hortaliça de importância econômica e social no contexto do agronegócio brasileiro.

Todavia, alguns fatores limitam a produtividade desta cultura no Brasil, como as fitomoléstias, que reduzem a produtividade e oneram os custos de produção (JONES et al., 1991; LOPES; ÁVILA, 2005). Tal situação proporciona um desestímulo para os tomatocultores, haja vista que o controle químico é de baixa eficiência. Adicionalmente, grande parte das perdas é ocasionada por doenças de natureza bacteriana, o que torna relevante o estudo de alternativas para o sucesso do controle desses fitopatógenos no campo, em viveiro e em casa de vegetação.

As estratégias de controle das fitobacterioses são baseadas em combinações de práticas de manejo, como a aquisição de sementes sadias, o uso de cultivares resistentes e a eliminação de plantas voluntárias e doentes (JONES et al., 1991; LOPES; ÁVILA, 2005). Mas, sobretudo, se realizam aplicações intensivas de antibióticos, compostos à base de cobre e misturas contendo

mancozeb (SHERF; MACNAB, 1986; JARDINE; STEPHENS, 1987; SAAD; ABUL HASSAN, 2000; MCMANUS et al., 2002). No entanto, estes agroquímicos ocasionam o aumento da frequência de populações bacterianas resistentes, diminuição ou eliminação de populações antagonicas e contaminação do meio ambiente. Dessa forma, é importante buscar estratégias para o manejo desses patógenos, com a premissa de reduzir ou eliminar o uso abusivo desses pesticidas.

O estudo de bactérias benéficas tem ganhado destaque como alternativa para o controle de doenças que acometem a produtividade de culturas agronomicamente importantes (GNANAMANICKAM, 2006; CAMPOS SILVA et al., 2008; PUSEY; STOCKWELL; MAZZOLA, 2009; ROMEIRO et al., 2010). Nesse panorama, as bactérias endofíticas têm se destacado como micróbios promissores no biocontrole de enfermidades em plantas, bem como em tomateiro (M'PIGA et al., 1997; BENHAMOU; KLOEPPER; TUZUN, 1998; CAMPOS SILVA et al., 2008; BARRETTI et al., 2009).

Habitantes comuns dos tecidos internos de diversas espécies de plantas (STROBEL et al., 2004), as bactérias endofíticas, em grande maioria, sobrevivem internamente sem causar efeitos deletérios em plantas (SCHULZ; BOYLE; SIEBER, 2006; RYAN et al., 2008). Mas, algumas podem proporcionar efeitos benéficos, favorecendo a promoção de crescimento, a indução de resistência e o controle biológico contra patógenos (RYAN et al., 2008). Em se tratando do controle biológico, esses micróbios são capazes de diminuir ou prevenir efeitos deletérios de certos organismos patogênicos, devido à ação dos mecanismos de biocontrole. Estes mecanismos foram estudados intensivamente por Kloepper et al. (1999), Gray e Smith (2005) e Compant et al. (2005a).

Como exemplos desses mecanismos citam-se a síntese de sideróforos, voláteis e substâncias antimicrobianas, a competição por espaço e nutrientes e a

indução de resistência sistêmica (ISR) (ROSENBLUETH; MARTÍNEZ-ROMERO, 2006; CAMPOS; PINHO; FREIRE, 2010). A capacidade das bactérias endofíticas de controlar doenças possibilita o emprego desses micróbios em tomateiro contra fitobacterioses, cujo controle é, muitas vezes, ineficaz com os agroquímicos utilizados.

Alguns estudos direcionam a essa possibilidade devido aos bons resultados do uso de bactérias endofíticas contra as fitobactérias *Xanthomonas vesicatoria*, *Pseudomonas syringae* pv. *tomato*, *Ralstonia solanacearum* e *Clavibacter michiganensis* subsp. *Michiganensis* (LONG et al., 2003; CAMPOS SILVA, 2004; BARRETTI; SOUZA; POZZA, 2008; CAMPOS SILVA et al., 2008). Adicionalmente, isso abre possibilidades para a ampliação dos estudos de bactérias endofíticas nativas que desempenhem o papel de proteger a planta contra o sucesso da patogênese de patógenos bacterianos e fúngicos. Dessa maneira, pode ser idealizado um bioformulado para posterior comercialização contra enfermidades dessa cultura, principalmente fitobacterianas.

Contudo, muitos isolados bacterianos citados em literatura como promissores não chegam ao mercado devido a problemas na formulação dessas culturas (BASHAN, 1998), notadamente devido à instabilidade das células vivas desses micróbios diante das condições desfavoráveis nas quais os mesmos foram isolados. Isso pode ser evitado com o isolamento de metabólitos sintetizados por esses micróbios que atuem direta e ou indiretamente contra fitopatógenos, o que pode proporcionar o desenvolvimento de bioprodutos com maior estabilidade, viabilidade e atividade em plantas (TJALSMA et al., 2004; ROMEIRO et al., 2005; ONGENA et al., 2007). No caso de macromoléculas, isso já é realidade para proteínas harpinas sintetizadas por bactérias do gênero *Erwinia*, *Ralstonia* e *Pseudomonas* (WEI; KIM; BEER, 2000; CUI et al., 2001; TAMPAKAKI; PANOPOULOS, 2001; DAYAN; CANTRELL; DUKE, 2009), as quais geraram os bioprodutos Messenger® e ProAct®, com amplo espectro de ação contra

fitopatógenos de diversas culturas (WEI et al., 2000b; DAYAN; CANTRELL; DUKE, 2009).

A busca por potenciais agentes de biocontrole para uso agrícola em substituição a agroquímicos tem se tornado uma realidade crescente nos principais centros de pesquisa do mundo. Nesse contexto, pesquisas com bactérias endofíticas podem trazer benefícios agrônômicos importantes, tais como aumento de produtividade, redução ou eliminação de doenças recorrentes, menor distúrbio do meio ambiente e obtenção de alimentos saudáveis. Embora estudos importantes estejam sendo realizados com esses micróbios, há muito para ser investigado sobre os mesmos, principalmente em se tratando da síntese de moléculas que podem ser promissoras para utilização em condições de casa de vegetação, viveiro e campo. A busca por essas moléculas, além da sua caracterização estrutural, abre possibilidades para se encontrar no mercado moléculas análogas com potencial para tornarem-se bactericidas e terem sua produção ampliada em escala industrial.

Neste contexto, este trabalho foi realizado com os objetivos de transformar o tipo selvagem de *Pseudomonas syringae* pv. *tomato* (ex OKABE) Young; Dye e Wilkie (1978) com o plasmídeo integrativo pNKGFP, bem como realizar estudos com os isolados dessa fitobactéria expressando o gene *GFP* em plantas de tomate (*Solanum lycopersicum* L.) como desafiantes às bactérias endofíticas *Bacillus pumilus* e *Bacillus amyloliquefaciens*. Adicionalmente, macromoléculas de natureza proteica sintetizadas pelas bactérias endofíticas foram testadas em tomateiro como eliciadoras de indução de resistência contra *Xanthomonas vesicatoria* (ex. DOIDGE) Vauterin et al. (1995)

## **2 REFERENCIAL TEÓRICO**

### **2.1 Manchas bacterianas em tomateiro**

Há múltiplas enfermidades que dificultam o cultivo do tomate em nosso país, com destaque para as manchas foliares que representam o principal problema nas lavouras. Dentre as principais manchas foliares, as incitadas por bactérias apresentam relevância pelo difícil controle, causando grandes perdas em condições ambientais favoráveis. Das doenças bacterianas de natureza foliar mais frequentes em campos de produção de tomate merecem destaque a mancha-bacteriana e a mancha-bacteriana pequena, que podem causar perdas estimadas em 60% e 30%, respectivamente (BASHAN; OKON; HENIS, 1982; LOPES; QUEZADO-SOARES, 1997).

#### **2.1.1 Mancha-bacteriana do tomateiro**

A mancha-bacteriana do tomateiro, causada por *X. vesicatoria* (*Xv*), é uma das doenças mais importantes da cultura, com ocorrência frequente em áreas produtoras irrigadas, ou não, por sistema de pivô-central. As perdas devido a esta doença são resultantes da redução da produção em decorrência direta dos sintomas e dos custos dos produtos químicos utilizados em seu controle, notadamente fungicidas cúpricos (JARDINE; STEPHENS, 1987; SAAD; ABUL HASSAN, 2000). Além de aumentar os custos da produção, o controle químico é de baixa eficiência (GOODE; SASSER, 1980a; MARINGON et al., 1986; JARDINE; STEPHENS, 1987; SAAD; ABUL HASSAN, 2000). Soma-se a isso a pouca durabilidade dos cultivares resistentes à mancha-bacteriana devido à alta variabilidade genética dos agentes causadores da doença (GOODE; SASSER, 1980; JONES et al., 2004).

A mancha-bacteriana pode ser observada em *Solanum* spp. e *Capsicum* spp., mas pode ocorrer em outras espécies hospedeiras, como *Datura stramonium*, *Hyoscyamus* spp., *Lycium* spp., *Nicotiana rustica*, *Nicandra physalodes*, *Physalis minima* e *Solanum* spp. A bactéria sobrevive em tecidos infectados da parte aérea da planta e em restos culturais, podendo ser isolada de sementes de tomate e pimentão, em que a incidência pode chegar a 60% (BASHAN; OKON; HENIS, 1982).

A doença pode se manifestar em qualquer estágio fenológico da cultura (GITAITIS; MCCARTER; JONES, 1992). Nas folhas, os primeiros sintomas aparecem na forma de pequenas áreas encharcadas de formato irregular, porém, com bordos definidos, que se tornam deprimidas, passando de uma coloração amarelada ou verde-clara para uma marrom-escura, até a necrose dos tecidos (GOODE; SASSER, 1980).

Distintamente do que ocorre em pimentão, em tomateiro, a mancha-bacteriana não leva à queda de folhas. Todavia, com o coalescimento das manchas necróticas foliares, verificam-se o secamento e a destruição da folhagem a partir da parte baixa das plantas. Nos frutos, as lesões iniciam-se na forma de pequenas áreas encharcadas a amarelas que se tornam marrom-acinzentadas e de textura áspera (JONES, 1991). Estas lesões tendem a ser deprimidas no centro e elevadas nas margens, variando entre 2 a 10 mm de diâmetro, podendo ser circundadas por um halo amarelo a esbranquiçado (GOODE; SASSER, 1980). A ocorrência da doença durante a floração causa quedas de flores, resultando em redução da produção (LOPES; QUEZADO-SOARES, 1997; LOPES; ÁVILA, 2005).

A mancha-bacteriana foi relatada, pela primeira vez, no Brasil, em 1959, em municípios do estado de São Paulo (RODRIGUES NETO; SUGIMORI; MALAVOLTA-JÚNIOR, 1984). As perdas de produtividade em condições experimentais de campo foram observadas em até 52% (LOPES; QUEZADO-

SOARES, 1997; LOPES; ÁVILA, 2005). Outros aspectos que mostram a importância da mancha-bacteriana para a cultura do tomateiro, tanto para consumo *in natura* quanto para o processamento industrial, são: eficiência variável do controle químico, ausência de fontes de resistência adequada, rápida disseminação em condições favoráveis de altas temperaturas (24-30° C) associadas à precipitação e disseminação à longa distância por sementes contaminadas (GOODE; SASSER, 1980; JONES, 1991). Estas representam um meio para a sobrevivência da bactéria por longos períodos (BASHAN; OKON; HENIS, 1982).

### **2.1.2 Mancha-bacteriana pequena do tomateiro**

A fitobactéria *Pseudomonas syringae* pv. *tomato* (*Pst*) foi originalmente descrita por Okabe (1933) e nomeada de *Bacterium tomato*. Mais tarde, foi renomeada como *Pseudomonas tomato*, por Alstatt (1944). No entanto, somente em 1984 definiram o nome como *Pseudomonas syringae* pv. *tomato* (OKABE) Young; Dye e Wilkie (1978), com publicação no Manual de Bergey de *Systematic Bacteriology*.

No Brasil, a mancha-bacteriana pequena foi observada pela primeira vez em plantios de tomate (*Solanum lycopersicum* L.) no estado de São Paulo (ROBBS, 1962). Nos últimos anos, a doença tornou-se importante nas principais regiões produtoras de tomate industrial, cultivado ou não sob pivô central, em condições de temperaturas entre 20° e 25° C, com umidade elevada. Há estimativas de que as perdas possam chegar a 30% (LOPES; QUEZADO-SOARES, 1997). Embora haja dados mais alarmantes, com perdas de 75%, quando a doença se manifesta no início do cultivo (YUNIS et al., 1980).

A sintomatologia aparente dessa enfermidade ocorre na parte aérea, com manchas pequenas de aspecto necrótico, circundada por um halo amarelado

(JONES et al., 1991). Em condições favoráveis, o progresso da doença leva à queima e à desfolha precoce do tomateiro (SRISINK; SIVASITHAMPARAM, 1987). O surgimento da doença no campo pode estar diretamente relacionado com a principal fonte de inóculo, a semente, pois, quando infestadas, plântulas podem desenvolver sintomas e iniciar epidemias severas (DEVASH; OKON; HENIS, 1980; YUNIS et al., 1980). Outras fontes possíveis de inóculo são restos culturais, plantas voluntárias e solo (CHAMBERS; MERRIMAN, 1975; BONN; GITAITIS; MACNEILL, 1985; JARDINE; STEPHENS; FULBRIGHT, 1988).

A principal medida de controle desta enfermidade é a aquisição de sementes sadias (RAT, 1988), acompanhada do uso de variedades resistentes portadoras do gene *Pto*. No entanto, ainda são utilizados agroquímicos cúpricos e ditiocarbamatos em grande escala (DEVASH; OKON; HENIS, 1980; GOODE; SASSER, 1980), que apresentam baixa eficiência na redução da doença. Isso tem contribuído para o aumento de metabolitos nocivos, como o ETU em frutos (ZAVATTI; ABAKERLI, 1996). Dessa forma, alternativas de controle têm sido pesquisadas, visando reduzir à severidade desta enfermidade, como o uso de agentes de biocontrole (BAIS; FALL; VIVANCO, 2004; CAMPOS SILVA, 2004; 2008).

Com o advento das técnicas moleculares a *Pst* tem se destacado como fitobactéria modelo (DE WIT; JOOSTEN, 2002; FOUTS et al., 2002; VAN DER HOORN; WRIGHT; BEATTIE, 2004; KABISCH et al., 2005; MELOTTO et al., 2006; TORRES et al., 2006) para a elucidação das interações entre fitobactéria-planta, servindo de base para interpretações dos fenômenos envolvidos na patogênese de outros patossistemas.

## 2.2 Bactérias endofíticas como agentes de biocontrole

A interação de bactérias associadas a tecidos internos de plantas sadias tem sido objeto de estudo, proporcionando, dessa forma, a elucidação dos mecanismos de proteção e crescimento de plantas, resultantes desta relação (ROSENBLUETH; MARTÍNEZ-ROMERO, 2006; RYAN et al., 2008). Notadamente, a observação da colonização dos tecidos internos de plantas sadias por micro-organismos surgiu em meados de 1866, com De Bary, o qual atribuiu o nome a esses organismos de endofíticos, ou endófitos, palavra derivada do grego *endon* = dentro e *phyton* = planta (BALDANI; SALLES; OLIVARES, 2002; SAIKKONEN et al., 2004). No entanto, há autores que atribuem a Perotti, em 1926, a descrição dessa relação natural entre as bactérias nativas e seus hospedeiros, bem como a sua denominação de endófitos (HALLMANN et al., 1997).

Apesar da controvérsia entre alguns autores sobre a primeira autoria da observação e denominação desses organismos, há muito vem se adotando o termo endofítica em diversos estudos (MICHEREFF et al., 1994; HALLMANN et al., 1997; SAIKKONEN et al., 2004; MASTRETTA et al., 2006; ROSENBLUETH; MARTÍNEZ-ROMERO, 2006; PRIETO; MERCADO-BLANCO, 2008; RYAN et al., 2008). Assim, o conceito mais recente e corrente é o de que as bactérias endofíticas são aquelas que colonizam o interior dos tecidos de plantas sadias sem causar efeitos deletérios ao hospedeiro e tampouco apresentam sinais externos visíveis provenientes de sua colonização (SCHULZ; BOYLE; SIEBER, 2006; RYAN et al., 2008).

O isolamento dessas bactérias vem sendo relatado por diversos autores em plantas de importância econômica, como algodão (QUADT-HALLMANN; KLOPPER, 1996), arroz (STOLTZFUS et al., 1997), soja (KUKLINSKY-SOBRAL et al., 2004), batata (LONG et al., 2003), cana-de-açúcar (OLIVEIRA

et al., 2002; BODDEY et al., 2003; LOIRET et al., 2004), tomate (PILLAY; NOWAK, 1997; BARRETTI; SOUZA; POZZA, 2008), citros (ARAÚJO et al., 2001; ARAÚJO et al., 2002), milho (ARAÚJO et al., 2002; ESTRADA et al., 2002), videira (BELL et al., 1995) e outras. Há relatos também de bactérias endofíticas em plantas medicinais, estando, muitas vezes, envolvidas em complexas relações de síntese, acúmulo e degradação de metabólitos secundários de interesse econômico (NETO; AZEVEDO; CAETANO, 2004).

Em geral, a alta densidade populacional de bactérias endofíticas é observada nas raízes e decresce no sentido do caule para as folhas (LAMB; TONKYN; KLUEPFEL, 1996; QUADT-HALLMANN; KLOEPPER, 1996; MASTRETTA et al., 2006). Dessa forma, a origem, a penetração, a colonização e a transmissão de bactérias endofíticas são muito discutidas. As mesmas podem ser provenientes de sementes, da rizosfera, da filosfera e de material propagado vegetativamente (ROSENBLUETH; MARTÍNEZ-ROMERO, 2006; RYAN et al., 2008). Atualmente, muito se tem elucidado com o emprego de novos métodos para o estudo da interação planta-bactéria endofítica, como o uso de proteínas autofluorescentes (AFPs) e  *$\beta$ -glucuronidase (GUS)* (LARRAINZAR; O' GARA; MORRISSEY, 2005; RYAN et al., 2008). Estas ferramentas permitem o monitoramento da bactéria endofítica no interior dos tecidos da planta, bem como a exploração de sítios específicos de colonização e formação de biofilme (MASTRETTA et al., 2006; RYAN et al., 2008), possibilitando, assim, inferir com exatidão a origem desses micro-organismos, bem como os mecanismos que governam o processo de penetração, colonização e transmissão.

Em relação à penetração e à colonização dos tecidos por bactérias endofíticas, de modo geral, sabe-se que as mesmas penetram passivamente através de aberturas naturais da planta, como estômatos, hidatódios, lenticelas, radículas, raízes laterais e ferimentos (JACOBS; BUGBEE; GABRIELSON, 1985; KIJIMA et al., 1993; LAMB; TONKYN; KLUEPFEL, 1996;

HALLMANN et al., 1997; MASTRETTA et al., 2006). Adicionalmente, Hallmann et al. (1997) consideram como principal via de acesso às endofíticas os ferimentos ocorridos naturalmente durante o crescimento da planta, como, por exemplo, a alongação do sistema radicular devido à diferenciação meristemática. Outro aspecto importante a comentar é o mecanismo de penetração ativo desses organismos, que sintetizam enzimas que degradam a parede celular dos tecidos radiculares (MASTRETTA et al., 2006; ROSENBLUETH; MARTÍNEZ-ROMERO, 2006). Este fenômeno é somente observado durante a penetração da epiderme radicular, mas nunca depois da colonização dos espaços intercelulares do córtex. Isso sugere que as bactérias endofíticas induzem a produção de celulases e pectinases somente para servir da penetração no hospedeiro (MASTRETTA et al., 2006).

Outra possibilidade para a penetração dessas bactérias na planta hospedeira é o uso de organismos vetores que proporcionam o favorecimento da entrada nos espaços apoplásticos, como foi demonstrado por alguns autores (ASHBOLT; INKERMAN, 1990; FRANKE et al., 2000). Uma vez dentro do tecido da planta, bactérias endofíticas tendem a localizar-se em tecidos específicos da mesma, como no córtex radicular ou colonizar a planta sistemicamente por transporte através do sistema vascular ou apoplástico (HUREK et al., 1994; JAMES et al., 1994). Outros autores também relatam a colonização dos espaços intercelulares e vasos do xilema (ROSENBLUETH; MARTÍNEZ-ROMERO, 2006). Tanto assim que Dong et al. (1994) detectaram a presença de endofíticas em espaços intercelulares no parênquima de caule de cana-de-açúcar e Compant et al. (2005b) encontraram um isolado de *Burkholderia* sp. nos vasos do xilema e câmaras subestomáticas em plantas de *Vitis vinifera*. Notadamente, a colonização intracelular também já foi relatada, embora não seja comum (JACOBS; BUGBEE; GABRIELSON, 1985; HUREK et al., 1994; QUADT-HALLMANN; BENHAMOU; KLOEPPER, 1997).

O fato de bactérias endofíticas colonizarem os tecidos internos das plantas confere a elas vantagens sobre outros micro-organismos, pois ocupam nichos que oferecem proteção ao ambiente externo (RYAN et al., 2008), possibilitando sobreviver em condições mais uniforme, evitando a exposição direta à temperatura, potencial osmótico e radiação ultravioleta (LODEWYCKX et al., 2002). Assim, tem-se uma relação entre planta-bactéria endofítica, podendo esta ser simbiótica, mutualística, comensalista e trofobiótica (RYAN et al., 2008). Neste contexto, as bactérias endofíticas podem estimular a promoção de crescimento em plantas e atuar como agentes de biocontrole. Também podem apresentar atividade importante na fitorremediação e constituírem efetivo papel na fertilidade do solo, por meio da solubilização de fosfato e fixação de nitrogênio (MASTRETTA et al., 2006; RYAN et al., 2008).

A promoção de crescimento em bactérias endofíticas apresenta mecanismos similares encontrados em rizobactérias. Isso inclui a atividade na solubilização de fosfato (VERMA; LADHA; TRIPATHI, 2001; WAKELIN et al., 2004), a produção de ácido indol-acético (AIA) (LEE et al., 2004), sideróforos (COSTA; LOPER, 1994) e síntese de fitormônios (MASTRETTA et al., 2006; ROSENBLUETH; MARTÍNEZ-ROMERO, 2006). As mesmas também podem suprir vitaminas essenciais para as plantas (PIRTTILA et al., 2004). Além disso, inúmeros outros benefícios ao crescimento das plantas são atribuídos às bactérias endofíticas, incluindo o ajustamento osmótico, regulação estomatal, modificação da morfologia do sistema radicular, aumento da percepção de minerais e alterações no metabolismo e acúmulo de nitrogênio (COMPANT et al., 2005a; RYAN et al., 2008). Atualmente estão sendo empregadas bactérias endofíticas promotoras de crescimento em áreas de regeneração florestal e para a fitorremediação de solos contaminados (RYAN et al., 2008).

Outro importante papel das bactérias endofíticas na engenharia agrônômica é seu emprego no biocontrole de fitomoléstias em uma diversidade de patossistemas. Para tal, as mesmas são capazes de diminuir ou prevenir efeitos deletérios de certos organismos patogênicos, devido à ação dos mecanismos de biocontrole. Estes foram estudados intensivamente por Kloepper et al. (1999) e, posteriormente, por Gray e Smith (2005) e Compat et al. (2005a). Como exemplos, têm-se a síntese de sideróforos, voláteis e substâncias antimicrobianas; a competição por espaço e nutrientes e ISR (ROSENBLUETH; MARTÍNEZ-ROMERO, 2006). A ação efetiva dos mecanismos de biocontrole somente é possível porque bactérias endofíticas colonizam nichos ecológicos similares àqueles ocupados por fitopatógenos, especialmente patógenos vasculares (BERG; EBERL; HARTMANN, 2005).

Numerosos estudos vêm mostrando que bactérias endofíticas são capazes de controlar fungos e bactérias (STURZ; MATHESON, 1996; DUIJFF et al., 1997; KRISHNAMURTHY; GNANAMANICKAM, 1997), insetos (AZEVEDO et al., 2000) e nematoides (HALLMANN et al., 1997; 1998). No tocante aos fitopatógenos, estudos vêm sendo realizados utilizando bactérias endofíticas em diversos patossistemas, tais como *Gaeumannomyces graminis* em trigo (COOMBS; MICHELSEN; FRANCO, 2004), *Xylella fastidiosa* em citros (ARAÚJO et al., 2002), *Meloidogyne incognita* em *Arabidopsis* (HALLMANN et al., 2001), *Xanthomonas campestris* pv. *campestris* em repolho (ASSIS et al., 1998), *Phytophthora nicotianae* em tabaco (COVENTRY; DUBERY, 2001) e outros.

O emprego de bactérias endofíticas no controle de doenças depende de inúmeros fatores relacionados à interação do trinômio endofítica-planta-fitobactéria, pois a competição existente entre os micro-organismos em habitats em comum pode reduzir a eficiência do controle, inviabilizando a sua utilização. Segundo Raupach e Kloepper (1998), na maioria dos casos, o controle biológico

que ocorre naturalmente é devido à mistura de micro-organismos antagônicos, sendo, portanto, importante avaliar a interação de diferentes bactérias para o controle de patógenos.

### **2.3 Macromoléculas-MAMPs**

Estudos dos produtos sintetizados por bactérias benéficas têm se tornado crescentes nas pesquisas em metabolômica e proteômica (CHEN; NAGARAJAN, 1994; CAO et al., 2006; DONG-HAI et al., 2008; WU et al., 2009), principalmente por promoverem o controle direto ou indireto em plantas contra fitopatógenos (STANGHELLINI; MILLER, 1997; LIGON et al., 2000; RYU et al., 2004; ROMEIRO et al., 2005; TRAN; KRUIJT; RAAIJMAKERS, 2008). No entanto, a maioria dos estudos trata de moléculas de baixo peso, como sideróforos, compostos voláteis (VOCs), surfactantes, antibióticos e outras (NEILANDS; LEONG, 1986; STANGHELLINI et al., 1996; LIM; KIM, 1997; STANGHELLINI; MILLER, 1997; SILO-SUH et al., 1998; LIGON et al., 2000; PRESS; LOPER; KLOPPER, 2001; COOK et al., 2002; SOUZA et al., 2003; RYU et al., 2003; 2004; MEZIANE et al., 2005; TRAN; KRUIJT; RAAIJMAKERS, 2008; RAAIJMAKERS; VLAMI; SOUZA, 2002). No caso de moléculas de alto peso, estas podem ser tanto constituintes da célula bacteriana (FELIX et al., 1999; MEYER; PÜHLER; NIEHAUS, 2001; KUNZE et al., 2004; ZIPFEL et al., 2004; MISHINA; ZEIER, 2007) quanto sintetizadas e liberadas por elas (CHEN; NAGARAJAN, 1994; ROMEIRO et al., 2005; CAO et al., 2006; DONG-HAI et al., 2008; WU et al., 2009; ROMEIRO et al., 2010).

As macromoléculas presentes na célula bacteriana podem desencadear reações de defesa em plantas contra diversos fitopatógenos (FELIX et al., 1999; MEYER; PÜHLER; NIEHAUS, 2001; KUNZE et al., 2004; ZIPFEL et al.,

2004; MISHINA; ZEIER, 2007), atuando como eliciadores inespecíficos (BOLLER, 1995; NÜRNBERGER et al., 2004; VORWERK; SOMERVILLE; SOMERVILLE, 2004). Isso significa que induzem reações em diferentes espécies ou variedades de plantas, promovendo a resistência de natureza basal. Os principais eliciadores constitutivos da célula bacteriana são: flagelina (flg) (FELIX et al., 1999; ZIPFEL et al., 2004), fator de alongação (EF-Tu) (KUNZE et al., 2004), lipopolissacarídeo (LPS) (MEYER; PÜHLER; NIEHAUS, 2001; MISHINA; ZEIER, 2007), harpinas (HrpZ) (WEI et al., 1992; WEI; BEER, 1993; 1995; TANAKA et al., 2001; CAO et al., 2006; DONG-HAI et al., 2008), proteínas de choque frio (CSPs) (THIERINGER et al., 1998; FELIX; BOLLER, 2003), superóxido dismutase (SodM) (WATT et al., 2005; 2006) e peptídeoglicano (PGN) (FELIX; BOLLER, 2003; GUST et al., 2007; ERBS et al., 2008). A essas macromoléculas eliciadoras dos mecanismos gerais de defesa em plantas dá-se o nome de padrões moleculares associados a patógenos (PAMPs) ou, atualmente, de padrões moleculares associados a micróbios (MAMPs).

PAMPs são moléculas evolutivamente conservadas provenientes principalmente do patógeno. No entanto, como organismos não patogênicos também sintetizam essas moléculas, o termo “padrão molecular associado ao patógeno” se torna impróprio, sendo o termo “associado a micróbio” mais preciso (AUSUBEL, 2005). Em plantas, as MAMPs ativam diferentes respostas de defesa, incluindo alterações na concentração do  $\text{Ca}^{2+}$  citoplasmático, ativação da cascata de MAP-quinases (MAPKs, proteínas quinases), indução de genes relacionados à defesa, produção de fitoalexinas, produção de espécies ativas de oxigênio (EAOs) e óxido nítrico (NO), deposição de calose para reforçar a parede celular, fechamento estomatal e, conseqüentemente, prevenção da entrada de fitobactérias (HE; SHAN; SHEEN, 2007).

A flg encontra-se presente em todas as bactérias móveis, podendo desencadear resposta imune em diferentes espécies de plantas (HE; SHAN; SHEEN, 2007). Notadamente, o reconhecimento molecular entre planta-flg dá-se pela região de 22 aminoácidos representados pelo peptídeo flg22 (FELIX et al., 1999). Em estudo realizado por Zipfel et al. (2004) foi demonstrado que plantas de *A. thaliana* deficientes na percepção da flagelina apresentaram resistência basal reduzindo a infecção por *Pst*, indicando que as reações de defesa mediadas por esta macromolécula diminuem a severidade de doenças em plantas hospedeiras suscetíveis.

Kunze et al. (2004) demonstraram a ocorrência de um novo eliciador bacteriano, o N-terminal do EF-Tu, capaz de ativar respostas de defesa em plantas de *A. thaliana* e outras brássicas. Estes autores demonstraram ainda que, pela utilização de um isolado bacteriano sem o gene da flg (*FLC*), o EF-Tu, a mais abundante e conservada proteína bacteriana, funciona como MAMP. Com a utilização de métodos combinados de cromatografia, digestão enzimática e MALDI-TOF/MS, Kunze et al. (2004) atribuíram a atividade eliciadora a um epitopo de 18 aminoácidos. O peptídeo que representa este domínio, Elf18, é capaz de induzir “explosão” oxidativa, aumentar a biossíntese de etileno e levar a mudanças em vários genes em concentrações subnanomolares, interessantemente, tão potente e quase equivalente a flg (flg22). Além disso, o sinalizador efg18 age através da cascata de sinalização baseada em MAP-quinase. O pré-tratamento de plantas de *A. thaliana* com elf18 induz maior resistência a infecções causadas por *Pst*.

O espectro de ativação de respostas de defesa mediada pelos MAMPs pode ser diferente, de acordo com as espécies hospedeiras. Assim, a flg das fitobactérias pode induzir respostas de defesa em uma variedade de hospedeiros, incluindo *A. thaliana*, arroz e solanáceas (KUNZE et al., 2004). Em contrapartida, o EF-Tu tem induzido respostas de defesa restritas a membros da

família *Brassicaceae*. Esse tipo de informação é altamente relevante para o desenvolvimento de potenciais indutores de resistência com amplo espectro de ação.

Os LPS são componentes indispensáveis da parede celular de bactérias gram-negativas. Os mesmos, em diversas fontes bacterianas, induzem a produção de H<sub>2</sub>O<sub>2</sub> em fumo (MEYER; PUHLER; NIEHAUS, 2001), expressão de genes *PR* em fumo e *A. thaliana* e mobilização mais rápida de diversas respostas de defesa em folhas de pimenta (MISHINA; ZEIER, 2007). A aplicação de LPS purificado de *Pseudomonas aeruginosa* ou *Escherichia coli* em folhas de *A. thaliana* levou à resistência sistêmica contra *P. syringae* pv. *maculicola* com acúmulo de ácido salicílico (AS) e aumento da expressão sistêmica de *PR-1* e *PR-5*, indicando que o LPS contribui para a resistência sistêmica adquirida (SAR) em *A. thaliana* (MISHINA; ZEIER, 2007).

A molécula de PGN confere à parede celular de bactérias gram-negativas e positivas rigidez e estrutura e são exclusivamente encontradas nesses micro-organismos. Erbs et al. (2008) demonstraram que os PGNs de *Xanthomonas campestris* pv. *campestris* (*Xcc*) e *Agrobacterium tumefaciens* (*At*) atuam como eliciadores e alvos da resposta da imunidade inata em *A. thaliana*. Adicionalmente constataram que muropeptídeos foram significativamente mais eficientes na eliciação das respostas do que a intacta molécula de PGN. As CSPs são induzidas em condições de baixas temperaturas, condicionando à célula bacteriana o preparo para manter o crescimento naquelas condições (THIERINGER et al., 1998). A CSP de *Micrococcus lysodeikticus* (= *Staphylococcus aureus*) foi identificada como MAMP induzindo respostas de defesa em células de tabaco (FELIX; BOLLER, 2003). O peptídeo de 22 aminoácidos da região N-terminal da CSP, *csp22*, foi sintetizada baseada em sequências consenso da CSP bacteriana e provou ser um domínio ativo. Adicionalmente, estudos usando várias solanáceas revelaram que folhas tratadas

com *csp15*, a mais conservada parte da *csp22*, a qual inclui um motivo de ligação a RNA (RNA-1), induziu “explosão” oxidativa, bem como um aumento na biossíntese de etileno, indicando um sistema de percepção para CSP nessas plantas. A SodM é a mais abundante proteína do proteoma extracelular da *Xcc*, capaz de induzir “explosão” oxidativa em células de tabaco (WATT et al., 2005), além de estar envolvida na redução de ânions superóxidos para peróxido de hidrogênio (WATT et al., 2006).

Em se tratando de macromoléculas de natureza proteica, sintetizadas e liberadas por bactérias que promovam resistência sistêmica em plantas, as mais estudadas são as harpinas (WEI et al., 1992; WEI; BEER, 1993; 1995; TANAKA et al., 2001; CAO et al., 2006; DONG-HAI et al., 2008). A primeira harpina foi descoberta em *Erwinia amylovora*, codificada pelo gene *hrpN* (WEI et al., 1992). Os autores observaram que a exposição de plantas a esta harpina ativava resistência a fitopatógenos e promovia crescimento. Após esta descoberta, outras harpinas foram sendo isoladas e caracterizadas. Tanaka et al. (2001) encontraram harpinas em patovares de *Pseudomonas syringae* eliciadoras da resposta de defesa em ervilha. Tampakaki e Panopoulos (2001) descreveram a harpina HrpZPsph em *P. syringae* pv. *phaseolicola*, que induzia resistência em linhagens de fumo. As harpinas são potentes indutoras da SAR, levando a planta ao “estado de indução” contra diversos fitopatógenos.

As inúmeras investigações realizadas com a harpina codificada pelo gene *hrpN* de *Erwinia amylovora* levaram ao desenvolvimento do bioproduto comercial Messenger® (WEI et al., 1992; WEI; BEER, 1993; 1995), o qual tanto contribui para o estímulo do crescimento em plantas quanto para o controle de fitopatógenos. Outro bioformulado com as mesmas propriedades é o ProAct®, um combinado das harpinas: harpinN, harpinW e harpinZ de, *Erwinia amylovora*, *Ralstonia solanacearum* e *Pseudomonas syringae*, respectivamente (DAYAN; CANTRELL; DUKE, 2009).

A investigação da síntese de macromoléculas liberadas por espécies de *Bacillus* indicam efeito eliciador para resistência sistêmica em plantas (ROMEIRO et al., 2005; CAO et al., 2006; DONG-HAI et al., 2008; WU et al., 2009; ROMEIRO et al., 2010). Romeiro et al. (2005; 2010) demonstraram haver ISR em plantas de tomate expostas a macromoléculas de *Bacillus cereus*. Notadamente, a resistência sistêmica culminou no controle dos fitopatógenos *Alternaria solani*, *Corynespora cassiicola*, *Xv* e *Pst*. Cao et al. (2006) constataram o efeito direto da proteína cry1Ab sintetizada por *B. thuringiensis* contra *Plutella xylostella*, bem como a indução da ISR em plantas de brócolis, com aumento dos níveis da proteína PR-1. Adicionalmente, Wu et al. (2009) estudaram a expressão da proteína HpaG de *B. subtilis* que induz resistência sistêmica em tabaco. Interessantemente, macromoléculas proteicas sintetizadas por espécies de *Bacillus* como eliciadoras da resistência sistêmica em plantas carecem de maiores estudos. No entanto, estudos desta natureza envolvendo esses micro-organismos têm aumentado substancialmente, haja vista a importância deste gênero como agentes de biocontrole.

Para finalizar, o estudo sistemático de macromoléculas de natureza proteica sintetizadas por agentes de biocontrole pode proporcionar a elucidação das respostas de defesa em planta contra fitopatógenos, além de conduzir para o desenvolvimento de bioformulados que proporcionem maior atividade, estabilidade e viabilidade, em comparação com células vivas de bactérias antagonicas. Assim, evita-se o uso intensivo de agroquímicos e possibilita o controle de doenças de forma mais inócua possível ao trinômio ambiente-homem-animais.

## 2.4 Identificação e caracterização das proteínas autofluorescentes

O nome proteínas autofluorescentes (AFPs) é genericamente utilizado para designar alguns grupos de proteínas que emitem fluorescência sobre determinado espectro de luz sem necessitarem de substrato específico para se expressarem. Assim, o primeiro relato de uma proteína emitindo fluorescência adveio de estudos coordenados por Shimomura, Johnson e Saiga (1962). Esses pesquisadores realizaram estudos bioquímicos com a água-viva *Aequorea victoria* e caracterizaram a proteína bioluminescente aequorina. Esta, por sua vez, emitia fluorescência azulada quando complexada com íons de cálcio ( $\text{Ca}^{+2}$ ). Notadamente, também isolaram uma proteína que emitia luz esverdeada atualmente denominada de *green fluorescent protein* (GFP) (MORIN; HASTINGS, 1971; MORISE et al., 1974). Adicionalmente, as GFPs ocorrem em uma grande variedade de água-viva, em espécies das classes *Hydrozoa*, tais como *Aequorea*, *Obelia* e *Phialidium*, e *Anthozoa*, como a *Renilla* (MORIN; HASTINGS, 1971; WARD; CORMIER, 1979).

Outra importante proteína fluorescente a destacar é a descoberta por Matz et al. (1999) em coral, denominado de *Discosoma striata*. A proteína foi originalmente nomeada de drFP583, mas renomeada como DsRED, por finalidades comerciais. Interessantemente, esta proteína emite luz vermelha, diferindo do espectro de fluorescência emitido pela GFP. Como na GFP, variantes da DsRed com melhores propriedades para aplicações na biologia celular e microbiologia estão disponíveis comercialmente. Como exemplo, a variante E5 da DsRed apresenta mudanças de fluorescências do verde ao vermelho ao longo do tempo. Dessa forma, funciona como um indicador, dando informações temporais e espaciais (TERSKIKH et al., 2000). Na atualidade, podem ser encontradas AFPs que emitem diferentes colorações, excitadas em comprimentos de ondas distintos (SHANER; PATTERSON; DAVIDSON,

2007; TKACZYK et al., 2008). Como exemplos, têm-se a *blue fluorescent protein* (BFP), *cyan fluorescent protein* (CFP) e *yellow fluorescent protein* (YFP), que são originadas da GFP. Dessa forma, amplia-se a aplicabilidade dessas proteínas em diversos estudos.

#### **2.4.1 As AFPs em bacteriologia de plantas**

Os avanços tecnológicos no emprego das AFPs nas diversas áreas da biologia têm proporcionado acréscimos importantes nos estudos microbiológicos. Nas associações entre micro-organismo-planta, muitas pesquisas vêm possibilitando a elucidação dos mecanismos que permeiam essas interações. No tocante a bactérias benéficas, estudos importantes com filobactérias, endofíticas e rizobactérias têm crescido em diversos institutos de pesquisa (MONIER; LINDOW, 2003; 2005ab; LACAVA; ARAÚJO; AZEVEDO, 2007; FERREIRA et al., 2008) com a premissa de preencher as lacunas existentes sobre os fenômenos que permeiam as interações antagonistas-plantas.

Em se tratando de filobactérias, aspectos ecológicos dessas populações vêm aumentando a curiosidade de muitos pesquisadores (LEVEAU; LINDOW, 2001; MONIER; LINDOW, 2005a), pois são micro-organismos que se encontram expostos a condições adversas do meio externo e, ainda sim, conseguem manter uma uniformidade populacional na superfície foliar. Dessa forma, pesquisadores, como Monier e Lindow (2003; 2005ab) e Leveu e Lindow (2001; 2002), realizaram estudos importantes sobre o sequestro de nutrientes, padrões de agregação, imigração e distribuição espacial, em filobactérias portadoras do gene *GFP*. Interessantemente, a GFP possibilitou a visualização desses micro-organismos em microhabitats distintos, como câmeras subestomáticas, depressões das paredes anticlinais, hidatódios e na base de

tricomas, mostrando os esconderijos que servem como micro-habitats em condições adversas.

Bactérias endofíticas têm constituído o maior foco das pesquisas com o uso das AFPs (COMPANT et al., 2005b; MASTRETTA et al., 2006; LACAVAL; ARAÚJO; AZEVEDO, 2007; FERREIRA et al., 2008; RYAN et al., 2008), certamente, por se tratar de micro-organismos que ocupam quase sistematicamente os tecidos internos da planta, sem causar danos ao hospedeiro. Aliás, conferem-lhes benefícios contra a invasão de fitopatógenos e condições adversas. Assim, o uso das AFPs nestes micro-organismos objetiva investigar a distribuição espacial no sistema vascular, espaços intercelulares, transmissibilidade via semente, formação de biofilme, etc. (MASTRETTA et al., 2006; RYAN et al., 2008), possibilitando inferir com exatidão o movimento e a origem desses micro-organismos.

Como exemplo, Tombolini et al. (1999) observaram, em sementes de cevada, o padrão de colonização da *Pseudomonas chlororaphis* MA342, agente de biocontrole. Os mesmos autores demonstraram a agregação do antagonista próximo à região do embrião, na base do coleóptilo e próximo ao ponto de emergência da raiz, o que é possível devido à alta concentração de nutrientes dos tecidos em diferenciação. Outro estudo importante foi realizado por Compat et al. (2005b) com a bactéria endofítica *Burkholderia* sp. em plantas de *Vitis vinifera*. Os autores demonstraram a colonização sistemática deste antagonista em toda planta, após exposição da raiz. Curiosamente, células da bactéria endofítica foram encontradas próximo ao ostíolo estomatal.

O uso das AFPs em bactérias fitopatogênicas necessita de maiores pesquisas, pois há poucos trabalhos disponíveis (BOGS et al., 1998; NEWMAN et al., 2003; NJOLOMA et al., 2005; MELOTTO et al., 2006; BELIMOV et al., 2007; LACAVAL; ARAÚJO; AZEVEDO, 2007; RIGANO et al., 2007). Mas, trabalhos como o de Newman et al. (2003) e Melotto et al. (2006) se destacam

pelo emprego inteligente dessa técnica na investigação da associação fitobactéria-planta. Newman et al. (2003) realizaram estudos pormenorizados da colonização de plantas de *Vitis vinifera* por *Xylella fastidiosa*, destacando sítios específicos de colonização, como cálice e estilete. Melotto et al. (2006), utilizaram a *Pst* DC3000 expressando o gene *GFP* em plantas de *Arabidopsis*, objetivando investigar a abertura e o fechamento de estômatos pela síntese de coronatina, fitotoxina sintetizada pela fitobactéria.

As limitações no uso das AFPs estão prestes a serem sanadas, principalmente em relação à ineficiência da técnica em bactérias gram-positivas, que constitui um dos maiores gargalos no uso das AFPs. Olubajoa e Bacon (2008) obtiveram sucesso na transformação de *Bacillus mojavensis* com biomarcadores *GFP*, *CFP* e *YFP*, abrindo possibilidades para a adoção da técnica em outras espécies de *Bacillus*. Outro aspecto relevante no uso das AFPs ainda pouco esclarecida diz respeito ao sucesso e ao insucesso da técnica em isolados da mesma espécie, quando se usa o mesmo vetor ou transposon, mesmo quando estes são submetidos à mesma metodologia.

### 3 CONSIDERAÇÕES GERAIS

O uso de bactérias endofíticas pode promover benefícios substanciais na engenharia agrônômica, desencadeando melhorias econômicas e ambientais. No entanto, um dos maiores desafios seria manter a ação efetiva dessas populações de bactérias endofíticas em diferentes condições e ou manter sua viabilidade. Isso apenas pode ser alcançado com maiores conhecimentos sobre a ecologia dessas populações e suas interações moleculares. Dessa maneira, o uso de genes repórter como *GFP* e *GUS* permite o monitoramento da bactéria endofítica no interior dos tecidos da planta, bem como a exploração de sítios específicos de colonização e formação de biofilme (MASTRETTA et al., 2006; RYAN et al., 2008b), elucidando a ecologia desses micro-organismos. Adicionalmente, o estudo e a investigação de proteínas ou glicoproteínas eliciadoras de IRS sintetizadas por bactérias endofíticas permitem, eventualmente, idealizar a formulação de um bioproduto efetivo no controle de fitopatógenos.

Neste contexto, Romeiro et al. (2005; 2010), trabalhando com um isolado *Bacillus cereus* UFV-101, mostraram que macromoléculas sintetizadas e exportadas pela rizobactéria levavam as plantas de tomate ao estado de indução de resistência, contribuindo para o melhor entendimento de como *Plant Growth-Promoting Rhizobacteria* (PGPRs) induzem IRS.

Por fim, é necessário investigar com persistência métodos alternativos para o controle de enfermidades de plantas que sejam, ao mesmo tempo, eficientes e menos agressivos à saúde humana e ao ambiente. Encontrar uma forma, o mais inócua possível, de ativar os mecanismos de defesa da planta, promovendo sua própria proteção contra patógenos, ao invés de saturá-la e intoxicá-la com defensivos, por certo será a estratégia politicamente correta do futuro. Adicionalmente, há que se ter em mente que a busca por alimentos produzidos sob um sistema de manejo capaz de agredir menos o ambiente e a

saúde do consumidor vem interessando cada vez mais aos produtores (BROWNE et al., 2000; RIGBY et al., 2001; MÄDER et al., 2002), posto já existir mercado crescente para produtos orgânicos, os quais podem chegar a preços 30% superiores aos pagos por produtos não orgânicos, tornando viável o investimento nestes sistemas de produção (JUNQUEIRA; LUENGO, 2000). O controle biológico de doenças de plantas vem sendo cada vez mais explorado, com um aumento crescente das pesquisas nesta área, gerando, atualmente, cerca de 40 produtos comerciais nos Estados Unidos da América (PAULITZ; BELANGER, 2001).

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**SEGUNDA PARTE – ARTIGOS**

**ARTIGO 1**

**Biocontrol activity of *Bacillus* against a GFP-marked *Pseudomonas syringae*  
*pv. tomato* on tomato phylloplane**

Prepared in accordance with the Australasian Plant Pathology  
(Preliminary version)

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## ABSTRACT

We report the biocontrol activity of endophytic bacteria *Bacillus pumilus* and *Bacillus amyloliquefacies*, autochthonous of tomato, against the plant pathogenic bacterium *Pseudomonas syringae* pv. *tomato* strain NS4 constitutively expressing the *GFP* gene. The strain NS4 was obtained from the transformation of *Pseudomonas syringae* pv. *tomato* wild-type strain NW with the plasmid pNKGFP containing GFP-cassette for chromosomal integration. The GFP-marked phytobacteria was submitted to the pathogenicity and compatibility tests, as well as to population studies on the phylloplane, to determine its survival. In all of the bioassays the strain NS4 presented similar characteristics to the wild-type, being chosen as phytobacteria model for studies with the endophytic strains. For the biocontrol experiments, tomato plants were previously sprayed with the endophytic bacteria, and after four days, inoculated with the NW and NS4. On the tomato phylloplane the challenger pathogen had their populations drastically reduced, and tomato leaves presented low number of bacterial speck lesions, compared to the treatment with copper oxychloride. Additionally, under epifluorescence microscopy, few isolated GFP-tagged cells of the strain NS4 were observed colonizing important niches on the tomato phylloplane. However, non treated leaves with the antagonists presented a large number of GFP-tagged cell aggregates. Our results demonstrated that the endophytic bacteria can also act efficiently on the biocontrol of the bacterial speck, even sprayed on tomato plants. In addition to highlighting the use of the strain NS4, constitutively expressing the *GFP* gene, as a model to elucidate phenomena involved in the phytobacteria-antagonist-tomato or phytobacteria-tomato interactions.

Keywords: Bacterial speck, biological control, endophytic bacteria, *Bacillus pumilus*, *Bacillus amyloliquefaciens*, green fluorescent protein.

## RESUMO

Neste estudo, verificou-se o biocontrole exercido pelas bactérias endofíticas *Bacillus pumilus* e *Bacillus amyloliquefacies*, autóctones de tomateiro, contra a bactéria fitopatogênica *Pseudomonas syringae* pv. *tomato* isolado NS4, constitutivamente expressando o gene *GFP*. O isolado NS4 foi obtido da transformação de um isolado tipo-selvagem NW de *Pseudomonas syringae* pv. *tomato* via plasmídeo integrativo pNKGFP. O isolado gfp-marcado foi submetido a testes de patogenicidade e compatibilidade, bem como a estudos populacionais em filoplano, para determinar a sua sobrevivência. Em todos os bioensaios, o isolado NS4 apresentou características semelhantes ao tipo-selvagem, sendo escolhido como modelo para estudos contra as bactérias endofíticas. Para os experimentos de biocontrole, plantas de tomate foram pulverizadas previamente com as bactérias endofíticas e, após quatro dias, inoculadas com os isolados NW e NS4. Em filoplano de tomateiro, os patógenos desafiantes tiveram suas populações drasticamente reduzidas e as folhas de tomate apresentaram baixo número de lesões da pinta bacteriana, comparado ao tratamento com oxiclóreto de cobre. Adicionalmente, sob microscopia de epifluorescência, poucas células solitárias GFP-alvo do isolado NS4 foram observadas colonizando nichos importantes em filoplano de tomateiro. No entanto, folhas não tratada com os antagonistas apresentaram grande número de agregados de células GFP-alvo. Os resultados demonstraram que as bactérias endofíticas podem atuar eficientemente no biocontrole da pinta-bacteriana em plantas de tomate e destacar o uso do isolado NS4, constitutivamente expressando o gene *GFP*, como modelo para elucidar fenômenos envolvidos na interação fitobactéria-antagonista-tomateiro ou fitobactéria-tomateiro.

Palavras-chave: Pinta bacteriana, controle biológico, bactérias endofíticas, *Bacillus pumilus*, *Bacillus amyloliquefaciens*, proteínas autofluorescentes.

## INTRODUCTION

*Pseudomonas syringae* pv. *tomato* (ex Okabe) Young et al. (1978) is a Gram-negative bacterium that causes bacterial speck disease on tomato leave (*Solanum lycopersicum* L.) and necrotic symptoms in stems and fruits (Jones et al. 1991). The bacterial infection in tomato young plants may result in 75% of losses whereas if it occurs later the losses reach about 5% in yield (Yunis et al. 1980). This plant pathogen has been found growing epiphytically on plant foliage without causing disease symptoms (Hirano and Upper 2000). Due to its importance, this phyto-bacteria has been the target of numerous studies to understand the molecular mechanisms and co-evolution of pathogenesis and plant disease resistance.

The management of bacterial speck is usually accomplished by copper-based pesticides, mancozeb and some antibiotics (Jardine and Stephens 1987; Saad and Abul Hassan 2000). However, under environmental favorable disease conditions the bacterial development and inoculum concentration are high, and the standard treatment becomes considerably less effective (Jardine and Stephens 1987). Therefore, the biocontrol appears as an ecologically correct alternative for the management of bacterial speck, reducing the use of pesticides. Currently, several works have demonstrated the biocontrol potential by the genus *Bacillus*, acting as phyllobacteria, rhizobacteria and endophytic bacteria (Silva et al. 2004; Halfeld-Vieira et al. 2006; Campos Silva et al. 2008; Lanna Filho et al. 2010). In the case of endophytic bacteria, numerous reports have shown that these microorganisms are capable of inhabiting also different environments, such as the phyllosphere and rhizosphere (Compant et al. 2005). Therefore, microorganisms are versatile to efficiently adapt to plants, allowing range of possible uses in important crops.

In this context, here we report the transformation of *Pseudomonas syringae* pv. *tomato* wild-type strain NW with pNKGFP plasmid containing GFP-cassettes by chromosomal integration and expression of *GFP* gene. In addition, strain of *P. syringae* pv. *tomato* expressing *GFP* gene were subjected to population studies on tomato (*Solanum lycopersicum* L.) phylloplane, as well as to pathogenicity and compatibility tests. Additionally, we also described the antagonistic effect of the endophytic bacteria *B. pumilus* and *B. amyloliquefaciens* on the population of *P. syringae* pv. *tomato* strain NW and strains NS1, NS2, NS3 and NS4 constitutively expressing the *GFP* gene, and biocontrol of bacterial speck by spraying those endophytes on tomato plants.

## MATERIALS AND METHODS

### Microorganisms and growth condition and plants

The *Pseudomonas syringae* pv. *tomato* wild-type strain NW was grown in 523 medium (Kado and Heskett 1970) and single colonies were suspended in 30 % glycerol solution and stored at -80 °C (Gerhardt 1994). The bacterium *Escherichia coli* DH5a pir was also used as host for the integrative plasmid pNKGFP (Ferreira et al. 2008), which contain the Green Fluorescent Protein (*GFP*) gene into a mini-Tn10 derivative with a kanamycin (kn) resistance gene.

The endophytic bacteria *Bacillus amyloliquefaciens* and *Bacillus pumilus* were previously isolated from tomato stem and tested as good biocontrol agents in previous work (Campos Silva et al. 2008). They were then grown in 523 medium (Kado and Heskett 1970) and preserved in deep freezer at -80°C, emulsified once in 30 % (v/v) glycerin (Gerhardt 1994).

All *in vivo* experiments were carried out at the Laboratory of Plant Bacteriology, Department of Plant Pathology of the Federal University of Lavras, Minas Gerais State, Brazil. Santa Cruz 'Kada' tomato plants were grown in a non-sterilized mixture of soil, sand and manure (2:1:1, v/v/v) in a greenhouse at 28°C ± 4°C and relative humidity of 70 %.

### *Pseudomonas syringae* pv. *tomato* strain NW transformation

The NW was grown for 18 hours at 28 °C in 5 mL SOB liquid medium (Sambrook et al. 1989). After growth, this culture was added in 250 mL SOB liquid medium and incubated at 28 °C under continuous shaking for 12 hours (DO<sub>600</sub> = 7.0). The cells were harvested by centrifugation (3.000 × g; 10 min; 4 °C), resuspended with 250 mL of cold ultrapure water and centrifuged again.

The supernatant was discarded and the bacterial pellet resuspended in 10 % glycerol and centrifuged. The pellet was resuspended in 10 % glycerol for electroporation. Following electroporation (Gene Pulser, BioRad - 2,5 kV, 25  $\mu$ F, 200  $\Omega$ ) in an electroporation cuvette (0,2 cm) within a mixture of 100  $\mu$ L cell suspension and 1  $\mu$ g of pNKGFP plasmid. After transformation, 1 mL of Luria-Bertani (LB) medium was added, incubated for 1 h at 28 °C and plated on LB medium supplemented with kanamycin (50 mg.mL<sup>-1</sup>). The identification of clones carrying the *GFP* gene was carried out on UV light.

#### Pathogenicity assay

For the pathogenicity or hypersensitivity test, soybean (*Glycine max*) and tobacco (*Nicotiana benthamiana*) plants were inoculated with four gfp-marked bacteria *P. s. pv. tomato* (NS1, NS2, NS3 and NS4) and a wild-type strain NW (20  $\mu$ L; OD<sub>540</sub> = 0.2; suspension cells  $\sim 10^8$  cfu.mL<sup>-1</sup>) by infiltration into the abaxial side of intact leaves with a plastic syringe without a needle, and the inoculation points were labeled by ink marks on the upper surface. The control was composed by water infiltration into the abaxial side of intact leaves.

In the compatibility test, tomato plants fifteen days after sowing, were sprayed with a GFP-marked strains (NS1, NS2, NS3 and NS4) and a wild-type strain NW suspensions (OD<sub>540</sub> = 0.2; suspension cells  $\sim 10^8$  cfu.mL<sup>-1</sup>). Inoculated plants were maintained in a greenhouse and lesions counted after disease symptoms have fully developed. For each treatment, 10 replicates were used, with one plant per pot considered as one replicate.

### Bacterial population dynamics on phylloplane

Tomato plants, fifteen days after sowing, were sprayed with suspension of GFP-marked cells (NS1, NS2, NS3 and NS4) and wild-type strain NW ( $OD_{540} = 0.2$ ; suspension cells  $\sim 10^8$  cfu.mL<sup>-1</sup>). Leaves were collected randomly and placed in flasks containing 100 mL of sterile phosphate buffer (0.1 M; pH 7.0; containing 0.05 % Tween-80) and sonicated for 8 min in an ultrasonic cleaning bath to recover bacterial cells. Bacterial populations were estimated from three grams of healthy leaves randomly sampled from each plant pot. Appropriate dilutions of leaf washings were plated on 523 semi-selective medium containing cycloheximide (50  $\mu$ g.mL<sup>-1</sup>) and cephalixin (50  $\mu$ g.mL<sup>-1</sup>). The wild-type strain NW and four GFP-marked bacteria *P. s. pv. tomato* were constitutively resistant to the cephalixin.

In parallel, endophytic bacteria also were sprayed ( $OD_{540} = 0.2$ ; suspension cells  $\sim 10^8$  cfu.mL<sup>-1</sup>) on tomato plants. Leaves were collected randomly and placed in flasks containing 100 mL of sterile phosphate buffer (0.1 M; pH 7.0; containing 0.05% Tween-80) and sonicated for 8 min in an ultrasonic cleaning bath to recover bacterial cells. Bacterial populations were estimated from three grams of symptomless leaves randomly sampled from each plant pot. Appropriate dilutions of leaf washings were plated on 523 medium containing cycloheximide (50  $\mu$ g.mL<sup>-1</sup>) and neomycin (50  $\mu$ g.mL<sup>-1</sup>). The *B. pumilus* and *B. amyloliquefaciens* endophytic were constitutively resistant to the neomycin.

The leaves were collected at intervals of 24 hours and population sizes were estimated from plate counts made after 2 to 3 days incubation at 28 °C. For each treatment, four replicates were used, with three grams of leaves per pot considered as one replicate.

### Phylloplane biocontrol

Fifteen days after sowing, tomato plants were sprayed with suspension of live endophytic cells ( $OD_{540} = 0.2$ ; suspension cells  $\sim 10^8$  cfu.mL<sup>-1</sup>). Four days later, plants were inoculated with *P. s. pv. tomato* strain NW and NS4 ( $OD_{540} = 0.2$ , suspension cells  $\sim 10^8$  cfu.mL<sup>-1</sup>). Leaves were collected randomly and placed in flasks containing 100 mL of sterile phosphate buffer (0.1 M; pH 7.0; containing 0.05% Tween-80) and sonicated for 8 min in an ultrasonic cleaning bath to recover bacterial cells. Bacterial populations were estimated from three grams of symptomless leaves randomly sampled from each plant pot. Appropriate dilutions of leaf washings were plated on 523 medium containing cycloheximide (50  $\mu$ g.mL<sup>-1</sup>) and cephalixin (50  $\mu$ g.mL<sup>-1</sup>). The population sizes were estimated from plate counts made after 2 to 3 days incubation at 28 °C. For each treatment, four replicates were used, with three grams of leaves per pot considered as one replicate.

### Control of bacterial speck

Tomato plants fifteen days after sowing, were sprayed with suspension of live endophytic cells ( $OD_{540} = 0.2$ ; suspension cells  $\sim 10^8$  cfu.mL<sup>-1</sup>), copper oxichloride (CO) (2 g.mL<sup>-1</sup>) (positive control) and water (negative control). Four days later, plants were inoculated with *P. s. pv. tomato* strain NW and NS4 ( $OD_{540} = 0.2$ ; suspension cells  $\sim 10^8$  cfu.mL<sup>-1</sup>). Inoculated plants were kept in greenhouse, and after the symptoms of the disease were fully developed, the number of lesions per leaf was counted within all the leaflets. For each phyto-bacteria, there were used ten plants per treatment, with one plant per pot considered one replicate. The bioassay was repeated three times.

### Fluorescence microscopy

Ten-day-old plants were sprayed with suspension of live endophytic cells and water. Four days later, plants were inoculated with *P. s. pv. tomato* strain NS4. After five days, 1 cm diameter leaflet discs were removed and mounted on a slide bridged with 30 % (v/v) glycerol (microscope grade). Fluorescence microscopy was carried out on an Axio Observer Z1 microscope, and the images were taken using the *Axivision 4.6* Image Program, Zeiss. GFP-tagged bacterial cells were excited with the 488 nm filter.

### Scanning electron microscopy (SEM)

Ten-day-old plants were sprayed with suspension of live endophytic cells and water. After four days, three leaves per treatment were cut, and submitted to a fixative procedure in a modified Karnovsky solution (glutaraldehyde 2.5 % and paraformaldehyde 2.5 % in sodium cacodylate buffer 0.05 mol L<sup>-1</sup>; pH 7.2; CaCl<sub>2</sub> 0.001 mol L<sup>-1</sup>), for 24 hours (at 4 °C), infiltrated with a cryoprotection solution (glycerol 30 % in water) for 30 min, and cross-sectioned with a scalpel blade after being immersed in liquid nitrogen. Sections were transferred to a 1 % aqueous solution of osmium tetroxide for 1 hour at room temperature, and subsequently dehydrated for 10 min each in a crescent series of acetone solutions (25, 50, 75, 90 and 100 %). After that, they were dried in a critical-point drier CPD 030, (Balzers, Jundiaí, SP, Brazil). Processed materials were mounted on aluminum stubs, fractured side up, sputter coated with gold SCD 050, (Balzers, Jundiaí, SP, Brazil) and observed in a scanning electron microscopy (SEM) LEO EVO 40 XVP (K. E. Developments, Cambridge, England). Leaves of healthy tomato plants exposed to PBS were used as controls. Two images were generated and three leaflets were used for

each treatment. Images of the phylloplane region were generated at random for each sample, at several magnifications, and digitally recorded. Images were processed using the software Corel Draw 12, with which comparisons among treatments were done.

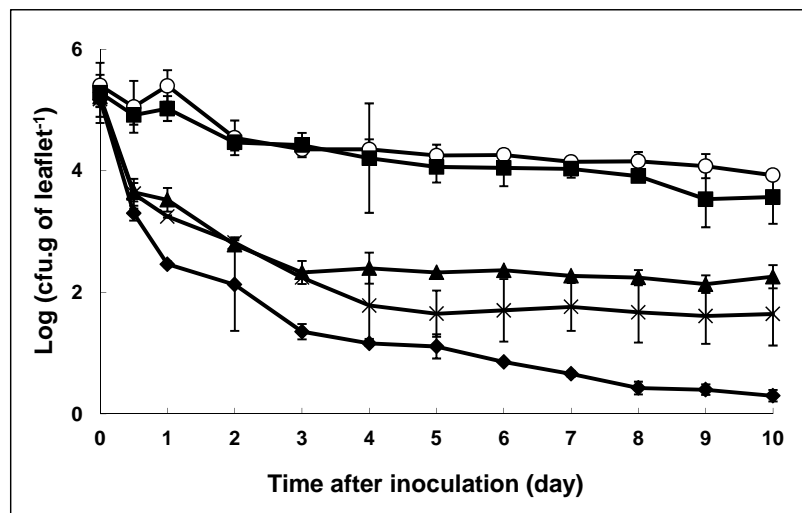
#### Statistical Analysis

All experiment was performed in a completely randomized design. The results were subjected to analysis of variance and means were compared by the Tukey test ( $p < 0.05$ ) using the software Statistica®, version 7.0 (Statsoft, 2005).

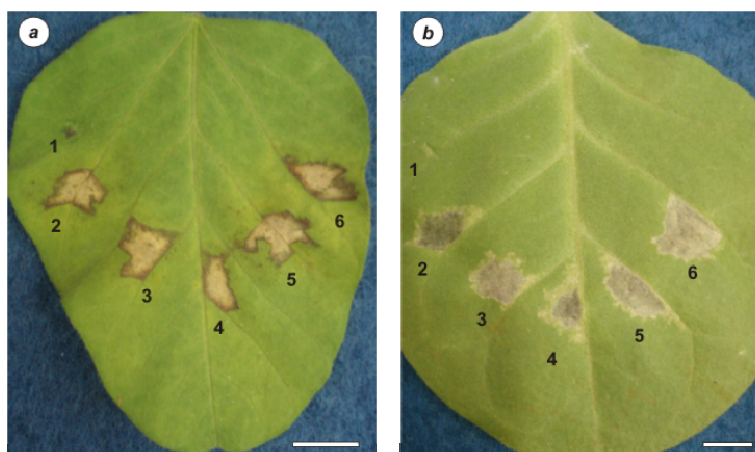
## RESULTS

### Electroporation, compatibility and pathogenicity tests

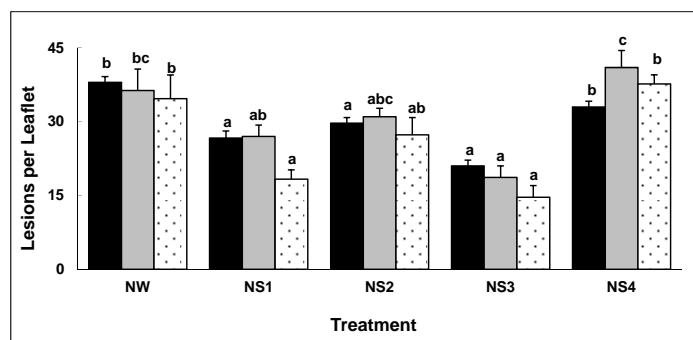
The transformation of *P. s. pv. tomato* wild-type strain NW by pNKGFP plasmid containing GFP-cassettes for chromosomal integration and expression of *GFP* gene, generated four GFP-marked bacteria that under fluorescence microscopy expressed the *GFP* gene. The GFP-marked strains NS1, NS2, NS3 and NS4 had their population behavior tested on the tomato phylloplane, and that which presented a population dynamics similar to the wild-type for 10 days was the strain NS4 (Fig. 1). In addition, at 10 days the population of the NW and NS4 strains on the phylloplane remained at elevated levels with  $3.9 \times 10^6$  and  $3.5 \times 10^6$  (CFU.g of leaves<sup>-1</sup>), respectively. The pathogenicity test was positive for all test GFP-marked strain, showing hypersensitive response (HR) in soy leaves and tobacco, inducing necrotic lesions after 24 h of infiltration of the bacterial suspensions (Fig. 2). In the compatibility test all of the GFP-marked strains caused disease in tomato plants. But only the strain NS4 presented the highest disease severity levels, compared with the control (NW) (Fig. 3). For presenting similar characteristics to the wild-type strain NW in the results described above, the plant pathogenic bacterium *P. s. pv. tomato* strain NS4 was chosen as challenge pathogen against the endophytic bacteria *B. amyloliquefaciens* and *B. pumilus* for the biocontrol assays.



**Fig. 1** Population dynamic of *P. syringae* pv. *tomato* strains on tomato inoculated by spraying ( $OD_{540} = 0.2$ ; suspension cells  $\sim 10^8$  cfu.mL $^{-1}$ ): NW (○) NS1 (×), NS2 (▲), NS3 (◆) and NS4 (■). Each point represents the mean  $\pm$  standard error of the mean of four replicates



**Fig. 2** Photography, tobacco (a) and soy leaves (b) showing hypersensitive response to *P. s. pv. tomato* GFP-marked strains. The leaves were infiltrated with the 1 = water (control), 2 = strain NS1, 3 = strain NS2, 4 = strain NS3, 5 = strain NS4 and 6 = strain NW and photographed after 48 h. The bacterial cell suspensions were infiltrated at a concentration of  $1.0 \times 10^8$  cfu.mL $^{-1}$ . (Bars = 2 cm)

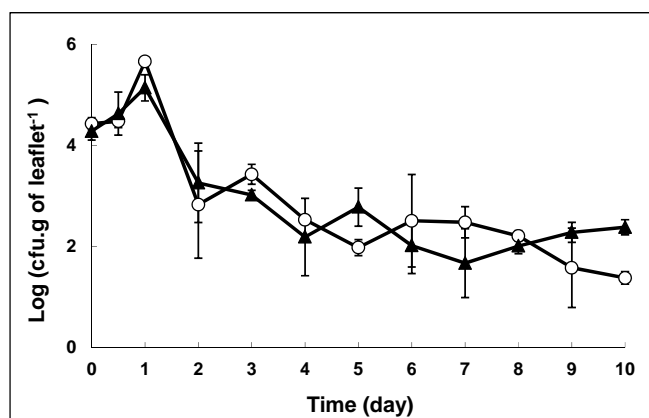


**Fig. 3** Severity of *P. s. pv. tomato* GFP-marked strains and NW (control) artificially inoculated on Santa Cruz 'Kada' tomato plants. Three replicate were performed for each treatment, represented by black, dark grey and white bars. The columns represent the mean and vertical bars represent the standard error of the mean. Means followed by same letter do not differ by the Tukey test ( $p < 0.05$ )

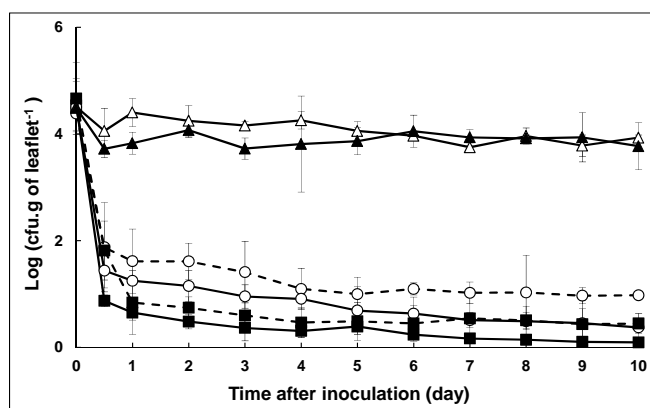
#### Phylloplane biocontrol

The biocontrol agents *B. pumilus* and *B. amyloliquefaciens* survived on the tomato phylloplane after 10 days of spraying, presenting population levels of  $1.3 \times 10^6$  and  $2.3 \times 10^6$  (CFU.g of leaves<sup>-1</sup>), respectively (Fig. 4). Additionally, tomato leaves sprayed with the endophytic bacteria and inoculated with the NS4 and NW after four days, presented lower challenge pathogen population levels (Fig. 5), compared with leaves without the antagonist spraying. Leaves previously exposed to *B. pumilus*, after 10 days of inoculation of the challenge pathogens, presented a reduction of 75 and 84 % in the populations of the NW and NS4, respectively. Additionally, previously exposed *B. amyloliquefaciens* leaves presented a population reduction of the NW and NS4 of 90 and 97 %, respectively. The plant sprayed with the antagonists, and inoculated with the phytobacteria after four days, presented lower severity of the bacterial speck, compared to the treatment with copper oxichloride (positive control). The

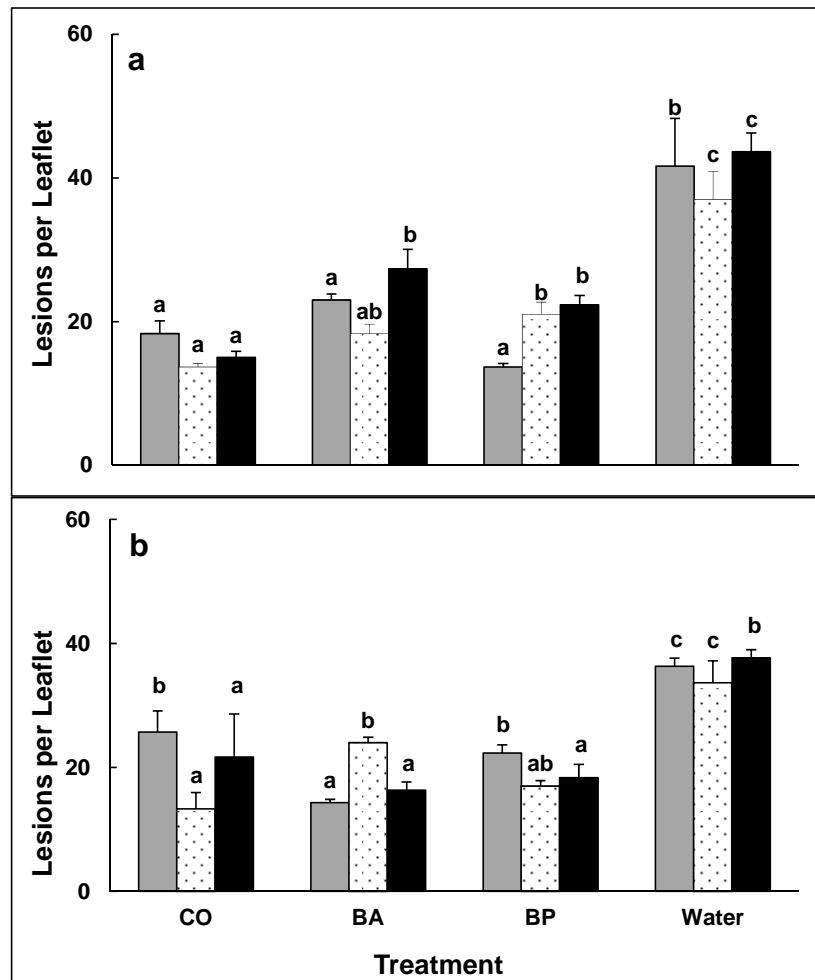
highest disease severity levels occurred in plants sprayed with water (negative control) (Fig. 6).



**Fig. 4** Population dynamics of *B. pumilus* (○) and *B. amyloliquefaciens* (▲) sprayed on tomato leaves. Each data point indicates the mean of the log (cfu.g of leaflet<sup>-1</sup>) bacterial population. Each point represents the mean ± standard error of the mean



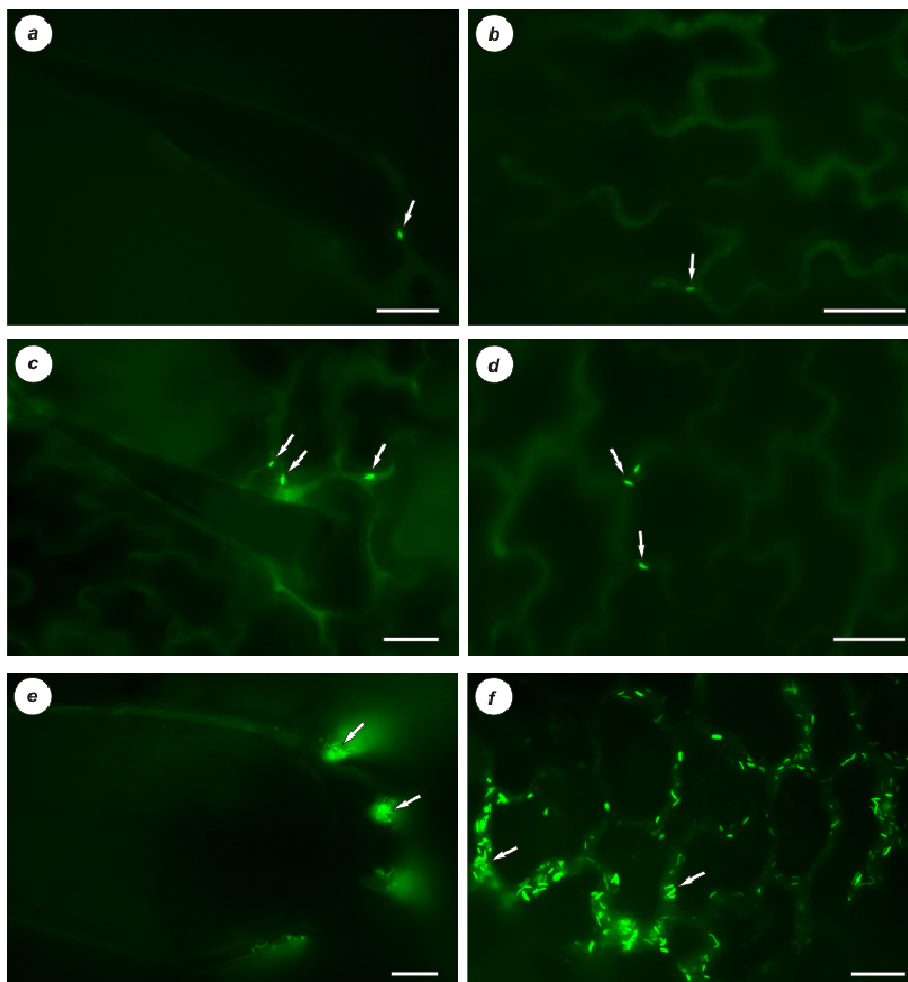
**Fig. 5** Population dynamics of NW (○) and NS4 (■) on tomato leaves four days after exposure to treatments with *B. pumilus* (---) and *B. amyloliquefaciens* (—). For control, tomato leaves were sprayed with water and after four days inoculated with NW (Δ) and NS4 (▲). Each data point indicates the mean of the log-transformed bacterial population. Each point represents the mean ± standard error of the mean



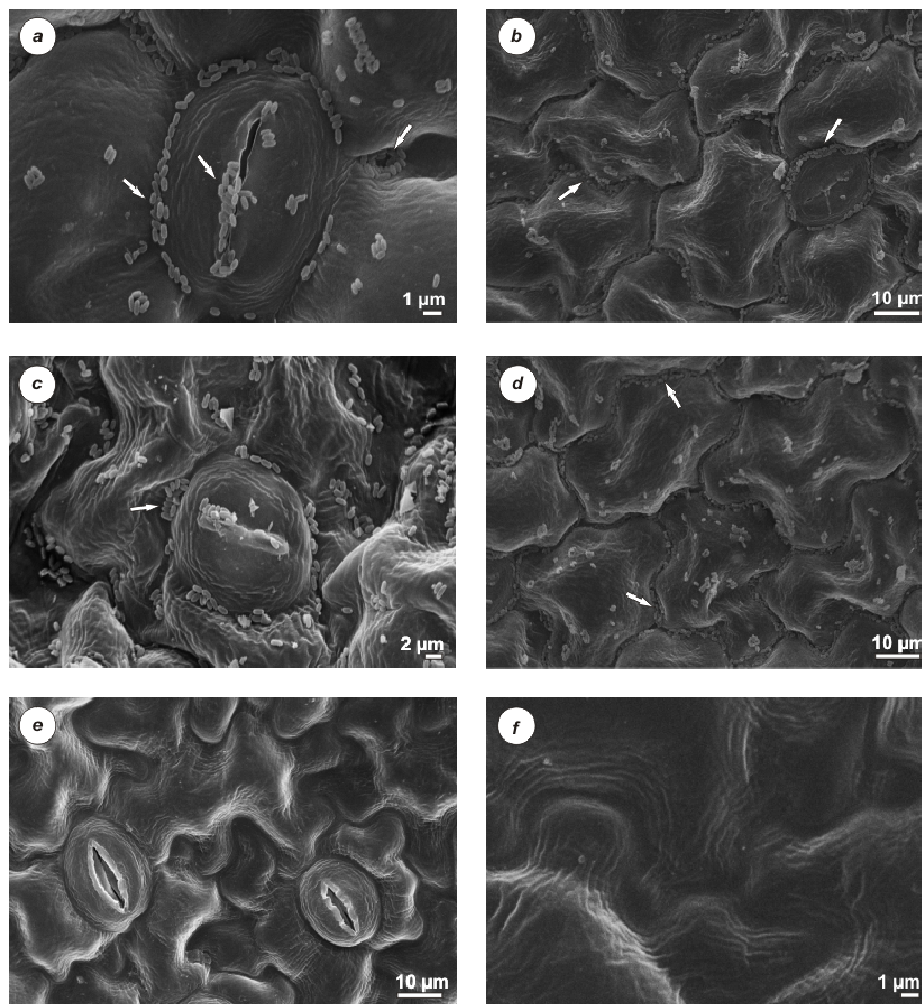
**Fig. 6** Severity of bacterial speck caused by *P. s. pv. tomato* wild-type strain NW (a) and strain NS4 (b), artificially inoculated on Santa Cruz 'Kada' tomato plants, four days after exposure to treatments: copper oxichloride (CO), *B. amyloliquefaciens* (BA), *B. pumilus* (BP), and water (negative control). The dark grey, white and black bars represent the experiment replicates. Means followed by same letter do not differ by the Tukey test ( $p < 0.05$ ). The columns represent the mean and vertical bars represent the standard error of the mean

## Fluorescence microscopy and scanning electron microscopy

The studies under epifluorescence microscopy showed that on the tomato phylloplane treated with the endophytic bacterium, the GFP-tagged cells of *P. s. pv. tomato* strain NS4 were found isolated and in small quantities in important colonization niches, such as in the trichome base and between the depressions along the junctions of adjacent epithelial cells (Fig. 7). On the other hand, on the phylloplane not treated with the antagonists, the strain NS4 was found in high amounts, efficiently colonizing the foliar surface, forming large clusters at the trichome base and between the depressions along the junctions of adjacent epithelial cells (Fig. 7f). The observation of the colonization pattern of the epiphytic bacteria on the phylloplane by SEM confirmed their ability in establishing at specific sites on the foliar surface (Fig. 8). Notably, the formation of cell aggregates could be observed in the depressions along the junctions of adjacent epithelial cells (Fig. 8A and B), besides the colonization of the stomatal region (Fig. 8b and c). As expected, the treatment with water did not present bacterial colonization (Fig. 8e and f).



**Fig. 7** Photomicrographs under epifluorescence microscope of living cells of *P. s. pv. tomato* strain NS4 expressing the GFP, on tomato leaf surfaces. Single bacterial cell at trichome base (a) and between the depressions along the junctions of adjacent epithelial cells (b) on leaf sprayed with *B. amyloliquefaciens*; single bacterial cells at trichome base (c) and between the depressions along the junctions of adjacent epithelial cells (d) of leaf sprayed with *B. pumilus*; large bacterial cluster formation at trichome base (e) and between the depressions along the junctions of adjacent epithelial cells (f), on tomato leaf surface not sprayed with endophytic bacteria. (Bars = 20  $\mu$ m)



**Fig. 8** Scanning electromicrographs on abaxial tomato leaves four days after exposure to treatments with *B. amyloliquefaciens*, *B. pumilus* and water (control). *B. amyloliquefaciens* aggregates at the stomatal region, ostiole (a) and between the depressions along the junctions of adjacent epithelial cells (b); *B. pumilus* aggregates at the stomatal region and ostiole (c), as well as between the depressions along the junctions of adjacent epithelial cells (d); e and f represent the control treatments

## DISCUSSION

The *P. syringae* pv. *tomato* transformation by electroporation using the plasmid pNKGFP originated four GFP-marked strains. However, our studies demonstrate that the transformed phyto bacteria on the tomato phylloplane presented lower population levels over 10 days, compared to the wild-type. Especially we observed variations in the survival capacity over time of each phyto bacteria transformed. Probably that is due the random integration of GFP-cassettes in the chromosome of the transformed phyto bacteria, therefore causing interference in the adaptive capacity of those strains when associated to the host plant surface. Our assumption becomes more solid when compared to the studies of Monier and Lindow (2003) on the bean plant phylloplane. The authors observed that for *P. syringae* strain B728a constitutively expressed GFP marker gene, presented a lower survival rate in comparison with the wild-type strain B728a. We also observed a fast population reduction after four days of inoculation of the transformed phyto bacteria on the tomato phylloplane. That is probably related to the depletion of nutrients on the foliar surface, given that other studies have shown that bacterial growth on plants is limited by carbon availability (Wilson and Lindow 1994; Mercier and Lindow 2000; Leveu and Lindow 2001).

All of the GFP-marked strains submitted to the pathogenicity test caused hypersensitive response, characterized by necrosis resulting from localized plant cell death at the infection site (Klement 1982; Dixon and Lamb 1990). The major hypothesis to explain resistance proposes that a receptor present in the plant (encoded by *R* genes) specifically recognizes the molecule called an elicitor, which is produced by an *avr* gene in the pathogen (Klement 1982; Yang et al., 1997; Lanna Filho and Resende 2009). In the compatibility test all of the GFP-marked strains were capable of causing the bacterial speck. However,

variation in the virulence of each *GFP* gene bearing phyto-bacteria caused different disease severity levels (Fig. 3). Our bioassays with the GFP-marked strains showed that only the strain NS4 was similar to the wild-type strain NW, suggesting that this strain can be used as a model for elucidation of phenomena involved in the pathogenesis of tomato plants. The antagonists probably acted on the reduction population of the NW and NS4 by multiple mechanisms, such as competition for space and nutrients and antibiosis. (Lindow and Brandl 2003; Monier and Lindow 2004, 2005; Lanna Filho et al. 2010). In addition, Campos Silva et al. (2008) reported the antibiosis effect by these antagonists against *P. s. pv. tomato*. Considering, species of *Bacillus* synthesize large amounts of metabolites that can act as antimicrobials against other microbes and, or act as elicitors of the resistance induction (Ongena et al. 2007). Additionally, one cannot discard the hypothesis of the occurrence of competition for space and nutrients by those antagonists, it even taking place on the phylloplane by bacteria of an endophytic nature. That hypothesis can be sustained by the study of Compant et al. (2005), which demonstrated large colonization by the endophytic *Burkholderia* sp. strain PsJN in the stomatal region and between depressions along the junctions of adjacent epithelial cells of leaves of *Vitis vinifera*. Therefore, the endophytic bacteria could occupy niches that could be occupied by phyto-bacteria and other pathogens, because the nutrients unavailable for the survival of those phytopathogens. Although the bacteria *B. pumilus* and *B. amyloliquefaciens* are of endophytic nature, it was observed high population levels of those bacteria on the tomato phylloplane, after 10 days of spraying (Fig. 4). It could show that those microbes are highly adaptable to the environmental conditions the phylloplane. That observation can raise new discussion on the function of the endophytic bacteria in the biocontrol of plant diseases, because it was not known that those microbes could survive so long on phylloplane as observed with epiphytic bacteria in other studies (Monier and

Lindow 2004, 2005). Therefore, those microbes present wide physiological versatility, could survive on phylloplane, as well as penetrating through natural openings, such as stomates and hydathodes, arriving at the apoplast and systemically colonizing the plant.

This study evidenced the reduction of the bacterial speck by the endophytic, with only the spraying of the tomato plants with a suspension of their live cells. As observed in Figure 6, the disease levels of plants exposed to the two antagonists are similar to the copper based chemical treatment, the chemical compound most used in agriculture against plant pathogenic bacteria. Possibly the reduction of the disease severity by the antagonists is related to the occupation of niches important for survival and penetration of the phyto-bacteria, such as: substomatal chambers, hydathodes, trichome base and cavities between the depressions along the junctions of adjacent epithelial cells (Fig. 8) or the phenomenon of resistance induction could also be occurring, leading the plant to an “induction state”, given that other studies have shown that endophytic bacteria act as an elicitor of this phenomenon (Kloepper and Ryu 2006).

On the phylloplane treated with the endophytic bacteria, we observed low density of NS4 GFP-tagged cells confirming the biocontrol potential of the tested antagonists. Additionally, in the substomatal chambers the presence of NS4 GFP-tagged cells was also not observed (data not shown). This fact suggests that the endophytic bacteria may be competing for space and/or nutrients, as well as antimicrobial synthesis that inhibit the growth of the strain NS4. Campos Silva et al. (2008) report the antibiosis mechanism by the endophytic *B. pumilus* and *B. amyloliquefaciens* against the plant pathogenic bacterium *P. syringae* pv. *tomato* supporting our statement on the involvement of the antimicrobial synthesis by the endophytic that could inhibit the growth of the strain NS4 on the phylloplane. Our studies clearly demonstrate that endophytic strains efficiently colonize the surface of tomato leaves occupying

specific niches, such as: ostiole, stomatal region and cavities between the depressions along the junctions of adjacent epithelial cells, probably interfering the colonization of *P. syringae* pv. *tomato* strain NS4 (Fig. 8).

The studies present in this work evidenced the important action of the endophytic bacteria *B. pumilus* and *B. amyloliquefaciens* in the biocontrol of the bacterial speck, as well as the useful of a new *P. s.* pv. *tomato* GFP-marked that may be used as a model for the elucidation of the interactions among phytobacteria-antagonist-host, or phytobacteria-host. We also reported the capacity of the endophytic *Bacillus* strains to survive under the environmental conditions of the tomato phylloplane, increase the knowledge of those microorganisms in association with plant hosts, as well as eventually lead to the development of a bioproduct. Finally, our data showed in this work strongly contribute to the better understanding of how the endophytic bacteria act on bacterial speck biocontrol, besides proposing the use of the plant pathogenic bacterium *P. s.* pv. *tomato* strain NS4 as model to elucidate phenomena involved in the ecology, epidemiology and pathogenesis of tomato plants.

#### **ACKNOWLEDGEMENTS**

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## ARTIGO 2

### **Proteins synthesized by two endophytic bacteria inducing resistance in tomato against bacterial spot**

Prepared in accordance with the Antonie van Leeuwenhoek  
(Preliminary version)

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## ABSTRACT

The fractions 42 and 75 corresponding to protein peaks of *Bacillus pumillus* (BPP2) and *Bacillus amyloliquefaciens* (BAP2) showed to act as elicitors of IR (Induced Resistance) in tomato plants against the plant pathogenic bacterium *Xanthomonas vesicatoria*. The endophytic bacteria were cultivated in a nutrient poor medium until they reached the inflection point of the exponential growth phase and soon afterwards were precipitated by centrifugation. The precipitate was discarded and the supernatant lyophilized, filtered in a 0.22 µm membrane, dialyzed in a 12 kDa cut-off point membrane and fractionated in a column containing Sephacryl S-300 HR equilibrated with PBS buffer. Fractions that presented the highest protein concentrations were sprayed on tomato plants, and after four days, the test pathogen was inoculated. The protein peaks BPP2 and BAP2 corresponding to the fractions 42 and 75 controlled the phyto-bacteria efficiently, in comparison to the control (water). Additionally, the protein peaks promoted the increase of the POX and PPO enzyme activity in the plant, characterizing the phenomenon of resistance induction. In SDS-PAGE stained with silver nitrate, the protein fractions 42 and 75 showed as simple bands with estimated molecular mass at 28 and 43 kDa, respectively. Thus for the first times, we reported the synthesis of two macromolecules of proteic nature from endophytic bacteria that act as resistance induction elicitors in tomato plants against the *X. vesicatoria*.

**Keywords:** Biological control, induction resistance, *Xanthomonas vesicatoria*, *Bacillus pumilus*, *Bacillus amyloliquefaciens*.

## RESUMO

As frações 42 e 75, correspondentes aos picos proteicos de *Bacillus pumilus* (BPP2) e *Bacillus amyloliquefaciens* (BAP2), atuaram como eliciadoras de indução de resistência em plantas de tomate contra *Xanthomonas vesicatoria*. As bactérias endofíticas foram cultivadas em meio pobre até atingirem o ponto de inflexão na fase exponencial de crescimento e, em seguida, sedimentadas por centrifugação. O sedimento foi descartado e o sobrenadante liofilizado, filtrado em membrana de 0,22 µm, dializado em saco de diálise com 12 kDa de ponto de corte e fracionado em coluna contendo Sephacryl S-300 HR equilibrada com tampão PBS. Frações que apresentaram as maiores concentrações de proteína foram pulverizadas em plantas de tomate e, após quatro dias, foi inoculado o patógeno teste. Os picos proteicos BPP2 e BAP2, correspondentes as frações 42 e 75, controlaram eficientemente a fitobactéria, comparados ao controle (água). Adicionalmente, os picos proteicos promoveram, em planta, o aumento da atividade das enzimas POX e PPO, caracterizando o fenômeno de indução de resistência. Em SDS-PAGE corado com nitrato de prata, as frações proteicas 42 e 75 foram reveladas como bandas simples com massa molecular estimada em 43 e 28 kDa, respectivamente. Dessa forma, reporta-se, pela primeira vez, a síntese de duas macromoléculas de natureza proteica por bactérias endofíticas que atuam como eliciadoras de indução de resistência em plantas de tomate contra a *X. vesicatoria*.

Palavra-chave: Biological control, indução de resistência, *Xanthomonas vesicatoria*, *Bacillus pumilus*, *Bacillus amyloliquefaciens*.

## INTRODUCTION

Endophytic bacteria or, endophytes, are common inhabitants of the internal tissues of various plants species (Strobel et al., 2004). The vast majority survive internally without causing harmful effects to the plant and some can provide beneficial effects, favoring phytohormones synthesis, resistance induction and the biological control against pathogens (Ryan et al., 2008). Many endophytic bacteria are common members of the genus *Bacillus* (Lodewyckx et al., 2002). This genus is known by the wide synthesis of low molecular weight secondary metabolites, including antibiotics, volatile organic compounds (VOCs), siderophores and surfactants. However, very little is known about the macromolecule synthesis by those microbes that can promote disease control in plants, mainly when dealing with proteins with antimicrobial activity and, or elicitors defense responses.

Recent studies demonstrated that macromolecules of proteic nature synthesized by rhizobacteria of the genus *Bacillus* can have an important antifungal function (Kavitha et al., 2005) and induce resistance against phytopathogens (Romeiro et al., 2005; 2010). Those works are important to understand the biological control mechanisms at molecular and biochemical levels, and they can elucidate phenomena that controls at molecular the operation of the secretory system of Gram-positive bacteria in the endophytic-plant interactions (Tjalsma et al., 2000; 2004).

The defense responses in plants elicited by macromolecules of proteic nature are usually related to systemic acquired resistance (SAR) (Wei et al., 1992; 1995; Tanaka et al., 2001). However, Romeiro et al. (2005; 2010) evidenced there is induced systemic resistance (ISR) in tomato by a protein synthesized by the rhizobacteria *B. cereus*. The studies with proteins synthesized

by endophytic bacteria can provide bioproduct development with higher stability, viability and activity in plants. That is the reality for harpin protein synthesized by bacteria of the genera *Erwinia*, *Ralstonia* and *Pseudomonas* (Wei et al., 1992; 1995; 2000; Tanaka et al., 2001; Dayan et al., 2009), which generated Messenger® and ProAct® bioproducts with wide spectrum action against phytopathogens of various crops (Wei et al., 1992; 1995; 2000; Dayan et al., 2009).

The present study report the synthesis of macromolecules of proteic nature by the endophytic bacteria *B. pumilus* and *B. amyloliquefaciens* that induce resistance in tomato plants (*Solanum lycopersicum* L.) against the bacterial spot caused by *X. vesicatoria* (ex. Doidge) Vauterin et al. (1995).

## **MATERIALS AND METHODS**

Experiments were carried out at the Laboratory of Plant Bacteriology, Department of Plant Pathology, at Federal University of Lavras, Minas Gerais, Brazil. Santa Cruz 'Kada' tomato plants were grown in a non-sterilized mixture of soil, sand and manure (2:1:1, v/v/v) in a greenhouse at room temperature ( $28 \pm 2$  °C) and relative humidity of 70 %.

### **Microorganisms, their origin and cultivation**

Endophytic bacteria *Bacillus pumilus* (BP) and *Bacillus amyloliquefaciens* (BA) were previously isolated from tomato stem and acted as efficient biocontrol agents (Campos Silva et al., 2008). Then, they were grown in 523 medium (Kado and Heskett, 1970) and preserved in a deep freezer at -80 °C, emulsified once in 30 % (v/v) glycerin (Gerhardt, 1994). *X. vesicatoria* was obtained from the collection of the Plant Bacteriology Lab and was grown in 523 medium and preserved at -80°C, emulsified once in 30 % glycerin (Gerhardt, 1994).

### **Endophytic bacteria cultivation in poor medium**

For this experiment, the endophytic BP and BA were grown in a modified minimal liquid medium of Simmons (SM) (Simmons, 1926) in which citrate was replaced by 0.1 % (w/v) glucose as the sole carbon source (Gijsegem et al., 1995; 2000) who point out that detection of macromolecules given off by bacteria in supernatant is easier after their cultivation in minimal culture media. The turbidity of liquid cultures in side-arm flasks under continuous shaking, at

room temperature ( $28 \pm 1$  °C), was recorded at different time intervals to determine the inflection point in the exponential phase of the growth curve.

### **Preparation of supernatant**

Endophytic cells were grown in a SM. At the inflection point of the exponential growth phase, cells were removed from suspension by centrifuging once ( $10.000 \times g$  for 15 min.) in a RC-5C Sorvall refrigerated centrifuge. The supernatant was filtered under sterile conditions through a cellulose membrane of 0.22  $\mu\text{m}$  pore size, transferred into dialysis bags (cut-off = 12 kDa) and dialyzed with continuous stirring against 400 volumes of phosphate-buffered saline (PBS) (0.1 M; adjusted to pH 7.0) at 4 °C. The PBS was changed several times during the 48 h of dialysis at 4 °C. Dialysates were lyophilized and stored at -20 °C for later usage.

### **In vitro bioassays**

The putative toxic activity of supernatants against tomato pathogenic bacteria was verified by the overlay diffusion method (Vidaver et al., 1972). Melted semisolid culture media [0.8 % (w/v) agar, 48 °C] containing propagules ( $10^8$  cfu.mL<sup>-1</sup>) of every pathogenic bacteria [*Clavibacter michiganensis* subsp. *michiganensis* (Cmm), *Pseudomonas syringae* pv. *tomato* (Pst), *Pseudomonas syringae* pv. *syringae* (Pss), *Pseudomonas corrugate* (Pc), *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc), *Ralstonia solanacearum* (Rs) and *X. vesicatoria* (Xv)], was poured into Petri dishes containing a basic layer of solid water-agar [2 % (v/v)], in an amount enough for a 1 mm-thick overlay. After solidification, a cork borer was used to produce a 0.5 cm<sup>2</sup> well in the central part of the overlay and 30  $\mu\text{L}$  of supernatant (2 mg.mL<sup>-1</sup>) placed in the cavity.

Additionally, as negative control we used PBS (0.1 M, pH = 7.0) and SM. Bioassay plates were moved to an incubator (28 °C) and checked daily for one week for inhibition halo presence. For each bacterial pathogen the bioassay was repeated thrice.

### **Exposure of tomato plants to the supernatants**

Fifteen days after sowing tomato plants, cv. Santa Cruz 'Kada', were sprayed with a suspension of *BP* and *BA* ( $OD_{540} = 0.3$ , corresponding to approximately  $10^8$  cfu.mL<sup>-1</sup>), supernatants of endophytic *B. pumilus* (SBP) and *B. amyloliquefaciens* (SBA) (2 mg.mL<sup>-1</sup>), acibenzolar-S-methyl (ASM) (0.05 g.L<sup>-1</sup> water), SM and PBS (0.1 M; pH 7.0) used as controls. Four days later, plants were inoculated with *Xv* by spraying with an inoculum suspension ( $OD_{540} = 0.2$ , corresponding to approximately  $10^8$  cfu.mL<sup>-1</sup>). In all cases, plants were kept in a moist chamber for 24 h before and after inoculation. Inoculated plants were maintained in a greenhouse and lesions counted after disease symptoms had fully developed. All experiment was performed in a completely randomized design with five replicates per treatment. The results were subjected to analysis of variance and means were compared by Tukey' test ( $p < 0.05$ ) using the software Statistica®, version 7.0 (Statsoft, 2005).

### **Partial resolution of supernatants by gel filtration**

The purification system was established in a refrigerator at 4 °C and composed by a peristaltic pump P-1 from Pharmacia Biotech. The Sephacryl S-300 HR column 60 cm long and 2 cm in diameter was equilibrated with PBS (0.1 M; pH 7.0). The flow rate was set at 15 drops.min<sup>-1</sup>, [equivalent to 0.37 mL.min<sup>-1</sup> (0.22 mL.h<sup>-1</sup>)]. The column void volume was calculated with Blue

Dextran ( $2 \text{ mg.mL}^{-1}$ ). Successive 2 mL aliquots ( $2 \text{ mg.mL}^{-1}$ ) of the lyophilized supernatants of each endophyte was applied into the column, from which 2 mL fractions were collected by means of a BioRad model 2110 fraction collector, and protein monitored by the absorbance at 280 nm. Protein in the supernatants was quantified according to Bradford (1976) by means of a Microplate Spectrophotometer PowerWave™ XS, and the results were expressed as bovine serum albumin (BSA) equivalents.

### **Exposure of plants to the protein peaks**

The fractions corresponding to protein peaks of *B. pumilus* 1 (BPP1), *B. pumilus* 2 (BPP2), *B. amyloliquefaciens* 1 (BAP1), *B. amyloliquefaciens* 2 (BAP2) and *B. amyloliquefaciens* 3 (BAP3) obtained by gel filtration in a Sephacryl S-300 HR column, were sprayed ( $1.0 \text{ mg.mL}^{-1}$ ) on tomato plants, cv. Santa Cruz 'Kada', ten days after germination. At the same time endophyte cells ( $10^8 \text{ cfu.mL}^{-1}$ ), ASM ( $0.05 \text{ g.L}^{-1}$  water), and PBS (0.1 M; pH 7.0) were also sprayed. Four days later, plants were inoculated with *Xv* by spraying with an inoculum suspension ( $10^8 \text{ cfu.mL}^{-1}$ ). In all cases, plants were kept in a moist chamber for 24 h before and after inoculation. Inoculated plants were maintained in a greenhouse and lesions counted after disease symptoms had fully developed. The experiment was performed in a completely randomized design with four replicates per treatment. The results were subjected to analysis of variance and means were compared by the Tukey test ( $p < 0.05$ ) using the software Statistica®, version 7.0 (Statsoft, 2005).

### **Determination of enzyme activities**

To evaluate the activity of guaiacol peroxidase (POX) and polyphenol oxidase (PPO), tomato seeds were sowed in 500 mL pots containing non-sterilized mixture of soil, sand and manure, maintained in a greenhouse under the same environmental conditions as noted above. Ten-day-old tomato plants were sprayed with *BP*, *BA*, *BPP2*, *BAP2*, *ASM*, *PBS* and water (control). Treated and control plants were harvested at 0.5, 1, 2, 4, 5, 6 and 7 days after spraying. In an additional trial, 10-day-old plants were sprayed with the test treatments and 4 days later they were inoculated with 100 mL *X. vesicatoria* cell suspension ( $10^8$  cfu.mL<sup>-1</sup>). Leaves were harvested at 4, 5, 6 and 7 days after spraying. The leaves were excised and frozen in liquid nitrogen and immediately utilized. Fresh leaf material (2.0 g) from treated and control tomato plants was homogenized with a mortar and a pestle in 3mL of ice-cold 50 mM sodium acetate buffer, pH 5.2. After filtration through cheesecloth, the homogenate was centrifuged at  $13.000 \times g$  for 15 min. and the supernatant (crude extract) used as the source of enzymes. All the steps were carried out at 0-4 °C. Protein content of the crude extracts was determined using the Bradford (1976) protein assay, using BSA as a standard.

The activity of POX was determined according Urbanek et al. (1991), adding 25  $\mu$ L of the crude extract preparation to 2 mL of a solution containing 50 mM sodium acetate buffer, pH 5.2, 20 mM guaiacol, and 20 mM hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). After incubation at 30 °C for 10min, the absorbance was read at 480 nm. One POX unit of activity (UA) was expressed as the variation of 1U of absorbance at 480 nm.mg<sup>-1</sup> of soluble protein per minute (UA mg P<sup>-1</sup> min<sup>-1</sup>). PPO was determined by adding 50  $\mu$ L of the crude extract to 3 mL of a solution containing 100 mM potassium phosphate buffer, pH 6.5, and 25 mM pyrocatechol. The increase of absorbance at 410 nm, for 10 min at 30 °C, was

measured (Gauillard et al., 1993). One PPO unit was expressed as the variation of absorbance at 410 nm.mg<sup>-1</sup> of soluble protein per minute and expressed as UA per milligram protein per minute (UA mgP<sup>-1</sup> min<sup>-1</sup>). All experiments were arranged in a randomized block design with three blocks and one experimental unit (plot) consisted of a 500 mL pot containing a single plant. The results were subjected to analysis of variance and means were compared by the Tukey test ( $p < 0.05$ ) using the software Statistica®, version 7.0 (Statsoft, 2005).

### **Determination of molecular weight by SDS-PAGE**

The fractions 42 and 75 corresponding to BPP2 and BAP2 obtained by gel filtration in a Sephacryl S-300 HR column were electrophoresed in a discontinuous buffer system using 5 % stacking gel (pH 6.8) and 10 % separating gel (pH 8.8) in Tris-glycine buffer (pH 8.3) according to Laemmli (1970). Prior to SDS-electrophoresis, the protein peaks (10 µg in 30 µL) was mixed with an equal volume of sample buffer (100 mM Tris-HCl buffer, 4% SDS, pH 6.8) containing 5 % β-mercaptoethanol. A mixture of standard marker proteins (Genei Pvt. Ltd.) was used. The electrophoresis was conducted at a constant current of 90 V per sample in a slab gel measuring 170 x 150 x 1.5 mm. The gel was silver stained and scanned by the Vilber Lourmat Gel documentation system with Bioimage software.

## RESULTS

The growth of the endophytic bacteria *BP* and *BA* in minimal medium reached the stationary phase in approximately 14 hours. Additionally, in the exponential phase, the inflection point was reached at 0.45 of absorbance after 10 hours of microbial growth (Figure 1). According to Gijsegem et al. (1995; 2000) the cultivation of bacteria in nutrient-poor medium facilitates detection of macromolecules liberated in the supernatant by the bacteria. Thus, the inflection point at 0.45 of absorbance was chosen as ideal for the detection of a macromolecule candidate for a resistance induced elicitor.

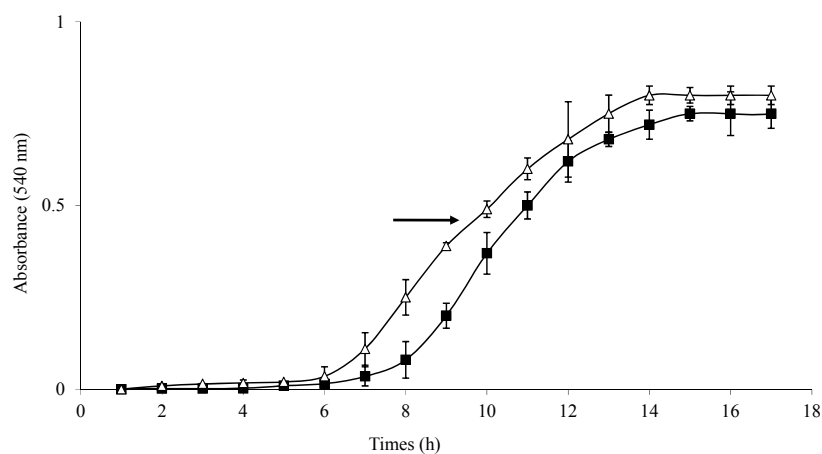


Figure 1 Growth curve of *B. pumilus* (Δ) and *B. amyloliquefaciens* (■) on modified liquid Simmons medium at 28 °C. The inflection point in the exponential growth phase was reached at 0.45 absorbance, as shown by the arrow (→). Each data point indicates means and vertical bars represent the standard error of the means

The concentrated supernatant (2 mg.mL<sup>-1</sup>) of each endophytic bacterium submitted to the *in vitro* bioassay by the overlay diffusion method (Vidaver et al., 1972) did not show toxic activity against the phyto-bacterias *Cmm*, *Pst*, *Pss*,

*Pc*, *Pcc*, *Rs* and *Xv* (data not shown). Additionally, the PBS used as control and the Simmons medium also did not present toxic activity against the tested phyto-bacteria, as was expected. Experiments repeated three times in a greenhouse demonstrated that tomato plants of cv. Santa Cruz 'Kada' sprayed with 2.0 mg.mL<sup>-1</sup> of SBP and SBA reduced the severity of the bacterial spot caused by *Xv*, compared to the SM and the PBS (control) (Figure 2). SBP and SBA reduced the severity of the bacterial spot by 56.6 and 58 %, on mean, compared with the PBS-pre-treated controls. The best mean protection against *Xv* was conferred upon tomato plants by *BA* which reached 67.2 % protection in comparison with PBS-pre-treated controls. Soon afterwards, the best protection means against the phyto-bacteria were provided by *BP* and ASM with protection of 63.4 and 62.2 %, respectively. The SM was tested in order to eliminate any doubt as to its effect on the control of the disease. In the greenhouse bioassays it did not differ from the control treatment, presenting high levels of disease severity. As ASM is a well-known plant protection inducer of resistance, it was used as a standard to allow comparison of the effectiveness and action mode of SBP, SBA, *BP* and *BA* used in this current study.

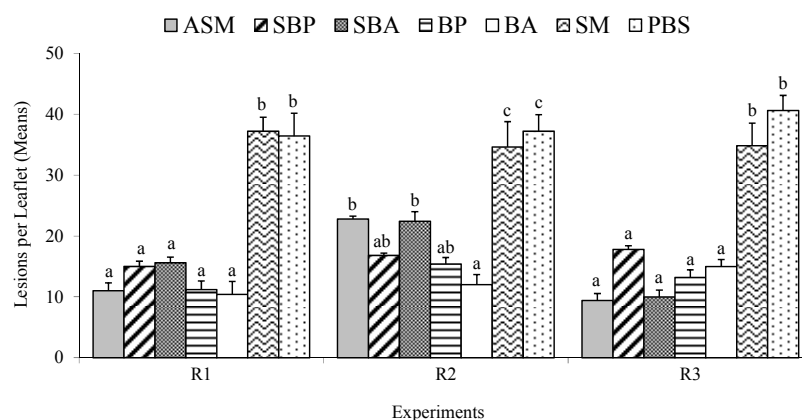


Figure 2 Severity of bacterial spot (*Xv*) inoculated on Santa Cruz 'Kada' tomato plants, four days after exposure to ASM, SBP, SBA, BP, BA, SM and PBS (control) treatments. The bioassay was repeated three times (R1, R2 and R3). Means followed by same letter do not differ by the Tukey test ( $p < 0.05$ ). The columns represent the means and vertical bars represent the standard error of the means

In the gel filtration chromatography profile containing Sephacryl S-300 HR the SBP presented the protein peaks BPP1 and BPP2, while SBA showed three protein peaks BAP1, BAP2 and BAP3 (Figure 3). Each proteic peak was sprayed on tomato plants under a greenhouse, in an experiment repeated twice. Thus, we observed that the BPP2 and BAP2 peaks corresponding to SBP and SBA presented activity against the bacterial spot, when compared to the PBS control (Figures 4A and B). Figure 4A shows that ASM presented the highest bacterial spot control mean, with 65.2 % protection compared with PBS-pre-treated controls. In addition, the treatments with BPP2 and BP also obtained control averages of 63.5 and 59.6 %, respectively. Figure 4B shows that the best control averages against the phytobacteria were provided by BAP2 and BA with protection of 56.6 and 54.7 %, respectively. ASM also presented good results in the reduction of the severity of the disease, with 44.4 % control compared with PBS (control). The protein peaks BPP1, BAP1 and BAP3 did not protect tomato

plants against the bacterial disease, not differing from the controls treatment (PBS). In SDS-PAGE the protein peaks BPP2 and BAP2 appeared as a single protein band in gel stained red with silver nitrate. The proteins presented an estimated molecular mass of 28 and 43 kDa, respectively (Figure 5). The proteins correspond to the fractions 75 and 42, collected after gel filtration (Figure 3).

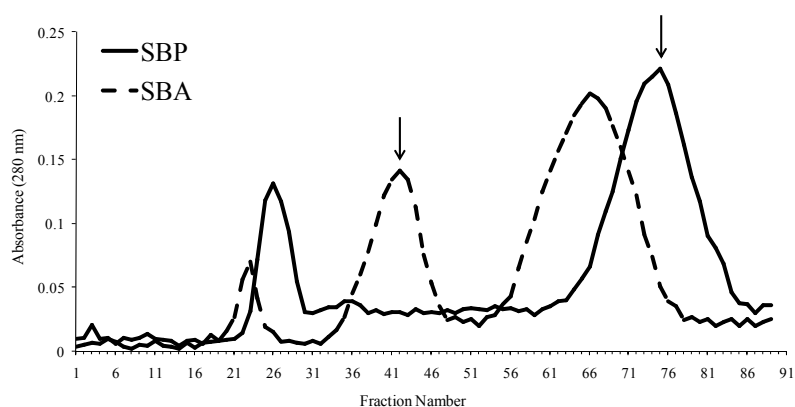


Figure 3 Purification of supernatants of SBP and SBA by gel filtration. Two milliliters of each supernatant containing  $2 \text{ mg.mL}^{-1}$  BSA equivalents was loaded on a Sephacryl S-300 HR (0.5 x 60 cm) column. The protein peaks BPP2 and BAP2 indicated by the arrows ( $\rightarrow$ ) correspond to the fractions 42 and 75, respectively. The purification of the supernatants was repeated seven times

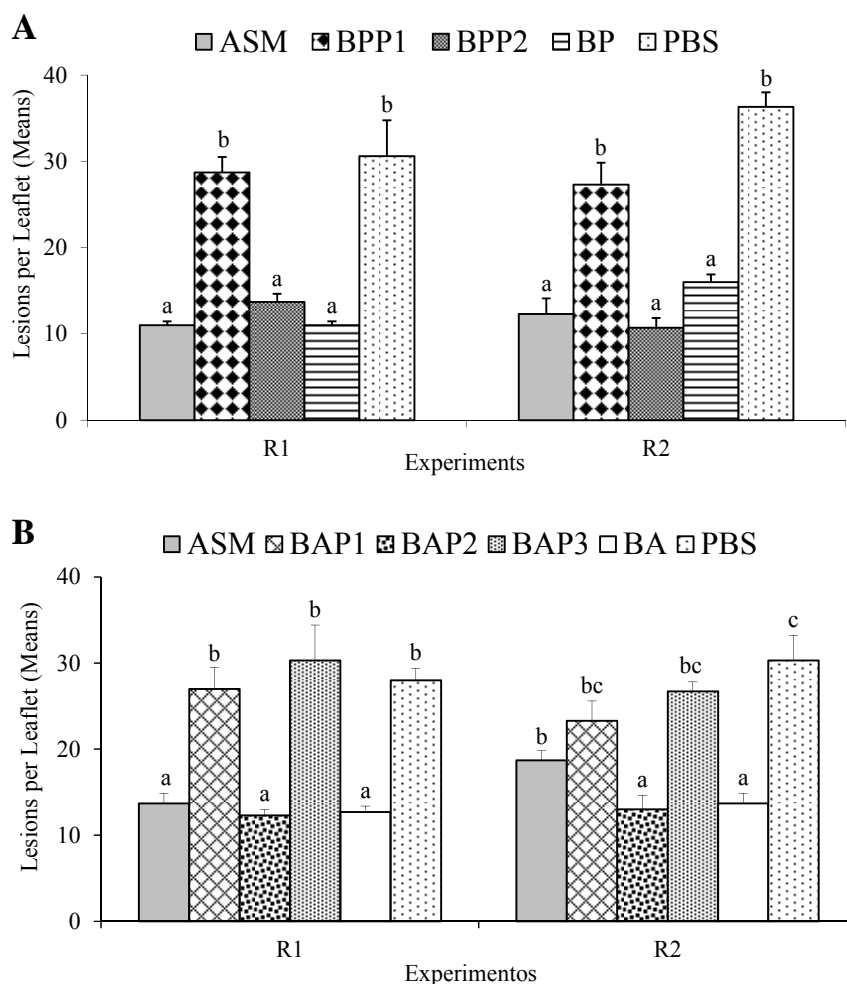


Figure 4 Severity of bacterial spot (*Xv*) inoculated on Santa Cruz 'Kada' tomato plants, four days after exposure to treatments: (A) ASM, BPP1, BPP2, BP and PBS (control); (B) ASM, BAP1, BAP2, BAP3, BP and PBS (control). The bioassay was repeated twice (R1 and R2). Means followed by same letter do not differ by the Tukey test ( $p < 0.05$ ). The columns represent the means and vertical bars represent the standard error of the means

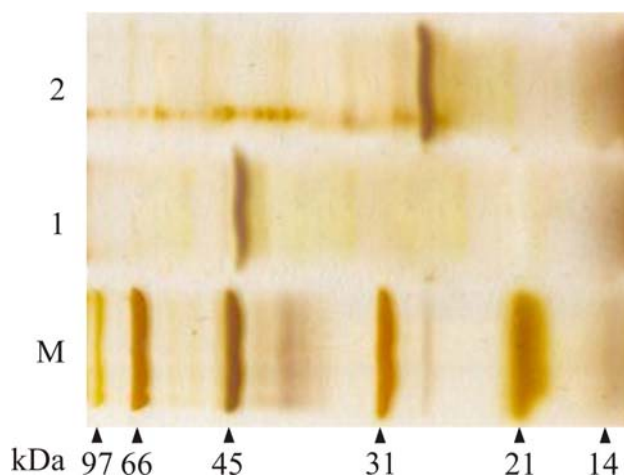


Figure 5 Gel electrophoresis analysis of protein fractions 42 (BAP2) and 75 (BPP2). Line M, broad range standard protein markers (phosphorylase, 97.4 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; soyabean trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa); lines 1 and 2, correspond to fractions 42 (43 kDa) and 75 (28 kDa). Samples of the protein peaks were submitted to SDS-PAGE and stained for proteins with silver nitrate

After spraying on tomato plants, the protein peaks BPP2 and BAP2, clearly induced the POX and PPO enzyme increase, but among the treatments, ASM was that which induced the highest accumulation of enzymes tested in the tomato tissue, in comparison to the control (water) (Figures 6 and 7A-C). The PBS was sprayed on tomato plants in order to eliminate any suspicion of its inductor effect, because it was used to elute the proteins in the gel filtration column. The results of the buffer were similar to the control, there was no observed increase of the POX and PPO enzymatic activity after pulverization (Figures 6 and 7D). The increase of the activity of the tested enzymes was also observed in plants previously sprayed with BPP2, BAP2 and ASM, and 4 days later inoculated with *Xv* (Figures 6 and 7 A-C). Plants sprayed with ASM and inoculated with the phyto bacteria after 4 days, presented a significant increase of

POX after 48 h (Figure 6C). In plants pre-sprayed with PBS and later inoculated with the phyto bacteria, the increase of the activity of the tested enzymes was not observed, being similar to the control (Figures 6 and 7D). The activity of PPO in plants inoculated with *Xv* increased after 48 h, for all of the treatments (Figure 7 A-D). However, the highest accumulation of that enzyme was observed in plants previously sprayed with BPP2, BAP2 and ASM, when compared to PBS and the control (water).

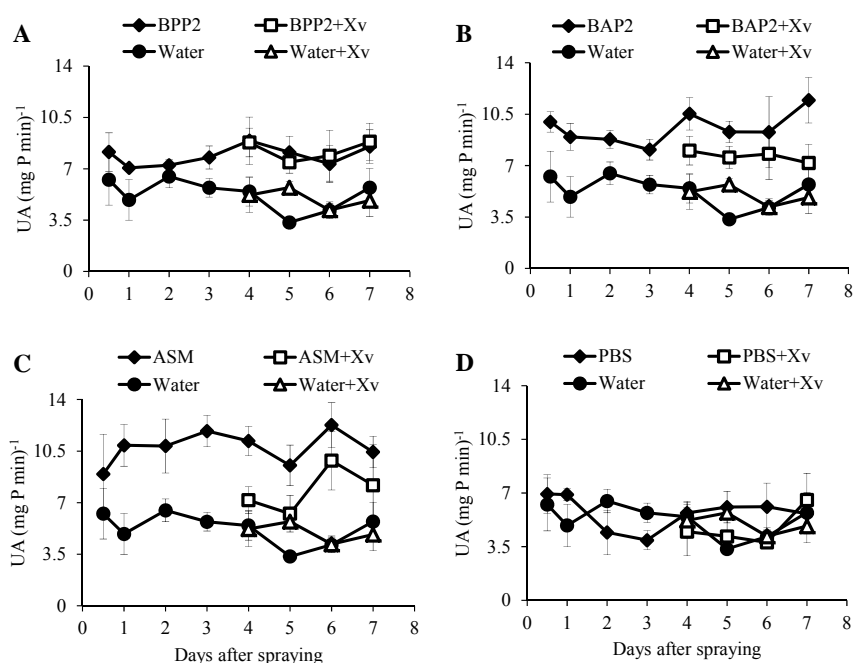


Figure 6 Activity of phenol peroxidase (POX) in leaves of tomato cv. Santa Cruz 'Kada'. Ten days after sowing tomato plants, cv. Santa Cruz 'Kada' were sprayed with treatments: (A) peak of *B. pumilus* (BPP2), (B) peak of *B. amyloliquefaciens* (BAP2), (C) Acibenzolar-S-Methyl (ASM) and (D) Water (control). Enzymatic responses were evaluated at 0.5, 1, 2, 3, 4, 5, 6 and 7 days (◆ ; ●) and 5, 6, 7 and 8 days (□ ; Δ) after inoculation of phyto bacteria *Xv*. Each data point indicates the means and vertical bars represent the standard error of the means

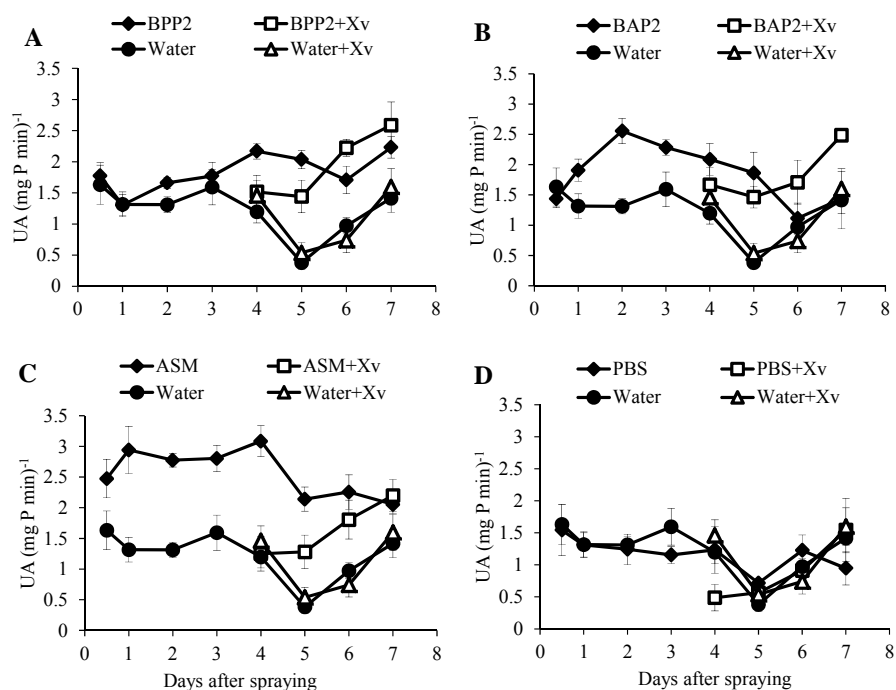


Figure 7 Activity of polyphenol oxidase (PPO) in leaves of tomato cv. Santa Cruz 'Kada'. Ten days after sowing tomato plants, cv. Santa Cruz 'Kada' were sprayed with treatments: (A) peak of *B. pumilus* (BPP2), (B) peak of *B. amyloliquefaciens* (BAP2), (C) Acibenzolar-S-Methyl (ASM) and (D) PBS Buffer and Water (control). Enzymatic responses were evaluated at 0.5, 1, 2, 3, 4, 5, 6 and 7 days (◆ ; ●) and 4, 5, 6 and 7 days (□ ; △) after inoculation of phyto-bacteria *X. vesicatoria*. Each data point indicates the means and vertical bars represent the standard error of the means

## DISCUSSION

The Simmons medium was selected as a basal minimal medium, based on the observation by Gijsegem et al. (1995; 2000) that macromolecules synthesized by bacteria and exported into the environment are easily detected in the supernatants of a poor culture medium. Our study confirmed a higher ease of macromolecule detection in the modified Simmons medium, as also observed by Romeiro et al. (2005) in studies with a plant growth promoting rhizobacteria (PGPR). Additionally, the inflection point at 0.45 absorbance in the exponential growth phase was chosen in the growth curve of endophytic bacteria. Before this point, the target macromolecules might not have been synthesized and exported from the bacterial cells, whereas afterwards, although export processes might have occurred, cells could have used these molecules as carbon and nitrogen sources, as they were growing in a minimal medium (Madigan et al., 2003).

The supernatants of the endophytic bacteria did not present toxic activity, *in vitro*, against the tested pathogens. However, when sprayed on plants, they drastically reduced the severity of the bacterial spot of the tomato. That phenomenon can be explained by one of the criteria of Steiner and Schönbeck (1995), which deals with the absence of toxic effects of the inducer agent on the defiant pathogen. Therefore, it can be evidenced that products synthesized and released by the endophytic bacteria in the poor medium would act as elicitors of the defense response in tomato, impeding the pathogenesis of the phytobacteria. In that way, plants in “induction state” would have the defense system on alert, prepared for possible pathogen invasion. Compared to our studies, Romeiro et al. (2005) report the absence, *in vitro*, of toxic effects of the proteic supernatant of PGPR *Bacillus cereus* strain UFV-101 against tomato pathogens. However the authors observed that when immersing the root system of tomato plants in the proteic supernatant of PGPR, the plants became resistant

to the pathogens, characterizing the phenomenon of resistance induction. This result sustains our evidence; although the supernatant of the endophytes does not present toxic activity against pathogens, it acts as a resistance inducer.

The previous spraying of SBP and SBA on tomato plants clearly reduced the severity of the bacterial spot. This result sustains the fact that there are macromolecules synthesized and released in the poor medium that are promoting resistance induction. That can be affirmed only because the supernatant was submitted to the dialysis inside the bags with cut-off = 12 kDa and filtered in 0.22  $\mu\text{m}$  membrane. We understood that the protecting effect of the supernatant is intimately related to contact among macromolecules that are recognized by receptors in the plant tissue, and later they trigger biochemical signals that culminate in the defense against pathogen invasion (Choudhary et al., 2007; Hammond-Kosack and Jones, 2002). Our studies compare to those conducted by Romeiro et al. (2010), which report that there is resistance induction in tomato leaves previously sprayed with supernatants of the PGPR *B.cereus* strain UFV-101, against the pathogen *Corynespora cassicola*, thus sustaining our evidence that macromolecules present in the supernatant of the endophytic bacteria are elicitors of resistance induction.

Our study also reports the biocontrol of the endophytic bacteria against the bacterial spot on tomato phylloplane (Figures 2 and 4). Although endophytic bacteria have the interior of plant tissue as a habitat, they can perfectly adapt to conditions on the foliar surface, surviving those environmental conditions for a certain amount of time (Compant et al., 2005). This characteristic leads us to believe that the endophytic *BP* and *BA*, in contact with the plant surface, can continuously synthesize and release proteins with defense response eliciting action in the environment, and at the same time exercise biocontrol mechanisms, such as competition for space and nutrients. These facts explain the success of those microbes in the control of the phyto-bacteria on the phylloplane, as shown

in our studies (Figure 2). The SM was tested by us in the spraying of the tomato plants. However, no effect of the culture medium in the control of the bacterial disease in tomato plants was observed as reported by Romeiro et al. (2005). Our studies with ASM only confirm its protecting effect on tomato plants against *Xv*, as reported in numerous works (Silva et al., 2003; Cavalcanti et al., 2006ab)

The fractions 42 and 75 corresponding to protein peaks BPP2 and BAP2 obtained by gel filtration demonstrated a control of the bacterial spot severity in tomato plants in greenhouse. Additionally the molecular weight of those proteins was determined by SDS-PAGE with silver nitrate stain, presenting masses of 28 and 43 kDa (Figure 5). We can affirm that the purified proteins sprayed on plants promoted the resistance induction, controlling the disease. This fact can be compared to studies carried out by Romeiro et al. (2010), who attributed the control of the target spot by a protein synthesized by a PGPR elicitor. Although the authors did not determine the precise molecular weight of the protein, they only affirmed that it had a molecular weight above 12 kDa. Additionally the molecular weights of the proteins purified in that work are in agreement with most of the proteins synthesized by antagonistic bacteria that have effect against pathogens (Gaudriault et al., 1998; Kavitha et al., 2005; Wong et al., 2008). However, those works do not report the resistance induction eliciting activity of proteins synthesized by *Bacillus* species, but only the toxic activity against pathogens. The exception only occurs for the harpin group of proteins that are synthesized by Gram-negative bacteria (Wei et al., 1992; Gaudriault et al., 1998; Wei et al., 2000). Our data clearly demonstrate two new proteins with induction action synthesized by endophytic bacteria of the genus *Bacillus*, whose control efficiency compares to ASM, the main resistance inducer used in the tomato crop.

The highest accumulation of the POX and PPO enzymes in tomato plants after spraying of the BPP2, BAP2 and ASM treatments demonstrates the

SAR phenomenon (Hammond-Kosack and Jones, 2002). Notably, SAR is quickly triggered after the plant tissues enter in contact with the elicitor agent, and is characterized by the increased expression of the large number of pathogenesis-related genes (*PR* genes), in both local and systemic tissues (Loon, 1997; Durrant and Dong, 2004). ISR occurs in response to a non-pathogenic inductor and the expression of PR proteins is delayed, in a *priming* effect (Loon et al., 1998; Heil and Bostock, 2002; Choudhary et al., 2007). Another important observation of our study is reported to the high activity of the POX and PPO enzymes in plants pre-sprayed with BPP2 and BAP2 and 4 days later, inoculated with *Xv*. That high enzymatic activity clearly demonstrates that the plants were in the “induction state”, because the activation of SAR had already occurred 4 days before the spraying of the treatments, as shown in the times of 0.5, 1, 2, 3 and 4 days, at Figures 6 and 7 A-C. In that way, the plant, when noticing the presence of the pathogen intruder, quickly triggered defense responses to impede the success of pathogenesis. Our studies demonstrate for the first time the inducing activity of two proteins synthesized by endophytic bacteria of the genus *Bacillus*, on the control of bacterial spot. That discovery can elucidate important phenomena in the biocontrol of endophytic bacteria against economically important diseases, as well as supply a basis for the development of new bioproducts with molecules potentially more efficient in the control of diseases.

ASM used in our studies as a base for comparison of BPP2 and BAP2, has been exhaustively studied for years as potential chemical resistance inducers and SAR priming (Cavalcanti et al., 2006b). Here, it demonstrated efficient control over bacterial spot, inducing a large increase of the POX and PPO enzymes in tomato plants, as seen in other works (Silva et al., 2003; Cavalcanti et al., 2006b). POX is involved in numerous plant physiological processes along the plant life cycle, among them, lignin and suberin deposition associated with

enhanced resistance toward pathogen attack (Boudet, 1998). It is also involved in the biosynthetic route of lignin promoting the generation of hydroxyls (OH<sup>•</sup>), which act directly against pathogens. Another important function of this enzyme is the elimination of free radicals toxic to the plant with the conversion of H<sub>2</sub>O<sub>2</sub> into H<sub>2</sub>O and O<sub>2</sub> (Resende et al., 2007). PPO also plays an important role in the events against the invasion of pathogens, they act as ‘scavengers’ and catalyze the oxidation of phenolic compounds to *o*-quinones, which possess antibiotic properties (Mohammadi and Kazemi, 2002).

In conclusion, the systematic study of macromolecules of a proteic nature synthesized by endophytic bacteria that act as systemic induction resistance elicitors can lead to the elucidation of the phenomena involved in the defense response of plants against phytopathogens. Our studies raise a new discussion on the involvement of those microbes in the biocontrol of diseases, since they synthesize resistance inducing proteins. Studies of this nature are not common for Gram-positive bacteria, especially for *Bacillus*, but stand out in Gram-negative bacteria, as in the case of harpins (Wei et al., 1992b; Gaudriault et al., 1998; Wei et al., 2000). The first publication of a resistance inducing protein synthesized by a type of *Bacillus* (Romeiro et al., 2005; 2010), opened the doors for deeper investigations on the biocontrol exercised by Gram-positive beneficial bacteria, thus making it possible to elucidate phenomena involved in protein synthesis that eventually can lead to bioproduct development with higher efficiency in the control of phytobacteria and other pathogens.

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