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AISLAMIENTO Y CARACTERIZACIÓN DE ELEMENTOS GENÉTICOS INVOLUCRADOS EN LA FORMACIÓN DE LA AGALLA INDUCIDA POR EL INSECTO *Iatrophobia brasiliensis* EN PLANTAS DE *Manihot esculenta* (YUCA)

Tesis sometida a la consideración de la Comisión del Programa de Doctorado en Ciencias para optar al grado y título de Doctorado Académico en Ciencias

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RESUMEN

Las agallas se definen como desviaciones del patrón normal de desarrollo de las plantas, formadas como resultado de una reacción específica a la presencia y actividad de un organismo “foráneo”. Las agallas de plantas inducidas por insectos son tejidos especializados que presentan un arreglo ordenado de diferentes capas de células y un crecimiento predeterminado. En estas estructuras, su tamaño, forma y metabolismo están bajo el control del respectivo insecto inductor. Se han propuesto una serie de hipótesis para tratar de explicar el mecanismo de inducción de las agallas de plantas. La hipótesis más relevante plantea que las agallas son inducidas por la acción de sustancias químicas secretadas por los respectivos insectos inductores, tales sustancias incluirían reguladores de crecimiento vegetal como las auxinas, citoquininas, ácido indolacético u otros tipos de compuestos. Sin embargo, el modo de acción de estas sustancias químicas, los procesos de desarrollo que estas alteran y su mecanismo específico de inducción son aún desconocidos. Para estudiar el mecanismo de inducción, se seleccionó como sistema de estudio la agalla inducida por el insecto *Iatrophobia brasiliensis* (Diptera: Cecidomyiidae) en plantas de la especie *Manihot esculenta* Crantz (yuca). En esta tesis, se proponen varios enfoques experimentales para estudiar el proceso de inducción y formación de las agallas de insectos. El planteamiento del problema y las hipótesis principales se abordan en tres artículos científicos con el respaldo de una amplia revisión crítica de literatura, misma que a su vez, respalda el diseño experimental y el enfoque metodológico empleado. Este planteamiento constituye la base del primer artículo, del cual se derivan además una serie de predicciones que se describen a partir de los resultados obtenidos en los dos artículos publicados posteriormente. En el segundo artículo, se describe el registro e inventario de morfotipos de agallas realizado en el Área de Conservación Guanacaste, material genético que sirvió de base para analizar, en esos morfotipos de agallas, la presencia y secuencia de bases del posible marcador identificado y caracterizado inicialmente en la agalla de yuca. Luego, en el tercer artículo, marcadores moleculares basados en la reacción en cadena de la polimerasa y datos provenientes de secuenciación genómica profunda fueron utilizados para identificar posibles elementos genéticos involucrados en la formación de la agalla de yuca. Además, se evaluó la hipótesis de que las células de la agalla están transformadas genéticamente y se analizó el microbioma de las agallas y del tejido sano circundante. Se aplicó un análisis discriminante de secuencias para excluir selectivamente entre el ADN exógeno y el genoma de referencia de la planta huésped. A partir de este enfoque experimental, se identificaron varias secuencias candidatas de inserción asociadas con secuencias conocidas de genes bacterianos, siendo las más relevantes las relacionadas con el factor regulador de la transcripción CadR, la ATPasa transportadora de cadmio, codificada por el gen *cadA*, una proteína permeasa involucrada en el transporte de nitrato (gen *nrtB*) y la enzima ATPasa arsénica (gen *arsA*). Además, se caracterizó un fragmento de ADN específico de agallas que podría constituir un elemento genético accesorio involucrado en el mecanismo de inducción, el cual mostró homología parcial con la enzima conjugadora de ubiquitina E2 Q2 del hongo *Fulvia fulva*. Finalmente, el tercer artículo proporciona también evidencias relacionadas con la modificación del microbioma endofítico, resultados que conjuntamente con la transformación genética de las células vegetales en *M. esculenta,* se hipotetiza constituyen dos requisitos esenciales para la formación de las agallas en las plantas de yuca. Adicionalmente, se obtuvo experimentalmente una estructura inicial similar a una agalla en los tejidos cultivados in vitro de *M. esculenta* que fueron inoculados utilizando una cepa bacteriana del género *Rhodococcus,* aislada a partir del insecto inductor y que se plantea podría estar relacionada con el proceso de inducción de la agalla. En conclusión, los resultados obtenidos en esta investigación aportan evidencias en relación con la transformación genética de las células que conforman el tejido de la agalla inducida en plantas de yuca. Considerando la evidencia aportada en relación con la presencia del posible marcador molecular (gen de ubiquitina) en diferentes morfotipos de agallas inducidas en otras especies de plantas, la transformación genética de las células de la agalla podría ser parte de un mecanismo de inducción de este tipo de estructuras ampliamente distribuido en la naturaleza.

ABSTRACT

Galls are defined as modifications of the normal developmental design of plants, produced by a specific reaction to the presence and activity of a foreign organism. Insect-induced plant galls are specialised plant tissues with an organised arrangement of cells and predetermined growth. The size, structure, and metabolism of galls are under the control of gall-forming insects and host plant species. The most general hypothesis suggests that gall formation is triggered by the action of chemical substances secreted by the gall inducer, including plant growth regulators such as auxins, cytokinins, indole-3-acetic acid (IAA), and other types of compounds. However, the mode of action of these chemical substances and the general mechanism by which the insect could control and manipulate plant development and physiology is still not known. To study the induction mechanism of insect galls, we selected the gall induced by *Iatrophobia brasiliensis* (Diptera: Cecidomyiidae) in cassava (Euphorbiaceae: *Manihot esculenta* Crantz) as our model. In this thesis, different experimental approaches are proposed to study the process of induction and formation of insect galls. The problem statement and main hypotheses are addressed in three scientific articles supported by an extensive critical literature review, which supports the experimental design and methodological approach used. This approach constitutes the basis of the first article, from which some predictions are described in the two articles published subsequently. In the second article, the registry and inventory of gall morphotypes carried out in the Guanacaste Conservation Area are described. This genetic material is used to detect and analyze the potential DNA sequence from the cassava gall marker characterized in the third article. Also, in this third publication, molecular markers and deep metagenomic sequencing data were employed to analyse the gall microbiome and to test the hypothesis that gall cells are genetically transformed by insect vectored bacteria. A shotgun sequencing discrimination approach was implemented to selectively discriminate between foreign DNA and the reference host plant genome. Several known candidate insertion sequences were identified, the most significant being DNA sequences found in bacterial genes related to the transcription regulatory factor CadR, cadmium-transporting ATPase encoded by the cadA gene, nitrate transport permease protein (nrtB gene), and arsenical pump ATPase (arsA gene). In addition, a DNA fragment associated with ubiquitin-like gene E2 was identified as a potential accessory genetic element involved in gall induction mechanism in the fungus *Fulvia fulva*. Finally, in the third article, evidence supporting that the modification of the endophytic microbiome and the genetic transformation of plant cells in *M. esculenta* are two essential requirements for insect-induced gall formation is provided. An initial gall-like structure was experimentally obtained in *M. esculenta* cultured tissues through inoculation assays using a *Rhodococcus* bacterial strain that originated from the inducing insect, which we related to the gall induction process. Results provide evidence suggesting an insect-induced gall formation mechanism mediated by genetic transformation events in Cassava plants. Based on these findings and having observed the same potential DNA marker in galls from other plant species (ubiquitin-like gene E2), bacterially mediated genetic transformation of plant cells may represent a more widespread gall induction mechanism found in nature.

LISTA DE ABREVIATURAS

AIA: Ácido Indolacético.

TBE: Buffer Tris-Borato EDTA.

TE: Buffer Tris-EDTA.

SDS: Sodium Dodecyl Sulfate (Dodecil sulfato de sodio)

PVP: Polivinil Pirrolidona.

RAPD: Random Amplified Polymorphic DNA (Polimorfismo a lo largo de los Fragmentos Amplificados).

PCR: Polymerase Chain Reaction (Reacción en cadena de la polimerasa).

MPM: Marcador de Peso Molecular.

PPO: Polifenol oxidasa.

UPS: ubiquitin-proteosome system.

ICGMC: International Cassava Genetic Map Consortium.

THG: Transferencia horizontal de genes.

FQRs: factores químicos reguladores.

GO: Gene Ontology.

COG: cluster of gene ontology approach.

SGF: Specific Gall Fragment, por sus siglas en inglés.

HTS: high-throughput sequencing.

UFC: Unidades formadoras de colonias.

PUBLICACIONES GENERADAS:

La presente tesis está basada en los siguientes artículos de investigación original publicados.

I. Gätjens-Boniche, O. (2019). The mechanism of plant gall induction by insects: revealing clues, facts, and consequences in a cross-kingdom complex interaction. *Rev. Biol. Trop.*, 67 (6), 1359–1382. doi: 10.15517/rbt.v67i6.33984

II. Gätjens-Boniche, O., Sánchez-Valverde, M., Trejos-Araya, C., Espinoza-Obando, R., Pinto-Tomás, A. A., and Hanson, P. E. (2021). Plant galls recorded from Guanacaste Conservation Area-Costa Rica as an integrated concept of a biological database. *Biota Neotropica* 21 (3). doi:10.1590/1676-0611-bn-2020-1153

III. Gätjens-Boniche, O., Jiménez-Madrigal, J. P., Whetten, R. W., Valenzuela-Diaz, S., Alemán-Gutiérrez, A., Hanson, P. E., and Pinto-Tomás, A. A. (2023). Microbiome and plant cell transformation trigger insect gall induction in cassava. *Frontiers in Plant Science*, 14, 1237966. doi: 10.3389/fpls.2023.1237966

I. INTRODUCCIÓN GENERAL

Diferentes especies de insectos tienen la habilidad de cambiar y manipular el desarrollo normal de las plantas para inducir una estructura altamente organizada llamada agalla. Las agallas de plantas inducidas por insectos son entidades biológicas constituidas por crecimientos anormales que se desarrollan por el estímulo y presencia del insecto inductor en la planta. Al respecto, el tamaño, estructura y metabolismo de la agalla están bajo el control del respectivo insecto inductor y a su vez también por el tipo de planta hospedera (Tooker et al., 2008; Raman, 2011; Raman, 2021; Li et al., 2017; Ferreira et al., 2018; Ferreira et al., 2019). En este contexto, el proceso o mecanismo por medio del cual el insecto controla o manipula el desarrollo y fisiología de la planta es aún desconocido (Takeda et al., 2021; Raman, 2021; Desnitskiy et al., 2023; Hirano et al., 2023).

La capacidad de inducir agallas en las plantas ha surgido varias veces entre y dentro de diferentes órdenes de insectos, con representantes de especies inductoras de estas estructuras en Diptera, Hymenoptera, Hemiptera, Coleoptera, Lepidoptera y Thysanoptera, entre otros (Mani, 1992; Espı́rito-Santo y Fernandes, 2007). En cada uno de tales ordenes de insectos, la capacidad de formar agallas parece tener un origen independiente (Nyman y Julkunen, 2000; Ronquist et al., 2015; Desnitskiy et al., 2023).

Además de los insectos, las agallas de plantas pueden ser inducidas por ácaros y nemátodos (de Lillo et al., 2018; Harris y Pitzschke, 2020; Olmo et al., 2020; Favery et al., 2020; Desnitskiy et al., 2023). Algunas especies de hongos y bacterias e incluso algunos virus también pueden inducir crecimientos primarios similares a agallas o, simplemente, la formación de neoplasias con bajos niveles de diferenciación celular (Raman, 2011; Harris y Pitzschke, 2020).

Un gran número y diversidad de morfotipos de agallas de plantas e insectos inductores han sido reportados alrededor del mundo (Araújo 2017, Bergamini et al. 2017, Coelho et al., 2017, Martins et al. 2018; Ansaloni et al. 2018; Ley-López et al. 2019; Menezes et al., 2023). A pesar de que los himenópteros y dípteros son los mayores inductores de agallas, una gran diversidad se puede encontrar también en las agallas formadas por trips, áfidos**,** cochinillas y otros grupos de insectos (Raman, 2011; Labandeira, 2021). Al respecto, nuevas especies de insectos inductores se describen periódicamente, a la vez que otros estudios sobre abundancia y diversidad, tanto de los morfotipos de agallas como de sus respectivos inductores, contribuyen a ampliar el conocimiento existente en este campo (Urso-Guimarães et al. 2017, Araújo 2017, Liu et al. 2018, Flor et al. 2018, Ascendino y Maia 2018, Silva et al. 2018, Araújo et al. 2019, Ribeiro et al. 2019, Soares et al., 2021, entre otros).

El hecho de que diferentes grupos de insectos posean la capacidad de formar agallas en una gran variedad de plantas, ha motivado un sinnúmero de investigaciones tendientes a dilucidar el mecanismo de inducción de este tipo de estructuras, pues además constituyen modelos para comprender una serie de procesos fundamentales en el desarrollo de las plantas. A pesar de lo anterior, los mecanismos de inducción y el contexto evolutivo de este tipo de estructuras son aún poco entendidos (Stone y Schönrogge, 2003, Raman, 2021; Takeda, 2021).

En este trabajo se seleccionó como modelo de estudio el sistema de agallas cilíndricas inducidas en plantas de yuca (*Manihot esculenta*) por el insecto *Iatrophobia brasilensis,* perteneciente a la familia Cecidomyiidae (Montaldo, 1977; Rivera-Hernández, 2011). La yuca (*Manihot esculenta* Crantz) es un cultivo ampliamente cultivado en África, Asia, América Central y América del Sur. *M. esculenta* es una fuente de alimento importante para millones de personas en todo el mundo y puede cultivarse durante todo el año en los trópicos. Además, la yuca se puede propagar fácilmente en condiciones de invernadero y bajo condiciones *in vitro*. Debido a su versatilidad y potencial, los programas de fitomejoramiento y los consorcios internacionales han invertido importantes recursos económicos en la comprensión de la genética de la yuca. Esto ha permitido la creación de mapas de ligamiento genético y ensamblaje del genoma a escala cromosómica (Wang et al., 2014; ICGMC , 2015; Lyons et al., 2022). Las plantas de yuca están sujetas a la formación de agallas, particularmente agallas cilíndricas inducidas por el Cecidomyiidae *I. brasiliensis* (Montaldo, 1977; Rivera-Hernández, 2011), modelo de estudio de éste trabajo. A pesar de la falta de estudios relacionados con el proceso de inducción y formación de las agallas de yuca, un estudio reciente realizado por de Souza et al. (2024), identificó con un alto nivel de detalle, cambios metabólicos y estructurales a nivel de la pared celular tanto en hojas como en las agallas de *M. esculenta* inducidas por *I. brasiliensis*.

II. MARCO TEÓRICO

2.1 Inducción y estructura de las agallas

Los tejidos que forman las agallas inducidas por insectos exhiben modificaciones bioquímicas y citológicas que les proporcionan una mayor calidad nutricional que el tejido vegetal circundante, facilitando así una fuente continua de alimento y beneficios adicionales al insecto inductor (Ferreira et al., 2017; Favery et al., 2020). La mayoría de las agallas contienen un tejido altamente especializado conocido como tejido nutritivo, caracterizado por altas concentraciones de azúcares (Nogueira et al., 2018), lípidos, proteínas, nitrógeno y una variedad de otros compuestos (Huang et al., 2015; Isaías et al., 2018).

Se han propuesto una serie de hipótesis para tratar de explicar los mecanismos de inducción de las agallas de plantas; una de estas hipótesis sugiere que las agallas son el resultado de una irritación mecánica producida por la presencia y actividad del respectivo insecto en el tejido vegetal. Se ha planteado la hipótesis de que moléculas elicitoras de fitohormonas suministradas por el respectivo insecto inductor (Rohfritsch y Shorthouse, 1982; Tooker y Helms, 2014; Ponce et al., 2021) o indirectamente por un microorganismo asociado (Giron et al., 2013; Bartlett y Connor, 2014; Giron et al., 2016), así como proteínas efectoras secretadas por el insecto inductor de agallas (Zhao et al., 2015; Cambier et al., 2019; Zhao et al., 2019; Korgaonkar et al., 2021) podrían ser los principales estímulos responsables del proceso de inducción de las agallas de insectos. Estas sustancias inductoras estarían presentes en los fluidos salivales, estructuras ovipositoras o bien en los excrementos del respectivo insecto inductor. Algunas de estas sustancias químicas incluirían a las auxinas, citoquininas, ácido indolacético (AIA) y otros tipos de compuestos. Sin embargo, el modo de acción de estas sustancias químicas y los procesos de desarrollo que estas alteran son aún desconocidos y es motivo de un amplio debate entre los investigadores del tema (Hori, 1992; Raman, 2021; Takeda et al., 2021). Este panorama se complica aún más en el caso de las agallas prosoplásmicas, como las formadas por los insectos de las familias Cecidomyiidae y Cynipidae, debido a la complejidad de su desarrollo y estructura, y a la diversidad de estrategias adaptativas que presentan (Sinnott, 1960; Raman, 2011; Raman, 2021; Desnitskiy et al., 2023). Finalmente, otra hipótesis propone un mecanismo mediado por la acción de virus mutualistas u otro tipo de elemento genético asociado con el insecto, circunstancia que implicaría por lo tanto la transferencia de ADN hacia las células de la planta hospedera (Ananthakrishnan, 1998; Nieves-Aldrey, 1998; Stone y Schönrogge, 2003). Por otra parte, la naturaleza fisiológica del estímulo generado por el insecto y la respuesta de la planta, son preguntas que aún permanecen abiertas (Stone y Schönrogge, 2003; Favery et al., 2020; Raman, 2021; Takeda et al., 2021; Hirano et al., 2023).

Durante el proceso de inducción de las agallas, se ha propuesto que las células de la planta deben ser condicionadas para producir un estado fisiológico particular (Raman, 2011, Raman, 2021). Al respecto, diferentes estudios han mencionado que los aminoácidos presentes en las secreciones salivales de los insectos inductores de agallas, esencialmente lisina, histidina y triptófano, podrían funcionar como “pre-acondicionadores” para la inducción de las agallas (Hori, 1992). Al parecer, los aminoácidos secretados en la saliva provocarían una mayor plasticidad e incrementarían la sensibilidad de los tejidos de la planta a la acción del respectivo insecto inductor. Aunque la presencia de pectinasas en la saliva de los insectos no se ha correlacionado con la inducción de las agallas, estas enzimas podrían degradar las paredes celulares y con ello contribuirían a condicionar el tejido para la acción del insecto. En el mismo sentido, se ha especulado que la polifenol oxidasa (PPO), presente también en las secreciones salivales de los insectos y los compuestos fenólicos derivados de su acción enzimática, podrían incrementar la sensibilidad de los tejidos de la planta al estímulo por parte del respectivo insecto inductor. No obstante, se ha especulado que la compleja interacción entre los tejidos de la planta hospedera y la polifenol oxidasa podría tener una importancia fundamental en la formación de las agallas (Miles, 1968; Hori, 1992; Ananthakrishnan, 1998). Al respecto, Miles (1968) menciona que la interacción y balance entre el sistema de lapolifenol oxidasa de los insectos y la planta hospedera y más aún la modulación del potencial redox, concerniente a la acumulación de radicales de oxígeno (Isaias et al., 2015), podría determinar si el “ataque” por parte del insecto produce una lesión (necrosis) o bien el desarrollo de la agalla.

Diferentes estudios han reportado concretamente que el Ácido Indolacético (AIA) podría ser un poderoso agente inductor de las agallas, también se ha especulado que este compuesto podría interactuar con otros reguladores de crecimiento vegetal, como citocininas y giberelinas, o de forma sinérgica con otras sustancias para promover el proceso de inducción y maduración de este tipo de estructuras (Tooker et al., 2014; Bedetti et al., 2014; Bailey et al., 2015; Raman, 2021).

En insectos, los órdenes Hymenoptera y Diptera forman las agallas más complejas estructuralmente y cuentan con el mayor número reportado de especies inductoras de agallas. No obstante, una gran diversidad de agallas es formada también por trips, pulgones e insectos de otros órdenes (Ananthakrishnan, 1998; Hanson y Gómez-Laurito, 2005; Harris y Pitzschke, 2020).

En el caso particular de los insectos inductores de agallas pertenecientes a la familia Cecidomyiidae, se ha reportado que el huevo o la hembra al ovipositar podrían generar el estímulo inicial y que la larva secretaría sustancias que promueven el crecimiento del tejido vegetal bajo su acción, provocando la formación de la agalla. No obstante, debido a la complejidad del proceso de inducción y desarrollo de este tipo de agallas, esta hipótesis química, en toda su amplitud, es una explicación poco probable para comprender en su totalidad el mecanismo de morfogénesis de este tipo de estructuras. Sin embargo, Boysen-Jensen (1952, citado por Hori, 1992), Miles (1968) y más recientemente Ferreira (2019), proponen que la larva se mueve instintivamente y secreta sustancias reguladoras en sitios apropiados del tejido "atacado" y en tiempos específicos, generando así un ambiente propicio que favorece el desarrollo de la agalla.

Con respecto a los himenópteros de la familia Cynipidae, diferentes trabajos han asociado tanto a las auxinas como a las citoquininas con el proceso de inducción y morfogénesis de las agallas. Más aún, se ha correlacionado la morfogénesis e inducción de estas estructuras con la actividad de oviposición de la hembra, secreciones del huevo y la actividad y secreción de sustancias a partir de la larva (Miles, 1968; Hori, 1992; Raman, 2011). Al igual que para las agallas formadas por los insectos de la familia Cecidomyiidae, el mecanismo de morfogénesis de las agallas formadas por los cinípidos no puede explicarse simplemente por la acción de algún regulador de crecimiento vegetal. Sin embargo, se ha postulado que el micro ambiente generado por la larva durante el proceso de alimentación podría contribuir al proceso de morfogénesis, tal como se mencionó también para las agallas formadas por los dípteros pertenecientes a la familia Cecidomyiidae.

Estructuras o síntomas similares a agallas fueron obtenidos en pruebas realizadas por medio de la inoculación de extractos totales de hemípteros y homópteros inductores de agallas en sus respectivas plantas hospederas. Así mismo, por medio de la inyección adecuada de AIA u otros tipos de auxinas, o bien auxinas mezcladas con aminoácidos, se han logrado obtener resultados similares, pero con menos éxito. Aunque los insectos de estos ordenes producen agallas poco diferenciadas, los resultados obtenidos por medio de estos experimentos han apoyado la hipótesis sobre la inducción química de este tipo de estructuras (McCalla et al., 1961; Miles, 1968; Hori, 1992). Más recientemente, Hirano et al. (2023) obtuvieron la formación de una estructura inicial tipo agalla al realizar ensayos de inoculación usando como planta modelo a *Arabidopsis thaliana*, utilizando para ello extractos del áfido *Schlechtendalia chinensis*.

Aunque en términos generales se ha aceptado el hecho de que algún tipo de "estímulo" químico por parte del insecto está involucrado en la inducción y morfogénesis de las agallas (McCalla et al., 1961; Miles, 1968; Rohfritsch y Shorthouse, 1982; Hori, 1992; Leitch, 1994; Ananthakrishnan, 1998; Raman, 2011; Tooker y Helms, 2014; Bailey et al., 2015; Oates et al., 2016, Giron et al., 2016; Ponce et al., 2021; Takeda et al., 2021; Desnitskiy et al., 2023), la posibilidad de que exista un mecanismo de inducción molecular que involucre la transferencia de elementos genéticos no ha sido considerada ni explorada ampliamente. Algunos investigadores como Ananthakrishnan (1998), Nieves-Aldrey, (1998), así como Stone y Schönrogge (2003), han considerado la posibilidad de que un mecanismo a nivel genético molecular pueda estar mediando el proceso de formación y control de la morfogénesis de la agalla. Específicamente se ha sugerido que el insecto inductor podría insertar plásmidos, viroídes o virus mutualistas en el genoma de la planta, los que regularían y controlarían el proceso de formación de las agallas. Sin embargo estos autores no ofrecen ninguna evidencia o trabajo que pueda sustentar esta afirmación. Las bases moleculares del proceso de inducción de las agallas de plantas inducidas por insectos es aún desconocido (Stone y Schönrogge, 2003; Raman, 2021; Ponce et al., 2021; Desnitskiy et al., 2023). Por otra parte, la naturaleza fisiológica del estímulo dado por el insecto inductor y la influencia de su propia constitución genómica, así como la reacción generada por la planta, son preguntas que permanecen abiertas y sugieren la necesidad de realizar investigaciones más profundas en este campo (Ananthakrishnan, 1998; Raman, 2021; Takeda et al., 2021, Desnitskiy et al., 2023).

En la literatura consultada, de forma recurrente se mencionan tres grandes problemas o retos en la identificación de las moléculas responsables del proceso de formación de las agallas. Primero, la dificultad de establecer ensayos apropiados para los tejidos de la planta involucrados en el proceso de inducción. En segundo lugar, las posibles moléculas inductoras utilizadas por los insectos podrían ser químicamente similares a aquellas presentes normalmente en la planta. Finalmente, debido a que se espera que las señales provenientes del insecto generen una cascada de respuestas en la planta, es difícil separar el primer impacto morfogenético originado por el inductor, de la respuesta secundaría generada por la planta (Stone y Schönrogge, 2003; Raman, 2021).

El proceso de morfogénesis de las agallas es un fenómeno complejo, que involucra la recanalización o reorientación del desarrollo de la planta por parte del insecto inductor (Ananthakrishnan, 1998; Ferreira et al., 2019; Raman, 2021). El grado en el cual el insecto manipula el crecimiento de la planta para formar la agalla varía considerablemente, e involucra desde la inducción de la proliferación celular, hasta la formación de una estructura compleja que la planta no produce en condiciones normales. Al igual que otros órganos y estructuras normales de las plantas, las agallas inducidas por insectos presentan características anatómicas e histológicas propias, las cuales varían enormemente en diversidad y en el grado de complejidad (Mani, 1992, en Shorthouse y Rohfritsch 1992; Stone y Schönrogge, 2003; Raman, 2011; De Oliveira et al., 2011; Raman, 2021).

Shorthouse y Rohfritsch (1992) separan la morfogénesis de las agallas inducidas por insectos en dos categorías distintas: permanente, que se mantiene aun cuando el respectivo insecto es removido de la agalla o muere, y transitoria, donde el efecto es generado por medio de una estimulación continúa por parte del insecto inductor, el cual desaparece si el insecto es removido de la agalla o si este muere. El proceso de morfogénesis de las agallas podría dividirse a su vez para una mayor simplificación en tres fases principales. La primera de estas fases involucra el "condicionamiento" de las células del respectivo tejido de la planta por parte del insecto, para hacerlas más susceptibles y apropiadas a su acción como inductor. En la siguiente fase se da el proceso de inducción de la agalla como tal, producto del alargamiento y la división celular, que tiene como resultado la formación de una agalla "primaria". La fase final consiste en la maduración de la agalla, en la cual la agalla primaria crece hasta completar su morfogénesis (Ferreira et al., 2019; Raman, 2021). Algunos de los aspectos más relevantes relacionados con la biología de las agallas incluyen la extraordinaria diversidad de formas, colores, tamaños y estructuras internas que estas presentan.

2.2 Bacterias y agallas

Un gran número de bacterias endosimbióticas han sido identificadas en diferentes grupos de insectos, incluidos los insectos inductores de agallas (Campbell et al., 2015; El-Sayed and Ibrahim, 2015; Gutzwiller et al., 2015; Michell and Nyman, 2021; Yang et al., 2021; Coolen et al., 2022; Yang et al., 2022). Asimismo, los microorganismos asociados a insectos podrían ser importantes mediadores en las interacciones entre insectos y plantas (Hammer y Bowers, 2015; Sugio et al., 2015; Wielkopolan y Jakubowska, 2021; Coolen et al., 2022). Algunos investigadores han informado que la infección simultánea con diferentes especies de endosimbiontes en el mismo organismo huésped es un fenómeno común en varios grupos de insectos (Thao et al., 2000; Thao, 2002; Russell et al., 2003; Krawczyk et al., 2015; El-Sayed et al., 2015; Ghosh et al., 2015; Brentassi et al., 2017). Diferentes tejidos en el mismo huésped constituyen diferentes microambientes para los organismos endosimbiontes. Algunos tejidos podrían ser, por ejemplo, nutricionalmente favorables, inmunotolerantes o simplemente fáciles de colonizar (Mouton et al., 2003; Kondo, 2005; Koga et al., 2012; Hansen y Moran, 2014; Sugio et al., 2015).

En los últimos años ha surgido un creciente interés por la biología reproductiva de los parásitos endosimbiontes que se transmiten a través de la madre y manipulan la reproducción de su organismo huésped. La evidencia acumulada muestra que muchas especies de artrópodos están infectadas por diferentes tipos de organismos endosimbiontes transmitidos por la madre a través de mecanismos de transmisión vertical y que tienen una gran influencia en la biología de sus huéspedes. Algunos de estos microorganismos endosimbiontes incluyen especies pertenecientes a los géneros *Wolbachia*, *Spiroplasma*, *Rickettsia*, *Arsenophonus* y *Cardinium*, entre otros (Weeks et al., 2003; Zchori-Fein y Perlman, 2004; Goto et al., 2006; Casper-Lindley et al., 2011; Goodacre y Martin, 2012; Kageyama et al., 2012; Koga et al., 2012; Kremer et al., 2012; Herren et al., 2013; Ma et al., 2014; Boivin et al., 2014; Ma et al., 2015; Sugio et al., 2015; Brentassi et al., 2017; Mariño et al., 2017; Ma y Schwander, 2017).

Se ha especulado que las relaciones simbióticas entre insectos inductores y microorganismos están involucradas en el desarrollo de agallas en las plantas (Tooker y Helms, 2014; Gätjens-Boniche, 2019; Klimov et al., 2022). Se ha reportado que muchos insectos inductores de agallas tienen asociados microoganismos que pueden estar involucrados en el desarrollo de las agallas o bien podrían facilitar el proceso de herbivoría, este es el caso de ciertos Cecidomyiidae ("ambrosia gall midges") asociados con simbiontes fúngicos; no obstante, los estudios que exploran el papel de esos microorganismos asociados con los ciclos de vida de los insectos agalladores son escasos (Hansen y Moran, 2014; Tooker y Helms, 2014; Huang et al., 2015). Bacterias del género *Wolbachia* han sido asociadas con la aparición del fenómeno conocido como islas verdes, generado por la polilla minadora de la hoja del manzano, *Phyllonorycter blancardella* (Gracillariidae), un fenómeno similar al observado en algunos tipos de agallas de plantas inducidas por insectos (Kaiser et al., 2010; Gutzwiller et al., 2015). Fenómeno en el cual las agallas y sus tejidos circundantes se mantienen verdes por periodos de tiempo prolongados, aún después de que la hoja ha sido desprendida de la planta, ha sido asociado con la manipulación de los niveles de citoquininas por bacterias endosimbiontes presentes en el insecto inductor (Zhang et al., 2017) y más específicamente en bacterias del género *Wolbachia* (Kaiser et al., 2010).

La transmisión de bacterias bajo condiciones naturales o artificiales por insectos vectores ha sido reportada en algunas interacciones entre insectos y plantas (Zeidan y Czosnek, 1994; Galambos et al., 2021; Wielkopolan y Jakubowska, 2021; Ratcliffe et al., 2022).

La transferencia de bacterias por insectos en plantas se ha descrito en sistemas patológicos bien conocidos como en la enfermedad del Dragón Amarillo de los cítricos (Huanglongbing, HLB), causada por la bacteria fitopatógena *Candidatus Liberibacter asiaticus* (CLas), que es transmitida por el psílido *Diaphorina citri*. Asimismo, la adquisición y transmisión efectiva de *A. tumefaciens* por la mosca blanca (*Bemisia tabaci*) fue demostrada por Zeidan y Czosnek (1994).

Ejemplos de crecimientos similares a agallas inducidos por microorganismos incluyen a *Agrobacterium tumefaciens* ("crown gall"), *Rhodococcus fascians*, *Pseudomonas savastanoi*, *Xanthomonas citri*, *Pantoea agglomerans*, *Taphrina betulina* (witches broom), y *Ustilago esculenta* (Swarup et al., 1991; Jump y Woodward, 1994; Chalupowicz et al., 2009; You et al., 2011; Dolzblasz et al., 2018; Harris y Pitzschke, 2020). De estos, *A. tumefaciens* es el mejor estudiado debido a su capacidad de realizar la transformación genética de células vegetales, lo que ha promovido su uso extenso en el campo de la biotecnología de plantas (Kavipriya et al., 2019; Song et al., 2019; Lian et al., 2022). Debido a que la transformación de células vegetales mediada por *A. tumefaciens* es el mecanismo de formación de estructuras similares a agallas más estudiado (Chou et al., 2022; Hopp et al., 2022; Azizi-Dargahlou y Pouresmaeil, 2023), este sistema constituye el mejor referenciado para proponer un mecanismo alternativo en el cual la inducción de agallas complejas como las causadas por insectos de las familias Cynipidae (Hymenoptera) y Cecidomyiidae (Diptera) (Sinnott, 1960; Raman, 2011), son también el resultado de un mecanismo de trasformación genética de las células vegetales.

Se ha propuesto que los insectos formadores de agallas han adquirido genes de microorganismos simbióticos mediante transferencia horizontal de genes (Giron y Glevarec, 2014; Bartlett y Connor, 2014). La transferencia horizontal de genes (THG) es el movimiento y transferencia de información genética entre diferentes organismos, y es un fenómeno común entre patógenos de animales y plantas, así como entre simbiontes y patógenos (De la Cruz y Davies, 2000; Suzuki et al., 2015). Por lo tanto, bajo el escenario anterior, la transferencia horizontal de genes (THG) podría jugar un papel fundamental en la inducción y evolución de las agallas en las plantas. Diferentes estudios han mostrado evidencias de que los mecanismos moleculares implicados en los diferentes procesos de simbiosis y patogénesis presentan una serie de vías comunes que han revelado similitudes existentes en la modulación e interacciones entre patógenos y simbiontes con sus huéspedes (De la Cruz y Davies, 2000; Hentschel et al., 2000; Rankin et al., 2011). Además, la información generada por estudios de secuenciación del genoma microbiano ha demostrado que la transferencia horizontal de genes es un proceso importante y ampliamente distribuido dentro del escenario evolutivo de los organismos procariotas (Nikoh y Nakabachi, 2009; Jayaprakashvel et al., 2017). Los procariotas poseen múltiples elementos genéticos móviles intra y extra-cromosomales, como islas genómicas, plásmidos, transposones, secuencias de inserción o bacteriófagos, que les permiten inducir cambios estructurales y fisiológicos, así como la adquisición o pérdida de regiones genómicas. Además, el hecho de que un gran número de determinantes patogénicas y simbióticas se localicen en elementos genéticos móviles permite que se genere una fuente de variación permanente dentro de estos organismos. De igual manera, algunos autores han sugerido que la adquisición e incorporación de plásmidos en bacterias podría constituir un proceso clave para la adaptación de estos microorganismos a nuevos nichos ecológicos y para su desarrollo como simbiontes o patógenos (Vivian et al., 2001; Suzuki et al., 2015; Jayaprakashvel et al., 2017). La variabilidad genética juega un papel muy importante al generar las condiciones que permiten la evolución de nuevos tipos de interacciones entre organismos, por lo que la TGH entre diferentes especies podría representar un poderoso mecanismo a través del cual el resultado final de la interacción entre un patógeno o simbionte y su huésped podría ser alterado permanentemente (Hentschel et al., 2000; Suzuki et al., 2015; von Wintersdorff et al., 2016; Porse et al., 2018). Más aún, el mecanismo por el cual los eucariotas adquieren genes de organismos emparentados lejanamente sigue siendo poco entendido (Suzuki et al., 2015).

2.3 Trabajo previo realizado por Gätjens-Boniche

Un estudio previo realizado por Gätjens-Boniche (2007) como parte de su trabajo de tesis de maestría, mostró evidencias previas que apoyan la hipótesis relacionada con la presencia de elementos genéticos exógenos en los tejidos que forman la agalla.

En dicho estudio se exploró la presencia de posible ADN exógeno, posibles secuencias de inserción de ADN presentes únicamente en las células que forman la agalla y no en el tejido vegetal sano de las plantas de *M. esculenta*, inicialmente utilizando iniciadores de PCR específicos como un posible marcador molecular de agallas. El par de iniciadores de PCR se diseñó tomando como base la secuencia consenso de ADN resultante del alineamiento de fragmentos amplificados diferencialmente a partir de los tejidos de la agalla que fueron obtenidos previamente mediante un ensayo de RAPDs modificado. Se realizaron un gran número de ensayos de RAPDs utilizando diferentes iniciadores aleatorios; sin embargo, los cebadores decámeros derivados de secuencias conservadas de los genes ipt e iAAM alojados en el ADN de transferencia del plásmido Ti de especies de *Agrobacterium* y otras bacterias relacionadas, fueron los que generaron el mayor número de fragmentos amplificados diferencialmente a partir de muestras de agallas. La calidad del ADN purificado, así como las condiciones de amplificación del RAPD modificado, así como los perfiles térmicos, permitieron obtener alta reproducibilidad y confiabilidad en los perfiles de ADN a diferentes concentraciones del ADN analizado. La detección y análisis de los fragmentos de RAPDs amplificados se realizó mediante electroforesis en gel y por medio del sistema de electroforesis de ADN/ARN por microchip (MultiNA). Los perfiles obtenidos mostraron fragmentos amplificados diferencialmente de varios tamaños en todas las muestras de agallas analizadas, a menudo de 100 a 4500 pares de bases (pb). Utilizando este enfoque experimental, se obtuvieron y aislaron las secuencias de nucleótidos de cuatro de las muestras de los fragmentos amplificados diferencialmente más comunes derivados del ADN genómico de la agalla. Tres de estas secuencias mostraron la misma secuencia de 500 nucleótidos. La secuencia de ADN consenso obtenida se utilizó como plantilla para el diseño de iniciadores de PCR específicos. El fragmento de ADN amplificado diferencialmente a partir de muestras de agallas (que llamamos fragmento de agalla específico) se aisló y secuenció posteriormente.

El fragmento de PCR esperado de acuerdo al diseño de los iniciadores de PCR se amplificó específicamente sólo en muestras de agallas de *M. esculenta.* El fragmento de ADN amplificado diferencialmente a partir de las muestras de agallas no mostró similitud estadísticamente significativa con ningún otro gen reportado según el “Basic Local Alignment Search Tool” (BLAST, base de datos NCBI Genbank y el sistema integrado de genomas y microbiomas microbianos (IMG/M, https://img.jgi.doe.gov) y de acuerdo con los análisis de anotaciones. Sin embargo, varios resultados mostraron similitudes parciales con una cobertura de baja a media y a menudo discontinua, con algunos genes similares al gen de la ubiquitina E2, un componente del sistema ubiquitina-proteosoma (UPS). Específicamente, la enzima de conjugación de la ubiquitina (E2) juega un papel crítico en el transporte de la enzima activadora de ubiquitina (E1) a la enzima ubiquitina-ligasa (E3), probablemente determinando de esta forma si la proteína marcada será degradada o bien es reclutada en procesos no proteolíticos (Liu et al., 2020). Adicionalmente, algunas evidencias sugieren que estas enzimas podrían estar involucrados en procesos de reparación del ADN, especialmente en la reparación post-replicación del ADN (Wen et al., 2008; Andersen et al., 2008). Sin embargo, hasta ahora se desconoce la función de las enzimas E2 en las plantas. Se ha reportado con frecuencia en diferentes especies de hongos un gen E2 similar a ubiquitinas que podría ser un componente del sistema ubiquitina-proteosoma (UPS). El fragmento identificado muestra una similitud parcial con la enzima ubiquitina conjugada E2 Q2 del hongo *Fulvia fulva*, que fue uno de los resultados más similares obtenidos en BLAST (E- value = 6.37e−29, identidad = 71.8%, 266 pares de bases de longitud, número de acceso CP090172). Estos hallazgos permiten hipotetizar que la posible proteína codificada por el fragmento específico de agalla podría representar un nuevo tipo de elemento genético regulador similar a la ubiquitina asociada con la manipulación del sistema ubiquitina-proteosoma, utilizado indirectamente por el insecto inductor a través de bacterias para manipular y redirigir el desarrollo de las plantas durante la formación de la agalla en la planta de yuca.

Por lo tanto, esta investigación plantea como hipótesis de trabajo que la secuencia identificada como marcador específico en las agallas de *M. esculenta* inducidas por *I. brasilensis* forma parte de un elemento genético de inserción mayor, el cual proviene a su vez de una bacteria endosimbionte presente en el insecto inductor. Esta hipótesis es respaldada por varias líneas de evidencia. Los genes de ubiquitina codifican péptidos implicados en la señalización y el destino proteína-proteína como un componente básico de la maquinaria de regulación a nivel celular. Este tipo de proteínas regulan la expresión genética a nivel transcripcional (Adams y Spoel, 2018) y a nivel post-transcripcional (Xu y Xue, 2019; Liu et al., 2020). Se ha reportado que los genes pertenecientes a la familia de las ubiquitinas constituyen elementos esenciales en los mecanismos de manipulación a nivel celular en la interacción entre patógenos y simbiontes y sus organismos hospederos (Janjusevic et al., 2006; Vierstra, 2009; Park et al., 2012; Singer et al., 2013; Banfield, 2015; Kud et al., 2019). Los genes similares a ubiquitinas de moléculas efectoras secretadas por bacterias con similitud estructural y/o funcional a los componentes de la vía para del sistema de ubiquitina-proteosoma (ubiquitin-proteosome system (UPS), por sus siglas en inglés) imitan y modifican el sistema UPS del huésped (Ramachandran et al., 2021), permitiendo el secuestro de la maquinaria celular del hospedero, como ha sido reportado previamente en las agallas de corona inducidas por *A. tumefaciens* (Magori y Citovsky, 2012; Lacroix y Citovsky, 2015). El sistema UPS se considera la principal vía de recambio de proteínas presente en todos los dominios de organismos vivos y es especialmente importante en la regulación de casi todas las vías de señalización del desarrollo de las plantas, incluido el crecimiento y desarrollo de las plantas mediado por hormonas, así como en las respuestas de estas al estrés (Santner y Estelle, 2010; Sadanandom et al., 2013; Shu y Yang, 2017; Adams y Spoel, 2018; Xu y Xue, 2019). Aunque se sabe poco en relación con la manipulación del sistema de ubiquitinación (UPS) en insectos inductores de agallas, más evidencias se acumulan sugiriendo que el desarrollo de las plantas puede ser manipulado a través del sistema UPS mediante moléculas efectoras secretadas a partir de las glándulas salivales del insecto inductor de la agalla (Zhao et al., 2015).

III. HIPÓTESIS

3.1. Planteamiento de hipótesis

Se propone que la hiperplasia que caracteriza la fase inicial de inducción de la agalla en *M. esculenta* es generada por el estímulo y acción de un elemento genético móvil insertado en el genoma de las células que forman las agallas inducidas por el insecto *Iatrophobia brasiliensis*. Este elemento genético podría ser similar o análogo al plásmido Ti presente en bacterias del género *Agrobacterium.* Este plásmido a su vez sería transferido al tejido vegetal, probablemente por alguna bacteria endosimbionte presente en algunos tipos de fluidos del respectivo insecto inductor. Estos fluidos incluirían las secreciones salivales, excrementos, e inclusive secreciones producidas durante el proceso de oviposición, según sea el caso. En una segunda etapa del proceso de formación de la agalla, el insecto estaría en capacidad de regular y modular el proceso de hiperplasia e hipertrofia inicial, por medio de algún factor químico regulador (análogos a reguladores de crecimiento vegetal, factores de trascripción, metabolitos, etc). Estos factores químicos reguladores, que en lo sucesivo se denominarán como FQRs, podrían ser análogos a los compuestos descritos en las investigaciones realizadas por Doss y colaboradores (2000). Estos investigadores encontraron que cierto tipo de sustancias secretadas por insectos de la especie *Bruchus pisorum* (gorgojo del guisante) y llamadas por ellos conjuntamente como "bruchinas", tienen la capacidad de alterar el desarrollo en plantas de guisante. Específicamente estas sustancias aparentemente tienen la capacidad de actuar como estimuladores del crecimiento vegetal, produciendo un estado de neoplasia en el tejido afectado (Doss *et al*., 2000**).** Al respecto, las bruchinas (Weber, 2002) y otros compuestos derivados de los insectos como proteínas efectoras (Zhao et al., 2015; Cambier et al., 2019; Zhao et al., 2019; Korgaonkar et al., 2021), son moléculas inductoras especializadas que pueden alterar el desarrollo de las plantas y podrían ser los estímulos desencadenantes del proceso de inducción de la agalla. Estos compuestos podrían contener por lo tanto señales similares a otras moléculas presentes en las vías que conducen a una determinada respuesta biológica en la planta huésped.

En función de lo anterior, podría interpretarse desde otra perspectiva, que las bruchinas o bien compuestos de naturaleza similar, podrían tener la capacidad de regular la expresión de genes involucrados en la síntesis de reguladores de crecimiento endógenos en las plantas (fitohormonas). Además, dado que se trata de moléculas relativamente pequeñas, estas podrían difundir a través de las paredes celulares de la planta. Por otra parte, debido a la naturaleza lipídica de las bruchinas, se propone que las mismas podrían tener un mecanismo de acción similar al de otras moléculas derivadas de ácidos grasos u hormonas esteroideas en animales.

Por lo tanto, tomando en consideración todo lo expuesto anteriormente, las bruchinas o moléculas similares podrían convertirse en candidatos apropiados para actuar como factores químicos reguladores en la formación de las agallas. Estos factores químicos reguladores (FQRs) podrían regular entonces la actividad y expresión de los genes presentes en las secuencias de inserción del plásmido u otro tipo de elemento genético, insertado en el genoma de las células vegetales que conforman la agalla. Los genes presentes en las secuencias de inserción y que en principio podrían estar regulados por los FQRs, pueden ser genes que codifican para enzimas involucradas en la síntesis de reguladores de crecimiento vegetal, tales como las auxinas, citocininas, giberelinas u otras. Por su parte, estos FQRs podrían ser secretados por la hembra durante el proceso de oviposición, por el huevo o bien por la larva del respectivo insecto inductor. Al respecto, actualmente se conocen algunos genes que realizan esta función en sistemas como el descrito para el género *Agrobacterium*. En estos organismos se conoce claramente la acción de los genes *ipt*, *iaaM* e *iaaH*, como precursores de las enzimas involucradas en la síntesis de citocininas y auxinas; sustancias esenciales para mantener el estado de neoplasia en los “tumores vegetales” inducidos por este tipo de bacterias. Los genes *ipt, iaaM* e *iaaH*, están presentes en la secuencia de inserción del plásmido Ti de *Agrobacterium* y son insertados conjuntamente en el genoma de las células vegetales (Alberts et al., 2002; Watson et al., 1996**;** Hopp et al., 2022; Azizi-Dargahlou y Pouresmaeil, 2023**).**

Por consiguiente, las células transformadas involucradas en la síntesis de los reguladores de crecimiento vegetal generarían un gradiente de reguladores desde las capas más próximas a la cámara interna de la agalla, hasta el exterior de la misma. Así, las capas de células transformadas más cercanas a la cámara interna de la agalla estarían más expuestas a la acción de los FQRs secretados por las larvas del insecto. El gradiente de reguladores de crecimiento generado provocaría a su vez una diferenciación celular en la dirección del gradiente, fenómeno muy conocido en sistemas vegetales, lo que explicaría en principio la diferenciación de tejidos y capas celulares alrededor de la cámara interna de la agalla.

Un proceso alternativo por medio del cual algunos insectos modularían la morfogénesis de la agalla, especialmente en agallas que muestran una menor complejidad, es por algún tipo de acción mecánica llevada a cabo por parte de la larva del insecto. Así, el ámbito de acción, el tipo de planta hospedera y por otra parte el grado de regulación y modulación del proceso de formación de la agalla por parte del insecto inductor, explicaría en parte el extraordinario nivel de complejidad y diversidad encontrado en los diferentes tipos de agallas.

En función de los supuestos mencionados, se propone además un posible escenario evolutivo, en el cual un crecimiento similar al que se observa actualmente por la infección de *Agrobacterium* en plantas, sería causado probablemente por alguna línea ancestral de esta bacteria. Esta circunstancia a su vez podría haber generado un microambiente o nicho apropiado para la alimentación de “antiguos” insectos. Este escenario hipotético pudo haber propiciado que estos insectos adquiriesen la capacidad de transportar e inocular esta posible bacteria ancestral de *Agrobacterium* de una planta a otra. Al amparo de esta suposición y dado la factibilidad de la transferencia horizontal de genes, tal como se ha mencionado en los trabajos de Hentschel *et al*. (2000) y De la Cruz y Davies (2000), se sugiere la posibilidad de una transferencia de información genética por medio de plásmidos u otro tipo de elementos genéticos móviles, entre una cepa ancestral de *Agrobacterium* y alguna bacteria endógena o endosimbionte presente en los insectos. La adquisición y transferencia efectiva de *Agrobacterium* por insectos ha sido demostrada por Zeidan y Szosneck (1994). Estos investigadores demostraron que la mosca blanca (*Bemisia tabaci*) puede adquirir y trasmitir *Agrobacterium tumefaciens*, al alimentarse de cultivos líquidos conteniendo la bacteria o directamente a partir de la alimentación de agallas de corona.

En síntesis, tomando en consideración la complejidad morfológica y fisiológica de las agallas de plantas inducidas por insectos, que involucra entre otros aspectos un gran control del proceso de desarrollo, se propone la hipótesis de que los tejidos que forman esta estructura están transformados genéticamente. Específicamente, se plantea que la hiperplasia característica en la fase inicial de la inducción de agallas puede ser desencadenada por medio de la inserción de elementos genéticos exógenos en el genoma de las células vegetales, esto a través de una bacteria endosimbiótica presente en el insecto inductor. Para probar esta hipótesis, se emplearon una combinación de análisis de marcadores genéticos, análisis metagenómicos e inducción experimental de agallas en plantas de yuca. En conjunto, los resultados obtenidos respaldan el papel de los microorganismos y la transformación genética en los mecanismos de inducción de las agallas inducidas en plantas de yuca por *I. brasiliensis.* Este último regularía la actividad de estas secuencias de inserción presentes en el genoma de la planta a través de la secreción de factores químicos reguladores.

IV. OBJETIVOS

4.1 Objetivo general

Aislar y caracterizar elementos genéticos involucrados en la formación de la agalla inducida por el insecto *Iatrophobia brasilensis* en plantas de *Manihot esculenta (*yuca) y determinar su presencia en otros morfotipos de agallas.

4.2 Objetivos específicos

1. Establecer a partir del problema y de las hipótesis de trabajo establecidas, la fundamentación y lineamientos que conduzcan a un planteamiento experimental integral para el desarrollo y posterior consecución de los objetivos del trabajo de investigación.

2. Realizar un registro sistemático de morfotipos de agallas de plantas y sus organismos asociados en el Área de Conservación Guanacaste que permita el establecimiento de un inventario de este tipo de estructuras como material base para el análisis experimental de la presencia de un posible marcador molecular específico de agallas en diferentes plantas hospederas.

3. Confirmar la presencia de posibles secuencias de inserción en el ADN genómico de los tejidos que conforman la agalla inducida por el insecto *L. brasilensis* en las plantas de *Manihot esculenta* y determinar el papel de bacterias endosimbióticas involucradas en el proceso de inducción, formación y crecimiento de esta estructura.

V. PRÓLOGO

El trabajo de investigación de tesis está dividido en tres capítulos, a partir de los cuales se generan las publicaciones relacionadas dentro del marco del programa de Doctorado en Ciencias.

En el primer artículo, evidencias previas y recientes relacionadas con el sistema de agallas inducidas por insectos fueron analizadas de forma integral desde un nuevo enfoque. A partir de un planteamiento profundo del problema y sustentado en una amplia revisión de literatura, se refutan las hipótesis relacionadas con el proceso de inducción de las agallas. El resultado de este ejercicio académico es el planteamiento de una hipótesis alternativa que rompe los paradigmas previamente establecidos y reenfoca el abordaje experimental seguido en el trabajo de tesis. El análisis realizado sugiere que, como resultado de la complejidad del proceso de inducción y desarrollo de las agallas de insectos, la hipótesis química es muy poco probable como una explicación completa del mecanismo de inducción y morfogénesis de estas estructuras. Por lo tanto, se propone que un mecanismo de inducción mediado por la inserción de elementos genéticos exógenos en el genoma de las células que forman la agalla podría estar involucrado en la formación de estas estructuras, proceso que estaría a su vez mediado por una bacteria endosimbionte del insecto inductor. En resumen, en este primer capítulo se realiza el planteamiento formal del problema de investigación y la fundamentación de la hipótesis principal de trabajo, finalizando en los lineamientos que sustentan el enfoque experimental.

En el capítulo II, se exploran y colectan nuevos morfotipos de agallas en el Área de Conservación Guanacaste (ACG). Los morfotipos de agallas fueron registrados, caracterizados y depositados dentro de un herbario especializado establecido con esta finalidad. Además, los organismos asociados con los morfotipos de las agallas se incluyeron en el inventario cuando fue posible obtenerlos e identificarlos. Este trabajo es el primer y más detallado inventario de agallas vegetales realizado hasta el momento en el ACG. En total, fueron registrados cuarenta y cuatro familias, setenta géneros y ochenta y siete especies de plantas hospederas. Ciento treinta y un morfotipos de agallas de plantas fueron identificados en el Área de Conservación Guanacaste. Ciento veinte de nuestros registros de campo (91.6%) de agallas de plantas fueron morfotipos nuevos, no sólo para Costa Rica, sino también para el mundo. Como consecuencia de esta investigación el inventario resultante de estas entidades biológicas se colocó dentro de un herbario especializado que se estableció con este objetivo (cecidiarium). Este repositorio representa una forma estandarizada y completa para el manejo de los datos y materiales biológicos asociados con las agallas de las plantas. También como resultado del trabajo realizado se sugiere una nomenclatura para estandarizar los registros e identificaciones de morfotipos de agallas. Algunos de los morfotipos de agallas registrados se utilizaron posteriormente en las pruebas llevadas a cabo para caracterizar un posible marcador universal asociado a estas estructuras.

Por último, el capítulo III está enfocado en demostrar experimentalmente la principal hipótesis derivada del trabajo de tesis. El estudio permitió identificar y validar un posible marcador molecular presente de forma específica en el ADN genómico de las agallas. La presencia de una secuencia exógena al genoma de referencia de la planta de yuca *Manihot esculenta* Crantz en la agalla formada por el insecto *Iatrophobia brasiliensis* fue establecida por medio del uso de marcadores basados en la metodología de PCR, tanto de PCR de punto final como de PCR en Tiempo Real por medio de la utilización de sondas TaqMan. No obstante, no fue posible determinar con certeza si dicha secuencia estaba insertada en el genoma de las células que forman la agalla en las plantas de yuca. Adicionalmente, a partir de los datos generados de la secuenciación de las muestras de agallas y tejidos sanos de yuca, por medio de la utilización de herramientas bioinformáticas se identificaron y caracterizaron secuencias de inserción candidatas en las muestras de agallas. Asimismo, a partir de los datos generados por la secuenciación de los metagenomas completos de dos muestras de hojas sanas y dos de agallas, se determinó la presencia a nivel de especie de una microbiota específica asociada con los tejidos de las agallas de yuca, pues se observó un grupo de microorganismos que parecen estar enriquecidos o presentes de forma específica en dichos tejidos.

VI. DESARROLLO DE LA INVESTIGACIÓN

Capítulo 1

Planteamiento del problema, fundamentación de la hipótesis principal y lineamientos del enfoque experimental.

Descripción

Se propone que la hiperplasia que caracteriza la fase inicial de inducción de la agalla en plantas de yuca, es generada por el estímulo y acción de un elemento genético similar o análogo al plásmido Ti presente en líneas del género *Agrobacterium.* El razonamiento que fundamenta el artículo se desarrolló ampliamente en la sección de planteamiento de la hipótesis.

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SPECIAL ARTICLE

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The mechanism of plant gall induction by insects: revealing clues, facts, and consequences in a cross-kingdom complex interaction

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Abstract: Galls are defined as modifications of the normal developmental design of plants, produced by a specific reaction to the presence and activity of a foreign organism. Although different organisms have the ability to induce galls in plants, insect-induced galls are the most elaborate and diverse. Some hypotheses have been proposed to explain the induction mechanism of plant galls by insects. The most general hypothesis suggests that gall formation is triggered by the action of chemical substances secreted by the gall inducer, including plant growth regulators such as auxins, cytokinins, indole-3-acetic acid (IAA), and other types of compounds. However, the mode of action of these chemical substances and the general mechanism by which the insect could control and manipulate plant development and physiology is still not known. Moreover, resulting from the complexity of the induction process and development of insect galls, the chemical hypothesis is very unlikely a complete explanation of the mechanism of induction and morphogenesis of these structures. Previous and new highlights of insect gall systems with emphasis on the induction process were analyzed on the basis of the author’s integrated point of view to propose a different perspective of gall induction, which is provided in this article. Due to the extraordinary diversity of shapes, colors, and complex structures present in insect galls, they are useful models for studying how form and structure are determined at the molecular level in plant systems. Furthermore, plant galls constitute an important source of material for the study and exploration of new chemical substances of interest to humans, due to their physiological and adaptive characteristics. Considering the finely tuned control of morphogenesis, structural complexity, and biochemical regulation of plant galls induced by insects, it is proposed that an induction mechanism mediated by the insertion of exogenous genetic elements into the genome of plant gall cells could be involved in the formation of this kind of structure through an endosymbiotic bacterium.

Key words: insect galls, inductor insect, induction mechanism, plant morphogenesis, effectors.

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The term plant gall has been applied to different systems, and although there is no consensus about the definition of the term, the same has been used as a generalized expression more than a precise scientific term or concept (Williams, 1994). Nonetheless, in general terms, galls could be defined as deviations in the normal plant development pattern, produced by a specific reaction to the presence and activity of a foreign organism (Shorthouse & Rohfritsch, 1992; Inbar et al., 2009; Huang et al., 2015). Gall-inducing insects, also called gall inducers, gall makers, or simply gallers, live within the plant tissue, which supplies food, low levels of potentially harmful chemical substances, protection against unfavorable environmental factors (Nogueira, Costa, Silva, & Isaias, 2018), and shelter against natural enemies (Mani, 1992; Ananthakrishnan, 1998; Raman, Schaefer, & Withers, 2005; Tooker & De Moraes, 2008; Tooker, Rohr, Abrahamson, & De Moraes, 2008; Huang et al., 2015; Isaias et al., 2018).

The meaning of the adaptive value of galls and the kind of biological interaction existing between gall-inducing insects and their host plants is the subject of a continuous debate among the different groups of researchers that work in the field (Nyman & Julkunen 2000; Stone & Schönrogge, 2003). Some groups established that galls originated as a mechanism of defense developed by insects against attack by their natural enemies. Moreover, the main function of the gall is to give shelter and food to the larvae of the galling insect; however, this and other related ideas are still the target of extensive debate (Ananthakrishnan, 1998; Stone & Cook 1998; Price, Waring & Fernández, 1986; Stone & Schönrogge, 2003; Tooker et al., 2008; Giron, Huguet, Stone, & Body, 2016). Different lines of thought relate galls with processes of pathogenesis, symbiosis, and defense mechanisms in plants (Hartnett & Abrahamson, 1979; Price et al., 1986). Regardless of the type of specific interaction between gall-inducing insects and their host plants, natural selection consequently operates on the insect to stimulate the development of protective and/or nutritive tissues in the plant; on the other hand, in the plant, natural selection acts to resist the stimulus generated by the insect (Ananthakrishnan, 1998).

Hymenoptera and Diptera are two orders with a particularly large number of gall inducers, but great diversity can also be found in galls formed by thrips, aphids, and insects from other orders (Ananthakrishnan, 1998; Hanson & Gómez-Laurito, 2005). A large number and diversity of plant gall morphotypes and inducing insects have been reported worldwide (Espírito-Santo & Fernandes, 2007). New species of inducing insects are periodically described, while other studies on the abundance and diversity of gall morphotypes, as well as their corresponding inducers, has helped to broaden the existing knowledge in this field (Shorthouse & Rohfritsch, 1992; Williams, 1994; Ronquist & Liljeblad, 2001; Hanson & Gómez-Laurito, 2005; Dalbem & Mendonça, 2006; Güçlü, Hayat, Shorthouse, & Göksel, 2008; Coelho et al., 2009; Maia, Fernandes, Magalhãcs, & Santos, 2010a; Maia, Fleury, Soares, & Isaias, 2010b; Medianero, Paniagua, & Castaño-Meneses, 2010; Maia & Oliveira, 2010; Santos, Almeida-Cortez & Fernandes, 2011; Sano, Havil, & Ozaki, 2011; Maia, 2014; Santos de Araújo, 2017; Martins dos Santos, Pereira Lima, Souza Suares, & Calado, 2018).

Besides insects, plant galls are also induced by a great variety of organisms such as bacteria, fungi, nematodes, and mites (Leitch, 1994; Williams, 1994; Ananthakrishnan, 1998, Raman, 2011). Galls induced by insects are distinct from those induced by fungi and bacteria in their form, organization, and complexity. More complex and diverse galls are induced by insects such as those of the Cynipidae and Cecidiomyiidae families, which show extreme examples of radial symmetry, belonging to the orders Hymenoptera and Diptera, respectively (Raman, Cruz, Muniappan, & Reddy, 2007; Sinnott, 1960; Raman, 2011). A general scheme for the structural complexity of plant galls and the taxonomic groups of their inducers is proposed by the author from the reviewed literature (Fig. 1) (Rohfritsch & Shorthouse, 1982; Mani, 1992; Davey, Curtis, Gartland, & Power, 1994; Gómez & Kisimova-Horovitz, 1997; Williams, 1994; Valentine, 2003; Sá et al., 2009; Raman 2011; Álvarez, Molist, González-Sierra, Martínez, & Nieto-Nafría, 2014; Formiga, Silveira, Fernandes, & Isaias, 2015; Muñoz-Viveros et al., 2014; Guimarães, Neufeld, Santiago-Fernandes, & Viera, 2015; Hernández-Soto et al., 2015; Suzuki, Moriguchi, & Yamamoto, 2015; Mellah, Enhassaïni, & Álvarez, 2016; Oliveira et al., 2016; Richardson, Body, Warmund, Schultz, & Appel, 2016; Ferreira, Álvarez, Avritzer, & Isaias, 2017; Palomares-Rius, Escobar, Cabrera, Vovlas, & Castillo, 2017; Cotrim Costa, Gonçalves da Silva Carneiro, Santos Silva, & Isaias, 2018;

Nogueira, Costa, Silva, & Isaias, 2018); however, a consensus on this approach does not exist, and substantial variation can be observed within each group.

The fact that different groups of insects possess the capacity to form galls in a wide variety of plants has motivated a great number of investigations attempting to elucidate the mechanism of induction of this type of structure. Nevertheless, considering the importance of galls as models for understanding a series of fundamental processes in the development of plants, the induction mechanisms and the evolutionary context of this type of structure is still poorly understood (Stone & Schönrogge, 2003, Raman, 2011; Oates, Denby, Myburg, Slippers, & Naidoo, 2016).

The aim of this paper is to provide an updated general description of plant galls induced by insects, focused on the induction process as well as how, according to an integrated interpretation by the author, the associated characteristics of these structures and the biological processes they regulate could be the basis for an alternative induction hypothesis mediated by the insertion of exogenous genetic elements into the plant gall cells through some endosymbiotic bacteria originating from the insect.

Plant gall development and diversity of plant gall-inducing insects

The association between galls and their inducing organisms has likely been recognized since the study of these systems began (Mani, 1992). However, it was not until the 17th century that Malpighi described, in the Western World, that the growth and development of these structures was correlated to the activity of feeding, oviposition, and particular nutritional requirements of the inducing insect (Fagan, 1918; Hough, 1953).

Gall morphogenesis is a complex phenomenon, which involves reorientation of the

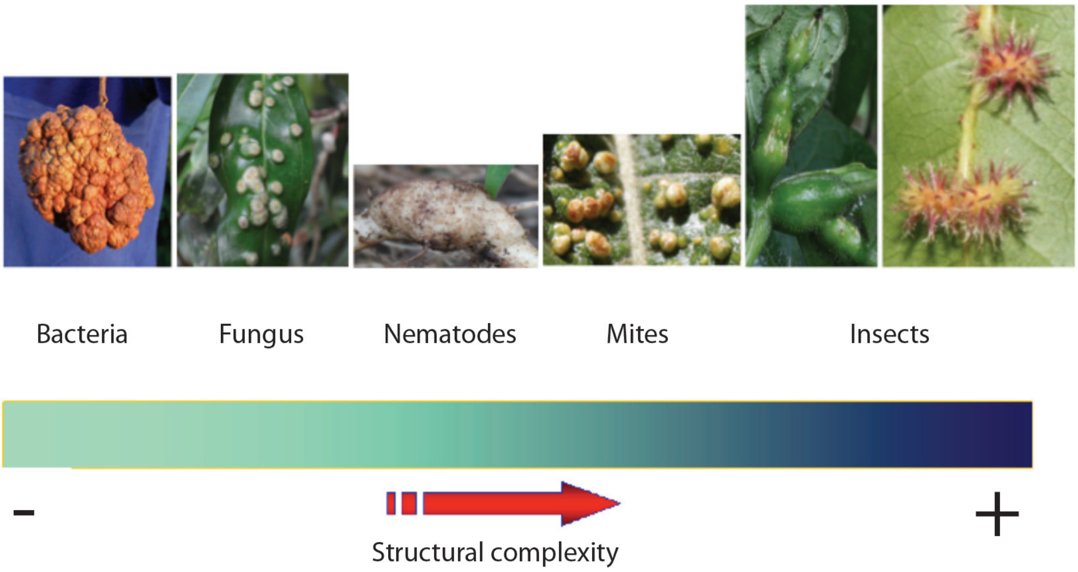

Fig. 1. Proposed general scheme for the structural complexity of plant galls and the taxonomic groups of gall inducers. Images show some examples of galls. Bacteria crown gall found on *Pittosporum sp*. (Pittosporaceae), induced by *Agrobacterium tumefaciens*. Fungus gall on *Satyria warszewiczii* (Ericaceae), induced by *Exobasidium emeritense*. Nematode gall induced by *Meloidogyne incognita* on *Solanum lycopersicum* (tomato, Solanaceae). Mite gall induced on *Acnistus arboresens* (Solanaceae). Insect gall induced on *Cissus fuliginea* (Vitaceae) by an unknown diptera Cecidomyiidae and on *Hirtella racemosa* (Chrysobalanaceae) by an unknown diptera Cecidomyiidae. Taxonomic identification of host plants of insect galls was performed by Roberto Espinoza, and inductor insects were identified by Paul Hanson. Photo credit: taken from Patrick Roper (bacteria crown gall), Omar Gätjens-Boniche (fungus gall, nematode gall, mite gall and insect galls).

plant's development by the inducing insect (Ananthakrishnan, 1998; Raman, 2011; Oates, Külheim, Myburg, Slippers, & Naidoo, 2015; Agudelo et al., 2018). The degree to which the insect manipulates the plant's growth to form the gall varies considerably and involves changes ranging from the induction of cell proliferation (Agudelo et al., 2018) to the formation of a complex structure that the plant does not produce under normal conditions. Just like normal plant organs and structures, galls induced by insects present anatomic and histologic characteristics of their own, which vary greatly in their diversity and degree of complexity (Fig. 2) (Nyman & Julkunen, 2000; Mani, 1992; Ananthakrishnan, 1998; Stone & Schönrogge, 2003; Oliveira & Isaias, 2010; Raman, 2011; Oliveira, Carneiro, Magalhães, & Isaias, 2011; Oliveira et al., 2016). Tissues near the inducing insect show cytological and morphological changes that benefit its feeding process and development. This tissue, also known as "nutritive tissue", commonly presents high concentrations of sugar (Nogueira et al., 2018), lipids, proteins, nitrogen, and other nutrients that provide a continuous source of food for the insect and show intense phosphatase activity (Miles, 1968; Rohfritsch & Shorthouse, 1982; Shorthouse & Rohfritsch, 1992; Raman, 2011, Oliveira & Isaias, 2010; Oliveira et al., 2011; Nabity, Haus, Berenbaum, & Delucia, 2013; Huang et al., 2015; Oates et al., 2016; Ferreira et al., 2017; Isaias et al., 2018). Typical nutritive cells show a dense cytoplasm with abundant cell organelles, fragmented vacuoles, a hypertrophied nucleus and nucleolus, and dedifferentiated plastids clustered around the nucleus, as well as chloroplasts modified

Fig. 2. Some plant galls from Costa Rican flora. **A)** Gall induced on *Pisonia macranthocarpa* (Nyctaginaceae) by an unidentified insect species (Diptera, Cecidomyiidae). **B)** Gall induced on *Vitis tiliifolia* (Vitaceae) by an unidentified insect species (Diptera, Cecidomyiidae). **C)** Gall induced on *Hirtella racemosa* (Chrysobalanaceae) by an unidentified insect species (Diptera, Cecidomyiidae). **D)** Gall induced on *Semialarium mexicanum* (Hippocrateaceae) by an unknown inducer. Taxonomic identification of the host plants was carried out by Roberto Espinoza, and the inductor insects were identified by Paul Hanson. Photo credit: Omar Gätjens-Boniche (A, C, and D) and Gregorio Dauphin (B).

to varying degrees and modified cell walls (Shorthouse & Rohfritsch, 1992; Raman, 2011; Carneiro & Isaias, 2015). Ferreira et al. (2017) compared six gall systems with different levels of structural complexity (aphids, mites, and Nematoda), using histometric and histochemical analyses. Based on the types of storage tissue, the authors proposed a classification of three types of storage tissues: typical nutritive tissues (TNT), common storage tissues (CST), and nutritive-like tissues (NLT). TNT and NLT present cells with a dense cytoplasm and a large nucleus; TNT serve as a direct food source for gall inducers. CST have vacuolated cells, and may store starch and other types of energy-rich molecules, as do the cells of NLT. Likewise, several studies have demonstrated that insects generally feed on a reduced area of the gall (Nyman & Julkunen, 2000).

The inducing insect can modify the expression of genes within restricted areas of the host plant, thereby producing new developmental events in the tissues under its influence. Gall morphogenesis occurs in a relatively short time; however, this fact apparently does not influence the complexity observed in such morphological entities (Ananthakrishnan, 1998, Nabity et al., 2013; Oates et al., 2015).

In many kinds of abnormal growth or deviation from normal organismal development, there are alterations in the mechanisms that regulate cell proliferation and differentiation. Within this context, “crown galls” induced by the genus *Agrobacterium* are an example of structures formed due to the proliferation of cells with a low level of differentiation; hence, they are considered the simplest and least derived plant gall within the wide variety of these structures found in Nature. On the other hand, galls induced by insects are very well-organized structures showing different degrees of differentiation, the reason why they are considered as the most complex and derived structures. Nonetheless, in spite of the clear differences between these two extremely diverse groups of plant galls, they show important similarities. For instance, both systems require a previous state of “conditioning” towards the development of the structure. In the case of insect galls, the “conditioner” is the insect itself, which modulates the tissue that will form the structure through mechanical action and the secretion of chemical substances. In crown galls the conditioning factor is given by a series of metabolic events prior to the genetic transformation of plant cells by the bacterium (Rohfritsch & Shorthouse, 1982; Davey et al., 1994; Piñol, Palazón, Cusidó & Serrano, 1996; Valentine, 2003; Suzuki et al., 2015).

In a similar manner as in so-called “tumor cells” of crown galls, cells from insect-induced galls acquire a certain autonomy and independence from their normal tissue development pattern. From the induction process, cell development is redirected due to the influence of the inducing stimulus. However, unlike insect galls, crown galls have an unlimited capacity to grow without a defined pattern of development. After the initial stimulus, cell proliferation in both systems develops in a different way; in the case of bacteria-induced crown galls, cell proliferation occurs in an uncontrolled way and does not require the continuous presence of bacteria once the process is initiated. In contrast, for adequate and complete development of galls induced by insects, in general, the continuous, active presence of the insect is required (Rohfritsch & Shorthouse, 1982; Davey et al., 1994; Valentine, 2003; Suzuki et al., 2015).

According to Rohfritsch & Shorthouse (1992), Arduin & Kraus (1995), and Sá et al. (2009), plant galls present four basic stages of development, but significant differences between gall inducers can occur. These stages of development involve the processes of initiation, growth and differentiation, maturation, and finally, dehiscence (Fig. 3). The state of growth and development is a continuous process of cell division and differentiation that generally depends on the feeding activity of the larva, which in turn is mainly responsible for molding the shape of the gall’s inner chamber. After the nutritive tissue is formed around the inner chamber, a mass of cells binds the vascular tissue of the gall to the plant. Frequently, sclerenchymatous tissue is also formed around

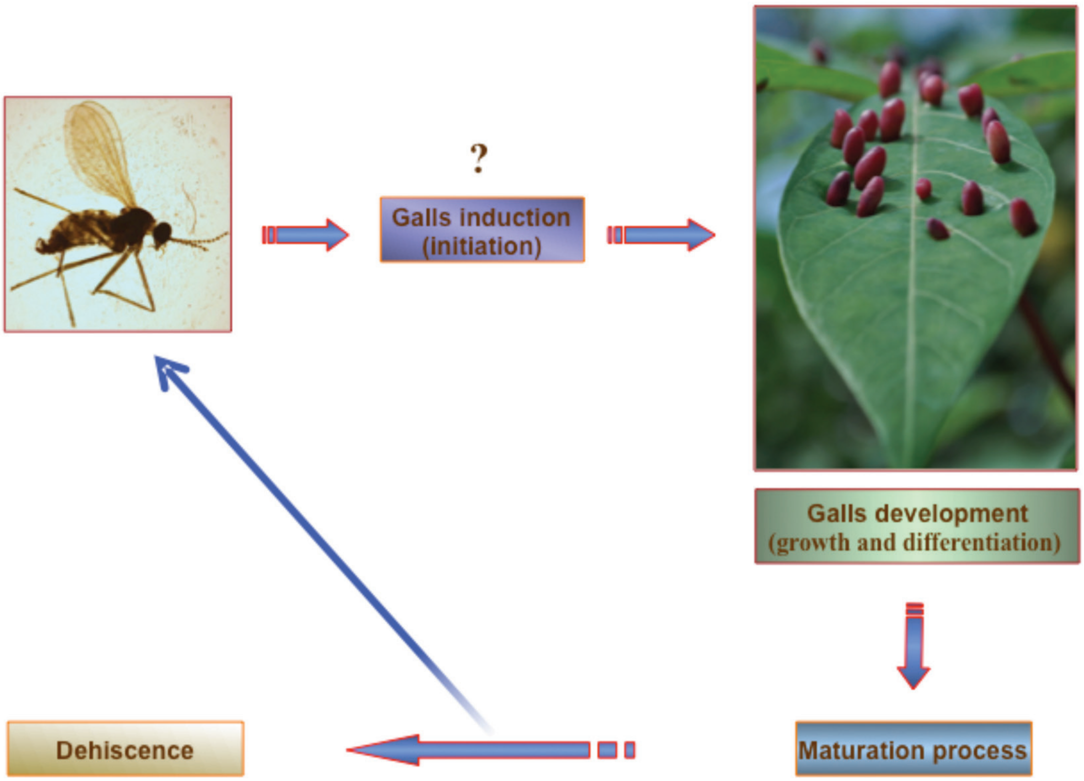

Fig. 3. General scheme for the life cycle of insect-induced galls. This figure is based on the gall induced by the Cecidomyiidae *Latrophobia brasiliensis* in *Manihot esculenta* Crantz (Cassava). The scheme was synthesized from the reviewed literature. Photo credit: Omar Gätjens-Boniche.

the inner chamber and near the vascular tissue of the gall, which causes its separation into internal and external regions. The internal region is considered to be influenced by the activity of the larva, whereas the external region or outer cortex of the gall is under the influence of the plant. The opening and dehiscence of galls occurs towards the end of the maturation stage and represents the period of greatest chemical and physiological change in the tissues that comprise it. Not only has it been demonstrated that the meristematic tissues react to the stimuli but also that young stems of various species of plants can be stimulated and modified to make these structures.

Shorthouse & Rohfritsch (1992) separate the morphogenesis of galls induced by insects into two processes. The first is a permanent effect, which remains even when the corresponding insect is removed or dies. A second process implies that the effect is generated through continuous stimulation by the inducing insect, which disappears if the insect is removed from the gall or if it dies.

Several authors have tried to classify galls according to a series of morphological criteria that have, in spite of being arbitrary, established the groundwork for the development of a great number of studies. Shorthouse & Rohfritsch (1992) and Williams (1994) distinguished two basic types of galls: organoids and histoids. The first one results from organ proliferation or modification, maintaining the basic organ structure. Histoid galls, in contrast, originate from the proliferation of modified cells leading to the formation of new tissue. Plant galls are also classified according to more strict morphological criteria. Galls called “kataplasmic” are irregular in size and shape, presenting an irregular growth pattern with little differentiation in their tissues. On the other hand, “prosoplasmic” galls are more complex and differentiated and are formed as a result of the formation of a new structure (Miles, 1968; Williams, 1994). Nonetheless, it is important to emphasize that any attempt at classification turns into a difficult task, mainly because of the great quantity of existing intermediate states and shapes among the different gall morphotypes. Therefore, the shape and structure of galls depends on a large number of factors, including the species to which the host plants belong, the species of the inducing insects, the type of organ attacked, the state of development of the plant and, in some cases, even the sex of the insect. According to Raman (2011), about 90 % of all the known galls show bilateral or radial symmetry. As reported by the same authors, in the specific case of galls formed by insects belonging to the families of Cecidomyiidae (Diptera) and Cynipidae (Hymenoptera), they show surprising levels of radial symmetry. Moreover, some factors that affect gall size include the number of larvae present, the structural diversity of the galls, the percentage of tissue infected, the physiological state of the host plant, environmental conditions, and the genotype of the plant (Mani, 1992; Ananthakrishnan, 1998; Stone & Schönrogge, 2003; Raman, 2011).

Some galls are simply “swellings”—undifferentiated cell masses or those with a low level of differentiation, while others show a surprisingly high degree of differentiation, organization and specialization within their cells and tissues, frequently with characteristics exclusively associated with the gall from which they originate. Based on the above, this last type of gall, called “prosoplasmic”, presents an anatomy and histology very characteristic of its own. Prosoplasmic galls induced by some families of insects are, due to their high degree of complexity and organization, the ones that generate a higher interest (Mani, 1992).

The order Hymenoptera includes the most complex and organized galls described so far for the Class Insecta. In this group, gall-inducing insects are classified in the Suborder Symphyta, family Tenthredinidae and the Suborder Apocrita, with two Superfamilies: Chalcidoidea, which includes the families Pteromalidae, Eurytomidae, and Agaonidae and the superfamily Cynipoidea, represented by the family Cynipidae. Gall-inducing Hymenoptera species present a wide distribution over several areas of the planet and can be found in various groups of dicotyledonous plants and even in monocotyledonous plants, especially in gramineous species (Shorthouse & Rohfritsch, 1992). According to Rohfritsch & Shorthouse (1982) and Shorthouse & Rohfritsch (1992), Cynipidae contains the main gall inducers of the order Hymenoptera, and they can cause the development of different kinds of galls in distinct organs of the plant. Most representatives of this family induce the formation of galls in leaves, sprouts, stems, and roots. Females lay eggs on the surface of the tissue, and the egg itself induces an initial gall, even though the larvae of these insects are the main inducing agents of these structures. Cynipidae eggs have a lytic effect on the cells that surround them, which leads to the formation of a chamber that protects the young larva. Larvae have mouth structures that allow them to break the plant cell wall to suck on the contents of the nutritive cells. Many species of Cynipids are characterized by complex life cycles, commonly accompanied by an alternation of generations. Individuals of both generations can attack the same organ or different types of organs in the plant, resulting in the formation of radically different galls, and in some cases, even the individuals of the two generations are morphologically different. The surface of the galls formed by the Cynipids can be coated by trichomes, scales, thorns, or other types of outgrowths. Nevertheless, one of the most important characteristics of these structures is the formation of concentric areas of differentiation and an area of sclerenchymatous cells around the larval chamber.

The order Diptera grouped in the suborder Nematocera, which includes the family Cecidomyiidae (gall midges), and the suborder Brachycera, which includes Tephritidae (fruit flies) and Agromyzidae. Gall inducers belonging to this order present a broad global distribution, and in contrast to other orders of gall- inducing insects, they can also be found in monocotyledonous plants, especially grasses. Nonetheless, the majority of arthropod-induced galls occur on dicotyledons, and at least 66 % of the dicotyledon families harbor galls (Shorthouse & Rohfritsch, 1992; Hanson & Gómez-Laurito, 2005). Hanson & Gómez-Laurito (2005) suggested that possibly more than 90 % of dicotyledon species in all major biogeographic regions harbor gall-inducing by cecidomyiids. Around 80 % of plant galls induced by insects from the Neotropical region are induced by the Cecidomyiidae family, and new species of inducing insects belonging to this family are constantly being described around the world. Moreover, according to Hanson & Gómez-Laurito (2005), about 70 % of the gall-inducing arthropods in Costa Rica are Cecidomyiidae.

Galls formed by Diptera, especially those induced by individuals of the family Cecidomyiidae, are characterized by a high degree of tissue differentiation; on the other hand, the insects are characterized by the complexity of their life cycles. Another important characteristic present in gall-inducing Diptera species is their capacity to pupate inside the gall. In the family Cecidomyiidae only the larvae have the capacity to induce galls; these have a poorly developed mouth structure and feed by sucking on fluids exuded from the gall cells, without causing any damage or necrosis (Rohfritsch & Shorthouse, 1982; Shorthouse & Rohfritsch, 1992). In those galls, nutritive tissue is present throughout the development of the structure. At the same time, for the development and maintenance of nutritive tissue, the active presence of the larva of the inducing insect is necessary (Ananthakrishnan, 1998).

Galls induced by the orders Thysanoptera and Hemiptera appear as small bumps or abnormal growths, whose tissues are essentially made of parenchymatous cells. Some species can also cause a leaf roll accompanied by cellular hypertrophy. In the case of the hemipterans, they induce a variety galls types, which vary from simple forms to very sophisticated complex structures. Several species of coleopteran gall-inducing larvae produce tunnels in different parts of the plants, and the eggs are placed in the interior of cavities prepared by insect females. Although plant galls induced by Coleoptera have been described as characterized by the absence of nutritive tissue (Shorthouse & Rohfritsch, 1992), there are descriptions detailing the presence of this type of tissue or nutritive-like tissue in coleopteran galls (Raman et al., 2007; Barnewall & De Clerck-Floate, 2012). Nevertheless, little is known about gall-inducing Coleoptera, especially in tropical ecosystems (Korotyaev, Konstantinov, Lingafelter, Mandelshtam, & Volkovitsh, 2005). Many galls formed by Lepidoptera do not develop nutritive tissue, and larvae of these insects are fed by chewing the tissue that surrounds the internal chamber, producing a large amount of detritus (Rohfritsch & Shorthouse, 1982; Shorthouse & Rohfritsch, 1992). However, recent studies on lepidopteran-induced galls suggest that these structures may also present nutritive tissue and are not as simple as they have traditionally been described. For example, a true nutritive tissue that showed metabolite concentration gradients, which seem to be specific for lepidopteran galls, was described by Ferreira & Isaias (2013). Nutritive tissue was described in *Bauhinia ungulata* L. (Fabaceae) by Bedetti, Ferreira, de Castro, and dos Santos Isaias (2013). Moreover, nutritive cells in the galls induced on the leaves of *Tibouchina pulchra* (Cham.) Cogn. (Melastomataceae) have a large amount of rough endoplasmic reticulum, ribosomes, polyribosomes, and mitochondria, which are evidence of the high metabolic status of these cells. Likewise, vascular cambium-like, with high protein synthesis and lipid storage, are characteristic of that nutritious tissue. The nutritive cells are stimulated by the activity of galling larvae, consequently generating a new tissue type (Vecchi, Menezes, Oliveira, Ferreira, & Isaias, 2013).

The induction mechanism of plant galls by insects: What do we know?

The capacity of a large number of insects to form galls in different groups of plants has motivated a great deal of research with the aim of elucidating the mechanism of induction of this type of structure. Hori (1992) describes four main hypotheses that could explain the formation of plant galls. The first of these hypotheses suggests the injection of a fluid from the insect during the oviposition process, which would mediate gall induction. A second hypothesis proposes that gall formation is the result of mechanical irritation due to the presence of a foreign body on the plant tissue. An extension of this hypothesis suggests that galls are induced at a "reactive site" with particular traits of available meristematic regions by the action of the inductor insect, probably in stem cell areas (Weis, Waltonanand, & Crego, 1988; Abrahamson & Weis, 1997; Espírito-Santo, Neves, Andrade-Neto, & Fernandes, 2007; Silvia & Connor, 2017). The third hypothesis proposes that the formation of galls is induced by the secretion of active components from the saliva of the insect. Finally, a fourth hypothesis purports that the formation of galls is mediated by the excretion of metabolic products from the insect.

For simplicity, the morphogenic process of plant gall induction by insects can be divided into three main phases. The first one involves "conditioning" of the cells of the corresponding plant tissue by the insect, to make them more susceptible and suited to its action as inductor. In the following phase, induction of the gall as such takes place, whereby cell division and elongation results in the formation of a "primary" gall. The final phase consists of gall maturation, in which the primary gall grows to complete its morphogenesis (Shorthouse & Rohfritsch, 1992; Raman, 2011).

As mentioned above, in the plant gall induction process, plant cells should be conditioned to produce a particular physiological state (Raman, 2011). In this respect, different studies have mentioned that the amino acids present in the salivary secretions of gall-inducing insects, essentially lysine, histidine, and tryptophan, could function as "preconditioners" for gall induction. It seems that these amino acids could cause major plasticity and would increase the sensitivity of the plant tissue to the action of the corresponding inducing insect. Although the presence of pectinase in the saliva of insects has not been correlated with gall induction, such enzymes could degrade the cell walls and in turn contribute to tissue preconditioning to the action of the insect. Likewise, it has been speculated that polyphenol oxidase (PPO), also present in the saliva secretions of insects and the phenolic compounds derived from its enzymatic action, could increase plant tissue sensitivity to the stimulus of the inductor insect. It has also been suggested that the complex interaction between the host plant tissue and polyphenol oxidase might be of fundamental value in gall formation (Miles, 1968; Hori, 1992; Ananthakrishnan, 1998; Saltzmann, Giovanini, Zheng, & Williams, 2008). In this respect, Miles (1968) indicated that interactions and the balance between insect polyphenol oxidase and the host plant could determine whether the "attack" of an insect causes injury (necrosis) or gall development. Moreover, the modulation of redox potential has been related to gall initiation and establishment, especially concerning the accumulation of reactive oxygen species (Isaias, Oliveira, Moreira, Soares, & Carneiro, 2015).

Different studies have reported that indoleacetic acid (IAA) could be a powerful gall-inducing agent, and it has also been speculated that this compound could interact with other plant growth regulators, like cytokinins and gibberellins, or in a synergistic way with other chemical substances, to promote the induction and maturation of these structures (McCalla, Genthe, & Hovanitz, 1961; Miles, 1968; Hori, 1992; Leitch, 1994; Ananthakrishnan, 1998; Mapes & Davies, 2001; Stone & Schönrogge, 2003; Raman, 2011, Tooker, & Helms, 2014; Bartlett & Connor, 2014; Bedetti, Modolo, & dos Santos, 2014; Bailey, Percy, Hefer, & Cronk, 2015). However, the mechanism of action through which these substances act to promote the development of plant galls is very poorly understood and is currently a subject of discussion. This scenario becomes more complicated in the case of prosoplasmic galls, like the ones formed by insects of the Cecidomyiidae and Cynipidae families, because of the complexity of their development and structure.

Symbiotic relationships between gall-inducing insects and microorganisms have been hypothesized to be involved in plant gall development (Hansen & Moran, 2014; Tooker and Helms, 2014). Several studies have demonstrated the presence of a great number of endosymbiotic bacteria in different insect groups (Degnan, Lazarus, & Wernegreen, 2005; Kikuchi, Meng & Fukatsu, 2005; Delmotte, Rispe, Schaber, Silva, & Moya, 2006; Fukatsu et al., 2007; Jaenike, Polak, Fiskin, Helou, & Minhas, 2007; Goto, Anbutsu, & Fukutsa, 2006; Xi, Gavotte, Xie, & Dobson, 2008; Toft, Williams, & Fares, 2009; Gutzwiller, Dedeine, Káiser, & Giron, 2015; Krawczyk, Szymańczyk, & Obrępalska-Stęplowska, 2015; El-Sayed & Ibrahim, 2015; Campbell et al., 2015), as well as bacteriocytes (Nikoh & Nakabachi, 2009; Braendle et al., 2009). Some of these symbiont microorganisms are mutualistic and contribute to the viability of their hosts, while others are parasites, which tend to affect their corresponding hosts in a negative way. Insect-associated microorganisms could be important mediators of interactions between insects and plants (Sugio, Dubreuil, Giron, & Simon, 2015; Hammer & Bowers, 2015, Wielkopolan & Obrępalska-Stęplowska, 2016). Some researchers have reported that simultaneous infection with different species of endosymbionts in the same host organism is a common phenomenon in several insect groups (Thao et al., 2000; Thao, Gullan, & Baumann, 2002; Russell et al., 2003; Ishii, Matsuura, Kakizawa, Nikoh, & Fukatsu, 2013; Krawczyk et al., 2015; El-Sayed et al., 2015; Ghosh, Bouvaine, & Maruthi, 2015; Brentassi et al., 2017). Different tissues in the body of the same host constitute different microenvironments for endosymbiont organisms. Some tissues could be, for instance, nutritionally favorable, immunotolerant, or simply easy to colonize (Mouton, Henri, Bouletreau, & Vavre, 2003; Kondo, Shimada, & Fukatsu, 2005; Koga, Meng, Tsuchida, & Fukatsu, 2012; Hansen & Moran, 2014; Sugio et al., 2015).

In recent years, a growing interest has emerged regarding the reproductive biology of endosymbiont parasites that are transmitted through the mother and manipulate the reproduction of their host organism. Accumulated evidence shows that many species of arthropod are infected by different kinds endosymbiont organisms transmitted from the mother through vertical transmission, which have a great influence on the biology of their hosts. Some of these endosymbiont microorganisms include *Wolbachia*, *Spiroplasma*, *Rickettsia*, *Arsenophonus*, and *Cardinium*, among others (Weeks, Velten & Stouthamer, 2003; Zchori-Fein & Perlman, 2004; Goto et al., 2006; Casper-Lindley et al., 2011; Goodacre & Martin, 2012; Kageyama, Narita, & Watanabe, 2012; Koga et al., 2012; Kremer et al., 2012; Herren, Paredes, Schüpfer, & Lemaitre, 2013; Ma, Vavre & Beukeboom, 2014; Boivin et al., 2014; Ma et al., 2015; Sugio et al., 2015; Brentassi et al., 2017; Mariño, Verle Rodrigues, & Bayman, 2017; Ma & Schwander, 2017).

Many galling insects are known to have microbial associates that may be involved in gall development or could facilitate herbivory, such as *Ambrosia* gall midges associated with fungal symbionts, but studies exploring the role of microbial associates in the lifecycles of insect gallers are scarce (Hansen & Moran, 2014; Tooker & Helms, 2014; Huang et al., 2015). Bacteria of the genus *Wolbachia* have been associated with green-island formation by the apple leaf-mining moth *Phyllonorycter blancardella*, a similar phenomenon to the one observed in some types of plant galls induced by insects (Kaiser, Huguet, Casas, Commin, & Giron, 2010; Gutzwiller et al., 2015). Their results suggest that bacteria impact green-island induction by manipulating cytokinin levels. In addition, secretions of phytohormones, such as cytokinins, by endosymbiotic microorganisms have also been associated with the plant–galling insect interaction (Spíchal, 2012). Likewise, Bartlett and Connor (2014) hypothesized that the inducing insects obtained their ability to induce galls via endosymbiotic microbes, which have acquired the biosynthetic pathways to produce IAA and trans-zeatin family cytokinins from plants. It is not surprising then that the control of cytokinins constitutes an important selection factor for arthropods and pathogens because of the importance of these phytohormones in the regulation of plant morphology, senescence, and defense, especially with regard to the mobilization of nutrients in each of these processes (Giron, Frago, Glevarec, Pieterse, & Dicke, 2013; Tooker & Helms, 2014; Naseem, Wölfling, & Dandekar, 2014; Giron et al., 2016).

It has been proposed that galling insects acquired genes from symbiotic microorganisms through horizontal gene transfer (Giron & Glevarec, 2014; Bartlett & Connor, 2014). Horizontal gene transfer (HGT) is the movement and transference of genetic information between different organisms, and it is a common phenomenon between pathogens of animals and plants, and between symbionts and pathogens (De la Cruz & Davies, 2000; Suzuki et al., 2015). Indirect evidence supporting the previous hypothesis is provided by works such as those carried out by Nikoh et al. (2008). Molecular analyses performed by these authors in *Wolbachia*, one of the most abundant intracellular bacteria described in arthropods, as well as nematodes, suggested that approximately 30 % of *Wolbachia* genes are present in the nuclear genome of host insects. In this study, through fluorescence in situ hybridization techniques, they located the transferred genes of *Wolbachia* in the proximal region of the short arm of the insect X chromosome. The collected evidence indicated that this process of horizontal gene transfer was probably generated from an individual event. In another study, it was determined that the genome of *Wolbachia pipientis* contains high levels of repetitive sequences of DNA and also mobile genetic elements (Wu et al., 2004). In spite of its wide distribution and the effects of *Wolbachia* on the biology of its hosts, little is known about the molecular mechanisms that mediate the interaction between this bacterium and its invertebrate hosts (Wu et al., 2004; Chrostek & Teixeira, 2015).

Therefore, under the previous scenario, horizontal gene transfer (HGT) could play a fundamental roll in plant gall induction and evolution. In recent years, more evidence has shown that the molecular mechanisms involved in the different processes of symbiosis and pathogenesis present a series of common pathways which have revealed existing similarities in the modulation and interactions between pathogens and symbionts with their hosts (De la Cruz & Davies, 2000; Hentschel, Steiner, & Hacker, 2000; Rankin, Rocha, & Brown, 2011; Suzuki et al., 2015). Furthermore, the information generated by microbial genome sequencing studies has demonstrated that horizontal transference of genes is an important process and widely distributed within the evolutionary scenario of prokaryote organisms (Nikoh & Nakabachi, 2009; Jayaprakashvel, Bhrathi, Muthezhilan, & Hussain, 2017).

In addition to chromosomes, prokaryotes possess mobile genetic elements, such as genomic islands, plasmids, transposons, insertion sequences or bacteriophages, which allow them to induce structural and physiological changes, as well as the acquisition or loss of genomic regions. Moreover, the fact that a great number of pathogenic and symbiotic determinants are located in mobile genetic elements allows a source of permanent variation to be generated within these organisms. In addition, some authors have suggested that the acquisition and incorporation of plasmids into bacteria could constitute a key process to the adaption of these microorganisms to new ecological niches and to their development as symbionts or pathogens (Vivian, Murillo, & Jackson, 2001; Suzuki et al., 2015; Jayaprakashvel et al., 2017). Genetic variability plays a very important role by generating the conditions that allow the evolution of new types of interactions among organisms, thus HGT between different species could represent a powerful mechanism through which the final result of the interaction between a pathogen or symbiont and its host could be altered permanently (Hentschel et al., 2000; Suzuki et al., 2015; von Wintersdorff et al., 2016; Porse, Schou, Munck, Ellabaan, & Sommer, 2018).

Not only is HGT responsible for speciation and subspeciation in bacteria; it also constitutes an important mechanism in eukaryote organisms. There are sufficient information related to the role of conjugation processes in the transference of genetic information from bacteria to eukaryote cells. Such eukaryote cells include yeasts, filamentous fungi, and plant cells (De la Cruz & Davies, 2000; Rankin et al., 2011; Suzuki et al., 2015). For example, the mechanism through which *Agrobacterium tumefaciens* transfers genes from the bacterium to plant cells is well known: it occurs through the action of the T-DNA segment present in the Ti plasmid (Suzuki et al., 2015). Stable natural transgenic plants of sweet potato containing *Agrobacterium* T-DNA sequences with their foreign genes expressed at detectable levels in different tissues were reported by Kyndt et al. (2015). Likewise, the work of Diao, Freeling, & Lisch (2006), provides evidence of HGT through the transposons of superior plants. In this regard, some bacteria, retroviruses, and DNA viruses constantly integrate different kinds of genetic elements into the chromosomes of animal and plant cells through mechanisms such as conjugation and transformation (De la Cruz & Davies, 2000, Oliver et al., 2006; Klasson, Kambris, Cook, Walter, & Sinkins, 2009; Nikoh & Nakabachi, 2009; Suzuki et al., 2015). Moreover, the mechanism by which eukaryotes acquire genes from distantly related organisms remains obscure (Suzuki et al., 2015).

Although, in general terms, it has been accepted that some kind of "chemical stimuli" (very likely a phytohormone) from the insect is involved in the induction and morphogenesis of galls (McCalla et al., 1961; Miles, 1968; Rohfritsch & Shorthouse, 1982; Hori, 1992; Leitch, 1994; Ananthakrishnan, 1998; Raman, 2011; Yamaguchi et al., 2012; Connor et al., 2012; Erb, Meldau & Howe, 2012; Giron et al., 2013, Tooker & Helms, 2014; Bailey et al., 2015; Oates et al., 2016, Giron et al., 2016), up to now it has not been possible to determine with certainty whether insects could synthesize phytohormones. However, Yamaguchi et al. (2012) found abnormally high concentrations of a type of zeatine in the glands of the "sawfly" *Pontania sp.* (Hymenoptera, suborder Symphyta) which, according to these researchers' criteria could be strong evidence that this insect can synthesize cytokinins as well as IAA. Likewise, Shih, Lin, Huang, Sung, and Yang (2018) found evidence that gall induction could be related to the secretion of phytohormones like cytokinin and auxin, as well as Brassinosteroids (steroids hormones), from the inductor insect. In a similar direction, Bartlett & Connor (2014) showed evidence consistent with the hypothesis that exogenous cytokinins, in combination with IAA from the gall-inducing insect, lead to gall induction. Additionally, Brütting et al. (2018) demonstrated, using ^{15}N-isotope labeling, the transference of the cytokinin N^{6}-isopentenyladenine (IP) from the free-living herbivore and non-galling insect *Tupiocoris notatus* to *Nicotiana attenuata* plants via their oral secretions.

On the other hand, the possibility of a molecular induction mechanism in insect-induced plant galls that involves the transference of genetic elements has neither been considered nor explored extensively. Cornell (1983) suggested the possibility that the gall-inducing insect could insert some genetic elements, mutualistic viroid, or virus into the plant genome, which would regulate and control the process of gall formation. However, this author did not offer any evidence that could support this statement. The molecular basis of the induction of plant galls by insects is still unknown (Stone & Schönrogge, 2003; Raman, 2011; Oates et al., 2016; Bailey et al., 2015; Giron et al., 2016). Moreover, the physiological nature of the stimuli given by the inducing insect and the influence of its own genomic constitution, as well as the reaction generated by the plant, are questions that remain completely open.

Stone & Schönrogge (2003) mentioned three great problems or challenges in identifying the molecules responsible for the process of gall formation. First is the difficulty of establishing an appropriate assay for the plant tissues involved in the process of induction. Second, the possible inducing molecules used by insects could be chemically similar to those normally present in the plant. Third, since it is expected that the signals coming from the insect generate a cascade of responses in the plant, it would be very difficult to separate the first morphogenetic impact originated by the inductor from the secondary responses generated by the plant.

In the particular case of gall-inducing insects belonging to Cecidomyiidae, it has been reported that either the egg or the ovipositing female could generate the initial stimulus and that the larva, by secreting substances that promote the growth of plant tissue under its action, could cause the formation of the gall (Hori, 1992).

Regarding the family Cynipidae (Hymenoptera), different studies have associated both auxins and cytokinins with the processes of gall induction and morphogenesis. Moreover, the morphogenesis and induction of these structures have been correlated with the activity of oviposition of the female, secretions of the insect egg, and the activity and secretion of chemical substances from the larva (Miles, 1968; Hori, 1992, Shorthouse & Rohfritsch, 1992; Raman, 2011). As with the galls formed by insects from the family Cecidomyiidae, the mechanism of morphogenesis of galls formed by cynipids cannot be explained simply by the action of plant phytohormones. Nevertheless, Boysen-Jensen (1952) and Miles (1968) support the hypothesis of chemical induction, arguing that the larva moves instinctively and secretes regulatory substances in the proper locations of the “attacked” tissue at specific times, thereby generating a suitable environment that favors the development of the gall.

“Omics” is an informal term that refers to fields of study in biology ending in -omics, such as genomics, proteomics, or metabolomics, among others. Emerging work conducted with new omics technologies is expanding our understanding of some relevant aspects relating to plant gall induction and morphogenesis. In a recent paper regarding the identification of the galling effector repertoires of the Hessian fly, it was shown that around 7 % of its genome encodes putative effector proteins, which include the secreted salivary gland protein (SSGP)-71, a known member of an arthropod protein family (Zhao et al., 2015). Moreover, these authors showed that although SSGP-71 lacks sequence homology with other proteins, its structure resembles both ubiquitin E3 ligases from plants and E3-ligase-mimicking effectors from plant pathogenic bacteria. Protein analyses indicate that the mature SSGP-71 protein contains a cyclin-like F box domain near the N-terminus and a series of leucine-rich repeats (LRRs). F box domains are frequently associated with LRRs, and both domains mediate protein-protein interactions, according to Ho, Tsai, and Chien (2006). These types of proteins are associated with the transfer of ubiquitin to target proteins destined for degradation in the proteasome. In addition, they play essential roles in phytohormonal signaling, plant development, and plant immunity. Zhao et al. (2015) also proposed that SSGP-71 proteins are a novel class of F-box-LRR mimics that enable the insect to hijack the plant proteasome in order to directly produce nutritive tissue and additionally defeat basal plant immunity. These authors further propose that their results prove that these effectors are the agents responsible for arthropod-induced plant gall formation. Likewise, Shih et al. (2018) demonstrated, by using transcriptome analysis, the modification of normal plant tissue to form galls. Moreover, they indicated that the manipulation of genes related to gall formation might be induced by auxin, cytokinin, and even steroid hormones (Brassinosteroids) secreted by gallers of Hemiptera, Lepidoptera, and Diptera. Similarly, other transcriptomic and genomic studies provide evidence leading to altered gene expression in galled plant tissues. These altered genes and effector proteins could be involved in several aspects of gall insect biology, including feeding, metabolic alterations, suppression of defense responses, and developmental manipulation of the host plant tissue (Rawat, Neeraja, Nair, & Bentur, 2012; Hearn, 2013). Even more interesting, Hearn (2013) also determined that genes expressed in gall wasp genomes encode plant-cell-wall-degrading enzymes that could originate from plant pathogenic bacteria. Pawłowski, Staszak, Karolewski, and Giertych (2017), using a proteomic approach to compare the galls induced by three oak gall species, *Cynips quercusfolii*, *Cynips longiventris*, and *Neuroterus quercus-baccarum*, with non-gall plant tissue in the host plant *Quercus robur*, described several proteins that could potentially be related to plant gall formation. On the other hand, for non-insect galls, a transcriptomic approach by Olszak et al. (2018) showed evidence that galls induced by *Plasmodiophora brassicae* in *Arabidopsis* reprogram critical steps of the host cell cycle. That distortion leads to initial cell hyperplasia, which increases the number of cells, followed by overgrowth of cells colonized by the pathogen. The authors showed that *P. brassicae* infection stimulates the formation of the E2Fa/RBR1 complex and upregulation of MYB3R1, MYB3R4, and A- and B-type cyclin expression. Those cell cycle factors were previously described as important regulators of the G2-M cell cycle checkpoint.

An interesting survey in nematode galls (*Meloidogyne incognita*), using high throughput sequencing for small non-coding RNAs, identified siRNA clusters that were differentially expressed in infected roots of *Arabidopsis thaliana*. Those siRNAs were overrepresented in infected tissue, with a size 23 - 24 nt, corresponding to heterochromatic siRNAs (hc-siRNAs), which are known to regulate the expression of transposons and probably genes at the transcriptional level, by an RNA-directed DNA methylation (RdDM) pathway that induces the silencing of transposable elements (Medina et al., 2018).

Insect-induced plant galls and phytochemistry

An interesting aspect of some plant galls is the particular or even radical phytochemistry between these structures and normal plant tissues. Research conducted on galls of different species of plants have revealed that the composition and concentration of chemical substances in these structures can differ from those of other plant tissues and organs (Tooker & de Moraes, 2008; Saltzmann et al., 2008; Giron & Huguet, 2011; Huang et al., 2015; Oates et al., 2015; Hall, Carrol, & Kitching, 2017; Kot, Jakubczyk, Karaś, & Złotek, 2017). Tissues near the outside of the gall frequently accumulate high levels of tannins and other chemical compounds related to the process of defense of the gall and, in consequence, of the insect (Ananthakrishnan, 1998; Li et al., 2017; Chen et al., 2018; Nogueira et al., 2018). A study by Vereecke et al. (1997) revealed that the chemical composition of ethanol and aqueous extracts of galls produced in the leaves of *Nicotiana tabacum* differs drastically from that of non-infected plant tissue extracts. It has been reported that the concentrations of some carbohydrates such as hemicellulose, xylose, and arabinose increase during gall development in the tree *Zelcowa* (Yeo, Chae, So, Lee, & Sakurai, 1997). Other authors have also reported differences in the concentrations of certain secondary compounds as well as certain types of phytohormones in plant gall tissue (Kraus & Spiteller, 1997; Pinkwart, Diettrich, & Luckner, 1998). Kot et al. (2017), Li et al. (2017) and Hall et al. (2017) demonstrated that galls induced by cynipid species and the wasp *Leptocybe invasa* (Hymenoptera: Eulophidae), respectively, contain high levels of phenolic compounds compared with control tissues. Moreover, increased production of waxes in the gall induced by the insect *Baccharopelma* spp. (Hemiptera: Psyllidae) in leaves of *Baccharis spicata* (Lam) Baill has been related to a protective function against desiccation by Agudelo et al. (2018).

Several authors have reported high concentrations of certain nutritive substances in gall tissues; some of those substances include sugars, proteins, phosphates, lipids, and nitrogen compounds (Tooker et al., 2008; Giron & Huguet, 2011; Huang et al., 2015; Li et al., 2017). In contrast, some researchers have described that galls can present low levels of certain chemical compounds related to the processes of plant defense, such as some phenolic compounds (Price et al., 1986; Agudelo et al., 2018).

Due to the fact that many galls present high quantities of certain nutrients and low levels of other chemical substances that are damaging to insects, a hypothesis has been proposed related to the galler being able to manipulate the development of its host plant by generating a tissue with a higher nutrient value (nutritional hypothesis). Nevertheless, several studies conducted with the goal of proving this hypothesis revealed that the concentrations of certain chemical compounds considered as defensive in plants are higher in the gall tissues, which is contrary to the above-mentioned hypothesis and suggests the need for a reconsideration of the same (Nyman & Julkunen, 2000).

Taking into consideration studies such as the those carried out by Nyman & Julkunen (2000), Tooker & De Moraes (2008), Tooker et al. (2008), Giron & Huguet (2011), Huang et al. (2015), Oates et al. (2015), Li et al. (2017), Kot et al. (2017), Chen et al. (2018), and Agudelo et al. (2018), comparing the chemical composition of galls with that of normal plant tissue, the conclusion would be that gall-inducing insects could control some the chemical properties of these structures.

CONCLUSIONS AND PERSPECTIVES

Although the chemical induction hypothesis has been accepted with some discretion and questioning as the general mechanism of plant gall induction, there are, so far, no related studies on a putative induction mechanism involving exogenous genetic elements in the process of insect gall formation. Moreover, little has been speculated in relation to this topic. A possibility exists that the control of induction and morphogenesis of insect galls could be under strict genetic control, possibly mediated by the insertion of mobile genetic elements into the genome of plant gall cells. Likewise, that process could be mediated by means of an endosymbiotic bacteria from the insect. Thus, due to the demonstrated ability of the inductor to manipulate the process of morphogenesis in insect galls, the galling insect should be able to control the regulation and expression of those exogenous insertion sequences at different levels. Consequently, under this hypothetical scenario, the insertion sequences would function as mediators of the molecular interaction between animal and plant systems. Genes contained in these possible insertion sequences could be those related to the control of the host cellular machinery and analogous phytohormones genes to those present in the host plant, among others. Virtually no work has been conducted in this direction, probably because of insufficient knowledge and the complexity of insect-plant-gall system relationships.

On the other hand, if a relatively simpler plant gall induced by *Agrobacterium* species involves a complex interaction between the inductor organism and its host plant, which is mediated by the insertion of genetic elements into the genome of the host cells, why is it not then assumed that a similar or even more complex mechanism exists for the induction of more complex plant galls, which could also be induced by the delivery of genetic elements from the cecidogenic organism? We could also rephrase the question as follows: is cellular self-proliferation an essential requirement or condition for the genetic transformation of plant cells, as occurs in the case of "crown galls" induced by *Agrobacterium*?

It is essential to conduct studies to understand, at the molecular level, the mechanism of induction and morphogenesis of plant galls induced by insects, exploring the presence of any possible symbiotic organism and some kind of external genetic element to the plant gall cells, associated with any of the symbionts. With this goal in mind, an appropriate gall induction model system should be chosen. The choice of an insect–plant gall system to be used as an experimental model should take into account gall diversity and morphologic complexity in order to include, in the same plant species, prosoplasmic and kataplasmic gall types. The next step would be to compare the similarities and differences at the molecular level, among different kinds of galls and how these could affect the extraordinary morphology and diversity observed in nature. Due to the diversity of shapes, colors, and complex structures displayed by insect galls, these systems could constitute ideal models to study how form and structure are determined at the molecular level in biological systems, more specifically, taking as a parameter plant morphology.

Due to their physiological and biochemical particularities, the identification of chemical substances or even specific genes in plant galls that could be of interest or have practical applications, according to the genetic transformation hypothesis postulated in this article, could result in these tissues becoming real germplasm sources, which may have a great impact on conservation policies and offer a promising background for the development of applied biotechnologies. Considering all the above information, it is clear that plant galls represent an important germplasm sink and a promissory gene bank that should be explored, used, and preserved as an authentic treasure of our biodiversity.

RESUMEN

El mecanismo de inducción de agallas de plantas por insectos: revelando claves, hechos y consecuencias en una interacción compleja entre reinos. Las agallas se definen como modificaciones del diseño y desarrollo normal de las plantas debido a una reacción específica a la presencia y actividad de un organismo foráneo. Aunque diferentes grupos de organismos tienen la habilidad de inducir agallas en plantas, las agallas inducidas por insectos son las más elaboradas y diversas. Algunas hipótesis han sido propuestas para explicar el mecanismo de inducción de las agallas de insectos. La hipótesis más general sugiere que la formación de las agallas es disparada por la acción de sustancias químicas secretadas por el insecto inductor, incluyendo reguladores de plantas como auxinas, citoquininas, ácido-3-indolacético (AIA) o bien otros tipos de compuestos. No obstante, el modo de acción de estas sustancias químicas y el mecanismo general por medio del cual el insecto podría controlar y manipular el desarrollo y fisiología de la planta es aún desconocido. Más aún, como resultado de la complejidad del proceso de inducción y desarrollo de las agallas de plantas inducidas por insectos, la hipótesis química es una explicación insuficiente e incompleta en relación con el mecanismo de inducción y morfogénesis de estas estructuras. Previas y nuevas evidencias relacionadas con el sistema de agallas de insectos, con énfasis en el proceso de inducción, fueron analizadas desde un punto de vista integral del autor para proponer en este artículo una perspectiva diferente sobre la inducción de este tipo de estructuras. Debido a la extraordinaria diversidad de formas, colores y estructuras complejas presentes en las agallas de insectos, las mismas constituyen modelos útiles para estudiar cómo la forma y la estructura son determinadas a nivel molecular en los sistemas vegetales. Además, las agallas de plantas son un importante origen de material para el estudio y exploración de nuevas sustancias químicas de interés humano, debido a las características fisiológicas y adaptativas que presentan. Considerando el control fino del proceso de morfogénesis, regulación bioquímica y complejidad estructural de las agallas de insectos, se propone que un mecanismo de inducción mediado por la inserción de elementos genéticos exógenos dentro del genoma de las células de la planta que forman la agalla podría estar involucrado en la formación de este tipo de estructuras, vía una bacteria endosimbiótica.

Palabras clave: agallas de insectos, insecto inductor, mecanismo de inducción, morfogénesis vegetal, efectores.

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Capítulo 2

Inventario de morfotipos de agallas como material base para el análisis experimental de la presencia del posible marcador específico para agallas.

Descripción

Para la obtención de muestras de plantas y agallas y el establecimiento del herbario, se realizó un muestreo preliminar de las agallas presentes en dos ecosistemas diferentes, bosque tropical seco y bosque tropical húmedo. Los muestreos se realizaron en el Área de Conservación Guanacaste (ACG), provincia de Guanacaste y en el Parque Nacional Arenal (La Fortuna, San Carlos, Alajuela, Costa Rica). Estas dos regiones forman parte del Área de Conservación Arenal Tempisque, por lo que los permisos para realizar la investigación y colecta de los materiales vegetales se tramitaron en las oficinas del MINAE pertenecientes a esta región. Las agallas colectadas se clasificaron de acuerdo al sitio de procedencia, grupo taxonómico más cercano de la planta hospedera y tipo morfológico básico de la agalla, esto es kataplásmica o prosoplásmica. Además, se registró la especie del insecto inductor y otros organismos inquilinos cuando fue posible.

Para el establecimiento del herbario, las muestras de plantas con agallas se colocaron en prensas de madera con varias capas de papel periódico y cartón. Las muestras prensadas posteriormente se secaron en un secador diseñado con este propósito y luego las muestras ya secas se colocaron en cartulina de papel especial para herbario. Una vez preparadas las muestras se depositó un registro en el herbario del museo Nacional y las restantes muestras se colocaron en un herbario establecido en las instalaciones del ITCR en la Sede San Carlos (Herbario de Cecidias, Escuela de Ciencias Naturales y Exactas, Sede San Carlos del ITCR).

El estudio realizado como parte del segundo artículo publicado, mismo que se enmarca dentro del objetivo dos de la tesis, no solo permitió realizar por primera vez un registro ordenado y exhaustivo de agallas en una región geográfica especifica de Costa Rica (Área de Conservación Guanacaste, ACG), lo anterior bajo una propuesta de nomenclatura descriptiva nueva para este tipo de estructuras. También el estudio incluye descripciones morfológicas detalladas de las agallas registradas, junto con la distribución geográfica de las respectivas especies de plantas hospederas. Estos registros y el inventario en físico establecido permitieron contar con las muestras necesarias debidamente identificadas y caracterizadas para los posteriores estudios con marcadores de ADN.

El trabajo realizado es un primer intento para establecer un registro e inventario detallado de los morfotipos de agallas de plantas y sus organismos asociados en el Área de Conservación Guanacaste, utilizando para ello un enfoque integrado que consiste en una base de datos biológica contemplada dentro de inventarios físicos de colecciones de materiales de plantas secas y muestras húmedas preservadas en etanol al 70%, así como de los posibles insectos inductores y otros asociados preservados en viales con etanol al 70% para su identificación taxonómica. Estas colecciones de materiales biológicos se organizaron y preservaron bajo condiciones de ambiente controlado dentro de un herbario especializado, el cual debido a sus condiciones únicas se le dio el nombre de cecidiarium.

Los inventarios existentes a la fecha sobre la ocurrencia y diversidad de morfotipos de agallas en las regiones tropicales son escasos e incompletos. En el caso de la Región Neotropical, la mayoría de los registros de morfotipos de agallas vegetales provienen de registros realizados en Brasil por parte de una comunidad de investigador activos en este tema. Particularmente para Costa Rica, no existen registros previos sistemáticos de agallas de plantas por regiones o ecosistemas específicos.

Tomando como referencia la literatura disponible, 120 de los morfotipos descritos y registrados en este estudio son registros nuevos no solo para el ACG y para Costa Rica, sino también para el mundo. Aunque las muestras fueron recolectadas en época de lluvias, cuando las plantas tienen mayor cantidad de biomasa verde, se estima que las agallas aquí registradas representan una pequeña porción del total de agallas de plantas presentes en el Área de Conservación Guanacaste. Esto se debe a la mayor diversidad vegetal y características topográficas del AGC, así como a las dificultades para buscar y obtener muestras del dosel, particularmente en árboles altos como los del Bosque Viejo.

El supuesto extendido aunque cada vez menos respaldado, de que la diversidad de agallas en las regiones tropicales es menor que en las regiones templadas xéricas no es sostenible al tomar en consideración la biodiversidad existente en los ecosistemas tropicales y los resultados de los últimos estudios sobre la diversidad de agallas en las regiones tropicales. Al respecto, la segunda publicación realizada aporta también evidencias que sugieren que la diversidad de agallas estaría directamente correlacionada con la riqueza de especies de plantas hospederas en las áreas bajo estudio.

A pesar del creciente número de nuevos morfotipos de agallas reportados continuamente, aún persisten problemas relacionados con una clasificación estandarizada para estas estructuras. Los problemas incluyen una mala caracterización del morfotipo de la agalla, la falta de fotografías referenciadas o imágenes de mala calidad y la inexistencia de una nomenclatura estandarizada. El segundo artículo publicado propone un sistema de codificación estandarizado para la clasificación de los morfotipos de agallas de plantas con el objetivo de evitar confusiones en el registro de estas estructuras.

Por primera vez, un mapa detallado con coordenadas geográficas, distribución altitudinal, cobertura forestal, información hídrica y otros datos asociados a las agallas colectadas, fueron incluidos como parte de los resultados de la segunda publicación realizada. Este tipo de mapa georreferenciado es una herramienta útil que permite comprender el contexto ecológico de la aparición de agallas ambientes naturales, permitiendo así identificar y contrastar la distribución de las plantas hospedantes con la distribución y el hábitat de los insectos inductores.

Por lo tanto, el artículo publicado permitió contar con materiales vegetales y sus respectivas agallas debidamente registrados, organizados, almacenados e identificados a nivel de especie para los estudios posteriores con marcadores moleculares de ADN. A partir de la presencia de un potencial marcador de ADN (gen E2 similar a la ubiquitina) en diferentes morfotipos de agallas (o con una identidad similar) en distintas especies de plantas hospederas, e incluso detectado en plásmidos silvestres purificados a partir de especies de bacterias endófitas al tejido de la agalla de yuca, sumado a los hallazgos descritos en el tercer artículo derivado del trabajo de tesis, se planteó la posibilidad que la transformación genética de células vegetales mediada por bacterias representa un mecanismo de inducción de agallas de insectos ampliamente extendido en la naturaleza.

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Article

Plant galls recorded from Guanacaste Conservation Area-Costa Rica as an integrated concept of a biological database

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Abstract: Galling insects are specialist herbivorous that have the ability of manipulating plant tissue to form complex biological structures called galls. Even though different organisms have the ability to induce galls in plants, insect galls have the highest degree of structural complexity. The main goal of this study was to obtain a preliminary systematic record of plant gall morphotypes from the Guanacaste Conservation Area in Costa Rica and integrate the information into a biological database. Plant gall morphotypes were recorded, characterized and deposited into a specialized herbarium established as a reference for the inventory. Moreover, organisms associated with gall morphotypes were included in the inventory when it was possible to obtain and identify them. Galls were collected in the rainy season over a period of three years. In total, we recorded forty-four families, seventy genera, and eighty-seven host plant species. One hundred thirty-one morphotypes of plant galls were identified in the Guanacaste Conservation Area. The family with the highest number of gall morphotypes was Fabaceae (8.4%). Leaves were the organ with the largest number of galls (71%), followed by stems (17.6%), and apical buds (6.9%). The predominant gall shape was globular (25.2%), followed by discoid (18.3%). Fifty-nine percent of the galls had a glabrous texture, which was most common on leaves, with 77%. One hundred twenty of our field records (91.6%) of plant galls were new morphotypes not only for Costa Rica but also the world. As a consequence of this research and considering the prospect of future increases in new gall records (and associated organisms), we proposed having the biological entities resulting from the inventory placed in a cecidiarium. This repository represents a standardized and comprehensive way to manage the data and biological materials associated with the plant galls. We also suggest a nomenclature for standardizing gall morphotype registries and identifications. This work is the first and most detailed inventory of plant galls carried out thus far in the Guanacaste Conservation Area.
Keywords: *Plant gall, morphotype, tropical dry forest, biological database, cecidiarium.*

Registro de galhas em plantas da Área de Conservação Guanacaste, Costa Rica, como conceito integrado de um banco de dados biológico

Resumo: Os insetos galhadores são herbívoros especializados, que têm a habilidade de manipular os tecidos vegetais, formando uma complexa estrutura biológica. Diferentes organismos têm a capacidade de induzir galhas, porém as de insetos têm maior grau de complexidade estrutural. O principal objetivo desse estudo foi realizar um levantamento sistemático preliminar das galhas de insetos na Área de Conservação Guanacaste, na Costa Rica, e inserir as informações em uma base de dados biológicos. Os morfotipos de galhas foram registrados, caraterizados e depositados em um herbário estabelecido como base de referência deste inventário. Além disso, quando foi possível obter e identificar os organismos associados a cada morfotipo de galha, eles foram incluídos no inventário. As amostras de galhas foram coletadas na estação chuvosa, durante três anos. No total, foram registradas 44 famílias, 70 gêneros e 87 espécies de plantas hospedeiras. Cento e trinta e um morfotipos de galhas foram identificados na Área de Conservação de Guanacaste. A família com o maior número de morfotipos de galhas foi Fabaceae (8.4%). Os órgãos vegetais com o maior número de galhas foram as folhas (71,0%), seguidas dos caules (17,6%), e das gemas apicais com 6,9%. A forma predominante das galhas foi globoide (25,2%), seguida da lenticular (18,3%) e 59% das galhas apresentaram textura glabra, observada mais frequentemente folhas (77%). Cento e vinte morfotipos de galhas (91.6%) representaram novos registros não só na Costa Rica como também no mundo. Como consequência desta pesquisa e considerando as perspectivas de aumentos futuros de novos registros de galhas (e organismos associados), nós propomos que as entidades biológicas resultantes deste inventário sejam depositadas em um cecidiário. Este repositório representa uma maneira padronizada e abrangente de gerenciar e integrar os dados e os materiais biológicos associados às galhas das plantas. Também sugerimos uma terminologia para padronizar os registros e identificações dos morfotipos de galhas. Este inventário de galhas em plantas é o primeiro e o mais detalhado já realizado na Área de Conservação Guanacaste.

Palavras-chave: *morfotipo, floresta seca tropical, banco de dados biológicos, cecidiarium.*

Introduction

Plant galls are atypical plant tissue structures induced by the action and activity of a foreign organism. Although several organisms have the ability to induce galls in plants, the most diverse and complex galls are formed by insects (Shorthouse & Rohfritsch 1992). Gall-inducing insects are highly specialized sedentary herbivores, which feed specifically on certain specialized cells that are found within the plant structure whose formation they have induced (Shorthouse & Rohfritsch 1992, Tooker et al. 2008, Raman 2011).

Gall-inducing insects generally have specific host plants (Cuevas-Reyes et al. 2014). Gall biology is closely associated with the respective inducing insect, in such a way that galls induced by a particular insect species are basically always the same shape and can differ distinctly from others induced by related species. On the other hand, there are some gall morphotypes induced by different species of insects that present similar shapes (Raman 2011). Distinctive characteristics of each type of gall are probably due to slight variations in the way that each insect species stimulates the development of the gall in the corresponding plant tissue (Shorthouse & Rohfritsch 1992). Li et al. (2017) suggested that gall development is influenced by the gall-inducing insects as well as by the tissue developmental stage and plant genotypes.

The use of gall morphotypes is a commonly used and reliable parameter because evidence indicates that each gall is unique to a particular gall-inducing insect (Stone and Schönrogge 2003), and each galling species is specific to a particular host plant (Abrahamson et al. 1998). According to Isaias et al. (2013), a gall morphotype could be defined as a characteristic phenotypic variation in a neo-formed plant organ, which is produced by the species-specific interaction between the inducing organism and a specific host plant. Due to the fact that each gall inducer is able to alter the morphogenesis in a predetermined organ (Rohfritsch 1992), and because specificity of galler taxa is strongly linked to an appropriate oviposition site in the host plant (Eigenbrode & Jetter 2002), gall morphotype is widely used as a way to refer to types of plant galls. The vast majority of gall-inducing arthropods are restricted to a single host plant species, thus corroborating the idea that the gall morphotype can be used as reliable substitute of gall-inducing species. In addition, gall polymorphism, which could lead to failures in the identification of galls, appears to be a rather rare phenomenon (Carneiro et al. 2009).

Thousands of gall-inducing insects have been identified around the world, for the most part belonging to the orders Thysanoptera, Hemiptera, Lepidoptera, Coleoptera, Diptera, and Hymenoptera (Rohfritsch & Shorthouse 1982, Leitch 1994, Williams 1994, Hanson & Gómez-Laurito 2005, Espírito-Santo & Fernandes 2007, Ansaloni et al. 2018). Gall-inducing insects induce the formation of galls in leaves, buds, flowers, stems, roots and other organs of the plant, depending on the gall-inducing species. A growing number of studies on the diversity and abundance of plant gall morphotypes, and their respective inducing insects, allowed us to build upon the existing information. With a certain regularity reports are made of records and descriptions of new species of gall-inducing insects (Ronquist & Liljeblad 2001, Dalbem & Mendonça 2006, Güçlü et al. 2008, Coelho et al. 2009, Maia & Oliveira 2010, Maia et al. 2010a, Maia et al. 2010b, Medianero et al. 2010, Sano et al. 2011, Santos et al. 2011a, Maia 2014, De Araújo 2017, Bergamini et al. 2017, Coelho et al., 2017, Martins et al. 2018, Ley-López et al. 2019).

Inventories of biological specimens are an important tool to know and preserve biological diversity. To date, for tropical regions, detailed inventories on the occurrence and diversity of gall morphotypes are scarce and incomplete. In the Neotropical Region, the majority of plant gall morphotype records are from Brazil (Urso-Guimarães & Scarelli-Santos 2006, Saito & Urso-Guimarães 2012, Isaias et al. 2013, Carvalho-Fernandes et al. 2016, Urso-Guimarães et al. 2017, Araújo 2017, Liu et al. 2018, Flor et al. 2018, Ascendino & Maia 2018, Vieira et al. 2018, Silva et al. 2018, Araújo et al. 2019, Ribeiro et al. 2019, among others) and some of them were conducted in different types of tropical dry forests or semi-arid ecosystems (Coelho et al. 2009, Santos et al. 2011b, Maia & Souza 2013, Costa & Araújo 2019). Likewise, other reports have been done for the Mexican dry forest (Cuevas-Reyes et al. 2004, Cuevas-Reyes et al. 2014). For the Costa Rican flora there are no systematic and detailed records of plant galls for specific regions or ecosystems. Studies are restricted to sporadic or general reports (Gómez & Kisimova-Horovitz 1997, Hanson & Gómez-Laurito 2005, Retana-Salazar & Nishida 2007, Retana-Salazar & Sánchez-Chacón 2009, Hanson et al. 2014). Hanson et al. (2014) reported 1,100 morphospecies of gall-inducers, distributed in six orders of insects, from Costa Rica. Nevertheless, this survey was restricted to the identification of the insect gall inducers and their associated parasitoids, using plant gall morphotypes as indirect indicators of insect morphospecies, and therefore lacking detailed descriptions of the gall morphotypes found, usually without associated images. The work carried out by Ley-López et al. (2019) in the area of Sarapiquí, in northern Costa Rica provides primarily a checklist of the native vascular plants found to harbor galls.

The Guanacaste Conservation Area (Área de Conservación Guanacaste, ACG for its acronym in Spanish) is located in the northwest region of Costa Rica, in the life zone categorized as tropical dry forest (Holdridge & Tosi 1967). ACG is a conglomeration of several national parks and other natural areas (sectors), among which are Santa Rosa National Park, Guanacaste National Park, Rincón de la Vieja National Park, Murciélago Sector, among others (SINAC 2014). ACG protects the most emblematic tropical dry forest in Central America (Quirós-Arias 2017). This Costa Rican national park was created to favor the restoration process of old pasture areas into the primary and secondary forest through natural succession. Since 1999 it it has been proclaimed a World Heritage Site by UNESCO (Quirós-Arias 2017).

The present work is a first attempt to establish a detailed record and inventory of plant gall morphotypes and their associated organisms in the Guanacaste Conservation Area utilizing an integrated approach consisting of a biological database with a specialized herbarium (cecidiarium).

Materials and Methods

1. *Study area*

Fieldwork and sample collection were carried out predominantly in the Pacific area of Guanacaste Conservation Area (Área de Conservación Guanacaste, ACG), Guanacaste province, Costa Rica, located in the northwest region of the country (10.839366, -85.618176, administrative area) (Figure 1A). Plants and gall morphotypes were collected in different ecosystems in ACG. The Guanacaste Conservation Area is characterized by having a long dry season, generally from late November to mid-May, with a rainy season from mid-May to November (Borchert 1994, Lobo-Segura 2019). The sampled sites are located in the tropical dry forest ecosystem, as well as areas in the premontane wet transition, according to the Holdridge & Tosi (1967) classification. In the last decades, ACG has been affected by long periods of drought, being one of the areas most affected by the *El Niño* phenomenon and climate change projections (Quirós-Arias 2017).

The Guanacaste Conservation Area includes 296,526 acres on land and 106,255 acres of marine habitats. Approximately 65% of all Costa Rican species are present in this area, representing 2.6% of the world's biodiversity (SINAC 2014).

2. *Field and laboratory work for gall morphotypes inventory and sampling associated insects*

Galls were sampled by randomly walking the trails and examining the vegetation along the pathways by two people searching simultaneously on both sides of the road. All plants and their aerial organs were observed to a maximum height of approximately 3 meters. Each gall and host plants found were collected and then packed in plastic bags for further processing. The photographic record of each gall morphotype, when possible, was made directly in the field. Randomized trials that were perpendicular to existing trails were used occasionally for sampling galls, with distances varying from 50 to 400 meters, but without a pre-established experimental design.

Plant galls were collected and processed from May 2010 to December 2012 in different types of vegetation that comprised the ACG biome. Field sampling was carried out every 3 or 4 months per collection season, for a period of three days each, covering approximately 6-8 hours of field work per day. Sampling was conducted especially during the rainy season, when there is a greater probability of finding leaves on the plants, due to the marked dry season and a predominantly deciduous forest. Gall samples were collected mainly at lower-medium altitudes of 0 to 1100 m above sea level (m.a.s.l.): Santa Rosa Sector (La Casona area), Murciélago Sector, Santa Elena Sector, Pocosol Sector (Góngora Area, road to Cacao Volcano), and Rincón de la Vieja National Park. Moreover, gall samples and their corresponding host plants were collected mostly from deciduous plant species typical of non-riparian environments as well as near sites with original-natural forest with evergreen species. More typical riparian sites or similar environments followed comparable field zonification criteria of Frankie et al. (1974) and Borchert (1994). Two *Bosque Viejo* (Old Forest) patches were sampled: close to La Casona (near the administrative area of Santa Rosa National Park) and from the route to Playa Naranjo Sector. The *Bosque Viejo* patches are considered remnants of the original forest with little human disturbance and is characterized by an abundance of evergreen plant species.

Specimens of each plant harboring galls were prepared for reference as herbarium vouchers to be identified later. Plant gall specimens with floral or fruit parts for host identification were dried when possible, otherwise the insects obtained, and relevant gall samples were preserved in 70% ethanol. Since gall traits changed in dried samples, especially their morphology and color, a printed photographic registry of each specimen is included in the inventory. All samples were deposited at the cecidiarium (specialized herbarium) established at the facilities of Technological Institute of Costa Rica (Instituto Tecnológico de Costa Rica, Campus Tecnológico Local San Carlos, Escuela de Ciencias Naturales y Exactas). Also, some selected gall samples were preserved in FAA (formalin, acetic acid, alcohol) for later morphological studies. Collected galls were classified according to place of origin, family, genus, and species of the host plant, and according to the basic morphological type. Sampling site description was registered for all samples, but when there were more than two accessions of plant materials in the inventory, only one description of the sampling site for each morphotype was included here. Collected samples were geo-referenced by Global Positioning System (GPS). Each gall morphotype location was geo-referenced using the Decimal Degrees (DD) format. A map to show the reference morphotypes collected per site at ACG was made with QGIS version 3.10.5 (QGIS.org 2020) Development Team and using the following layers from Atlas Digital CR 2008-2014: Conservation Areas (AConservacionSINAC2014; SINAC 1998), Wild Protected Areas (Areassilvestresprotegidas2014crtm05; SINAC 1999), elevation level (Relieve 2008crtm05; CATIE N.D.), Rivers (Rios150000crtm05; Soto-Montoya 2007), and land cover (Cober2005crtm05; Earth Observation System Laboratory et al. 2006). To facilitate visualization of geographical location, some neighboring galls collected from nearby areas (i.e., spaced between 5 m to 400 m linear meters) were considered as a cluster, depending on the geographic characteristics of the site; for these clusters the same geographic position was assigned.

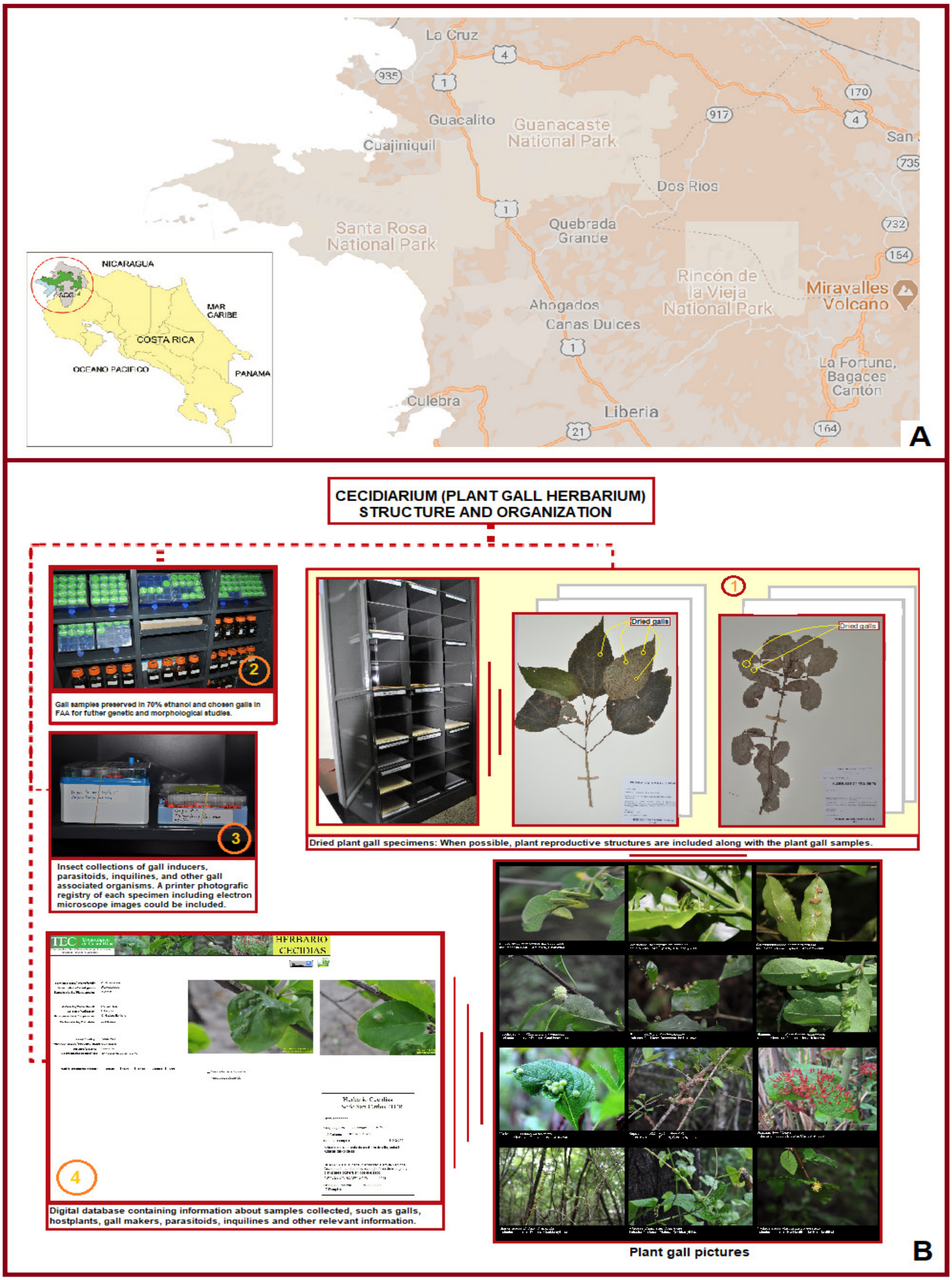

Figure 1. 1A) Guanacaste Conservation Area (Área de Conservación Guanacaste, ACG) map showing the Santa Rosa National Park, Guanacaste National Park, and Rincón de la Vieja National Park sectors. Taken and modified from Área de Conservación Guanacaste- SINAC (https://www.acguanacaste.ac.cr/index.php), and Google Earth®. 1B) Plant Gall Herbarium (cecidiarium) organization. 1) Dried plant gall specimens, 2) Plant gall samples preserved in 70% ethanol and FAA, 3) Insect collection of gall inducers, parasitoids, and inquilines preserved in 70% ethanol vials, 4) Digital database containing information about gall morphotypes, host plants, gall makers, associated organisms, sampling sites, etc.

In addition to the list of host plants harboring galls, the morphological characterization of all gall morphotypes found is provided. Gall morphotypes were used to register plant galls, as well as a reference for the galling insect collection, assuming that each morphotype is unique and each galling species is specific to its host plant (Espírito-Santo & Fernandez 2007, Abrahamson et al. 1998, Carneiro et al. 2009). The morphological characteristics used in gall differentiation were basic gall form, external color and epidermial structure. Morphotype shape was established according to the most usual morphological classifications found in the literature on this topic (Maia 2001, Urso-Guimarães et al. 2003, Carneiro et al. 2009, Santos et al. 2011a, Saito & Urso-Guimarães 2012, Isaias et al. 2013). Since these classification criteria could be ambiguous, we classified galls based on their basic general shapes considering that gall morphology could be a mixture of different shapes.

Gall morphotypes were named according to the two first letters from the binomial scientific name, followed by the numerical order of appearance in each plant species, and if necessary, the third letter of the specific epithet might also be used. We propose this nomenclature designation to avoid confusion in the registry of gall morphotypes. Moreover, only detailed literature descriptions with photographs were considered for previous records of plant gall morphotypes.

Adult stages of the gall-inducers and their parasitoids were obtained by rearing galls in plastic bags until the adult emerged. Gall inducers, parasitoids, and inquilines were preserved in plastic vials containing 70% ethanol and deposited in the cecidiarium. Roberto Espinoza carried out the taxonomic identification of the host plants, and the inductor insects were identified by Paul Hanson as much as possible.

Data from external gall epidermis lignification and trichome-covered galls from deciduous forest areas and evergreen tree areas such as *Bosque Viejo* were statistically analyzed by a Chi-square test.

3. *Gall morphotype inventory and collection of associated organisms in a specialized herbarium as an integrated biological database*

Inventories of biological specimens are a valuable tool to know and preserve biological diversity; for plant specimens, for instance, a traditional herbarium is an appropriate way to carry out this task. A specialized herbarium of plant galls was created to become a reference collection for Costa Rican plant galls. This herbarium began operating in 2012 and currently has around 400 sample accessions. The collection started with plant galls from the Guanacaste Conservation Area. To date, the herbarium is made up of four basic units: the plant gall collection of dried specimens, selected gall samples preserved in 70% ethanol (some of them stored at -70ºC), associated insects preserved in 70% ethanol, and a digital database with all the collected information (Figure 1B). Due to the fact that gall traits change in dried samples, a printed photographic registry of each specimen is included in the collection, and for further morphological studies, a collection of selected galls in FAA (formalin-acetic acid-alcohol) is expected to be included in the future. A database with plant gall data and photographs, as well as information related to their associated organisms, is expected to be available using FileMaker-Pro software or another similar program. This specialized herbarium functions according to appropriated technical standard and collections are maintained in a controlled environment at 20 C° with relative humidity between 40-60%.

Results

A total of eighty-seven species, in seventy genera and forty-four families, of plants that host galls were recorded in the Guanacaste Conservation Area (Table 1). We found one hundred thirty-one morphologically distinct types of plant galls in ACG. The plant families with the highest number of gall morphotypes were Fabaceae (8.4%), Rubiaceae (7.6%), Malvaceae (6.1%), Sapindaceae (5.3%), Boraginaceae (4.6%), and Nyctaginaceae with 4.6% (Table 2). Sixty plant species harbored one gall morphotype, fifteen had two associated morphotypes, six plant species harbored three gall morphotypes, two species contained four gall morphotypes and three species harbored five morphotypes. The species with the greatest number of galls were *Acalypha diversifolia* (Euphorbiaceae) and *Psychotria horizontalis* (Rubiaceae) with four gall morphotypes, as well as *Pisonia macranthocarpa* (Nyctaginaceae), *Sideroxylon obtusifolium* (Sapotaceae), and *Stegnosperma cubense* (Stegnospermataceae) with five morphotypes each.

Some gall samples were so rare that there were not enough to obtain insects, but in many cases, although we had enough plant material, it was not possible to obtain adult insects for identification purposes. Gall-inducer identification to family level was possible in many cases based on the larval stages encountered during the dissections of some selected galls when enough material was available. Nevertheless, even when adult stages were obtained, identification of most insects beyond the family level was complicated by the lack of appropriate taxonomic references, a limitation described by Hanson et al. (2014). Therefore, most of the collected insects remain as unidentified species. The inducing insects that were identified belong to the family Cecidomyiidae (Diptera). Some parasitoids/inquilines (all belonging to the order Hymenoptera) were identified to the family, subfamily or genus level.

Plant gall morphotype description, name and characteristics are presented below under host plant families, genera and species in alphabetical order. They included gall morphology classification, color, epidermial structure, organs attacked, associated organisms as well as host plant description, location and geographical coordinates.

Acanthaceae

Aphelandra scabra (Vahl) Sm. Morphotype Ap_sc_1 (Figure 2A). Gall description: Irregular shape, white, induced on bud, hairy epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Shrub, nearly 2 m tall, on top of rock by the river. Location: Guanacaste, Liberia, Curubandé. Guanacaste National Park, Las Pailas Area, gallery forest with remnant trees. Coordinates/Altitude: 10,7749444 N 85,35025 W, 955 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Avicennia germinans L. Morphotype Av_ge_1 (Figure 2B). Gall description: Discoid shape, green or yelowish-brown, induced on leaves and veins, glabrous epidermis, protruding on both surfaces of the leaf. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Shrub, nearly 3 m tall, barren. Location: Guanacaste, Liberia, Nacascolo. Guanacaste National Park, Naranjo Beach Area, mangrove and beach area, coastal area in dry-forest. Coordinates/Altitude: 10,78335 N 85,6644861 W, 14 m. Registry comments: Gall morphotype resembles the one described by Oliveira dos Santos et al. (2013).

Table 1. Number of gall morphotypes per plant family in ACG (Guanacaste, Costa Rica).

PLANT FAMILY	NUMBER OF GALL MORPHOTYPES	PLANT FAMILY	NUMBER OF GALL MORPHOTYPES
Acanthaceae	2	Nyctaginaceae	6
Anacardiaceae	2	Ochnaceae	1
Annonaceae	2	Olacaceae	2
Apocynaceae	1	Phytolaccaceae	1
Asteraceae	3	Picraminiaceae	2
Bignoniaceae	4	Piperaceae	3
Boraginaceae	6	Poaceae	1
Burseraceae	1	Polygonaceae	2
Chrysobalanaceae	1	Primulaceae	3
Erythoroxylaceae	2	Rubiaceae	10
Euphorbiaceae	4	Rutaceae	1
Fabaceae	11	Sabiaceae	1
Fagaceae	2	Salicaceae	2
Hippocrateaceae	3	Sapindaceae	7
Krameriaceae	1	Sapotaceae	5
Lauraceae	3	Simaroubaceae	1
Malpighiaceae	3	Smilacaceae	1
Malvaceae	8	Solanaceae	1
Melastomataceae	1	Stegnospermataceae	5
Meliaceae	1	Urticaceae	1
Moraceae	5	Verbenaceae	3
Myrtaceae	3	Vitaceae	3
	Total		131

Table 2. Number of gall morphotypes on each plant organ in ACG (Guanacaste, Costa Rica).

PLANT ORGAN	N° GALL MORPHOTYPES	%
Bud	9	6.87
Fruit	1	0.76
Inflorescence	1	0.76
Leaf midvein	5	3.82
Leaf midvein and petiole	1	0.76
Leaf veins	4	3.05
Leaves	80	61.07
Leaves and buds	2	1.53
Leaves and petiole	1	0.76
Petiole	4	3.05
Stem	23	17.56
Total	**131**	**100**

Anacardiaceae

Astronium graveolens Jacq. Morphotype As_gr_1 (Figure 2C). Gall description: Globular shape, yellowish green to brown at maturity, induced on leaves, lignified epidermis. Glabrous, on the adaxial surface of the leaves. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Sapling, nearly 5 m tall, barren. Location: Guanacaste, Liberia, Nacascolo. Santa Rosa National Park, Bosque Viejo Area between the entrance of the park and *La Casona*. Coordinates/Altitude: 10,85072 N 85,60796 W, 321 m. Registry comments: Gall recorded by Hanson & Nishida (2014).

Spondias mombin L. Morphotype Sp_mo_1 (Figure 2D). Gall description: Globular shape, green galls, brown at maturity, located on the adaxial surface of the leaf. Glabrous and lignified epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Tree, nearly 4 m tall. Location: Guanacaste, La Cruz. Cañas Castilla Country Estate, along the Sonzapote riverside, area of farms and gallery forests. Coordinates/Altitude: 11,11379167 N 85,57459722 W, 200 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world, although two galls induced on this plant species by Cecidomyiidae were reported, without a reference image, by Medianero et al. (2010).

Annonaceae

Guatteria diospyroides Baill. Morphotype Gu_di_1 (Figure 2E). Gall description: Fusiform shape, brown, induced on stem, lignified epidermis. On the steam nearly the leaf. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Small tree, nearly 1.5 m tall, barren. Alternate leaves, elongated (linear in shape), Woody

Figure 2. Plant Gall morphotypes recorded in Área de Conservación Guanacaste (ACG), Guanacaste, Costa Rica. 2A) Gall induced in *Aphelandra scabra* (Vahl) Sm., morphotype Ap_sc_1, 2B) Gall induced in *Avicennia germinans* L., morphotype Av_ge_1, 2C) Gall induced in *Astronium graveolens* Jacq., morphotype As_gr_1, 2D) Gall induced in *Spondias mombin* L., morphotype Sp_mo_1, 2E) Gall induced in *Guatteria diospyroides* Baill., morphotype Gu_di_1, 2F) Gall induced in *Sapranthus palanga* R.E.Fr., morphotype Sa_pa_1, 2G) Gall induced in Sapranthus palanga R.E.Fr., morphotype Sa_pa_1, 2H) Gall induced in *Koanophyllon albicaule* (Sch. Bip. ex Klatt) R.M. King & H. Rob., morphotype Ko_al_1, 2I) Gall induced in *Porophyllum punctatum* (Mill.) S.F. Blake, morphotype Po_pu_1, 2J) Gall induced in *Wedelia* sp. Jacq., morphotype We_sp_1, 2K) Gall induced in *Arrabidaea patellifera* (Schltdl.) Sandwith, morphotype Ar_pa_1, 2L) Gall induced in *Cydista diversifolia* (Kunth) Miers, morphotype Cy_di_1, 2M) Gall induced in *Cydista diversifolia* (Kunth) Miers, morphotype Cy_di_2, 2N) Gall induced in *Pleonotoma variabilis* (Jacq.) Miers, morphotype Pl_va_1, 2O) Gall induced in Cordia alliodora (Ruiz & Pav.) Oken., morphotype Co_al_1, 2P) Gall induced in *Cordia alliodora* (Ruiz & Pav.) Oken., morphotype Co_al_2.

stemleaves, and hardened, lustrous dark green in color. Location: Guanacaste, Liberia, Curubandé. Rincón de la Vieja National Park, road to active crater, Los Gemelos Area, rainforest zone. Coordinates/ Altitude: 10,80032 N 85,35 W, 1000 m; 10,93202778 N 85,46 W, 1421 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Sapranthus palanga R.E.Fr. Morphotype Sa_pa_1 (Figure 2F). Gall description: Spherical shape, green and yellow, induced on leaves, hairy epidermis. Yellowish green galls on the leaf underside, with trichomes, brown at maturity. Gall inducer: unknown. Parasitoids/ Inquilines: unknown. Plant description: Small tree, 4 m tall, barren. Location: Guanacaste, Liberia, Nacascolo. Lookout point between the main entrance of Santa Rosa National Park and *La Casona*, old secondary forest area. Coordinates/Altitude: 10,85648 N 85,6106 W, 623 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Apocynaceae

Forsteronia spicata (Jacq.) G. Mey. Morphotype Fo_sp_1 (Figure 2G). Gall description: Irregular shape, green to yellowish at maturity, induced on leaves, glabrous epidermis, protruding on the surface of the leaves. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Semi-scandent shrub, nearly 5 m tall, barren. Woody stem. Opposite leaves, orbicular to ovate in shape. Location: Guanacaste, Liberia, Nacascolo. Santa Rosa National Park, Santa Rosa area, between the Inter-American highway and *La Casona*, secondary oak forest area. Coordinates/Altitude: 10,86577222 N 85,60990278 W, 290 m; 10,83581 N 85,62347 W, 306 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Asteraceae

Koanophyllon albicaule (Sch. Bip. ex Klatt) R.M. King & H. Rob. Morphotype Ko_al_1 (Figure 2H). Gall description: Conical shape, green, induced on leaves, glabrous epidermis. Wider at the middle, green, and protruding from the midvein. Gall inducer: Cecidomyiidae. Parasitoids/Inquilines: unknown. Plant description: Shrub, nearly 3 m tall, barren, acuminated leaves. Location: Guanacaste, Liberia, Nacascolo. Santa Rosa National Park, Bosque Viejo Area between the entrance of the park and *La Casona*. Coordinates/Altitude: 10,85072 N 85,60796 W, 321 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Porophyllum punctatum (Mill.) S.F. Blake. Morphotype Po_pu_1 (Figure 2I). Gall description: Globulous shape, green, induced on leaves, glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Shrub, nearly 50 cm tall, white flowers. Opposite simple leaves, 1-1.5cm in length. Location: Guanacaste, La Cruz, Santa Elena. Santa Rosa National Park, Nancite Beach Area, open area over a serpentinite rock. Coordinates/Altitude: 10,80485833 N 85,69909167 W, 10 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Wedelia sp. Jacq. Morphotype We_sp_1 (Figure 2J). Gall description: Discoid shape, greenish or yellow, induced on leaves, glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Herbaceous plant, nearly 0.75 m tall, pistillate yellow flowers, leaves with serrate margin. Location: Guanacaste, La Cruz. Guanacaste National Park, on the way to the Maritza Biological Station, secondary growth open area, near high-voltage towers. Coordinates/Altitude: 10,95072222 N 85,59705556 W, 272 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Bignoniaceae

Arrabidaea patellifera (Schltdl.) Sandwith. Morphotype Ar_pa_1 (Figure 2K). Gall description: Conical shape, elongated galls, green, induced on leaves, pubescent epidermis. Gall inducer: Cecidomyiidae. Parasitoids/Inquilines: unknown. Plant description: Vine, barren. Bifoliate leaves with cuspidate apex. Location: Guanacaste, Liberia, Nacascolo. Santa Rosa National Park, Bosque Viejo Area between the entrance of the park and *La Casona*. Coordinates/Altitude: 10,85072 N 85,60796 W, 321 m; 10,83581 N 85,62347 W, 306 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world. However, one gall induced by Cecidomyiidae on this plant species was reported, without a reference image, by Medianero et al. 2010.

Cydista diversifolia (Kunth) Miers. Morphotype Cy_di_1 (Figure 2L). Gall description: Fusiform shape, green or yellow, green to brown color at maturity. Induced on stem, lignified and glabrous epidermis. Gall inducer: Cecidomyiidae. Parasitoids/Inquilines: unknown. Plant description: Understory vine. Leaves with acute apex, bifoliate. Location: Guanacaste, Liberia, Mayorga. Santa Rosa National Park, Bosque Viejo Area between the main entrance of the park and *La Casona*. Coordinates/Altitude: 10,85072 N 85,60796 W, 321 m; 10,85648 N 85,6106 W, 323 m; 10,95072222 N 85,59705556 W, 272 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world, although a gall induced by Cecidomyiidae on this plant species was reported, without a reference image, by Medianero et al. (2010).

Cydista diversifolia (Kunth) Miers. Morphotype Cy_di_2 (Figure 2M). Gall description: Discoid shape, yellow and red, yellowish spots, brown center, induced on leaves, glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Understory vine. Leaves with acute apex, bifoliate. Location: Guanacaste, Liberia, Nacascolo. Santa Rosa National Park, start of the road to Naranjo Beach, old secondary growth area with clearings. Coordinates/Altitude: 10,83581 N 85,62347 W, 306 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world, although a gall induced by Cecidomyiidae on this plant species was reported, without a reference image, by Medianero et al. (2010).

Pleonotoma variabilis (Jacq.) Miers. Morphotype Pl_va_1 (Figure 2N). Gall description: Globular shape, yellowish, induced on leaves, glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Shrub of 2 m tall. Location: Guanacaste, Liberia, Nacascolo. Santa Rosa National Park between the Inter-American highway and *La Casona*, secondary oak forest area. Coordinates/Altitude: 10,86577222 N 85,60990278 W, 290 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Boraginaceae

Cordia alliodora (Ruiz & Pav.) Oken. Morphotype Co_al_1 (Figure 2O). Gall description: Spherical shape, green, induced on leaves, hairy epidermis. Tomentose, on the surface of the leaf, brownish. Gall inducer: Cecidomyiidae. Parasitoids/Inquilines: *Cecidellis* (Pteromalidae); Entedoninae (Eulophidae). Plant description: Shrub, nearly 0,30m-2.5 m tall, barren. Alternate, simple leaves, lanceolate and pubescent. Location: Guanacaste, Liberia, Nacascolo. Santa Rosa National Park, start of the road to Naranjo Beach, old secondary growth area with clearings. Coordinates/Altitude: 10,83581 N 85,62347 W, 306 m; 10,89222222 N 85,47077778 W, 701 m; 10,92638889 N 85,7292 W, 45 m; 10,83422222 N 85,6115 W, 324 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world, although a gall induced by Cecidomyiidae on this plant species has been reported, without a reference image, by Medianero et al. (2010), Cuevas et al. (2014), and Ley-López et al. (2019).

Cordia alliodora (Ruiz & Pav.) Oken. Morphotype Co_al_2 (Figure 2P). Gall description: Spherical shape, green and brown, induced on bud, hairy epidermis. Gall inducer: Cecidomyiidae. Parasitoids/Inquilines: *Cecidellis* (Pteromalidae); Entedoninae (Eulophidae). Plant description: Shrub, nearly 0,30m -2.5 m tall, barren. Alternate, simple leaves, lanceolate and pubescent. Location: Guanacaste, Liberia, Nacascolo. Guanacaste Conservation Area, Santa Rosa National Park, monument to the heroes of 1856. Coordinates/Altitude: 10,83422222 N 85,6115 W, 324 m. Registry comments: Gall morphotype resembles the one described by Medianero et al. (2010).

Cordia collococca L. Morphotype Co_co_1 (Figure 3A). Gall description: Irregular shape, yellow or white, induced on bud, glabrous epidermis. Gall inducer: Cecidomyiidae. Parasitoids/Inquilines: unknown. Plant description: Tree, nearly 15 m tall, discoid green fruits. Location: Guanacaste, Liberia, Mayorga. Guanacaste Conservation Area, Góngora, roadside to the Cacao Volcano. Coordinates/Altitude: 10,88683333 N 85,47311111 W, 597 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Cordia collococca L. Morphotype Co_co_2 (Figure 3B). Gall description: Discoid shape, green (brown at maturity), induced on leaves, glabrous epidermis. Gall inducer: Cecidomyiidae. Parasitoids/Inquilines: unknown. Plant description: Tree, nearly 15 m tall, discoid green fruits. Location: Guanacaste, Liberia, Mayorga. Guanacaste Conservation Area, Cacao Volcano Sector, Cacao Biological Station. Coordinates/Altitude: 10,92658333 N 85,47 W, 1129 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Cordia collococca L. Morphotype Co_co_3 (Figure 3C). Gall description: Globular shape, green, induced on petiole, glabrous epidermis. Gall inducer: Cecidomyiidae. Parasitoids/Inquilines: unknown. Plant description: Tree, nearly 15 m tall, discoid green fruits. Location: Guanacaste, Liberia, Mayorga. Guanacaste Conservation Area, Cacao Volcano Sector, Cacao Biological Station. Coordinates/Altitude: 10,92658333 N 85,47 W, 1129 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Cordia sp. L. Morphotype Co_sp_1 (Figure 3D). Gall description: Conical shape, green, induced on leaves, glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Tree, nearly 15 m tall, discoid green fruits. Location: Guanacaste, Liberia, Mayorga. Guanacaste Conservation Area, Góngora, roadside to the Cacao Volcano. Coordinates/Altitude: 10,88683333 N 85,47311111 W, 597 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Burseraceae

Bursera graveolens (Kunth) Triana & Planch. Morphotype Bu_gr_1 (Figure 3E). Gall description: Cylindrical shape, greenish or yellow-brown, yellow apex, brownish black at senescence, induced on leaves, glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Shrub, nearly 1 m tall, procumbent branches, and barren. Location: Guanacaste, La Cruz, Santa Elena. Guanacaste Conservation Area, Murciélago Sector, El Hachal Bay, rocky beach and dry-forest area. Coordinates/Altitude: 10,93408333 N 85,73 W, 15 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Chrysobalanaceae

Hirtella racemosa Lam. Morphotype Hi_ra_1 (Figure 3F). Gall description: Cylindrical shape, yellow and red, induced on leaves, soft spines covered with hairs epidermis. With white appendages, reddish pilosity, on the upper or lower surface of the leaf. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Shrub, 2,5-3 m tall, by the side of the road. Alternate leaves, with cuspidate apex and entire margin, pilose stipules. Location: Guanacaste, Liberia, Nacascolo. Entrance to the old forest (*Bosque Viejo*) between the welcome booth and *La Casona* at Santa Rosa National Park. Coordinates/Altitude: 10,85072 N 85,60796 W, 321 m; 10,85245 N 85,600727 W, 335 m; 10,85072 N 85,60796 W, 321 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Erythoroxylaceae

Erythroxylum macrophyllum Cav. Morphotype Er_ma_1 (Figure 3G). Gall description: Spherical shape, beige, induced on leaves, glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Shrub, nearly 2m tall, barren. Leaves with acute apex and entire margin. Location: Guanacaste, Liberia, Curubandé. Guanacaste National Park, Las Pailas Area, in the old secondary forest after the pasture, on the way to the crater. Coordinates/Altitude: 10,78427778 N 85,3484167 W, 955 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world, although a gall on this plant species was reported, without a reference image, by Ley-López et al. (2019).

Erythroxylum macrophyllum Cav. Morphotype Er_ma_2 (Figure 3H). Gall description: Spherical shape, white-yellow, induced on the upper and lower surface of leaves, glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Shrub, nearly 2m tall, barren. Leaves with acute apex and entire margin. Location: Guanacaste, Liberia, Curubandé. Guanacaste National Park, Las Pailas Area, in the old secondary forest after the pasture, on the way to the crater. Coordinates/Altitude: 10,78427778 N 85,3484167 W, 955 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Euphorbiaceae

Acalypha diversifolia Jacq. Morphotype Ac_di_1 (Figure 3I). Gall description: Irregular shape, greenish and yellow, induced on leaves, more or less terminal on branch, glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Shrub, nearly 1.70-2,5 m tall, barren, with scandent branches, in understory, barren. Leaves with cuspitade apex and crenate margin. Location: Guanacaste, Liberia, Mayorga. Guanacaste Conservation

Figure 3. Plant Gall morphotypes recorded in Área de Conservación Guanacaste (ACG), Guanacaste, Costa Rica. 3A) Gall induced in *Cordia collococca* L., morphotype Co_co_1, 3B) Gall induced in *Cordia collococca* L., morphotype Co_co_2, 3C) Gall induced in *Cordia collococca* L., morphotype Co_co_3, 3D) Gall induced in *Cordia* sp. L., morphotype Co_sp_1, 3E) Gall induced in *Bursera graveolens* (Kunth) Triana & Planch., morphotype Bu_gr_1, 3F) Gall induced in *Hirtella racemosa* Lam., morphotype Hi_ra_1, 3G) Gall induced in *Erythroxylum macrophyllum* Cav., morphotype Er_ma_1, 3H) Gall induced in *Erythroxylum macrophyllum* Cav., morphotype Er_ma_2, 3I) Gall induced in *Acalypha diversifolia* Jacq., morphotype Ac_di_1, 3J) Gall induced in *Acalypha diversifolia* Jacq., morphotype Ac_di_2, 3K) Gall induced in *Acalypha diversifolia* Jacq., morphotype Ac_di_3, 3L) Gall induced in Acalypha diversifolia Jacq., morphotype Ac_di_4, 3M) Gall induced in *Haematoxylum brasiletto* H. Karst., morphotype Ha_br_1, 3N) Gall induced in *Inga* punctata Willd., morphotype In_pu_1, 3O) Gall induced in *Inga* sp. Mill., morphotype In_sp_1, 3P) Gall induced in Inga sp. Kunth, morphotype In_sp_2.

Area, Góngora, roadside to the Cacao Volcano. Coordinates/Altitude: 10,88683333 N 85,47311111 W, 597 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world, alhtough a gall on this plant species was reported, without a reference image, by Ley-López et al. (2019).

Acalypha diversifolia Jacq. Morphotype Ac_di_2 (Figure 3J). Gall description: Globular shape, yellow and red, induced on bud, glabrous epidermis. Red-yellowish inflorescense galls. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Shrub, nearly 1.70-2,5 m tall, barren, with scandent branches, in understory, barren. Leaves with cuspitade apex and crenate margin. Location: Guanacaste, Liberia, Mayorga. Guanacaste Conservation Area, Góngora, roadside to the Cacao Volcano. Coordinates/Altitude: 10,88683333 N 85,47311111 W, 597 m; 10,89222222 N 85,47077778 W, 701 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world, although a gall on this plant species was reported, without a reference image, by Nieves-Aldrey et al. (2008).

Acalypha diversifolia Jacq. Morphotype Ac_di_3 (Figure 3K). Gall description: Spherical shape, greenish and yellow, induced on leaves, hairy epidermis. Galls with spiny projections, green to brownish at senescence, Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Shrub, nearly 1.70-2,5 m tall, barren, with scandent branches, in understory, barren. Leaves with cuspitade apex and crenate margin. Location: Guanacaste, Liberia, Mayorga. Guanacaste Conservation Area, Góngora, road to the Cacao Volcano, secondary rainforest area, next to the road. Coordinates/Altitude: 10,89222222 N 85,47077778 W, 701 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Acalypha diversifolia Jacq. Morphotype Ac_di_4 (Figure 3L). Gall description: Globular shape, green, induced on stem, soft spines epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Shrub, nearly 1.70-2,5 m tall, barren, with scandent branches, in understory, barren. Leaves with cuspitade apex and crenate margin. Location: Guanacaste, Liberia, Mayorga. Guanacaste Conservation Area, Góngora, road to the Cacao Volcano, secondary rainforest area, next to the road. Coordinates/Altitude: 10,89222222 N 85,47077778 W, 701 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Fabaceae

Haematoxylum brasiletto H. Karst. Morphotype Ha_br_1 (Figure 3M). Gall description: Conical shape, red, induced on leaves, glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: *Torymus* (Torymidae); Pteromalidae. Plant description: Woody sapling, nearly 5 m tall. Location: Guanacaste, La Cruz, Santa Elena. Santa Rosa National Park, Nancite Beach Area, serpentinite rock. Coordinates/Altitude: 10,80485833 N 85,69909167 W, 10 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Inga punctata Willd. Morphotype In_pu_1 (Figure 3N). Gall description: Globular shape, brown, induced on stem, lignified epidermis. Gall inducer: unknown. Parasitoids/Inquilines: Pteromalidae. Plant description: Tree, nearly 10 m tall, barren. Leaves elliptic in shape. Location: Guanacaste, Liberia, Mayorga. Guanacaste Conservation Area, Cacao Volcano Sector, pasture area next to the forest, start of the trail to the biological station. Coordinates/Altitude: 10,92286111 N 85,46375 W, 1018 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Inga sp. Mill. Morphotype In_sp_1 (Figure 3O). Gall description: Elliptical shape, green and brown, induced on leaf midvein, lignified epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Shrub, nearly 4 m tall, barren. Location: Guanacaste, Liberia, Curubandé. Rincón de la Vieja National Park, road to active crater, Los gemelos Area, forest zone. Coordinates/Altitude: 10,80032 N 85,35 W, 1000 m; 10,78427778 N 85,3484167 W, 955 m. Registry comments: Gall morphotype resembles the one described by Rodriguez et al. (2014).

Inga sp. Kunth. Morphotype In_sp_2 (Figure 3P). Gall description: Spherical shape, brown, induced on the adaxial surface of the leaf, hairy epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Shrub, nearly 4 m tall, barren. Location: Guanacaste, Liberia, Mayorga. Guanacaste Conservation Area, Cacao Volcano Sector, pasture area next to the forest and start of the trail to the biological station. Coordinates/Altitude: 10,92286111 N 85,46375 W, 1018 m; 10,78427778 N 85,3484167 W, 955 m. Registry comments: Gall morphotype resembles the one described by Rodriguez et al. (2014).

Lonchocarpus felipei N. Zamora. Morphotype Lo_fe_1 (Figure 4A). Gall description: Cylindrical shape, green and yellowish, induced on leaves, glabrous epidermis. Protruding on the underside, densely covering the entire leaf. Gall inducer: Cecidomyiidae. Parasitoids/Inquilines: unknown. Plant description: Sapling, nearly 4 m tall, barren. Opposite and ovated in shape, with glabrous underside and pubescent surface. Location: Guanacaste, Liberia, Nacascolo. Santa Rosa National Park, entrance to the lookout point of Naranjo Beach, Bosque Viejo Area. Coordinates/Altitude: 10,80586111 N 85,64 W, 250 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Lonchocarpus felipei N. Zamora. Morphotype Lo_fe_2 (Figure 4B). Gall description: Cylindrical shape, green and yellowish, induced on leaves, hairy epidermis. Protruding on the underside, densely covering the entire leaf. Gall inducer: Cecidomyiidae. Parasitoids/Inquilines: unknown. Plant description: Sapling, nearly 4 m tall, barren. Opposite and ovated in shape, with glabrous underside and pubescent surface. Location: Guanacaste, Liberia, Nacascolo. Santa Rosa National Park, Bosque Viejo Area between the park entrance and *La Casona*. Coordinates/Altitude: 10,85072 N 85,60796 W, 321 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Lonchocarpus paviflorus Benth. Morphotype Lo_pa_1 (Figure 4C). Gall description: Discoid shape, green and yellowish, induced on leaves, hairy epidermis. Truncated at apex, pilose, on the underside of the leaf. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Shrub, nearly 2 m tall, barren. Imparipinnate, opposite compound leaves. Location: Guanacaste, Liberia, Nacascolo. Guanacaste National Park, Santa Rosa area, entrance to the lookout point of Naranjo Beach, secondary forest area. Coordinates/Altitude: 10,80586111 N 85,64 W, 250 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Lonchocarpus phaseolifolius Benth. Morphotype Lo_pha_1 (Figure 4D). Gall description: Discoid shape, green, induced on leaves, hairy

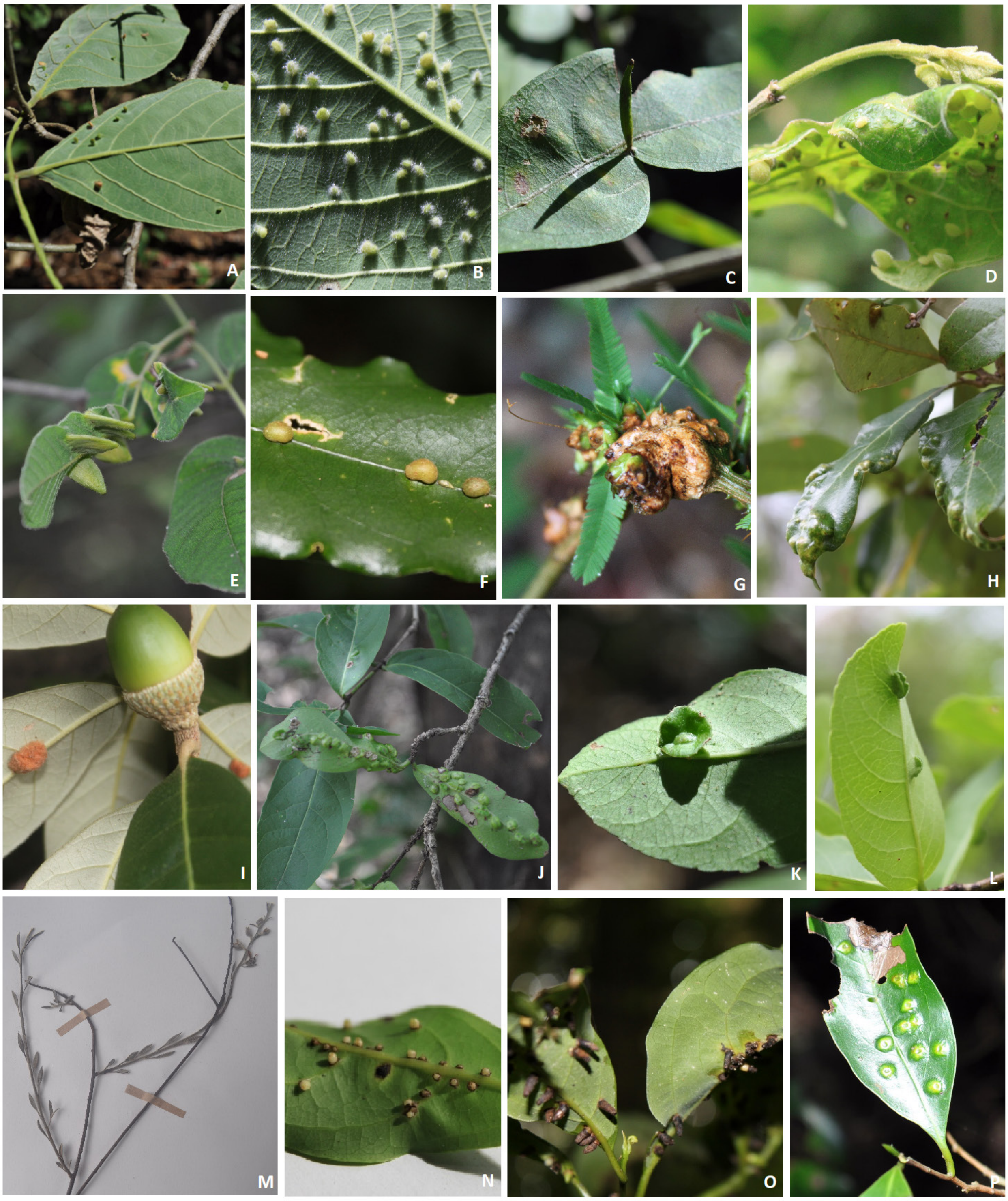

Figure 4. Plant Gall morphotypes recorded in Área de Conservación Guanacaste (ACG), Guanacaste, Costa Rica. 4A) Gall induced in *Lonchocarpus felipei* N. Zamora, morphotype Lo_fe_1, 4B) Gall induced in *Lonchocarpus felipei* N. Zamora, morphotype Lo_fe_2, 4C) Gall induced in *Lonchocarpus paviflorus* Benth., morphotype Lo_pa_1, 4D) Gall induced in *Lonchocarpus phaseolifolius* Benth., morphotype Lo_pha_1, 4E) Gall induced in *Lonchocarpus phlebophyllus* Standl & Steyerm., morphotype Lo_phl_1, 4F) Gall induced in *Lonchocarpus sp.* Kunth, morphotype Lo_sp_1, 4G) Gall induced in *Senegalia tenuifolia* (L.) Britton & Rose., morphotype Se_te_1, 4H) Gall induced in *Quercus oleoides* Schltdl. & Cham., morphotype Qu_ol_1, 4I) Gall induced in Quercus oleoides Schltdl. & Cham., morphotype Qu_ol_2, 4J) Gall induced in *Semialarium mexicanum* (Miers) Mennega, morphotype Se_me_1, 4K) Gall induced in *Semialarium mexicanum* (Miers) Mennega, morphotype Se_me_2, 4L) Gall induced in *Semialarium mexicanum* (Miers) Mennega, morphotype Se_me_3, 4M) Gall induced in *Krameria revoluta* O. Berg, morphotype Kr_re_1, 4N) Gall induced in *Belischmiedia costaricensis* (Mez & Pittier) C.K. Allen, morphotype Be_co_1, 4O) Gall induced in Belischmiedia costaricensis (Mez & Pittier) C.K. Allen, morphotype Be_co_2, 4P) Gall induced in *Nectandra salicina* C.K Allen, morphotype Ne_sa_1.

epidermis. Truncated at apex, pilose, on the underside of the leaf. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Tree, nearly 20 m tall, fallen, racemes with green fruits, unripe fruits. Location: Guanacaste, La Cruz, Santa Elena. Guanacaste Conservation Area, Murciélago Sector, old secondary growth, road between El Hachal Bay and Murciélago Station. Coordinates/Altitude: 10,92638889 N 85,7292 W, 45 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Lonchocarpus phlebophyllus Standl & Steyerm. Morphotype Lo_phl_1 (Figure 4E). Gall description: Irregular shape, green, induced on leaves, hairy epidermis. Flat on the underside of the leaf. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Tree, nearly 4 m tall, by the side of the river. Location: Guanacaste, La Cruz, Santa Elena. Guanacaste National Park, in the Murciélago Sector, old secondary forest area. Coordinates/Altitude: 10,89686111 N 85,7301111 W, 126 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Lonchocarpus sp. Kunth. Morphotype Lo_sp_1 (Figure 4F). Gall description: Globular shape, yellow and brown, induced on leaf midvein, glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Sapling, nearly 1 m tall, barren. Leaves with cuspidate apex. Location: Guanacaste, Liberia, Mayorga. Guanacaste Conservation Area, Cacao Volcano Sector, pasture area next to the forest and start of the trail to the biological station. Coordinates/Altitude: 10,92286111 N 85,46375 W, 1018 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world, although a gall on this plant species was reported, without a reference image, by Cuevas et al. (2014) and de Souza Mendoca et al. (2014).

Senegalia tenuifolia (L.) Britton & Rose. Morphotype Se_te_1 (Figure 4G). Gall description: Irregular shape, brown, induced on apical buds, lignified epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Herbaceous plants, nearly 0.4 m tall, barren. Paripinnate, compound leaves. Location: Guanacaste, Liberia, Nacascolo. Guanacaste Conservation Area, Santa Rosa National Park, roadside near the *Bosque Viejo*, next to the park entrance. Coordinates/Altitude: 10,85263889 N 85,607472 W, 310 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Fagaceae

Quercus oleoides Schltdl. & Cham. Morphotype Qu_ol_1 (Figure 4H). Gall description: Irregular shape, green, yellow at maturity, induced on leaaves, glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Small tree, nearly 5 m tall, by the side of the road. Alternate, simple leaves, with entire margin, underside heavily pubescent. Location: Guanacaste, Liberia, Nacascolo. Santa Rosa National Park, between the Inter-American highway and *La Casona*, secondary oak forest area. Coordinates/Altitude: 10,86577222 N 85,60990278 W, 290 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world, although a gall on this plant species was reported, without a reference image, by Pascual-Alvarado et al. (2017).

Quercus oleoides Schltdl. & Cham. Morphotype Qu_ol_2 (Figure 4I). Gall description: Globular shape, orange and brown, induced on the underside of the leaf, hairy epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Small tree, nearly 5 m tall, by the side of the road. Alternate, simple leaves, with entire margin, underside heavily pubescent. Location: Guanacaste, Liberia, Nacascolo. Lookout point between the main entrance of the Santa Rosa National Park and *La Casona*, old secondary forest area. Coordinates/Altitude: 10,85648 N 85,6106 W, 623 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world, although a gall on this plant species was reported, without a reference image, by Pascual-Alvarado et al. (2017).

Hippocrateaceae

Semialarium mexicanum (Miers) Mennega. Morphotype Se_me_1 (Figure 4J). Gall description: Discoid shape, light green, induced on the underside of the leaf, glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: Pteromalidae, Tetrastichinae Eulophidae), *Torymus* (Torymidae). Plant description: Shrub, nearly 3 m tall, barren. Location: Guanacaste, Liberia, Nacascolo. Guanacaste National Park, Santa Rosa Area, entrance to the lookout point of Naranjo Beach, secondary forest area. Coordinates/Altitude: 10,81105556 N 85,64 W, 246 m; 10,80485833 N 85,69909167 W, 10 m; 10,86577222 N 85,60990278 W, 290 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Semialarium mexicanum (Miers) Mennega. Morphotype Se_me_2 (Figure 4K). Gall description: Discoid shape, green, induced on leaves, glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: Pteromalidae, Tetrastichinae (Eulophidae) *Torymus* (Torymidae). Plant description: Shrub, nearly 3 m tall, barren. Location: Guanacaste, Liberia, Nacascolo. Guanacaste National Park, Santa Rosa area, entrance to the lookout point of Naranjo Beach, secondary forest area. Coordinates/Altitude: 10,81105556 N 85,64 W, 246 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Semialarium mexicanum (Miers) Mennega. Morphotype Se_me_3 (Figure 4L). Gall description: Discoid shape, green, induced on the underside of the leaf, like a shell, glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: Pteromalidae, Tetrastichinae (Eulophidae) *Torymus* (Torymidae). Plant description: Shrub, nearly 3 m tall, barren. Location: Guanacaste, La Cruz, Santa Elena. Santa Rosa National Park, Nancite Beach Area, serpentinite rock. Coordinates/Altitude: 10,80485833 N 85,69909167 W, 10 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Krameriaceae

Krameria revoluta O. Berg. Morphotype Kr_re_1 (Figure 4M). Gall description: Elliptical shape, green and brown, induced on stem, hairy epidermis. Gall inducer: unknown. Parasitoids/Inquilines: Tetrastichinae Entedoninae (Eulophidae). Plant description: Herbaceous plant, 30 cm tall, lilac flowers with white filaments. Alternate, simple leaves, heavily pubescent, approx. 1 cm in legth. Small lilac flowers. Location: Guanacaste, La Cruz, Santa Elena. Santa Rosa National Park, Nancite Beach Area, open area over a serpentinite rock. Coordinates/Altitude: 10,80485833 N 85,69909167 W, 10 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Laureaceae

Belischmiedia costaricensis (Mez & Pittier) C.K. Allen. Morphotype Be_co_1 (Figure 4N). Gall description: Spherical shape, yellowish and brown, induced on the underside of the leaf, glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Sapling, nearly 1.70 m tall, barren, alternate leaves with entire margin. Location: Guanacaste, Liberia, Mayorga. Guanacaste Conservation Area, Cacao Volcano Sector, pasture area next to the forest, start of the trail to the biological station. Coordinates/Altitude: 10,92286111 N 85,46375 W, 1018 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Belischmiedia costaricensis (Mez & Pittier) C.K. Allen. Morphotype Be_co_2 (Figure 4O). Gall description: Conical shape, yellow or brown, induced on the underside of the leaf, glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Sapling, nearly 1.70 m tall, barren. Alternate leaves with entire margin. Location: Guanacaste, Liberia, Mayorga. Guanacaste Conservation Area, Cacao Volcano Sector, pasture area next to the forest, start of the trail to the biological station. Coordinates/Altitude: 10,92286111 N 85,46375 W, 1018 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Nectandra salicina C.K. Allen. Morphotype Ne_sa_1 (Figure 4P). Gall description: Discoid shape, yellowish in the centre and green on the borders, induced on leaves, glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: Braconidae, Entedoninae (Eulophidae). Plant description: Shrub, nearly 1.5 m tall, barren. Location: Guanacaste, Liberia, Curubandé. Guanacaste National Park, Las Pailas Area, in the forest after the pasture, on the way to the crater. Coordinates/Altitude: 10,76955556 N 85,34519444 W, 744 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Malpighiaceae

Banisteriopsis cornifolia (Kunth) C.B. Rob. Morphotype Ba_co_1 (Figure 5A). Gall description: Discoid shape, yellowish, induced on leaves, glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Shrub, nearly 3 m tall, with procumbent branches, barren. Location: Guanacaste, Liberia, Nacascolo. Santa Rosa National Park, Bosque Viejo Area between the entrance of the park and *La Casona*. Coordinates/Altitude: 10,85072 N 85,60796 W, 321 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Banisteriopsis cornifolia (Kunth) C.B. Rob. Morphotype Ba_co_2 (Figure 5B). Gall description: Elliptical shape, green or yellowish, induced on leaves, glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Shrub, nearly 3 m tall, with procumbent branches, barren. Location: Guanacaste, Liberia, Nacascolo. Santa Rosa National Park, Bosque Viejo Area between the entrance of the park and *La Casona*. Coordinates/Altitude: 10,85072 N 85,60796 W, 321 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Byrsonima crassifolia (L.) Kunth. Morphotype By_cr_1 (Figure 5C). Gall description: Conical shape, green or yellowish, induced on leaves, glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Small tree, nearly 2m tall, barren. Opposite, simple leaves, medium size, with trichomes on the underside. Location: Guanacaste, La Cruz. Guanacaste National Park, on the way to the Maritza Biological Station, secondary growth area along side the river. Coordinates/Altitude: 10,88683333 N 85,47311111 W, 272 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Malvaceae

Guazuma ulmifolia Lam. Morphotype Gu_ul_1 (Figure 5D). Gall description: Globular shape, yellow and brown, induced on bud, glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: *Sycophila*, "*Eurytoma*" (Eurytomidae), Tetrastichinae (Eulophidae). Plant description: Sapling, nearly 3 m tall, barren. Ovate leaves with dentate margin. Location: Guanacaste, Liberia, Mayorga. Guanacaste Conservation Area, Góngora, roadside to the Cacao Volcano. Coordinates/Altitude: 10,88683333 N 85,47311111 W, 597 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Guazuma ulmifolia Lam. Morphotype Gu_ul_2 (Figure 5E). Gall description: Irregular shape, green and yellow, distributed on the glabrous adaxial surface of the leaf, glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Sapling, nearly 3 m tall, barren. Ovate leaves with dentate margin. Location: Guanacaste, Liberia, Mayorga. Guanacaste Conservation Area, Góngora, roadside to the Cacao Volcano. Coordinates/Altitude: 10,88683333 N 85,47311111 W, 597 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world, although a gall on the same plant organ for this plant species was reported, without a reference image, by Coelho et al. (2014) but gall description doesn't match.

Guazuma ulmifolia Lam. Morphotype Gu_ul_3 (Figure 5F). Gall description: Globular shape, green, induced on stem, hairy epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Sapling, nearly 3 m tall, barren. Ovate leaves with dentate margin. Location: Guanacaste, Liberia, Mayorga. Guanacaste Conservation Area, Góngora, roadside to the Cacao Volcano. Coordinates/Altitude: 10,89222222 N 85,47077778 W, 701 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Hampea appendiculata (Donn. Sm.) Standl. Morphotype Ha_ap_1 (Figure 5G). Gall description: Irregular shape, yellow, induced on leaves, glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Sapling, nearly 1.5 m tall, barren. Location: Guanacaste, Liberia, Mayorga. Guanacaste Conservation Area, Cacao Volcano Sector, pasture area next to the forest and start of the trail to the biological station. Coordinates/Altitude: 10,92286111 N 85,46375 W, 1018 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Malvaviscus arboreus Dill. ex Cav. Morphotype Ma_ar_1 (Figure 5H). Gall description: Spherical shape, green, induced on leaves, hairy epidermis. Gall inducer: Cecidomyiidae. Parasitoids/Inquilines:

Figure 5. Plant Gall morphotypes recorded in Área de Conservación Guanacaste (ACG), Guanacaste, Costa Rica. 5A) Gall induced in *Banisteriopsis cornifolia* (Kunth) C.B. Rob., morphotype Ba_co_1, 5B) Gall induced in *Banisteriopsis cornifolia* (Kunth) C.B. Rob., morphotype Ba_co_2, 5C) Gall induced in *Byrsonima crassifolia* (L.) Kunth, morphotype By_cr_1, 5D) Gall induced in *Guazuma ulmifolia* Lam., morphotype Gu_ul_1, 5E) Gall induced in *Guazuma ulmifolia* Lam., morphotype Gu_ul_2, 5F) Gall induced in *Guazuma ulmifolia* Lam., morphotype Gu_ul_3, 5G) Gall induced in *Hampea appendiculata* (Donn. Sm.) Standl., morphotype Ha_ap_1, 5H) Gall induced in *Malvaviscus arboreus* Dill. ex Cav., morphotype Ma_ar_1, , 5I) Gall induced in *Malvaviscus arboreus* Dill. ex Cav., morphotype Ma_ar_2, 5J) Gall induced in *Ochroma pyramidale* (Cav. ex Lam.) Urb., morphotype Oc_py_1, 5K) Gall induced in *Waltheria indica* L., morphotype Wa_in_1, 5L) Gall induced in *Miconia sp.* Ruiz & Pav., morphotype Mi_sp_1, 5M) Gall induced in Guarea glabra Kunth. , morphotype Gu_gl_1, 5N) Gall induced in *Brosimum alicastrum* Swartz, morphotype Br_al_1, 5O) Gall induced in *Ficus croata* (Miq.) Miq., morphotype Fi_cr_1, 5P) Gall induced in *Ficus ovalis* Desf. ex Willd., morphotype Fi_ov_1.

unknown. Plant description: Shrub, 1.5 m tall, red flowers, on top of rock by the river. Location: Guanacaste, Liberia, Curubandé. Guanacaste National Park, Las Pailas Area, in the forest after the fumaroles. Coordinates/Altitude: 10,85072 N 85,60796 W, 321 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Malvaviscus arboreus Dill. ex Cav. Morphotype Ma_ar_2 (Figure 5I). Gall description: Globular shape, brown, induced on stem, lignified epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Shrub, 1.5 m tall, red flowers, on top of rock by the river. Location: Guanacaste, Liberia, Nacascolo. Santa Rosa National Park, around *La Casona* at Santa Rosa National Park. Coordinates/ Altitude: 10,76955556 N 85,34519444 W, 745 m; 10,77722222 N 85,35025 W, 955 m; 10,88683333 N 85,47311111 W, 597 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Ochroma pyramidale (Cav. ex Lam.) Urb. Morphotype Oc_py_1 (Figure 5J). Gall description: Irregular shape, reddish-brown on the adaxial surface of the leaf and white on the underside of the leaf, induced on leaves, hairy epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Small tree, nearly 1.7 m tall, barren. Location: Guanacaste, Liberia, Mayorga. Guanacaste Conservation Area, Góngora, roadside to the Cacao Volcano, next to the Góngora River. Coordinates/ Altitude: 10,83422222 N 85,6115 W, 569 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world, although a gall on this plant species was reported, without a reference image, by Ley-López et al. 2019.

Waltheria indica L. Morphotype Wa_in_1 (Figure 5K). Gall description: Globular shape, green galls, yellowish/redish at maturity, brown, on the underside of the leaf and petiole, hairy epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Small tree, nearly 5 m tall, by the side of the road. Location: Guanacaste, La Cruz. Guanacaste National Park, on the way to the Maritza Biological Station, secondary growth open area, near high-voltage towers. Coordinates/Altitude: 10,95072222 N 85,59705556 W, 272 m. Registry comments: Gall recorded by Figueiredo et al. 2014.

Melastomataceae

Miconia sp. Ruiz & Pav. Morphotype Mi_sp_1 (Figure 5L). Gall description: Irregular shape, green and brown, induced on bud and leaves, glabrous epidermis. Gall inducer: unknown. Parasitoids/ Inquilines: *Torymus* (Torymidae), Eupelmidae, Pteromalidae. Plant description: Shrub, nearly 2.5 m tall, barren. Location: Guanacaste, Liberia, Mayorga. Guanacaste Conservation Area, Cacao Volcano Sector, pasture area next to the forest, start of the trail to the biological station. Coordinates/Altitude: 10,93202778 N 85,46 W, 1277 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world, although a gall on this plant species has been reported, without a reference image, by De Souza (2014), Medianero et al. (2014), and Ley-López et al. (2019).

Meliaceae

Guarea glabra Kunth. Morphotype Gu_gl_1 (Figure 5M). Gall description: Cylindrical shape, green, on adaxial and abaxial surface of the leaf, glabrous epidermis. Gall inducer: unknown. Parasitoids/ Inquilines: unknown. Plant description: Shrub, nearly 4 m tall, barren. Location: Guanacaste, Liberia, Mayorga. Guanacaste Conservation Area, Góngora, road to the Cacao Volcano, secondary rainforest area, next to the road. Coordinates/Altitude: 10,89222222 N 85,47077778 W, 701 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Moraceae

Brosimum alicastrum Swartz. Morphotype Br_al_1 (Figure 5N). Gall description: Cylindrical shape, yellowish and brown, induced on leaves, glabrous epidermis. Gall inducer: unknown. Parasitoids/ Inquilines: unknown. Plant description: Small tree, nearly 3 m tall, barren. Location: Guanacaste, Liberia, Nacascolo. Guanacaste National Park, Santa Rosa area, entrance to the lookout point of Naranjo Beach, secondary forest area. Coordinates/Altitude: 10,85072 N 85,60796 W, 292 m; 10,80586111 N 85,64 W, 250 m; 10,85072 N 85,60796 W, 321 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world, although a gall on this plant species has been reported, without a reference image, by Cuevas et al. (2004), Cuevas et al. (2014), and Ley-López et al. (2019).

Ficus croata (Miq.) Miq. Morphotype Fi_cr_1 (Figure 5O). Gall description: Discoid shape, yellowish or red, induced on leaves, glabrous epidermis. Gall inducer: Cecidomyiidae. Parasitoids/Inquilines: unknown. Plant description: Tree, nearly 15 m tall, barren. Location: Guanacaste, Liberia, Nacascolo. Santa Rosa National Park, around the historic *Hacienda La Casona*, isolated trees alongside the old corral. Coordinates/Altitude: 10,83382 N 85,61269 W, 307 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Ficus ovalis Desf. ex Willd. Morphotype Fi_ov_1 (Figure 5P). Gall description: Globular shape, green, induced on leaves, glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Tree, nearly 15 m tall, pedunculated infructescence, yellow, one or more by leaf bud. Alternate leaves, in several different sizes. Location: Guanacaste, Liberia, Nacascolo. Santa Rosa National Park, around the historic *Hacienda La Casona*, isolated trees alongside the old corral. Coordinates/Altitude: 10,83382 N 85,61269 W, 307 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Pseudolmedia glabrata (Liebm.) C.C. Berg. Morphotype Ps_gl_1 (Figure 6A). Gall description: Conical shape, green and yellow, induced on leaves, glabrous epidermis. Gall inducer: unknown. Parasitoids/ Inquilines: unknown. Plant description: Small tree, nearly 3 m tall, milky secretion, barren. Location: Guanacaste, Liberia, Mayorga. Guanacaste Conservation Area, Cacao Volcano Sector, next to the road. Coordinates/ Altitude: 10,88683333 N 85,47311111 W, 597 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Pseudolmedia mollis (Liebm.) C.C. Berg. Morphotype Ps_mo_1 (Figure 6B1/6B2). Gall description: Globular shape, green on the adaxial surface of the leaf, greenish-white and pilose on the abaxial surface of the leaf, glabrous epidermis. Gall inducer: unknown. Parasitoids/ Inquilines: unknown. Plant description: Sapling, nearly 3 m tall, with milky secretion, barren. Location: Guanacaste, Liberia, Mayorga.

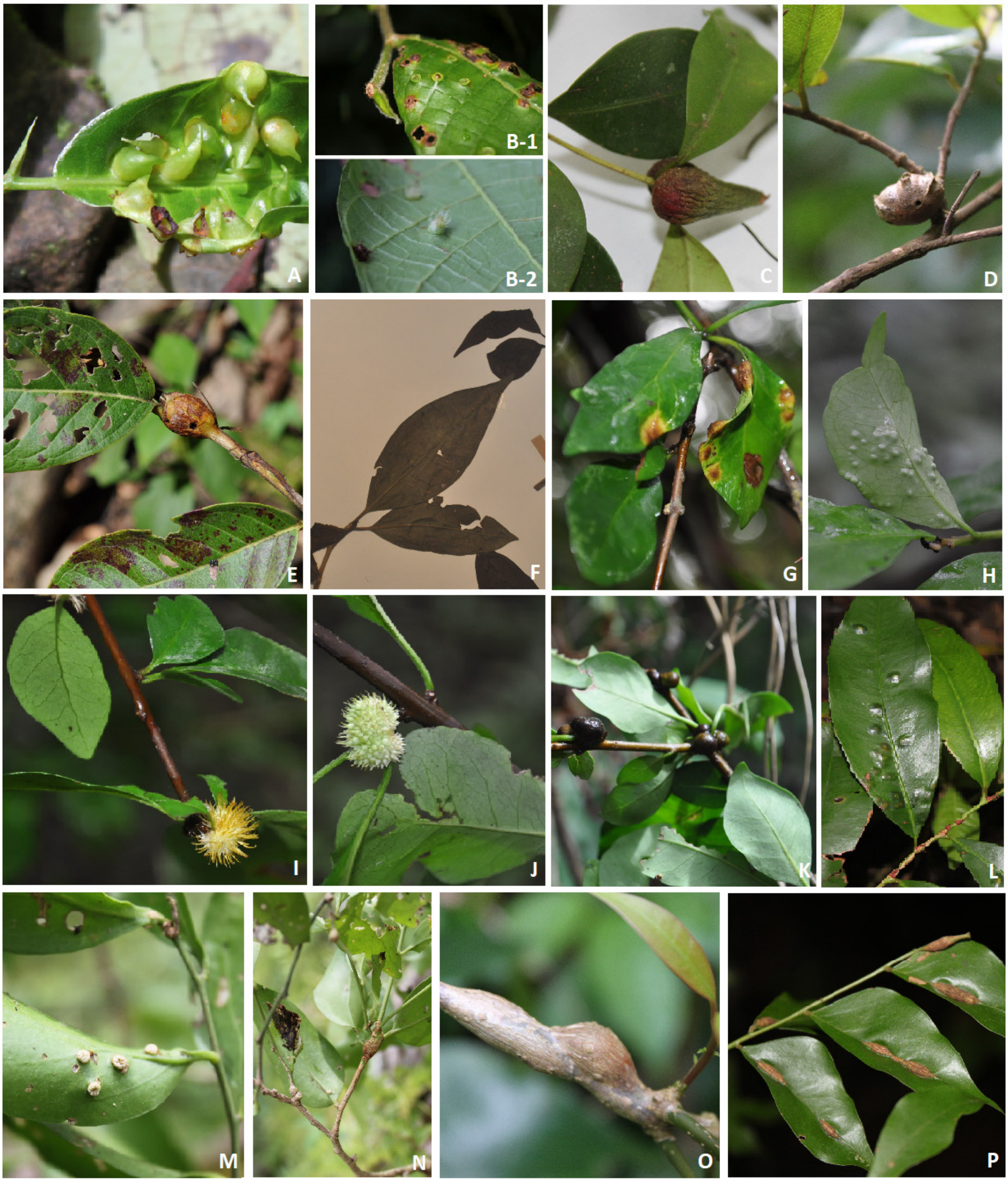

Figure 6. Plant Gall morphotypes recorded in Área de Conservación Guanacaste (ACG), Guanacaste, Costa Rica. 6A) Gall induced in *Pseudolmedia glabrata* (Liebm.) C.C. Berg, morphotype Ps_gl_1, 6B1/6B2) Gall induced in *Pseudolmedia mollis* (Liebm.) C.C. Berg, morphotype Ps_mo_1, 6C) Gall induced in *Eugenia* sp. (Miq.) Miq., morphotype Eu_sp_1, 6D) Gall induced in *Myrcia splendens* (Sw.) DC., morphotype My_sp_1, 6E) Gall induced in *Psidium guajava* L., morphotype Ps_gu_1, 6F) Gall induced in sp. Ruiz & Pav., morphotype Ne_sp_1, 6G) Gall induced in *Pisonia macranthocarpa* (Donn. Sm.) Donn. Sm., morphotype Pi_ma_1, 6H) Gall induced in *Pisonia macranthocarpa* (Donn. Sm.) Donn. Sm., morphotype Pi_ma_2, 6I) Gall induced in *Pisonia macranthocarpa* (Donn. Sm.) Donn. Sm., morphotype Pi_ma_3, 6J) Gall induced in *Pisonia macranthocarpa* (Donn. Sm.) Donn. Sm., morphotype Pi_ma_4, 6K) Gall induced in *Pisonia macranthocarpa* (Donn. Sm.) Donn. Sm., morphotype Pi_ma_5, 6L) Gall induced in Ouratea lucens (Kunth.) Engl., morphotype Ou_lu_1, 6M) Gall induced in *Schoepfia schreberi* J.F. Gmel., morphotype Sc_sc_1, 6N) Gall induced in *Schoepfia schreberi* J.F. Gmel., morphotype Sc_sc_2, 6O) Gall induced in *Trichostigma polyandrum* (Loes.) H. Walter, morphotype Tr_po_1, 6P) Gall induced in *Picramnia antidesma* Sw., morphotype Pi_an_1.

Guanacaste Conservation Area, Cacao Volcano Sector, pasture area next to the forest, start of the trail to the biological station. Coordinates/Altitude: 10,92286111 N 85,46375 W, 1018 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Myrtaceae

Eugenia sp. (Miq.) Miq. Morphotype Eu_sp_1 (Figure 6C). Gall description: Conical shape, greenish and reddish, induced on stem, glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Shrub, nearly 0,5 m tall, barren. Location: Guanacaste, Liberia, Mayorga. Guanacaste Conservation Area, Cacao Volcano Sector, Cacao Mountain range. Coordinates/Altitude: 10,93202778 N 85,46 W, 1421 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Myrcia splendens (Sw.) DC. Morphotype My_sp_1 (Figure 6D). Gall description: Globular shape, brown, induced on stem, lignified epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Small tree, nearly 2 m tall, barren. Location: Guanacaste, Liberia, Mayorga. Guanacaste Conservation Area, Góngora, road to the Cacao Volcano, sedimentary rock area next to the Góngora River. Coordinates/Altitude: 10,83422222 N 85,6115 W, 629 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world, although a gall on this plant species was reported, without a reference image, by Ley-López et al. (2019).

Psidium guajava L. Morphotype Ps_gu_1 (Figure 6E). Gall description: Elliptical shape, brown, induced on stem, lignified epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Sapling, nearly 0.5 m tall, barren. Location: Guanacaste, La Cruz. Guanacaste National Park, on the way to the Maritza Biological Station, secondary growth open area, near high-voltage towers. Coordinates/Altitude: 10,95072222 N 85,59705556 W, 272 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world, although a gall on this plant species was reported, without a reference image, by Maia (2012).

Nyctaginaceae

Neea sp. Ruiz & Pav. Morphotype Ne_sp_1 (Figure 6F). Gall description: Fusiform shape, green, induced on leaves, glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Shurb, nearly 1.5 m tall, barren. Location: Guanacaste, Liberia, Curubandé. Rincón de la Vieja National Park, forest area on the way to active crater. Coordinates/Altitude: 10,78427778 N 85,3484167 W, 955 m. Registry comments: Similar to gall recorded by Maia (2014).

Pisonia macranthocarpa (Donn. Sm.) Donn. Sm. Morphotype Pi_ma_1 (Figure 6G). Gall description: Irregular shape, yellow and brown, induced on leaves, glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Shrub, nearly 2.5 m tall, barren. Location: Guanacaste, La Cruz, La Cruz. Guanacaste National Park, on the way to the Maritza Biological Station, secondary growth area alongside the river. Coordinates/Altitude: 10,95072222 N 85,59705556 W, 272 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Pisonia macranthocarpa (Donn. Sm.) Donn. Sm. Morphotype Pi_ma_2 (Figure 6H). Gall description: Irregular shape, green, induced on leaves, glabrous epidermis. Gall inducer: Cecidomyiidae. Parasitoids/Inquilines: unknown. Plant description: Shrub, nearly 4 m tall, unripe fruit are light green. Location: Guanacaste, La Cruz, La Cruz. Guanacaste National Park, on the way to the Maritza Biological Station, secondary growth area along the river. Coordinates/Altitude: 10,95072222 N 85,59705556 W, 272 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Pisonia macranthocarpa (Donn. Sm.) Donn. Sm. Morphotype Pi_ma_3 (Figure 6I). Gall description: Spherical shape, yellow, induced on bud, soft spines on epidermis. Gall inducer: Cecidomyiidae. Parasitoids/Inquilines: unknown. Plant description: Shrub, nearly 2 m tall, barren. Location: Guanacaste, La Cruz, La Cruz. Guanacaste National Park, on the way to the Maritza Biological Station, secondary growth area along the river. Coordinates/Altitude: 10,95072222 N 85,59705556 W, 272 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Pisonia macranthocarpa (Donn. Sm.) Donn. Sm. Morphotype Pi_ma_4 (Figure 6J). Gall description: Globular shape, white, induced on bud, soft spines on epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Shrub, nearly 2 m tall. Location: Guanacaste, La Cruz. Guanacaste National Park, on the way to the Maritza Biological Station, secondary growth area alongside the river. Coordinates/Altitude: 10,95072222 N 85,59705556 W, 272 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Pisonia macranthocarpa (Donn. Sm.) Donn. Sm. Morphotype Pi_ma_5 (Figure 6K). Gall description: Globular shape, brownish, induced on stem, lignified epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Shrub, nearly 2 m tall. Location: Guanacaste, La Cruz. Guanacaste National Park, on the way to the Maritza Biological Station, secondary growth area along the river. Coordinates/Altitude: 10,95072222 N 85,59705556 W, 272 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Ochnaceae

Ouratea lucens (Kunth.) Engl. Morphotype Ou_lu_1 (Figure 6L). Gall description: Discoid shape, green (concolorous) to brownish yellow galls on leaves, glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Understory sapling, with red stipules. Location: Guanacaste, Liberia, Nacascolo. Entrance to the old forest (*Bosque Viejo)*, between the welcome booth and *La Casona* at Santa Rosa National Park. Coordinates/Altitude: 10,85072 N 85,60796 W, 321 m. Registry comments: Similar to gall recorded by Bergamini et al. (2017).

Olacaceae

Schoepfia schreberi J.F. Gmel. Morphotype Sc_sc_1 (Figure 6M). Gall description: Discoid shape, white, induced on leaves, glabrous epidermis. Gall inducer: Cecidomyiidae. Parasitoids/Inquilines: unknown. Plant description: Shrub, 3 m tall, by the side of the road. Location: Guanacaste, Liberia, Nacascolo. Entrance to the old forest (*Bosque Viejo*), between the welcome booth and *La Casona* at Santa Rosa National Park. Coordinates/Altitude: 10,8336278 N 85,6132333 W, 292 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Schoepfia schreberi J.F. Gmel. Morphotype Sc_sc_2 (Figure 6N). Gall description: Elliptical shape, brown, induced on stem, lignified-glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Shrub, 3 m tall, by the side of the road. Location: Guanacaste, Liberia, Nacascolo. Entrance to the old forest (*Bosque Viejo*), between the welcome booth and *La Casona* at Santa Rosa National Park. Coordinates/Altitude: 10,8336278 N 85,6132333 W, 292 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Phytolaccaceae

Trichostigma polyandrum (Loes.) H. Walter. Morphotype Tr_po_1 (Figure 6O). Gall description: Fusiform shape, brown, induced on stem, lignified, and glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Shrub, nearly 1.5 m tall, procumbent branches, reddish petioles, and barren. Location: Guanacaste, Liberia, Mayorga. Guanacaste Conservation Area, Góngora, road to the Cacao Volcano, side the road, next to the Góngora River. Coordinates/Altitude: 10,83422222 N 85,6115 W, 569 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Picraminiaceae

Picramnia antidesma Sw. Morphotype Pi_an_1 (Figure 6P). Gall description: Fusiform shape, brown, induced on leaves, lignified epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Shrub, barren. Alternate leaves, with entire margin. Location: Guanacaste, Liberia, Nacascolo. Entrance to the old forest (*Bosque Viejo*), between the welcome booth and *La Casona* at Santa Rosa National Park. Coordinates/Altitude: 10,85072 N 85,60796 W, 321 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Picramnia antidesma Sw. Morphotype Pi_an_2 (Figure 7A). Gall description: Globular shape, brown, induced on stem, lignified epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Shrub, barren. Alternate leaves, with entire margin. Location: Guanacaste, Liberia, Nacascolo. Santa Rosa National Park, *Bosque Viejo* area between the park entrance and *La Casona* Coordinates/Altitude: 10,85072 N 85,60796 W, 321 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Piperaceae

Piper sp. L. Morphotype Pi_sp_1 (Figure 7B). Gall description: Spherical shape, green and yellowish on the surface, whitish pubescence, on petiole and leaf rachis. On abaxial or abaxial leaf position, hairy epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Herbaceous plant, nearly 1m tall, by the side of the road. Location: Guanacaste, Liberia, Nacascolo. Entrance to the old forest (*Bosque Viejo*), between the welcome booth and *La Casona* at Santa Rosa National Park. Coordinates/Altitude: 10,85072 N 85,60796 W, 321 m. Registry comments: Gall morphotype resembles the one described by Maia & Mascarenhas (2017) on *P. richardiifolium*.

Piper sp. L. Morphotype Pi_sp_2 (Figure 7C). Gall description: Spherical shape, green and yellow, induced on Petiole, hairy epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Herbaceous plant, nearly 1m tall, by the side of the road. Location: Guanacaste, Liberia, Nacascolo. Entrance to the old forest (*Bosque Viejo*), between the welcome booth and *La Casona* at Santa Rosa National Park. Coordinates/Altitude: 10,85072 N 85,60796 W, 321 m. Registry comments: Gall morphotype resembles the one described by Bergamini et al. (2017) on *Piper arboreum* Aubl.

Piper yucatanense C. DC. Morphotype Pi_yu_1 (Figure 7D). Gall description: Globular shape, green, induced on leaves, glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Herbaceous plant, nearly 1 m tall, barren. Location: Guanacaste, Liberia, Mayorga. Guanacaste Conservation Area, Cacao Volcano Sector, pasture area next to the forest, start of the trail to the biological station. Coordinates/Altitude: 10,92286111 N 85,46375 W, 1018 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Poaceae

Paspalum sp. L. Morphotype Pa_sp_1 (Figure 7E). Gall description: Globular shape, brown, on the inflorescense, glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Herbaceous plants, nearly 50 cm tall, by the side of the road, purple glumes. Location: Guanacaste, Liberia, Nacascolo. Santa Rosa National Park, between the Inter-American highway and *La Casona*, secondary oak forest area. Coordinates/Altitude: 10,86577222 N 85,60990278 W, 290 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Polygonaceae

Coccoloba tuerckheimii Donn. Sm. Morphotype Co_tu_1 (Figure 7F). Gall description: Globular shape, orange and brown, induced on on the underside of the leaf, hairy epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Shrub, nearly 3 m tall, spiral leaves at the end of the branch, barren. Location: Guanacaste, Liberia, Mayorga. Guanacaste Conservation Area, Góngora, road to the Cacao Volcano, secondary forest area, next to the road. Coordinates/Altitude: 10,89222222 N 85,47077778 W, 701 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world, although a gall induced on this plant species was reported, without a reference image, by Ley-López et al. 2019.

Coccoloba venosa L. Morphotype Co_ve_1 (Figure 7G). Gall description: Cylindrical shape, Yellow/brown, induced on the adaxial and abaxial surface of the leaf, glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Shrub, nearly 1.5 m tall, on rocky area, flowers with yellow petals and white

Figure 7. Plant Gall morphotypes recorded in Área de Conservación Guanacaste (ACG), Guanacaste, Costa Rica. 7A) Gall induced in *Picramnia antidesma* Sw., morphotype Pi_an_2, 7B) Gall induced in *Piper* sp. L., morphotype Pi_sp_1, 7C) Gall induced in *Piper* sp. L., morphotype Pi_sp_2, 7D) Gall induced in *Piper yucatanense* C. DC., morphotype Pi_yu_1, 7E) Gall induced in *Paspalum* sp. L., morphotype Pa_sp_1, 7F) Gall induced in *Coccoloba tuerckheimii* Donn. Sm, morphotype Co_tu_1, 7G) Gall induced in *Coccoloba venosa* L., morphotype Co_ve_1, 7H) Gall induced in *Ardisia compressa* Schltdl., morphotype Ar_co_1, 7I) Gall induced in *Ardisia compressa* Kunth, morphotype Ar_co_2, 7J) Gall induced in *Ardisia revoluta* Kunth, morphotype Ar_re_1, 7K) Gall induced in *Psychotria deflexa* DC., morphotype Ps_de_1, 7L) Gall induced in *Psychotria horizontalis* Sw., morphotype Ps_ho_1, 7M) Gall induced in *Psychotria horizontalis* Sw., morphotype Ps_ho_2, 7N) Gall induced in *Psychotria horizontalis* L., morphotype Ps_ho_3, 7O) Gall induced in *Psychotria horizontalis* Sw., morphotype Ps_ho_4, 7P) Gall induced in *Psychotria quinqueradiata* Pol., morphotype Ps_qu_1.

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anthers. Location: Guanacaste, Liberia, Nacascolo. Guanacaste National Park, Santa Rosa Area, old secondary forest area, road to Naranjo Beach. Coordinates/Altitude: 10,79383333 N 85,66 W, 15 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Primulaceae

Ardisia compressa Schltdl. Morphotype Ar_co_1 (Figure 7H). Gall description: Discoid shape, green, induced on leaves, glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Small tree, 2 m tall, barren. Location: Guanacaste, Liberia, Curubandé. Guanacaste National Park, *Las Pailas* Sector, in the forest after the pasture, on the way to the crater. Coordinates/Altitude: 10,78427778 N 85,3484167 W, 955 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Ardisia compressa Kunth. Morphotype Ar_co_2 (Figure 7I). Gall description: Discoid shape, green, brown, and red at maturity, induced on leaves, glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: *Torymus* (Torymidae). Plant description: Small tree, 2 m tall, barren. Location: Guanacaste, Liberia, Mayorga. Guanacaste Conservation Area, Cacao Volcano Sector, Cacao mountain range. Coordinates/Altitude: 10,93202778 N 85,46 W, 1421 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Ardisia revoluta Kunth. Morphotype Ar_re_1 (Figure 7J). Gall description: Fusiform shape, green, induced on leaf midvein, glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Small tree, nearly 4m tall, barren. Location: Guanacaste, Liberia, Nacascolo. Santa Rosa National Park, *Bosque Viejo* area between the entrance of the park and *La Casona*. Coordinates/Altitude: 10,85072 N 85,60796 W, 321 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Rubiaceae

Psychotria deflexa DC. Morphotype Ps_de_1 (Figure 7K). Gall description: Conical shape, green and yellow, induced on leaves, glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Shrub, nearly 1m tall, barren. Location: Guanacaste, Liberia, Curubandé. Guanacaste National Park, *Las Pailas* Sector, in the old secondary forest after the pasture, on the way to the crater. Coordinates/Altitude: 10,78427778 N 85,3484167 W, 955 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Psychotria horizontalis Sw. Morphotype Ps_ho_1 (Figure 7L). Gall description: Fusiform shape, brown, induced on stem, lignified epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Herbaceous plant, nearly 50 cm tall, by the side of the road, greenish unripe fruits. Location: Guanacaste, Liberia, Nacascolo. Santa Rosa National Park, between the Inter-American highway and *La Casona*, secondary oak forest area. Coordinates/Altitude: 10,86577222 N 85,60990278 W, 290 m. Registry comments: Gall morphotype resembles the one described by Bergamini et al. (2017) on a Rubiaceae sp.

Psychotria horizontalis Sw. Morphotype Ps_ho_2 (Figure 7M). Gall description: Spherical shape, green, induced on leaves, presence of trichomes epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Herbaceous plant, nearly 50 cm tall, by the side of the road, greenish unripe fruits. Location: Guanacaste, Liberia, Curubandé. Guanacaste National Park, Las Pailas Sector, in the old secondary forest after the pasture, on the way to the crater. Coordinates/Altitude: 10,78427778 N 85,3484167 W, 955 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Psychotria horizontalis Sw. Morphotype Ps_ho_3 (Figure 7N). Gall description: Globular shape, brown, induced on stem, corrugated and lignified epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Small tree, nearly 1.5 m tall, barren. Location: Guanacaste, LaCruz. Parque Nacional Guanacaste, on the way to the Maritza Biological Station, secondary growth area along the river. Coordinates/Altitude: 10,95072222 N 85,59705556 W, 272 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Psychotria horizontalis Sw. Morphotype Ps_ho_4 (Figure 7O). Gall description: Globular shape, green, induced on petiole, glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Small tree, nearly 2.5 m tall, barren. Location: Guanacaste, Liberia, Curubandé. Guanacaste National Park, Las Pailas Sector, in the old secondary forest after the pasture, on the way to the crater. Coordinates/Altitude: 10,76955556 N 85,34519444 W, 744m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Psychotria quinqueradiata Pol. Morphotype Ps_qu_1 (Figure 7P). Gall description: Fusiform shape, brown, induced on stem, glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Shrub, nearly 1 m, greenish fruits Location: Guanacaste, Liberia, Curubandé. Guanacaste National Park, *Las Pailas* Sector, in the old secondary forest after the pasture, on the way to the crater. Coordinates/Altitude: 10,78427778 N 85,3484167 W, 955 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Psychotria valerioana Standl. Morphotype Ps_va_1 (Figure 8A). Gall description: Globular shape, green, induced on leaves and buds, glabrous epidermis. Gall inducer: Cecidomyiidae. Parasitoids/Inquilines: Entedoninae (Eulophidae). Plant description: Shrub, nearly 1 m tall, greenish fruit, white flowers. Location: Guanacaste, Liberia, Curubandé. Guanacaste National Park, Las Pailas Sector, in the old secondary forest after the pasture, on the way to the crater. Coordinates/Altitude: 10,78427778 N 85,3484167 W, 955 m; 10,80669 N 85,35 W, 1074 m; 10,80032 N 85,35 W, 1000 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Psychotria valerioana Standl. Morphotype Ps_va_2 (Figure 8B). Gall description: Conical shape, green on the base and the apex, yellowish in the middle, induced on leaves, glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Shrub, nearly 1 m tall, greenish fruit, white flowers. Location: Guanacaste, Liberia, Curubandé. Rincón de la Vieja National Park, road to active crater, Los gemelos Sector, forest zone. Coordinates/Altitude: 10,80669 N 85,35 W, 1074 m; 10,92286111 N 85,46375 W, 1018 m.

Figure 8. Plant Gall morphotypes recorded in Área de Conservación Guanacaste (ACG), Guanacaste, Costa Rica. 8A) Gall induced in *Psychotria valerioana* Standl., morphotype Ps_va_1, 8B) Gall induced in *Psychotria valerioana* Standl., morphotype Ps_va_2, 8C) Gall induced in *Psychotria valerioana* Standl., morphotype Ps_va_3, 8D) Gall induced in *Randia monantha* Benth., morphotype Ra_mo_1, 8E) Gall induced in *Zanthoxylum sp.* L., morphotype Za_sp_1, 8F) Gall induced in *Meliosma glabrata* (Liebm.) Urb., morphotype Me_gl_1, 8G) Gall induced in *Casearia arguta* Kunth, morphotype Ca_ar_1, 8H) Gall induced in *Casearia arguta* Kunth, morphotype Ca_ar_2, 8I) Gall induced in *Allophylus racemosus* (Sw.), morphotype Al_ra_1, 8J) Gall induced in *Allophylus racemosus* (Sw) , morphotype Al_ra_2, 8K) Gall induced in *Allophylus racemosus* (Sw) , morphotype Al_ra_3, 8L) Gall induced in Paullinia cururu L., morphotype Pa_cu_1, 8M) Gall induced in *Serjania pyramidata* Radlk., morphotype Se_py_1, 8N) Gall induced in Serjania schiedeana Schltdl., morphotype Se_sc_1, 8O) Gall induced in *Thouinidium decandrum* (Hump & Band), morphotype Th_de_1, 8P) Gall induced in *Sideroxylon obtusifolium* (Roem & Schult), morphotype Si_ob_1.

Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Psychotria valerioana Standl. Morphotype Ps_va_3 (Figure 8C). Gall description: Globular shape, greenish and brown, induced on leaf midvein, glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Shrub, nearly 1 m tall, greenish fruit, white flowers. Location: Guanacaste, Liberia, Mayorga. Guanacaste Conservation Area, Cacao Volcano Sector, pasture area next to the forest and start of the trail to the biological station. Coordinates/Altitude: 10,92286111 N 85,46375 W, 1018 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Randia monantha Benth. Morphotype Ra_mo_1 (Figure 8D). Gall description: Irregular shape, yellowish green (concolorous) on the surface, pale green on the underside, near veins on the underside, protruding from the abaxial surface, hairy epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Small tree, nearly 6 m tall, barren. Location: Guanacaste, Liberia, Nacascolo. Entrance to the old forest (*Bosque Viejo*), between the welcome booth and *La Casona* at Santa Rosa National Park. Coordinates/Altitude: 10,85072 N 85,60796 W, 321 m; 10,83581 N 85,62347 W, 306 m; 10,83382 N 85,61269 W, 307 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Rutaceae

Zanthoxylum sp. L. Morphotype Za_sp_1 (Figure 8E). Gall description: Irregular shape, green and yellow, induced on leaves, bulging on the leaf border, glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Tree, nearly 3 m tall, barren, and fallen. Location: Guanacaste, Liberia, Curubandé. Rincón de la Vieja National Park, road to active crater, Los Gemelos Sector, forest zone. Coordinates/Altitude: 10,80032 N 85,35 W, 1000 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Sabiaceae

Meliosma glabrata (Liebm.) Urb. Morphotype Me_gl_1 (Figure 8F). Gall description: Discoid shape, green and yellow, induced on leaves, sometimes with black dots, rough, on both the surfaces of the leaves, glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Sapling, nearly 1.5 m tall, barren. Oblong shaped leaves, with cuspidate apex. Location: Guanacaste, Liberia, Curubandé. Rincón de la Vieja National Park, road to active crater, Los gemelos Sector, forest zone. Coordinates/Altitude: 10,80032 N 85,35 W, 1000 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world, although a gall on this plant species was reported, without a reference image, by Ley-López et al. (2019).

Salicaceae

Casearia arguta Kunth. Morphotype Ca_ar_1 (Figure 8G). Gall description: Irregular shape, green, induced on leaves, glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: Entedoninae (Eulophidae). Plant description: Sapling, nearly 4 m tall, barren. Alternate, simple leaves, with crenate margin. Location: Guanacaste, Liberia, Curubandé. Rincón de la Vieja National Park, road to active crater, Los Gemelos Sector, forest zone. Coordinates/Altitude: 10,80669 N 85,35 W, 1074 m; 10,92286111 N 85,46375 W, 1018 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Casearia arguta Kunth. Morphotype Ca_ar_2 (Figure 8H). Gall description: Elliptical shape, light brown mixed with green, induced on stem, lignified epidermis. Gall inducer: unknown. Parasitoids/Inquilines: Entedoninae (Eulophidae). Plant description: Sapling, nearly 4 m tall, barren. Alternate, simple leaves, with crenate margin. Location: Guanacaste, Liberia, Curubandé. Rincón de la Vieja National Park, road to active crater, Los Gemelos Sector, forest zone. Coordinates/Altitude: 10,80669 N 85,35 W, 1074 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Sapindaceae

Allophylus racemosus Sw. Morphotype Al_ra_1 (Figure 8I). Gall description: Globular shape, green-white, induced on leaves, hairy epidermis. Gall inducer: Cecidomyiidae. Parasitoids/Inquilines: unknown. Plant description: Small tree, nearly 4 m tall, barren. Location: Guanacaste, La Cruz, Santa Elena. Guanacaste Conservation Area, Murciélago Sector, old secondary growth, road between *El Hachal* bay and *Murciélago* Station. Coordinates/Altitude: 10,92638889 N 85,7292 W, 45 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Allophylus racemosus Sw. Morphotype Al_ra_2 (Figure 8J). Gall description: Spherical shape, green or yellowish, induced on leaf midvein and petiole, hairy epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Shrub, nearly 2.5 m tall, barren. Location: Guanacaste, La Cruz, La Cruz. Guanacaste National Park, on the way to the Maritza Biological Station, secondary growth area along the river. Coordinates/Altitude: 10,95072222 N 85,59705556 W, 272 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Allophylus racemosus Sw. Morphotype Al_ra_3 (Figure 8K). Gall description: Globular shape, green and yellowish, induced on petiole, hairy epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Shrub, nearly 4 m tall, barren. Location: Guanacaste, La Cruz, La Cruz. Guanacaste National Park, on the way to the Maritza Biological Station, secondary growth area along the river. Coordinates/Altitude: 10,95072222 N 85,59705556 W, 272 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Paullinia cururu L. Morphotype Pa_cu_1 (Figure 8L). Gall description: Discoid shape, green-yellowish and brown, induced on leaves, glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Understory, scandent, herbaceous plant, nearly 3 m tall. Location: Guanacaste, Liberia, Curubandé. Guanacaste National Park, Las Pailas Sector, in the forest after the fumaroles. Coordinates/Altitude: 10,76955556 N 85,34519444 W, 744 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world, although a gall on this plant species was reported, without a reference image, by Cuevas et al. (2014).

Serjania pyramidata Radlk. Morphotype Se_py_1 (Figure 8M). Gall description: Globular shape, brown, induced on stem, lignified epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Vine, barren. Location: Guanacaste, Liberia, Nacascolo. Santa Rosa National Park, between the Inter-American highway and *La Casona*, secondary oak forest area. Coordinates/Altitude: 10,86577222 N 85,60990278 W, 290 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Serjania schiedeana Schltdl. Morphotype Se_sc_1 (Figure 8N). Gall description: Discoid shape, yellow or brown, induced on leaves, glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Sapling, nearly 25 cm tall, barren. Rhomboid shaped leaves, with cuspidate apex and denticulate margin, trifoliate/ternate leaves. Location: Guanacaste, Liberia, Nacascolo. Santa Rosa National Park, next to the research laboratories, old secondary forest area. Coordinates/Altitude: 10,83926 N 85,61808 W, 310 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Thouinidium decandrum Hump & Band. Morphotype Th_de_1 (Figure 8O). Gall description: Discoid shape, green to black, induced on leaves, glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Small tree, nearly 3 m tall, barren. Imparipinnate, alternate, compound leaves, with slightly serrate margin. Location: Guanacaste, Liberia, Nacascolo. Guanacaste National Park, Naranjo Beach Area, mangrove and beach area, coastal area at dry-forest. Coordinates/Altitude: 10,77928 N 85,6654 W, 14 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Sapotaceae

Sideroxylon obtusifolium (Humb. ex Roem. & Schult.) T.D. Penn. Morphotype Si_ob_1 (Figure 8P). Gall description: Fusiform shape, brown, induced on stem, lignified epidermis. Gall inducer: Cecidomyiidae. Parasitoids/Inquilines: unknown. Plant description: Small tree, nearly 3 m tall, simple axillary white flowers, unripe fruits (green). Location: Guanacaste, La Cruz, Santa Elena. Guancaste National Park, Murciélago Sector, old secondary forest area, on serpentinized peridotite on the edge of the Murciélago River. Coordinates/Altitude: 10,89686111 N 85,7301111 W, 126 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Sideroxylon obtusifolium (Humb. ex Roem. & Schult.) T.D. Penn. Morphotype Si_ob_2 (Figure 9A). Gall description: Irregular shape, green, induced on leaves (midvein), glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Small tree, nearly 3 m tall, simple axillary white flowers, unripe fruits (green). Location: Guanacaste, La Cruz, Santa Elena. Guanacaste National Park, Murciélago Sector, old secondary forest area, on serpentinized peridotite on the edge of the Murciélago River. Coordinates/Altitude: 10,89686111 N 85,7301111 W, 126 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Sideroxylon obtusifolium (Humb. ex Roem. & Schult.) T.D. Penn. Morphotype Si_ob_3 (Figure 9B). Gall description: Conical shape, green and yellowish, induced on fruit, glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Small tree, nearly 3 m tall, simple axillary white flowers, unripe fruits (green). Location: Guanacaste, La Cruz, Santa Elena. Guancaste National Park, Murciélago Sector, old secondary forest area, on serpentinized peridotite on the edge of the Murciélago River. Coordinates/Altitude: 10,89686111 N 85,7301111 W, 126 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Sideroxylon obtusifolium (Humb. ex Roem. & Schult.) T.D. Penn. Morphotype Si_ob_4 (Figure 9C). Gall description: Elliptical shape, brown and yellow-green, induced on stem, bulging galls on thorns, lignified epidermis. Gall inducer: unknown. Parasitoids/Inquilines: *Cecidellis* (Pteromalidae). Plant description: Small tree, nearly 3 m tall, simple axillary white flowers, unripe fruits (green). Location: Guanacaste, La Cruz, Santa Elena. Guancaste National Park, Murciélago Sector, old secondary forest area, on serpentinized peridotite on the edge of the Murciélago River. Coordinates/Altitude: 10,89686111 N 85,7301111 W, 126 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Sideroxylon obtusifolium (Humb. ex Roem. & Schult.) T.D. Penn. Morphotype Si_ob_5 (Figure 9D). Gall description: Globular shape, green, induced on inflorescence, glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Small tree, nearly 3 m tall, simple axillary white flowers, unripe fruits (green). Location: Guanacaste, La Cruz, Santa Elena. Guancaste National Park, Murciélago Sector, old secondary forest area, on serpentinized peridotite on the edge of the Murciélago River. Coordinates/Altitude: 10,89686111 N 85,7301111 W, 126 m. Registry comments: Gall morphotype resembles the one described by Rodrigues et al. (2014).

Simaroubaceae

Simarouba glauca DC. Morphotype Si_gl_1 (Figure 9E). Gall description: Irregular shape, green and yellowish, induced on leaves (foliar midrib), glabrous epidermis. Sometimes twisting the leaf. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Small tree, nearly 4 m tall, barren. Location: Guanacaste, Liberia, Mayorga. Santa Rosa National Park, lookout point between the main entrance of the park and *La Casona*. Coordinates/Altitude: 10,85648 N 85,6106 W, 323 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Smilacaceae

Smilax spinosa Mill. Morphotype Sm_sp_1 (Figure 9F). Gall description: Discoid shape, green and yellowish, induced on leaves, glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: Eurytomidae. Plant description: Vine on shrubs, barren. Location: Guanacaste, Liberia, Mayorga. Guanacaste Conservation Area, Cacao Volcano Sector, Cacao Mountain range. Coordinates/Altitude: 10,93202778 N 85,46 W, 1421 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Solanaceae

Lycianthes multiflora. Morphotype Ly_mu_1 (Figure 9G). Gall description: Fusiform shape, brown, induced on leaf veins, glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Herbaceous vine on shrub. Alternate leaves. Location:

Figure 9. Plant Gall morphotypes recorded in Área de Conservación Guanacaste (ACG), Guanacaste, Costa Rica. 9A) Gall induced in *Sideroxylon obtusifolium* (Roem & Schult), morphotype Si_ob_2, 9B) Gall induced in *Sideroxylon obtusifolium* (Roem & Schult), morphotype Si_ob_3, 9C) Gall induced in *Sideroxylon obtusifolium* (Humb. ex Roem. & Schult.) T.D. Penn., morphotype Si_ob_4, 9D) Gall induced in *Sideroxylon obtusifolium* (Humb. ex Roem. & Schult.) T.D. Penn., morphotype Si_ob_5, 9E) Gall induced in *Simarouba glauca* DC, morphotype Si_gl_1, 9F) Gall induced in *Smilax spinosa* Mill., morphotype Sm_sp_1, 9G) Gall induced in *Lycianthes multiflora*, morphotype Ly_mu_1, 9H) Gall induced in Stegnosperma cubense A. Rich, morphotype St_cu_1, 9I) Gall induced in Stegnosperma cubense A. Rich, morphotype St_cu_2, 9J) Gall induced in Stegnosperma cubense A. Rich, morphotype St_cu_3, 9K) Gall induced in Stegnosperma cubense A. Rich, morphotype St_cu_4, 9L) Gall induced in *Stegnosperma cubense* A. Rich, morphotype St_cu_5, 9M) Gall induced in *Urera simplex* Wedd., morphotype Ur_si_1, 9N) Gall induced in *Lippia oxyphyllaria* (Donn. Sm.) Standl., morphotype Li_ox_1, 9O) Gall induced in *Rehdera trinervis* (S.F. Blake) Moldenke, morphotype Re_tr_1, 9P) Gall induced in *Rehdera trinervis* (S.F. Blake) Moldenke, morphotype Re_tr_2.

Guanacaste, Liberia, Curubandé. Rincón de la Vieja National Park, road to active crater, Los Gemelos Sector, rainforest zone. Coordinates/Altitude: 10,80032 N 85,35 W, 1000 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world, although a gall induced on this plant species was reported, without a reference image, by Ley-López et al. (2019).

Stegnospermataceae

Stegnosperma cubense A. Rich. Morphotype St_cu_1 (Figure 9H). Gall description: Fusiform shape, grayish, induced on stem, lignified epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Decumbent shrub, nearly 2.5 m and 1m tall, barren. Location: Guanacaste, Liberia, Nacascolo. Guanacaste National Park, Naranjo Beach Area, mangrove and area, coastal area in dry-forest. Coordinates/Altitude: 10,7791389 N 85,66594444 W, 14 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Stegnosperma cubense A. Rich. Morphotype St_cu_2 (Figure 9I). Gall description: Discoid shaped galls, green, induced on leaves, glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: Eulophidae. Plant description: Decumbent shrub, nearly 2.5 m and 1m tall, barren. Location: Guanacaste, Liberia, Nacascolo. Guanacaste National Park, Naranjo Beach Area, mangrove and beach area, coastal area in dry-forest. Coordinates/Altitude: 10,77928 N 85,6654 W, 14 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Stegnosperma cubense A. Rich. Morphotype St_cu_3 (Figure 9J). Gall description: Globular shape, light green, induced on leaves, glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Decumbent shrub, nearly 2.5 m and 1m tall, barren. Location: Guanacaste, Liberia, Nacascolo. Guanacaste National Park, Naranjo Beach Area, mangrove and beach area, coastal area in dry-forest. Coordinates/Altitude: 10,7791389 N 85,66594444 W, 14 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Stegnosperma cubense A. Rich. Morphotype St_cu_4 (Figure 9K). Gall description: Discoid shape, yellow to greenish, induced on leaves, glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Decumbent shrub, nearly 2.5 m and 1m tall, barren. Location: Guanacaste, La Cruz, Santa Elena. Guanacaste Conservation Area, Murciélago Sector, old secondary growth, road between El Hachal Bay and Murciélago Station. Coordinates/Altitude: 10,92638889 N 85,7292 W, 45 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Stegnosperma cubense A. Rich. Morphotype St_cu_5 (Figure 8L). Gall description: Globular shape, white to greenish, induced on the underside of the leaf, glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Decumbent shrub, nearly 2.5 m and 1m tall, barren. Prominent globularl galls,. Location: Guanacaste La Cruz, Santa Elena. Guanacaste Conservation Area, Murciélago Sector, old secondary growth, road between El Hachal Bay and Murciélago Station. Coordinates/Altitude: 10,92638889 N 85,7292 W, 45 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Urticaceae

Urera simplex Wedd. Morphotype Ur_si_1 (Figure 9M). Gall description: Irregular shape, green and yellow, induced on leaves, distributed throughout the upper leaf surface and underside, glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Shrub, nearly 3 m tall, inflorescence with white stamens, pink petals and pedicels. Location: Guanacaste, Liberia, Curubandé. Rincón de la Vieja National Park, road to active crater, Los gemelos Area, rainforest zone. Coordinates/Altitude: 10,80032 N 85,35 W, 1000 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Verbenaceae

Lippia oxyphyllaria (Donn. Sm.) Standl. Morphotype Li_ox_1 (Figure 9N). Gall description: Spherical shape, light green, induced on leaves (midvein or secondary veins), hairy epidermis. Gall inducer: Cecidomyiidae. Parasitoids/Inquilines: unknown. Plant description: Herbaceous plant or shrub, up to 2 m tall, barren. Location: Guanacaste, Liberia, Nacascolo. Santa Rosa National Park, Santa Rosa Area, between the entrance of the Inter-American highway and *La Casona*, secondary oak forest area. Coordinates/Altitude: 10,86577222 N 85,60990278 W, 290 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Rehdera trinervis (S.F. Blake) Moldenke. Morphotype Re_tr_1 (Figure 9O). Gall description: Elliptical shape, green and brown, induced on leaf midvein, glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Small woody sapling, nearly 3 m tall, strong secondary growth. Location: Guanacaste, Liberia, Nacascolo. Santa Rosa National Park between the Inter-American highway and *La Casona*, secondary oak forest area. Coordinates/Altitude: 10,86577222 N 85,60990278 W, 290 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Rehdera trinervis (S.F. Blake) Moldenke. Morphotype Re_tr_2 (Figure 9P). Gall description: Discoid shape, green and yellowish, induced on leaves, glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Small woody sapling, nearly 3 m tall, strong secondary growth. Location: Guanacaste, Liberia, Nacascolo. Santa Rosa National Park between the Inter-American highway and *La Casona*, secondary oak forest area. Coordinates/Altitude: 10,86577222 N 85,60990278 W, 290 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Vitaceae

Cissus fuliginea Croat. Morphotype Ci_fu_1 (Figure 10A). Gall description: Fusiform shape, green to yellowish green, induced on stem and leaf veins, hairy epidermis. Gall inducer: Cecidomyiidae. Parasitoids/Inquilines: unknown. Plant description: Vine, near by the mud pits. Tropical dry forest area. Trifoliate leaves with serrate margin and tendrils. On top of *Sweetenia*, barren. Location: Guanacaste, Liberia, Nacascolo. Administrative area of the National Park. Coordinates/Altitude: 10,83694444 N 85,62 W, 406 m; 10,76955556 N 85,34519444 W, 744 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

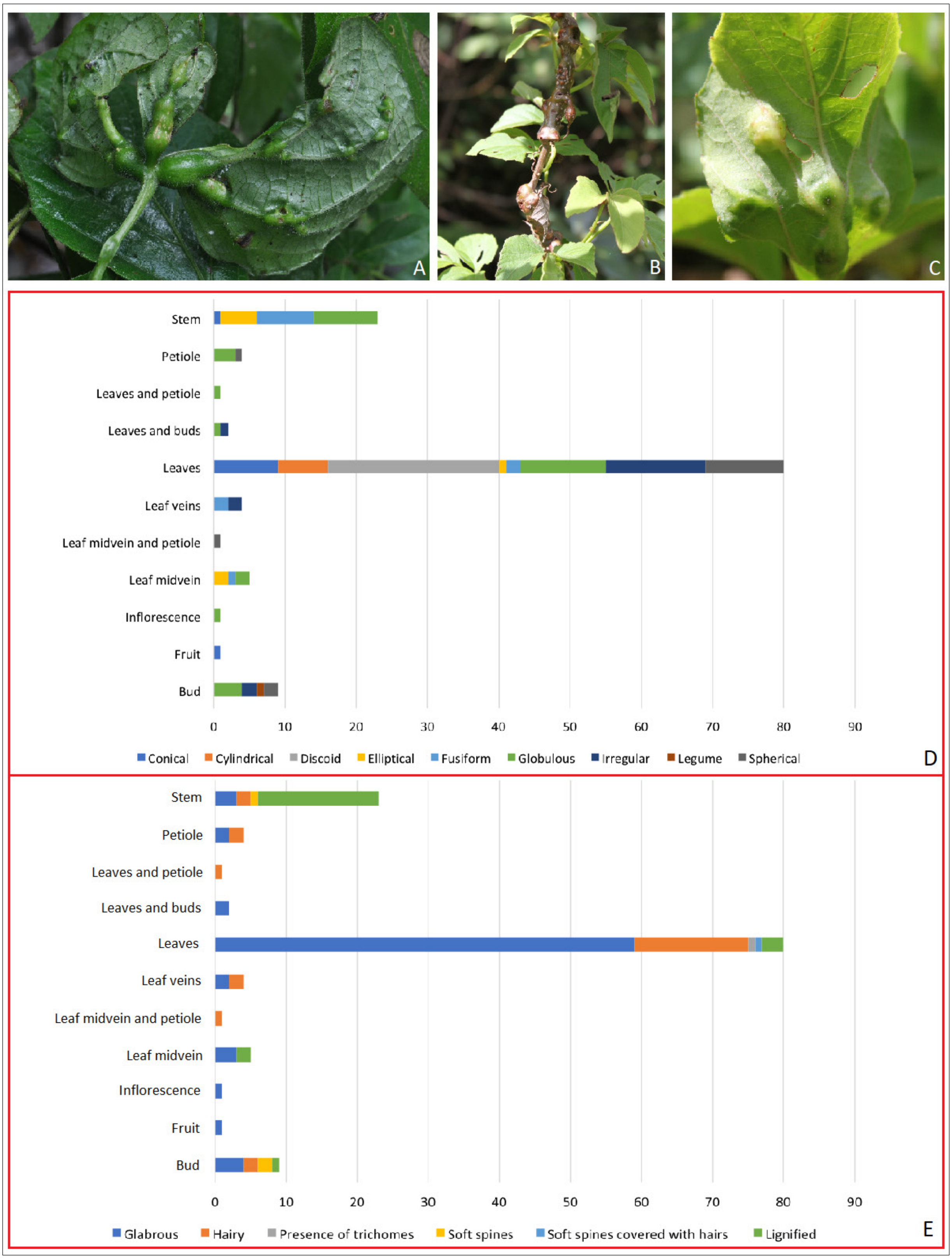

Figure 10. Plant Gall morphotypes recorded in Área de Conservación Guanacaste (ACG), Guanacaste, Costa Rica. 10A) Gall induced in *Cissus fuliginea* (Croat.), morphotype Ci_fu_1, 10B) Gall induced in *Cissus fuliginea* (Croat.), morphotype Ci_fu_2, 10C) Gall induced in *Cissus fuliginea* (Croat.), morphotype Ci_fu_3, 10D) Gall morphology vrs incidence in plant organs, 10E) Gall epidermis texture and their incidence by plant organ. For 10D and 10E, the values on the X-axis represent the total number of morphotypes.

Cissus fuliginea Croat. Morphotype Ci_fu_2 (Figure 10B). Gall description: Fusiform shape, reddish-brown or green, induced on stem, lignified epidermis. Gall inducer: unknown. Parasitoids/Inquilines: unknown. Plant description: Vine, near by the mud pits. Tropical dry forest area. Trifoliate leaves with serrate margin and tendrils. On top of *Sweetenia*, barren. Location: Guanacaste, Liberia, Mayorga. Guanacaste Conservation Area, Góngora, road to the Cacao Volcano, sedimentary rock area next to the Góngora River. Coordinates/Altitude: 10,83422222 N 85,6115 W, 569 m; 10,76955556 N 85,34519444 W, 744 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

Cissus fuliginea Croat. Morphotype Ci_fu_3 (Figure 10C). Gall description: Irregular shape, green to yellowish green, induced on leaf veins, glabrous epidermis. Gall inducer: unknown. Parasitoids/Inquilines: Tetrastichinae (Eulophidae). Plant description: Vine, near by the mud pits. Tropical dry forest area. Trifoliate leaves with serrate margin and tendrils. On top of *Sweetenia*, barren, exposed. Location: Guanacaste, Liberia, Nacascolo. Santa Rosa National Park, start of the road to Naranjo Beach, old secondary growth area with clearings. Coordinates/Altitude: 10,83581 N 85,62347 W, 306 m; 10,83422222 N 85,6115 W, 569 m. Registry comments: First gall morphotype record for Guanacaste Conservation Area, Costa Rica, and the world.

From the 131 gall morphotypes recorded, the leaves were the organ with the largest number of galls with sixty-one percent (n=80), followed by stems (17.6%), and apical buds (6.9%) (Figure 10D). Eight different morphotype forms of galls were identified: conical, cylindrical, discoid, elliptical, fusiform, globular, irregular, and spherical. The most frequent gall shape was globular (*globulous*) (25.2%), followed by discoid (18.3%), irregular (14.5%), spherical (11.5%) and conical with 8.4%. The greatest number of gall shapes on leaves were discoid (30%), followed by irregular (17.5%), globular (15%), and spherical (14%) (Figure 10D). We also described six different types of gall epidermal texture: hairy, presence of trichomes, glabrous, soft spines, soft spines covered with hairs, and lignified. Fifty-nine percent of galls were glabrous, twenty-one percent were hairy and eighteen were lignified, which were among the most abundant. The most common epidermal texture on leaves was glabrous (77%), as well as hairy about 61%). Lignified epidermis (74% prevalence) was the most frequent texture on stems (Figure 10E). Green was the predominant color of galls, with 27%.

Except for 11 records, 120 of all recorded galls are new records for ACG, Costa Rica and the world. The collected plant gall morphotypes and their associated organisms from the Guanacaste Conservation Area, were characterized and recorded in a biological database within a specialized herbarium. We named this integrated herbarium a cecidiarium.

When we compare trichome-like structures and lignified external texture in the recorded gall morphotypes between the deciduous and evergreen forest (*Bosque Viejo*), 55% of the total morphotypes exhibit trichome-like structures and 43.5% has lignified texture in the evergreen forest. The chi-square test did not show significant statistical differences between the two types of forest for these two phenotypic variables (p>0.05, $\chi^{2}_{(\alpha=0,.05,\ df=1)=}$ 3.84).

Discussion

A large number of galling insects have been described in recent years and estimates clearly suggest that the number of species yet to be described could significantly exceed the number of known species (Espírito-Santo & Fernandes 2007). New gall morphotypes have been reported continuously, especially from Brazil, mainly as a consequence of an active community of researchers in that country.

Based on the reviewed literature, it appears that 120 morphotypes described and registered in this study are new records, not only for Costa Rica, but also for the world. Although samples were collected in the rainy season, when plants have a greater amount of green biomass, we estimate that the galls recorded here represent a small portion of the total plant galls present in the Guanacaste Conservation Area. This is based on the higher plant diversity and topographic characteristics of the AGC, as well as the difficulties in searching and obtaining samples from the canopy, particularly in tall trees like those in the *Bosque Viejo* (old forest). The canopy is the forest stratum with the greatest active growth, and so this is an area where it would be more probable to find galls. Additionally, many galls are hidden inside fruits and other plant organs such as roots (Ley-López et al. 2019), and the high plant density interferes with searching for and finding galls.

Most of the galling species in the Mexican tropical dry forest, for instance, occurred on trees and shrubs, with fewer on herbs and climbing plants (Cuevas-Reyes et al. 2014). For the ACG tropical dry forest, the panorama was similar, based on our results. Moreover, in the tropical dry forest of the Caatinga (Pernambuco, Brazil), most galls were induced on leaves (73.44%), stems (20.31%), and on apical buds (6.25%) (Santos et al. 2011b). These results were similar to data obtained for ACG, where 61% of galls were formed on leaves, followed by stems (17.6%), and on apical buds (6.9%) (Figure 10D). In contrast to the Caatinga, where the most frequent gall shape was spheroid (32.81%), followed by discoid with 25% (Santos et al. 2011b), for ACG the most frequent gall shape was globular (25.2%), but, the percentage of discoid galls was similar with 18.3% (the second one in frequency). Furthermore, glabrous epidermis shows the highest frequency in both tropical dry forests, with 78% for the Caatinga and 77% for the ACG, surprisingly similar values. Additionally, green was the predominant surface color of galls with 27% for ACG, while it was 73% for the Caatinga (Santos at al. 2011b). Moreover, as in the ACG, for the tropical dry forest of Parque da Sapucaia-Brazil (Costa & Araújo 2019), the plant family with the greatest richness of gall morphotypes was Fabaceae. Similarly, the most affected plant organ was the leaf (82.2%). Otherwise, globular (20.6%) and discoid (13.7%) were the most abundant gall shapes in the aforementioned study, which are comparable with the values for ACG (globular 25.2% and discoid with 18.3%). Glabrous was the predominant external texture with 82.7% and green the most frequent gall color (44.8%) in the tropical dry forest of Parque da Sapucaia, while in the ACG 59% of the galls were glabrous and 27% were green.

The presence of trichomes on gall epidermis ("hairy" or pilose) could be important due to the known properties of these anatomical structures in protecting plants against insect attack (especially the phytophagous insects), temperature regulation, and drought resistance by reducing water loss and moisture retention. Moreover, accumulated evidence suggests that trichomes can absorb UV radiation and reduce the damage by UV-B to photosystem II, preventing stomatal closure (Xiao et al. 2017). Lignins have been associated with plant mechanical support, growth, resistance to insect pests, temperature regulation, and drought tolerance, among other functions (Liu et al.2018). Leaving aside the hypothesis that tissue lignification protects the plant against natural enemies, in galls lignification has also been associated with water conduction (Guedes et al. 2019), as well as protection from UV and oxidative damages generated by excess light exposure and water deficiency (Detoni et al. 2011, Arriola et al. 2018). Our data from ACG shows that only 21% of morphotypes exhibit trichomes on the epidermis while 17.6% of gall morphotypes were lignified, which represents less than expected according to the adaptive advantages attributed to both phenotypic traits. Nevertheless, the lignified epidermis was the most frequent texture in stem galls with 74% (Figure 10E). Given the function that both anatomical traits could have as a selective response to abiotic conditions, when we compare the deciduous and evergreen forest (e.g. *Bosque Viejo*), our results contrast with what might be expected, since in the evergreen forest 55% of the total morphotypes showed trichome-like structures and 43.5% of all galls exhibit lignified external texture ($p>0.05$, $\chi^{2}_{(\alpha=0,.05,\ df=1)} = 3.84$). Hence, the values for these two phenotypic variables did not show significant statistical differences between the two types of forest. The role of lignified and trichome-covered galls in different biomes of the tropical dry forest are still open questions that should be studied in greater detail.

The low success rate in obtaining adult gall-inducers from a given sample can be explained by the lack of knowledge of insect and gall phenologies. The most practical way of increasing the success rate of rearing adults is probably by collecting the same gall morphotype throughout the year since many galls are seasonal. Furthermore, multiple samples would be needed from each plant species, a task that is frequently not possible because of the time and resources required.

In recent years new gall morphotypes are being reported continuously (Nieves-Aldrey et al. 2008, Hanson et al. 2014), Goetz et al. 2018, Araújo et al. 2019, Costa & Araújo 2019). The evidence from these new reports suggests that gall diversity is directly correlated with the richness of plant species in the areas under study (Cuevas-Reyes et al. 2014, Araújo 2017, Coelho et al. 2017). Thus, the previous assumption that gall diversity in tropical regions is less than that in xeric temperate regions is not sustainable considering the biodiversity in tropical ecosystems and the results of the latest studies on the diversity of galls in tropical regions. Cuevas-Reyes et al. (2004) found a significant positive correlation between gall-inducing insect species richness and plant species abundance in a Mexican tropical dry forest.

Given the above evidence that future inventories in tropical regions will continue to discover an increasing diversity of gall morphotypes, we proposed organizing the plant galls and associated organisms in a specialized herbarium. This allowed us to have wet and dry collections of plant samples, and preserved specimens of the organisms associated with the galls, in combination with physical records and digital databases providing detailed information about the collected morphotypes, including proper image registration. A detailed repository for the inventory will avoid failures in gall descriptions as well as prevent mixing samples and information from plant galls with other unrelated data. For instance, Ley-López et al. (2019), reported that around 25% of plant specimens previously recorded with galls in the herbarium were ambiguous as to whether the tissue alteration was a gall or not. The specialized herbarium established as a direct consequence of this research represents a unique kind of biological collection different from a traditional plant herbarium, and for this reason the name cecidiarium was suggested as a general concept for summarizing these types of biological records. Considering the diversity of plant galls, this effort represents an important reservoir of germplasm that should be conserved under proper conditions.

Despite the increasing number of new gall morphotypes being reported, some problems still remain beforeachieving a standardized classification. Problems include poor gall morphotype characterization, lack of referenced photos or poor quality images, and the non-existence of a standardized nomenclature. Here, we propose a standardized codification system for plant gall morphotype classification, with the aim of avoiding confusion in the registry of these structures. Gall morphotypes were named using the two first letters from the binomial scientific name of the host plant, followed by the numerical order in which they were discovered (Figures 2-10).

Detailed maps with geographic coordinates, altitudinal distribution, forest cover, and other data are useful tools for understanding the ecological context of gall occurrence. Therefore, georeferenced maps with an appropriate data will allow us to pinpoint and contrast host plant distribution with insect-inducer distribution and habitat (Figure 11).

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Author Contributions

Omar Gätjens-Boniche: substantial contribution in the concept and design of the study; contribution to data collection; contribution to data analysis and interpretation; contribution to the data registration and processing; obtained the financial support and wrote the article.

Paul Hanson: contribution in the concept and design of the study; contribution to critical revision, adding intellectual content; contribution to manuscript preparation; contribution to identify the inductor and insects associated with plant galls.

Marilyn Sanchez-Valverde: contribution to the data registration and processing; contribution to manuscript preparation.

Carla Trejos-Araya: contribution to data analysis and interpretation; contribution to critical revision; contribution to manuscript preparation.

Roberto Espinoza: substantial contribution in the taxonomic identifications of the host plants.

Adrian Pinto-Tomás: contribution to critical revision, adding intellectual content.

Conflicts of Interest

The authors declare that they have no conflict of interest related to the publication of this article.

Ethics

Host plants and their galls were collected under the permission of the Comisión Nacional para la Gestión de la Biodiversidad (CONAGEBIO) of Costa Rica.

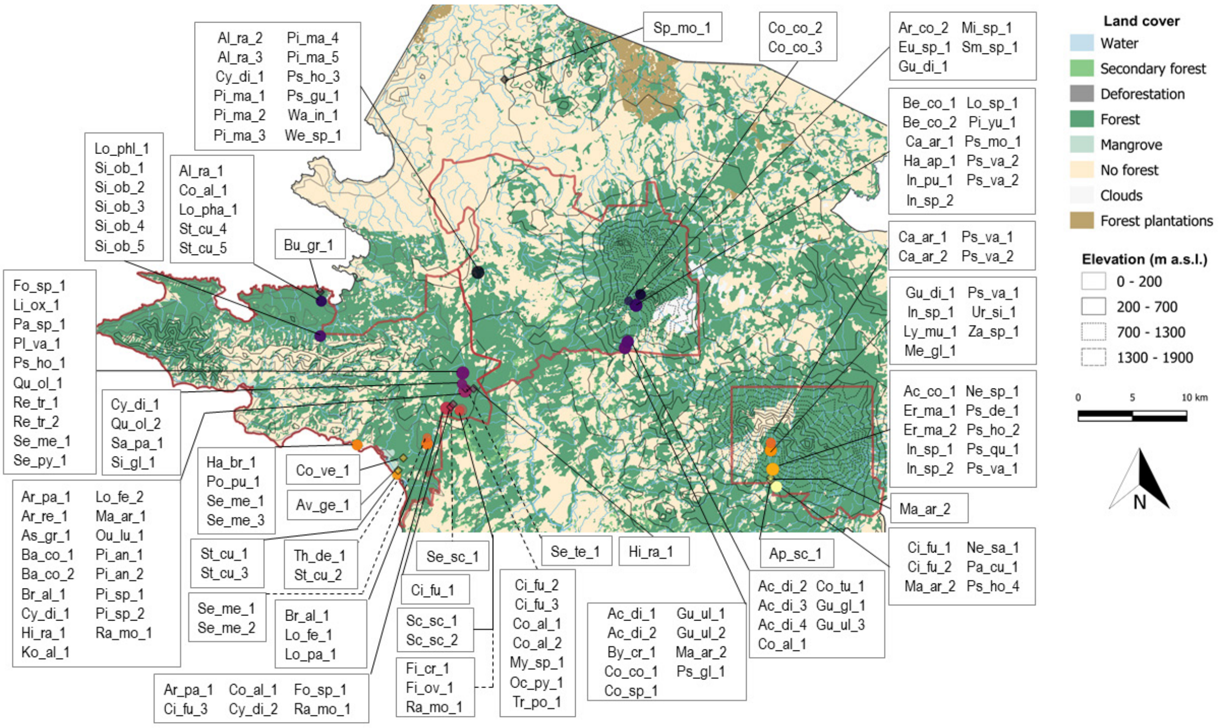

Figure 11. Spatial distribution of gall morphotypes collected in Área de Conservación Guanacaste (ACG), Guanacaste province, northwest region of Costa Rica. Diamond symbols represent sites were only one gall morphotype was collected. Circle symbols represent cluster sites where two or more gall morphotypes were found; circle diameters vary according to the number of gall morphotypes collected in this area. Larger circles mean that more gall morphotypes were found at a specific area. Cluster size varies between 5-400 linear meters.

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Capítulo 3

Mecanismo de inducción de las agallas de plantas inducidas por insectos: evidencias que respaldan un proceso de transformación genética y el papel de bacterias endofíticas involucradas en el proceso de inducción, formación y crecimiento de esta estructura.

Descripción

Si bien es cierto que diferentes organismos tienen la capacidad de inducir agallas en las plantas, las agallas inducidas por insectos son las más elaboradas y diversas de todas las reportadas (Shorthouse & Rohfritsch 1992; Raman, 2011; Raman, 2021). Por otro lado, la hipótesis que involucra una posible inducción química a través de fitohormonas o sustancias similares (McCalla et al., 1961; Miles, 1968; Rohfritsch y Shorthouse, 1982; Hori, 1992; Leitch, 1994; Ananthakrishnan, 1998; Raman, 2011; Tooker y Helms, 2014; Bailey et al., 2015; Oates et al., 2016, Giron et al., 2016; Cambier et al., 2019; Korgaonkar et al., 2021; Ponce et al., 2021; Takeda et al., 2021; Desnitskiy et al., 2023), o bien por medio de proteínas efectoras por parte del insecto (Zhao et al., 2015; Cambier et al., 2019; Zhao et al., 2019; Korgaonkar et al., 2021), ha sido ampliamente aceptada como el probable mecanismo general de inducción de las agallas de plantas inducidas por insectos. Sin embargo, en la literatura revisada sobre este tema a la fecha, no existen estudios que relacionen el proceso de formación de las agallas de insectos con un posible mecanismo de inducción mediado por la inserción de elementos genéticos exógenos al genoma de la planta hospedera.

Existe la posibilidad de que el proceso de inducción y morfogénesis de las agallas de los insectos pueda estar bajo un estricto control genético, posiblemente mediado por la inserción de elementos genéticos móviles en el genoma de las células de las agallas de las plantas, elementos genéticos tales como plásmidos o transposones. Asimismo, ese proceso podría estar mediado por una bacteria endosimbiótica del insecto.

En este trabajo de investigación, se evaluó la hipótesis de que la hiperplasia característica de la fase inicial de la inducción de agallas podría desencadenarse por la inserción de elementos genéticos exógenos al genoma de las células vegetales, esto a través de una bacteria endosimbiótica que se originaría desde el insecto inductor. Para probar esta hipótesis, se empleó una combinación de análisis que involucran el uso de marcadores genéticos, secuenciación metagenómica y la inducción experimental de agallas bajo condiciones controladas. Los datos provenientes de la secuenciación genómica profunda de dos muestras de hojas sanas y dos grupos de agallas provenientes de las mismas plantas, fueron utilizados para identificar posibles elementos genéticos involucrados en la formación de la agalla de yuca y para analizar el microbioma de las agallas y del tejido sano circundante. Para ello se implementó un enfoque de discriminación de secuencias de ADN con el objetivo de excluir selectivamente entre el ADN exógeno y el genoma de la planta huésped de referencia (yuca). A partir de este enfoque experimental y la utilización de marcadores moleculares basados en la reacción en cadena de la polimerasa, se identificaron varias secuencias de inserción candidatas relacionadas con secuencias de genes conocidos. Las secuencias más significativas están relacionadas con genes asociados con bacterias, como el factor regulador de la transcripción CadR, la ATPasa transportadora de cadmio codificada por el gen *cadA*, una proteína permeasa involucrada en el transporte de nitrato (gen *nrtB*) y el gen *arsA* que codifica para la enzima arilsulfatasa A, involucrada en el procesamiento de esfingolípidos del subgrupo de las sulfátidas. Adicionalmente, por medio de marcadores de PCR se caracterizó un fragmento de ADN específico de agallas que podría constituir un elemento genético accesorio involucrado en el mecanismo de inducción de la agalla de yuca, el cual muestra similitud con el gen de la ubiquitina *E2*. Los datos metagenómicos generados proporcionaron también evidencias relacionadas con la modificación del microbioma endofítico en los tejidos que forman las agallas de *M. esculenta*.

Los resultados publicados como parte de este capítulo, aportaron por lo tanto evidencias que sugieren un mecanismo de inducción de las agallas de plantas inducidas por insectos que podría estar mediado por eventos de transformación genética en las células de la planta huésped. Los datos aluden a que un elemento genético móvil alojado en plásmidos de una bacteria del género *Rhodococcus* sp. podría estar involucrado en el mecanismo de inducción de la agalla inducida en plantas de yuca por el insecto *Iatrophobia brasiliensis*. Los resultados obtenido de la cuantificación de genes presentes en las secuencias específicas de los tejidos de las agallas, indican que las categorías COG (Categorías de grupos ortólogos funcionales, COG por sus siglas en inglés) de transporte y metabolismo de aminoácidos, transporte y metabolismo de carbohidratos, producción y conversión de energía, transporte y metabolismo de nucleótidos, modificación postraduccional, la traducción de la estructura ribosómica y la biogénesis, así como la replicación, recombinación y reparación del ADN fueron las principales categorías funcionales representadas. Estos resultados podrían indicar que las funciones relacionadas con el crecimiento, el transporte de metabolitos de las plantas a los tejidos internos de las agallas, la replicación y la expresión de ácidos nucleicos, además de la regulación genética en diferentes niveles, podrían ser promovidos o aumentados por el ADN exógeno detectado en las células de las agallas. La inducción de una estructura inicial similar a una agalla en tejidos cultivados de *M. esculenta* fue posible mediante ensayos de inoculación utilizando un aislamiento bacteriano del género *Rhodococcus*, obtenido a partir del insecto inductor. Además, se aportan evidencias que respaldan el papel de bacterias endofíticas involucradas en el proceso de inducción, formación y crecimiento de esta estructura.

En conjunto, los hallazgos encontrados respaldan el papel de la transformación genética y los microorganismos en la inducción de la agalla de yuca inducida por el insecto *I. brasiliensis*.

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Microbiome and plant cell transformation trigger insect gall induction in cassava

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Several specialised insects can manipulate normal plant development to induce a highly organised structure known as a gall, which represents one of the most complex interactions between insects and plants. Thus far, the mechanism for insect-induced plant galls has remained elusive. To study the induction mechanism of insect galls, we selected the gall induced by *Iatrophobia brasiliensis* (Diptera: Cecidomyiidae) in cassava (Euphorbiaceae: *Manihot esculenta* Crantz) as our model. PCR-based molecular markers and deep metagenomic sequencing data were employed to analyse the gall microbiome and to test the hypothesis that gall cells are genetically transformed by insect vectored bacteria. A shotgun sequencing discrimination approach was implemented to selectively discriminate between foreign DNA and the reference host plant genome. Several known candidate insertion sequences were identified, the most significant being DNA sequences found in bacterial genes related to the transcription regulatory factor CadR, cadmium-transporting ATPase encoded by the *cadA* gene, nitrate transport permease protein (*nrtB* gene), and arsenical pump ATPase (*arsA* gene). In addition, a DNA fragment associated with ubiquitin-like gene *E2* was identified as a potential accessory genetic element involved in gall induction mechanism. Furthermore, our results suggest that the increased quality and rapid development of gall tissue are mostly driven by microbiome enrichment and the acquisition of critical endophytes. An initial gall-like structure was experimentally obtained in *M. esculenta* cultured tissues through inoculation assays using a *Rhodococcus* bacterial strain that originated from the inducing insect, which we related to the gall induction process. We provide evidence that the modification of the endophytic microbiome and the genetic transformation of plant cells in *M. esculenta* are

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two essential requirements for insect-induced gall formation. Based on these findings and having observed the same potential DNA marker in galls from other plant species (ubiquitin-like gene *E2*), we speculate that bacterially mediated genetic transformation of plant cells may represent a more widespread gall induction mechanism found in nature.

KEYWORDS

***Iatrophobia brasiliensis*, *Manihot esculenta*, plant galls, metagenomics, induction mechanism, genetic transformation, endophytes**

Introduction

Insect galls are abnormal structures developed by the presence and stimuli of insects in the host plant. Insect-induced plant galls are specialised plant tissues with an organised arrangement of cells and predetermined growth. The size, structure, and metabolism of galls are under the control of gall-forming insects and host plant species (Rohfritsch and Shorthouse, 1982; Leitch, 1994; Raman, 2011).

Insect gall tissues exhibit biochemical and cytological modifications that provide them with a higher nutritional quality than the surrounding plant tissue, thus facilitating a continuous source of food and additional benefits to the inducing insect (Koyama et al., 2004; Nabity et al., 2013; Ferreira et al., 2017). Most galls contain highly specialised tissue known as nutritive tissue, characterised by high concentrations of sugar (Nogueira et al., 2018), lipids, proteins, nitrogen, and a variety of other compounds (Shorthouse and Rohfritsch, 1992; Huang et al., 2015; Isaias et al., 2018).

The ability to induce galls in plants has emerged several times among and within insect orders, with representatives of gall-inducing species currently known in Diptera, Hymenoptera, Hemiptera, Coleoptera, Lepidoptera, and Thysanoptera (Mani, 1992; Espírito-Santo and Fernandes, 2007). In each order, the ability to form such structures appears to have an independent origin (Nyman and Julkunen, 2000; Ronquist et al., 2015).

In addition to insects, plant galls can be induced by mites and nematodes (Favery et al., 2016; de Lillo et al., 2018; Harris and Pitzschke, 2020; Olmo et al., 2020; Desnitskiy et al., 2023). Some fungi and bacteria species and even some viruses can also induce primary gall-like growths or, simply, neoplasm formation with low levels of cell differentiation (Ananthakrishnan, 1998; Raman, 2011; Gätjens-Boniche, 2019; Harris and Pitzschke, 2020). Examples of gall-like growths induced by microorganisms include *Agrobacterium tumefaciens* (crown gall), *Rhodococcus fascians*, *Pseudomonas savastanoi*, *Xanthomonas citri*, *Pantoea agglomerans*, *Taphrina betulina* (witches broom), and *Ustilago esculenta* (Swarup et al., 1991; Jump and Woodward, 1994; Chalupowicz et al., 2009; You et al., 2011; Dolzblasz et al., 2018; Harris and Pitzschke, 2020). Of these, *A. tumefaciens* is the best studied, as its habit of genetically transforming plant cells has found extensive use in plant biotechnology (Kavipriya et al., 2019; Song et al., 2019; Lian et al., 2022). Because *A. tumefaciens*-mediated genetic transformation of plant cells is the most understood mechanism of plant gall formation (Chou et al., 2022; Hopp et al., 2022; Azizi-Dargahlou and Pouresmaeil, 2023), this constitutes the best referenced system to propose alternative induction mechanisms in which complex gall formation caused by insects such as cynips (Hymenoptera) and cecidomyiids (Diptera) (Sinnott, 1960; Raman, 2011) is also the result of a based plant cell genetic transformation.

A large number of endosymbiotic bacteria in different insect groups, including gall-inducing insects, have been reported in several studies (Campbell et al., 2015; El-Sayed and Ibrahim, 2015; Gutzwiller et al., 2015; Michell and Nyman, 2021; Yang et al., 2021; Coolen et al., 2022; Yang et al., 2022). Insect-associated microorganisms could be important mediators of interactions between insects and plants (Hammer and Bowers, 2015; Sugio et al., 2015; Wielkopolan and Jakubowska, 2021; Coolen et al., 2022). Symbiotic relationships between inducing insects and microorganisms have been hypothesised to be involved in plant gall development (Hansen and Moran, 2014; Tooker and Helms, 2014; Gätjens-Boniche, 2019; Klimov et al., 2022). Delivery of bacteria under natural or artificial conditions by insect vectors has been reported in many insect–plant interactions (Zeidan and Czosnek, 1994; Galambos et al., 2021; Wielkopolan and Jakubowska, 2021; Ratcliffe et al., 2022). Insect-vectored bacteria in plants have been described in well-known systems such as the Huanglongbing (HLB) disease of citrus caused by the phytopathogenic bacterium Candidatus *Liberibacter asiaticus* (CLas), which is transmitted by the psyllid *Diaphorina citri.* Likewise, acquisition and effective delivery of *A. tumefaciens* by the whitefly *Bemisia tabaci* was demonstrated by Zeidan and Czosnek (1994).

It has been hypothesised that phytohormone elicitor molecules delivered by the insect inducer (Rohfritsch and Shorthouse, 1982; Tooker and Helms, 2014; Ponce et al., 2021) or indirectly by an associated microorganism (Giron et al., 2013; Bartlett and Connor, 2014; Giron et al., 2016), and effector proteins secreted by the gall-inducing insect (Zhao et al., 2015; Cambier et al., 2019; Zhao et al., 2019; Korgaonkar et al., 2021) may be the main triggering stimuli responsible for the gall induction process.

Cassava (*Manihot esculenta* Crantz) is a widely cultivated crop in Africa, Asia, and Central and South America that provides an important food source for millions of people worldwide. Cassava plants can be grown yearlong in the tropics. Moreover, it can be easily propagated in greenhouse and *in vitro* conditions. Owing to its versatility and potential, plant breeding programmes and international consortiums have invested significant resources in understanding cassava genetics. This has led to the creation of genetic linkage maps and chromosome-scale genome assembly (Wang et al., 2014; ICGMC (International Cassava Genetic Map Consortium), 2015; Lyons et al., 2022). Cassava plants are subject to gall formation, particularly cylindrical galls induced by the Cecidomyiidae, *Iatrophobia brasiliensis* (Montaldo, 1977; Rivera Hernández, 2011). However, detailed studies are lacking on the induction and formation processes of this gall.

Here, we tested the hypothesis proposed by Gätjens-Boniche (2019), who postulated that characteristic hyperplasia in the initial phase of gall induction can be triggered by the insertion of exogenous genetic elements into the genome of plant cells through an endosymbiotic bacterium originating from the inducing insect. To test this hypothesis, we employed a combination of genetic marker analyses, metagenomic analyses, and experimental gall induction in cassava plants. Taken together, our findings provide support for the role of microorganisms and genetic transformation in gall induction mechanisms.

Materials and methods

Galling insect model

We chose the cylindrical gall induced in Euphorbiaceae *M. esculenta* Crantz (cassava) (Figure 1A) by the Cecidomyiidae *I. brasiliensis* (Montaldo, 1977; Rivera Hernández, 2011) as our biological model (Figure 1B). The cassava genome has been well documented (Wang et al., 2014; ICGMC (International Cassava Genetic Map Consortium), 2015; Lyons et al., 2022), allowing the identification of potential alterations or insertions from exogenous sources.

Additionally, six prosoplasmic gall morphotypes (greater structural complexity) and two kataplasmic galls (low structural complexity) were selected to validate the general induction hypothesis (Figure 1D). For details, see Supplementary Text.

Samples were obtained from several plant specimens collected in the Guanacaste Conservation Area, Guanacaste, Costa Rica, as well as around the Santa Clara community, San Carlos, Alajuela, Costa Rica. A voucher specimen of each host plant species and galls was deposited at the Cecidiarium (specialised gall herbarium), established at the Instituto Tecnológico de Costa Rica.

Microdissection of salivary glands from *Iatrophobia brasiliensis* larvae

Larval salivary glands from *I. brasiliensis* were extracted by microdissection in a biosafety flow-hood (High Ten, Model 3BH-24), under sterile conditions (Figure S1). Larvae were surface decontaminated according to the protocol established by Zahner et al. (2008) with some modifications. Consecutive rinses were done for 2 min in 3% sodium hypochlorite and then 70% ethanol, followed by a rinse in sterile nuclease-free water. All dissected salivary glands were collected and employed in the DNA extraction. All life stages were surface cleaned.

Isolation and characterisation of endosymbiotic insect bacteria

Two colony-forming units (CFUs) were preliminarily isolated from the larval head of *I. brasiliensis* under sterile conditions. The dissected larvae were in instars one or two. The larvae were extracted live from the inner chamber of the gall, and only active larvae were collected. Different parts of the larva, such as the head and segments of the digestive system, were placed in YEB 1× culture medium (Piñol et al., 1996) to obtain a primary bacterial culture. Serial dilutions were made from the original culture to obtain single colonies (CFUs). The cells were recovered and maintained in YEB 1× solid and liquid suspension to 4–8°C and in glycerol (20% w/v) at −80°C. Each of the CFUs was named isolated symbiotic bacteria (ISB).

ISB 2 formed short rods and macroscopically coccoid-like elements and produced round, entire, convex, orange/pinkish colonies with smooth matte surfaces on YEB 1× Bacto agar medium after 4–5 days of incubation at 26°C. The bacterium showed features of a pinkish, flat, circular, slightly irregular border, variable length with curved shapes in some of them, and a mucous texture; it was a Gram-positive bacillus (Figure S2).

Molecular characterisation of bacteria was done through 16S sequencing using the procedures described by Rainey et al. (1996) and Hanshew et al. (2013), with some modifications. Taxonomic identification of the endosymbiotic bacteria was done using Kraken2 (Wood et al., 2019), the same for the endophytic bacteria, as described later. Details of the analytical pipeline are described below. Bacterial strains were identified by 16S gene sequencing before each inoculation assay to induce gall formation in the selected cassava tissues. We prepared templates for PCR either by total DNA extraction or by lysis of CFU bacteria growing in a solid culture medium. Bacterial cells were diluted in Elution Buffer (10 mM Tris, pH 8.0) (Qiagen, Hilden, Germany). Samples were lysed by vortexing and heating at 95°C for 5 min. The filtrate was then centrifuged at 8,000 *g* for 5 min.

Isolation of culturable microorganisms associated with plant gall tissue

We isolated CFUs that potentially represent endophytic bacterial strains and one fungal growth from surface-sterilised internal sections of *M. esculenta* gall tissue sections. Seven were selected for high-throughput sequencing (HTS). Each of these CFUs was named an isolate of endophytic bacteria (IEB).

Surface tissue was sterilised following the methodology described for DNA purification. CFUs grown directly from gall slice explants were carefully collected by a sterile bacteriological

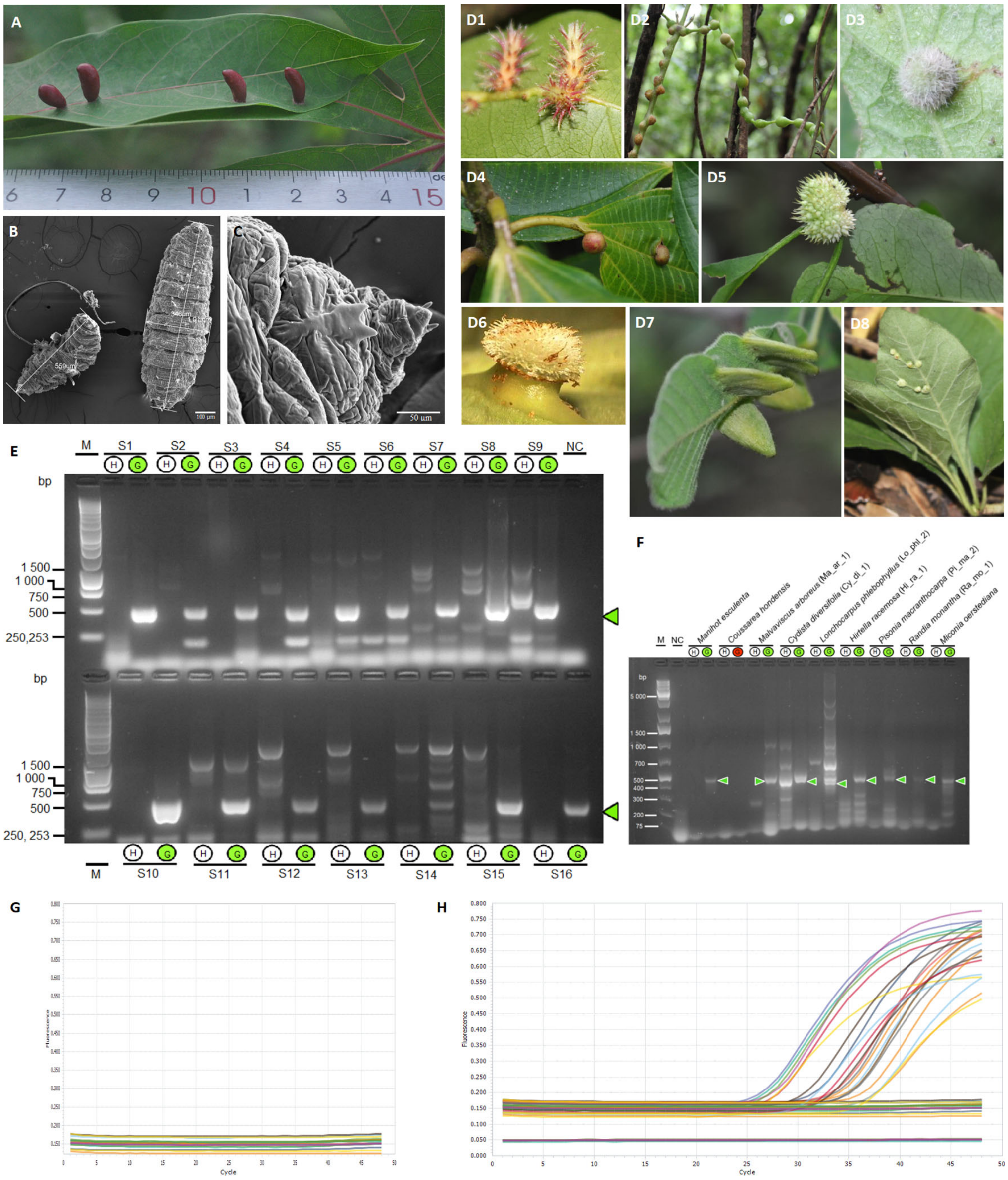

FIGURE 1

Identification of a potential universal marker in different gall systems. **(A)** Gall induced by the Cecidomyiidae *Iatrophobia brasiliensis* in *Manihot esculenta* (Montaldo, 1977). **(B)** Scanning electron micrograph of *I. brasiliensis* larva. **(C)** Scanning electron micrograph of the ventral area near the head of *I. brasiliensis* larva. **(D1)** Gall induced in *Hirtella racemosa* Lam. (Chrysobalanaceae), morphotype Hi_ra_1, by an unidentified Cecidomyiidae. **(D2)** Gall induced in *Cydista diversifolia* (Kunth) Miers (Bignoniaceae), morphotype Cy_di_1 by an unidentified Cecidomyiidae. **(D3)** Gall induced in *Malvaviscus arboreus* Dill. ex Cav. (Malvaceae), morphotype Ma_ar_1, by an unidentified Cecidomyiidae. **(D4)** Gall induced in *Miconia oerstediana* (Melastomataceae) by an unidentified Cecidomyiidae. **(D5)** Gall in *Pisonia macranthocarpa* (Donn. Sm.) Donn. Sm. (Nyctaginaceae), morphotype Pi_ma_4, induced by an unknown insect species. **(D6)** Gall in *Coussarea hondensis* (Standl.) C.M. Taylor & W.C. Burger (Rubiaceae) induced by an unknown insect. **(D7)** Gall induced in *Lonchocarpus phlebophyllus* Standl & Steyerm. (Fabaceae), morphotype Lo_phl_1, induced by a Psyllidae. **(D8)** Gall induced in *Randia monantha* Benth. (Rubiaceae), morphotype Ra_mo_1, induced by an unknown insect species. **(D2–D8)** Gätjens-Boniche et al. (2021). **(E)** Agarose gel electrophoresis of DNA fragments (specific gall fragment marker, SGF) amplified by PCR, comparing healthy leaf tissue DNA samples **(H)** and gall tissue DNA samples **(G)**. Lane M, molecular weight marker (1 kb ladder); line NC, negative control (reagents only); lines S1–S16, samples of healthy leaf and gall tissues growing in the same plant organ (pair-compared). **(F)** Gel electrophoresis of PCR products using primers for the specific gall fragment marker (SGF) in gall morphotypes of different host plant species. Lane M, molecular weight marker (Gene Ruler 1 KB Plus); line NC, negative control (reagents only); lines 3–20, samples of healthy leaf and gall tissues of different plants growing in the same plant organ (pair-compared). The green circle indicates a positive sample for the specific gall fragment amplification **(E, F)**. **(G, H)** Real-time PCR by Taq Man Probe showing the detection of the specific gall fragment from gall DNA samples of *Manihot esculenta* (amplification plot H) and from healthy leaf samples (amplification plot G). Each trace shows the ΔRn (normalised net fluorescence signal of the PCR product) plotted against the number of PCR cycles.

loop in a sterile biosafety chamber. Primary cultured bacteria were diluted in liquid medium YEB 1× (Piñol et al., 1996) until CFUs were obtained on solid medium. This process was repeated two to three times. Bacteria in cultured media were grown for 3–4 days at 26°C. Subsequently, bacterial cells were recovered and maintained in YEB 1× solid medium and suspended in liquid medium YEB 1× at 4–8°C and in glycerol (20% w/v) at −80°C.

DNA purification

Isolation of total genomic DNA from plant tissues

All tissues were sampled from plants morphologically identifiable as *M. esculenta*. Healthy leaf tissue from *M. esculenta* was checked in a stereoscope to avoid visible microgalls or some other type of foliar damage or disturbance. Prior to DNA extraction, the galls were dissected by carefully splitting them in half without damaging the larva present in the internal chamber, which was removed from the tissue. Healthy leaves and galls were washed with alkaline liquid soap and Triton X-100 detergent (dissolved in sterile water). The plant material was vortexed three times with 70% ethanol (v/v) and sterile water. Subsequently, the leaves and galls were surface sterilised according to the methodology suggested by Meyer and Hoy (2008) and Mueller et al. (2004), with some modifications. Plant materials were immersed for less than 30 s in sodium hypochlorite sequential solutions (3% NaOCl, 6% NaOCl, and 3% NaOCl) and rinsed with sterile water. The sterilisation process was conducted in a biosafety flow-hood (High Ten, Model 3BH-24). After cleaning and sterilisation, all samples were immediately stored at −80°C until DNA extraction.

Genomic DNA extraction was performed according to the methodology established by Dellaporta et al. (1983), with modifications and following column precipitation steps as described by the Dneasy Power Plant Pro Kit protocol (Mobio/QIAGEN, Carlsbad, CA, USA). The phenolic separation solution (PSS) in the Dneasy Power Plant Pro Kit was used during maceration to avoid DNA methylation. Total genomic DNA was extracted from both healthy leaf and gall tissues (different morphotypes). In the case of galls, DNA samples were collected from individual and pooled galls growing on the same leaf.

Genomic DNA isolation from salivary glands of inducing insects, *Iatrophobia brasiliensis*

Larval samples for DNA extraction were collected by dissecting galls from *M. esculenta*. Pools of 10 larvae of *I. brasiliensis* were decontaminated by being exposed to 3% NaOCl for 3–4 min, followed by successive 70% alcohol and water rinses under a biosafety flow-hood (High Ten, Model 3BH-24). Genomic DNA purification from the salivary glands of the insect larvae was performed using the Power Soil DNA Isolation Kit (Mobio/QIAGEN, Carlsbad, CA, USA) with the additional steps, mainly K proteinase (100 mg/mL) treatment and incubation at 65°C for 1 h. Furthermore, salivary gland DNA samples were extracted three times with phenol-chloroform isoamyl alcohol (PCI).

DNA isolation from endophyte and putative insect endosymbiotic bacteria

Total genomic DNA from endosymbiotic bacteria isolated from *I. brasiliensis* insects was extracted using a Dneasy Blood and Tissue Kit (Qiagen, Hilden, Germany). The same DNA extraction protocol was used for endophytic bacteria strains grown from surface-sterilised internal sections of *M. esculenta* gall tissue sections. Plasmid DNA from the isolated bacteria strains was carried out by alkaline lysis, as described by Li et al. (1995) with some modifications. The integrity and yield of bacterial genomic DNA and wild-type plasmids were checked by 0.8% agarose gel electrophoresis.

For all genomic DNA samples (plant, insect, and bacteria), quality was assessed by 260/280 and 260/230 ratios measured on a NanoDrop 8000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

Modified RAPD methodology for discovering gall-associated molecular markers

Samples of healthy leaf and gall tissues growing in the same plant organ were compared to detect differentially amplified DNA fragments in the DNA of the gall and not present in the same healthy tissue. The theoretical approach of this methodology is shown in Figure S3A. Assays were made under standard RAPD methodology conditions with the commercial random primers OPC-06, OPI-04, OPA-03, OPD-18, OPD-03, OPE-06, OPA-17, and OPB-04 (Operon Technologies, Alameda, California, USA). In these analyses, a modified methodological approach was also carried out by simultaneously using a combination of two non-random primers of conserved sequences from *A. tumefaciens* genes along with a non-standard RAPD thermal profile. Primers from conserved *A. tumefaciens* genes were used because the genetic transformation of plant cells mediated by this bacterium is the best-studied genetic transformation in a plant system. Decamer primers derived from conserved sequences of the Isopentyl Transferase Gene (ipt) and from the iaaM gene (Tryptophan 2-monooxygenase) harboured in the transfer DNA of the Ti plasmid of *Agrobacterium* species generated the highest number of differentially amplified fragments in previous RAPD assays. Primers were designed from the alignment of conserved regions in different species that harbour these genes using DNA Star Lasergene 99 (Madison, Wisconsin, USA) and BioEdit version 4.8.10.1 (Hall, 1999). Nucleotide sequences for these genes were obtained from the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/pubmed/). Accessions for the iaaM genes were M91609, Z18270, X77327, U04358, and L33867, and for the ipt genes, they were X77327, X53945, X17428, M91610, and Z46375. RAPD reactions were performed on 200-µL sterile, pyrogen-safe, thin-walled plastic tubes for PCR RNase-DNase (Axygen, CA, USA), using 0.5 units of Dream Taq (Fermentas Life Sciences, Lithuania), PCR 1× (750 mM Tris-HCl [pH 8.8], 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 0.5 µM of each primer, and 10 ng of

DNA), adjusted to a final volume of 25 µl with nuclease-free sterile water (Promega Corporation, Madison, Wisconsin, USA). The primer set used was ipt forward, 5′-CGGTGAACGA-3′ and iaaM reverse, 5′-TCCAATTTCT-3′. DNA was initially denatured for 3.5 min at 95°C, followed by 15 cycles of 95°C for 30 s, 34.5°C for 30 s, and 72°C for 2 min. This was followed by 35 cycles of 30 s denaturation at 95°C, 30 s annealing at 46°C, and 2 min elongation at 72°C, with a final elongation step of 72°C for 7 min. Reactions were carried out in a thermocycler PTC-200 DNA Engine (MJ-Research, Waltham, Massachusetts, USA). Samples of healthy and gall tissues growing in the same plant were compared in pairs. Each primer set and RAPD condition was carefully tested more than three times using 20 to 30 samples. Reactions were performed in a flow-hood (High Ten, Model 3BH-24).

RAPD products were analysed by 1%–1.5% agarose gel electrophoresis with 0.5 × TBE and 1× Gel Red at 75 V (Electrophoresis chamber and Power Pac 300 Bio-Rad, Hercules, California, USA). Amplicons were separated in a MultiNA automated system for DNA and RNA microchip analysis (MultiNA-Shimadzu, Tokyo, Japan). PCR products were cleaned using Promega Wizard SV gel and the PCR clean-up system, as per the manufacturer's directions (Promega, Madison, WI).

The DNA gall fragments differentially amplified from the insect gall tissue of cassava by modified RAPD methodology were sequenced at the Centro de Investigaciones en Biología Celular y Molecular, Universidad de Costa Rica, San José, Costa Rica. Sequencing reactions were performed using dideoxynucleotide chain termination with the BigDye^{TM} Terminator Kit (Applied Biosystems, USA) and 5 pmol of each ipt forward and iaaM reverse sequencing primers and analysed with an ABI Prism® 3700 Automated Sequencer (Applied Biosystems, USA). Sequenced fragments were aligned using DNA Star Lasergene 99 (Madison, Wisconsin, USA) and BioEdit version 4.8.10.1 (Hall, 1999).

PCR amplification and sequencing for the specific gall marker

The DNA gall fragments differentially amplified from gall samples were used as templates to design a potential gall molecular marker based on our RAPD results. Primers were designed using Primer3 v.4.1.0 (Koressaar and Remm, 2007; Untergrasser et al., 2012). The primer sequences were 5′-CTT GAC ATG TTC TGG AGC GG-3′ for the forward primer (Primer_Gall-Forward) and 5′-AAC GAG CGT GGT ACT GTG AT-3′ for the reverse primer (Primer_Gall-Reverse) (Invitrogen, Carlsbad, CA, USA). The expected amplicon size was 471 bp. Primers were tested with DNA samples from both healthy and gall tissues from *M. esculenta* plants and other gall morphotypes, as well as with DNA extracted from insect salivary glands. Subsequently, the target gene was amplified in the isolated wild-type plasmids from two putative insect endosymbiotic bacteria of the genus *Rhodococcus* and *Pseudomonas* and in wild-type plasmids of all endophytic bacteria isolated from the cassava gall tissue. PCR was carried out with 1× PCR buffer (750 mM Tris-HCl [pH 8.8], 200 mM $(NH_4)_2SO_4$, and 0.1% Tween 20), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 2 U Taq Polymerase (Thermo Fisher Scientific, Wilmington, DE, USA), 0.5 µM forward and reverse primers (Invitrogen, Carlsbad, CA, USA), 10 ng of sample DNA, and H_2O (Promega Corporation, Madison, Wisconsin, USA) for a final reaction volume of 25 µL. Amplification was performed in a thermal cycler PTC-200 DNA Engine (MJ-Research, Waltham, Massachusetts, USA) using the following cycling conditions: initial denaturation at 95°C for 3,5 min.; 40 cycles of 95°C for 30 s, 62°C for 45 s, and 72°C for 1 min; and final extension at 72°C for 5 min. The transition temperature between each step was 1°C/s. The assays were repeated multiple times for 170 galls and healthy tissue samples. Three samples with varying amounts of salivary glands were used in total, as were two bacterial plasmid samples. PCR reactions for these samples were repeated at least six times.

The PCR products were separated on a 1.5% agarose gel with 1 × TAE and 1× Gel Red at 75 V (Electrophoresis chamber and Power Pac 300 Bio-Rad, Hercules, California, USA). The expected amplicons were excised from the gel and purified using the QIAquick Gel Extraction Kit, following the manufacturer's directions (QIAGEN, Hilden, Germany). Samples were sequenced using the Sanger method through a Macrogen service provider (Macrogen Inc., Seoul, Korea). Samples were purified and prepared for sequencing according to the methodology established by Macrogen. Sequencing of nonspecific amplified fragments for PCR was attempted; however, good quality and long base sequences could not be obtained, except for two of the samples. Sequenced fragments were aligned using DNA Star Lasergene 99 (Madison, Wisconsin, USA) and BioEdit version 4.8.10.1 (Hall, 1999).

All amplified fragments were aligned to the SGF consensus sequence using BioEdit version 4.8.10.1 (Hall, 1999) and Jalview version 2-a, 2.11.1.5 (Waterhouse et al., 2009). For salivary gland amplified fragments, an overlap of bases was frequent in several of the sequenced PCR fragments, which could show variants of the target DNA sequence in this insect tissue. A similar trend was observed in one of the colonies from endosymbiont *Rhodococcus* isolated from the inducer insect when their wild-type plasmids were analysed and linked to the DNA amplification profile, which could indicate polymorphic variants of their wild-type plasmids.

Both DNA fragments amplified from gall samples (RAPD modified technique) and the SGF were analysed using BLAST [National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov/pubmed/), Integrated Microbial Genomes & Microbiomes (IMG/M) system (https://img.jgi.doe.gov)].

Detection of the specific gall fragment by real-time qPCR

A real-time PCR marker was designed and tested in healthy and gall tissue using a TaqMan probe with homology to the consensus of differentially amplified DNA from galls. TaqMan-based qPCR was carried out in a 20-µL reaction mixture containing Go Taq Master Mix 2X (Promega, USA) and 1µL of 20X TaqMan®Gene Expression Primer/Probe Mix (Applied Biosystem, CA, USA).

Primers and the TaqMan probe were designed and synthesised from the specific gall fragment target with 0.5 µM of each primer (AI7ZYBP_Forward: 5′-TGTTCGCTGCACAGAGTTCT-3′ and AI7ZYBP_Reverse: 5′-GGCTTGAGTGCTTCGATTTCG-3′), 0.25 µM of the MGB probe, labelled with FAM reporter dye at the 5′ and non-fluorescence Quencher TAMRA at the 3′ end (AI7ZYBP_M_TCTGCCACCGGACCCT_NFQ), and 2.5µL of 5 ng/µL of extracted DNA. qPCR cycling conditions included initial denaturation at 95°C for 3 min and 35 cycles of denaturation at 95°C for 30 s and primer annealing and extension steps together at 60°C for 60 s. Three replicates of the negative template control consisting of nuclease-free water for molecular biology grade reactions were included in each amplification assay. Assays were performed on a LightCycler 96 Real-Time PCR System (Roche Diagnostics, Risch-Rotkreuz, Switzerland). We tested over 30 healthy tissue and gall samples and obtained an amplification average of 90% for the PCR marker or amplification signal in the case of qPCR. The assays were repeated more than six times for the same healthy leaf and gall samples.

Bioinformatic analysis

Library preparation and sequencing

Short-read libraries were prepared using Illumina's DNA TruSeq Nano Library preparation kit, following the manufacturer's instructions. For the leaf and gall genomic DNA samples, 150-bp single-ended (SE) libraries were prepared and sequenced on the Illumina HiSeq2500 platform using the SBS sequencing kit version 4. For the bacterial isolates' genomic DNA samples, 250-bp paired-ended (PE) libraries were prepared and sequenced in the Illumina MiSeq platform using Reagent kit version 2. Both library preparation and next-generation sequencing were performed by the NC State University Genomic Sciences Laboratory (Raleigh, NC, USA). Raw data can be retrieved from the Short Read Archive (SRA) under the Bioproject accession number: PRJNA905450.

No sequencing was performed for any endosymbiotic or endophytic bacteria wild-type plasmids due to the stability and integrity of the plasmid DNA during the fragmentation procedure.

Host discriminant genomic analysis

The aim of this analysis was to identify potential genes or insertion sequences from bacteria in the cassava genomic DNA samples (genotype Valencia); these sequences were believed to induce gall formation in otherwise healthy plants. To do so, a stepwise filtering strategy was implemented. First, sequence read quality was assessed with FastQC (Andrews, 2010); when needed, index, adapters, and low-quality sequences were eliminated. After quality control, reads from both healthy and gall tissues were mapped against the cassava reference genome CV AM560-2 (Phytozome genome ID: 520; Bredeson et al., 2016) using BBmap, part of the BBTools suite (Bushnell, 2015), with the semiperfect mode option. Unmapped reads from healthy leaf tissue and leaf galls were compared against each other, and the shared reads were filtered using BBduk, part of the BBTools suite. The remaining gall reads, unmapped to the reference genome and unmatched to healthy leaves according to defined parameters, were *de novo* assembled into contigs using SPAdes (Prjibelski et al., 2020). A MegaBlast of the selected contigs was performed, setting the minimum length to 200 bp and the percentage of homology to ≥95%. The resulting contigs, the product of gall exclusive reads, were then mapped against the cassava reference genome using the Burrows–Wheeler Aligner (BWA-MEM) (Li and Durbin, 2009). Alignments with low mapping quality (≥20) were filtered using SAMtools (Li et al., 2009). Contigs with matching sequences in either their 5′, 3′, or both ends but non-matching sequences in their core, based on the CIGAR string, were selected as potential foreign DNA insertion sites. Reads were mapped back to the gall hybrid/fusion contigs containing potential foreign DNA insertion sites to assess coverage. Contigs with less than three cover reads were discarded, except for three of them that showed significant annotations with known gene sequences.

We also analysed the putative gall insertion sequence marker (named gall fragment) experimentally in plasmids isolated from all seven isolated endophytic bacteria and the two bacteria isolated from the larval insect head of *I. brasiliensis* and *in silico* by bioinformatic tools in the assembled genomes of the same sequenced bacteria species. In particular, for the *in silico* analysis, isPCR and the primer sequences designed for the gall fragment marker did not show any amplification from the cassava reference genome, nor did it produce amplification products for the insect endosymbiotic bacteria genomes.

Metagenomic sequence analysis and taxonomic profile assignment

Shotgun metagenomic sequencing approach was used to analyse the microbiome associated with cassava galls and healthy leaf tissues. Taxonomic profiles were performed using Kraken2 (Wood et al., 2019) and its standard database, which includes bacteria, archaea, viruses, and eukaryotic genomes (https://benlangmead.github.io/aws-indexes/k2). Analyses were applied to reads data (QC filtered reads) from the sequenced gall and healthy tissue samples with an abundance filter of 10K reads.

Taxonomic profiles were also carried out to identify unique reads in the gall tissue following the host discriminant genomic analysis (HDGA) methodological approach to determine their taxonomic origin. Reads were mapped against the cassava genome, retaining only unmapped reads. We then clustered the sequences to obtain unique reads by sample only. This finding was further confirmed by contrasting the resulting taxonomic profiles of healthy samples with gall samples.

After a detailed analysis of the taxonomic profiles was carried out to identify the likely contaminants introduced during processing or due to any potential contaminant event during the final stage of DNA purification of cassava healthy leaves and gall samples, five bacteria taxa were removed as possible external contamination in all sequenced samples.

Bacteria endophytic condition determination by synteny analysis

We applied synteny bioinformatic analysis to confirm whether isolated bacteria from both gall tissue and the inducing insect were components of the endophytic microbiome in this structure. To carry out this analysis, exclusive gall reads and bacteria samples were *de novo* assembled using SPAdes (Bankevich et al., 2012) in their metagenome mode for gall reads and isolate mode for bacteria samples. To confirm synteny, contigs from both sides were compared using BLAST+ (Camacho et al., 2009).

Genome annotation and functional analyses

To analyse potential differences in terms of function in bacterial groups (endophytic vs. endosymbiont), an enrichment analysis was performed using the Gene Ontology (GO) terms included in the TopGO R package (Alexa, 2022) and a cluster of gene ontology approach (COG) (Galperin et al., 2019). To carry out the analysis, bacterial genomes previously assembled were annotated using prokka (Seemann, 2014). To obtain their corresponding GO and COG terms, the proteins were further annotated using eggnog (Huerta-Cepas et al., 2019). To obtain the raw counts of genes, original assembly reads were mapped back to the annotated genes using BWA MEM (Li, 2013) and filtered with SAMtools (Li et al., 2009), including flags 0 × 8 for non-paired reads and 0 × 2 for properly paired reads. Then, raw counts were obtained using the HTSeq-count from the HTSeq framework (Anders et al., 2015). Finally, differential abundance analysis was performed on shared genes using DESeq2 (Love et al., 2014), allowing their differentiation in abundance post-analysis of their GO terms.

Ab initio predictions

Ab initio prediction was performed on the differentially amplified fragments from *M. esculenta* gall tissues (consensus DNA sequence) and on amplified fragments from purified plasmids of the endophytes IEB 1-2, IEB 3-1, and IEB 5-1, as well as purified plasmids from ISB 1 and ISB 2 isolated from the larval head of the inducing insect. These fragments were amplified with specific gall primers as previously described in the PCR methodology section. *Ab initio* prediction was also carried out on the hybrid sequence fasta files. Gene predictions and their functions were performed using the pipelines of Rapid Prokaryotic Genome Annotation (Prokka) (Seemann, 2014) on the web server Galaxy (www.usegalaxy.org). The pipeline of Prokka uses Prodigal and UniProt for the prediction of coding regions and functional similarity, respectively.

Identification of gene segments

The identification of conserved genetic regions in all target sequences obtained by PCR and in the possible hybrid sequences of interest, obtained by high-throughput sequencing, was performed using the PipMaker program (http://pipmaker.bx.psu.edu/cgi-bin/pipmaker?advanced) to produce local alignments of the genetic sequences using BlastZ (Schwartz et al., 2003), and dot plots of the gap-free segments of the alignments were generated as unbroken diagonal lines, indicating a high degree of identity across the genetic sequences (Schwartz et al., 2000).

Inoculation assays for primary gall induction

Two bacteria grown in solid and liquid YEB 1× culture medium (Piñol et al., 1996) were used to inoculate emerging leaves from apical buds and young leaves of *M. esculenta* (Cassava). Bacterial strains isolated from the larval head of *I. brasiliensis*, subsequently identified as *Rhodococcus* (related to *Rhodococcus* sp. P-2 and *Rhodococcus erythropolis* species, according to the taxonomic profile), were cultured in YEB 1× solid medium and cultured at 26 ± 1°C in a shaker to 110 rpm (Hotech® model 721-2T, Hotech Instruments Corp., New Taipei City, Taiwan) for 3–4 days in the dark. Subsequently, a single clone was used to inoculate 5 mL of YEB 1× liquid medium. After culturing under shaking conditions at 26 ± 1°C for 2–3 days in the dark, a small bacterial suspension was transferred to 10 mL of YEB 1× liquid medium and transferred once again under a sterile biosafety flow-hood to YEB 1× solid medium and cultured for 2–3 days under the above conditions. Then, a single clone was used to inoculate 100 mL of YEB 1× liquid medium to the same preconditions. The endophytic bacterium *Pantoea ananatis*, one of the endophytic bacteria isolated from gall tissue, was used as a control bacterium. The management and culture of this bacterium were carried out under the same conditions described above.

In vitro micropropagated *M. esculenta* plants were used for bacterial inoculation assays under sterile conditions. Apical bud and leaf sections of 0.5–1.0 cm were used for inoculation. Explants were maintained first in solid MS medium for different periods of time. Prior to plant material co-cultivation with bacterial cultures, all explants were treated with slight mechanical abrasion using wet filter paper impregnated with ground glass. Just before inoculation, bacterial biomass was measured with a Lambda 25 spectrophotometer (Perkin Elmer-Applied Biosystems, Foster City, California, USA) and set to a OD_{600} value of 0.5–1.0. Then, *in vitro* plant material was submerged into bacteria cultured in YEB 1× liquid medium and shaken in the dark at 28 ± 1°C, for 16–24 h. Inoculated explants were placed in sterile glass Erlenmeyer flasks and washed three times with sterile water. Excess water was removed, and explants were grown separately on MS solid (agar 5.7 g/L) or liquid medium without plant growth regulators. Half of the cultures were supplemented with 3 mg L^{-1} Kathon (only in solid medium), a bacteriostatic reagent used to inhibit bacterial growth in the cultured medium, under white, fluorescent light with an irradiance of 27 µmol m^{-2} s^{-1} at 28 ± 1°C and 110 rpm in a shaker for the liquid medium (Hotech® model 721-2T, Hotech Instruments Corp., New Taipei City, Taiwan). Both types of explants not co-cultured with the bacteria but treated with mechanical abrasion were employed as controls. Thirty to fifty explants were used in each experiment, which was repeated five times in solid medium and two times in liquid medium. Kathon reagent was used only in the first assay carried out in a solid medium.

Inoculation of plants grown in soil under controlled greenhouse conditions was also attempted. The experimental conditions and bacterial growth were carried out using the same methodology. Groups of 50 plants per treatment grown in pots with soil were used to manually inoculate one to two new emerging leaves. Before bacterial inoculation, the leaf surface was carefully cleaned with 70% ethanol. Then, the emerging leaves were treated with a slight mechanical abrasion using a sterilised YEB 1× liquid medium containing ground glass, followed by rubbing the leaf surface by hand using clean latex gloves. After this, sterilised cotton impregnated with liquid medium containing the bacterial growth was placed and fixed on the prepared leaf surface for 1–2 days. The inoculated leaf area was covered with sterile gauze and sterile insulating plastic. This inoculation test was repeated three times.

A randomised block design was followed in this assay. However, because of the viability decline observed in cassava leaves under the established experimental conditions, only presence–absence results for gall-like structure and abnormal tissue development were displayed graphically. Infostat software version 2020p (http://www.infostat.com.ar) was employed to graph the results obtained from the experiments (Di Rienzo et al., 2020). Daily evaluations were performed in all treatments for 6 weeks. Data are shown as the mean and standard error (±SE) of tissue response due to inoculation with the bacteria. Taxonomic identification of the alleged endosymbiotic bacteria strain from the inducing insect (*Rhodococcus*, isolation ISB 2), used in these gall induction assays, was determined before each test by 16S gene sequencing and then confirmed by a taxonomic profile using genomic bioinformatics tools, as also performed for endophytic bacteria.

Sample preparation for scanning electron microscopy and transmission electron microscopy

Samples of healthy leaves and galls of different sizes from *M. esculenta* plants, as well as the larvae of the inducing insect, were prepared as described by Sánchez et al. (2006), with the modification of fragmentation in liquid nitrogen and dehydration by passage through a gradient of acetone solutions. Sample preparation was performed at the Center for Research in Microscopic Structures, University of Costa Rica, San José, Costa Rica. Samples were observed using a scanning electron microscope (Hitachi S-3700, Tokyo, Japan) with an acceleration voltage of 15 kV.

For transmission electron microscopy (TEM) analyses, samples were fixed with a modified Karnowsky solution, then post-fixed with 2% osmium tetroxide for 1 h, submitted to contrast, and dehydrated in a gradient of acetone solutions (i.e., 30%, 50%, 70%, 90%, and 100%). After washing with 100% acetone, the samples were submitted to pre-infiltration with 1:1 Spurr resin/100% acetone for 5 h with shaking. The subsequent filtration of the samples was performed with pure resin for 12 h. The samples were then shaped and polymerised at 70°C for 3 days. The blocks were then cut into 70-nm-thick sections on an ultramicrotome (Leica Power Tome PC, Leica Microsystems GmbH, Wetzlar, Germany) with a diamond blade (45°), mounted on uncoated 200-mesh copper grids, and stained with uranyl acetate and lead citrate. TEM observations were performed using a Hitachi model HT-7700 microscope (Tokyo, Japan) operating at 100 kV.

Results and discussion

Characterising a potential gall molecular marker

To assess our hypothesis, DNA samples from healthy leaf and gall tissues from the same plant were purified (Figures 1A, D1–D8). Samples were collected from healthy leaves of *M. esculenta* and galls induced by Cecidomyiidae *I. brasiliensis* (Figures 1B, C), and these were carefully cleaned and sterilised to guarantee total epidermis disinfection, including the inner gall chamber.

Possible exogenous DNA, or a DNA insertion sequence present only in gall cells but not in the healthy plant tissue of *M. esculenta* plants, was initially explored using specific PCR primers as a potential gall marker. The primer pair was designed based on the consensus DNA sequence resulting from the alignment of differentially amplified fragments obtained through a previously carried out modified RAPD assay.

A high number of RAPD assays were performed using different random primers; however, decamer primers derived from conserved sequences of the ipt and iAAM genes harboured in the transfer DNA of the Ti plasmid of *Agrobacterium* species and other related bacteria generated the highest number of differentially amplified fragments from gall samples. The quality of purified DNA, as well as the modified RAPD amplification conditions and thermal conditions, allowed us to obtain high reproducibility and reliability in the DNA profiles at different concentrations of analysed DNA. Analytical detections of the RAPD amplicons by gel electrophoresis and the Microchip Electrophoresis System for DNA/RNA (MultiNA) showed differentially amplified fragments of several sizes in all gall samples tested, often from 100 to 4,500 base pairs (bp) (Figures S3B, C). Using these approaches, we isolated and obtained the nucleotide sequences of four samples from the most common differentially amplified fragments derived from gall genomic DNA. Three of these sequences were identical, showing the same nucleotide sequence as the fragment of approximately 500 bp (Supplementary Data 1). The consensus DNA sequence obtained was used as a template to design and test specific PCR primers. For further information, see Supplementary Text.

The gall DNA fragment differentially amplified from gall samples (which we call a specific gall fragment, SGF) was isolated and sequenced. The expected PCR fragment was specifically amplified only in gall samples from *M. esculenta* (Figure 1E), total DNA from the inducing-insect salivary glands, and in the isolated wild-type plasmids from two putative insect endosymbiotic bacteria of the genera *Rhodococcus* (ISB 2 bacterial isolate) and *Pseudomonas* (ISB 1 bacterial isolate) (Figures 2A, S4). Moreover, PCR products with sizes between 350 and 600 bp were also amplified from wild-type plasmids of all endophytic bacteria isolated from cassava gall tissue (Figures 2A, S4). Additionally, specific gall PCR fragments showing a size similar to that expected

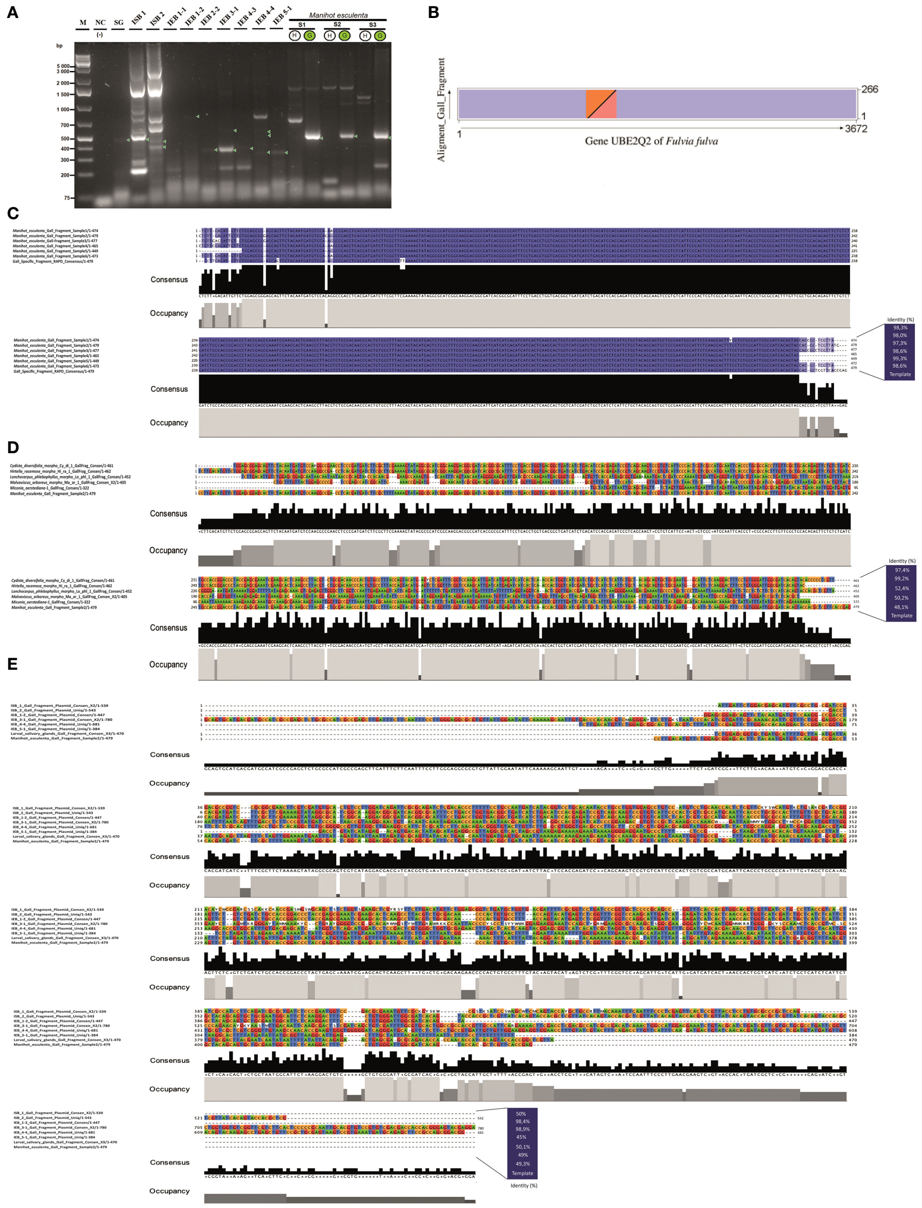

FIGURE 2

Genetic characterisation of the specific gall fragment marker. **(A)** Gel electrophoresis of PCR products using primers for the specific gall fragment marker (SGF) from purified wild-type plasmids of two putative endosymbiotic bacteria, *Pseudomonas* and *Rhodococcus*, isolated from the larval head of the inducing insect *Iatrophobia brasiliensis* (isolates ISB 1 and ISB 2), as well as from purified wild-type plasmids of seven possible endophytic bacteria isolates selected from the cassava plant gall tissue (IEB). PCR amplicons are also shown for the inducing insect salivary gland sample (SG). Samples of DNA purified from healthy leaf and gall cassava tissues were used as positive reaction controls (lines S1–S3). Lane M, molecular weight marker (Gene Ruler 1 KB Plus); line NC, negative control (reagents only). Green circles indicate positive samples for the specific gall fragment amplification (S1–S3). **(B)** Dot plot representation of the aligned and annotated specific gall fragment sequence showing overlapping regions with the *UBE2Q2* gene of *Fulvia fulva*. The overlapping region between the sequences is shown as coloured triangles in each of the represented axes. **(C)** Alignments of specific gall fragments amplified and sequenced from six gall samples compared to the 479 consensus bp DNA reference sequence. **(D)** Alignments among the 479 consensus DNA sequences of the specific gall fragment from cassava and five sequenced gall morphotypes of different host plant species. **(E)** Alignment of sequenced PCR amplicons performed using specific gall fragment primers over purified wild-type plasmids of two alleged endosymbiotic bacteria isolated from the larval head of the inducing insect *I. brasiliensis* (colony-forming units ISB 1 and ISB 2) and from purified wild-type plasmids of seven possible endophytic bacteria isolations selected from the cassava plant gall tissue (colony-forming units IEB), as well as from PCR sequenced fragments from the inducing insect salivary gland (SG). The consensus sequence of the specific gall fragments (SGF) of cassava was used as a template sequence. Grey bar plots show the occupancy within each sequence position, and the black bar shows the base consensus within each sequence position in the resulting alignment.

from cassava galls were selectively amplified from gall morphotype samples of eight different host plant species (Figure 1F). Many of these fragments were sequenced and then aligned to the consensus sequence of the differentially amplified fragment from the cassava gall. The aligned sequences showed high identity with the reference DNA fragment in all *M. esculenta* gall samples (99%–100%) (Figure 2C) and with the two IEB isolates 1-2 isolated from inner plant gall tissue (98.9%) and ISB 2 isolated from the larval insect head (98.4%) (Figure 2E). Furthermore, samples of other galls from *Cydista diversifolia* (97.4%) and *Hirtella racemosa* (99%) also showed high identity (Figure 2D), but a less conspicuous homology (between 48% and 53%) was detected from galls on *Lonchocarpus phlebophyllus*, *Malvaviscus arboreus*, and *Miconia oerstediana* (Figure 2D). This, in turn, suggests a diversification of the coding genetic element under different selection pressures for functional adaptation. Moreover, a repeatedly amplified DNA fragment from the salivary glands of a similar size to that expected showed an identity mean value comparable to the previous reference (49.3%), which could suggest that other bacteria in the salivary glands or an insect homologous gene might harbour a similar DNA sequence (Figure 2E).

A real-time PCR marker by the TaqMan probe was designed and tested following a similar approach used for end-point PCR assays with corresponding modifications. The results showed amplification signals only in the gall tissue (Figures 1G, H) and in plasmids purified from the isolated colonies of the insect endosymbiotic bacteria *Pseudomonas* sp. and *Rhodococcus* sp. (results not shown).

The differentially amplified gall DNA fragment from gall samples (SGF) did not show statistically significant similarity with any other reported gene according to the Basic Local Alignment Search Tool [BLAST, NCBI Genbank database, and Integrated Microbial Genomes & Microbiomes (IMG/M) system, https://img.jgi.doe.gov] and annotation analysis. Nevertheless, several results showed partial pairings with low to medium and often discontinuous length coverage with some ubiquitin-like genes, more specifically, the ubiquitin-like gene *E2*, a component of the ubiquitin-proteosome system (UPS), which has frequently been reported in different fungus species. This fragment showed partial homology to the Ubiquitin-conjugating enzyme E2 Q2 of the fungus *Fulvia fulva*, which was among the most significant (E-value = 6.37e−29, identity = 71.8%, 266 bp of length, accession number CP090172) (Figure 2B). However, we predicted that it was different enough to represent a new ubiquitin-like regulatory genetic element associated with the manipulation of the ubiquitin–proteasome system, used indirectly by the inducing insect via the bacteria to manipulate and redirect plant development during gall formation.

The ubiquitin gene family encodes peptides involved in protein–protein signalling and destination as a component of the basic cellular regulation machinery. These types of proteins regulate gene expression at the transcriptional (Adams and Spoel, 2018) and post-translational levels (Xu and Xue, 2019; Liu et al., 2020). The ubiquitin gene family has been reported as an essential part of molecular cell manipulation mechanisms in different pathogen and endosymbiotic–host interactions (Janjusevic et al., 2006; Vierstra, 2009; Park et al., 2012; Singer et al., 2013; Banfield, 2015; Kud et al., 2019). Ubiquitin-like genes from bacterial secreted effector molecules with structural and/or functional similarity to UPS pathway components mimic and modify the host UPS system (Ramachandran et al., 2021), allowing the hijacking of the cellular machinery, as has been previously reported in the crown gall system induced by *A. tumefaciens* (Magori and Citovsky, 2012; Lacroix and Citovsky, 2015). The UPS system is considered the major protein turnover pathway found across all domains of life and is especially important in regulating almost all plant development signalling pathways, including hormone-mediated plant growth and development, as well as plant responses to stress (Santner and Estelle, 2010; Sadanandom et al., 2013; Shu and Yang, 2017; Adams and Spoel, 2018; Xu and Xue, 2019). Although less is known about UPS manipulation in galling insects, there is emerging evidence that plant development can be manipulated through the UPS system by effector molecules in the salivary glands of the gall inducer (Zhao et al., 2015).

Genetic insertion events inferred from bioinformatic analysis provide evidence for plant cell transformation

Through a discrimination approach using shotgun metagenomic sequencing, we showed the presence of potential foreign-exclusive DNA within plant gall tissue. We named this methodological approach host discriminant genomic analysis (HDGA) (Figure S5). HTS data from healthy leaves and gall tissue (16× sequencing depth) were processed to separate, assemble, and analyse the DNA sequence reads different from the referenced host plant genome of *M. esculenta*. On average, 4.6% of the raw reads from both healthy leaf and gall tissues did not map to the reference genome. Furthermore, of those unmapped reads, 12.4% were unique to the gall tissue samples. *De novo* assembly of these specific reads produced 17,148 contigs. Eight of these assembled contigs produced fragments with sizes between 2,000 and 2,501 bases, 306 between 1,000 and 2,000 bases, 2,161 contigs of 500–1,000 bases, 14,664 fragments between 200 and 500 bases, and 8 fragments smaller than 200 bases (Supplementary Data 2). Using this approach, reads that did not map to the cassava reference nor were they shared between healthy and gall tissue, represent potential foreign DNA from endophytic organisms unique to galls, such as bacteria or fungi, or possible foreign DNA integrated into the genome of gall cells (see Figure S5 for the general pipeline-flow diagram approach). The resulting assemblies were compared against the reference *M. esculenta* genome to identify possible hybrid/fusion fragments, which must harbour homologous sequences with the host plant, along with external sequences without any homology to the host reference genome. When the assembled contigs were filtered once again following this approach, 407 contigs were retained, 59 of which had 300–571 bases, and 348 had 229–300 bases (Supplementary Data 3). Our analysis found 130 of the reported hybrid/fusion contigs with coverage from 10 to 30 reads. A total of 124 showed coverage ranging from six to nine. Only 74 displayed five to four reads, and 63 had three reads. Moreover, seven contigs showed the largest number of reads, with more than 95 (Supplementary Data 3). These hybrid sequences represent potential insertion regions for foreign DNA integrated into the

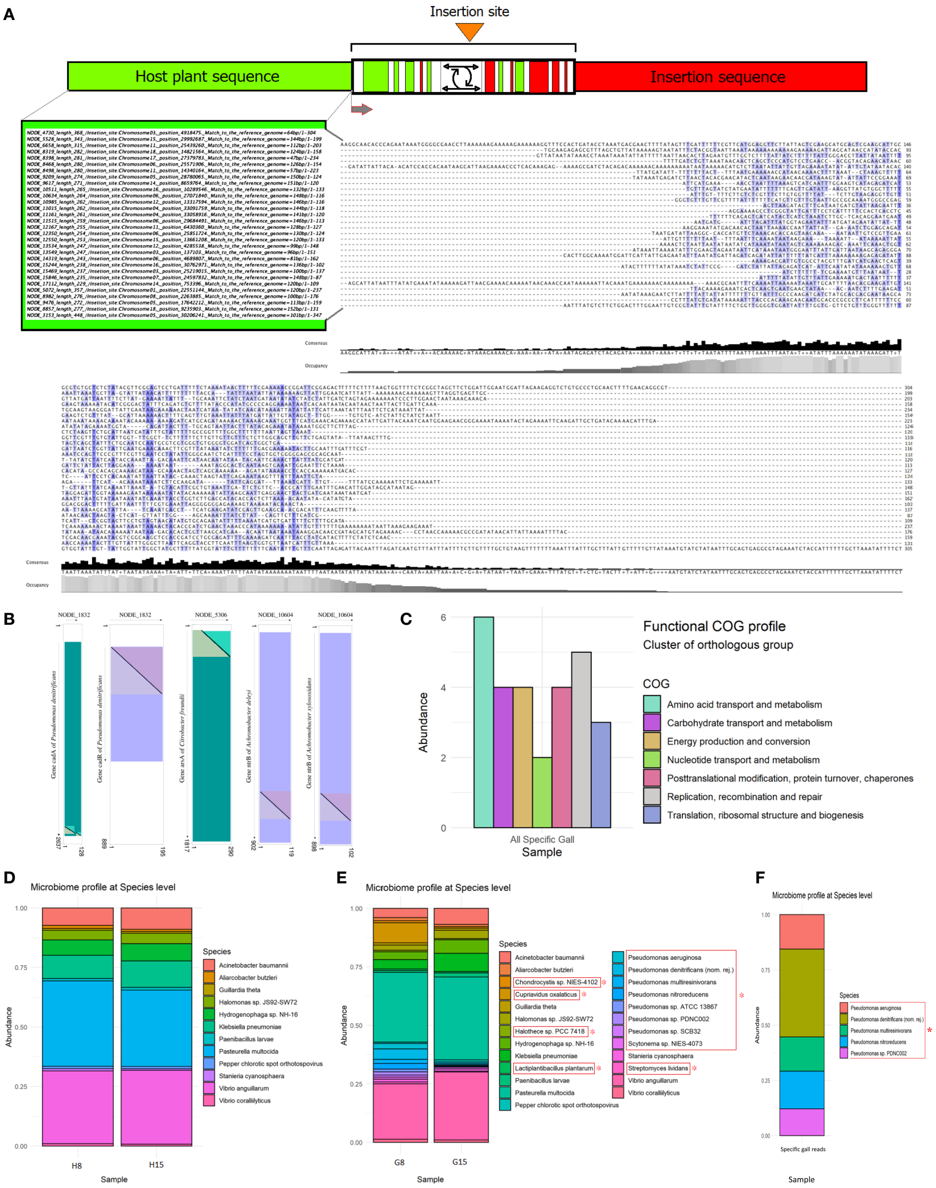

FIGURE 3
Bioinformatic analysis expands the catalogue of gall-specific sequences. **(A)** Theoretical diagram of the insertion regions according to the methodological approach applied. Position of the insertion sites into each host chromosome in the *Manihot esculenta* reference genome is shown on the left in a selected subgroup of hybrid contigs, mostly in forward orientation. Only the alignment framework of unmatched sequences to the cassava reference genome from hybrid/fusion contigs is shown on the right. Grey bar plots show the occupancy within each sequence position, and the black bar shows the base consensus within each sequence position in the resulting alignment scheme. **(B)** Dot plot representation of the aligned and annotated hybrid contigs region of unmatched sequences to the cassava reference genome, showing candidate insertion sequences in several of the hybrid contigs assembled, harbouring known DNA sequences revealing partial significant identity matches and covering with reported genes. Overlapping regions between the contigs and the annotated genes are shown as coloured triangles in each of the represented axes. Purple colour in the referenced gene represents forward strain orientation, and the blue-green colour (viridian) represents reverse strain orientation. **(C)** COG function classification histogram. Count of genes belonging to the COG categories related to exclusive gall reads involved in essential metabolic pathways and biological functions. **(D, E)** Microbiome profile of healthy plant tissue and gall tissue samples. Relative abundance of microorganism taxa identified in the microbiome of healthy leaves and gall samples of cassava. Taxonomic profiles were carried out to a 10K filter using raw reads generated by the shotgun sequencing approach. Each bar represents the organism taxon detected in one sample. The profile showed a similar abundance between both healthy samples **(D)**, but a different relative abundance of microorganisms between gall samples. **(E)** Asterisks indicate enriched or exclusive microorganism species present only in gall tissue according to the taxonomic profile carried out, comparing sequenced heathy leaf samples with gall samples. The most common core endophyte taxa between leaf and gall tissues are also shown. **(F)** Taxonomic identity profile (10K) associated with some of the selected gall-specific reads. Gall-specific reads were bioinformatically filtered from the sequenced gall samples, which mismatched with the cassava reference genome and filtered against shared reads from healthy tissue samples.

genome of gall cells in the sample of mixed galls used for library preparation. Thirty hybrid contigs showing the highest alignment parameters when compared with the cassava reference genome, mainly mapped to the forward strand of this reference genome, are shown in Figure 3A. The estimated insertion position within the assigned chromosomes in the host *M. esculenta* genome is also shown, evidencing multiple possible insertion sites in the genome of plant cells (see Supplementary Data 3 for all candidate hybrid assemblies).

Structural variations, such as repeated sequences, including retrotransposons (Bartlett et al., 2014), inversions, in tandem sequences (Gang et al., 2019), deletions, duplications, and other

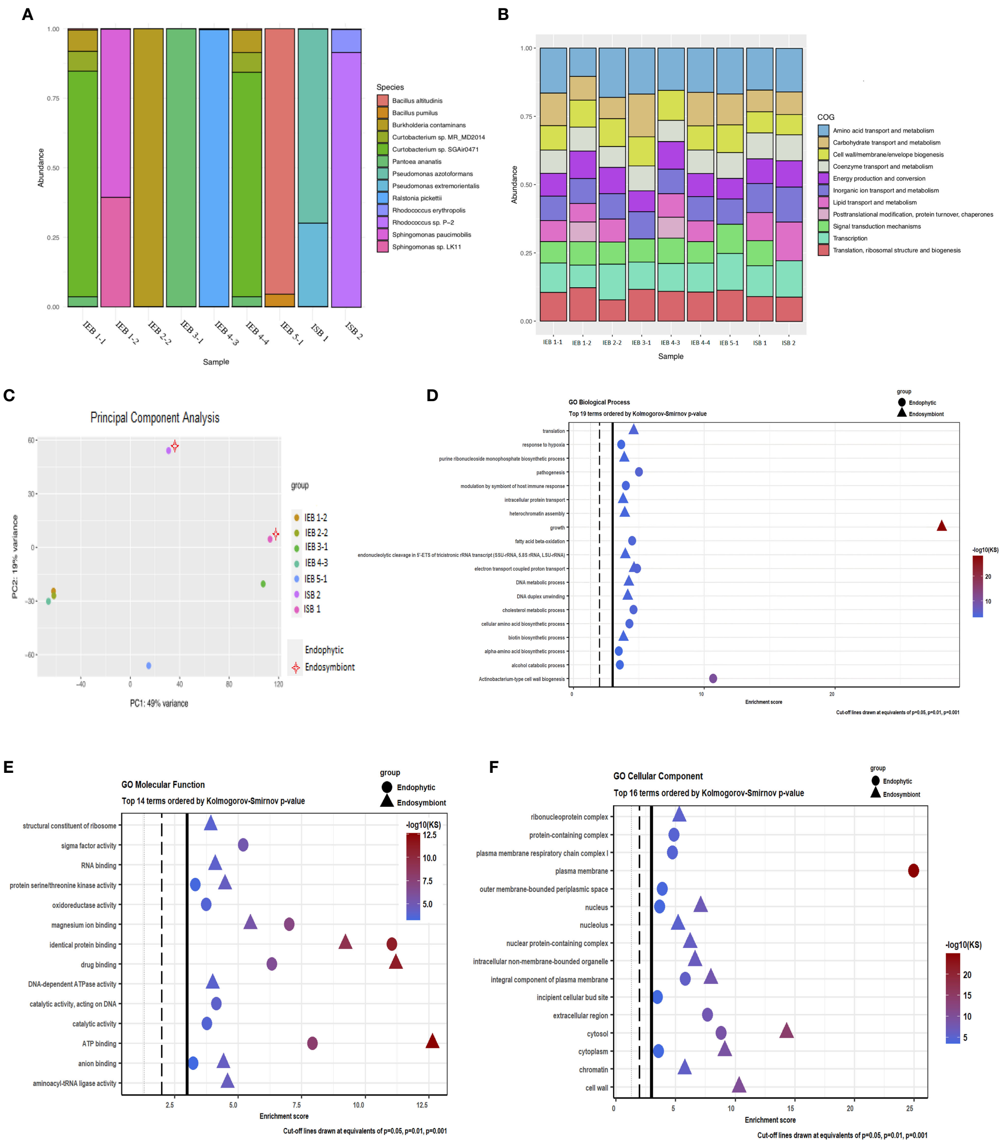

FIGURE 4
Microbiome taxonomic profile and functional analysis of gall endophytes and putative endosymbionts of the inducing insect. **(A)** Relative abundance of bacterial taxa identified in each of the samples of colony-forming units (CFUs) obtained from gall tissue of *Manihot esculenta* and from the larva head of the inducing insect *Iatrophobia brasiliensis*. The figure displays the most abundant taxa individually, with the remainder grouped together. Each bar represents the bacterial taxa detected in one sample. Each CFU from the insect head was called an isolated symbiotic bacteria (ISB). Each of the CFUs isolated and grown from internal sections of sterilised gall epidermis tissue was called an isolated endophytic bacteria (IEB). **(B)** Distribution of COG functional categories for CFU isolates from gall tissue and from the larval head of the inducing insect. **(C)** Genetic identity comparison of sequenced bacterial isolates classified up to genus by principal component analysis. **(D–F)** Functional analysis showing the top Gene Ontology enriched pathways of endophytic and alleged endosymbiont bacteria genomes. A high score indicates a high degree of enrichment.

complex rearrangements, including vector backbone or chromosome sequences carried over together with insertion sequences, may occur around or near the insertion point regions bordering the exogenous DNA and in the host plant DNA. A similar phenomenon has been reported in the flanking regions of the T-DNA insertion fragment harboured in the Ti plasmid of *A. tumefaciens* (Krizkova and Hrouda, 1998; Brunaud et al., 2002; Bartlett et al., 2014; Kleinboelting et al., 2015). They could also occur due to the activity of transposable elements (Wicker et al., 2016), such as *Mariner*-like elements in the genomes of seven species of *Rhus* gall aphids (Ahmad et al., 2021). The integration sites within plant genomes seem to be largely randomly distributed under non-selective conditions (Kim and Gelvin, 2007). Small areas of microhomology in insertion sites between T-DNA and neighbouring plant genomic DNA have also been reported (Brunaud et al., 2002; Kleinboelting et al., 2015). The analyses of microhomology indicate that this type of sequence could be, most of the time, a prerequisite for integration events (Gorbunova and Levy, 1999). Furthermore, a depth analysis of the host plant flanking sequences revealed a high proportion of the characterised T-DNAs inserted into or close to repetitive elements in transgenic barley lines without causing negative effects on transgene expression (Bartlett et al., 2014).

Because each insertion process is a single event that potentially generates a structurally hypervariable region in the DNA around the specific insertion site, finding a consensus sequence or motif around these sites is a challenge (Figure 3A). Those regions containing hypervariable sequences around the integration sites could certainly limit the assembly of reads towards the exogenous DNA sequence, thus restricting the length in hybrid/fusion contigs reported in this survey. Likewise, the extension of read assemblies towards the plant genome orientation in the hybrid contigs is also restricted because all common reads between the sequenced genomes of healthy plants and galls were filtered out, primarily with the reference cassava genome and then by pairing against themselves. The alignment quality with regard to the reference cassava genome, filtering stringency, and the read coverage obtained in our analysis provide evidence in support of the hypothesis that genetic material is inserted into the genomes of plant gall cells (Figure 3A, Supplementary Data 3). Technical artifacts, such as sequencing errors, formation of chimeric DNA during library preparation, and nonspecific assemblies, are unlikely to account for all our observations of putative hybrid/fusion fragments, nor is the presence of hypothetical orthologues of genes from an endophytic microorganism in the plant genome.

BLAST and annotation analysis of the unmatched sequences in some hybrid contigs not associated with the *M. esculenta* reference genome did not show a high identity frequency with reported DNA sequences, while others showed low identity and coverage. However, following our methodological approach, we located known candidate insertion sequences in several assembled hybrid contigs. Some of these candidates harbour DNA sequences revealing partially significant identity matches and cover with reported genes (Figure 3B, Supplementary Data 4). Among the outstanding associated genes, transcription regulatory factor CadR and CadA (Cd^{2+} transporting ATPase enzyme) were found in the hybrid contig NODE_1832_length_571_cov_2.279352_0 (E-value = 8E−89, identity = 96%, 194 bp of length, accession number CP043626 and E-value = 6E−33, identity = 81%, 127 bp of length, accession CP043626, respectively) (Supplementary Data 4, Figure 3B), located at the distal end to the possible insertion site in the region not associated with the cassava reference genome, which showed identity with *Pseudomonas* species, such as *Pseudomonas nitroreducens* strain HBP1, *Pseudomonas denitrificans* strain BG1, and *Pseudomonas multiresinovorans* strain populi, which will be reported later as exclusive or enriched components of the gall microbiome (Figures 3D, E). Likewise, significant putative insertion sequences associated with the *arsA* gene (arsenical pump ATPase), NODE_5306_length_349_cov_1.058824_1 (E-value = 9E−112, identity = 100%, 215 bp of length, accession number CP070545), GTP-binding protein encoded by the *obg* gene, NODE_9526_length_272_cov_0.441026_16 (E-value = 1E−12, identity = 76%, 107 bp of length, accession AP018162), nitrate transport permease protein encoded by the *nrtB* gene, NODE_10604_length_264_cov_0.786096_0 (E-value = 4E−46, identity = 92%, 118 bp of length, accession CP065997), assimilatory nitrite reductase enzyme encoded by the *nasE* gene involved in biological nitrate assimilation, NODE_10604_length_264_cov_0.786096_0 (E-value = 2E−44, identity = 91%, 118 bp of length, accession LT976871), and bicarbonate transport system permease protein encoded by the *cmpB* gene, NODE_10604_length_264_cov_0.786096_0 (E-value = 3E−35, identity = 85%, 118 bp of length, accession LR594671) were also found (Figure 3B, Supplementary Data 4). Interestingly, despite its similarity to reported genes in bacteria, all of them are essential for rapid plant growth, maintenance, and survival under unfavourable conditions and are also associated with gene regulation mechanisms (Supplementary Data 4).

Additionally, the association among the 17,148 gall-specific contigs as potential components of an integrated DNA fragment in the gall cells could be inferred based on their annotated function. However, for many hypothetical proteins, a function could not be assigned (Supplementary Data 5). The presence of different transcriptional, post-translational, and cell cycle regulatory factors, as well as exogenous polymerases, transposases (transposase A), or the integration host factor subunit alpha of bacteriophage lambda (which plays a crucial role in the insertion process of lambda DNA into the *Escherichia coli* chromosome), among others, within the gall-specific contigs, provide indirect evidence and are particularly revealing.

Functional Cluster of Orthologous Group (COG) profile analysis, using gall exclusive contigs, showed that the COG categories of amino acid transport and metabolism, carbohydrate transport and metabolism, energy production and conversion, nucleotide transport and metabolism, posttranslational modification, the translation of the ribosomal structure and biogenesis, and DNA replication, recombination, and repair were the main functional categories represented. Although a relatively small number of contigs were used due to bioinformatic processing, these results could indicate that functions related to growth, transport of plant metabolites to inner gall tissues, replication, and expression of nucleic acids, in addition to gene regulation at different levels, might be promoted or increased by the exogenous DNA detected in gall cells (Figure 3C).

Metagenomes reveal an enriched microbial community in galls

We used HTS to analyse the metagenomes of two gall samples, each consisting of a pool of galls and two healthy tissue samples. The average percentage of assigned reads to any species-level taxon for healthy tissue samples was 4.64%, whereas 4.77% was the average for gall samples, according to reference databases. We showed only the significant microorganisms found at a filter resolution of 10K. The resulting taxonomic profile showed a common microbiome between gall and healthy tissue samples, including a core community dominated by 12 taxa, 9 of which were bacteria, namely, *Acinetobacter baumannii*, *Aliarcobacter butzleri*, *Halomonas* sp. JS92–SW72, *Hydrogenophaga* sp. NH–16, *Klebsiella pneumoniae*, *Paenibacillus larvae*, *Pasteurella multocida*, *Vibrio anguillarum*, and *Vibrio coralliilyticus*, in addition to two cyanobacteria, *Guillardia theta* and *Stanieria cyanosphaera*, and the Pepper chlorotic spot orthotospovirus (Figures 3D, E). Furthermore, an exclusive or enriched microbial community was detected in samples of gall tissues. The taxonomic composition included a community dominated by eight species belonging to the genus *Pseudomonas*, two species of cyanobacteria (*Chondrocystis* sp. and *Halothece* sp. PCC 7418), and three other bacteria species, *Cupriavidus oxalaticus*, *Lactiplantibacillus plantarum*, and *Streptomyces lividans* (filamentous bacterium) (Figure 3E). A brief overview of the relevant functional characteristics reported for these microorganisms and their possible role in gall development and maintenance can be found in the Supplementary Text section.

From the sequencing data exclusive to gall tissue, up to 69.2% of the reads were unassigned to any taxon. These may correspond to microorganisms not included in the available databases at the time of our analysis, or even indicate the possibility of new species as part of this unique microbial community. Nevertheless, a significant amount of exclusive gall reads was associated with several of the same *Pseudomonas* species reported as exclusive or enriched by metagenomic analysis in gall tissue (Figures 3E, F).

Isolation and genomic analysis of putative gall-inducing symbionts and gall endophytes

Two isolates (CFUs) from the original culture grown from the larval head of the inducing insect, *I. brasiliensis*, as well as seven endophytic bacteria isolates selected from cassava gall tissue, were sequenced using high-throughput platforms. Taxonomic profile analysis applied to all isolates revealed that four corresponded to a single species (IEB 2-2 = *Burkholderia contaminans*, IEB 3-1 = *P. ananatis*, IEB 4-3 = *Ralstonia pickettii*, and IEB 5-1 = *Bacillus altitudinis*). Moreover, three isolates showed association with two bacteria species of the same genus (IEB 1-2 = *Sphingomonas* sp. LK11 and *Sphingomonas paucimobilis*), and the two isolates from the original culture grown from the inducer insect, isolate ISB 1, which was *Pseudomonas azotoformans* and *Pseudomonas extremorientalis*, and isolate ISB 2, which was *Rhodococcus* sp. P-2 (which could not be classified to the species level, major component) and *R. erythropolis*. Likewise, isolates IEB 1-1 and IEB 4-4 were assigned to the same bacteria species: *Burkholderia contaminans*, *Curtobacterium* sp. MR_MD2014, *Curtobacterium* sp. SGAir0471, and *P. ananatis* (Figure 4A). However, none of the bacteria isolated from either the gall or the inducing insect larva were precisely detected in the resulting taxonomic profiles belonging to the sequenced samples of healthy plants and gall tissues (Figures 3D, E). Nevertheless, through synteny comparison analysis using specific gall contigs as target sequences compared to each of the sequenced bacterial genomes, the endophytic condition was determined only for the bacteria species isolated from gall tissue, not for those from the inducing insect (Figure S6). Thus, these endophytes might be a marginal component of the gall microbiome, but many of them could also be exclusive components of the microbial community within gall tissues. None of the gall-specific fragment consensus sequences (obtained experimentally) shared homology or aligned with any of the bacterial genomes. The isolation, identification, and subsequent characterisation of microbiome components are difficult tasks due to their low abundance in tissue. This is especially true when using whole-host genome sequencing techniques. However, this barrier may often be overcome using different culture media for growth.

Interestingly, Yang et al. (2021) reported the same bacterial genera sequenced in this study (both gall and inducing insect), except for *R. pickettii*, in their bacterial community of *Lithosaphonecrus arcoverticus* (Hymenoptera: Cynipidae) and in their gall (galled twigs) in *Lithocarpus glaber* (Fagaceae). *Pseudomonas* and *Sphingomonas* have also been identified as members of a common core bacterial community in willow-galling sawflies (Michell and Nyman, 2021). Moreover, *R. erythropolis*, *P. ananatis*, and *Pseudomonas* spp. were recently identified from the bacterial communities by Yang et al. (2022) as predominant species in chestnut tree galls induced by *Dryocosmus kuriphilus*. These bacteria were also identified as components of the microbial communities of the inducing insect and in *Torymus sinensis*, a host-specific parasitoid of *D. kuriphilus*.

Microbial genome comparison by COG functional categories is shown in the COG profile for each isolated CFU (Figure 4B). Also, genetic identity comparison by principal component analysis of sequenced bacterial isolates is shown in Figure 4C. Gene Ontology enrichment analysis of endophytic bacteria genomes showed that the most relevant enriched terms were those related to *Actinobacterium*-type cell wall biogenesis in the category of biological processes and plasma membrane in the category of cellular components. Of the 14 most significant molecular functions identified, sigma factor activity, magnesium ion binding, identical protein binding, drug binding, and ATP binding were the most significantly enriched. Moreover, growth factors for biological processes, in addition to the nucleus, intracellular non-membrane-bounded organelle, nuclear protein-containing complex, integral components of the plasma membrane, cytosol, cytoplasm, and cell wall of GO cellular component, were significantly increased in putative larval endosymbiotic bacteria.

Similarly, magnesium ion binding, identical protein binding, drug binding, and ATP binding were the most significant molecular functions in the enrichment values for these bacteria (Figures 4D–F). Significant discrete GO terms from endophytic bacteria and possible insect endosymbiotic bacteria are shown in Supplementary Data 6. Thus, functional analyses carried out on the endophytic bacteria and on the presumed endosymbiotic bacteria isolated and sequenced from the gall tissue and insect head, respectively, as well as functions associated with the exclusive or enriched microbial community identified in cassava galls by metagenomic analysis (Figure 3E), suggest that the microbial community may play an important role in gall induction, growth, and maintenance. The inferred metabolic pathways and biological functions show that several endophytic bacterial species have a range of different potential functions, including biodegradation of phenolic and potentially harmful metabolic compounds, nutrient supplementation, synthesis of plant hormones, and secondary metabolite degradation. Moreover, this endophytic bacterial community has the potential to synthesise essential amino acids and vitamins, and some of them could be involved in nitrogen and phosphate metabolism, suggesting that these bacterial species could contribute significantly to the nutritional quality of gall tissue. Hence, from an ecological, evolutionary, and functional point of view, our data show that some components of this microbiome can play important roles, both in the host plant itself and in galls. Furthermore, we argue that an unrevealed, induced microbial community that seems to be evident in our findings might have a critical impact on insect gall induction and maintenance despite their low abundance in the gall tissue.

Artificial gall induction by potential bacterial symbionts

An initial gall-like structure was obtained using the *Rhodococcus* bacterial strain, which was related to the gall induction process (Figures 5A, I–P). Putative insect endosymbiotic bacterial lines isolated from the inducing insect (larva head), initially classified as *Rhodococcus* sp. by sequencing the 16S gene and then identified as the genus *Rhodococcus* based on the taxonomic profile (related to *Rhodococcus* sp. P-2 and *R. erythropolis*), were used in these assays. The endophytic bacterium *P. ananatis* isolated from gall tissue was used as a control bacterium.

Plant tissue culture inoculation assays on *M. esculenta* were carried out under controlled laboratory conditions. Most of the gall-like structures were formed in leaves and leaf sections of *M. esculenta* rather than in micro-stakes with apical shoots (apical buds with small leaf primordia), where their formation was the lowest in the first 2 weeks. However, a greater increase in tissue necrosis was observed after 2 weeks of culture (Figure 5A). Moreover, since the culture medium used was not optimised for the propagation and maintenance of leaves, it was not possible to maintain the structure for more than 4–5 weeks. Necrosis and early fall of the inoculated leaves did not allow long-term monitoring of *in vitro* materials (with a few exceptions), an effect also observed in inoculated greenhouse plants.

The inoculation of plants grown in soil under greenhouse conditions was also attempted. However, except for the rare event of neoplasm formation over the inoculated leaf primordia, no gall-like structures were clearly observed (results not shown). This could be the consequence of an increased level of control by the plant in its growth process or unaccounted for environmental factors. Moreover, strong tissue chlorosis was observed after the first week, when the bacteria *P. ananatis* was used in the assay (control inoculation bacterium), observing broad bacterial growth on the tissues without tissue folding or gall-like structures (Figure 5C). Likewise, no significant tissue folding or gall-like structure was observed in the control material treated only with mechanical abrasion using the ground glass (Figure 5B). The different success rates of inducing gall-like structures in the *in vitro* assays and under controlled greenhouse conditions indicate that specific inoculation conditions have yet to be optimised.

After inoculation and incubation with cassava explants, bacterial colonies on plant tissue were frequently observed and were usually associated with invaginations and folds of the leaf blade. However, these leaf deformations and invaginations were not frequently associated with macroscopically visible bacterial growth (Figures 5I, K, L, N, P). Furthermore, a few showed differentiated neoplastic tissue that visually differed from the surrounding tissue (Figures 5l, N, P). An increased tissue reaction was observed when a high bacterial density grew over the plant tissues. This increased reaction induced a higher rate of deformation and invagination of the leaf blade, which usually triggered an earlier necrosis condition in the explants (Figure 5A, images not shown), probably generated by the hypersensitive reaction of the plant tissue. Initial gall formation induced under natural field conditions on young leaf primordia and on medium-mature young leaves is showed in Figures 5D–H.

The ability to induce abnormal gall-like growth has also been reported in *Rhodoccocus fascians* (Stes et al., 2011; Dolzblasz et al., 2018; Harris and Pitzschke, 2020). The development of plant growth is closely related to a linear virulence plasmid harbouring an array of cytokinin genes encoded by the fasciation (fas) operon in most pathogenic isolates (Francis et al., 2012; Creason et al., 2014; Radhika et al., 2015; Jameson et al., 2019). Furthermore, despite the absence of authentic leafy galls in Pistachio Bushy Top Syndrome (PBTS), synergistic coinfection has been reported between *Rhodococcus corynebacterioides* and *R. fascians* by Vereecke et al. (2020).

Bacterial strains belonging to the genus *Rhodococcus* (Figure 4A), isolated from the inducing insect (ISB 2 isolate) and used in gall induction assays, were not detected with certainty at the species level in the sequenced samples of galls and healthy plant tissues when metagenomic analysis was applied (Figures 3D–F). Therefore, we suggest three possible explanations for our findings. The first scenario proposes that the bacterial lines used in our gall induction assays were not conclusively detected because the bacteria had a lower relative abundance within the gall tissue. Thus, the target bacterial DNA would not have a representative fraction in the gall samples purified

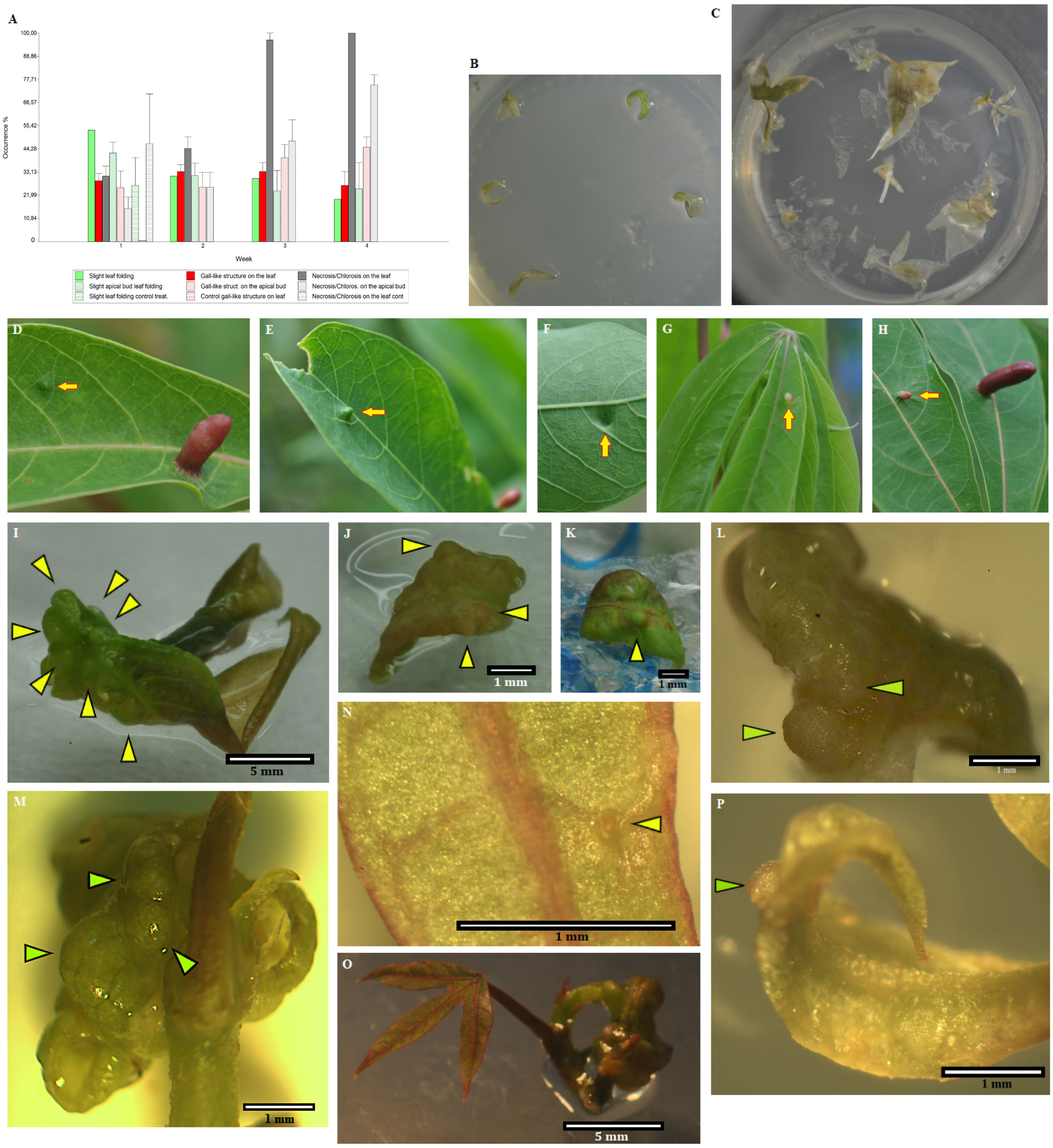

FIGURE 5

Bioassays show that potential insect endosymbiotic bacteria of the genus *Rhodococcus* induce gall-like structures in *Manihot esculenta* plants. **(A)** Graph of primary gall induction on leaves and micro-stakes with apical buds. Data for bacterial inoculation control are not shown due to the tissue damage caused since the first week of data collection. **(B)** Control inoculation, with only slight mechanical abrasion (without bacteria inoculation) in solid medium. **(C)** Leaf and apical buds control culture, inoculated with *Pantoea ananatis* (IEB 3-1) control bacterium. **(D–F, H)** Initial gall formation induced under natural field conditions on medium-mature young leaves. **(G)** Gall induced under natural field conditions on young leaf primordia. **(I–L)** Gall-like structure induced on leaves by inoculation with the isolated *Rhodococcus* strain (1–2 weeks of culture). The solid medium was supplemented with 3 mg L^{-1} Kathon, a bacteriostatic reagent used to inhibit bacterial growth in the culture medium. **(M–P)** Gall-like structure induced on apical buds by inoculation with the isolated *Rhodococcus* strain (**M**, 4 weeks of culture; **N–P**, 3 weeks of culture). Green or yellowish arrows show the formation of gall-like structures. Data are shown as the mean (± SE) of tissue response due to inoculation with the bacteria.

for sequencing. An additional explanation could be that the putative bacterium is involved in the gall induction process, but it is not a component of the gall cell endophytic microbiome, according to our results, thus performing its action externally to the plant cell. Furthermore, the bacterium could exert its action at the initial stage of gall induction. Third, another taxonomically related bacterium sharing a similar molecular mechanism might also induce the formation of this structure in its initial stage.

Detection of the specific gall marker fragment in sequenced plant material and sequenced bacterial isolates

The non-detection of the differentially amplified gall fragment (specific gall fragment experimentally obtained) in the sequenced *M. esculenta* gall genome and specifically in the sequenced *Rhodococcus* spp., which we argue is related to the gall induction process, could be explained by the sequencing of large low-copy-number wild-type plasmids harbouring large duplications, making it nearly impossible to correctly determine a plasmid genome sequence using a short-read sequencing platform, such as Illumina HiSeq and MiSeq (Smalla et al., 2015; Orlek et al., 2017). Moreover, large wild-type plasmids are difficult to reconstruct from whole-genome sequencing data; this arduous task usually requires a hybrid assembly approach that combines the long reads with the accuracy of short-read sequencing (Berbers et al., 2020). Consequently, localising genes in specific plasmids may be difficult (Orlek et al., 2017). Hence, based on our findings, no direct evidence that the specific gall fragment could be part of an insertion sequence was obtained, and we therefore argue otherwise that this specific gall fragment would be an accessory genetic component of the bacteria transformation machinery, harbouring a transformation plasmid, but this would not be part of the insertion sequence integrated into the genome of the plant cell, analogous to the configuration of the Ti plasmid in *A. tumefaciens* strains (Suzuki et al., 2015; Shao et al., 2018; Chou et al., 2022). Therefore, its specific detection in the DNA extracted from gall tissue by PCR-based methodologies could be the consequence of the transferred endophytic form of *Rhodococcus* spp. by the inducing insect, or another bacterium sharing the same genetic element, in which genomic and plasmid DNA are co-precipitated along with host plant DNA. Moreover, *in silico* PCR analysis of the specific gall fragment did not produce any amplicon from the cassava reference genome (results not shown), thus demonstrating that this DNA fragment is not a component of the host plant genome. However, further research on this specific topic should be carried out to provide more evidence.

Conclusion

We provide evidence suggesting an insect-induced gall formation mechanism mediated by genetic transformation events in host plant cells. Our data allude to a potential mobile genetic element harboured in *Rhodococcus* spp. bacteria isolated from the inducing insect that could be involved in the induction mechanism. Moreover, gall induction and growth could also be associated with a change in the microbiome composition in plant tissue. It is possible that the inducing insect injects components of the gall-specific endophytic community during female oviposition and larval feeding. Genetic transformation and microbiome modification of gall tissue in *M. esculenta* could be a more widely distributed induction mechanism in nature. This was supported by the detection of a potential accessory genetic component of the transformation machinery (ubiquitin-like gene *E2*) or with a similar identity in other insect galls in other plant species, and even detected in wild-type plasmids purified from endophytic bacteria species isolated from the same cassava gall tissue. While our results provide greater insight into plant–bacteria–insect interactions, further research is needed to fully understand the complex mechanisms of gall induction and formation. Moreover, the insertion of genetic elements from a putative bacterium could function as a switch in the molecular interaction between the inducing insect and the host plant.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Ethics statement

The manuscript presents research on animals that do not require ethical approval for their study.

Author contributions

OG-B: conceived the survey; substantial contribution to the concept and design of the bioinformatic approach; performed most of the molecular biology experiments; contribution to data analysis and interpretation; contribution to the data collection, registration, and processing; obtained and provided financial support; and wrote the article. JJ-M: substantial contribution to the concept, design, and analysis of the bioinformatic approach; participated in writing the manuscript. RW: contribution to the design and analysis of the bioinformatic approach; and critical revision of the manuscript. PH: contribution to the concept and design of the study; contribution to critical revision; and addition of intellectual content. SV-D: contribution to bioinformatic analysis and interpretation; contribution to critical revision and adding intellectual content; and contribution to manuscript preparation. AA-G: contribution to data analysis and interpretation; and contribution to manuscript preparation. AP-T: contribution to critical revision; adding intellectual content; and contribution to manuscript preparation. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1237966/full#supplementary-material

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Supplementary text for
Microbiome and Plant Cell Transformation Trigger Insect Gall Induction in Cassava

Supplementary Methods

Galling insect model

The morphotypes of galls include: *Cydista diversifolia* (Kunth) Miers (Bignoniaceae) morphotype Cy_di_1, induced by a Cecidomyiidae; *Malvaviscus arboreus* Dill. ex Cav. (Malvaceae) morphotype Ma_ar_1, induced by a Cecidomyiidae; *Hirtella racemosa* Lam. (Chrysobalanaceae) morphotype Hi_ra_1, induced by a Cecidomyiidae; *Pisonia macranthocarpa* (Donn. Sm.) Donn. Sm. (Nyctaginaceae) morphotype Pi_ma_4, induced by an unkonwn insect; *Randia monantha* Benth. (Rubiaceae) morphotype Ra_mo_1, induced by an unknown insect; *Lonchocarpus phlebophyllus* Standl & Steyerm. (Fabaceae) morphotype Lo_phl_1, induced by a Psyllidae; recorded and described by Gätjens-Boniche et al., 2021. In addition to the gall morphotype on *Coussarea hondensis* (Standl.) C.M. Taylor & W.C. Burger (Rubiaceae), induced by an unknown insect. These galls were recorded and described by Gätjens-Boniche et al. (2021) and the host plants were identified by Alonso Quesada (Herbario del Museo Nacional, San José, Costa Rica). The gall morphotype on *Miconia oerstediana* (O. Berg ex Triana) Michelang. (Melastomataceae), previously identified as *Conostegia oerstediana* and induced by a Cecidomyiidae, was also included; the host plant was identified by Mario Blanco Coto at Herbario de la Escuela de Biología, Universidad de Costa Rica.

Quantification of PCR inhibition level from genomic DNA purified using the specific endogenous gene UBQ-10 of *Manihot esculenta*

Strong PCR inhibition was previously detected in DNA extraction from cassava plant material using different genomic DNA purification protocols and commercial kits. For that reason, PCR reaction conditions were carefully tested. PCR inhibition was tested using the endogenous gene UBQ10 of *Manihot esculenta* according to reaction conditions described by Moreno *et al.* (2011). A serial dilutions factors of 1/10 (*i.e.,* 1X, 0.1X, 0.01X. 0.001X and 0.0001X) was used with genomic DNA purified from leaves and galls. 25 µl PCR reactions were carried out using Dream Taq DNA polymerase reagents (Fermentas Life Sciences, Lithuania), with component concentration as specified by the supplier, PCR quality H_2O (Promega Corporation, Madison, Wisconsin, USA), and primers: UBQ10 Fwd360 (5'-TGCATCTCGTTCTCCGATTG-3') and the reverse UBQ10 (5'-GCGAAGATCAGTCGTTGTTGG-3') (Integrated DNA Technologies, Redwood City, CA, USA). All tests were carried out in triplicates.

Purification of *Agrobacterium rhizogenes* Ri plasmid

The Ri *Agrobacterium rhizogenes* plasmid was used as positive control in RAPDs PCR reactions. Plasmids purified from *Agrobacterium rhizogenes*, line A4, were cultured in YEB 1X media (Piñol *et al.*, 1996) and incubated at room temperature (25 °C) for 3-4 days. Plasmid isolation was carried out as described by Li et al. (1995). Plasmids purified from *A. rhizogenes* line A4 were resuspended in 60 µl of sterile water. Extracted DNA was visualized in a 0.8% agarose gel, showing a high molecular weight and clearly defined fragment.

Purification and re-amplification of previously differentially amplified DNA fragments

Some of the differentially amplified DNA fragments obtained from RAPD assays were isolated and purified to re-amplify them. Purification was performed using the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany) and the Wizard PCR Preps DNA Purification Systems (Promega Corporation, Madison, Wisconsin, USA). These re-amplifications were performed to increase the concentration of purified fragments for cloning and sequencing. To verify the presence of the differentially amplified fragments from gall samples (from RAPD and re-amplification) PCR was performed following the same reaction conditions as above. Negative control for these PCR reactions did not amplify any band.

Cloning of differentially amplified DNA fragments from *Manihot esculenta* galls

Cloning of differentially amplified DNA fragments from *Manihot esculenta* galls were done in *Escherichia coli* XL1-Blue, transfected with pAMP1 cloning vectors (Invitrogen-Life Technologies). Cloning, ligation, transformation, and other DNA manipulations were performed according to standard procedures (Ausubel et al., 1995) or as recommended by the supplier. Fragments of different size and selected for cloning correspond to the ones purified and reamplified from different gall samples. Insertion of the target fragment was corroborated through PCR amplification. PCR reactions were performed using M13 PUC Forward and M13 PUC Reverse as well as SP6 and T7 primers. PCR products were separated and visualized by agarose gel electrophoresis to 1.5 %, TBE 0.5 X, Gel Red 1X, and 75 volts.

Bioinformatic analysis

Overview of Next Generation Sequencing analysis approach

We analyzed the presence of any endophytic microorganisms or possible DNA insertion sequences in the gall tissues comparing samples from healthy plant tissue and gall tissue from casava plants, using a high throughput sequencing platform. The generated data, composed of millions of reads, were analyzed using powerful computational tools. To identify possible exogenous DNA sequences in the gall tissues, a bioinformatic approach, which we named Host Discriminant Genomic Analysis (HDGA), was used. This methodological approach consists of a bioinformatic analysis in which the sequenced data were processed to separate, assemble, and analyze the DNA sequences different from those in the referenced host plant genome. The differing reads resulting from the matches between healthy tissue and gall tissue were separated, thus selecting only the specific reads presented in the DNA from gall tissue (readings having different bases). Sequenced reads between healthy cassava tissue samples, sequenced from genotype Valencia, and the cassava reference genome, CV AM560-2 (Phytozome genome ID: 520 Bredeson et al. 2016) were contrasted and mapping reads to the reference were filtered out. Polymorphic variants reads between the sequenced genomes of healthy plants and galls were filtered out, primarily with the reference cassava genome and then by pairing against themselves. Reads that did not map to the cassava reference represent potential foreign DNA from endophytic organism or foreign inserted DNA into the plant gall cell (see Figure S5 for general pipeline-flow diagram-approach). Differing reads detected only in gall tissue were assembled into contigs.

In addition, seven colony-forming units (CFUs) of possible endophytic bacteria from plant gall tissue and two CFUs from insect larval heads of *Iatrophofia brasiliensis* were isolated and sequenced in an Illumina MiSeq platform.

Supplementary results and discussion

Quantification of PCR inhibition level from genomic DNA purified using the specific endogenous gene UBQ-10 of *Manihot esculenta*

High PCR inhibition from *Manihot esculenta* DNA samples was detected and measured when the housekeeping gene UBQ10 was used, resulting in higher values in several gall samples, until an DNA dilution factor of 1X10 $^{-2}$ was used (data not shown). DNA extraction was performed to ensure high throughput with the least inhibition possible in subsequent PCR-based tests. Despite that effect, remnant inhibition and DNA degradation in cassava plant tissue was detected using as reference the endogenous ubiquitin gene UBQ-10 of *Manihot esculenta* (results not shown).

Characterizing a potential gall molecular marker

The presence of possible exogenous DNA sequences in gall tissue of *Manihot esculenta* plants was initially determined by the detection of differentially amplified fragments of DNA extracted from these structures by a modified RAPDs technique (Random Amplification Polymorphic DNA). Samples of healthy leaf and gall tissues, growing in the same plant organ, were compared. The aim of this approach was to detect amplification fragments present in the DNA of the gall and not present in the same healthy tissue. We noted the presence of a 550 bp fragment, amplified in many of the gall samples, as well as a clear fragment of approximately 1100 bp and another fragment with an approximated size of 1600 bp. The polymorphism observed when the ipt-Forward and iaaM-Reverse primer were used for RAPDs could indicate the preliminary evidence of foreign genetic material in the plant gall cells.

The consensus DNA sequence obtained from the alignment of RAPDs differentially amplified fragments was used as a template to design and test specific primers as PCR markers for the detection of a putative exogenous DNA or, a DNA insertion sequence present only in gall cells but not in the healthy plant tissues (Supplementary Data 1). PCR reaction was optimized to amplify the expected fragment under high stringency conditions, 6-celsius degrees above annealing temperature (62ºC). Nonetheless, some non-expected amplification bands were occasionally also amplified in leaves and galls of different samples, but the expected band was always present in the gall samples (Figure 1E), which we relate with the amplification of homologous DNA sequences from other endophytic bacteria in the host plant, that could share a similar colonization strategy. This was evidenced by the high and medium identity among DNA fragments amplified and sequenced from wild type plasmids of the putative endophytic bacteria (Figure. 2E), in addition to the amplification of fragments with different sizes than were expected from the same analyzed bacteria plasmids. Nonetheless, determining plasmid relationships is challenging due to the tendency of plasmids to lose, gain, and rearrange genetic content even in the same type of plasmids, therefore, with a tendency to share few phylogenetically concordant core genes (Tazzyman and Bonhoeffer, 2014; Orlek et al., 2017).

Occasionally, some healthy plant tissue samples showed an amplification signal, which we attribute to a possible degradation of the Taq Man probe by still active DNAses detected in DNA purifications (results not shown), which can lead to a false amplification signal. This was confirmed by no DNA amplification products through the PCR reactions using agarose gel electrophoresis and Microchip Electrophoresis System for DNA/RNA (results not shown). Occasional amplification of the expected PCR fragment was also observed in healthy tissue samples in end-point PCR assays, which we attribute to the presence of micro-galls not previously detected in healthy tissue. Furthermore, analysis of one of these unspecific amplified sequenced fragments showed a clear binding site for the forward primer and a less clear binding site for the reverse primer. However, this fragment showed less than 45% of discontinuous identity when compared to the expected sequence (results not shown).

The specific gall fragment (SGF) sequence was analized by BLAST (Basic Local Alignment Search Tool, NCBI) against relevant databases (NCBI GenBank and Integrated Microbial Genomes & Microbiomes (IMG/M) system (https://img.jgi.doe.gov), showing partial pairings with low-medium and often discontinuous length coverage with some ubiquitin-like genes, more specifically, the ubiquitin-like gene E2, a component of the ubiquitin-proteosome system (UPS). Specifically, the ubiquitin-conjugating enzyme (E2) plays a critical role in transporting the ubiquitin-activating enzyme (E1) to the ubiquitin-ligase enzyme (E3), probably determining whether the labeled protein would be degraded or involved in nonproteolytic processes (Liu et al., 2020). Moreover, some evidence suggests that they could be involved in DNA repair and especially in DNA post-replication repair (Wen et al., 2008; Andersen et al., 2008). However, the role of E2 enzymes in plants remains so far uncharacterized. Otherwise, Blast analysis showed high identity of the cloned RAPD fragment named 4F-2 with the 18S ribosomal RNA gene from several orchid mycorrhizal of the genus *Tulasnellacea*e sp. (Evalue:1.9e-147, Identity: 98% (307/314), Accession: KF266987.1).

Bioinformatic analysis expands the catalog of gall specific sequences and reveal genetic insertion events evidencing plant cell transformation

High throughput sequencing potentially allows full or partial sequencing of any particular genome component in a target sample with enough relative representation. Genome variants between the cassava genotypes sequenced and the reference genome were filtered and separated by comparing the reads of healthy tissues and those from galls. The differing reads resulting from the match between healthy leaf and gall tissues were separated, then selecting only the specific reads presented in the DNA from gall tissue (sequences having different bases when compared to the reference genome). Gall unique reads were assembled into contigs using SPAdes (Prjibelski et al., 2020). The resulting contigs were then mapped against the cassava genome reference to identify hybrid sequences, that is, contigs that contained cassava genome sequences in either the 5', 3' or both ends of the sequence. There were 216 (32%) hybrids fragments harboring sequences with interspersed homology to the host plant genome, which we associate once again with structural DNA rearrangements generated as a consequence of insertion events (Supplementary Data 3). Following this approach, reads that did not map to the cassava reference nor shared between healthy and gall tissue, represent potential foreign DNA from endophytic organisms, such as bacteria or fungi, or possible foreign DNA integrated into the genome of gall cells (see Figure S3 for general pipeline-flow diagram approach, Figure 3-A and Supplementary Data 3). Some of these endophytic organisms may be involved in gall induction and formation.

Metagenomes reveal an enriched microbial community in galls

Functional characteristics attributed to some of the exclusive or enriched gall microbiome components include the production of vitamin B12 (Fang et al. (2017), and the conversion of nitrate (NO3) into nitrogenous compounds (N2) by *Pseudomonas denitrificans,* while the plant growth-promoting bacteria *Pseudomonas nitroreducens* improves cell development and enhance nitrate uptake in plants (Trinh et al., 2018). Potassium-solubilizing bacteria (KSB) *Cupriavidus axalaticus* could play a fundamental role in solubilizing fixed potassium and consequently making this essential element accessible to plants. *Cupriavidus axalaticus* T2 strain of this bacterium has been related to the simultaneous degradation capability of phenolic compounds and denitrification under aerobic conditions to produce ammonium as well (Yan et al., 2021).

Meanwhile, shared species between gall and healthy tissue of the genus *Hydrogenophaga* (Gan et al., 2011), as well as *Pseudomonas* bacterial strains have been related to biodegradation of the potentially harmful compounds 4-Aminobenzenesulfonate, resulting in the release of ammonium and sulfate. Transformation and fixation of nitrogen compounds have been associated also with *Acinetobacter baumannii* along with the synthesis of IAA (Lin et al., 2018), and even this endophyte can help hosts to remove pollutants and withstand environmental stress (Khaksar et al., 2017). Endophytic species of *Halomonas* and *Pseudomonas* (detected only in gall tissue) have been associated with alleviation of the toxic effects of salinity (Zinniel et al., 2002; Zhang et al., 2020). *Guillardia theta* and *Stanieria cyanosphaera* cyanobacteria could simultaneously increase the photosynthesis carried out in the gall tissue (Figure 3B).

Achromobacter deleyi also appears to be an exclusive gall bacterium using a filter resolution of 5K, considered a significant but conservative value in the analysis (results not shown). This potentially exclusive gall bacterium was reported as a component of the microbiome in legume nodules in *Mimosa pudica* (Tapia-García, 2020). Likewise, *Phytobacter ursingii* for instance, has potentially been associated with nitrogen-fixation, despite no environmental isolates of this species being reported so far (Pillonetto, et al. 2017). Likewise, *Pseudomonas putida* produces and degrades IAA (Leveau and Gerards, 2008), and *Achromobacter xylosoxidans* is reported to have plant growth-promoting activity of IAA (Jha and Kumar, 2009). Moreover, this approach allows us to taxonomically classify read sequences, revealing a possible minor and exclusive community component of gall microbiome as well.

Analysis of taxa associated with exclusive gall reads did not map to the reference cassava genome was attempted. A significant amount of exclusive gall reads were associated with several of the same *Pseudomonas* bacterial species reported as exclusive or enriched in the gall tissue (Figure 3C, 3D). According to this result, we hypothesize that different regions of the bacterial genome could have been differentially sequenced between samples, but with sufficient relative abundance to be reflected in this taxonomic profile.

Moreover, the taxonomic profile carried out with the samples of healthy leaf tissue sequenced, and functional characteristics reported in the literature associated with some of these microorganisms, showed that some could be not only beneficial to the cassava plant, but may also include opportunistic pathogens in the microbiome (Figure 3D).

Particular functional analysis (GOs) of gall endophytes and putative endosymbionts of the inducing insect

Significant particular GOs of endophytic bacteria and from possible insect endosymbiotic bacteria are shown in Supplementary Data 6 as well. Among the specific functions and biological processes inferred from endophytic bacteria, the most intriguing are those related to pathogenesis (GO:0009405, value:1.3e-08), cellular amino acid biosynthetic process (GO:0008652, value:5,00E-06), biological process involved in symbiotic interaction (GO:0044403, value:5.1e-06), response to host immune response (GO:0052572, 6.6e-05), sulfur compound biosynthetic process (GO:0044272, value:0.00017), urea cycle (GO:0000050, velue:0.00018), response to nitrosative stress (GO:0051409, value:0.00044), regulation of immune response (GO:0050776, velue:0,0008), recombinational repair (GO: GO:0000725, value:0.00101), modulation by symbiont of host immune response (GO:0052553, value:0.00108), chromosome organization (GO:0051276, value:0.00128), double-strand break repair (GO:0006302, value:0.00202), positive regulation of DNA binding (GO:0043388, value:0.00215), organonitrogen compound biosynthetic process (GO:1901566, value:0.00316), regulation of DNA metabolic process (GO:0051052, value:0.0034), regulation of meiosis I (GO:0060631, value:0.0036), mitigation of host defenses by symbiont (GO:0030682, value:0.00362), chaperone-mediated protein folding (GO:0061077, value:0.00501), regulation of DNA-dependent DNA replication (GO:0090329, value:0.00643). Likewise, some relevant GOs associated with the putative endosymbiont genomes, include those related with drug metabolic process (GO:0017144, value:2.4e-06), cellular amino acid biosynthetic process (GO:0008652, value: 6.2e-06), urea cycle (GO:0000050, value:0.00011), organonitrogen compound biosynthetic process (GO:1901566, value:0.00065), response to host immune response (GO:0052572, value:0.00228), regulation of meiosis I (GO:0060631, value:0.00233), import into cell (GO:0098657, value: 0.00235), antibiotic catabolic process (GO:0017001, value:0.00294), regulation of DNA-dependent DNA replication (GO:0090329, value:0.00317), regulation of neurotransmitter levels (GO:0001505, value: 0.00328), double-strand break repair via homologous recombination (GO:0000724, value:0.00568), regulation of chromosome organization (GO:0033044, value:0.00634), positive regulation of nitrogen compound metabolic process (GO:0051173,value:0.00662), and DNA conformation change (GO:0071103, value:0.00662), among many others with significant value (Supplementary Data 6).

Moreover, direct wild type plasmid sequencing was attempted by purifying enrichment bacterial plasmid DNA using standard sequencing techniques from Illumina MiSeq sequencing platform. However, presumably due to the stability and integrity of plasmid DNA during the fragmentation procedure, no sequencing was done for any endosymbiotic and endophytic bacteria wild type plasmid.

Scanning electron microscopy (SEM) and Transmission electron microscopy (TEM) of gall tissues

Structures shown in Figure S7 by Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM), are consistent in morphology and size with intracellular bacterial cells (Lebsky and Poghosyan, 2014; Mensi et al., 2014; Zhou et al., 2014; War and Joshi, 2017; Esposito-Polesi et al, 2017). Moreover, some gall cells reveal bacteria added or embedded in a possible type of biofilm (Figures S7C, E), like those described by Esposito-Polesi et al. (2017) and Taufiq and Darah (2020). These possible endophytic microorganisms can also be observed along with possible plastids like amyloplasts of different sizes (Lebsky and Poghosyan, 2014; Gama et al., 2015; Olguin-Maciel et al., 2017) (Figures S7D, E). At least nine possible morphospecies of bacteria were observed in TEM images obtained from cassava gall tissues, some of them are shown in the Figures S7F-J. Although an exhaustive comparative search with respect to normal leaf tissue was not performed, the abundance of endophytic microorganisms seems to be greater in the gall tissues in relation to the surrounding leaf tissues (images not shown), which is consistent with the results of genomic analyses (Host Discriminant Genomic Analysis, HDGA).

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SUPLEMENTARY FIGURES

Figure S1. Inducing insect, *Iatrophobia brasiliensis*. (A) Larva. **(B)** Adult. **(C-D)** Salivary glands dissected from larva.

Figure S2. *Rhodococcus* bacterial strain isolated from the gall-inducing insect (larval head) associated with gall induction in *Manihot esculenta*. **(A)** Culture on YEB 1X media at 26 °C. **(B)** Cultured and storaged after more than one month refrigerated at 4-10 celsius degrees, showing pinksh color. **(C, D)** Gram-positive bacillus shown in light microscope.

Figure S3. Randomly Amplified Polymorphic DNA (RAPD) specific to gall samples. **(A)** Theoretical diagram for detection of differentially amplified fragments showing the hypothetical binding sites of primers and the respective amplified bands from healthy plant tissue and from gall tissue. Case1: differentially amplified band with one primer binding to plant DNA and the other binding to the inserted DNA sequence. Case 2: differentially amplified band with both primers binding sites to the inserted DNA sequence. Case 3: differentially amplified band with both primers binding to the genome of a potentially endophytic microorganism. **(B)** Agarose gel electrophoresis of amplified DNA fragments ranging from 100 to 4500 base pairs, comparing healthy leaf tissue DNA samples (H) and gall tissue DNA samples (G). M: Lamda DNA EcoRI/Hind-III Marker; NC: negative control (reagents only), lines S1-S8: samples of healthy leaf and gall tissues growing in the same plant (pair-compared). Arrows point to differentially amplified fragments in paired samples. PC: “positive” sample from *Agrobacterium rhizogenes* plasmids. **(C)** Analytical detections of the RAPDs amplicons through Microchip Electrophoresis System for DNA/RNA (MultiNA) show the amplification of differentially amplified fragments. Healthy leaf tissue DNA samples (H) and gall tissue DNA samples (G) pair-compared; MM: molecular markers ΦX174 DNA/Hae III marker (Promega) and 25 bp DNA marker (Invitrogen), NC: negative control (reagents only). Red arrows point to differentially amplified fragments in paired samples.

Figure S4. Gel electrophoresis of PCR amplicons using primers specific for the specific gall fragment marker (SGF). Samples correspond to purified wild type plasmids from two putative endosymbiotic bacteria of the genus *Rhodococcus* and *Pseudomonas,* isolated from the larval head of the inducing insect *Iatrophofia brasiliensis* (colony-forming units ISB 1 and ISB 2), as well as wild type plasmids purified from seven endophytic bacteria isolated from cassava gall tissue (IEB). PCR amplified fragments are also shown for the inducing insect salivary gland sample (SG). Samples of DNA purified from healthy leaf and gall tissues were used as positive controls (lines S1-S4). Green circles indicate positive amplification for SGF (S1-S3). M: molecular weight marker (Gene Ruler 1 KB Plus), NC: negative control (reagents only).

Figure S5. Host Discriminant Genomic Analysis (HDGA) workflow. High throughput sequencing data from samples of leaf healthy tissue and insect-induced galls were quality controlled (step 1) and compared against the cassava reference genome to separate reads with near perfect match sequences (step 2). The differing *reads* between healthy tissue and gall tissue were filtered-separated, thus selecting reads unique to DNA from gall tissue (step 3). Reads that did not map to the cassava reference represent potential foreign DNA from endophytic organism or foreign DNA inserted into the gall cell. Differing reads detected only in DNA from gall tissue were assembled into contigs (step 4). Contigs were then mapped to the cassava reference again (step 5) and the resulting alignments filtered (step 6), leaving only high-quality alignment of hybrid contigs: containing a fragment of both host DNA and foreign (possibly) inserted DNA. Annotation of the filtered alignments was done with MegaBlast, which revealed known candidate genes associated with the inserted sequences (step 7). Finally, taxonomic profiles of the gall unique contigs was conducted and the internal gall microbiome was determined (step 8).

Figure S6. Endophytic bacteria determined by synteny analysis. **(A-C)** Colony-forming units (CFUs) of potentially endophytic bacteria and one fungal growth isolated from surface-sterilized *Manihot esculenta* gall slices. Each of these CFUs were named IEB (isolates of endophytic bacteria). **(D-F)** Sequential isolation of CFUs from initial growths (2-3 sequential isolates). **(G-M)** Synteny analysis using specific galls contigs as target sequences versus each of the sequenced bacterial genomes.

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Supplementary Data

Supplementary Data 1: Alignment of RAPDs differentially amplified fragments of Specific Gall Fragment.

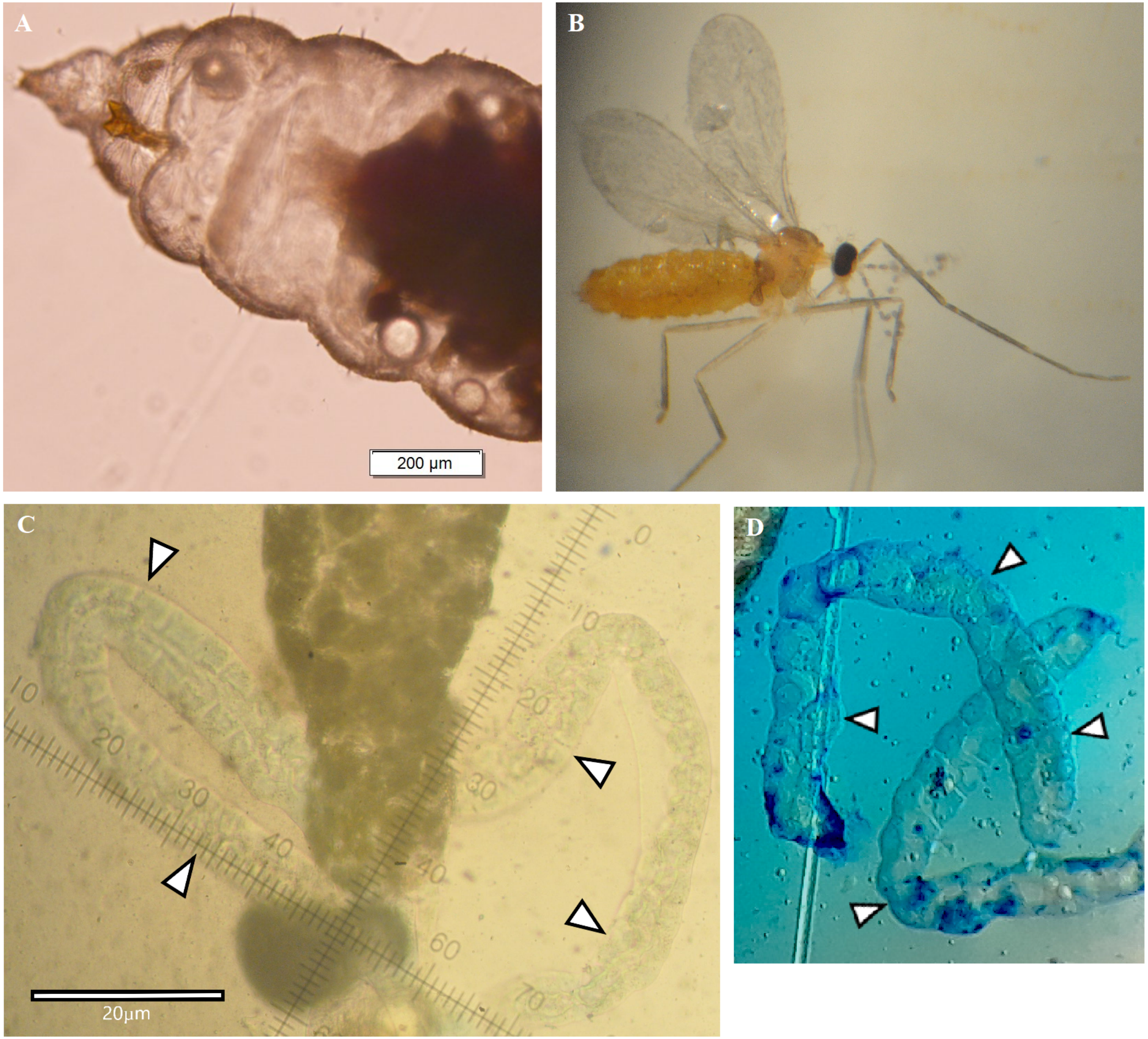
Supplementary Data 2: All Specific Gall Contigs sequences.

Supplementary Data 3: Selected Specific Gall Hybrid Contigs sequence information.

Supplementary Data 4: Blast Analysis and Annotation of Specific Gall Hybrid Contigs.

Supplementary Data 5: MegaBlast and Annotation of All Specific Gall Contigs.

Supplementary Data 6: Significant discrete GO terms from endophytic bacteria and possible insect endosymbiotic bacteria.



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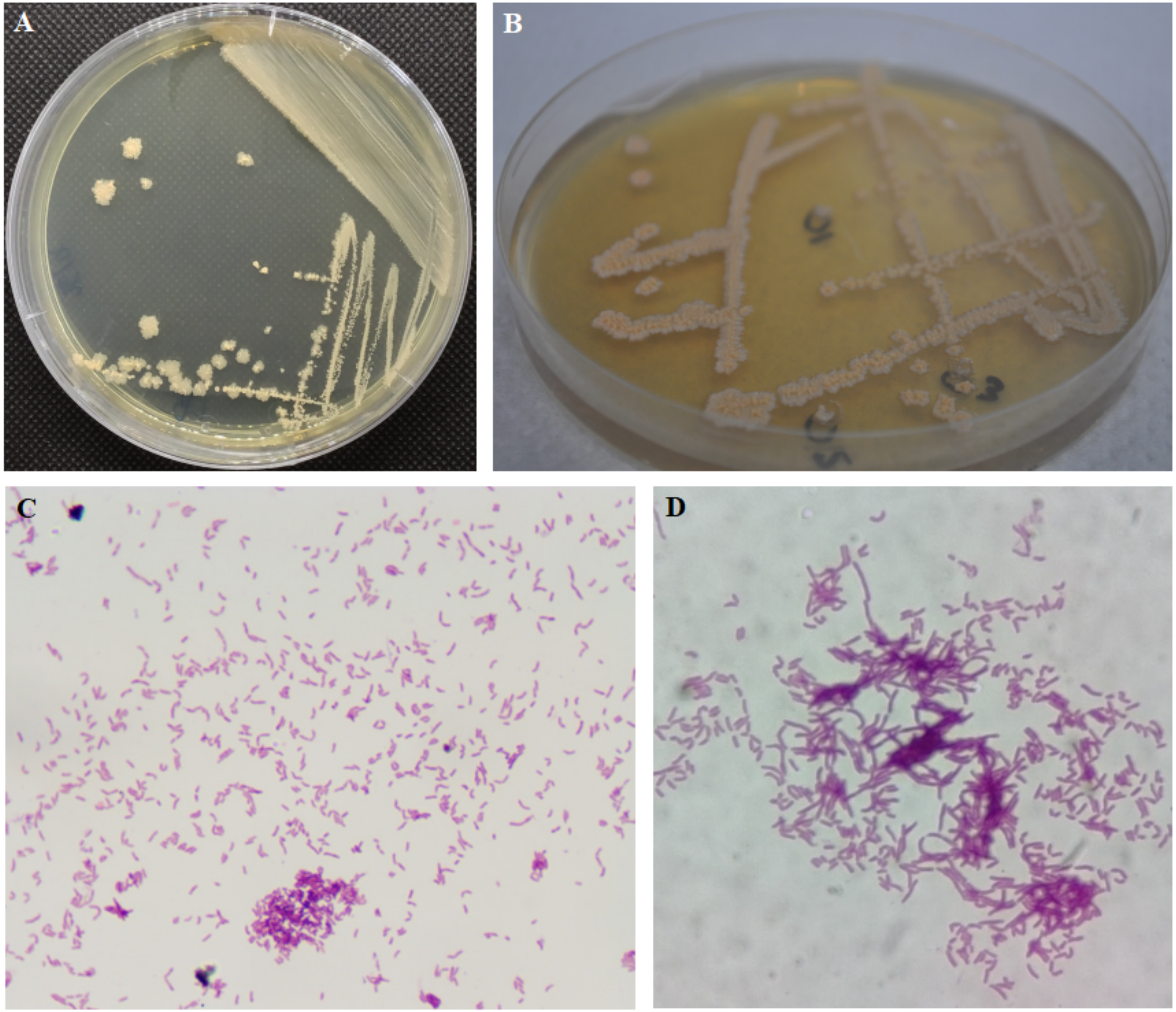

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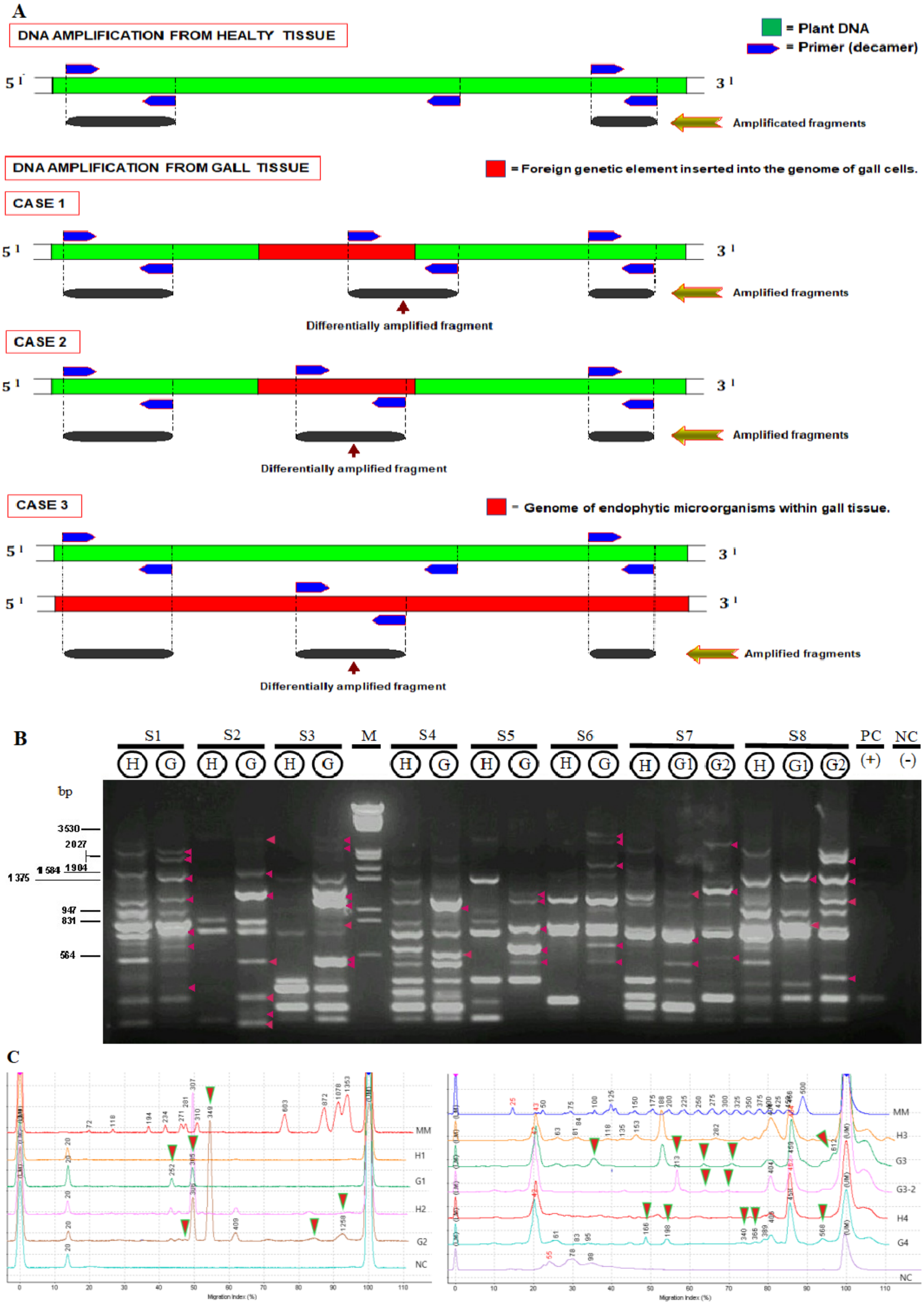

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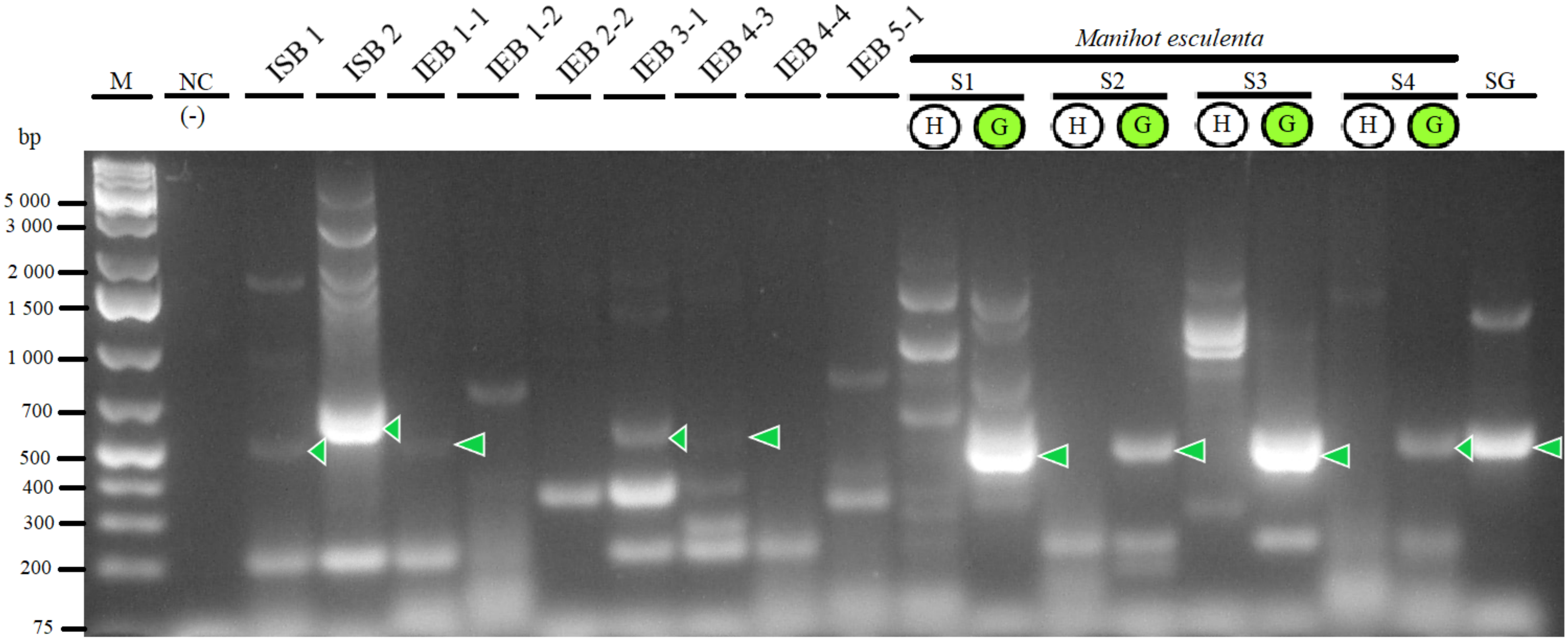

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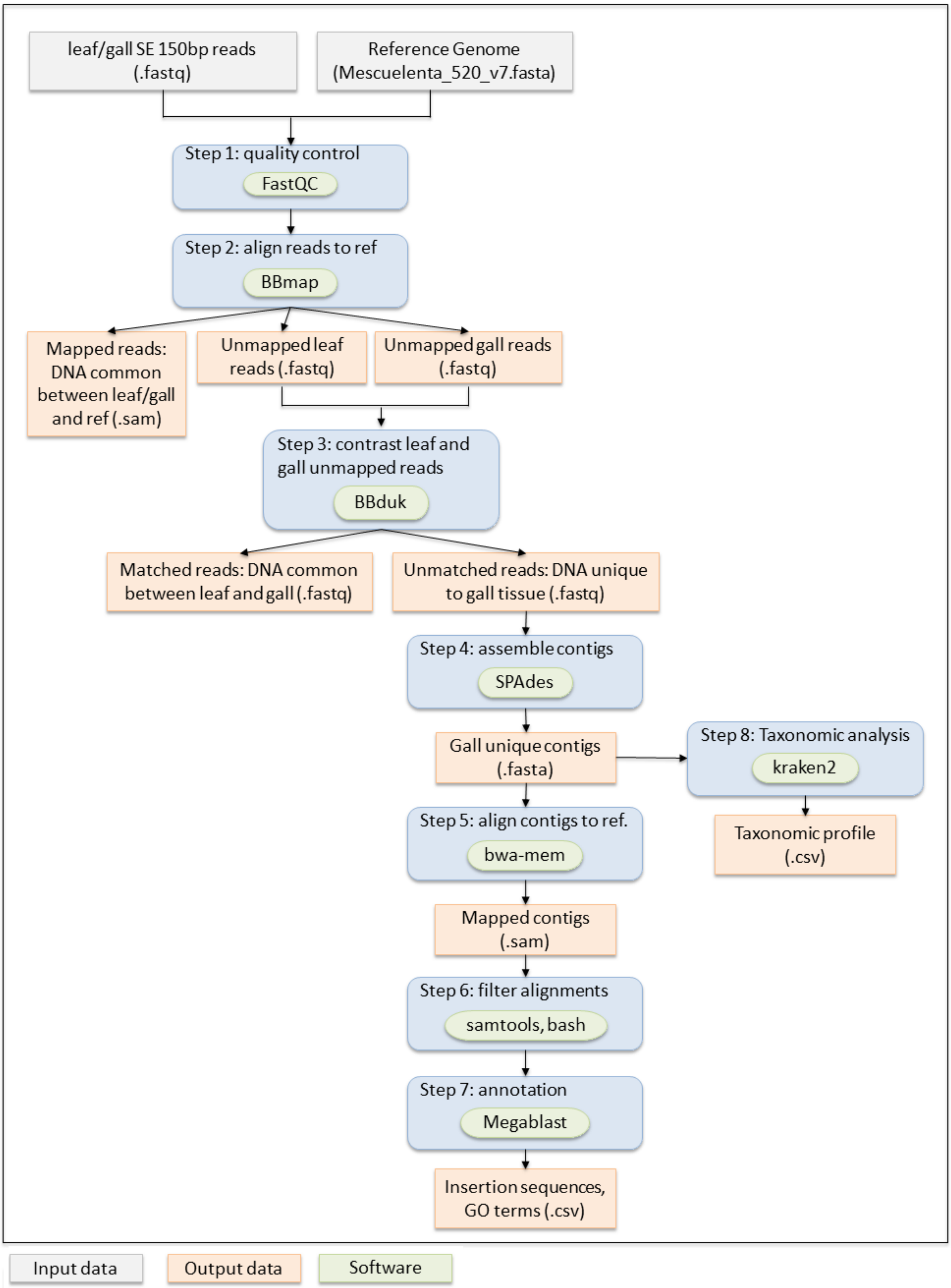

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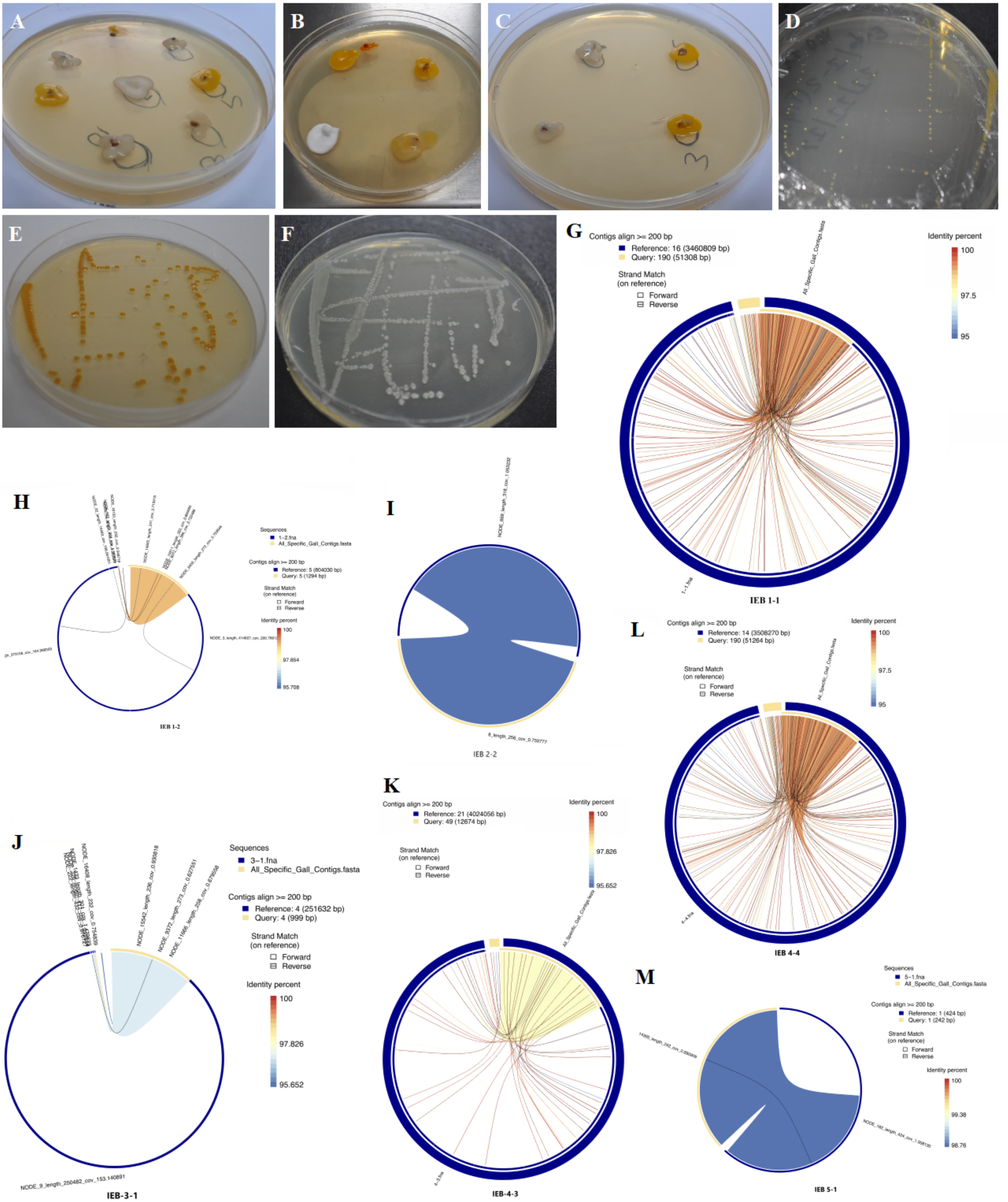

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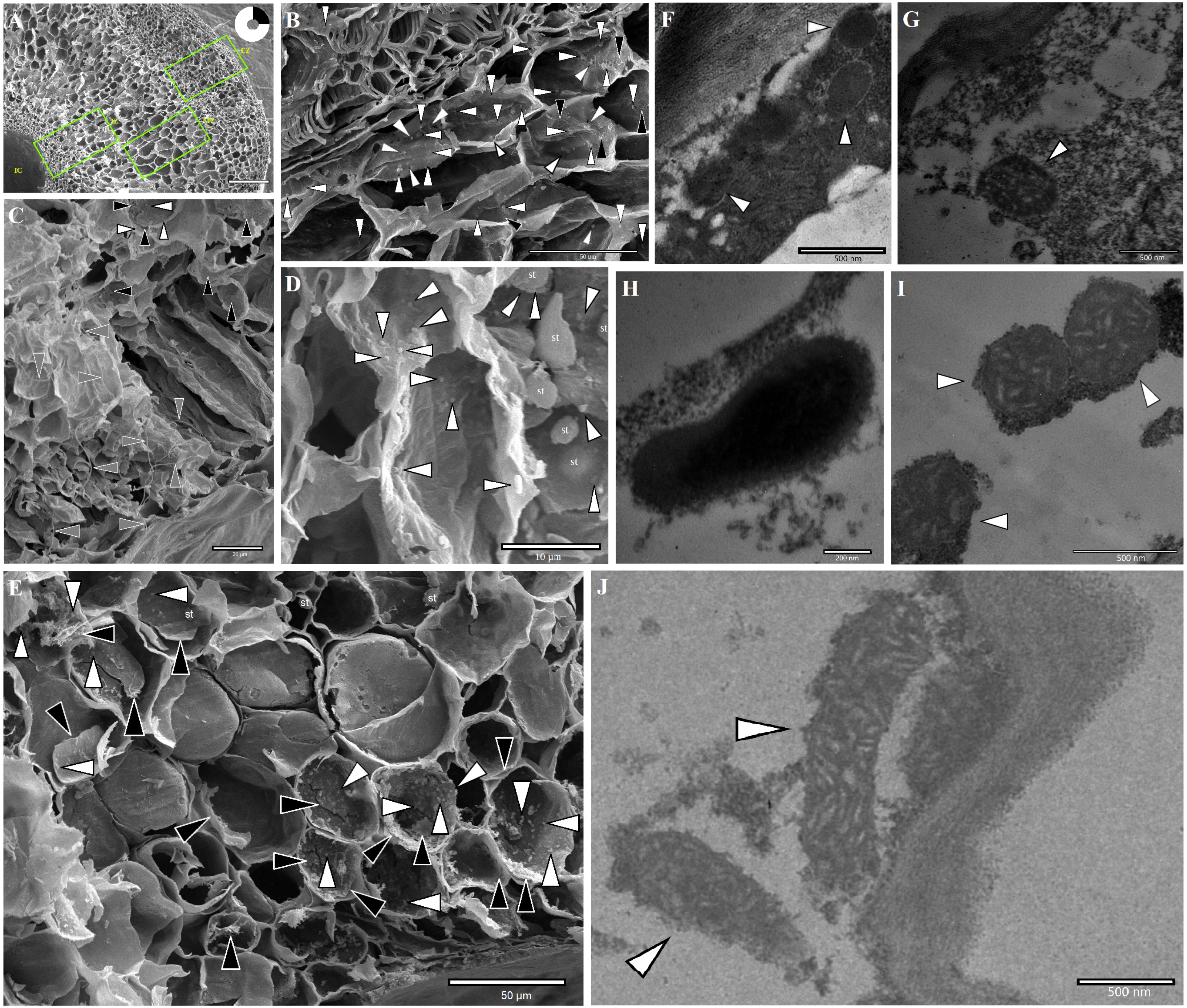

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```
#!/bin/bash

# Host Discriminant Genomic Analysis

######################################################################
#########
# Sample data: Bioproject PRJNA905450
# Reference genome: Bioproject PRJNA234389
######################################################################
#########

# STEP 1: Quality control
for file in ./*.fastq.gz; do fastqc $file; done
for file in ./*.fastq.gz; do name=$(basename $file .fastq.gz);
trimmomatic PE -threads 24  $file ${name}_R2.fastq.gz ${name}
_R1_paired.fastq.gz ${name}_R1_unpaired.fastq.gz ${name}
_R2_paired.fastq.gz ${name}_R2_unpaired.fastq.gz ILLUMINACLIP:TruSeq3-
PE.fa:2:30:10:2:True TRAILING:20 AVGQUAL:20 MINLEN:130

# STEP 2: Align reads to reference
bbmap ref=Mesculenta_520_v7.fasta
for file in ./*_paired.fastq.gz; do name=$(basename $file
_paired.fastq.gz); bbmap -Xmx200g in=$file outm=${name}_map.sam outu=$
{name}_unmap.fastq unpigz=t maxindel=80 strictmaxindel=t
semiperfectmode=t trimq=15 mintrimlength=80 subfilter=20 threads=24;
done
cat 8h_unmap.fastq >> unmap_leaf.fastq && cat 15h_unmap.fastq >>
unmapped_leaf.fastq
cat 13a_unmap.fastq >> unmap_gall.fastq && cat 15a_unmap.fastq >>
unmapped_gall.fastq

# STEP 3: Contrast leaf and gall unmapped reads
bioawk -c fastx '{print ">"$name"\n"$seq}' unmapped_leaf.fastq >
unmapped_leaf.fasta
bbduk -Xmx100g in=unmapped_gall.fastq.gz ref=unmapped_leaf.fasta
out=unmatch_gall.fastq outm=match_gall_leaf.fastq threads=24 qtrim=r
trimq=15 minlength=50 prealloc=t

# STEP 4: Assemble contigs
spades.py -o gall_unique_contigs -s unmatch_gall.fastq --only-
assembler --careful -t 24 -m 200

# STEP 5: Align contigs to reference
bwa index -p yuca Mesculenta_520_v7.fasta
bwa mem -t 24 yuca gall_unique_contigs.fasta >
gall_unique_to_yuca_ref.sam

# STEP 6: Filter alignments
samtools view -@ 24 -q 20 gall_unique_to_yuca_ref.sam >
only_mapped_contigs.sam
```

```
seqtk subseq gall_unique_contigs.fasta only_mapped_contigs.list >
gall_unique_contigs_mapped_to_ref.fasta
bbmap ref=gall_unique_contigs_mapped_to_ref.fasta
bbmap -Xmx100g in=unmatch_gall.fastq outm=unmatch_gall_map.sam
outu=unmatch_gall_unmap.fastq unpigz=t threads=24
scafstats=scafstats.txt
samtools view unmatch_gall_map.sam | cut -f3 >
contigs_with_reads_mapping_to_it.txt
sort contigs_with_reads_mapping_to_it.txt | uniq -c | sort -nr >
reads_per_contigs.txt
```

VII. DISCUSIÓN GENERAL

La presente tesis de doctorado se desarrolla en tres artículos científicos ya publicados. El primero de ellos aborda la problemática de fondo relacionada con el proceso de inducción y desarrollo de las agallas inducidas por insectos. A partir de una extensa revisión de literatura sobre el tema, se plantea y argumenta de forma amplia una hipótesis alternativa que pretende integrar las evidencias publicadas a la fecha con una serie de elementos inconexos que conforman el andamiaje teórico que conduce al abordaje experimental planteado para el presente trabajo de tesis doctoral.

En el artículo II se realizó un registro de morfotipos de agallas llevado a cabo en el Área de Conservación Guanacaste (Capítulo II). A estas muestras previamente registradas y colocadas en un herbario especializado al que se le dio el nombre de cecidiarium, se les realizaron pruebas de PCR para detectar la presencia del fragmento amplificado diferencialmente en las agallas inducidas en las plantas de yuca (artículo III). A partir de este inventario, se analizaron las secuencias de bases nitrogenadas de los fragmentos amplificados para determinar el grado de similitud de este posible elemento genético exógeno entre los diferentes sistemas insecto-planta-agalla incluidos en el estudio. Para someter a prueba esta hipótesis se purificó el ADN de algunas muestras seleccionadas provenientes del tejido sano de las hojas y de los tejidos que conforman las agallas en las diferentes especies de plantas. La escogencia de los sistemas insecto-planta-agalla utilizados como modelos experimentales, se realizó tomando en consideración la diversidad y complejidad morfológica de las agallas. Los resultados obtenidos mostraron la presencia de secuencias homólogas con la posible secuencia de inserción en las muestras de ADN purificado en los nueve morfotipos de agallas diferentes bajo estudio. Tres de los morfotipos de agallas secuenciadas y analizadas mostraron la misma secuencia de bases nitrogenadas con un porcentaje de identidad mayor al 97%: agallas en *Manihot esculenta*, *Cydista diversifolia* e *Hirtella racemosa*. Por otro lado, los otros 3 morfotipos mostraron un porcentaje de identidad media (48-53%): agallas de *Lonchocarpus phlebophyllus*, *Malvaviscus arboreus*, y *Miconia oerstediana*. Estos resultados podrían sugerir a su vez una diversificación del elemento genético codificante bajo diferentes presiones de selección en diferentes contextos de adaptación funcional.

En la publicación III también se aportan evidencias que sugieren un mecanismo de formación de agallas inducidos por insectos que podría estar mediado por eventos de transformación genética en las células de la planta huésped. Los datos obtenidos y los análisis realizados aluden a un potencial elemento genético móvil presente en aislamientos de la bacteria *Rhodococcus* sp., bacterias que por lo tanto podrían estar involucradas en el mecanismo de inducción de la agalla en las plantas de yuca.

A través de un enfoque discriminante utilizando secuenciación metagenómica completa (Shotgun metagenomics), se obtuvo evidencia consistente en relación con la presencia de ADN exclusivo exógeno dentro del tejido de las agallas. A este enfoque metodológico se le denominó Análisis Genómico Discriminante del Huésped (HDGA por sus siglas en inglés). A partir de este procedimiento metodológico, se procesaron datos de secuenciación de alto rendimiento de hojas sanas y tejidos de agallas, con una profundidad de secuenciación promedio de 16X, esto para separar, ensamblar y analizar las lecturas de la secuencia de ADN diferentes a las existentes en el genoma de la planta huésped referenciada de *Manihot esculenta* (yuca). Este enfoque metodológico consiste en un análisis bioinformático en el que los datos secuenciados se procesaron para separar, ensamblar y analizar las secuencias de ADN diferentes a las del genoma de *M. esculenta*. Por lo tanto, siguiendo este enfoque metodológico, las lecturas secuenciadas totales de muestras de tejido sano de hoja y muestras de tejido de agallas se compararon con el genoma de referencia de yuca para separar lecturas con secuencias de k-mers no coincidentes. Las diferentes lecturas resultantes de la coincidencia entre el tejido sano y el tejido de la agalla se separaron seleccionando así solo las lecturas específicas presentes en el ADN del tejido de la agalla (lecturas que tienen diferentes bases). Las lecturas de posibles variantes polimórficas entre las muestras de tejido sano de yuca del genotipo Valencia secuenciado y el genoma de yuca referenciado, se filtraron y separaron comparando las lecturas entre ambos. Las lecturas que no se asignaron a la referencia de yuca representan un ADN exógeno que potencialmente correspondería a un organismo endófito o bien a posibles secuencias de inserción presentes en el genoma de las células que forman el tejido de la agalla (ver la figura S3 de la publicación III, en donde se indica el diagrama de flujo general del procesamiento bioinformático utilizado). Las lecturas diferentes detectadas sólo en el ADN del tejido de la agalla se ensamblaron *de novo* para generar las secuencias de ADN (contigs) que resultaron específicas para este tipo de estructuras.

Adicionalmente, la inducción y el crecimiento de las agallas también podrían estar asociados con un cambio en la composición del microbioma en el tejido vegetal. Los resultados de los análisis metagenómicos incluidos en el artículo III aportan evidencias consistentes al respecto. Las características funcionales atribuidas a algunos de los componentes exclusivos o enriquecidos del microbioma reportado para las agallas incluyen la producción de vitamina B12 (Fang et al. (2017), y la conversión de nitrato (NO_3) en compuestos nitrogenados (N_2) por *Pseudomonas denitrificans*; mientras que *Pseudomonas nitroreducens,* considerada como una bacteria promotora del crecimiento vegetal, podría contribuir con el desarrollo celular y aumentar la absorción de nitrato en las plantas (Trinh et al., 2018). La bacteria solubilizadora de potasio (KSB) *Cupriavidus axalaticus*, también podría desempeñar un papel fundamental en la solubilización del potasio, permitiendo así que este elemento esencial sea más accesible para la planta. Además, la cepa T2 de la bacteria *Cupriavidus axalaticus* se ha relacionado con la capacidad simultánea de degradación de compuestos fenólicos y desnitrificación en condiciones aeróbicas, para producir también amonio (Yan et al., 2021). Una breve descripción de las características funcionales relevantes reportadas en la literatura para algunos de los principales componentes del microbioma tanto de la hoja sana como de la agalla y su posible papel en el desarrollo y mantenimiento de estos tejidos puede consultarse en la sección de texto complementario del artículo III.

En el tercer artículo también se discute la posibilidad de que el insecto inductor inyecte componentes de la comunidad endofítica específica de las agallas durante la oviposición de las hembras o durante la alimentación de las larvas. Además, como parte de los resultados incluidos en este artículo, también se analizaron y describieron los principales procesos biológicos y vías metabólicas asociadas con siete posibles bacterias endófitas del tejido de las agallas de plantas y dos bacterias obtenidas de la cabeza de larvas del insecto inductor *Iatrophofia brasiliensis*. Las vías metabólicas inferidas y las funciones biológicas asociadas con ambos grupos de microorganismos muestran que las bacterias endofíticas tienen una gama de diferentes funciones potenciales, incluida la biodegradación de compuestos fenólicos y compuestos potencialmente dañinos, la suplementación de nutrientes, la síntesis de hormonas vegetales y la degradación de metabolitos secundarios. Además, esta comunidad bacteriana endofítica tiene el potencial de sintetizar aminoácidos esenciales y vitaminas; algunos de ellos podrían estar involucrados en el metabolismo del nitrógeno y del fosfato, lo que sugiere que estas especies bacterianas podrían contribuir significativamente con la calidad nutricional del tejido que forma la agalla en yuca. Por lo tanto, desde un punto de vista ecológico, evolutivo y funcional, los análisis realizados sugieren que algunos componentes de este microbioma podrían desempeñar funciones importantes tanto en la planta huésped como en las agallas.

La no detección del fragmento amplificado diferencialmente a partir de las agallas (fragmento específico de agallas obtenido experimentalmente) en el genoma secuenciado de las agallas de yuca y específicamente en el genoma del aislamiento bacteriano de *Rhodococcus* sp. secuenciado, que bajo el sustento de los resultados obtenidos, se argumenta estaría relacionado con el proceso de inducción de la agalla inducida en las plantas de yuca, podría explicarse debido a que la secuenciación de plásmidos silvestres con un bajo número de copias es difícil mediante la plataforma de secuenciación de lectura corta como Illumina HiSeq y MiSeq. Lo anterior se complica aún más si tales plásmidos albergan un número importante de duplicaciones, lo que provoca que sea casi imposible determinar correctamente la secuencia nucleotídica completa de esos plásmidos (Smalla et al., 2015; Orlek et al., 2017). Además, los plásmidos de tipo silvestre de gran tamaño son difíciles de reconstruir a partir de datos de secuenciación del genoma completo. Sin embargo, esta ardua tarea requiere generalmente de un enfoque de ensamblaje híbrido, mediante la combinación de lecturas largas con la precisión de la secuenciación de lecturas cortas (Berbers et al., 2020). En consecuencia, localizar genes en plásmidos específicos puede ser una tarea en extremo difícil (Orlek et al., 2017). Por lo tanto, al margen de los resultados obtenidos, no se obtuvo evidencia directa de que el fragmento específico de agallas sea parte de una secuencia de inserción. Por ello se plantea que este fragmento específico sería un componente genético accesorio de la maquinaria de transformación utilizada por la bacteria, presente en un plásmido bacteriano, pero este no formaría parte de la secuencia de inserción integrada en el genoma de la célula vegetal, proceso que podría ser análogo a la configuración del plásmido Ti en cepas de *Agrobacterium tumefasciens* (Suzuki, et al., 2015; Shao et al., 2018; Chou et al., 2022). Por lo tanto, la detección específica de dicho fragmento en el ADN extraído de la agalla, podría ser una consecuencia indirecta de la presencia en el tejido de la agalla de la forma endofítica de *Pseudomonas* sp., la cual fue a su vez transferida por el insecto inductor, o bien otra bacteria que comparte el mismo elemento genético, en el que el ADN genómico y plasmídico se precipita conjuntamente con el ADN de la planta huésped.

Finalmente, también en el tercer artículo, se describe la obtención de una estructura similar a una agalla en su estadio inicial, por medio de ensayos de inoculación utilizando aislamientos de la bacteria *Rhodococcus* sp., mismos que fueron obtenidos a partir de cultivos primarios realizados con material de la cabeza del insecto inductor de la agalla de yuca (Fig. 5a-b, 5j-q, artículo III). No obstante, esta cepa bacteriana utilizada en los ensayos de inducción, no fue detectada con certeza en las muestras secuenciadas de agallas y tejidos sanos cuando se aplicó el análisis metagenómico (artículo III). Por lo tanto, se plantean tres posibles explicaciones para estos hallazgos. El primer escenario propone que los aislamientos bacterianos utilizados en los ensayos de inducción de agallas no se detectaron de manera concluyente debido a que las bacterias tienen una baja abundancia relativa dentro del tejido de las agallas. Por lo tanto, el ADN bacteriano blanco no tendría una fracción representativa en las muestras de agallas purificadas para la secuenciación. Una explicación adicional podría ser que la bacteria está involucrada en el proceso de inducción de las agallas, pero no es un componente del microbioma endófito de las células de las agallas (según los resultados obtenidos), por lo que la bacteria realizaría su acción externamente a la célula vegetal. Además, la bacteria podría ejercer su acción durante la etapa inicial de la inducción de agalla. En tercer lugar, otra bacteria taxonómicamente relacionada al género *Rhodococcus* y que comparte un mecanismo molecular similar, también podría inducir la formación de esta estructura en su etapa inicial.

VIII. CONCLUSIÓN GENERAL

El inventario de agallas realizado constituye el registro más completo, ordenado y sistemático realizado a la fecha en una región ecológica o geográfica concreta de Costa Rica, de forma más específica, es el primer estudio de este tipo realizado en el Área de Conservación Guanacaste. Además de proponer un nuevo sistema de clasificación de los morfotipos de agallas registrados, lo cual permitiría una identificación y registro más confiable de agallas de plantas, el estudio describe por primera vez 120 morfotipos no registrados previamente de agallas de plantas para Costa Rica y el mundo. El herbario especializado en donde se guardaron los materiales vegetales y los especímenes colectados de insectos asociados a las agallas, es único en su tipo y organización en Costa Rica; no se conoce la existencia de una instalación de características similares en ningún otro país.

La transformación genética y la modificación del microbioma del tejido de la agalla en las plantas de *Manihot esculenta* podría ser un mecanismo de inducción más ampliamente distribuido en la naturaleza para este tipo de estructuras. Esto fue respaldado por la detección de un posible componente genético de la maquinaria de transformación y/o manipulación de la célula vegetal (marcador de fragmento específico de agalla) en agallas formadas en otras especies de plantas, elemento genético que incluso fue detectado en plásmidos purificados a partir de bacterias endófitas aisladas a partir de la misma agalla de yuca. Si bien los resultados obtenidos en este estudio brindan una mayor comprensión de las interacciones planta-bacteria-insecto, se necesita una mayor investigación para comprender completamente los mecanismos complejos de inducción y formación de las agallas. Además, los elementos genéticos de inserción provenientes de una supuesta bacteria inductora, podrían funcionar como un interruptor en la interacción molecular entre el insecto inductor y la planta huésped. Los genes contenidos en estas posibles secuencias de inserción podrían estar relacionados con la manipulación de la maquinaria celular de la planta hospedera, genes análogos a fitohormonas y también aquellos que le confieren ventajas adaptativas al insecto inductor.

Los análisis funcionales realizados a partir de las bacterias endófiticas de las agallas y de los posibles endosimbiontes aislados del insecto inductor, al igual que el análisis obtenido de la cuantificación de genes presentes en las secuencias de ADN exclusivas aisladas de los tejidos de las agallas de *M. esculenta* (grupos ortólogos funcionales), sugieren que el enriquecimiento del microbioma o bien la adquisición de endófitos críticos, al igual que las posibles secuencias de inserción en el genoma de las células que forman las agallas, podrían regular vías metabólicas y funciones biológicas esenciales involucradas en la inducción y mantenimiento de la agalla, promoviendo a la vez una mayor calidad y rápido desarrollo de sus tejidos. Por tanto, desde un punto de vista ecológico, evolutivo y funcional, los resultados obtenidos muestran que algunos componentes del microbioma pueden desempeñar papeles importantes, tanto en la propia planta huésped como en las agallas.

Se obtuvo experimentalmente una estructura inicial similar a una agalla en tejidos cultivados de *M. esculenta* mediante ensayos de inoculación utilizando un aislamiento bacteriano del género *Rhodococcus*, obtenido a partir del insecto inductor. No obstante, a pesar del éxito relativo en la inducción de estructuras similares a agallas en los ensayos *in vitro* y en condiciones controladas de invernadero, las condiciones específicas de inoculación-inducción de la agalla de yuca deben ser aún optimizadas.

Finalmente, los resultados obtenidos como parte de esta investigación permiten concluir que los objetivos planteados originalmente como parte de este trabajo de tesis doctoral se cumplieron de forma satisfactoria, permitiendo a su vez establecer enfoques experimentales que abren la posibilidad a nuevas líneas de investigación para comprender el fenómeno de inducción de agallas vegetales por parte de insectos.

IX. REFERENCIAS

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