MICHELE CARLA NADAL

PLANT GROWTH PROMOTING BACTERIA ON *in vitro* ROOTING OF *Pyrus sp.*

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Dissertação apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Agronomia/Fitotecnia, área de concentração em Produção Vegetal, para a obtenção do título de Mestre.

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Ficha catalográfica elaborada pelo Sistema de Geração de Ficha Catalográfica da Biblioteca Universitária da UFLA, com dados informados pelo(a) próprio(a) autor(a).

Nadal, Michele Carla.
Plant growth promoting bacteria on *in vitro* rooting of *pyrus sp.* / Michele Carla Nadal. - 2019.
52 p. : il.

Orientador(a): Moacir Pascoal.
Coorientador(a): Joyce Dória Soares, Adalvan Daniel Martins.
Dissertação (mestrado acadêmico) - Universidade Federal de Lavras, 2019.
Bibliografia.

1. Identificação de bactérias. 2. Isolamento de bactérias. 3. Microrganismos. I. Pascoal, Moacir. II. Soares, Joyce Dória. III. Martins, Adalvan Daniel. IV. Título.

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APROVADA em 12 de novembro de 2019.

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Aos meus pais, Rozane e Leonir.

Aos meus irmãos, Michel e Ariele.

Ao meu namorado, José Henrique.

Dedico

AGRADECIMENTOS

Agradeço a Deus, por ter guiado meu caminho e colocado em minha vida pessoas boas, que me proporcionaram muitos momentos de alegria.

A todos os meus colegas de pós-graduação do laboratório, em especial à Eduarda, Graciele e Cecília, que foram as primeiras a me receberem. Carrego com muito carinho os momentos divididos com todos vocês.

Aos técnicos, Vantuil e Sulamara, muito obrigada por tudo, sem o trabalho que vocês exercem o laboratório não seria um dos melhores da universidade. O fato de, constantemente estarem buscando o estabelecimento de novas plantas, cultivando para a manutenção as espécies que existem nas salas de crescimento, treinando estagiários, e realizando experimentações, faz do laboratório um excelente lugar de aprendizagem.

Aos estagiários, especialmente ao Otávio, à Nathalia, Carla e Rejane, obrigada por me auxiliarem durante a execução e nas avalições dos experimentos. Aos demais estagiários, Lilian, Arthur, Ana e Bruna, também agradeço, pois sempre foram prestativos e me auxiliaram em determinadas tarefas. Desejo a vocês, futuros colegas, muito sucesso.

Aos pós-doutorandos Filipe e Gustavo, obrigada pelas trocas de ideias e pelo convívio agradável. Obrigada Filipe, pelo auxílio na execução de técnicas de cultura de tecidos, e pelos *feedbacks* dos trabalhos avaliativos. Gustavo, obrigada por ter sido meu parceiro, pelo auxílio no planejamento e execução de toda a metodologia relacionada à microbiologia. Sem a tua ajuda esse experimento não teria sido realizado.

Aos meus orientadores, professor Moacir, professora Joyce e Adalvan, muito obrigada pela oportunidade de trabalho, pelos esforços despendidos, pelo auxílio financeiro para a realização do meu mestrado. Tenho muita gratidão e admiração pela equipe de trabalho que vocês formam. Realizar meu mestrado sob a orientação de vocês me fez crescer muito como profissional. Sempre me lembrarei da frase dita pelo professor Moacir na minha qualificação: “para que dificultar, se podemos auxiliar e facilitar a vida das pessoas”. Acredito que esta frase resume a atitude de vocês frente à minha orientação, uma conduta de auxílio e apoio no desenvolvimento do trabalho. Obrigada por terem me recebido no laboratório, e terem sido sempre prontos a me ajudar.

Aos meus queridos amigos que me receberam em Lavras, Célio, Dimi, Rodrigo e Dona Dalva, obrigada por terem alegrado meus dias! Pelas boas conversas, risadas,

churrascos! Por terem me ajudado em tudo que precisei, me consolando, dado apoio, e torcido pelo meu sucesso. Vou carregá-los no meu coração para sempre.

Aos meus amigos de longa data, que mesmo longe pareciam estar perto (risos), sempre foram meus parceiros, Josi, Dianini e Luisa. Não tenho palavras para descrever o quanto vocês me animam. Ao Maurício e ao Pará, pela parceria, conselhos e boas risadas.

Aos meus amigos que conheci nesse um ano e meio de UFLA, Adalvan, Duda, Carol, Graci, Thiago e Ramon. Obrigada por dividirem comigo momentos tão divertidos, por me alegrarem, pelas conversas, pelos "memes", por serem seres humanos espetaculares!

À minha família, por sempre me apoiar e torcer por mim. Apesar da saudade, obrigada pai e mãe, por terem me criado para voar e por passarem segurança, de que se um dia eu precisar voltar, vocês me receberão. Obrigada Michel e Ariele, por serem os melhores irmãos do mundo. Obrigada aos meus sogros, dona Ana e seu José, por apoiarem a mim e ao José Henrique, e nos ajudarem em tudo que precisamos.

Ao meu namorado, José Henrique, obrigada por todo o apoio durante o mestrado. Sem você teria sido muito estressante. Obrigada pela imensa ajuda com as avaliações e as estatísticas do trabalho. Admiro-te imensamente pelo profissional que és, pela paciência que tens, pelo respeito e pela dedicação em tudo que faz. Fostes o maior presente que a vida me deu. Obrigada, do fundo do meu coração.

Ao CNPq, CAPES e FAPEMIG pelo apoio financeiro aos projetos de pesquisas e concessão de bolsas aos membros da equipe.

Ao professor Leo Rufatto da Universidade do Estado de Santa Catarina (CAV-UDESC) pela concessão do material vegetal utilizado no experimento.

À Universidade Federal de Lavras, ao Departamento de Agricultura, e a todos os professores que contribuíram para a minha formação acadêmica, assim como aos colegas de mestrado e doutorado que dividiram sala de aula comigo. Agradeço pelas experiências trocadas.

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Código de Financiamento 001.

MUITO OBRIGADA!

“Se tens fé, cumpre saberes que tudo é possível àquele que a tem.”

(Mateus, 17:20)

RESUMO

Plantas da família das Rosáceas, como as do gênero *Pyrus*, apresentam capacidade de enraizamento *in vitro* reduzida, sendo necessário o uso de reguladores de crescimento. Em muitos casos, também é verificada a presença de bactérias no meio de cultura, o que leva ao descarte de plantas, porém, sabe-se que inúmeras bactérias apresentam capacidade de promover crescimento vegetal. Buscou-se investigar a presença destes microrganismos na cultura de tecidos vegetais de porta-enxerto de *Pyrus communis*, isolando, identificando e selecionando bactérias produtoras de auxina. Além disso, os microrganismos selecionados foram inoculados *in vitro* verificando o efeito no processo de enraizamento das plantas e o comportamento na fase de aclimatização. O trabalho demostrou a existência de microrganismos endofíticos na cultura de tecidos vegetais das seleções 'OHxF87' e 'PDW'. Foi observada a predominância do gênero *Acinetobacter* e baixa diversidade da população de microrganismos oriundos de plantas *in vitro,* sendo isoladas bactérias das espécies *Acinetobacter ursingii, Bacillus subtilis,* e *Micrococus leteus*. A seleção dos microrganismos foi realizada através do teste de produção de auxina, visto a dificuldade que estas plantas apresentam no enraizamento. Dentre todos os microrganismos identificados, 30,36% apresentaram capacidade de produção de auxina. Ao realizar a inoculação *in vitro*, duas estirpes *A. ursingii* demonstraram eficiência semelhante à auxina sintética para o genótipo 'OHxF87'. Observou-se variação de resposta dependendo do genótipo utilizado. Os clones oriundos da seleção 'PDW' apresentaram enraizamento inferior quando inoculados com microrganismos, porém, ainda sim, ocorreu enraizamento utilizando estirpes de *A. ursingii*. Na fase de aclimatização, todas as plantas apresentaram um alto nível de enraizamento, favorecendo o desenvolvimento dos porta-enxertos.

Palavras-chave: Identificação de bactérias. Isolamento de bactérias. Microrganismos.

ABSTRACT

Plants of the Rosaceae family, such as those of the *Pyrus* genus, have reduced *in vitro* rooting ability, requiring the use of growth regulators. In many cases, bacteria are also observed in the culture medium, leading to plants being discarded. However, it is known that numerous bacteria have the ability to promote plant growth. This study sought to investigate the presence of these microorganisms in plant tissue culture of *Pyrus communis* rootstocks by isolating, identifying, and selecting auxin-producing bacteria. The selected microorganisms were also inoculated *in vitro* to assess their effect on the rooting process of plants and their behavior during the acclimation phase. The results showed the presence of endophytic microorganisms in plant tissue cultures of the 'OHxF87' and 'PDW' selections. A predominance of the genus *Acinetobacter* and low diversity of the microorganism population originating from *in vitro* plants were observed, and the bacterial species *Acinetobacter ursingii*, *Bacillus subtilis*, and *Micrococcus luteus* were isolated. Microorganism selection was performed using the auxin production test, given the difficulty of rooting these plants. Of all the identified microorganisms, 30.36% had auxin production ability. In the *in vitro* inoculation, two *A. ursingii* strains showed efficiency similar to that of synthetic auxin for genotype 'OHxF87'. A genotype-dependent variation in response was observed. The clones derived from the 'PDW' selection showed reduced rooting when inoculated with microorganisms; however, rooting occurred using the *A. ursingii* strains. During the acclimation phase, all plants showed a high level of rooting, favoring the development of rootstocks.

Keywords: Bacteria identification. Bacteria isolation. Microorganisms.

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PRIMEIRA PARTE

1 INTRODUÇÃO

Nas últimas décadas, o uso de microrganismos para promover crescimento vegetal tem sido objeto de estudo em diversas pesquisas. Estes trabalhos demonstram a existência de relações benéficas entre microrganismos e plantas, assim como, a capacidade que diferentes microrganismos têm em promover o crescimento vegetal.

O benefício para as plantas em sua relação com microrganismos pode ocorrer de maneira direta por meio da fixação biológica de nitrogênio, solubilização de fosfato e produção de hormônios, por exemplo; ou por meio de contribuições indiretas, como o controle biológico, a antibiose e a resistência sistêmica induzida.

Ao abordar técnicas utilizadas na cultura de tecidos vegetais, sabe-se que além do meio nutritivo adequado, é extremamente importante o balanço hormonal para a garantia de sucesso dos processos. Espécies lenhosas pertencentes à família das Rosáceas, como ameixeira, pessegueiro, macieira e pereira, são de difícil estabelecimento *in vitro*, apresentando desde problemas com contaminações, até dificuldade de obtenção de boas taxas de enraizamento e sobrevivência *ex vitro*.

Apesar da cultura de tecidos vegetais, buscar sempre plantas livres de qualquer microrganismo, não é totalmente conhecido o impacto que possíveis endofíticos podem gerar no processo de micropropagação de plantas.

Diante do exposto, pode-se perguntar: Apesar de todos os cuidados estabelecidos nos protocolos *in vitro*, é possível que existam microrganismos endofíticos em explantes *in vitro*? Ainda: Se existem microrganismos endofíticos colonizando plantas estabelecidas *in vitro*, quais as funções que exercem para o desenvolvimento vegetal?

Para estes questionamentos o número de respostas ainda é pequeno, fazendo-se necessários mais estudos. Este trabalho teve como objetivo geral investigar a presença e o impacto de microrganismos endofíticos em plantas micropropagadas de *Pyrus communis*. Os objetivos específicos foram: isolar, identificar e selecionar bactérias produtoras de auxina de plantas micropropagadas de *Pyrus sp.* e inoculá-las *in vitro* verificando o efeito no processo de enraizamento *in vitro* e o comportamento na fase de aclimatização.

2 REFERENCIAL TEÓRICO

2.1 Aspectos gerais sobre a cultura

A pera é uma fruta de clima temperado, com sabor agradável e delicado. Possui ampla aceitação pelo mercado consumidor, sendo consumida principalmente *in natura*, em pratos doces e salgados, além de possuir alto valor nutricional (SILVA et al., 2014).

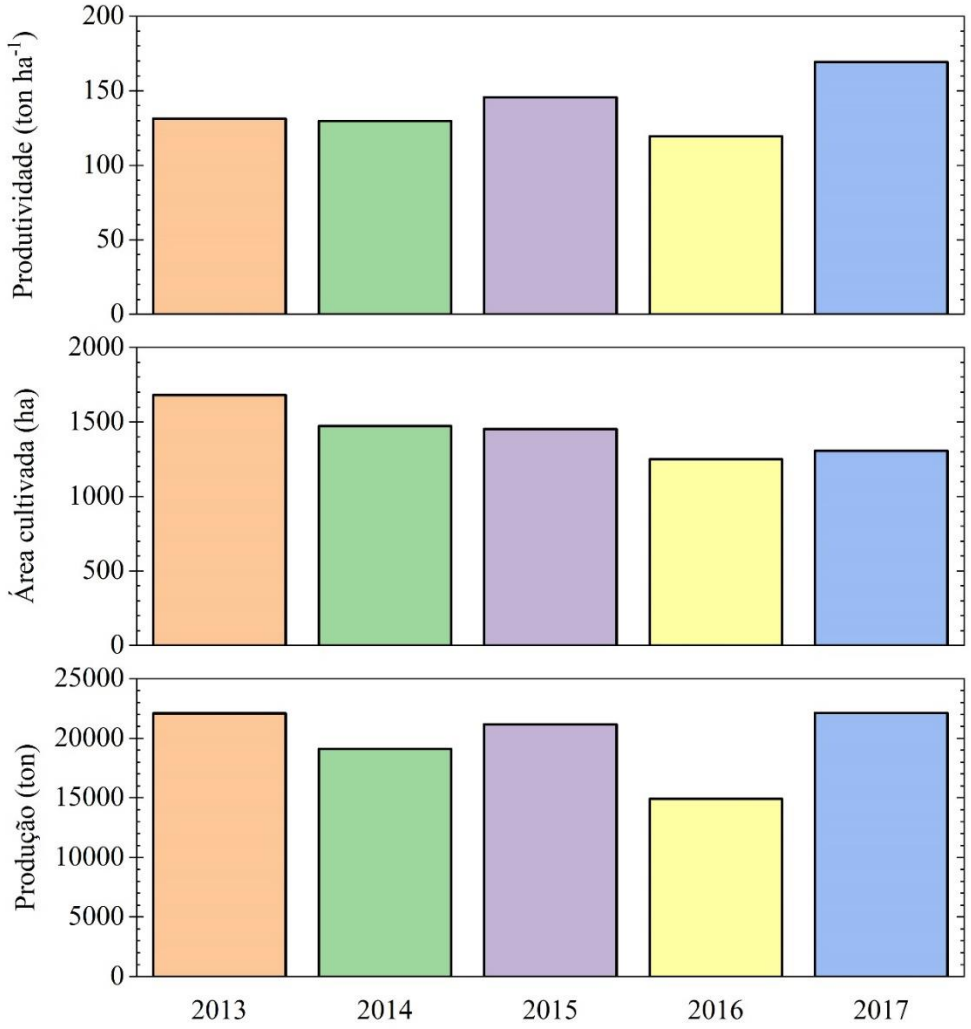
Pertencente ao gênero *Pyrus* e à família das Rosáceas, estudos mostram que a pereira é uma frutífera milenar. Pesquisas com base nas características estruturais e análise filogenética identificaram pares de genes duplicados, que datam ocorrência do gênero entre 30 a 45 milhões de anos atrás (HOU et al., 2018).

Comercialmente, o gênero é dividido em dois grandes grupos: as peras europeias e as peras asiáticas. O primeiro grupo apresenta textura encorpada e formato alongado, e o segundo grupo apresenta textura arenosa e corpo arredondado. As características organolépticas fazem da pera uma das principais frutas produzidas no mundo (FAO, 2019; LAYNE; QUAMME, 1975).

No Brasil, a produção de peras europeias é concentrada nos estados do Rio Grande do Sul e Santa Catarina, e de peras asiáticas nos estados do Paraná, Santa Catarina e São Paulo, sendo que as cultivares Housui, Nijisseiki e Kousui são as mais plantadas no sul do país, e as cultivares Atago e Okusankichi destacam-se no estado de São Paulo (FAORO; ORTH, 2010).

Nos últimos cinco anos ocorreram poucas variações na produção da fruta no Brasil. Em 2013, foram produzidas 22.078 toneladas e, na última safra, 2017, a produção foi de 22.108 toneladas, uma variação de apenas 100 kg. Houve redução na área produzida e aumento de produtividade da fruta, como pode ser observado na Figura 1 (FAO, 2019).

Figura 1 - Área cultivada, produção e produtividade de peras no Brasil.



Fonte: FAO (2019).

Apesar do grande mercado interno consumidor, a cultura não apresenta destaque entre as frutíferas de clima temperado cultivadas. A expansão desta frutífera no país, e a melhoria dos índices de produtividade, são limitados principalmente pela falta de material genético, os quais se incluem porta-enxertos adequados (RUFATO et al., 2012), além problemas técnicos, como a baixa frutificação efetiva da cultura no sul do país (HAWERROTH et al., 2011).

Os porta-enxertos são fundamentais no processo de formação de frutíferas, visto que interferem no desenvolvimento e vigor da copa, precocidade de produção, quantidade e qualidade da produção, bem como na capacidade de adaptação às condições edafoclimáticas desfavoráveis, e resistência a pragas e doenças (HARTMANN et al., 2002).

Comumente, o marmelo (*Cydonia oblonga* Mill.) é usado como porta-enxerto para pereira. No entanto, uma das características deste porta-enxerto é a incompatibilidade de enxertia com algumas cultivares comerciais importantes, além da suscetibilidade à clorose de

ferro induzida por calcário (MARINO et al., 2013). Além do marmeleiro, o *Pyrus calleryana* apresenta-se como um dos porta-enxertos mais utilizados no Brasil, principalmente por sua adaptação ao clima subtropical e tropical.

Novos materiais vêm sendo estudados nos programas de melhoramento vegetal brasileiros, como exemplo, os porta-enxertos 'OHxF87' e 'PDW', promissores em plantios de alta densidade.

A série de clones OHxF (Old Home x Farmingdale) é originária do *Pyrus communis*, amplamente utilizada na América do Norte, e como características essa série auxilia na precocidade, produtividade e qualidade de algumas cultivares europeias de pera (ERCISLI et al., 2006), sendo que o clone 'OHxF87' é um dos melhores da série. Como características particulares, possui porte semi-anão e compatibilidade com a maioria das variedades de pereiras europeias e asiáticas (APAL, 2019).

Da maneira similar, o clone 'PDW' ou Pyrodwarf (Old Home x Bonne Luise d'Avranches) também é originário do *Pyrus communis*, possui boa compatibilidade com as variedades de pereira europeia e asiática, baixa suscetibilidade à clorose do ferro (WSU, 2019).

No que se refere à propagação da espécie, o cultivo *in vitro* de plantas tem se tornado cada vez mais comum. Diferentemente de outros métodos de propagação vegetativa, a propagação *in vitro,* ou micropropagação, permite o controle de variáveis responsáveis pelo desenvolvimento da planta, como a formação de raízes. A técnica também propicia produção rápida e eficiente de mudas, mantendo-se as características agronômicas oriundas da planta matriz.

De maneira geral, a propagação *in vitro* de espécies lenhosas da família das Rosáceas apresenta menor adaptabilidade à cultura de tecidos *in vitro,* principalmente no processo de enraizamento, o que reduz o sucesso da micropropagação (GRIMALDI et al., 2008).

2.2 Enraizamento *in vitro* de *Pyrus* sp

A sinalização genética para indução de formação de raízes ocorre por diferentes mecanismos, dentre os principais estão concentrações especificas de auxina ou de seus análogos sintéticos. As auxinas são conhecidas como morfógenos, à medida que são utilizadas para induzir a formação de embriões de células somáticas, contribuindo para a formação e manutenção do meristema apical de raiz (TAIZ et al., 2017).

Concentrações deste hormônio nas formas de ácido indol-3-acético (AIA), ácido indol-3-butírico (AIB), ácido naftaleno-acético (ANA), têm garantido o sucesso no enraizamento de cultivares e genótipos de diferentes espécies de pereiras, como *Pyrus communis, P. pyrifolia, P. calleryana, P. amygdaliformis, P. pyraster, P. syriaca, P. betulifolia,* e *P. bretschneideri* (BELL; REED, 2002).

Além dos reguladores, os resultados de enraizamento podem variar dependo do meio de cultura utilizada e da exposição dos explantes à luminosidade. Estudo com *P. elaeagrifolia* mostra que o uso AIB, em meio MS, com permanência das plantas durante 10 dias no escuro, promove maior taxa de enraizamento para a espécie (AYGUN; DUMANOGLU, 2015), já para a espécie selvagem *P. ussuriensis,* as maiores taxas de enraizamento foram obtidas em meio MS ½ suplementado com AIB e AIA (YANG et al., 2017).

Em relação a *P. communis*, Silva et al. (2018) relatam que, para algumas seleções de porta-enxerto desta espécie, o meio QL modificado por Leblay promove resultados satisfatórios em todas as fases de cultivo *in vitro*. No entanto, também é relatado que para clones oriundos de cruzamento com *P. communis*, o meio MS suplementado com AIB, promove o enraizamento e o sucesso na aclimatização das plantas (LIZÁRRAGA et al., 2017), bem como, o meio MS½ suplementado com AIB e ácido giberélico (GA3), promove o enraizamento para clones de Pyrodwarf (RUŽIĆ et al., 2011).

Os resultados relatados na literatura recente demonstram que para diferentes espécies de *Pyrus*, o enraizamento *in vitro* não ocorre sem a utilização de reguladores hormonais sintéticos, assim como corroboram Sun et al. (2009), demonstrando que a eficiência do enraizamento é dependente do genótipo.

2.3 Bactérias endofíticas na cultura de tecidos vegetais

Nas últimas décadas o uso de microrganismos para promover crescimento vegetal tem sido objeto de estudo em diversas pesquisas. Esse fato mudou a forma como contaminações bacterianas eram vistas na cultura de tecidos vegetais, promovendo o interesse em investigar o impacto que microrganismos endofíticos podem gerar no processo de micropropagação das plantas (QUAMBUSCH; WINKELMANN, 2018).

Segundo Andrews e Hirano (1992), todos os microrganismos que habitam órgãos da planta, ou que em algum momento da vida podem colonizar tecidos internos dos vegetais, sem causar danos aparentes, são considerados microrganismos endofíticos. Estes

microrganismos podem ser fungos, bactérias, actinobactérias ou leveduras. Dentre suas características, está a modulação do metabolismo vegetal, podendo estimular o crescimento e desenvolvimento de maneira direta ou indireta.

Dentre os mecanismos diretos de promoção de crescimento, Olanrewaju et al. (2017) destacam a produção de hormônios vegetais como auxinas, citocininas e giberilinas, a liberação de enzimas como a ACC deaminase, a fixação de nitrogênio, a solubilização de fosfato, a produção de sideróforos, e a absorção de ferro. Dentre os indiretos, o controle biológico, a antibiose, e a resistência sistêmica induzida.

No que diz respeito à cultura de tecidos vegetais, existem alguns fatores que afetam o desenvolvimento dos explantes, os quais são cuidadosamente controlados, como temperatura, luminosidade e meio de cultura. O meio de cultura fornece às plantas todos os nutrientes necessários para seu desenvolvimento. Além dos nutrientes, acrescenta-se ao meio, sacarose, vitaminas, agente estruturante, e em muitos casos, hormônios vegetais sintéticos. Estes são utilizados para propiciarem melhor balanço hormonal aos explantes e promoverem multiplicação e enraizamento das plantas (ANDRADE, 2002).

Estudos relados por Orlikowska et al. (2017) têm demostrado que algumas bactérias endofíticas também beneficiam explantes *in vitro*, promovendo aumento da multiplicação e enraizamento, bem como qualidade dos processos de organogênese e embriogênese. Além disso, as bactérias podem ser benéficas durante o processo de aclimatização das plantas, encurtando o período de enraizamento, aumentando o crescimento de parte aérea, e o número de brotações (RUSSO et al., 2008; VETTORI et al., 2010).

Como o meio de cultura utilizado para propagação de plantas, apresenta condições ideais de desenvolvimento, com variações dependendo das espécies, dificilmente nota-se a presença de endofíticos. Esses microrganismos só são revelados a partir do momento que amostras do tecido vegetal são transferidas para meios de cultivo bacterianos específicos, como foi observado em *Prunus avium* e *Malus* sp., ambas pertencentes à família das Rosáceas (QUAMBUSCH; WINKELMANN, 2018).

2.4 Produção de auxina por bactérias promotoras de crescimento vegetal

A auxina é um dos hormônios vegetais indispensáveis para o crescimento e desenvolvimento das plantas, sendo que o ácido indol-3-acético (AIA) é a auxina natural mais comum produzida por plantas, bactérias e fungos.

A auxina foi inicialmente descoberta em plantas, no século XIX, pelo botânico alemão Julian Von Sachs, o qual observou o movimento de plantas em resposta à luz e à gravidade, propondo que esta resposta era dada devido à formação de substâncias endógenas. Esta ideia foi amparada por Charles e Francis Darwin e, posteriormente, identificada como AIA (TAIZ et al., 2017). Nas plantas, este hormônio desempenha papel central na divisão e alongamento celular, desenvolvimento de frutos e senescência. Além disso, a sua concentração é variável nos diferentes tecidos e espécies vegetais (TAIZ et al., 2017).

Atribuída especialmente à formação de raízes, em dicotiledôneas, a auxina induz a formação de raízes laterais, enquanto em monocotiledôneas a formação de raízes adventícias. Alguns estudos mostram que este hormônio está envolvido na coordenação espacial das divisões celulares do xilena, induzindo a formação dos elementos de vaso (HEO; BLOB; HELARIUTTA, 2017).

O mecanismo predominantemente utilizado para explicar os efeitos positivos de bactérias promotoras de crescimento vegetal é a capacidade de produção de auxina (OLANREWAJU et al., 2017). Diversas bactérias presentes na rizosfera apresentam essa capacidade, nesse caso, as moléculas de auxinas apresentam efeitos significativos na comunicação entre plantas e microrganismos, como parte da estratégia de colonização e, por consequência, promovem o crescimento das plantas, em uma relação de simbiose (SPAEPEN; VANDERLEYDEN, 2011; SPAEPEN; VANDERLEYDEN; REMANS, 2007)

A síntese de auxina, na maioria das bactérias, ocorre a partir do aminoácido L-triptofano, onde este é convertido em auxina por diferentes rotas. A rota conhecida como AIP é a mais comum, envolve uma reação de desaminação e descarboxilação para formação do ácido indol-3-ácético-pirúvico. Outras rotas são bastante estudas, como a rota TAM que tem como produto final a triptamina, a rota IAN com o ácido indol-3-acetonitrila (TSAVKELOVA et al., 2006). Além disso, outros compostos que estão ativamente envolvidos no anabolismo do AIA, em bactérias, foram relatados por terem atividade de auxina, como indol-3-acetamida, indol-3-piruvato, e o indol-3-acetaldeído (OLANREWAJU et al., 2017).

Recentemente, Rivera et al. (2018), estudando o metabolismo de cepas de *Azospirillum brasilense*, confirmaram produção de AIA somente na presença de L-triptofano, observando também que a biossíntese do AIA pode ser inibida pela presença de vários L-aminoácidos, provavelmente por desvio do metabolismo celular.

A inoculação com essas bactérias produtoras de auxina tem sido usada para estimular a germinação de sementes e acelerar o crescimento de raízes (MARTÍNEZ-VIVEROS et al., 2010). Também vem sendo aplicadas na cultura de tecidos vegetais com intuito de formar e aumentar o enraizamento de plantas (LARRABURU et al., 2007; PEÑAFIEL-JARAMILLO et al., 2016).

Como exemplo de algumas bactérias identificadas como promotoras de auxinas tem-se a *Azospirillum brasilense* (RIVERA et al., 2018), *Bacillus megaterium* (LÓPEZ-BUCIO et al., 2007), *Bacillus siamensis* (HOSSAIN et al., 2019), *Bacillus methylotrophicus* (PÉREZ-FLORES et al., 2017), *Pseudomonas veroni* (PEÑAFIEL-JARAMILLO et al., 2016), *Acinetobacter johnsonii* (SHI; LOU; LI, 2011), *Acinetobacter baumannii* (LIN; SHU; LIN, 2018).

3 CONSIDERAÇÕES FINAIS

Após o desenvolvimento dos experimentos, confirmou-se a existência de microrganismos endofiticos na cultura de tecidos vegetais em seleções de *Pyrus sp.* Foram isoladas bactérias das espécies *Acinetobacter ursingii*, *Bacillus subtilis* e *Micrococus leteus*, dentre as quais 30,36% apresentavam capacidade de produção de auxina.

Após a inoculação *in vitro*, verificou-se que duas estirpes selecionadas demonstram eficiência semelhante à auxina sintética para o genótipo ‘OHxF87’. As estirpes foram identificadas como *A. ursingii* strain 1 e 4.

Pôde-se observar que existe variação de resposta aos microrganismos dependo do genótipo utilizado, sendo que os clones oriundos da seleção ‘PDW’ apresentaram enraizamento inferior quando inoculados com microrganismos, porém, ainda assim, ocorre enraizamento utilizando as estirpes *A. ursingii* strain 1 e 2.

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SEGUNDA PARTE – ARTIGOS*

ARTIGO 1 MICROBIOLIZATION *IN VITRO* OF *PYRUS COMMUNIS* WITH *ACINETOBACTER URSINGII* TO STIMULATE RHIZOGENIC POTENTIAL

ABSTRACT

Plants of the Rosaceae family, such as those of the *Pyrus* genus, have reduced *in vitro* rooting ability, requiring the use of growth regulators. In many cases, bacteria are also observed in the culture medium, leading to plants being discarded. However, it is known that numerous bacteria have the ability to promote plant growth. This study sought to investigate the presence of these microorganisms in plant tissue culture of *Pyrus communis* rootstocks by isolating, identifying, and selecting auxin-producing bacteria. The selected microorganisms were also inoculated *in vitro* to assess their effect on the rooting process of plants and their behavior during the acclimation phase. The results showed the presence of endophytic microorganisms in plant tissue cultures of the 'OHxF87' and 'PDW' selections. A predominance of the genus *Acinetobacter* and low diversity of the microorganism population originating from *in vitro* plants were observed, and the bacterial species *Acinetobacter ursingii*, *Bacillus subtilis*, and *Micrococcus luteus* were isolated. Microorganism selection was performed using the auxin production test, given the difficulty of rooting these plants. Of all the identified microorganisms, 30.36% had auxin production ability. In the *in vitro* inoculation, two *A. ursingii* strains showed efficiency similar to that of synthetic auxin for genotype 'OHxF87'. A genotype-dependent variation in response was observed. The clones derived from the 'PDW' selection showed reduced rooting when inoculated with microorganisms; however, rooting occurred using the *A. ursingii* strains. During the acclimation phase, all plants showed a high level of rooting, favoring the development of rootstocks.

Keywords: Auxin. Bacteria identification. Bacteria isolation. Plant growth-promoting microorganisms.

1 INTRODUCTION

The pear tree is a temperate climate fruit tree belonging to the genus *Pyrus* and family Rosaceae. Micropropagation is a promising technique that is widely used for the production of seedlings of this species. However, woody species of the Rosaceae family have lower adaptability to plant tissue culture (GRIMALDI et al. 2008), and *in vitro* rooting is one of the main difficulties in the micropropagation process of these plants.

In addition to the difficulties related to rooting, the persistent presence of endogenous microorganisms is common in micropropagated plants, despite their rigorous establishment and subsequent *in vitro* culture protocols (ALI et al., 2018; ÖRGEÇ et al., 2018). This phenomenon has also been observed in *Prunus avium* and *Malus* sp. plants, both of which

belong to the family Rosaceae (QUAMBUSCH et al., 2016, 2014; QUAMBUSCH; WINKELMANN, 2018), and in tree plants such as *Pinus sylvestris* (PIRTTILÄ et al., 2008; POHJANEN et al., 2014).

Endophytic and epiphytic microorganisms may have the ability to modulate plant development and can be used to improve the multiplication and *in vitro* rooting of explants through increases in the quality of organogenesis and embryogenesis processes (ORLIKOWSKA et al., 2017). The use of plant hormones such as auxin in the form of indole-3-acetic acid (IAA) or indole-3-butyric acid (IBA) has typically ensured greater success in the rooting of this species (AYGUN; DUMANOGLU, 2015; YANG et al., 2017). However, synthetic auxins are classified as biochemical pesticides by the US Environmental Protection Agency and United States Department of Agriculture, and their use is controlled in some countries; thus, the use of microorganisms instead of synthetic hormones is an alternative to reduce the use of synthetic auxins in vegetative micropropagation (ERTURK et al., 2010; MONTERO-CALASANZ et al., 2013; PRETTY, 2008).

The mechanism predominantly used to explain the positive effects of growth-promoting bacteria is auxin production ability (OLANREWAJU et al., 2017). The synthesis of auxin by microbial metabolism usually requires the presence of the amino acid L-tryptophan, which can be converted to auxin via different pathways, such as IPyA, with indole-3-pyruvic acid as the final product, the TAM pathway, with tryptamine as the final product, and the IAN pathway, with indole-3-acetonitrile as the final product (TSAVKELOVA et al., 2006). In addition, other compounds that are actively involved in IAA synthesis in bacteria have been reported to possess auxin activity, such as indole-3-acetamide, indole-3-pyruvate, and indole-3-acetaldehyde (OLANREWAJU et al., 2017).

Several bacteria that are present in the rhizosphere produce phytohormones that regulate plant development (SPAEPEN et al., 2007; SPAEPEN; VANDERLEYDEN, 2011). Bacterial IAA is a reciprocal signaling molecule in plant-microorganism interactions, where the plant provides exudates containing nutrients and shelter for microorganisms, and the microorganisms provide auxin, which is essential for root development (MALHOTRA; SRIVASTAVA 2009, AHMED; HASNAIN, 2010).

Important plant growth-promoting bacteria (PGPB), such as *Azospirillum brasilense* or *Bacillus*, are commonly used in agriculture due to their abilities to promote plant growth (HOSSAIN et al., 2019; LÓPEZ-BUCIO et al., 2007; RIVERA et al., 2018). However, many other microorganisms, such as *Proteus vulgaris*, *P. mirabilis*, *Klebsiella pneumoniae*,

Escherichia coli, and some bacteria of the genus *Acinetobacter*, have the ability to stimulate plant growth via either indirect routes, such as solubilization of phosphate in the soil, production of siderophores, or biological control, or even direct routes, such as the production of plant growth regulators including auxin, cytokinins, gibberellins, or abscisic acid (KARADENIZ et al., 2006; LIN et al., 2018; SHI et al., 2011).

Despite their recognized capacity for promoting plant development, the use of microorganisms in a controlled manner in vegetative micropropagation in tissue culture is still incipient (QUAMBUSCH; WINKELMANN, 2018). However, some studies have shown that it is possible to develop the rhizogenic potential in the presence of microorganisms, avoiding the use of synthetic auxins (LARRABURU et al., 2007; PEÑAFIEL JARAMILLO ET al., 2016). Thus, the use of plant growth-promoting microorganisms may shorten the rooting period and increase the growth of shoots and number of sprouts during the acclimation process, which represents a decrease in cultivation time, reduced costs, and an environmentally friendly approach (RUSSO et al., 2008; VETTORI et al., 2010).

In this context, the objective of this study was to isolate, identify, and select auxin-producing endophytic bacteria in micropropagated pear (*P. communis*) plants and perform microbiolization of the isolates during *in vitro* micropropagation, assessing the rhizogenic potential and behavior of transplants during the acclimation phase

2 MATERIALS AND METHODS

2.1 Plant material, multiplication, and *in vitro* rooting using synthetic auxin

Pyrus communis rootstocks from the 'OHxF87' (Old Home x Farmingdale) and 'PDW' (Old Home x Bonne Luise d'Avranches) selections were used, which were provided by the Agro-veterinary Center, Santa Catarina State University, Brazil. The plant material was established in QL medium (LEBLAY et al. 1991) at the Laboratory of Plant Micropropagation at Santa Catarina State University. For the multiplication protocol, MS culture medium was used (MURASHIGE; SKOOG, 1962), containing 30 mg L^{-1} of sucrose and 5.5 mg L^{-1} of agar, supplemented with 1.5 mg L^{-1} BAP and 0.1 mg L^{-1} IBA, pH adjusted to 5.8. A total of 20 mL of nutrient solution was added to the test tubes, which were kept in a growth room at 24°C with a photoperiod of 16 hours (40 - 56 μmol m^{-2} s^{-1}) for 45 days.

For rooting, the exposure time of the explants to IBA was tested. For this purpose, 20 mg L^{-1} of the phytohormone at 0, 24, 48, 72, and 96 hours was used. Each treatment consisted of 12 replicates. The culture medium and growth conditions were the same as those described for the multiplication protocol. After 35 days, the percentage of survival and rooting, the number of leaves and roots, and the shoot length and longest root length were evaluated.

2.2 Isolation and identification of endophytic bacteria in *Pyrus communis* rootstocks

The microorganisms were isolated from rootstocks multiplied *in vitro*. Surface sterilization of the segments of leaves and stems was performed. For this process, the material was washed in distilled water and then 70% alcohol (30 seconds), followed by rinsing in sterile water (three times) and immersion in 1% hypochlorite solution (1 minute).

Plating was performed by the dilution method (1:10) in sterile peptone water (0.1% peptone). The plant material was macerated together with the peptone water and incubated in a shaker at 150 rpm for 30 minutes at 28°C. Subsequently, three serial dilutions were performed (1:10), and using the surface plating method, a 0.1-mL aliquot was inoculated into the culture media nutrient agar (NA) (5 g L^{-1} of peptone, 3 g L^{-1} of yeast extract, 15 g L^{-1} of agar), M9 (10 g L^{-1} of Na_2HPO_4, 3 g L^{-1} of KH_2PO_4, 0.6 g L^{-1} of NaCl, 20 g L^{-1} of NH_4Cl, 5 g L^{-1} of glucose, 15 g L^{-1} of agar), and Luria Bertani (LB) (10 g L^{-1} of tryptone, 5 g L^{-1} of yeast extract, 10 g L^{-1} of NaCl, 15 g L^{-1} of agar). The plates were incubated for 36 hours in a biochemical oxygen demand incubator at 28°C. After this period, dilutions with 30 to 300 colonies were selected and separated by different morphotypes.

2.2.1 Identification of isolates by the matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) technique

For identification, the bacterial isolates were reactivated with 18 hours of culture. Approximately 1 μg of biomass was added to a 500-μg tube containing 6 μL of organic solution (50% of water, 47.5% of acetonitrile, and 2.5% of 2.5% (v/v) trifluoroacetic acid). The samples were vortexed and centrifuged for 60 seconds each at 4000 g at room temperature. This method is based on mass spectrometry, wherein the samples are placed on a plate with a matrix, which is then evaporated under the incidence of a laser. The system ionizes the volatilized material, which, upon reaching the detectors, records the detection time and quantity. Thus, each microorganism has a characteristic spectrum that is then analyzed by

specific software. This method detects and identifies proteins by determining their individual molecular weights and specific fragments. The *Escherichia coli* K12 strain is normally used as a standard for the external calibration of MALDI-TOF following the method described by LIMA-NETO et al. (2014).

2.2.2 Selection of IAA-promoting bacteria

To determine the production of the auxin IAA, the strains were grown in nutrient broth (NB) (5 g L^{-1} peptone, 3 g L^{-1} yeast extract) supplemented with 0.1 g L^{-1} L-tryptophan and incubated in a shaker at 150 rpm for 24 hours at 28°C. One-milliliter aliquots were removed and centrifuged for 5 minutes at 5,000 g, and 0.5 mL of the supernatant was mixed with 0.5 mL of Salkowski reagent (GLICKMANN; DESSAUX, 1995). The mixture was incubated in the dark at room temperature for 20 minutes, and the absorbance was measured by a mass spectrophotometer (Multiskan GO) using the software SkanIt 5.0 Microplate Readers RE, version 5.0.0.42. To determine the auxin concentration, a standard curve of auxin concentrations of 0, 5, 10, 15, and 20 mg L^{-1} was obtained, and a linear regression equation was fitted.

2.2.3 *In vitro* microbiolization

The bases of the explants were dipped for 30 minutes in NB solution (5 g L^{-1} peptone and 3 g L^{-1} yeast extract) containing bacteria at a population density of approximately 10^8 CFU mL^{-1}. After 30 minutes, the bases of the explants were dried in sterile absorbent paper and transferred to tubes containing 20 mL of MS medium (30 mg L^{-1} of sucrose, 5.5 mg L^{-1} of agar, pH adjusted to 6.0) (MURASHIGE; SKOOG, 1962). The material was transferred to a growth chamber at 24°C with a photoperiod of 16 hours (40 - 56 μmol m^{-2} s^{-1}) for 35 days. Subsequently, the following parameters were evaluated: number of leaves and roots, length of shoots and roots, and total fresh weight and dry weight.

2.2.4 Ultrastructural analysis by scanning electron microscopy

Scanning electron microscopy was used to observe the presence of growing bacteria in the rootstocks after treatment. Plants were fixed in Karnovsky solution (2.5% glutaraldehyde,

2.0% paraformaldehyde in 0.05 M sodium cacodylate buffer, CaCl2 0.001 M, pH 7.2). After fixation, the standard protocol described by Bozzola and Russel (1999) was used with some modifications. The samples were collected from the fixative and placed in glycerol for 30 minutes. Subsequently, they were cut in liquid nitrogen (cryofracture), followed by three washes in distilled water and dehydration in acetone series (25%, 50%, 75%, 90% once, and 100% three times).

After dehydration, the samples were taken to a Balzers CPD 030 critical point dryer to replace the acetone with CO_2 and to complement drying. The samples were mounted on stubs with carbon tape on aluminum foil and covered with gold in a Balzers SCD 050 sputter coater for observation under a LEO EVO 40 scanning electron microscope (ALVES, 2005).

2.2.5 Acclimation

After 35 days *in vitro*, the rooted or unrooted plants from all treatments were removed from the tubes, and their base was washed with tap water to remove the agar remnants. Subsequently, they were transferred to 200-mL plastic containers containing vermiculite. Acclimation occurred at a temperature of 24 ± 2ºC and photoperiod of 16 hours (40 - 56 μmol m^{-2} s^{-1}) for 60 days.

The substrate was periodically irrigated with sterile distilled water. After this period, the following parameters were evaluated: number of leaves and roots, length of shoots and roots, and total fresh weight and dry weight.

2.2.6 Experimental design and statistical analyses

A completely randomized experimental design was used, with eight treatments containing 20 *in vitro* replicates and 20 replicates evaluated during the acclimation period. The treatments consisted of the five bacterial isolates with the highest IAA production (*Acinetobacter ursingii* strain 1, *Micrococcus luteus*, *Acinetobacter ursingii* strain 2, *Acinetobacter ursingii* strain 3, *Acinetobacter ursingii* strain 4) and three controls (NB, synthetic auxin, and zero control). Treatments were inoculated in the ‘OHxF87’ and ‘PDW’ selections of *P. communis*.

The Shapiro-Wilk test was performed to assess data normality at a significance level of 5%. ANOVA was applied to the data that exhibited a normal distribution, comparing the

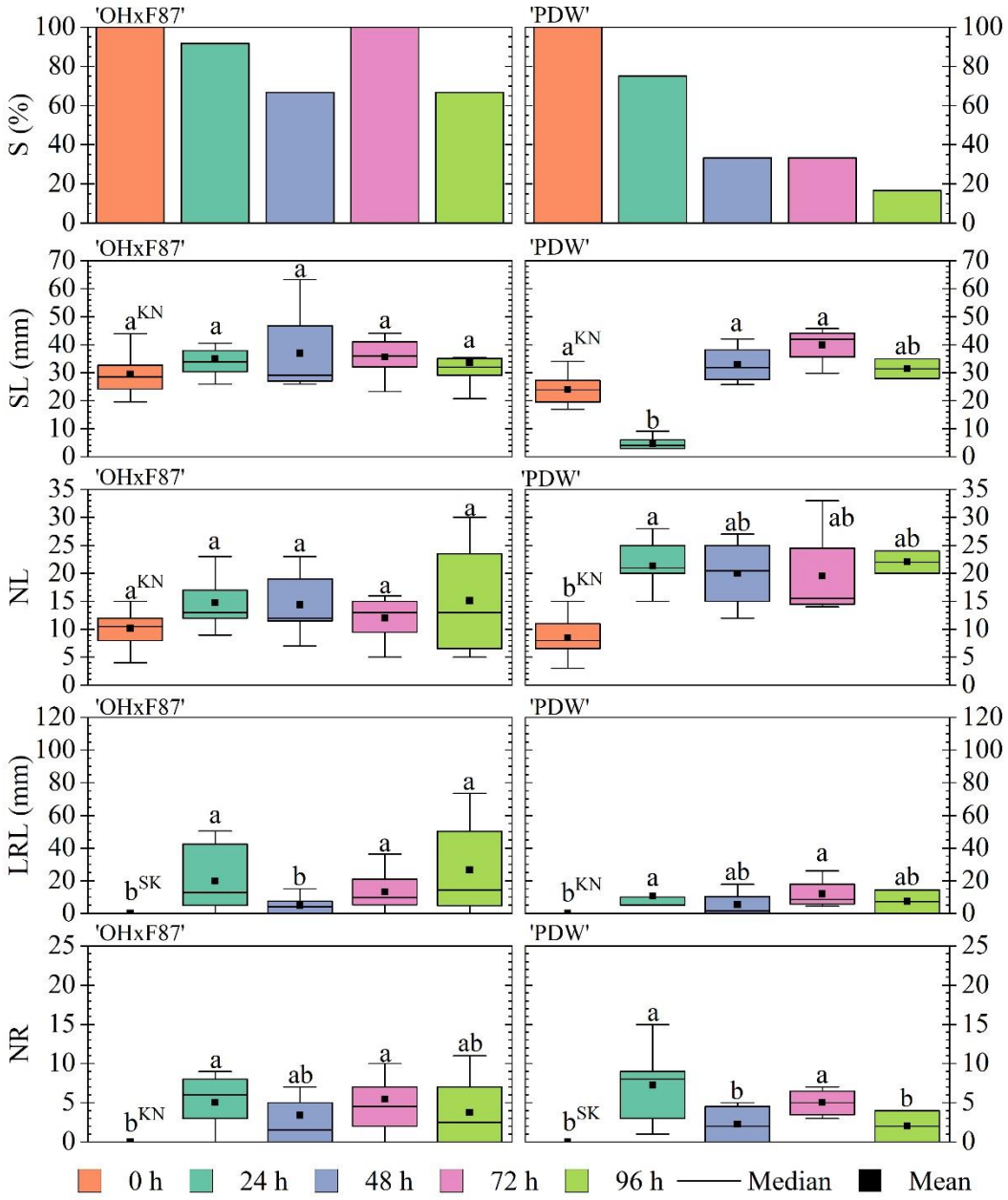
means by the Scott-Knott test (5%). For data that did not have a normal distribution, the Kruskal-Wallis test was used, and comparison was performed using the Nemenyi test. The software R was used for the statistical analyses.

3 RESULTS AND DISCUSSION

3.1.1 *In vitro* rooting using synthetic auxin

For both rootstocks, the exposure time of 24 hours at 20 mg L^{-1} IBA was sufficient to promote plant rooting (FIGURE 1). For the 'OHxF80' rootstock, the 24-hour treatment provided 95% survival, promoting an average of five roots with a length of 19 mm. The same treatment for rootstock 'PDW' resulted in the highest survival rate (75%), as well as the highest mean number of roots (seven roots per plants) with a length of 10 mm. In the absence of synthetic auxin, root formation did not occur (FIGURE 1).

Figure 1 - Boxplot and statistical test of the rooting of the ‘OHxF87’ and ‘PDW’ rootstocks exposed to synthetic auxin (IBA) for different times.



Variable: S - percentage of plant survival; SL - shoot length, in mm; NL - number of leaves; LRL - longest root length in mm; NR - number of roots. Treatment: Exposure times of 0, 24, 48, 72, and 96 hours. Statistics: SK - means followed by the same letter do not differ by the Scott-Knott test at the 5% confidence level; KN - means followed by the same letter do not differ by the Kruskal-Wallis-Nemenyi test at the 5% confidence level.

Source: From the author (2019).

In the literature, the protocols used for the rooting of *P. communis* selections differ regarding the culture medium, type, and concentration of hormones (SILVA et al., 2018; RUŽIĆ et al., 2011).

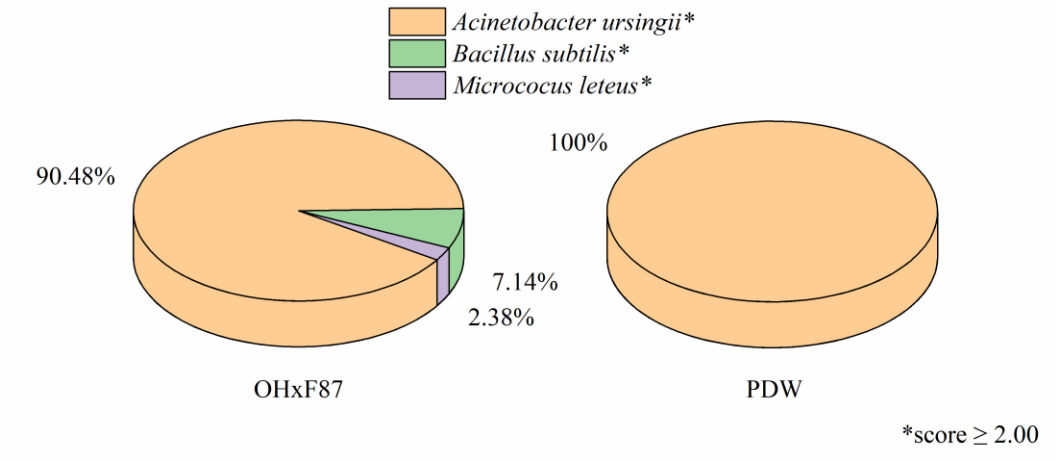
Regarding the culture medium, Silva et al. (2018) found that for the ‘OHxF’ and ‘PDW’ selections, QL medium modified by Leblay promotes satisfactory results in all stages of *in vitro* culture; however, in studies with PDW clones, Ružić et al. (2011) found the best results for *in vitro* rooting using ½MS medium (LIZÁRRAGA et al., 2017) in MS medium. Regarding the type and concentration of synthetic auxins, the forms IAA, IBA, and naphthalene-acetic acid (NAA) ensure successful rooting of cultivars and genotypes of different pear species (AYGUN; DUMANOGLU, 2015; BELL; REED, 2002; YANG et al., 2017), but with a very variable rooting rate according to the concentration of these hormones. These results show that the culture medium does not interfere with the rooting of these clones but rather the auxin concentration because auxins induce the formation of embryos from somatic cells, contributing to the formation and maintenance of the apical meristem of the roots (TAIZ et al., 2017).

In the present study, exposure to IBA resulted in rooting rates above 80%, surpassing some results found in the literature and confirming that *in vitro* rooting of clones occurs only in the presence of some type of treatment with a hormonal stimulus.

3.1.2 Isolation and identification of bacteria in *Pyrus communis*

In total, 56 isolates were identified belonging to the following species: *Acinetobacter ursingii, Bacillus subtilis*, and *Micrococcus luteus*. There was a predominance of the genus *Acinetobacter*, with low diversity of culturable microorganisms. For the ‘OHxF87’ rootstock, 94.48% of the microorganisms found were *Acinetobacter ursingii* strains, whereas only *Acinetobacter ursingii* strains were found for ‘PDW’ (FIGURE 2).

Figure 2 - Microbial community isolated from in vitro plants of *Pyrus communis* and identified using MALDI-TOF MS.



Source: From the author (2019).

In the process of plant micropropagation, the absence of microorganisms is expected, as it is guaranteed by the aseptic culture processes; however, the results demonstrated that even in these controlled conditions, microorganisms may be present, as also observed by Pirttilä et al. (2008) and Pohjanen et al. (2014) with *Pinus sylvestris* and by Quambusch et al. (2016, 2014) with *Prunus avium*.

The plants used in this study were established in QL medium and subsequently grown in MS medium. Bacterial growth could be observed on the surface of the culture medium after changes in the culture medium conditions. This phenomenon might be related to the release of exudates by plants after the first subculture for multiplying the plant material. In *P. avium* and *Malus* sp, the microorganisms were only revealed once the plant tissue samples were transferred to specific bacterial culture medium (QUAMBUSCH; WINKELMANN, 2018)

Because *in vitro* culture was performed under controlled conditions, there was no possibility of interactions with the complex microbial community that naturally occurs in the soil, which explains the low microbial diversity found in the studied genotypes. The same was observed in a study conducted by Quambusch et al. (2014) with six *P. avium* genotypes, where only five morphologically distinct isolates were identified from plants derived from tissue culture.

3.1.3 Selection of IAA-promoting bacteria

Among the identified microorganisms, 17 strains had auxin production ability (TABLE 1). The colonization of these microorganisms in plants might be related to the natural difficulty in rooting of the genus, as this substance has a significant effect on the process of bacterial colonization in plants because it promotes a symbiotic relationship between individuals (SPAEPEN et al., 2007; SPAEPEN; VANDERLEYDEN, 2011).

Table 1 - Production of auxin by bacteria isolated from pear (*Pyrus communis*) rootstocks 'OHxF87' and 'PDW'.

Microorganisms	**Rootstocks**	**AIA production (mg L^{-1})**
Acinectobacter ursingii 1	'OHxF87'	19,48
Micrococus leteus	'OHxF87'	10,82
Acinectobacter ursingii 2	'PDW'	10,16
Acinectobacter ursingii 3	'PDW'	8,99
Acinectobacter ursingii 4	'OHxF87'	8,60
Acinectobacter ursingii 5	'PDW'	7,61
Acinectobacter ursingii 6	'PDW'	6,50
Acinectobacter ursingii 7	'OHxF87'	5,55
Acinectobacter ursingii 8	'OHxF87'	5,16
Acinectobacter ursingii 9	'OHxF87'	4,00
Acinectobacter ursingii 10	'OHxF87'	3,44
Acinectobacter ursingii 11	'OHxF87'	2,89
Acinectobacter ursingii 12	'OHxF87'	2,45
Acinectobacter ursingii 13	'OHxF87'	2,06
Acinectobacter ursingii 14	'OHxF87'	1,78
Acinectobacter ursingii 15	'OHxF87'	1,39
Acinectobacter ursingii 16	'OHxF87'	1,28

Source: From the author (2019)

The mechanism predominantly used to explain the positive effects of growth-promoting bacteria is auxin production ability (OLANREWAJU et al., 2017). Different bacteria with this ability have been identified, such as *Azospirillum brasilense* (RIVERA et al., 2018), *Bacillus megaterium* (LÓPEZ-BUCIO et al., 2007), *Bacillus siamensis* (HOSSAIN et al., 2019), *Bacillus methylotrophicus* (PÉREZ-FLORES et al., 2017), *Pseudomonas veronii* (PEÑAFIEL JARAMILLO et al., 2016), *Acinetobacter johnsonii* (SHI et al., 2011), and *Acinetobacter baumannii* (LIN et al., 2018), among others.

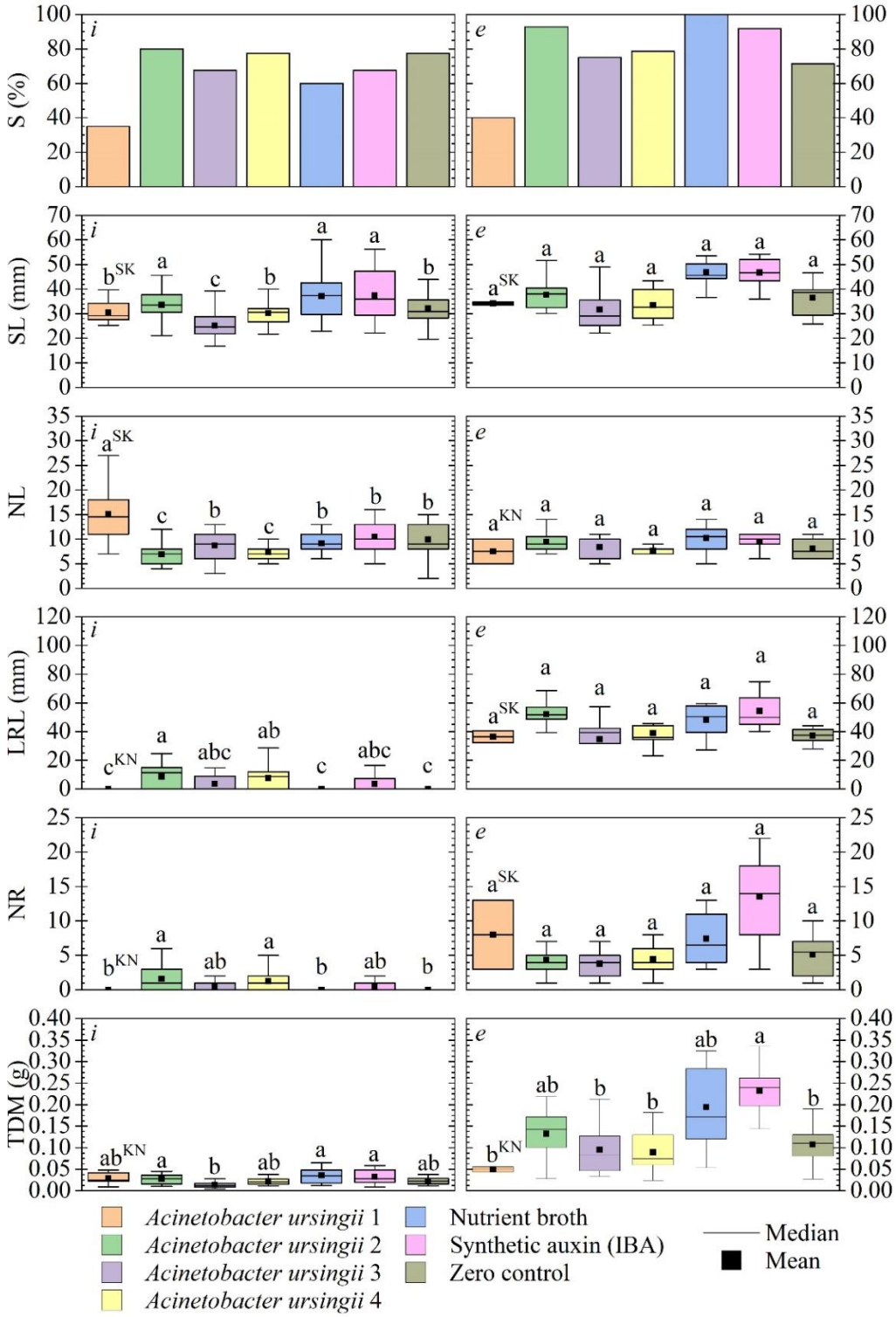
For *in vitro* inoculation, the five strains with the highest auxin production rate were selected. The selected IAA concentrations were between 19.48 mg L^{-1} and 8.60 mg L^{-1} after 24 hours of culture (TABLE 1).

The genus of bacteria predominantly identified in rootstocks (*A. ursingii*) is widely found in the environment. Species belonging to this genus have been described regarding their ability for nitrogen fixation, mineral solubilization, and production of siderophores, which are desirable characteristics for the direct and indirect promotion of plant growth (SACHDEV et al., 2010). However, these bacteria can also be frequently found as pathogenic opportunists (HARE et al., 2012), which can restrict their use without proper control. Bacteria *M. luteus* are also described in the literature as auxin-producing and having potential for maize rooting (Mike-Anosikes et al., 2018), and they can be considered endophytic because they have been isolated mainly from leaves of medicinal plants such as *Costus speciosus* (BARMAN; DKHAR, 2018).

3.1.4 Evaluation of *in vitro* rooting, acclimation, and ultrastructural analysis by scanning electron microscopy

In general, the results revealed an effect of the genotype for the strains tested and that, under the conditions tested, *in vitro* rooting could only occur using certain the *A. ursingii* strains (FIGURE 3). The treatment with *A. ursingii* isolates showed the same efficiency as the treatment with synthetic auxin for the different analyzed variables and allowed good development during the period of plant acclimation (FIGURE 3).

Figure 3 - Boxplot and statistical test of in vitro inoculation and acclimation of the ‘OHxF87’ rootstock.



Variable: S - percentage of plant survival; SL - shoot length, in mm; NL - number of leaves; LRL - longest root length in mm; NR - number of roots; TDM - total dry mass in g. Period: *i* - *in vitro*; *e* - acclimation. Statistics: SK - means followed by the same letter do not differ by the Scott-Knott test at the 5% confidence level; KN - means followed by the same letter do not differ by the Kruskal-Wallis-Nemenyi test at the 5% confidence level.

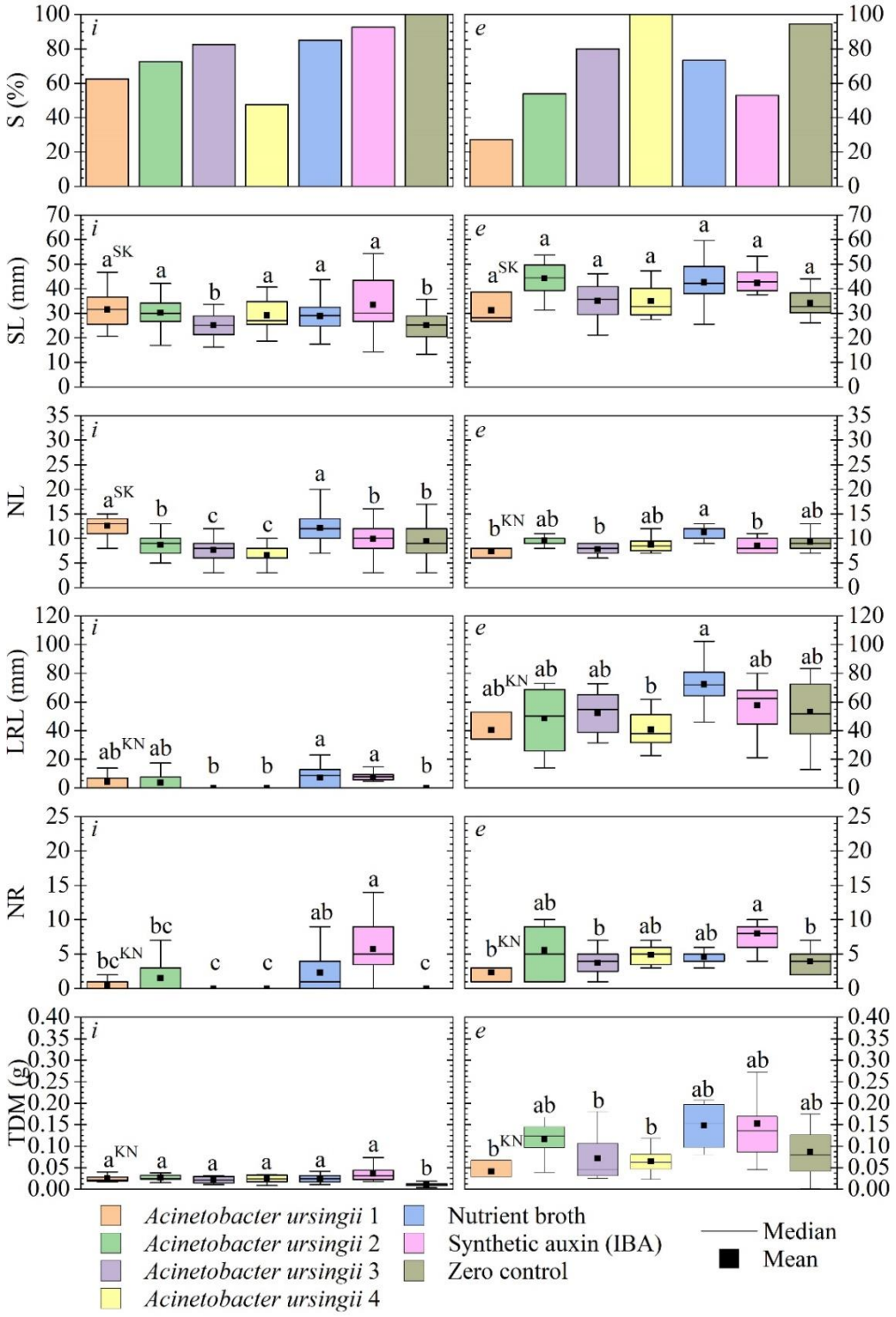
Source: From the author (2019)

Considering the results obtained for the ‘OHxF87’ rootstock, there were differences in the percentage of survival with and without inoculation of the microorganisms, where the survival of the plants with *in vitro* inoculation of strains 2, 3, and 4 was higher than with synthetic auxin. Strains 2 and 4 resulted in 80% survival *in vitro*, strain 3 in 70%, and IBA in 60% (FIGURE 3).

The high survival rates persisted during the acclimation period, and 95% of the plants originating from the inoculation with strain 2 and 80% of the plants inoculated with strains 3 and 4 survived during this phase. All plants from the NB treatment survived the acclimation period (FIGURE 3).

Regarding the ‘PDW’ rootstock (FIGURE 4), the addition of both the synthetic hormone and microorganisms *in vitro* negatively affected plant survival. However, although strain 4 showed a lower survival rate *in vitro* (50%), during the acclimation period, this strain provided 100% survival, which was higher than the other treatments. In addition, strain 3 provided 83% survival, which was equivalent to the NB control.

Figure 4 - Boxplot and statistical test of in vitro inoculation and acclimation of the 'PDW' rootstock.

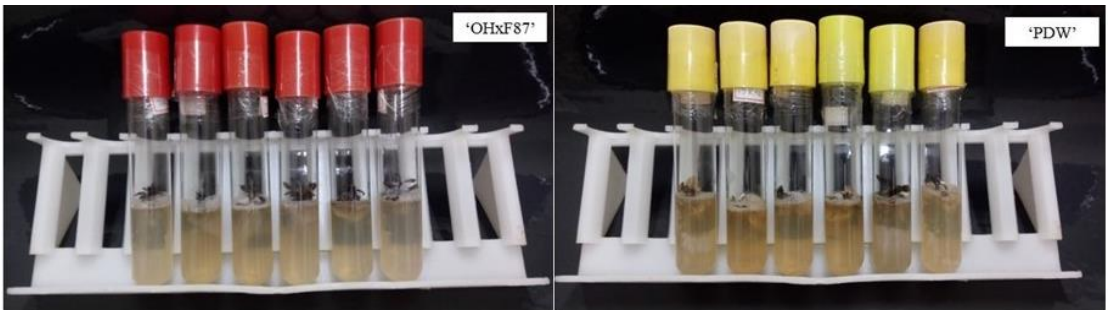


Variable: S - percentage of plant survival; SL - shoot length, in mm; NL - number of leaves; LRL - longest root length in mm; NR - number of roots; TDM - total dry mass in g. Period: i - *in vitro*; e - acclimation. Statistics: SK - means followed by the same letter do not differ by the Scott-Knott test at the 5% confidence level; KN - means followed by the same letter do not differ by the Kruskal-Wallis-Nemenyi test at the 5% confidence level.

Source: From the author (2019).

High survival rates *in vitro* and during the acclimation period showed that microorganisms could be beneficial for the development of this species, in addition to promoting biostimulation during the acclimation period of pear trees. However, in the present study, not all microorganisms could be inoculated *in vitro* despite producing auxin. In the treatment with *M. luteus*, the clones did not support the high population of this microorganism, which did not survive the inoculation (FIGURE 5).

Figure 5 - Results of inoculation with *Micrococcus luteus* after seven days in rootstocks 'OHxF87' and 'PDW'.



Source: From the author (2019).

Among the microorganisms, strain 2 promoted the greatest shoot growth at 38 mm, which exceeded control 0 during *in vitro* culture in the 'OHxF87' rootstock. Plants from the IBA and NB treatments showed an average length that was 10 mm longer than strain 2. Strain 1 promoted the growth of the greatest number of leaves, differing from the other treatments. In the acclimation phase, there were no differences between these variables (FIGURE 3).

However, for the 'PDW' rootstock, with the exception of strain 3, inoculation of the other bacteria provided a shoot length similar to that promoted by synthetic auxin and NB. Strain 1 promoted the greatest number of leaves in plants and was equivalent to the NB control. During the acclimation period, there were no differences in shoot length, and strains 2 and 4 did not differ from the NB control regarding the number of leaves (FIGURE 4).

These results suggested that, especially during the acclimation period, inoculation with the tested microorganisms enabled growth similar to that promoted by synthetic auxin in both rootstocks, which could reduce costs in the micropropagation process and even improve the propagation of these clones, given that survival during the acclimation period was higher with the use of some strains.

In a study investigating the vegetative growth of strawberries (Rosaceae), Andrade et al. (2019) also observed that the use of growth-promoting bacteria, such as *Azospirillum*

brasilense, *Burkholderia cepacia*, and *Enterobacter cloacae*, promoted shoot growth similar to that found in plants grown with synthetic fertilizer, enabling the use of microorganisms to reduce production costs.

Shoot growth and leaf formation is essential for plants, given that individuals with a greater number of leaves under ideal conditions have greater photosynthetic capacity, which benefits their growth and development (TAIZ et al., 2017). For these variables, strains 1, 2, and 4 contributed to shoot formation in the clones.

Auxin-producing bacteria have been used especially to stimulate seed germination and accelerate root growth (MARTÍNEZ-VIVEROS et al., 2010). Regarding root system formation, strain 2 promoted the greatest root length, which did not differ from strains 3 and 4 or IBA for the 'OHxF87' rootstock. Strain 1, NB, and C0 did not induce the formation of roots in this clone (FIGURE 3). In addition, the longest root length of plants inoculated with strain 2 was higher than that promoted by IBA, and strain 4 did not differ from the treatment with synthetic hormone. Strains 2 and 4 also provided a greater number of roots than synthetic auxin. During the acclimation phase, there were no differences in these variables, and root formation was observed in all treatments.

Strain 2 also promoted a greater accumulation of total dry mass of plants *in vitro*, which did not differ from IBA and NB and was higher than control 0. During the acclimation period, although strain 2 did not differ from IBA and NB, synthetic auxin resulted in the highest dry mass accumulation in 'OHxF87' clones (FIGURE 3).

However, for 'PDW', IBA and NB promoted better root formation *in vitro*, exceeding strains 3 and 4 and showing statistically equivalent results to strains 1 and 2. The same behavior was observed for the number roots. Strains 3 and 4 and control 0 did not induce root formation in this clone. During the acclimation period, plants from the NB control achieved a longer root length, and plants from the IBA treatment had the greatest number of roots (FIGURE 4). Similar to what was observed for the 'OHxF87' rootstock, all 'PDW' rootstock plants were rooted during the acclimation period.

Regardless of root formation ability, inoculation of microorganisms, NB, and IBA provided greater dry mass accumulation compared with the control treatment *in vitro*. During the acclimation stage, strain 2 promoted the same dry mass accumulation to that with the IBA, NB, and control treatments in the 'PDW' rootstock (FIGURE 4).

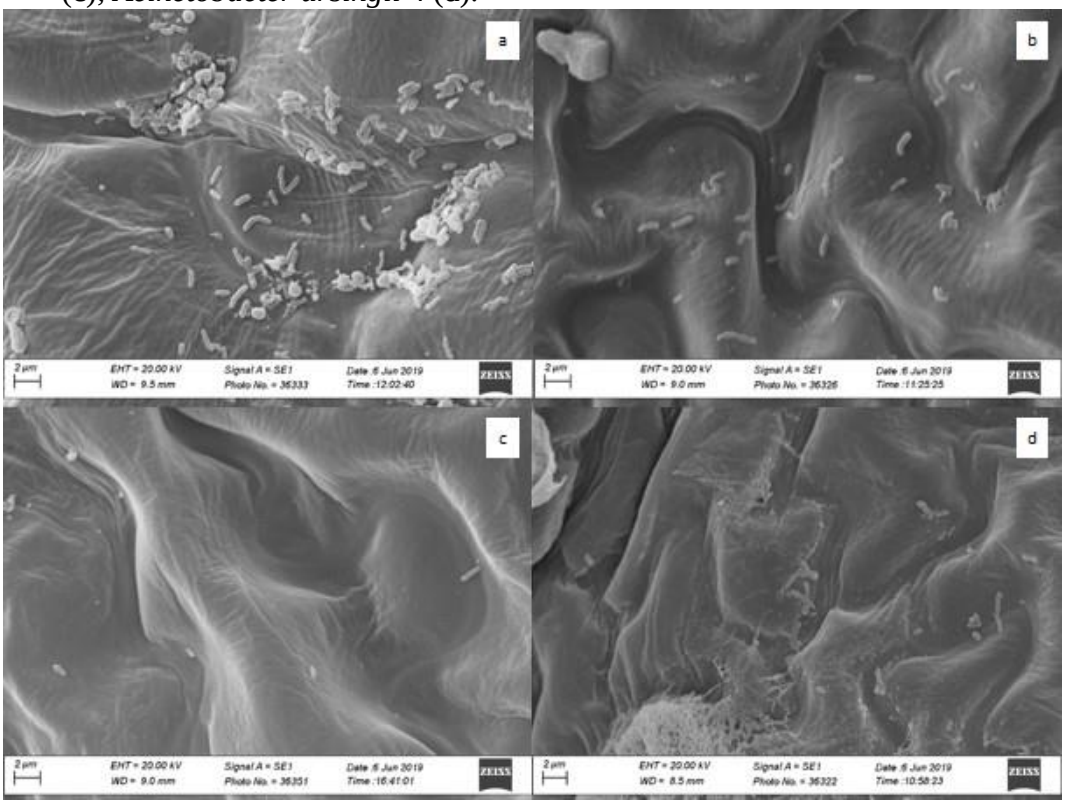
These results were similar to those found for shoot formation, demonstrating the potential use of these microorganisms in *in vitro* culture and their contribution in the *ex vitro*

rooting period, supporting their use as a viable alternative to the synthetic regulator. Some studies have also reported positive results for the *in vitro* rooting of woody plants from the Rosaceae family. For example, Larraburu et al. (2007) used a combination of synthetic microorganisms and auxins for rooting of Photinia. These authors observed an important role of microorganisms in root organogenesis of the plants, proposing an alternative protocol for micropropagation of the species. In *Prunus avium,* the use of *Rhodopseudomonas* sp. and *Microbacterium* sp. promoted *in vitro* rooting rates that were higher than control 0, ranging between 30 and 92.5%, and the formation of 0.7 to 7.2 roots depending on the cultivar studied (QUAMBUSCH et al. 2014). The use of microorganisms such as *Pseudomonas veronii* R4 and *P. fluorescens* CHA0, both of which are able to synthesize IAA, also enabled *in vitro* root formation in grape leaves (LARRABURU et al., 2007; PEÑAFIEL JARAMILLO et al., 2016).

Furthermore, *ex vitro* experiments have shown that the success of the acclimation of *P. communis* plants depends on hormone stimuli, as can be observed in the studies of (AYGUN; DUMANOGLU, 2015; LIZÁRRAGA et al., 2017). The present rooting results support this notion and confirm the findings of Quambusch et al. (2014), who showed that manipulation of the endogenous bacterial population through inoculation as growth promoters improves the survival and facilitates the growth of plants during critical stages of plant tissue culture.

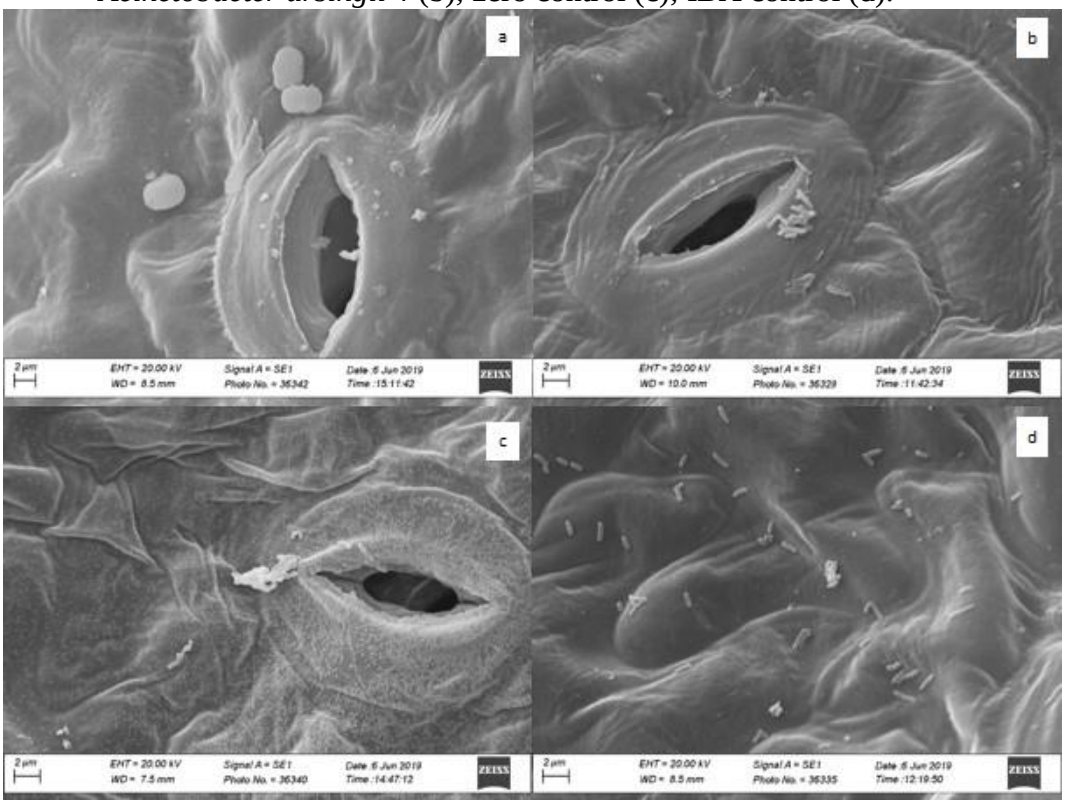
The best results were obtained for strains 2 and 3 of *A. ursingii* for the ‘OHxF87’ rootstock and strains 1 and 2 for ‘PDW’. The electron micrographs showed the presence of bacteria in ‘OHxF87’ rootstock plants that received these treatments (Figure 6) after *in vitro* micropropagation. No colonies were found in the NB control, synthetic IBA control, or negative control of this rootstock. However, for the ‘PDW’ rootstock, the electron micrographs revealed the presence of bacteria in treatments 2 - *A. ursingii* 2 and 4 - *A. ursingii* 4, in addition to colonies in the negative control and in synthetic IBA. No bacterial colonies were observed in treatments 1 - *A. ursingii* 1 and 3 - *A. ursingii* 3 or in the control with NB (FIGURE 7).

Figure 6 - Electron micrograph of the ‘OHxF87’ rootstock after in vitro micropropagation. *Acinetobacter ursingii* 1 (a); *Acinetobacter ursingii* 2 (b); *Acinetobacter ursingii* 3 (c); *Acinetobacter ursingii* 4 (d).



Source: From the author (2019)

Figure 7 - Electron micrograph of the ‘PDW’ rootstock. *Acinetobacter ursingii 2* (a); *Acinetobacter ursingii 4* (b); zero control (c); IBA control (d).



Source: From the author (2019).

The identification of microorganisms in plants from the negative control and IBA treatment in the ‘PDW’ rootstock strongly demonstrated the presence of endophytes in this genotype. Their presence was also demonstrated by the occurrence of rooting in the treatment with NB, given that this bacterial environment lacks components that stimulate rooting in plants. When the NB was absorbed by the plants, it might have contributed to the increase in the endophytic bacterial population already present in the plant tissue and, consequently, to the stimulation of plant rooting.

In addition, the acclimation results contributed evidence showing that growth-promoting bacteria could be present in plants that were not inoculated with the strains, because even plants that did not have roots exhibited good development in the *ex vitro* phase (FIGURES 4 and 5).

Bacteria were not visualized by scanning electron microscopy in treatments with strains 1 and 3, although the treatments were applied equally in the plants and in the NB for the ‘PDW’ clone, as well as in the control treatments for the ‘OHxF87’ clones. One possible reason for this finding was the choice of microorganisms based on their occurrence at the

stem base, as these structures were fragile as a result of the *in vitro* culture. In addition, the culture medium was rich in nutrients, which might obviate the need for cell colonization to obtain compounds of interest, or the symbiotic relationship. However, further studies are needed for additional clarification.

4 CONCLUSION

Bacteria of the species *Acinetobacter ursingii*, *Bacillus subtilis*, and *Micrococcus luteus* were isolated and identified, among which 16 *Acinetobacter ursingii* strains and the *Micrococcus luteus* strain showed auxin production ability. Strains *A. ursingii* 1 and 4 showed efficiency similar to that of synthetic auxin for the 'OHxF87' genotype. The clones originating from the 'PDW' selection showed lower rooting when inoculated with microorganisms; however, rooting occurred using *A. ursingii* strains 1 and 2. During the acclimation stage, all plants exhibited a high level of rooting, favoring the development of rootstocks.

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