Centro de Investigación en Alimentación y Desarrollo A.C.

MONITOREO DE *Erwinia amylovora* EN FUENTES DE INÓCULO PRIMARIO: OPTIMIZACIÓN DE PROTOCOLOS PARA LA DETECCIÓN DE FORMAS NO CULTIVABLES MEDIANTE UNA PCR DE VIABILIDAD SEMICUANTITATIVA

Por:

Daniel Alejandro de la Peña Baca

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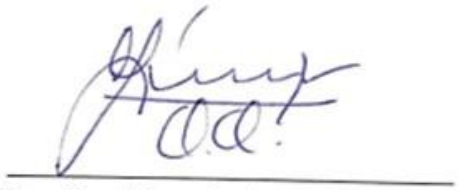
Los miembros de este comité designado para revisar la tesis de Daniel Alejandro de la Peña Baca, la han encontrado satisfactoria y recomiendan que sea aceptada como requisito parcial para obtener el grado de Doctor en Ciencias.

Dr. Carlos Horacio Acosta Muñiz
Director de Tesis

Dr. Claudio Rios Velasco
Integrante de Comité de Tesis



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Integrante de Comité de Tesis

Dr. José de Jesús Ornelas Paz
Integrante de Comité de Tesis

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"Si por alguna razón usted, el lector, está pensando en emprender un doctorado, debe saber que se trata de una labor francamente peligrosa y desafiante. No hay mejor ejemplo para entender a Einstein y la relatividad, que el tiempo invertido en las montañas de artículos leídos y el trabajo experimental. El costo serán noches en vela, fallos y frustraciones (será mejor pasarlos a sorbos de café) y un sin número de interrogantes. Si a pesar de ello decide continuar, seré sincero, vale totalmente la pena"

Alejandro de la Peña

CONTENIDO

RESUMEN

En los agroecosistemas, la inanición y la exposición de algunas especies bacterianas a compuestos citotóxicos inducen la formación de formas no cultivables como se ha documentado en *Erwinia amylovora*, agente causal del tizón de fuego. Esta situación dificulta el monitoreo y control del patógeno. Para superar esta limitación, se han desarrollado técnicas como la PCR de viabilidad (v-PCR), que permite detectar y cuantificar estas formas en matrices complejas. Sin embargo, aún no existe una técnica estandarizada, por lo que es necesario realizar modificaciones o adaptaciones que permitan determinar de forma directa, las poblaciones viables de *E. amylovora*, tanto en sus formas cultivables como no cultivables, a partir de muestras ambientales. Para ello, se colectaron doscientas muestras que incluían cancros remanentes activos, brotes jóvenes sintomáticos, frutos necrosados, tejido asintomático, tejido en fase de transición, suelo asociado a las raíces, flores de manzano, malezas no hospederas, abejas melíferas y agua de riego. Las muestras fueron procesadas de manera secuencial, comenzando con un pre-tratamiento (para liberar las células bacterianas contenidas en la muestra), seguido del conteo de células cultivables en medios selectivos, un tratamiento con DNasa y EMA (para discriminar el material genético libre o de células necrosadas), el aislamiento de ADNg y por último, la detección y cuantificación mediante v-sqPCR (correlacionando la concentración de dsDNA de los productos amplificados con la concentración celular conocida en muestras control). El 43.5 % de las muestras resultaron positivas en medio selectivo (con variaciones entre el 10 y el 90 % en el índice de positividad, según el tipo de muestra), siendo las muestras de tejido sintomático las que albergaron las poblaciones más abundantes de células cultivables (entre 10^5 y 10^7 UFC/g). Por otro lado, solo el 13 % de las muestras resultaron positivas mediante v-sqPCR (con un índice de positividad entre el 5 y el 75 %). Lo anterior, debido a un umbral mínimo de detección subjetivamente alto ($\approx 1 \times 10^4$ UFC). A pesar de ello, muestras específicas de cancros, brotes marchitos y frutos momificados mostraron conteos celulares significativamente mayores al ser analizadas con v-sqPCR, frente a lo registrado en placa de cultivo. Esto confirma la presencia de formas no cultivables de *E. amylovora* y su abundancia fluctúa entre 10^4 y 10^7 células/g. Esta investigación proporciona información útil sobre la dinámica de *E. amylovora* en fuentes de inóculo convencionales y no convencionales, evidencia la subestimación de las fuentes no convencionales y respalda la necesidad de modernizar

los actuales esquemas de monitoreo para mejorar los sistemas de respuesta y control antimicrobiano.

Palabras clave: tizón de fuego, cancros, frutos momificados, monoazida de bromuro de etidio.

ABSTRACT

In agroecosystems, starvation and exposure to cytotoxic compounds can induce the formation of non-cultivable forms in bacterial species such as *Erwinia amylovora*, the causal agent of fire blight. This complicates the monitoring and control of the pathogen. To address this challenge, techniques such as viability PCR (v-PCR) have been developed, enabling the detection and quantification of these forms in complex matrices. However, there is currently no standardized procedure for that, and modifications or adaptations are needed to directly determine the viable populations of *E. amylovora*, both in its cultivable and non-cultivable forms, in environmental samples. To achieve this, two hundred samples were collected, including active remnant cankers, symptomatic young shoots, necrotic fruits, asymptomatic tissue, tissue in the transition phase, soil associated with roots, apple blossoms, non-host weeds, honeybees, and irrigation water. The samples were processed sequentially, beginning with a pre-treatment step (to release bacterial cells from the samples), followed by culturing on selective media to count culturable cells, DNase and ethidium monoazide (EMA) treatments (to discriminate free genetic material or necrotic cells), DNA isolation, and finally, detection and quantification using v-sqPCR (correlating the concentration of dsDNA in the amplified products with known cell concentrations from control samples). 43.5% of the samples were positive in selective media (with positivity rates ranging from 10% to 90%, depending on the sample type), with symptomatic tissue samples containing the highest populations of culturable cells (between 10^5 and 10^7 CFU/g). In contrast, only 13% of the samples were positive by v-sqPCR (with a positivity rate ranging from 5% to 75%), due to a relatively high minimum detection threshold (≈1×10^4 CFU). However, specific samples from cankers, wilted shoots, and mummified fruits showed significantly higher cell counts when analyzed by v-sqPCR compared to those obtained from culture plates. This confirms the presence of non-cultivable forms of *E. amylovora*, with populations fluctuating between 10^4 and 10^7 cells/g. This research provides valuable insights into the dynamics of *E. amylovora* in both conventional and non-conventional inoculant sources, highlights the underestimation of non-conventional sources, and underscores the need to update current monitoring schemes to enhance antimicrobial response and control systems.

Keywords: fire blight, cankers, mummified fruits, ethidium bromide monoazide.

1. SINOPSIS

1.1. Justificación

A lo largo de doscientos años, el tizón de fuego ha sido considerada una de las patologías vegetales más temidas y complejas en el cultivo de frutales, particularmente (aunque no exclusivamente) en manzanos y perales. Con un cuadro evolutivo rápido, *Erwinia amylovora*, su agente causal, tiene la capacidad de diezmar huertos enteros en una sola temporada o de arraigarse crónicamente, lo que ocasiona pérdidas económicas y acorta la vida productiva de los árboles. El control químico es actualmente la herramienta principal para combatir esta enfermedad, mediante la supresión preventiva de las poblaciones bacterianas presentes en los huertos. La teoría más aceptada señala que los cancros remanentes son la fuente más probable de inóculo primario, por lo que el patógeno se monitorea en estas estructuras mediante técnicas de microbiología clásica basadas en la cultivabilidad usando medios selectivos. Otras fuentes no convencionales de inóculo primario (tales como brotes y frutos infectados en temporadas previas, el suelo asociado a las raíces e incluso plantas no hospederas), suelen ignorarse presuntamente por albergar poblaciones bacterianas epífitas no significativas, no obstante, la evidencia que respalde esta acción sigue siendo escasa y controvertida.

Investigaciones recientes han demostrado que condiciones de estrés en el agroecosistema, como la inanición o la exposición a citotóxicos (incluidos antibióticos), pueden inducir una fase no cultivable en *E. amylovora*. Como su nombre lo indica, estas formas viables no pueden detectarse mediante técnicas basadas en la cultivabilidad, lo que podría provocar la subestimación de las fuentes no convencionales de inóculo primario y, en consecuencia, llevar a un control ineficaz de la enfermedad. Incluir las formas no cultivables en los esquemas de monitoreo de la enfermedad representa un desafío significativo, ya que requiere la implementación de técnicas específicas basadas en la PCR de viabilidad (*v-PCR*), junto con procedimientos validados para la extracción de ADN a partir de muestras ambientales. A la fecha, no existe una técnica universalmente estandarizada para este fin, dado que el procedimiento debe adaptarse según las características de

la muestra, del huerto y del patógeno en cuestión.

1.2. Antecedentes

1.2.1. Viabilidad y Cultivabilidad: Dos Conceptos Diferentes

La inactividad celular como estrategia de supervivencia, ha sido un tema de especial interés para los microbiólogos. En ambientes naturales, la presencia de microorganismos plenamente viables pero que han cesado su capacidad reproductiva (de forma temporal o permanente) es más frecuente de lo que se puede pensar. Se estima que alrededor del 81 % de los géneros microbianos del planeta no han sido cultivados (Lloyd *et al*., 2018). Incluso, se estima que el 60 % de la biomasa de la Tierra corresponde a microorganismos en alguna fase de reposo (Gray *et al*., 2004). Como es de esperarse, es muy limitado el conocimiento de las formas de microorganismos no cultivables y, por ende, el papel que desempeñan en el sistema que habitan es prácticamente desconocido. La tasa de reproducción o desarrollo suele ser un buen indicador de la actividad celular en los organismos microscópicos, por lo que no es extraño asociar este parámetro con la viabilidad celular (o la mortandad, en su defecto). Durante décadas, la única forma de comprobar la viabilidad bacteriana era a través de su capacidad para multiplicarse en medios de cultivo, es decir, viabilidad y cultivabilidad celular eran considerados sinónimos. No obstante, en la década de 1980 se descubrió que la bacteria *Vibrio cholerae* enfrenta condiciones ambientales adversas mediante diversos mecanismos de adaptación, entre los cuales se incluye la incapacidad para multiplicarse (Colwell y Grimes, 2000). Posteriormente, se reportó que esta respuesta no era exclusiva de esta especie, sino que un gran número de géneros y especies bacterianas experimentaban una fase temporal de viabilidad no reproductiva. Las células en este estado fueron denominadas somnicélulas (Roszak y Colwell, 1987). El desarrollo de técnicas moleculares, particularmente las genómicas y metabolómicas, ha incrementado la evidencia sobre las fases no cultivables de las bacterias y ha planteado una interrogante que hoy en día sigue siendo objeto de estudio y debate entre los expertos: ¿Las formas no cultivables representan una estrategia de adaptación específicamente

regulada, o son una fase previa a la muerte irreversible? (Pinto *et al*., 2015).

1.2.2. Formas Bacterianas No Cultivables

Con base en evidencia y estadística, se estima que el número total de especies bacterianas puede ir desde las 300,000 mil (cifras conservadoras) hasta el millón, mientras que solo se han descrito y caracterizado unas 3,200 especies (Colwell y Grimes, 2000) y en su inmensa mayoría se trata de especies cultivables, pues si bien se conocen bacterias parásitos intracelulares obligados (como las de las familias Chlamydia, Rickettsia, Coxiella y algunas del género *Mycobacterium*) las cuales carecen de una fase cultivable en medios artificiales, el número de especies descritas e inventariadas en este grupo es abrumadoramente menor, en buena medida debido a su limitación en cuanto a cultivabilidad. Como se mencionó previamente, la incultivabilidad no es un fenómeno inusual en la dinámica poblacional de las bacterias. Aun dentro de las bacterias cultivables se han descrito adaptaciones o fases que inactivan el desarrollo en medios selectivos. Las esporas, por ejemplo (generalmente descrita en bacterias Gram positivas de los géneros *Bacillus* y *Clostridium*), son estructuras con una morfología y estructura diferente a las de las células vegetativas (Higgins y Dworkin, 2012). Están deshidratadas y son prácticamente inertes (Errington, 1993). Además, las esporas se caracterizan por altamente resistentes a las variaciones ambientales y condiciones extremas, como temperatura, radiación solar, humedad y citotóxicos, que otras formas no cultivables (Pinto *et al*., 2015). Por otro lado, las células persistentes son una subpoblación bacteriana que surge de forma espontánea, generalmente en especies de rápido crecimiento, como *Escherichia coli*, *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa*, *Salmonella enterica*, *Staphylococcus aureus* y *Streptococcus pyogenes* (Ayrapetyan *et al*., 2018). Estas células se caracterizan por ser altamente resistentes a citotóxicos (Gerdes y Maisonneuve, 2012). Aunque aún no se comprende completamente cómo ocurre su formación, ni cómo superan este estado, existen dos hipótesis al respecto: la primera sugiere que la exposición a citotóxicos induce la formación involuntaria de proteínas mal plegadas, lo que provoca estasis celular; la segunda plantea que se trata de un fenómeno epigenético mediado por el sistema toxina-antitoxina (Lewis, 2010). Por otro lado, se encuentran las células latentes y el estado viable no cultivable (VBNC), dos formas que se

caracterizan por mantener un metabolismo y respiración celular activos (Oliver *et al*., 1995) y una integridad completa de la membrana celular (Lloyd y Hayes, 1995), pero no forman colonias en medios nutritivos, no se dividen, pueden permanecer en esta forma durante periodos indefinidos y recuperan la cultivabilidad tras un período de reanimación específico (Kaprelyants *et al*., 1993; Kell y Young, 2000). Ambos estados son prácticamente indiferenciables, tanto que se ha sugerido que ambos términos se refieren al mismo estado fisiológico (Oliver, 2005), a diferencia del estado VBNC, las células latentes mantienen una actividad metabólica por debajo de los límites actuales de detección (Mukamolova *et al*., 2003).

1.2.3. Inducción y Reanimación de Fases No Cultivables

Para diversos autores no existen dudas de la ocurrencia de formas temporalmente no cultivables en ambientes naturales, sin embargo, las limitaciones técnicas para su estudio han acotado la mayor parte de evidencia experimental al área *in vitro* (Oliver, 2000). La capacidad de cultivo y actividad celular pueden ser reducidas o cesadas (y posteriormente reactivadas) a través de exposición a condiciones de estrés específicas entre las que se incluyen temperaturas y pH extremos, radiación UV, sustancias tóxicas, desecación, inanición y fluctuaciones en la concentración de oxígeno (Bjergbaek y Roslev 2005; Lleo *et al*. 2001).

La inanición y la exposición a citotóxicos son los factores de inducción de formas no cultivables más documentados (Ordax *et al*., 2009) y su efecto suele estar estrechamente relacionado con la temperatura. Por ejemplo, en ambientes cercanos a los 5 °C la respuesta no cultivable se retrasa aun cuando la bacteria se encuentre en inanición (Biosca *et al*., 2006). Esto ocurre presumiblemente debido a que las bajas temperaturas reducen por si sola la actividad metabólica de la bacteria, reduciendo el consumo de los pocos nutrientes disponibles en el medio, lo que retrasa la respuesta adaptativa (Biosca *et al*., 2009). Otro fenómeno que podría explicar este estado en condiciones de inanición es la aparición de rondas sucesivas de fenotipos de ventaja de crecimiento a partir de una fase estacionaria o GASP (por las siglas en inglés para “growth advantage in stationary pase”). Estas mutantes que suelen aparecer dentro de una misma población bacteriana poseen una mejor

aptitud para buscar y aprovechar los nutrientes que la cepa parental, lo que podría retrasar la aparición de formas no cultivables (Zambrano y Kolter, 1996).

La actividad metabólica celular, así como las condiciones de estrés, suelen dar origen a compuestos secundarios relacionados con el estrés oxidativo. En este sentido, el peróxido de hidrógeno (H_2O_2) se reconoce como un compuesto con un papel crucial en la inducción de respuestas no cultivables como el estado VBNC en una amplia gama de bacterias (Mizunoe *et al*., 1999). Mutantes deficientes de *Vibrio vulnificus* y *E. amylovora* incapaces de producir catalasa debido a la inactivación de los reguladores *katG/ocyR* no son cultivables en medios sólidos, presumiblemente debido a la incapacidad de la bacteria para desintoxicarse de la forma potencialmente fatal de H_2O_2 (Kong *et al*., 2004; Santander *et al*., 2018). No obstante, la temperatura también juega un papel crucial en la actividad de las catalasas bacterianas producidas de forma natural, pues se sabe que las bajas temperaturas inhiben la acción enzimática, lo que da como resultado la inducción de formas no cultivables aun en células no deficientes (Kong *et al*., 2004).

Ciertos compuestos citotóxicos también inducen la respuesta no cultivable. Algunas especies de enterobacterias pierden la capacidad de cultivo después de permanecer en una solución de sulfato de cobre (0.005 mM) por 36 días. Sin embargo, este tiempo se reduce tras exposiciones a concentraciones mayores (Ordax *et al*., 2006). Algo similar ocurre tras la exposición por al menos cinco minutos a compuestos como el hipoclorito de sodio (1 ppm), ácido acético (1 %) y peróxido de hidrógeno (5 %) (Santander *et al*., 2011). El mecanismo de respuesta de incultivabilidad bajo estrés por toxicidad no está del todo claro, pero podría estar relacionada con cambios en las proporciones de las biomoléculas que conforman la estructura o forma de la pared celular encargadas de regular la fluidez de las membranas (Clements y Foster, 1998), protegiendo así las células de altas o bajas presiones osmóticas y de compuestos citotóxicos como metales pesados, etanol, cloro, ácidos orgánicos e incluso antibióticos (Dong *et al*., 2020).

El programa genético que da lugar y mantiene las formas no cultivables es poco conocido. En *Escherichia coli* se ha identificado que el gen de la proteína de biosíntesis del cofactor de molibdeno A (*mobA*), el gen responsable de la biosíntesis del antígeno O157 y el gen de la toxina Shiga se mantienen muy activos durante las fases no cultivables (Yaron y Matthews, 2002).

Adicionalmente, se sabe que el agotamiento del factor sigma RpoS (y por ende los genes regulados por este) da como resultado una respuesta de incultivabilidad en *E. coli* y *S. enterica* (Boaretti *et al*., 2003; Kusumoto *et al*., 2012). Mutantes deficientes del gen *ppk1* (Polyphosphate Kinase 1) en *Campylobacter jejuni* redujeron significativamente la respuesta no cultivable (Gangaiah *et al*., 2009). De igual forma, mutantes de *E. coli* deficientes de la proteína sensora de osmolaridad EnvZ responsable de regular las porinas de membrana externa F y C, son incapaces de emitir una respuesta no cultivable frente al estrés osmótico, por pH e inanición (Darcan *et al*., 2009). En términos generales, el conocimiento respecto a los genes globales y específicos que regulan la aparición y mantenimiento de las formas no cultivables es muy limitado.

El factor principal para la reanimación o recuperación de células bacterianas en fase no cultivable es la inactivación del agente inductor. Este proceso solo ocurre bajo condiciones muy específicas y por un tiempo limitado, pues las células pierden la capacidad de cultivabilidad de forma permanente tras un periodo definido conocido como ventana de reanimación (Pinto *et al*., 2015). La ventana de reanimación depende de la especie bacteriana, del mecanismo de inducción y de las condiciones de reanimación utilizadas (Senoh *et al*., 2010). Estas condiciones van desde el aporte de nutrientes específicos que pongan fin a la inanición, hasta la utilización de quelatantes como el EDTA (Ordax *et al*., 2006) para inhibir iones tóxicos, neutralizadores de cloro como el tiosulfato de sodio o lavados con soluciones inocuas (Santander *et al*., 2011), permitiendo la reanimación celular tras específicos periodos de incubación.

1.2.4. Detección de Formas No Cultivables

La detección de una bacteria objetivo a partir de una muestra compleja requiere un paso de pre-enriquecimiento selectivo con el fin de aumentar la concentración del organismo objetivo hasta niveles detectables (Stevens y Jaykus, 2004). Sin embargo, este procedimiento no permite un monitoreo en términos de cuantificación real, ya que altera las poblaciones objetivo y es inútil para fases no cultivables. Para estos casos específicos, es necesario procesar directamente las muestras y recurirr a una v-PCR. Esta técnica combina la tradicional PCR cuantitativa con protocolos

adecuados para la extracción de ADN y el tratamiento con colorantes fotoreactivos, lo que resulta en una herramienta útil para cuantificar bacterias a partir de muestras complejas (Kiefer *et al*., 2022). Sin embargo, no existe una técnica estándar, por lo que es necesario implementar modificaciones o adaptaciones según la muestra a analizar, específicamente en lo que se refiere al pre-tratamiento para separar eficientemente las células objetivo de la matriz que las contiene y el tratamiento con el colorante fotoreactivo adecuado (Demeke y Jenkins, 2010).

1.2.5. Importancia de las Formas Bacterianas No Cultivables en la Fitopatología

El papel de las bacterias como agentes fitopatógenos se remonta a finales del siglo XVIII (con el tizón de fuego causado por *E. amylovora* como primer caso) y hasta la fecha su diagnóstico se basa en cotejar los signos y síntomas de la enfermedad con el aislamiento del agente causal en medios nutritivos (Pinto *et al*., 2015). Sin embargo, Xu *et al*., (1982) descubrieron y presentaron por primera vez un estado fisiológico bacteriano único de las especies no esporuladoras, cuya principal característica es la incapacidad para crecer en medios de cultivo convencionales tras permanecer por periodos variables en condiciones de estrés. Actualmente, el conocimiento profundo de las fases no cultivables está mayormente documentado en especies de importancia clínica, con estudios en al menos 85 especies bacterianas (Zhao *et al*., 2017) de las cuales solamente 12 son de interés agrícola (*Acidovorax citrulli*, *Agrobacterium tumefaciens*, *Clavibacter michiganensis*, *E. amylovora*, *Pantoea agglomerans*, *Pseudomonas syringae*, *Pseudomonas fluorescens*, *Ralstonia solanacearum*, *Rhizobium leguminosarum*, *Sinorhizobium meliloti*, *Xanthomonas axonopodis* y *Xanthomonas campestris*) (Pinto *et al*., 2015; Dong *et al*., 2020).

1.2.6. Implicaciones de las Formas No Cultivables en *E. amylovora*

El tizón de fuego, causado por *E. amylovora*, es una de las patologías vegetales más ampliamente documentada y afecta esencialmente a especies de pomáceas. Su fisiopatología compleja, rápida

evolución y esquemas de control poco eficientes, han hecho que se considere la enfermedad vegetal más temida entre los fruticultores. La infección primaria ocurre usualmente en primavera cuando *E. amylovora* es llevada desde la fuente de inóculo primario hasta las primeras flores (Van der Zwet y Keil, 1979). Dichas fuentes de inóculo primario son clasificadas con base en las poblaciones bacterianas que albergan, las cuales son estimadas a través de métodos basados en la cultivabilidad de la bacteria en medios selectivos. Esta información es crucial en la toma de decisiones para implementar la estrategia de control principal basada en la supresión de la bacteria a través de antibóticos como medida preventiva.

Se ha documentado que *E. amylovora* puede responder a condiciones de estrés como la inanición o exposición de citotóxicos a través de la inducción a formas no cultivables (Biosca *et al*., 2006; Ordax *et al*., 2006) sin embargo, la inmensa mayoría de la evidencia se ha obtenido de estudios *in vitro* y se conoce poco sobre la presencia de la bacteria bajo este estado en ambientes naturales. Como ya se ha mencionado, entornos naturales como los huertos, propician condiciones de estrés intermitentes (Roszak y Colwell, 1987) como la baja disponibilidad de nutrientes durante el invierno, cuando la actividad metabólica de los hospederos disminuye, y en consecuencia los nutrientes aprovechables por *E. amylovora* también son limitados. O bien, la presencia de compuestos citotóxicos que suelen acumularse en el suelo por periodos relativamente cortos, como los antibióticos sintéticos, o más estables como el sulfato de cobre (Colwell y Grimes, 2000), son inductores de formas no cultivables en *E. amylovora,* y de ocurrir en entornos naturales, implicaría que el actual esquema de monitoreo basado en cultivabilidad no considera las formas no cultivables, subestimando fuentes de inóculo potenciales y conduciendo a un manejo poco eficiente de la enfermedad.

1.3. Hipótesis

Fuentes convencionales y no convencionales de inóculo primario de *E. amylovora* albergan formas bacterianas no cultivables del patógeno.

1.4. Objetivo General

Diseñar y evaluar un procedimiento secuencial basado en v-PCR semicuantitativa, para detectar y estimar poblaciones viables, cultivables y no cultivables de *E. amylovora* a partir de fuentes de inóculo primario en huertos de manzano.

1.5. Objetivos Específicos

1. Estimar las poblaciones cultivables de *E. amylovora* en fuentes de inóculo primario en huertos comerciales de manzano.
2. Diseñar y estandarizar un pre-tratamiento eficiente para separar células bacterianas de matrices complejas.
3. Determinar y optimizar la concentración, condiciones de incubación y período de fotoactivación que permitan discriminar el material genético libre o de células necrosadas en muestras complejas, basado en una técnica enzimática combinada con monoazida de bromuro de etidio.
4. Estandarización de un protocolo para el aislamiento de ADNg a partir de muestras ambientales complejas.
5. Optimización de un protocolo de PCR semicuantitativo específico para *E. amylovora* a través de la correlación entre la concentración de dsDNA en los productos amplificados, con la concentración celular conocida en muestras control.
6. Estimación de las poblaciones viables cultivables y viables no cultivables mediante v-PCR semicuantitativa a partir de fuentes de inóculo primario en huertos comerciales de manzano.

1.6 Sección Integradora del Trabajo

En el primer artículo (pp.24) se aborda de forma detallada y actualizada la fisiopatología del tizón

de fuego, una enfermedad causada por la bacteria *E. amylovora*, que afecta a frutales, especialmente manzanos y perales. La enfermedad se caracteriza por una rápida propagación desde las flores a otros tejidos de la planta, como brotes, ramas y tronco, favorecida por la alta humedad y dispersada por insectos, viento y lluvia. Se explora el origen controvertido del inóculo primario, destacando los cancros en ramas y tallos como una fuente importante, pero también se muestra evidencia que señala a otros reservorios, como tejidos asintomáticos, frutos necrosados, plantas no hospedadoras y el suelo, como fuentes de inóculo significativos. La segunda mitad del documento profundiza en el concepto del estado viable no cultivable (VBNC) en *E. amylovora*, como una respuesta adaptativa ante condiciones de estrés, como inanición, bajas temperaturas o exposición a sustancias tóxicas. En este estado, la bacteria no puede multiplicarse en medios de cultivo convencionales, pero mantiene una viabilidad plena, actividad metabólica, virulencia y patogenicidad intactas. Se detalla el estado del arte en torno a los factores que permiten la inducción de esta respuesta en condiciones *in vitro* y las técnicas que permiten su reanimación bajo condiciones específicas. Por último, el artículo establece una serie de planteamientos e inferencias en torno a la relevancia del estado VBNC en el manejo del tizón de fuego, ya que podría llevar a subestimar ciertas fuentes de inóculo primario y concluye con la necesidad de diseñar e implementar técnicas de detección más avanzadas, como la v-PCR cuantitativa o la citometría de flujo, para mejorar las estrategias de control y manejo de la enfermedad.

El segundo artículo (pp.38) parte de la idea preconcebida y basada en evidencia de que la inanición y la exposición a compuestos citotóxicos son factores capaces de inducir una respuesta no cultivable en *E. amylovora*. Esto limita el monitoreo convencional de la enfermedad y por ende su control. Para probar esta hipótesis se determinaron las poblaciones viables de *E. amylovora* en sus formas cultivable y no cultivable, utilizando una v-PCR semicuantitativa aplicada a diferentes fuentes de inóculo primario disponibles en un huerto comercial de manzano, como cancros activos, brotes jóvenes y frutos visualmente necrosados, tejido asintomático, tejido en transición, suelo asociado a las raíces, flores del hospedero principal y de arvenses no hospederas, abejas melíferas y de agua destinada para riego. Un total de 200 muestras fueron procesadas de forma secuencial iniciando con un pre-tratamiento, el conteo de células cultivables, un tratamiento con DNasa y EMA, y la detección y cuantificación mediante v-sqPCR. El 43.5 % de las muestras resultaron positivas en medio selectivo. Las muestras de tejido sintomático albergaban poblaciones

abundantes de células cultivables, en rangos entre 10^5 y 10^7 UFC/g. Por otro lado, solo el 13 % de las muestras resultaron positivas mediante v-sqPCR, debido a un umbral mínimo de detección subjetivamente alto (≈1 × 10^4 UFC). Muestras específicas de cancros, brotes marchitos y frutos momificados arrojaron estimaciones mayores mediante v-sqPCR frente a los conteos en placa, lo que indica la presencia de formas no cultivables. En términos concretos, este estudio proporciona información útil y novedosa sobre la disponibilidad de inóculo en fuentes convencionales y no convencionales durante la prefloración y floración. Está claro que *E. amylovora* es capaz de sobrevivir al invierno en formas no cultivables, principalmente en estructuras necrosadas como cancros y frutos momificados, lo que insta a actualizar los esquemas de monitoreo actuales basados en cultivabilidad.

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2. PRIMARY INOCULUM OF *Erwinia amylovora*: ALTERNATIVE SOURCES AND VIABLE BUT NON-CULTURABLE STATE: A REVIEW

Daniel Alejandro de la Peña-Baca[1], Alejandro Romo-Chacón[1], Claudio Rios-Velasco[1], Guadalupe Isela Olivas-Orozco[1], José de Jesús Ornelas-Paz[1], Carlos Horacio Acosta-Muñiz[1]

[1]Centro de Investigación en Alimentación y Desarrollo A.C. Cuauhtémoc, Chihuahua, México

*Corresponding author: cacosta@ciad.mx

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REVIEW

Primary inoculum of *Erwinia amylovora*: alternative sources and viable but non-culturable state: A review

Daniel Alejandro de la Peña-Baca[1] · Alejandro Romo-Chacón[1] · Claudio Rios-Velasco[1] · Guadalupe Isela Olivas-Orozco[1] · José de Jesús Ornelas-Paz[1] · Carlos Horacio Acosta-Muñiz[1]

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Abstract

Fire blight is a complex and devastating plant disease caused by the necrotrophic bacterium *Erwinia amylovora*. One of the main factors that determine the occurrence of the disease is the infective inoculum from the primary sources in the orchard. The most accepted theory asserts that overwintering cankers are the most likely source of the primary inoculum. However, the origin remains controversial. Several studies have documented the infectious potential of alternative sources such as shoots and fruits infected in previous seasons, the soil, and even non-host plants. These alternative sources are underestimated because they harbor non-significant epiphytic populations of *E. amylovora*. However, the low populations of the pathogen could be related to the ability of the bacterium to induce a viable but non-culturable state (VBNC) in response to starvation or cytotoxic stress. The natural environment would favor these conditions, making it difficult to detect them through methods based on the cultivability of the bacteria. This review summarizes the main and alternate sources of primary inoculum of *E. amylovora*, its infectious potential, and the VBNC state as a survival mechanism during periods of stress under natural conditions.

Keywords Fire blight · Overwintering cankers · VBNC state · Alternate inoculum

Introduction

With a complex and rapidly evolving pathophysiology, fire blight caused by *Erwinia amylovora* is a widely documented disease and mainly affects pome trees. Primary infection usually occurs in spring when the bacteria travel from the primary inoculum source to the first flowers (van der Zwet and Keil 1979). The primary sources of inoculum are classified according to the number of bacterial populations and are studied mainly through methods based on the cultivability of bacteria in selective media and recently also through molecular techniques such as PCR. *E. amylovora* can respond to stress conditions such as starvation or exposure to cytotoxics through the viable but non-culturable state (VBNC) (Biosca et al. 2006; Ordax et al. 2006). This response has been reported in at least a hundred bacterial species (Dong et al. 2020). As its name indicates, the bacterium maintains complete viability and minimal metabolic activity but without the ability to grow in nutrient media (Roszak and Colwell 1987). VBNC state is a bacterial survival strategy in natural environments. However, the natural occurrence of *E. amylovora* in this state remains unknown.

This review details the conventional and unconventional sources of *E. amylovora* primary inoculum and its infectious potential. It also raises the possibility that the reported populations of culturable bacteria represent only a fraction of the real viable and potentially infectious inoculum present

✉ Carlos Horacio Acosta-Muñiz
cacosta@ciad.mx

Daniel Alejandro de la Peña-Baca
alejandro.pena17@estudiantes.ciad.mx

Alejandro Romo-Chacón
archacon13@ciad.mx

Claudio Rios-Velasco
claudio.rios@ciad.mx

Guadalupe Isela Olivas-Orozco
golivas@ciad.mx

José de Jesús Ornelas-Paz
ornelas@ciad.mx

[1] Centro de Investigación en Alimentación y Desarrollo A.C. (Cuauhtémoc Regional Coordination), Av. Río Conchos S/N, Parque Industrial, Z.C., 31570 Cuauhtémoc, Chihuahua, México

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in conventional and unconventional sources of primary inoculum, probably due to the presence of the bacteria in the VBNC state.

Fire blight and its causal agent

A phytopathogenic bacterium

The first report of fire blight comes from the Hudson Valley, New York, USA, in the late eighteenth century. It is currently present in at least fifty countries in the northern hemisphere and also in New Zealand (van der Zwet 2004; Zhao et al. 2019). This disease can reduce yields, shorten the productive life of fruit trees, and devastate entire orchards quickly when the incidence is high. The causative agent is *E. amylovora*, a Gram-negative, motile, necrogenic bacterium with a cell size ranging from 0.3 to $2.5 \times 0.8 - 3.0$ µm, depending on the strain, culture medium, and conditions, and observation technique (van der Zwet and Keil 1979). It grows in a wide temperature range (6–30 °C) but optimally between 25 and 27.5 °C (Billing 1974). Bacterial cells are protected by a layer of exopolysaccharides (EPSs), composed mainly of amylovoran and levan, which are closely related to the virulence and pathogenicity of the bacteria (Ayers et al. 1979). They also protect cells from environmental stresses such as desiccation, starvation, and cytotoxics (Jock et al. 2005; Ordax et al. 2010). Additionally, Roach et al. 2013 have suggested that the interactions between specific bacteriophages and *E. amylovora* are mediated by the presence and composition of EPSs.

Pathophysiology

Fire blight infection is rapid. Under favorable conditions (optimal temperature and high relative humidity), it can start in flowers and spread to new shoots, branches, trunk, and roots in a single season. In the winter, the bacteria hibernate in cankers and other tissues of the primary host or in alternate hosts near the orchard, where it remains inactive until the following year (Vanneste and Eden-Green 2000).

Rain, overhead irrigation, wind, and insects (presumably ants and some species of flies) disperse the pathogen from the primary inoculum source (discussed below) to the first flowers (van der Zwet and Keil 1979). The intercellular spaces of the floral stigmas provide an ideal environment for the rapid multiplication of *E. amylovora*, harboring epiphytic bacterial populations of up to 10^6 CFU/stigma (Johnson and Stockwell 1998). High humidity or precipitation favors the runoff of the bacteria from the stigma to the nectary through the stylar groove, where the diluted sugars of the nectary promote the multiplication of the bacteria, which makes it more likely to enter the vascular system of the plant through the nectar-secreting structures (nectarthodes) (Pusey 2000; Thomson 1986). Bees and other pollinators spread the pathogen among the flowers at this stage.

Floral infections result in partial (single flower) or complete (cluster) wilting, turning dark brown or black and remaining attached to the tree (Fig. 2a). The disease can progress rapidly from the peduncle to the terminal shoots, which turn dark brown and bend at the terminal end, causing the "shepherd's crook" (a characteristic symptom of fire blight) (Fig. 2b) (van der Zwet and Keil 1979). Tiny droplets of ooze can often be found on the surface of infected tissues. These are mainly composed of bacterial cells, EPSs, and sap. The ooze represents an essential source of easily dispersed infective cells (Eden-Green and Knee 1974).

Other host tissues (leaves, stems, fruits, and branches) can be infected by the bacteria entering through the stomatal openings or mechanical wounds caused by insects, phytophagous mites, hail, or injuries caused by pruning and other management tasks in the orchard (Steiner 2000; Thomson 2000).

Because of the infection, the stems, branches, and trunk crack and can eventually result in cankers. Furthermore, under ideal environmental conditions, the disease can progress to the crown and roots (van der Zwet and Keil 1979). The infective cycle described in this chapter is illustrated in Fig. 1 with solid lines.

Primary inoculum sources

The origin of the primary inoculum responsible for initiating fire blight infection in the spring is controversial. Overwintering cankers could be the most likely source of primary inoculum. However, other alternative sources have been proposed, such as shoots and fruits infected in previous cycles, which remain attached to the tree.

Overwintering cankers and bacterial ooze as the main source of primary inoculum

Severe endophytic infections of *E. amylovora* can cause cankers on branches and stems when vegetative growth ceases, usually during the summer. Cankers consist of dead bark, collapsed vascular tissues, and ooze (van der Zwet and Keil 1979) (Fig. 2c). The diameter of the cankers is 0.2–0.8 cm on young shoots and up to 20 cm on thick branches or trunks (van der Zwet and Beer 1992). It is possible to distinguish cankers with well-defined margins through simple inspections with a separation between the canker and healthy tissue. These cankers usually form at an early stage, when vegetative growth is active, and the tree responds to the infection by delimiting the damaged area with suberized tissue. On the other hand, there are cankers with indefinite margins,

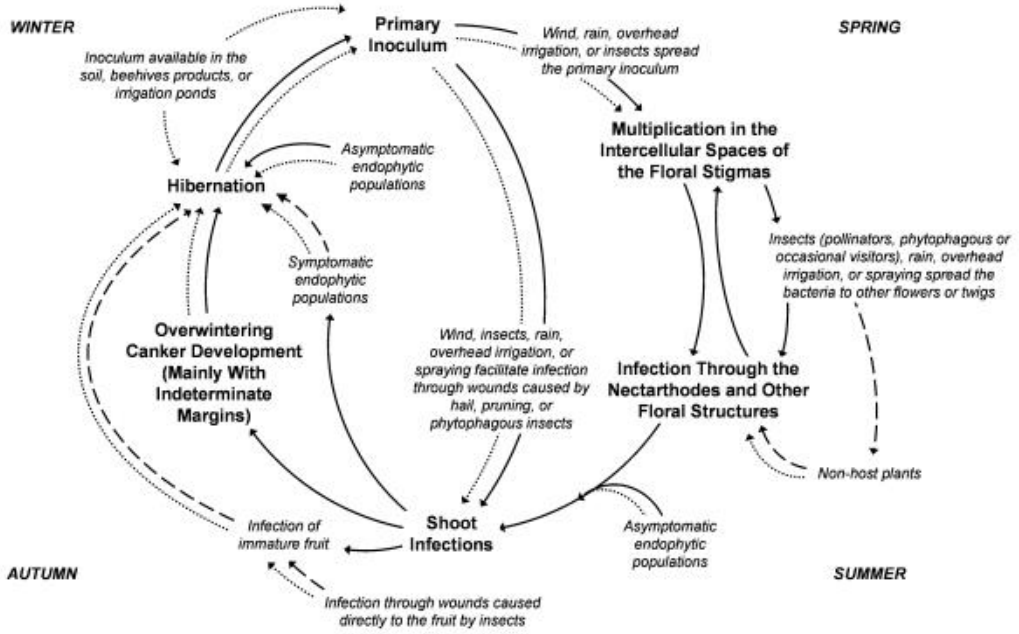

Fig. 1 Fire blight disease cycle scheme.

(Solid line): Conventional inoculum sources and routes of transmission that are widely documented and generally accepted. Refers to the culturable form of *E. amylovora*.

(Broken line): Alternative inoculum sources and routes of transmission that are controversial. This is a current issue that has triggered some research in recent years.

(Dotted line): Inoculum sources and transmission routes where the VBNC form of *E. amylovora* could hypothetically be involved

without a clear barrier between the canker and healthy tissue, these form in late summer or on young trees, especially on disease-susceptible cultivars (Steiner 2000). According to Beer and Norelli (1977), cankers with indefinite margins are the main source of primary inoculum in spring because they harbor a more significant number of viable bacterial cells in the transition tissue (disease progression zone).

At the beginning of spring, with the metabolic reactivation of the host and favorable environmental conditions (warm temperatures and high relative humidity), overwintering cankers are activated. Consequently, the populations of *E. amylovora* increase on their surface, and droplets of bacterial ooze of varying consistencies and colors can be observed (Thomson 2000).

The first controversy arises around the number of active cankers in the orchard and their infectious potential. Tullis (1927) detected viable populations of *E. amylovora* in 23 and 46% of cankers on pear and apple trees, respectively, and assumed that the percentage could be higher before winter. Miller (1929) documented that only 8% of overwintering cankers showed potentially infectious activity on apple trees. While Beer and Norelli (1977) reported twice that value (16%) in pear and apple cankers. In contrast, van der Zwet and Keil (1979) reported cankers without viable populations of *E. amylovora* on apple trees. Based on the values shown, it may seem unlikely that overwintering cankers are the main source of primary inoculum in spring. However, it should be noted that active cankers can be small (a few millimeters) and go unnoticed. These small cankers are just as important as the larger ones in spreading the bacteria, especially if they show ooze. (Ritchie and Klos 1974; Schroth et al. 1974; Thomson et al. 1975). The ooze can also emerge from any other infected tree tissue and in multiple places (which demonstrates the endophytic colonization capacity of the pathogen) (Gowda and Goodman 1970). It can harbor culturable populations of *E. amylovora* close to 10^8 CFU/µl (Slack et al. 2017). Moisture easily dissolves the ooze droplets, releasing the bacterial content. Therefore, rain,

Fig. 2 Signs and symptoms of fire blight. **a** Necrotic flower cluster. **b** Symptom known as shepherd's crook on a young shoot. **c** Active canker oozing. **d** Infection of immature apple fruit

dew, sprinklers to irrigate or control late frosts, and sprays of agrochemicals during flowering facilitate the dispersion of the bacteria from the ooze to other healthy tissues of the same tree or others nearby. Likewise, due to the thick consistency of the ooze, the wind can promote the formation of strands by disrupting and dragging them towards other tissues of the same tree or others nearby (van der Zwet and Keil 1979).

The primary dispersion of *E. amylovora* through insects is not entirely clear, and studies are needed to consider some behavioral aspects of insects and other arthropods associated with pome trees that could be potential disseminators of the disease. van der Zwet and Keil (1979) stated that dispersal of the pathogen by insects such as flies (Diptera: Muscidae) was unlikely, as they are attracted to ooze on infected tissues but rarely visit flowers. And conversely, bees, especially *Apis mellifera* L. (Hymenoptera: Apidae) used in pollination, are not attracted to cankers. However, the role of insects as disseminators of *E. amylovora* has been underestimated. Miller and Schroth (1972) showed that some flies of the genera *Pegomya* (Diptera: Anthomyiidae) and *Minettia* (Diptera: Lauxaniidae) can harbor the pathogen in a range between 10^1 and 10^5 CFU/insect. It has recently been shown that flies such as *Drosophila melanogaster* (Diptera: Drosophilidae) (Boucher et al. 2019) and *Delia platura* (Diptera: Anthomyiidae) (Boucher et al. 2021a) can acquire *E. amylovora* (by contact and ingestion) from primary inoculum sources such as bacterial ooze, and subsequently transmit disease to healthy tissues. To date, the exact factors that attract the insect to the cankers are unknown, but they could feed on the abundant sugars that make up the bacterial ooze (Slack et al. 2017).

On the other hand, Boucher et al. (2021b) report the presence of *E. amylovora* in phytophagous hemiptera of the species *Philaenus spumarius* (fam: Aphrophoridae), *Empoasca fabae* (fam: Cicadellidae) and the genus *Paraphlepsius* (fam: Cicadellidae). The authors postulate that the feeding wounds of these insects could be a route of entry for the bacteria, where some Diptera (attracted by the sap exuding from the wounds) could facilitate infection and suggest further research in this regard.

Alternative sources of primary inoculum

Severe fire blight infections can occasionally occur with the minimal presence or even absence of active cankers in the orchard. These events have led to the development of research on alternative sources of primary inoculum, which could be just as important as active cankers.

Asymptomatic tissue

Isolation of viable and virulent cells of *E. amylovora* from most symptomatic tissues is common. However, the presence of the bacteria in tissues without visible symptoms has also been documented.

Early studies, such as those by Nixon (1927) and (Rosen 1933), evidenced the endophytic presence of *E. amylovora* in fruit trees without causing symptoms. Dueck and Morand (1975) and Bonn 1979 recovered culturable cells of *E. amylovora* from asymptomatic tissues of apple and pear trees during winter. At the end of the twentieth century, the development of molecular identification techniques facilitated research in this area. For example, McManus and Jones (1995) detected *E. amylovora* in asymptomatic apple tree tissues (leaves, axillary buds, and fruit calyxes) through a nested PCR targeting a fragment of the plasmid pEA29 specific for *E. amylovora*. Similarly, Tancos et al. (2017) detected the pathogen in asymptomatic budwood of apple trees cvs. Gala and Topaz, using the same technique. Likewise, Llop et al. (2000) detected the pathogen by PCR in up to 25% of the samples analyzed from asymptomatic pear and apple trees. However, they also documented the occurrence of the pathogen in asymptomatic tissues of other host plants such as loquats (*Eriobotrya japonica*), quinces (*Cydonia oblonga*), *Pyracantha* sp., *Cotoneaster* sp., and *Crataegus* sp. The greater sensitivity of PCR compared to plate counts is evidenced in studies such as the one by Stöger et al. (2006), where the pathogen was detected in 15 of 26 asymptomatic apple and pear tissue samples, even though only five of the positive samples showed growth in selective culture media. In all the experiments mentioned, the samples were obtained from visibly symptomless trees. However, the authors reported symptoms of fire blight on other trees in the same orchard or in nearby plots, which could explain the origin of the inoculum.

Similar results were obtained by controlled experiments, where trees are artificially inoculated, and the bacteria is subsequently isolated from samples without visible symptoms. For example, Keil and van der Zwet (1972) recovered culturable cells in young shoots developed from axillary buds of apple and pear trees. Furthermore, the authors reported that the bacteria could be recovered in 60% of the young shoots even after six months without showing any symptoms. Similarly, at the beginning of spring, Crepel et al. (1995) detected the pathogen in about 30% of asymptomatic apple shoots by PCR. The shoots overwintered in natural conditions.

It seems clear that *E. amylovora* can naturally reside inside the plant without causing symptoms or activating the host's defense mechanisms. But as Keil and van der Zwet (1972) suggest, latent populations of the bacterium can trigger symptomatic infection if environmental conditions are right (Fig. 1). To a large extent, this could explain that occasionally there is a higher incidence of fire blight in the endophytic phase in branches and stems, with a minimum incidence in the floral phase (Bonn 1979).

The mechanisms through which *E. amylovora* remains endophytic and asymptomatic are poorly understood. However, the role of this phase in some spontaneous or unexpected infectious events should not be underestimated.

Symptomatic tissues

In addition to the margins of active cankers, the survival of *E. amylovora* in other necrotic areas such as buds, branches, or flowers is widely documented.

Kielak et al. (2001) using PCR, detected the pathogen in 70% of the samples (tissue from the margin of symptomatic shoots) collected during spring in apple trees cvs. Goldster and Elstar. However, the culturable form of the bacteria could only be confirmed in less than half of the samples. Similarly, Sobiczewski et al. (2006) using molecular techniques and after winter, detected *E. amylovora* in about 23 and 83% of the terminal shoot samples of apple cvs. Gala and Šampion (syn. Shampion), respectively, after being artificially infected the previous spring. Subsequently, Sobiczewski et al. (2017) documented the viability and survival of *E. amylovora* in apple trees cv. Idared in 81 and 30% of the symptomatic samples tested at five and eight months after infection, respectively. The pathogen was also detected in the peripheral veins of the leaf and the parenchymal tissue. Furthermore, the authors described an increased rate of positive samples when using PCR as the detection technique, compared to culture on selective media.

The detection of *E. amylovora* at the beginning of spring in previously infected tissues supports the hypothesis that the pathogen can hibernate in these tissues. One of the strategies to survive the winter is probably feeding on infected and necrotic tissue. That is, it subsists as necrotrophic (Kraepiel and Barny 2016; Sobiczewski et al. 2017; Weißhaupt et al. 2016). However, more studies are needed to explore the habits of *E. amylovora*.

In any case, the necrotic tissue that maintains viable populations of the pathogen at the beginning of spring becomes a potential source of the spread of the disease (Fig. 1). Regardless, studies are required to validate this approach.

Infected fruits as a source of inoculum

E. amylovora can infect the fruit at any stage of development. This infection can occur through mechanical injuries (such as those caused by hail) and can be favored by wind or rain (Billing 2000; Steiner 2000). It also occurs when the pathogen survives in dehydrated floral organs after pollination and persists in immature fruit (Hale et al. 1986) (Fig. 2d). The direct infection of the fruit through the phytophagous insects is not documented. For example, the role of some insect pests as vectors of fire blight, such as *Cydia pomonella* (Lepidoptera: Tortricidae) and some hemiptera of the genera *Lygus* (fam. Miridae), *Acrosternum* (fam. Pentatomidae), and *Euschistus* (fam. Pentatomidae) is unknown. These insects directly affect the fruit, therefore, we suggest more research on this topic (Fig. 1).

Azegami et al. (2006) inoculated *E. amylovora* through wounds on apple tree twigs bearing immature fruit. Subsequently, and after 17–30 days, fully mature fruits remained asymptomatic, but the pathogen could be detected in up to 10.8% of them. Based on this, the authors conclude that the bacterium can move from the fruit-bearing twigs, pass through the abscission layer, and invade the fruit during its maturation. In this sense, Jock et al. (2005) showed that *E. amylovora* persists asymptomatically in mature apple fruit tissue for at least two weeks at room temperature after inoculation.

The subsistence of *E. amylovora* in fruits is a widely discussed topic. Although there is a consensus regarding the practically null risk that asymptomatic fruits disperse the pathogen (Roberts and Sawyer 2008), controversy arises regarding fruits with evident symptoms. One of the common symptoms in infected fruits is the presence of drops of ooze emerging from it. This tissue rupture could be due to the pressure exerted by the EPSs when they hydrate and swell since enzymes produced by *E. amylovora* that degrade the plant cell wall are unknown (Slack et al. 2017; Zamski et al. 2006). Because of fire blight infections, immature fruit often remains attached to the tree for several months. These dehydrate, darken, and take on a mummified appearance (Fig. 3a). It has been documented that these mummified fruits can play an important role as a source of inoculum for various fungal diseases since they provide the conditions and nutrients for the subsistence of these organisms, even during

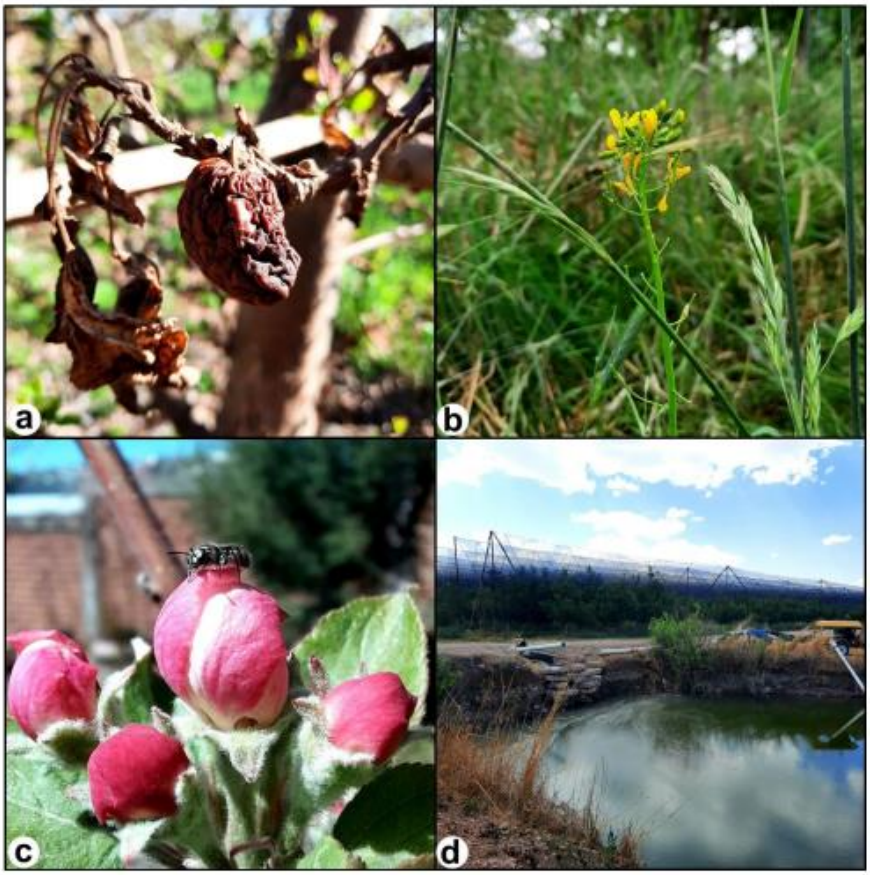

Fig. 3 Unconventional sources of primary inoculum. **a** Mummified fruit. **b** Weeds of the Brassicaceae family in the orchard. **c** Native pollinators. **d** Irrigation ponds

winter (Hong et al. 2000). It is very likely that this also occurs with fire blight, as Weißhaupt et al. (2016) detected populations of up to 10^9 cells/g of tissue in these mummified fruits even at the end of winter. Previously, Anderson (1952) dismissed its role as a source of spring inoculum. However, Voegele et al. (2010) and Weißhaupt et al. (2016) suggested a positive correlation between pathogen populations in mummified fruits and infection rates in apple trees, especially in their flowering stage. These fruits provide the bacteria with the ideal conditions to survive during the winter. Even more so if they present bacterial ooze since their composition can be used nutritionally by the bacteria (Ordax et al. 2010), and they also provide protection, keeping it viable for at least 20 months (Rosen 1938). This information suggests that populations of *E. amylovora* in mummified fruits should be monitored and considered to assess the risk of fire blight infection (Weißhaupt et al. 2016) (Fig. 1).

Non-host plants

The presence and persistence of *E. amylovora* in plant species considered as non-hosts is a poorly studied topic.

There are reports of natural infections in plum (*Prunus domestica*), Japanese rose (*Rosa rugosa*) (Vanneste et al. 2001), chokeberry (*Aronia melanocarpa*), strawberry (*Fragaria ananassa*) (Atanasova et al. 2005), and apricot (*Prunus armeniaca*) (Lee et al. 2020), among others. All are considered non-host species but are rosaceous, thus closely related to the recognized host plants for fire blight.

The presence of *E. amylovora* has also been documented in non-rosaceae plant species, especially in undergrowth and weeds of the families Brassicaceae, Poaceae, Polygonaceae, Ranunculaceae, Caryophyllaceae, Apiaceae, Asteraceae, Fabaceae, Adoxaceae, Sapindaceae, Malvaceae, Salicaceae, and Caprifoliaceae, commonly associated with pome orchards (Johnson et al. 2006; Weißhaupt et al. 2016) (Fig. 3b). In this sense, Johnson et al. (2006) report poor bacterial growth and low persistence in flowers of non-rosaceae plants of the genera *Acer*, *Brassica*, *Cytisus*, *Populus*, *Salix*, *Taraxacum*, *Trifolium*, and *Symphoricarpos*. In contrast, Weißhaupt et al. (2016) documented that although the multiplication of the pathogen in non-host plants is up to thirty times lower than that recorded in apple trees, *E. amylovora* is capable of persisting and multiplying in flowers of non-rosaceae plants. However, its presence is conditioned by the pressure of fire blight infection and the proximity to infected trees. These details are significant since it is demonstrated that the bacterium can develop in species classified with "dry stigmas" and not only in those with "wet stigma" as had been assumed until then (Johnson and Stockwell 1998). In any case, these non-rosaceae species are visited by bees and other pollinators (Fig. 3c). Therefore, non-rosaceae plants could be potential sites for the multiplication and dispersion of the pathogen that cannot be ruled out, even in periods longer than the flowering of fruit trees (Johnson et al. 2006; Weißhaupt et al. 2016) (Fig. 1).

Soil

The soil is probably the most varied and abundant reservoir of microorganisms due to its protection against stress factors such as high temperatures, UV radiation, and desiccation, favoring their persistence for long periods. The persistence of bacteria in agricultural soils is determined by various factors and physical characteristics such as humidity, texture (which affects water retention and aeration), temperature, pH, availability of nutrients, and the existing microbiota (Jamieson et al. 2002). However, the persistence and infectious capacity of *E. amylovora* from this environment are controversial.

Ark (1937) determined that *E. amylovora* can survive for 54 days in sterilized soil and only 38 days in untreated soil. In both cases, the bacterium maintained its infectious potential. Hildebrand et al. (2001) obtained similar results, who, using selective media, recovered culturable cells after eleven weeks of being inoculated in sterilized soil, while in untreated soil it persisted for four weeks. Additionally, the authors evidenced a significant increase in bacterial populations in the sterilized soil in the first week.

The presence of *E. amylovora* in the soil is attributable to infected and visibly necrotic organs of host and non-host plants that drop and rot rapidly, releasing viable bacterial cells into the soil (Taylor et al. 2003). This action suggests that the bacterium has necrotrophic habits or saprophytic-like behavior (Kraepiel and Barny 2016; Sobiczewski et al. 2017; Weißhaupt et al. 2016). This hypothesis is supported by studies that confirm a preference of *E. amylovora* for compounds such as sorbitol or sucrose (Bogs and Geider 2000; Suleman and Steiner 1994). These are present in the necrotic tissues of the host and could benefit the bacteria for a considerable period in the soil.

By relying mainly on simple molecules, *E. amylovora* faces biotic stress because of competition with other microorganisms better adapted to soil conditions (bacteria, actinobacteria, and fungi) (Hildebrand et al. 2001). In addition, the bacterium faces various antimicrobial compounds secreted by the native microbiota and the action of lytic bacteriophages, which are abundant in the soil (Vanneste and Paulin 1989).

Recently, Santander et al. (2020) through an experiment conducted in sterile soil, evidenced the formation of aggregates and biofilms by *E. amylovora* during root infection in pear trees, facilitating the entry of the pathogen into the vascular system. In this regard, Castiblanco and Sundin (2018) demonstrated that the formation of biofilms by *E. amylovora* is an elemental virulence factor attributable to the

structural complexity of the matrix. Additionally, it is known that these structures protect the bacteria from adverse biotic and abiotic factors (Flemming et al. 2016), and as already mentioned, the components of these biofilms are used by *E. amylovora* as a carbon source in the absence of nutrients (Ordax et al. 2010).

The role of the soil as a reservoir and source of primary inoculum for *E. amylovora* under natural conditions remains unclear. More research is needed on this topic, specifically, it is necessary to determine if the survival time of the bacteria under natural soil conditions is sufficient to initiate an infection in spring. In addition, possible routes through which the pathogen can reach the main sites of primary infection must be validated, as well as establishing whether wind, insects, or splashes from rain or irrigation are facilitators of infection.

VBNC state in *E. amylovora*

Bacteria activate various adaptation mechanisms in response to environmental stressors. One of these mechanisms that have become relevant lately is the viable but non-culturable state (VBNC). It consists of a period of latency where the bacterium remains intact and with detectable metabolic activity but cannot multiply in the culture media (Roszak and Colwell 1987). However, most bacterial species that show this adaptation can become reactivated by specific nutritional and temperature conditions (Oliver 2000). Generally, both VBNC state induction and reactivation can be induced by in vitro methods.

To date, induction of the VBNC state has been described in at least 100 bacterial and yeast species. Standing out for their global fruit and vegetable importance is *Acidovorax citrulli*, *Agrobacterium tumefaciens*, *Clavibacter michiganensis*, *Pseudomonas syringae*, *Ralstonia solanacearum*, *Rhizobium leguminosarum*, *Sinorhizobium meliloti*, *Xanthomonas axonopodis*, *Xanthomonas campestris* and of course, *E. amylovora* (Dong et al. 2020).

Inducing factors

The agents that induce the VBNC state are cytotoxic compounds such as copper, sulfur, chlorine, organic acids, and hydrogen peroxide. Also, desiccation conditions, low temperatures, and starvation.

Starvation is the most documented VBNC-inducing factor in *E. amylovora*. Ordax et al. (2009) observed that viable bacterial populations persisted for at least 35 days (with no significant variations) in calyxes of mature apples. However, the authors reported that between 7 and 28 days, the culturable cell counts were significantly reduced. The above is due to the VBNC state in response to starvation stress and desiccation in the fruit calyxes. Biosca et al. (2006) showed that this bacterium can survive for at least six months in oligotrophic environments such as water (drinking, deionized, and irrigation), reducing its ability to grow by up to four logarithms without compromising its viability. Subsequently, Biosca et al. (2007) demonstrated the survival of *E. amylovora* in natural (non-sterile) rainwater for at least 45 days. Furthermore, they reported that the cultivability of bacterial cells decreased rapidly in non-sterile water, contrary to what happened when the water was sterilized before inoculation. However, in both cases, the viability of the bacteria was maintained. Additionally, the authors reported a gradual increase in native bacterial populations in unsterilized rainwater showing greater competition for the scarce nutrients contained in the water, which exacerbated early starvation and consequently rapidly induced the VBNC state. Induction of the VBNC state in *E. amylovora* in response to starvation may be affected by environmental temperature. Biosca et al. (2006) reported that under sterile oligotrophic conditions, low temperatures (close to 5 °C) delayed the appearance of the VBNC state, maintaining the viability and cultivability of the bacteria. This behavior has also been documented in natural (non-sterile) oligotrophic microcosms, where low temperatures drastically reduce the metabolic activity of *E. amylovora* and thus reduce competition for nutrients from the medium. These factors could delay or prevent the induction of the VBNC state (Biosca et al. 2009).

Starved cells of *E. amylovora* show some morphophysiological changes such as a reduction in size and a rounded or elongated shape, easily distinguishable from the traditional bacillary shape (Santander et al. 2014). These changes may be associated with a stationary phase (Nyström 2004), also described in other enterobacteria such as *Salmonella enterica* (Gupte et al. 2003) and *Escherichia coli* (Wei and Zhao 2018). In addition, it has also been reported that *E. amylovora* under starvation conditions significantly reduces its motility (possibly to optimize nutrients) and shows small vesicles on the surface of the outer membrane. However, these changes seem to be independent of the activation of the VBNC state and do not influence the infectious potential or virulence of the bacteria (Santander et al. 2014).

Some cytotoxic compounds also induce the VBNC state in *E. amylovora* in response to toxicity stress. Among these substances is copper, which is used to manage fire blight. Chlorine, hydrogen peroxide, and acetic acid also induce this response, some of which are used to disinfect materials/tools and equipment in the orchard. Ordax et al. (2006) induced the VBNC state in *E. amylovora* by exposing it to a copper sulfate solution (0.005 mM). After 36 days of exposure, total viable cells remained, but agar plate counts fell below the limit of detection (1 CFU/mL). Additionally, the authors report that the induction time is reduced to less than a day when a tenfold higher concentration of copper sulfate is used. Subsequently,

Ordax et al. (2009) induced the VBNC state in *E. amylovora* inoculated in the calyxes of mature apples treated with copper sulfate (0.1 mM). After seven days, the culturable cell number was reduced below the detectable limit. However, viable cell populations remained close to the original regardless of assay temperature. Santander et al. (2011) showed that exposure of *E. amylovora* for at least 5 min to other compounds such as sodium hypochlorite (1 ppm), acetic acid (1%), and hydrogen peroxide (5%) induce the VBNC state and maintain good cell viability. The mechanism of VBNC response under toxicity stress in *E. amylovora* is not entirely clear. It could be related to changes in the proportions of the biomolecules that make up the structure of the cell wall and are responsible for regulating the fluidity of the membranes. In this way, cells in the VBNC state are protected from high or low osmotic pressures and cytotoxic compounds such as heavy metals, ethanol, chlorine, organic acids, and even antibiotics (Dong et al. 2020).

On the other hand, the role of beehives as a source of inoculum for *E. amylovora* is a topic worth mentioning. Although it is known that beehives can accumulate a great diversity of bacteria (Piccini et al. 2004), there is evidence that the survival of *E. amylovora* in the cultivable form does not extend beyond 48 h on the surface of bees *Apis mellifera* L. (Hymenoptera: Apidae), and around 72 h in products such as pollen or wax (Alexandrova et al. 2001). According to the authors, this is due to the subjectively high temperature of the beehive (about 35 °C), the low relative humidity (40–60%) and the antibacterial properties of the products. However, recently Choi et al. (2021) reported that in vitro, the VBNC form of *E. amylovora* persisted for up to 13 days on bees. This allows us to hypothesize that the persistence of the pathogen in products such as honey, wax or pollen could be greater due to the VBNC form, especially during winter, when the temperature inside the beehive is considerably lower (Meikle et al. 2017). However, more research is required on these approaches.

Resuscitation conditions

Generally, the inactivation/suppression of the inducing agent is sufficient for the cells in the VBNC state to recover their ability to grow. Chelating agents such as EDTA (Ordax et al. 2006), chlorine neutralizers such as sodium thiosulfate, or washing with buffer solutions (Santander et al. 2011) inactivates the inducer allowing cells to grow in enriched media and specific incubation conditions. Santander et al. (2012) reactivated previously induced *E. amylovora* cells to the VBNC state by inoculating them into pear seedlings. The authors suggest that this method is more effective in resuscitating cells than selective media (such as KB) or immature pear fruit.

However, the resuscitation of VBNC cells only occurs under specific conditions and for limited periods since the cells lose this ability gradually after a defined period. This stage is known as the resuscitation window (Pinto et al. 2015). The resuscitation window varies depending on the bacterial species, the inducers, and the resuscitation conditions required by the bacteria (Senoh et al. 2010). For *E. amylovora*, the resuscitation window is at least six months, representing a more extended period than that described for other bacteria (Biosca et al. 2006).

Importance of VBNC state in primary inoculum sources

Fruit growers and scientists agree that the main strategy for managing fire blight is to prevent the establishment of the disease in the orchard, preventing infectious events through the suppression of *E. amylovora* in the epiphytic phase. This strategy is achieved by spraying antibiotics, natural compounds with bactericidal activity, or antagonistic microorganisms. These actions are especially effective at critical moments (with a higher probability of infection) determined by prediction models (Steiner 2000). These models are based on climatic data, meteorological forecasts, and information concerning the host, such as the cultivar and the phenological stage. But the determining factor in decisions to control fire blight is the epiphytic populations and the infectious potential of the different sources of primary inoculum (Johnson and Stockwell 1998; Steiner 2000).

As described in this review, the infectious potential of conventional and unconventional sources of primary inoculum of *E. amylovora* is complex and controversial, with conflicting opinions and results. Based on this, we believe that research should delve into two approaches that together add to fire blight control strategies. The first of these is to obtain clear and convincing experimental evidence on the populations of *E. amylovora* in its cultivable form in sources of primary inoculum that have been ignored or underestimated. Subsequently, the real infectious potential of these sources must be evaluated, considering aspects such as environmental conditions, vectors, and dispersion mechanisms that could be participating in the infectious cycle of the disease.

Likewise, the second approach revolves around the role that the VBNC state may be playing as a determining phase in the infectious events of fire blight, mainly in the monitoring of bacterial populations in sources of primary inoculum.

Natural environments (for example, commercial orchards) favor intermittent stress conditions that regulate the interactions of the microbiota in the environment (Roszak and Colwell 1987). Stress factors, such as low nutrient availability, force *E. amylovora* to develop adaptive responses such as the VBNC state to survive for considerable periods (Santander et al. 2019). We believe that an example of this occurs during winter when the metabolic activity of the host decreases and, consequently, the nutrients available to *E. amylovora* are also limited. Nutrient limitation occurs in

other oligotrophic environments associated with the orchard, such as irrigation ponds (Fig. 3d) or non-host plants. Similarly, the more complex nutrient sources present in the soil and dead tissue may not be available to the bacteria, and once simple nutrients are depleted, they starve (Bogs and Geider 2000; Slack et al. 2017; Suleman and Steiner 1994). In this way, starvation could be acting as an inducer of the VBNC state in a natural environment.

With this hypothesis in mind, the bacterial inoculum available from conventional sources such as active cankers or bacterial exudates may be greater than estimated. But additionally, there is the possibility that some unconventional sources of primary inoculum addressed in this review could have a more prominent role in the disease cycle (the way in which these approaches could be integrated into the infective cycle of fire blight is illustrated in Fig. 1 with dotted lines.). To a certain extent, these hypotheses explain the discrepancies in the results obtained in the detection of *E. amylovora* from natural samples. Specifically, when comparing the count in selective media versus qPCR. However, these results must be analyzed with caution since, as will be discussed later, detection techniques based on the total DNA contained in the samples (such as PCR) also consider residual extracellular DNA and DNA from dead bacteria, which could cause false positives.

An important aspect that must be discussed is the limitations when detecting and quantifying bacterial populations in their VBNC form from samples obtained from the orchard, since to date there is no reliable technique that allows this task to be carried out. Due to the obvious limitation of the VBNC form, its detection and quantification must be performed using complex techniques such as flow cytometry or qPCR. Although flow cytometry can discriminate between viable and dead cells, it cannot differentiate between the abundant bacterial diversity contained in the sample. On the other hand, qPCR allows the detection and quantification of a specific bacterial species, but it is unable to discriminate the genetic material of non-viable cells, which can cause false positives (Santander et al. 2019). In this sense, the main advances have been made around qPCR. It is known that during DNA extraction it is possible to add a step by treating the sample with ethidium monoazide or propidium monoazide (Wagner et al. 2008). These compounds suppress the amplification of free extracellular DNA and DNA from dead cells during PCR, binding permanently to genetic material after photoactivation. In this way, it is possible to discriminate against non-viable cells and avoid false positives. However, the results obtained when analyzing complex natural samples are inconsistent and have questionable sensitivity (Santander et al. 2019; Wagner et al. 2008).

Designing and standardizing a methodology capable of accurately detecting and quantifying VBNC cells from natural samples is a significant challenge, and more research is required on this topic. This is a crucial step to evaluate the infectious potential of conventional and alternative sources of primary inoculum, elucidate the approaches presented, and, consequently, contribute to the development of more effective strategies for fire blight management.

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3. DETECTION AND QUANTIFICATION OF CULTURABLE AND NONCULTURABLE FORMS OF *Erwinia amylovora* IN PRIMARY INOCULUM SOURCES VIA SEMIQUANTITATIVE VIABILITY PCR

Daniel Alejandro de la Peña-Baca[1], América Jocelyn Pérez-Álvarez[1], Alejandro Romo-Chacón[1], Claudio Rios-Velasco[1], Guadalupe Isela Olivas-Orozco[1], José de Jesús Ornelas-Paz[1], Carlos Horacio Acosta-Muñiz[1]

[1]Centro de Investigación en Alimentación y Desarrollo A.C. Cuauhtémoc, Chihuahua, México

*Corresponding author: cacosta@ciad.mx

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Detection and quantification of culturable and nonculturable forms of Erwinia amylovora in primary inoculum sources via semiquantitative viability PCR

Daniel A. de la Peña-Baca, América J. Pérez Álvarez, Alejandro Romo-Chacón, Claudio Rios-Velasco, Guadalupe Isela Olivas-Orozco, José de Jesús Ornelas-Paz, Carlos Horacio Acosta-Muñiz*

Centro de Investigación en Alimentación y Desarrollo, A. C. Departamento de Microbiología y Biología Molecular. Av. Río Conchos S/N Parque Industrial. Z.C. 31570. Cd. Cuauhtémoc, Chihuahua, México.

ABSTRACT

In agroecosystems, starvation and exposure to cytotoxic compounds induce the formation of nonculturable states in bacterial species such as *Erwinia amylovora*, the causative agent of fire blight. This phenomenon complicates the monitoring and control of pathogens. To address this limitation, techniques such as viability PCR (v-PCR) have been developed, enabling the detection and quantification of nonculturable forms in complex matrices. In this study, viable populations of *E. amylovora* in both culturable and nonculturable forms were quantified via semiquantitative viability PCR (v-sqPCR) applied to various primary inoculum sources. The samples were subjected to four processing stages: preparation, culturable cell counting, treatment with DNase and ethidium monoazide bromide (EMA), and subsequent detection and quantification by v-sqPCR. The pathogen was detected in 43.5% of the samples using selective media, predominantly in necrotic tissues such as cankers, symptomatic shoots, and mummified fruits. Only 13% of the samples tested

positive by v-sqPCR, as they contained bacterial populations below the minimum detection threshold (≈1 × 10^4 CFU). The discrepancy between plate counts and v-sqPCR results suggests the presence of nonculturable forms, especially in cankers, symptomatic shoots, and mummified fruits. This study provides valuable insights into the dynamics of *E. amylovora* in both conventional and alternative inoculum sources, underscoring the need for more effective methods to detect nonculturable forms and enhance control strategies.

Keywords: Fire blight, cankers, mummified fruits, v-sqPCR, ethidium monoazide bromide

1. Introduction

The growth rate is a common indicator of cellular activity in microorganisms and is often associated with cell viability. For decades, bacterial viability was determined primarily by the ability of bacteria to multiply in culture media. However, fully viable microorganisms whose reproductive capacity has temporarily or permanently ceased are commonly found in natural environments. It is estimated that approximately 81% of microbial genera on the planet cannot be cultured (Lloyd et al., 2018). In these environments, bacteria adopt nonculturable forms as a survival strategy against stress factors such as unfavorable humidity and temperature conditions, as well as limited nutrient availability (Roszak & Colwell, 1987). While the viable but nonculturable state (VBNC) is the most extensively studied form (Oliver, 2005), there is ongoing debate regarding its differentiation from other unculturable forms, such as sporulation (Pinto et al., 2015), persistent cells (Ayrapetyan et al., 2018), and dormant cells (Mukamolova et al., 2003). Nevertheless, these forms share a common characteristic: the ability to enter a temporarily nonreproductive phase. In agroecosystems, the limited availability of usable nutrients and the presence of cytotoxic

compounds can induce a nonculturable state, which has been reported in at least ten agriculturally important species (Dong et al., 2020). For phytopathology, these nonculturable forms present a challenge, as despite the temporary cessation of multiplication, virulence and phytopathogenicity remain intact (Postnikova et al., 2015). An example of this is *E. amylovora*, the causative agent of fire blight, for which chemical control is based on the bacterial populations present in primary inoculum sources (Johnson & Stockwell, 1998). These sources are typically monitored at the end of winter and during flowering, mainly by cultivating samples on selective media. However, starvation (Biosca et al., 2006) and exposure to cytotoxic compounds (Ordax et al., 2006) have been shown to induce nonculturable forms of *E. amylovora*, which can evade classical monitoring methods, leading to an underestimation of primary inoculum sources and, consequently, inefficient chemical control. The presence of nonculturable forms of *E. amylovora* in the surrounding bark of bacterial cankers on apple trees has been reported (Dhar et al., 2024), but it remains unclear whether such forms also exist in other primary inoculum sources.

To address the need for direct and efficient detection and quantification of nonculturable bacterial forms, various procedures and techniques have been developed, primarily centered around viability PCR (v-PCR). This technique, along with appropriate DNA isolation protocols, provides a valuable tool for quantifying bacteria from complex matrices (Kiefer et al., 2022). However, as no single method is universally applicable, modifications and adaptations are required on the basis of the sample matrix, the target organism, and the use of photoreactive dyes such as ethidium monoazide bromide (EMA) and propidium monoazide (PMA) (Demeke & Jenkins, 2010). This study aimed to detect and quantify viable populations of *E. amylovora*, both culturable and nonculturable, in various primary inoculum sources in apple orchards. To achieve this goal, a series of techniques and sequential procedures were employed, including direct processing of environmental samples,

treatment with photoreactive dyes, isolation of genomic DNA, and subsequent semiquantitative PCR.

2. Materials and methods

Samples were collected during the preflowering (winter; January–February) and full-flowering (spring; March–April) stages of 2022 and 2023. The samples were obtained from a productive apple orchard with *Golden Glory* and *Royal Gala* cultivars located in the Cuauhtémoc region, northern Mexico (28°25'48.58" N, 106°55'49.3" W), covering an area of approximately 23 ha. In total, 200 samples from different sources and characteristics were obtained and processed, as detailed in Table 1.

The experiment was summarized in four stages: starting with sample preparation, followed by counting culturable populations on selective media. The samples were subsequently treated with DNase and EMA to remove extracellular genetic material and dead cells. Finally, the presence of the pathogen was detected and quantified via semiquantitative PCR.

2.1. Control samples

To evaluate the performance of the methodologies and before the analysis of the environmental samples, control samples were prepared for each stage. The first consisted of a suspension of bacteria in PBS (1X) - PVP (1% w/v) buffer, as well as soil and plant tissue samples inoculated with approximately 1×10^8 CFU/g·mL of *E. amylovora* (strain Ea7G), which were processed conventionally. To determine the sensitivity of the PCR, samples were prepared with cell

concentrations ranging from 10 to 10^8 CFU/g·mL, with increments of one logarithmic unit. In particular, to confirm that treatment with DNase and EMA eliminates DNA from dead cells or extracellular genetic material from the sample, thereby preventing its amplification in subsequent PCR, the methodology implemented by Nocker et al., (2006) was modified and adapted. The controls were added based on a suspension of Ea7G (10^8 CFU/mL). The suspension was previously lysed by two different methods: at 85 °C for 10 min and again with isopropanol (70%) for 10 min. Finally, gDNA was also added at a final concentration of 6 µg/mL. The control samples were processed in triplicate in independent assays.

2.2. Pretreatment of samples

The samples (target and control) were treated and prepared according to their origin and characteristics, as described below. Ten grams (of tissue and soil) or ten specimens per sample (flowers or insects) were weighed and resuspended in 90 mL of PBS-PVP, then ground with a food processor (Hamilton Beach® Model: 59766). For irrigation water, 1000 mL samples were collected, concentrated, and reduced to 100 mL by centrifugation for 10 min at 5,000 × g. With volumes close to 100 mL for each sample, they were homogenized by shaking at 50 rpm for 15 min. Subsequently, the samples were filtered and washed (with PBS/PVP buffer) sequentially using cellulose membranes: in the first step, through a 50 µm pore size (Whatman® Grade 2 W), followed by a 25 µm pore size (Whatman® Grade 114), and finally with a 10 µm pore size (Whatman® Grade 93). The filtrate was concentrated and reduced to 500 µL by centrifugation for 10 min at 5,000 × g.

2.3. Estimation of culturable cells

The quantification of culturable forms of *E. amylovora* was carried out by inoculation onto selective CCT media (Ishimaru & Klos, 1984). After 48 to 72 hours of incubation at 28 °C, colonies with the typical morphology of the pathogen were identified and quantified. As a control, colonies were randomly selected and confirmed by PCR.

2.4. Detection of nonculturable forms of *E. amylovora*

2.4.1. DNase and EMA treatment

After the enumeration of culturable cells, the sample pellet was resuspended in 1x DNase reaction buffer (10x reaction buffer, pH 7.5: 100 mM Tris-HCl, 25 mM magnesium chloride [$MgCl_2$], and 1 mM calcium chloride [$CaCl_2$]), and 0.4 U/µL of DNase I (Roche® Cat. No. 04536282001) was added, as reported by Lennon et al., (2018). The mixture was incubated at 37 °C for 1 hour with occasional shaking. After the incubation period, EMA was added to a final concentration of 100 µg/mL, as described by Nogva et al., (2003) and incubated in total darkness for 10 minutes on a cooling block, with occasional shaking. To photoactivate the EMA, the samples were exposed to a halogen lamp (500 W) at a distance of 20 cm for 3 minutes, always above the cooling block, to avoid heating the samples. The samples were subsequently washed with PBS/PVP buffer and centrifuged (10,000 × g for 5 minutes). To inactivate DNase, the samples were treated with 25 mM EDTA and incubated for 10 minutes at 65 °C, as reported by Reyneke et al., (2017).

2.4.2. DNA isolation

For the isolation of genomic DNA, the standardized protocol of Sahu et al., (2012) for plant samples with high contents of polysaccharides and phenolic compounds was used as a basis. In this case, the methodology was adapted and modified according to the research needs. The procedure is described as follows:

I. After treatment with DNase and EMA, the sample pellets were resuspended in 1 mL of Buffer I (pH 8.0: 50 mM EDTA, 120 mM Tris-HCl, 1 M NaCl, 0.5 M sucrose, 2% Triton X-100, 5% PVP, and 0.2% β-mercaptoethanol), previously heated to 60 °C. The mixture was homogenized with a vortex mixer for 1 minute and incubated at 60 °C for 1 hour, with occasional agitation.

II. The pellet was centrifuged at 10,000 × g for 10 minutes, the supernatant was discarded, and the pellet was resuspended in 1 mL of Buffer II (20 mM EDTA, 100 mM Tris-HCl, 1.5 M NaCl, 2% CTAB, and 1% β-mercaptoethanol), previously heated to 60 °C. The mixture was gently shaken with a micropipette and incubated at 60 °C for 30 minutes.

III. The mixture was centrifuged at 10,000 × g for 10 minutes, and the aqueous phase was transferred to a sterile tube. Two volumes of chloroform:isoamyl alcohol (24:1) were added, and the mixture was shaken by inversion and then centrifuged at 10,000 × g for 10 minutes. The surface aqueous phase was transferred to a sterile tube. This step was repeated.

IV. To precipitate the genetic material, two volumes of isopropyl alcohol (at -20 °C) were added, and the mixture was incubated at -20 °C for 15 minutes. The sample was then centrifuged at 10,000 × g for 10 minutes.

V. The pellet was washed with 70% ethanol (at -20 °C) and centrifuged at 10,000 × g for 10 minutes. The supernatant was discarded, and the pellet was left to air dry at room temperature for a few minutes.

VI. The pellet was resuspended in 100 µL of TE-sodium buffer (pH 8.0: 0.5 M NaCl, 10 mM Tris-HCl, and 1 mM EDTA), and RNase was added to a final concentration of 0.3 mg/mL. The mixture was incubated at 37 °C for 30 minutes. Two volumes of chloroform:isoamyl alcohol (24:1) were subsequently added, and the mixture was vortexed and then centrifuged at 10,000 × g for 10 minutes. The surface aqueous phase was transferred to a sterile tube.

VII. The genetic material was precipitated with absolute ethanol and 3 M sodium acetate (pH 5.2). The sample was subsequently centrifuged at 10,000 × g for 10 minutes. The pellet was washed with 70% ethanol (at -20 °C) and centrifuged at 10,000 × g for 10 minutes. The supernatant was discarded, and the pellet was allowed to dry at room temperature for a few minutes.

VIII. The pellet was resuspended in 30 µL of TE buffer (pH 8.0).

2.4.3. PCR and agarose gel electrophoresis

For PCR, the principle described by Obradovic et al., (2007) was used, where a specific sequence of 1269 bp in size is amplified from a chromosomal region conserved for this bacterium. The total PCR mixture was 25 µL, containing 15 µL of ultrapure water, 2.5 µL of 10X PCR buffer (100 mM Tris-HCl, 500 mM KCl, and 0.01% gelatin), 1.5 µL of $MgCl_2$ (25 mM), 1.0 µL of deoxynucleotide mix (each at 10 mM), 1.0 µL of oligo FER1-F (10 µM [5'-AGCAGCAATTAATGGCAAGTATAGTCA-3']), 1.0 µL of oligo FER1-R (10 µM [5'-AATTTAATCAGGTCACCTCTGTTCAAC-3']), 1.0 µL of Taq polymerase (5 U/µL), and 2.0 µL of DNA. The PCR program was run under the following conditions (Bio-Rad Inc. C1000® Touch Thermal Cycler): initial denaturation for 4 minutes at 95 °C; 28 cycles [30 seconds at 95 °C, 30 seconds at 60 °C, 1 minute and 25 seconds at 72 °C]; and a final extension of 5 minutes at 72 °C.

The PCR products were subsequently analyzed via electrophoresis in 1% agarose gels stained with ethidium bromide (0.5 µg/mL).

2.4.4. Semiquantification of PCR products

The concentration of the amplified product was determined using a Thermo Fisher Scientific™ NanoDrop One UV–Vis microvolume spectrophotometer. Data from three independent sensitivity assays were normalized and analyzed to obtain the regression equation using Minitab® software version 16.2.4. Since the level of sensitivity varies depending on the type of sample, a regression equation was obtained for the pure samples (PBS/PVP), plant tissue, and soil samples (Fig. 4). In this way, the concentration of dsDNA in the PCR products was correlated with the known cellular concentration in the control samples. The concentration of dsDNA in the PCR products of the positive field samples was then determined. Its value was substituted into the corresponding regression equation, and the approximate concentration of the pathogen in the processed field samples was obtained. All the above steps were performed under the same conditions, procedures, and equipment to minimize error.

3. Results

3.1. Performance of Methodologies on Control Samples

3.1.1. Cell recovery following sample pretreatment

Washing, filtration, and centrifugation resulted in a significant reduction ($\alpha = 0.05$) in plate counts for the plant tissue-based control samples (0.66 ± 0.04 log CFU/g). However, no significant reduction was observed in the PBS/PVP (0.11 ± 0.03 log CFU/mL) or soil samples (0.29 ± 0.09 log CFU/g) (Fig. 1).

3.1.2. Effectiveness of DNase and EMA

The preliminary treatment effectively eliminated DNA from the control samples, whether it was free DNA or derived from dead cells. Additionally, the enzymatic treatment with DNase and the use of EMA did not significantly impact the viability of the cells in the control samples.

3.1.3. PCR sensitivity

The sensitivity of the assay was significantly influenced by the nature of the sample. In the PBS/PVP control samples, well-defined PCR bands were obtained even at the lowest amplification concentration (≈1×10^4 CFU/mL or g). However, at the same concentrations, the soil and plant tissue control samples presented a loss of resolution, with thin and poorly defined bands (Fig. 2).

3.2. Monitoring of *E. amylovora* in Different Inoculum Sources

3.2.1. Culturable cells

A total of 43.5% of the processed samples contained culturable forms of *E. amylovora*. The positivity rate ranged from 10% to 90%, depending on the sample type (Fig. 3A). In all the cases,

at least two of the samples tested positive for the pathogen in its culturable form. The abundance of the pathogen showed significant variability, even within the same sample type. Symptomatic tissues, such as cankers and necrotic shoots, had the highest abundance, with concentrations of up to 10^7 and 10^5 CFU/g of tissue, respectively. In contrast, the irrigation water samples contained fewer than one culturable cell per milliliter. The year of sampling did not significantly affect the bacterial populations.

3.2.2. Total viable cells (sqPCR)

In contrast to the culturable samples, only 13% of the processed samples tested PCR positive. The positivity rate ranged from 5% to 75%, depending on the sample type (Fig. 3B). Notably, samples from asymptomatic tissue, irrigation water, honeybees, and nonhost flowers were PCR negative. Indirect quantification of *E. amylovora* using the regression equation indicated that cankers contained up to 10^7 cells/g, followed by symptomatic tissue samples with concentrations approaching 10^6 cells/g. The soil samples presented similar concentrations; however, only one of the samples tested positive by PCR. Similar to the culture plate counts, the abundance of the pathogen varied considerably even within the same sample type.

3.2.3. Nonculturable Cells

During data analysis, clear differences were observed between the concentrations of total viable cells (as determined by PCR) and culturable cells (determined by plate culture) in some samples (Fig. 5). On the basis of the results from the control assays, it can be inferred that these differences correspond to nonculturable forms. This is because the DNase and EMA treatments inhibit the PCR amplification of free DNA or dead cells, which helps distinguish nonculturable forms. However,

nonculturable forms can be confidently identified only when the difference between plate counts and v-sqPCR is greater than the potential errors introduced by pretreatment (see the "Cell recovery following sample pretreatment" section).

Five canker samples contained abundant populations of nonculturable forms, with concentrations ranging from 1×10^4 to 1×10^7 cells/g (Fig. 5A). Similarly, one symptomatic tissue sample contained at least 1×10^6 nonculturable cells/g (Fig. 5B), a concentration similar to that found in a transition zone tissue sample (Fig. 5D). Additionally, three mummified fruit samples contained nonculturable forms, with concentrations near 1×10^4 cells/g (Fig. 5C). Notably, canker samples CA2022-6 and CA2023-5, as well as mummified fruit sample FM2023-7, presented viable cell concentrations close to 1×10^4 cells/g of tissue, despite no culturable cells being recovered.

4. Discussion

Natural environments rarely provide optimal conditions for the abundant growth of bacteria. These environments are generally hostile and characterized by unfavorable humidity and temperature conditions, as well as a limited supply of nutrients necessary to sustain large bacterial populations (Roszak & Colwell, 1987). Agroecosystems are not immune to these conditions, as the availability of nutrients that can be utilized by the microbiota depends on the phenological stage of the host, which influences bacterial populations throughout the cycle (de la Peña-Baca et al., 2023). This, combined with abiotic stressors (such as desiccation, hypoxia, or exposure to intentional or residual cytotoxins), forces bacteria to develop nonculturable forms (Gauthier, 2000).

Monitoring nonculturable forms of a particular bacterial species in environmental samples is a significant challenge, requiring the use of complex molecular techniques in conjunction with sequential procedures that allow for discrimination between the genetic material of viable and nonviable cells. The present study demonstrated that the combined use of DNase I and EMA applied directly to environmental samples before DNA isolation enables the selective removal of genetic material from nonviable cells. However, further investigations into the specific details of this approach are warranted. According to the literature, the success of using EMA to eliminate nontargeted genetic material depends on several factors, such as the concentration of the photoreactive dye, the concentration of genetic material, the wavelength and exposure time during dye photoactivation, and the characteristics of the amplicon (Copin et al., 2021; Nocker et al, 2006; Petersen et al., 2021). This suggests that there is no definitive, universal technique for sample treatment; instead, the method must be tailored to the specific needs of each experiment. In our case, determining the optimal treatment conditions required preliminary testing (unpublished data), which, although no EMA toxicity was observed in viable *E. amylovora* forms, showed intermittent failures. These failures were related primarily to the type of sample (most frequently observed in plant tissue samples and occasionally in soil). This justified the inclusion of a DNase I pretreatment, which, under certain conditions, can be as effective as EMA (Reyneke et al, 2017). The addition of this step resulted in the complete suppression of nontargeted genetic material, even in environmental samples.

Pathogen detection often requires a selective preenrichment step to increase the concentration of the target organism to detectable levels (Stevens & Jaykus, 2004). However, this procedure does not allow for real-time quantification, and preenrichment may alter the proportion of cells in the nonculturable phase. In such cases, it is necessary to process the samples directly, which, in

practical terms, presents a significant challenge. Environmental samples are generally complex and heterogeneous, requiring treatments to separate the cells from the matrix in which they are contained, as well as to reduce the presence of substances that may inhibit subsequent procedures, such as PCR (Dester & Alocilja, 2022; Dhar et al, 2024). Homogenization techniques, such as stomaching and pulsating, have been reported to be effective when processing plant samples (Burnett & Beuchat, 2001; Kim et al., 2012). In this study, a combination of physical methods was employed, including plant tissue disruption via a processor, filtration to remove larger particles, washing, and centrifugation. Although these techniques are frequently used for similar purposes (Choi et al., 2017; Dwivedi & Jaykus, 2011; Zhou et al., 2020), their effectiveness depends on several factors, such as the affinity of the bacterial surface for the matrix that contains it (Stinson et al., 2021), the method used, and the characteristics of the target organism (Kim et al, 2012). In the case of *E. amylovora*, the exopolysaccharide capsule surrounding the pathogen, or the formation of biofilms can favor adhesion to matrix residues or other cells (Koczan et al., 2011), forming aggregates that may hinder separation by filtration or centrifugation. Successful sample homogenization during pretreatment is crucial for the sensitivity of direct detection and quantification of microorganisms. This step may account for the reduction in bacterial counts observed after pretreatment (Fig. 1 and Fig. 2). In this regard, (Kim et al, 2012) reported that after homogenization of fresh plant samples, between 0.2% and 99.8% of the initial inoculum was recovered, with a strong dependence on the homogenization technique and the type of sample used. Similarly, Wolffs et al., (2006) reported that adding a filtration step (with a pore size >40 μm) allowed the removal of larger particles from the sample, reduced contaminants that inhibit subsequent PCR, and facilitated the recovery of 79.1 ± 6.0% of the initial inoculum in biological samples without preenrichment.

The detection and quantification of *E. amylovora* via PCR is a standard procedure in plant pathology. Using the TaqMan real-time PCR technique, which targets the chromosomal DNA of *E. amylovora* (*amsC* gene and *ITS* region), in combination with an automated DNA isolation method based on magnetic beads (QuickPick™), Pirc et al., (2009) were able to detect populations as low as 10^3 cells/mL from suberized and visibly necrotic plant material. Similar results were obtained using the TaqMan minor-groove binder real-time PCR technique, which targets a specific chromosomal region in pure samples (Gottsberger, 2010). Hinze et al., (2016), subsequently detected and quantified the pathogen in floral samples processed directly by washing and centrifugation. For this purpose, real-time PCR was performed using specific primers (p29TF and p29TR), achieving reliable quantifications in the range of 10^3--10^7 cells/mL.

With respect to soil samples, Song et al., (2024) detected the presence of *E. amylovora* at concentrations as low as 10^4 cells/g via real-time PCR in combination with a commercial kit specifically designed for this type of sample. PCR has often been reported to have higher sensitivity than plate counting (Sobiczewski et al., 2017; Stöger et al., 2006). However, this approach is not without limitations, as PCR does not distinguish between genetic material from necrotic cells, nonculturable forms, or extracellular DNA (Santander et al., 2019). In light of this, viability PCR (v-PCR) has gained increasing interest in this field. Using this technique, *E. amylovora* has been detected and quantified at concentrations ranging from 10^3 to 10^7 cells/mL in canker samples (Santander et al, 2019). In another study employing a similar protocol, Santander et al., (2022) reported that the lowest reliable detection threshold was approximately 10^4 cells/g in canker samples. More recently, the procedure was optimized, improving the lower detection threshold to 10^2 cells/mL using the viability droplet digital PCR (v-ddPCR) variant (Dhar et al, 2024).

In this study, experimental evidence combined with a review of the literature suggests that the difference between the number of positive samples detected by PCR (13%) and plate counts (43.5%) is due primarily to pathogen populations being mostly below the minimum detection threshold (10^4 cells/mL or g) (Fig. 3). This finding also supports the view that cankers are the main source of *E. amylovora* inoculum (Beer & Norelli, 1977), with populations as high as 10^7 cells/g. More importantly, cankers harbor culturable forms of bacteria, and the differences between plate counts and PCR data indicate the presence of abundant nonculturable forms in at least five samples, two of which did not contain culturable cells (Fig. 5A). The reliable presence of nonculturable forms of this bacterium in apple cankers was first reported recently (Dhar et al, 2024), and the results of the present study confirm that such forms can survive the winter and act as prebloom inocula in the spring. Symptomatic tissue from infections in previous years is another source of primary inoculum that, although generally harboring low culturable populations, can also survive the winter in nonculturable forms (Fig. 5B). A similar situation is observed with mummified fruits, which are controversial in terms of their role as important sources of inoculum (Voegele et al., 2010; Weißhaupt et al., 2016). Although the maximum populations recovered (10^5 cells/g) are considerably lower than the 10^9 cells/g reported by (Weißhaupt et al, 2016), this study provides evidence that *E. amylovora* can survive the winter on these fruits in a nonculturable form, with populations as high as 10^4 cells/g, which could be undetected by conventional monitoring methods (Fig. 5C). Similarly, a transition zone tissue sample (15–20 cm from a visually active canker) analyzed by sqPCR presented bacterial populations up to 1.62 log units higher than those detected by plate counts, suggesting that the bacteria could survive the winter in an endophytic and nonculturable form (Fig. 5D). However, more evidence is needed, particularly to rule out the possibility that the tissue sample corresponds to an early-forming canker. For the remaining samples, no evidence was found that they harbored nonculturable forms of *E. amylovora*. However,

despite some experimental limitations, this study demonstrated that, at the end of winter, both asymptomatic tissue and soil contain sufficiently abundant culturable populations of *E. amylovora*, making them important inoculum sources.

During the flowering period, bacterial populations close to 10^6 CFU/flower were detected in apple flowers, similar to those reported by Slack et al., (2022). However, in dandelion flowers (*Taraxacum* sp.), the pathogen was detected in nearly one-third of the samples analyzed, with populations approaching 10^2 CFU/flower. The presence of *E. amylovora* in weed flowers of the *Taraxacum* genus has been previously reported (Johnson et al., 2006; Weißhaupt et al, 2016), and although its persistence is low, the presence of pollinators, such as honeybees, contributes to the spread of the disease. Additionally, the presence of *E. amylovora* in water from ponds adjacent to the orchards used for irrigation was analyzed. Although populations were less than 1 CFU/mL, owing to the minimum detection threshold, the presence of nonculturable forms cannot be ruled out, as these environments have been reported as inducers of such a response (Biosca et al, 2006; Biosca et al., 2007).

5. Conclusions

A key aspect in refining fire blight management strategies is a better understanding of the dynamics of bacterial populations within agroecosystems. In this context, our study provides valuable and novel insights into the availability of inoculum from both conventional and nonconventional sources during the preflowering and flowering periods. These results indicate that *E. amylovora* can survive the winter in nonculturable forms, primarily in necrotic structures such as cankers and mummified fruits. Given the limitations of culturability-based techniques for detecting and

quantifying pathogens, there is a need for faster and more effective alternatives that allow for the recovery of the maximum number of cells from complex matrices without compromising sample viability. These alternatives should also improve subsequent molecular procedures, such as DNA isolation and viability PCR (v-PCR). These advancements will ultimately facilitate the development of more efficient strategies for controlling fire blight.

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Conflict of interests

The authors have no relevant financial or nonfinancial conflicts of interest to disclose.

Author contributions

The conception, design, and planning of the research project were carried out by Daniel Alejandro de la Peña-Baca and Carlos Horacio Acosta Muñiz. The experimental work was conducted by Daniel Alejandro de la Peña-Baca and América Jocelyn Pérez-Álvarez. Data analysis and figure design were performed by Alejandro Romo-Chacón. The first version of the manuscript was

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Table captions

Table 1 Characteristics of the collected samples

Figures captions

Fig. 1 Changes in cell counts after pretreatment with washing, filtration, and centrifugation. ANOVA-Tukey ($\alpha = 0.05$)

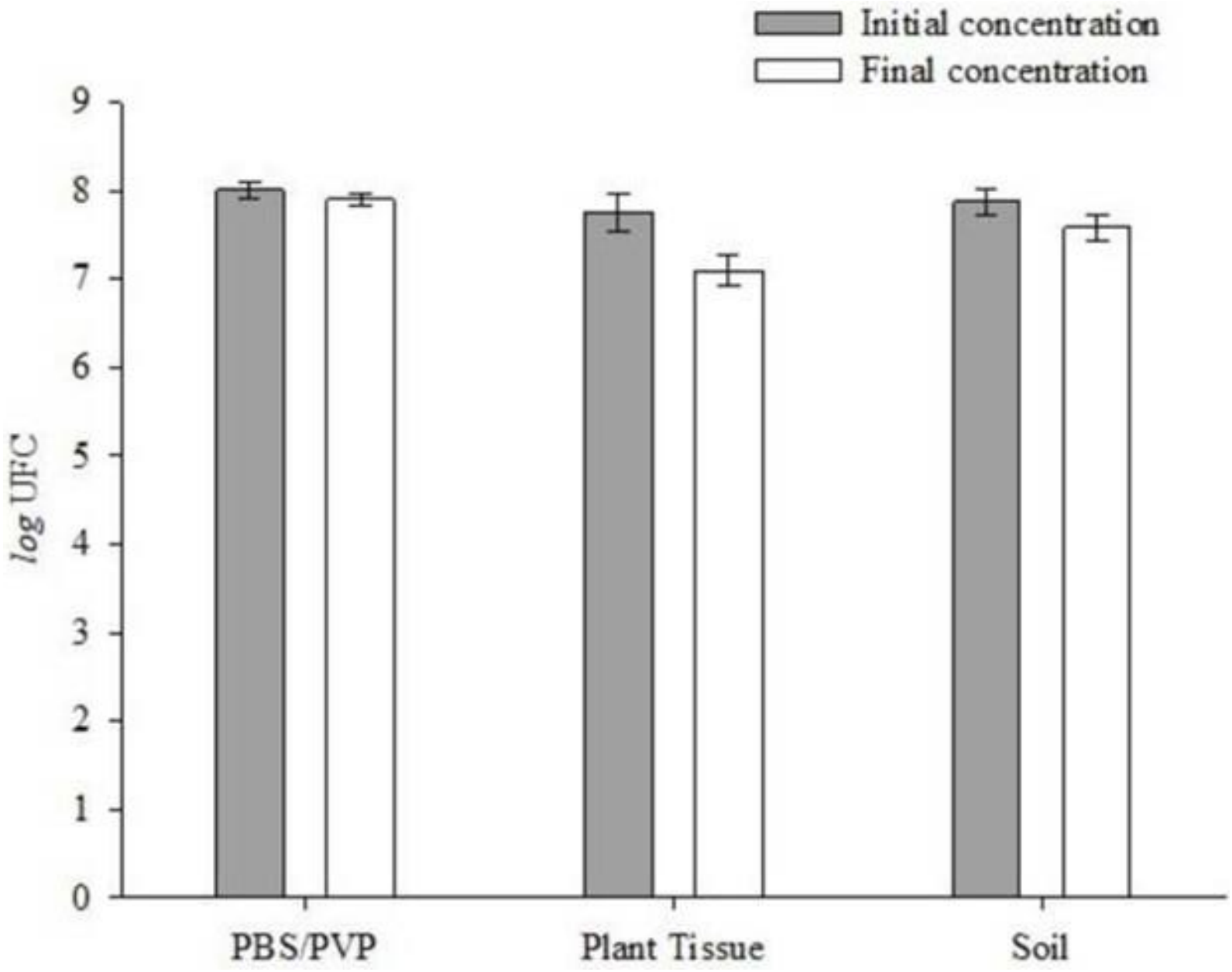
Fig. 2 Sensitivity of PCR in different control sample matrices for the detection of *E. amylovora*: PBS/PVP (a), plant tissue (b), and soil (c)

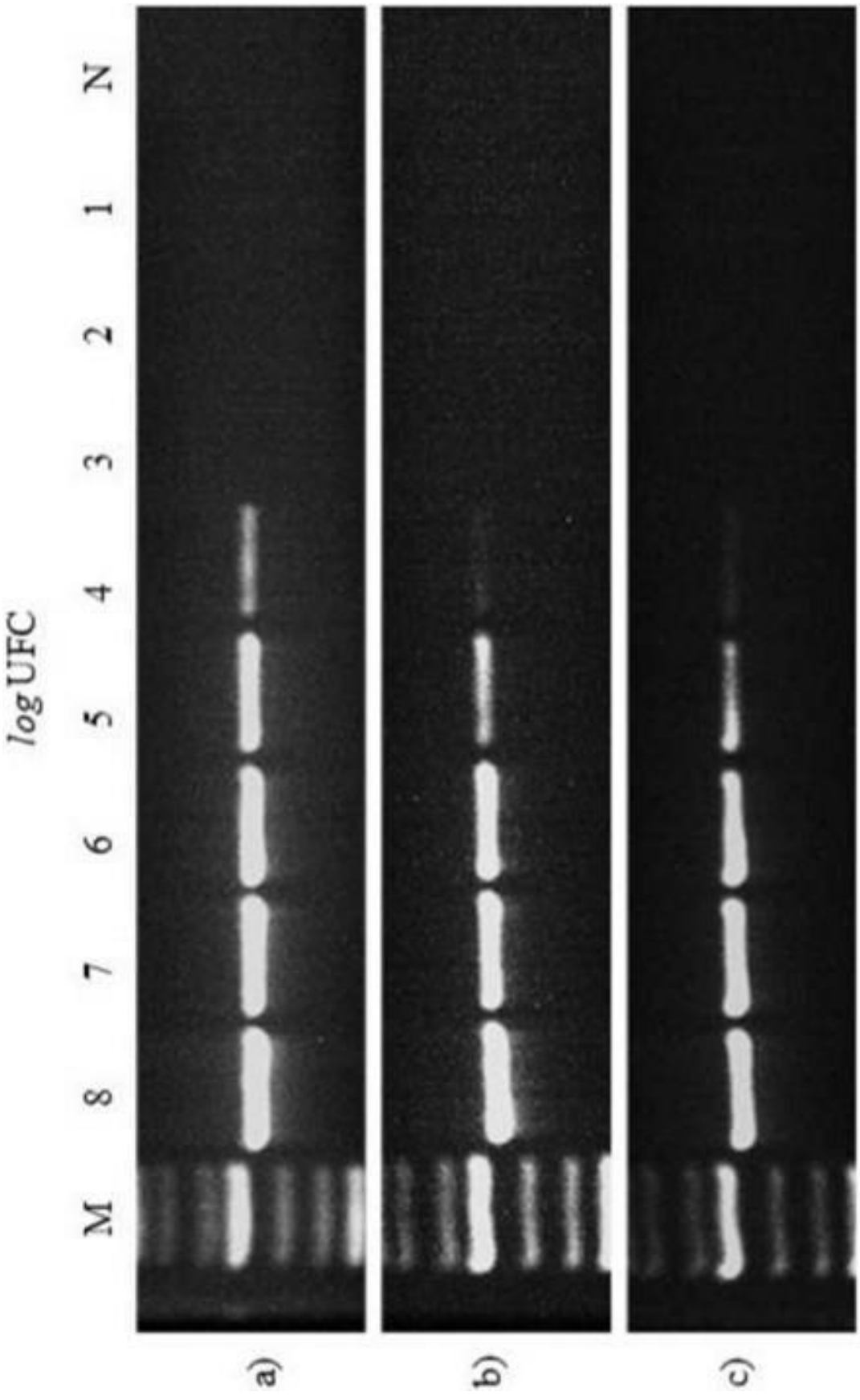
Fig. 3 Positivity and abundance of *E. amylovora*: a) Cultivable forms determined by CCT plate counting, and b) Estimates obtained by sqPCR. The horizontal line represents the lower detection limit of sqPCR

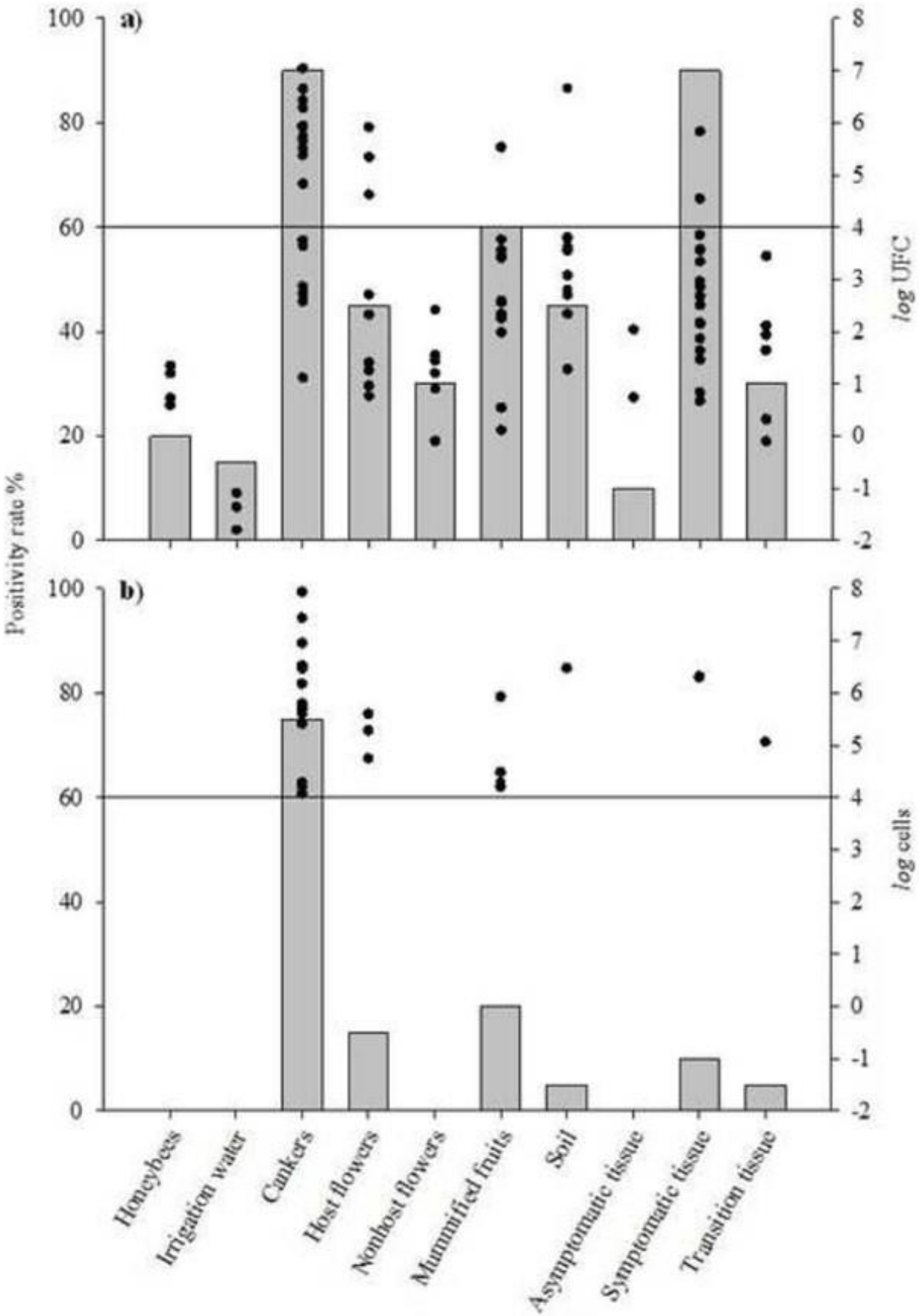
Fig. 4 Regression analysis correlating dsDNA concentration in PCR products with known cell concentrations in control samples: PBS/PVP (a), plant tissue samples (b), and soil samples (c)

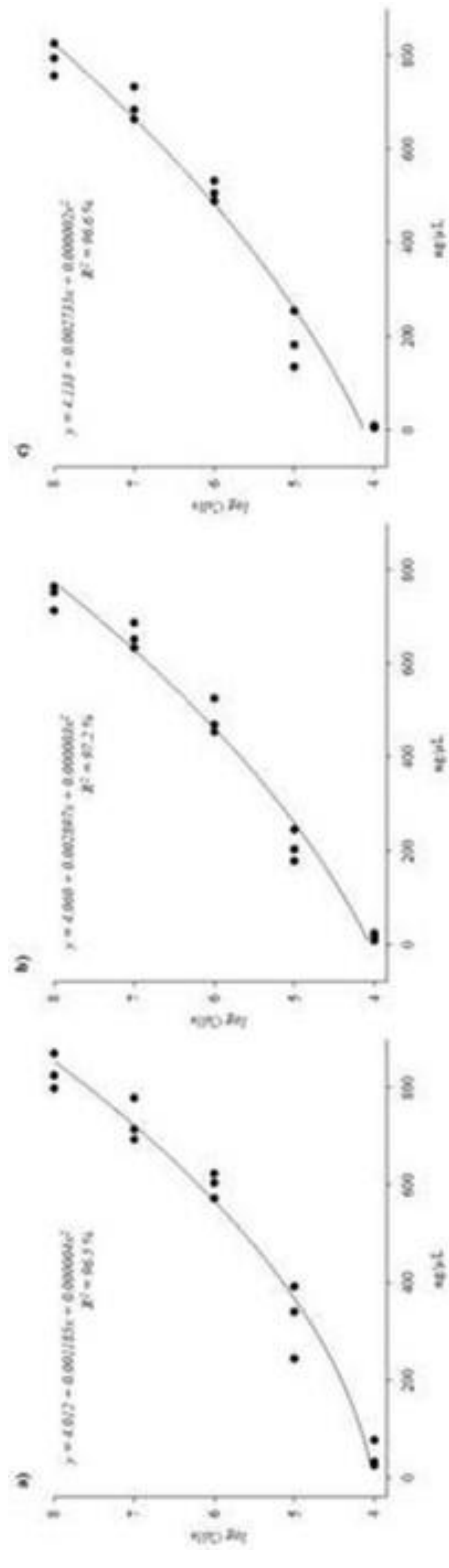
Fig. 5 Differences between plate counts (●) and sqPCR estimates (○). The graphs correspond to canker samples (a), symptomatic tissue (b), mummified fruits (c), and tissue from the transition zone (d). The positive bars represent the potential error introduced by the pretreatment of the samples. Nonculturable forms (♦) can be inferred only when the differences exceed this error. The horizontal line represents the minimum detection threshold of sqPCR.

Stage	Sample type	Characteristics
Prebloom (2022 y 2023)	Canker	Ranging from 3 to 12 cm in diameter, with undefined margins and minimal exudate presence
	Symptomatic tissue	Visually necrotic tissue from young shoots (previous year's growth)
	Mummified fruits	Ranging from 3 to 5 cm in diameter, dehydrated and visually necrotic
	Transition tissue	Transition zone tissue located 15 to 20 cm from a visually active canker
	Asymptomatic tissue	Visually healthy young shoots (from the previous year's growth) located on trees showing no symptoms
	Rhizospheric soil	Soil associated with roots at a depth of 15 to 20 cm and within a 50 cm radius from the trunk
Full bloom (2022 y 2023)	Host flowers	Apple blossoms 1 to 2 days old from opening and visually intact
	Nonhost flowers	Visually intact flowers from the genus *Taraxacum*
	Honeybees	Foraging bees of the species *Apis mellifera*
	Irrigation water	From a surface water catchment pond


Initial concentration
Final concentration
log UFC
9
8
7
6
5
4
3
2
1
0
PBS/PVP
Plant Tissue
Soil


log UFC
M 8 7 6 5 4 3 2 1 N
a)
b)
c)


a)
b)
Positivity rate %
log UFC
log cells
Honeybees
Irrigation water
Cankers
Host flowers
Nonhost flowers
Mummified fruits
Soil
Asymptomatic tissue
Symptomatic tissue
Transition tissue



log UFC or Cfu/g
AP2022-1
AP2022-2
AP2022-3
AP2022-4
AP2022-5
AP2022-6
AP2022-7
AP2022-8
AP2022-9
AP2022-10
TT2022-1
TT2022-2
TT2022-3
TT2022-4
TT2022-5
TT2022-6
TT2022-7
TT2022-8
TT2022-9
TT2022-10
CA2022-1
CA2022-2
CA2022-3
CA2022-4
CA2022-5
CA2022-6
CA2022-7
CA2022-8
CA2022-9
CA2022-10
ST2022-1
ST2022-2
ST2022-3
ST2022-4
ST2022-5
ST2022-6
ST2022-7
ST2022-8
ST2022-9
ST2022-10

4. CONCLUSIONES GENERALES

- El tejido sintomático visualmente necrosado resultó positivo a *E. amylovora* en formas cultivables con mayor frecuencia y abundancia que el resto de las muestras. No obstante, los medios selectivos descartan la fracción no cultivable.

El pre-tratamiento a base de lavados, filtraciones y centrifugaciones reducen significativamente la concentración bacteriana en las muestras a base de tejido vegetal (hasta 0.66 ± 0.04 log UFC/g).

- Un tratamiento conjunto a base de DNasa y EMA inhibió eficazmente la amplificación de productos en la PCR a partir de material genético libre o proveniente de células lisadas sin comprometer la viabilidad celular.

- El protocolo de extracción de ADNg a base de amortiguadores libres de fenol, permitió el aislamiento de material genético de buena calidad y pureza, sin importar las características de la muestra procesada.

- Debido a que el pre-tratamiento afecta negativamente la recuperación de células bacterianas a partir de muestras vegetales, también influencia negativamente la sensibilidad de la PCR. El protocolo solo fue capaz de detectar al patógeno en muestras que contenían concentraciones celulares superiores a 10^4 células/g.

- En términos concretos, los cancros albergan con mayor frecuencia y abundancia poblaciones no cultivables de *E. amylovora* a concentraciones entre 10^4 y 10^7 células/g. No obstante, también se pudo detectar la presencia de formas no cultivables en muestras de brotes marchitos, frutos momificados y tejido de transición. Lo anterior evidencia el riesgo potencial de estas fuentes de inóculo no convencionales para desencadenar un cuadro infeccioso.

5. RECOMENDACIONES

- Indagar respecto a la utilización de otras regiones conservadas de *E. amylovora* con menor tamaño, podría mejorar los resultados obtenidos en la PCR y reducir el umbral de detección mínimo.

- Probar otras técnicas de homogenización de tejidos que optimicen la recuperación de células a partir de matrices complejas permitiría aumentar la sensibilidad de la prueba.

- Es conveniente aplicar estas técnicas al monitoreo de la bacteria durante otras etapas fenológicas, no solo previo a la floración. Esto aportaría información valiosa para comprender de mejor forma la dinámica de las poblaciones bacterianas en el huerto.