UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO
DOCTORADO EN CIENCIAS BIOMÉDICAS
INSTITUTO DE ECOLOGÍA

IMPACTO EN EL FENOTIPO E INTERACCIONES ECOLÓGICAS DE LA DIVERSIDAD GENÉTICA DE PLANTAS CULTIVADAS CON ÉNFASIS EN EL ALGODÓN (*Gossypium hirsutum* L.)

TESIS
QUE PARA OPTAR POR EL GRADO DE:

DOCTORA EN CIENCIAS

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CIUDAD DE MÉXICO, ENERO 2020

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AGRADECIMIENTOS
INSTITUCIONALES

Al Programa de Doctorado en Ciencias Biomédicas, al Instituto de Ecología y a la Universidad Nacional Autónoma de México. Por convertirse en mi segunda casa durante estos años, y por hacer posible esta tesis.

Al Consejo Nacional de Ciencia y Tecnología (CONACyT), por brindarme la beca que me permitió realizar este doctorado (CVU: 660255).

A la CONABIO.

A mi comité tutor: Dra. Ana E. Escalante Hernández, Dra. Ana Wegier, Dra. Mariana Benítez Keinrad y Dr. Rafael Lira Saade. Estaré siempre agradecida por su tiempo, consejo y apoyo.

Al Laboratorio de Genética de la Conservación, Jardín Botánico, del Instituto de Biología.

A la Dra. Morena Avitia por su apoyo técnico en el Laboratorio de Ecogenómica del LANCIS, Instituto de Ecología.

A la Biól. Blanca Hernández por su ayuda con el proceso de cromatografía. A la Dra. Florencia García Campusano del INIFAP.

A la Biól. Tania Gabriela Sosa-Fuentes, por su apoyo en el trabajo de laboratorio a lo largo del proyecto.

Al Dr. Daniel Piñero, por el apoyo financiero brindado para una parte fundamental de la tesis.

A Eréndira García Ríos del Laboratorio de Cromatografía, Instituto de Química por permitirme trabajar en su laboratorio y el apoyo en el uso del HPLC.

Al CENID-COMEF, INIFAP por permitirme trabajar en el Laboratorio de Biotecnología.

AGRADECIMIENTOS
PERSONALES

El doctorado es un camino largo, lleno de baches donde uno se cuestiona por qué entró en primer lugar, pero también lleno de alegría y satisfacción, como cuando te sale la PCR, ganaste una batalla más contra R o por fin te aceptaron el paper. Puedo decir que termino esta etapa con mucho miedo del futuro pero también con mucho orgullo de lo que hemos logrado. Durante estos largos años muchas cosas sucedieron, no sólo a nivel académico sino también personal. Yo soy muy afortunada, en toda esta larga jornada tuve siempre personas en mi vida que me ayudaron a superar los obstáculos y a celebrar las victorias. Sin esas personas esto simplemente no habría podido suceder.

A mi comité tutor. Muchas gracias por escucharme siempre, por la paciencia, por hacer de cada tutoral una reunión de genuina discusión y por haberme enseñado tanto. Mariana, gracias además por las discusiones de pasillo, por las comidas compartidas y el café.

A Ana Wegier. Gracias por enseñarme tanto no sólo de la ciencia sino de la vida. Contigo me introduje por primera vez a un grupo de investigación, a los seminarios, a las reuniones y a la tarta de manzana. Gracias por creer en mi en momentos donde yo estaba segura de que fracasaría, gracias por estar siempre.

A Ana Escalante. Estaré siempre agradecida contigo por enseñarme de la mano a hacer ciencia, a escribir artículos, a enfocarme y no divagar. Gracias por las eternas reuniones donde no sólo se discutía el trabajo sino la vida, gracias por escucharme siempre y aconsejarme cuando me ganaba el estrés y la desesperación. Recordaré siempre con mucho cariño esta larga jornada contigo, donde estoy segura, las dos aprendimos muchísimo.

A mis compañeros de laboratorio. Pocas personas ven tan de cerca un doctorado como los que se sientan a tu lado, con los que comes día a día. Gracias a Karen, Morena, Juan, Vale, Karla, Gloria, Alberto, MaFer, Emilio por hacer más llevadera la rutina, por las comidas, las idas a Tigridia, los chistes, las terapias de grupo y por superar juntos los malos momentos.

A Marcelino. Gracias por siempre contestar mis preguntas y aguantar mis quejas, espero sepas que te admiro no sólo como científico loco sino como persona. Gracias por ser mi amigo todos estos años.

A Nat. Gracias por tu amistad y tus consejos. Por enseñarme que entre amigos también se puede trabajar y por introducirme al mundo del Eco-Evo-Devo. Por el chisme eterno, las caminatas por CU y los robos hormiga de queso.

A mis amigos de escalada, y de la vida. Javi, Fer, Jaqui, Betos. ¡Los quiero muchísimo! Gracias porque en más ocasiones de las que puedo recordar me salvaron de la locura. Por las tardes eternas de chisme y chela en Casa Boulder, que después fueron en la Pana, luego en América. Gracias por ser un respiro de aire fresco.

A las roomies de ayer y hoy. Ana, Anaid, Dianita. Gracias por darme un hogar cuando más lo necesitaba. Y aunque la vida nos ha llevado a todas a distintos puntos cardinales, me reconforta saber que siempre estamos ahí. Las quiero y las admiro, como mujeres y profesionistas.

A mi familia.

A los Hernández y a los Terán. Por estar lo largo de mi vida, por apoyarme siempre y por entender mi ausencia estos últimos años. A mis tías: Silvia, Hilda, Betty y primas: Dahlia, Dani, Ileana; que me han apoyado como pocos, siempre están en mi corazón.

A mi mamá, papá y hermano. Gracias por que todo lo que soy se lo debo a ustedes. Por sus enseñanzas, amor incondicional y esfuerzo. Esto es un logro de los cuatro.

A Chivis. ¡Lo logramos, ma! Gracias por enseñarme siempre fortaleza, resiliencia e inteligencia emocional con el ejemplo. Eres una mujer increíble y te admiro muchísimo. Gracias por hacerme lo que soy. Te quiero.

A Santi. Nadie estuvo tan cerca, tan presente como tú. Gracias por ser mi compañero y mi mejor amigo, por estar conmigo, por levantarme cada vez que todo valía y celebrar cuando las cosas salían bien. Esta tesis es tan mía como tuya. Te amo y quiero que sepas que sin ti esto no habría sido lo que fue, ni la mitad de divertido y seguro el doble de doloroso.

A Juan.

RESUMEN

La diversidad genética de plantas tiene un impacto directo no solamente en el fenotipo de los organismos sino también en cómo estos interactúan con el medio. El estudio de cómo la diversidad genética afecta distintos procesos de la vida de los organismos es fundamental para el desarrollo de procesos de mejoramiento, especialmente si hablamos de especies que forman parte de la cadena alimenticia o productiva humana. En esta tesis, seleccionamos parientes silvestres de los cultivos (CWR) de distintas especies de plantas para probar cómo afecta la diversidad genética en caracteres fenotípicos relacionados con la ***adecuación*** y con interacciones ecológicas. Seleccionamos a poblaciones de CWR ya que se sabe presentan altos niveles de diversidad genética, además de su importancia en términos de conservación. En primer lugar, en un esfuerzo por integrar la información disponible, realizamos un meta-análisis del efecto fenotípico de la modificación genética en cinco cultivos de importancia mundial. Encontramos que la modificación genética, tanto por domesticación como por ingeniería genética, tiene efectos más allá de los buscados debido a que en la modificación, por procesos a nivel genético pueden arrastrase caracteres no blanco. Algunos de estos caracteres pueden tener relevancia en la adecuación, llevando a las poblaciones a trayectorias evolutivas diferentes. En segundo lugar, realizamos un experimento sobre las consecuencias de la diversidad genética en el desempeño *in vitro*. Utilizando el algodón como modelo, probamos cómo la presencia de transgenes en poblaciones silvestres puede afectar el desempeño *in vitro*. Encontramos que la presencia de transgenes provoca costos ecológicos en los organismos que comprometen su desempeño, comprometiendo también el éxito que podrían tener estos en bancos de conservación de germoplasma. Finalmente, quisimos probar cómo la diversidad genética de poblaciones silvestres de algodón puede determinar la interacción con las comunidades microbianas de raíz. Encontramos que cada metapoblación de algodón en México, tiene la capacidad de seleccionar distinta composición y estructura de las comunidades microbianas, lo cual podría estar relacionado con la adaptación y sobrevivencia de los organismos a ambientes extremos. Finalmente, concluimos que la diversidad genética de plantas cultivadas es primordial en la determinación de diferentes aspectos de la vida de los organismos, no solamente de caracteres fenotípicos sino también en procesos que son parte de la construcción de nicho en plantas.

ABSTRACT

Plant genetic diversity has direct effects on the phenotype and in the way that the organisms interact with their environment. The study of the processes by which genetic diversity affects organisms is of special relevance when we talk about economically important crops, since the results of such studies are crucial for the development of novel genetic improvement events. In this thesis, we selected crop wild relatives (CWR) from different species to test the effect of genetic diversity in functional phenotypic traits and into ecological interactions. We chose CWR because of the high levels of genetic diversity that they usually exhibit besides their relevance for genetic conservation. First, to integrate the available information, we perform a meta-analysis of phenotypic consequences of genetic modification in five economically important crops. We found that genetic modification, either by domestication or genetic engineering, has non-target effects caused by the drag of phenotypic traits in the modification process. These phenotypic traits could also be related to plant fitness, leading to different evolutionary trajectories in the populations. Secondly, we experimented with the *in vitro* consequences of genetic diversity. Using cotton plants as a model, we test if the presence of transgenes could have effects in *in vitro* performance. We found ecological costs associated with transgene presence, that negatively impacted performance in *in vitro* conditions. This, in turn, could also have consequences for the success of such populations in germplasm bank conservation strategies. Finally, we wanted to test the effect of genetic diversity of the wild cotton populations into the microbial communities associated to plant roots. We found that each metapopulation selects microbial communities with different diversity and structure which could be related to the adaptation and survival of the populations in extreme environments. In conclusion, we demonstrate that crops genetic diversity is fundamental in the determination of different life aspects of the organisms, not only for phenotypic traits but also for processes related to niche construction.

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PRÓLOGO

A raíz de la inmensa diversidad de formas, tamaños y colores que observamos en los seres vivos, surge una de las preguntas más relevantes en biología: ¿cuáles son los procesos que dan origen a la diversidad biológica? En 1953, posterior a la publicación de la estructura del ADN por Watson y Crick, comienzan a surgir estudios centrados en responder esta pregunta desde la genética. Sin embargo, actualmente seguimos sin entender a profundidad los procesos que originan la diversidad, cómo surgen los fenotipos que observamos, y de estos, ¿cuáles tienen una base genética y cuáles una base ambiental? Es decir, seguimos sin entender completamente cómo afecta la diversidad genética al fenotipo de los organismos. Esto es relevante si pensamos en que todas esas preguntas sin resolver tienen consecuencias en la sobrevivencia y adaptación de los organismos que estudiamos. No solamente en medidas de adecuación, sino también en cómo estos organismos interactúan con otros en la naturaleza, afectando procesos tanto a nivel ecológico como evolutivo.

Esta tesis representa un esfuerzo por integrar y generar información que nos ayude a entender cómo la diversidad genética en plantas tiene consecuencias en el fenotipo y en la interacción con otras especies, lo cual a su vez, influye directamente en procesos ecológicos y evolutivos. Este fenómeno se abordará desde dos perspectivas: i) desde el estudio de las consecuencias en adecuación que ocurren cuando se altera la diversidad genética natural de una especie, enfocándonos en ambientes controlados (experimentos de jardín común y propagación *in vitro*), y ii) desde el estudio de las consecuencias que tiene la diversidad genética en las interacciones ecológicas, específicamente en cómo el genotipo puede determinar la interacción planta-microorganismo. La estructura de la tesis estará conformada entonces por una introducción general en la que se desarrollará el marco teórico compartido a lo largo de la tesis y tres artículos científicos identificados como capítulos: ***Capítulo I***; un meta-análisis en el que se

abordan los efectos fenotípicos que tiene la ingeniería genética en plantas domesticadas comparando contra sus parientes silvestres y las consecuencias de esto en términos de adecuación, ***Capítulo II***; un artículo con evidencia experimental de los efectos de la diversidad genética de plantas en ambientes de cultivo *in vitro* y las consecuencias de esto para la conservación y ***Capítulo III***; un artículo con evidencia experimental del efecto de la diversidad genética de plantas en interacciones ecológicas, específicamente en la interacción planta-rizósfera y las consecuencias de esto en términos de la adaptación de organismos en ambientes naturales. Finalmente, se desarrollan las conclusiones generales y perspectivas de este trabajo. Además, se incluyen los Apéndices I, II y III que corresponden al material suplementario de cada uno de los artículos científicos. Así como los apéndices IV, V y VI correspondientes a otros trabajos publicados a lo largo del doctorado.

Esta tesis fue realizada en el Laboratorio Nacional de Ciencias de la Sostenibilidad (LANCIS), del Instituto de Ecología de la UNAM, bajo la dirección de la Dra. Ana Elena Escalante Hernández. Fue financiada por una beca doctoral CONACyT (CVU: 660255) y diversos proyectos: "Programa para la Conservación de las poblaciones silvestres de *Gossypium hirsutum* en México" (DGAP003/WN003/18) de la Dirección General del Sector Primario (DGSPRNR) que pertenece a la SEMARNAT y CONABIO, y el proyecto UNAM-PAPIIT: IN214719, ambos de la Dra. Ana Wegier. Además, por el proyecto "Contribución de la Biodiversidad al Cambio Climático" de la Secretaría de Medio Ambiente y Recursos Naturales (SEMARNAT), del Dr. Daniel Piñero Dalmau.

INTRODUCCIÓN

1 DIVERSIDAD GENÉTICA

Los seres vivos, desde organismos unicelulares hasta los grandes mamíferos marinos que conocemos exhiben variación genética. Si pensamos, por ejemplo, en las flores de la especie *Ipomoea purpurea* que fácilmente vemos al caminar por la Ciudad de México, o por el campo, podemos recordar la amplia variedad de colores que presentan. Esta variación fenotípica en sus flores está dada por la estructura y variación genética de la especie que, en combinación con el ambiente en el que se desarrollan, pueden dar lugar a distintos colores de flores. La diversidad genética se puede entender como el número de características genéticas dentro de una especie que han resultado de procesos microevolutivos y demográficos que actúan en las poblaciones. En 1930, Ronald A. Fisher postuló, en el Teorema Fundamental de la Selección Natural, una correlación directa entre la variación genética de una población y la tasa evolutiva por selección natural. Hoy en día sabemos que la variación genética es una condición fundamental para la evolución, pues es el recurso sobre el cual actúa la selección natural (Fisher 1930). La diversidad genética de una población está fuertemente relacionada con la respuesta de los individuos ante variación ambiental, es decir, a mayor variación genética, mayor probabilidad de respuesta ante cualquier cambio. Es relevante entonces reconocer que la variación genética dentro de una población puede verse afectada por las cuatro fuerzas evolutivas: la ***selección natural***, la ***deriva génica***, el ***flujo génico*** y la ***mutación***.

1.1 DIVERSIDAD GENÉTICA EN POBLACIONES NATURALES

Las poblaciones naturales de plantas, generalmente, exhiben altos niveles de diversidad genética. Para ilustrar este fenómeno nos concentraremos en el caso particular de las poblaciones de parientes silvestres de los cultivos (CWR por el inglés: Crop Wild Relatives). En términos generales, un CWR se define como una especie silvestre emparentada con un cultivo al cual puede aportar material genético pero que, contrario al cultivo, no ha sido domesticada (Hunter & Heywood, 2011). Estas poblaciones se encuentran generalmente en los centros de origen y diversidad de las especies. En el caso de México, al ser centro de origen de cultivos como maíz, frijol, calabaza y algodón es posible encontrar sus poblaciones de CWR creciendo en el país (Acevedo et al, 2009).

Los CWR han sido utilizados históricamente como reservorios de diversidad genética y fenotípica a partir de los cuales se han seleccionado las características que presentan los cultivos que conocemos actualmente. Las poblaciones de CWR en condiciones silvestres presentan interacciones complejas con el ambiente donde crecen, que van desde interacciones con otras poblaciones de la misma especie, hasta interacciones con otros miembros de la cadena trófica. Estos procesos sumados a las tasas variables de cruzamiento, y a procesos adaptativos que surgen de la vida en ambientes cambiantes, han originado una vasta diversidad genética en las poblaciones CWR que les permite adaptarse constantemente a los cambios ambientales en condiciones silvestres (Amos & Harwood, 1998).

Altos niveles de diversidad genética en CWR también tienen consecuencias en la variación fenotípica que observamos en las poblaciones; por ejemplo, es común observar una gran variación en plantas silvestres, variación que se presenta en distintos tamaños, formas y colores de los frutos. Ante esto, en 1965 se consideró por primera vez a los CWR como un grupo blanco de conservación (Harlan 1965). Sin embargo, actualmente los esfuerzos de conservación no se limitan únicamente a la variación genética depositada en los CWR, sino a los procesos ecológicos y evolutivos que les dan origen (Moritz 2002 y Wegier 2013).

La necesidad de conservar no sólo la diversidad genética de poblaciones naturales sino los procesos que le dan origen resulta evidente cuando reflexionamos sobre las condiciones tan particulares en las que estos procesos ocurren. El ambiente tiene un fuerte impacto en la adaptación de los organismos, en donde la selección natural actúa localmente y puede resultar en diferenciación genética y fenotípica entre poblaciones geográficamente separadas (Slatkin & Hudson, 1991). En poblaciones naturales es frecuente notar este fenómeno en donde, a pesar de crecer en zonas geográficas relativamente cercanas, debido a procesos de adaptación local en cada uno de los microambientes en donde se desarrollan los organismos, podemos encontrar diferencias en las poblaciones de una especie. Es decir, podemos encontrar diferenciación genética y fenotípica a nivel intra-específico.

1.2 DIVERSIDAD GENÉTICA EN POBLACIONES MODIFICADAS

El mejoramiento genético en plantas es un proceso histórico que cambió sustancialmente el curso de la humanidad. Tanto la domesticación tradicional por selección artificial como las nuevas tecnologías de ingeniería genética son procesos que tienen fuertes consecuencias en los organismos. Estas consecuencias son inmediatas en forma de cambios genéticos y fenotípicos pero también pueden ser a largo plazo, provocando efectos evolutivos en las poblaciones modificadas. Ante estos procesos de alteración continua en los organismos, las interacciones entre el ***genotipo***, el fenotipo y el ambiente pueden ser también afectadas.

Domesticación

La domesticación es un proceso de mejoramiento que ha sido realizado históricamente por poblaciones humanas. Este proceso consiste en la selección artificial de características de los organismos basado en ventajas agronómicas (Diamond 2002) y tiene consecuencias genéticas, fenotípicas y evolutivas en las poblaciones de interés. Los procesos de selección artificial actúan sobre los CWR pues son los organismos que exhiben los rasgos fenotípicos que se busca

seleccionar, por lo tanto, la domesticación conlleva una diferenciación tanto genética como fenotípica entre los CWR y sus versiones domesticadas (Caja 1).

El proceso de domesticación en plantas tiene consecuencias más allá de la expresión de un rasgo deseado. En muchos casos, el ***entre-cruzamiento*** continuo para lograr el establecimiento de rasgos fenotípicos específicos a través de las generaciones, provoca un ***cuello de botella*** que puede reducir significativamente la diversidad genética (Gepts 2004 y Pickersgill 2007). Asimismo, dado que el desarrollo es un proceso complejo en el que existen interacciones a nivel genético, muchas veces la selección de características específicas puede provocar que otros caracteres se arrastren en la modificación, provocando lo que se conoce como efectos no esperados de la modificación (Filipecki & Malepszy, 2006, Burke et al, 2007). Uno de estos efectos no esperados mejor documentados es la convergencia fenotípica entre especies que fueron domesticadas con distinto propósito. A este fenómeno se le conoce como síndrome de domesticación y se define como el conjunto de características fenotípicas asociadas con el cambio genético desde la especie silvestre hasta su forma domesticada (Pickersgill 2007). Los tipos de síndrome de domesticación más comunes son la ***dominancia apical***, la pérdida de dispersión y ***dormancia de las semillas*** y la pérdida de mecanismos naturales de defensa (Vaughan et al, 2008 y Lu 2013). Estos efectos no esperados de la modificación, aunque no necesariamente comprometen el éxito de los organismos domesticados en términos de productividad, si comprometen su adaptación a condiciones naturales. Debido a esto, uno de los efectos más dramáticos de la domesticación es el estado en el que la planta domesticada es incapaz de sobrevivir sin intervención humana (Meyer & Purugganan, 2013).

Ingeniería genética

Gracias a los rápidos avances en genómica e ingeniería genética (IG), la humanidad ha sofisticado considerablemente los procesos de domesticación, pasando de procesos de selección artificial sobre caracteres existentes en los organismos a introducir rasgos novedosos en ellos. En 1973 Herbert Boyer y Stanley Cohen, a través de una serie de técnicas moleculares y de ingeniería, crearon una bacteria resistente al antibiótico kanamicina. Esta bacteria fue el primer Organismo Genéticamente Modificado (OGM) y el parteaguas para la modificación genética

tanto de plantas como de animales. Un OGM se define como un organismo al que se le confiere una nueva función o rasgo a través de la inserción de genes mediante ingeniería genética (Stewart et al, 2010). A través de esta tecnología se han transformado gran parte de los cultivos que conforman la base alimenticia mundial; en los que podemos encontrar en muchos casos, rasgos novedosos para la especie. Actualmente, se han utilizado otros métodos para realizar procesos de modificación genética en organismos, el más reciente y novedoso es el CRISPR-Cas9 (del inglés, Clustered Regularly Interspaced Short Palindromic Repeats) un sistema de defensa bacteriano utilizado como editor de genes (McManus & Sharp, 2002; Ma et al, 2017). Con esta nueva tecnología se han realizado procesos de modificación en plantas, sin embargo (Feng et al, 2013; Bortesi & Fischer, 2015) debido a su reciente uso estos mejoramientos aún no han sido comercializados o liberados al ambiente.

La IG en plantas se realiza generalmente sobre organismos previamente domesticados (Setlow 1991); es decir, un OGM es también un organismo domesticado. Sin embargo, y aunque tanto en la domesticación tradicional como en la ingeniería genética los organismos sufren modificación genética, los procesos que median esta modificación son cuantitativamente diferentes. Por un lado, en la domesticación tradicional, las nuevas combinaciones genéticas se obtenienen por cruzas sexuales entre organismos que presentan los rasgos buscados y que pertenecen a la misma especie. Mientras que, en la IG, los rasgos novedosos se obtienen mediante la inserción de genes de, en algunos casos, organismos no relacionados (Agrawal et al, 1999 y Nodari y Guerra, 2001).

A nivel molecular, la IG representa un proceso de caja negra. Para lograr la transformación de un organismo, múltiples copias del gen deseado son introducidas al genoma mediante diversas técnicas. Estas pueden ser desde ***biolística*** hasta la inserción mediada por la bacteria *Agrobacterium tumefaciens*, siendo esta última la más utilizada en transformación de cultivos (Zhang 2013). Durante el proceso, se espera que una o más copias del gen sean insertadas exitosamente y expresadas en la célula transformada, para después, crecer *in vitro* y convertirse en un organismo completo (Stewart 2010). Esto conlleva que al final del proceso de transformación no se sepa exactamente cuántas copias del gen se insertaron y en qué sitio del genoma ocurrió la inserción. Lo cual, tiene

consecuencias a nivel genético que, a pesar de la amplia utilización de la tecnología, siguen sin entenderse del todo.

Una de las premisas de la IG es que un OGM y su línea isogénica (versión genética y domesticada equivalente sobre la cual se hace la transformación) son sustancialmente equivalentes excepto por el rasgo novedoso que se insertó (Cellini et al, 2004). Sin embargo, al igual que en el proceso de domesticación tradicional, en la modificación por IG, la inserción de genes novedosos puede potencialmente provocar efectos no esperados al existir interacciones a nivel genético y ambiental entre el genoma receptor y los genes insertados. Los efectos no esperados entonces, podrían, al igual que en la domesticación tradicional, tener efectos cuantitativos en el genotipo, fenotipo y posiblemente en la adecuación de los organismos modificados.

CAJA 1. CONSECUENCIAS MORFOLÓGICAS DE LA DOMESTICACION

El proceso de domesticación tiene fuertes consecuencias morfológicas dadas por la constante selección artificial de caracteres. En el caso de la papaya, el proceso de domesticación ha sido tan extremo que la similitud con su pariente silvestre ha desaparecido casi por completo. El cambio en el tamaño del fruto, en el número de semillas y algunas características en el árbol son las consecuencias más evidentes del síndrome de domesticación en esta especie.

Imagen modificada de: Chávez-Pesqueira y Núñez-Farfán (2017).

2 DIVERSIDAD GENÉTICA Y SU RELACIÓN CON EL FENOTIPO

En 1909, Wilhelm Johannsen introdujo por primera vez la distinción entre genotipo y fenotipo (Caja 2). A partir de ese momento, se han realizado grandes esfuerzos en biología y en medicina para entender la relación entre ellos y cómo, y hasta en qué medida el genotipo determina al fenotipo. Sin embargo, seguimos sin entender a fondo los procesos por los cuales surgen los fenotipos, la relación que tienen con el genotipo (Venter et al, 2001) y cómo afectan la sobrevivencia de los organismos. Con el objetivo de contestar estas interrogantes es necesario desarrollar algunas cuestiones teóricas sobre lo que entendemos por fenotipo, y sobre los factores a nivel genético y ambiental que pueden alterar las trayectorias de los organismos, llevando a fenotipos novedosos que no se explican en su totalidad por una base genética.

CAJA 2. CONCEPTO DE FENOTIPO

El fenotipo se define como las características visibles de un organismo, que pueden incluir la fisiología, morfología y comportamiento, con una relación muy estrecha con la adecuación. Hasta antes de los avances en genómica y biología molecular se creía que la interacción genotipo-fenotipo guardaba una relación 1:1; es decir, a un gen siempre le correspondía un fenotipo. Ahora sabemos que el fenotipo es resultado de una compleja red de interacciones entre distintos genes y entre los genes y el ambiente y que hay pocos ejemplos en donde se conserva la interacción 1:1 (Lobo & Shawk, 2008). Como consecuencia, muchos organismos genéticamente equivalentes exhiben diferenciación fenotípica.

2.1 FACTORES QUE AFECTAN LA RELACIÓN GENOTIPO – FENOTIPO

Durante el estudio de cómo surgen los caracteres fenotípicos en los organismos, han existido distintos debates. Históricamente, uno de los más antiguos fue *natura vs nurtura*, o ¿qué determina los caracteres, la genética o el ambiente? Ahora

sabemos que la relación genotipo-fenotipo no es 1:1 y que la expresión de los fenotipos está dada por una combinación de procesos a nivel genético y ambiental. Procesos que a grandes rasgos pueden ser divididos en dos grupos: i) interacciones gen x gen (G x G) y ii) interacciones gen x ambiente (G x A).

Interacciones G x G

En el siglo XX, Reginald C. Punnet y William Bateson postularon por primera vez la existencia de interacciones entre genes (G x G) que pueden llevar a la aparición de fenotipos complejos. Existen distintos tipos de interacción G x G, tales como: epistasis, pleiotropía, ***genes modificadores*** y elementos transponibles (***transposones***), cada uno de los cuales tiene consecuencias fenotípicas y evolutivas para los organismos. En este trabajo nos centraremos en los dos primeros tipos: epistasis y pleiotropía, por estar estrechamente relacionados con nuestra pregunta general.

Epistasis

La epistasis es un fenómeno que ha recibido diferentes significados a lo largo de su estudio. Sin embargo, la primera definición, conocida como *epistasis clásica* y acuñada por Bateson en 1907, refiere al efecto del enmascaramiento de un alelo sobre otro encontrado en un ***locus*** diferente; y es un proceso que es considerado ubicuo en las redes regulatorias de genes en organismos vivos (Azpeitia et al, 2011). Se entiende entonces que las consecuencias fenotípicas de un gen dado cambiarán si existen fenómenos de epistasis. Esto tiene como consecuencia que uno de los principales efectos de la epistasis sea la generación de variación fenotípica y el cambio en la adecuación de las poblaciones (Wolf et al, 2000). Por lo tanto, la epistasis puede tener consecuencias evolutivas para los organismos que la presentan.

Pleiotropía

La pleiotropía es el fenómeno en donde un mismo gen puede dar lugar a caracteres fenotípicos diferentes y no relacionados (Paaby 2013) (Figura 1). Actualmente es un proceso bien estudiado en donde algunos mecanismos se han esclarecido, lo que ha llevado a la diferenciación, a grandes rasgos, de dos tipos de pleiotropía:

verdadera y espuria. La pleiotropía verdadera se da cuando dos rasgos fenotípicos surgen de un mismo *locus*, mientras que la espuria ocurre cuando un producto (que surge de un *locus*) se utiliza en diferentes vías o desencadena una cascada de señalización que termina en diferentes fenotipos (Solovieff et al, 2013). Asimismo, la pleiotropía juega un papel importante en los procesos evolutivos. Si, por ejemplo, existe selección (natural o artificial) sobre un rasgo fenotípico controlado por un gen que a su vez determina otros rasgos, estos se verán también afectados por el proceso de selección.

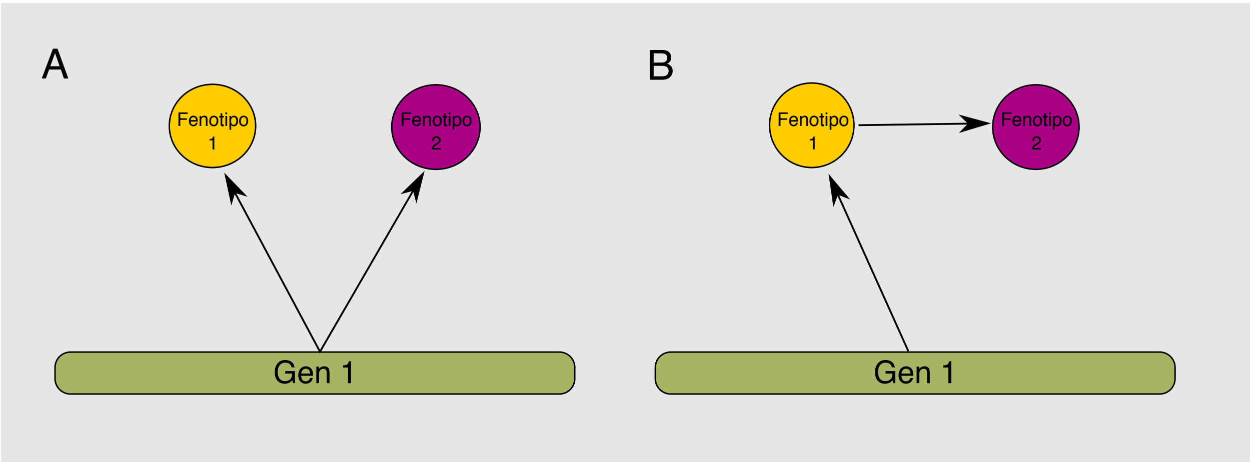

Figura 1. Representación gráfica de los tipos de pleiotropía. A: pleiotropía verdadera – proceso en el que un gen en un mismo *locus* determina dos fenotipos. B: pleiotropía espuria – proceso en el que un gen determina un fenotipo que es parte de la cadena regulatoria de otro, afectando ambos fenotipos indirectamente. Modificado de Solovieff et al. (2013).

Interacciones G x A

La respuesta diferencial de los genotipos ante variación ambiental se conoce como interacción organismo-ambiente. El estudio de este proceso se ha realizado utilizando lo que conocemos como ***norma de reacción***, que se entiende como el repertorio fenotípico de un genotipo a lo largo de una variable ambiental (Rivera-Yoshida, 2019) (Figura 2). Uno de los atributos más estudiados de la norma de reacción es la plasticidad fenotípica.

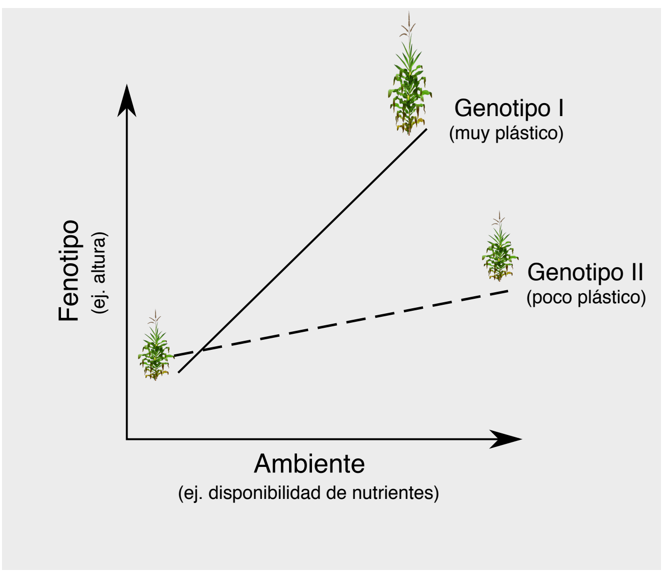

Figura 2. Norma de reacción. En un ambiente variable, un genotipo plástico (Genotipo I) puede modificar su fenotipo en respuesta a, por ejemplo, el aumento en la disponibilidad de nutrientes; mientras que un genotipo no plástico (Genotipo II) exhibe poca variación en el fenotipo sin importar la variación ambiental. Modificado de Pigliucci (2005).

Plasticidad

La plasticidad fenotípica se entiende como la habilidad de un genotipo para producir distintos fenotipos en distintos ambientes (Pigliucci, 2005), y es un proceso que juega un papel fundamental en la adaptación de los organismos. En muchos casos, la plasticidad representa la primera respuesta de los organismos ante ambientes cambiantes, y puede tener un valor adaptativo si permite a un genotipo tolerar mejor la variación ambiental, de modo que aumente su adecuación (Pigliucci, 2005). Algunos autores mencionan que este proceso, conocido como ***plasticidad adaptativa***, se ve favorecido cuando las poblaciones se enfrentan a escenarios particulares, como pueden ser la presencia de ambientes cambiantes; o bien, cuando por selección se favorecen fenotipos distintos en cada ambiente, o cuando ningún fenotipo es apto para todos los ambientes (Ghalambor et al, 2007, Pigliucci, 2001).

3 CONSECUENCIAS DE LA DIVERSIDAD GENÉTICA EN PLANTAS

A lo largo de las secciones anteriores desarrollamos, en primer lugar, cuáles son los procesos que pueden originar y afectar la diversidad genética, seguido de algunos de los mecanismos por los cuales ésta, a su vez, puede impactar en distintos procesos de la vida de los organismos. En esta sección nos concentraremos en desarrollar cómo la diversidad genética de las plantas tiene consecuencias en el fenotipo, entendiendo que el fenotipo no comprende únicamente caracteres morfológicos y fisiológicos, sino también procesos de interacción que pueden llegar a definir la sobrevivencia de los organismos en un ambiente determinado. Esta perspectiva a su vez nos permitirá reflexionar sobre las consecuencias de la diversidad genética en términos ecológicos, evolutivos y de conservación.

Consecuencias evolutivas

La diversidad genética de organismos y poblaciones tiene efectos directos sobre el fenotipo. En muchos casos, los caracteres fenotípicos afectados pueden tener relevancia evolutiva al ser componentes de la adecuación. En plantas, el estudio de las consecuencias de la diversidad genética en la adecuación se ha realizado mediante el monitoreo de distintos caracteres, tales como: altura, número de semillas, tasa de germinación, viabilidad de polen entre otros, bajo condiciones ambientales controladas. A este tipo de experimentos se les conoce como jardín común, y contempla la comparación de organismos genéticamente distintos bajo condiciones ambientales idénticas, con el objetivo de entender los efectos del genotipo y la variación ambiental sobre el fenotipo (Ballentine & Greenber, 2010). Es por esto que actualmente es una de las aproximaciones más utilizadas para probar la contribución del componente genético en una variable de interés.

A partir de estas estrategias experimentales hemos aprendido, por ejemplo, que los procesos de modificación genética (ver sección 1.2) al reducir la diversidad, pueden tener fuertes impactos en caracteres fenotípicos que son relevantes en la adecuación. Hemos aprendido también que, a través de los mecanismos descritos en la sección 2.1, en los procesos de modificación pueden

ser arrastrados caracteres no blanco, afectando de igual manera rasgos fenotípicos relacionados con la adecuación (Bergelson & Purrington 1996; Purrington & Bergelson 1997; Chun et al. 2013). A partir de estos hallazgos se puede entender que muchos de los procesos que pueden alterar la diversidad genética en plantas (naturales o mediados por el hombre) potencialmente afectarán también caracteres fenotípicos relacionados a la adecuación, llevando a las poblaciones a trayectorias evolutivas diferentes.

Consecuencias ecológicas

Todos los seres vivos que conocemos interactúan estrechamente con otros organismos y su ambiente. Estas relaciones se denominan interacciones ecológicas y son parte fundamental de la vida y adaptación de los organismos. Las relaciones ecológicas pueden ser benéficas o perjudiciales, a corto o largo plazo. De cualquier manera, son fundamentales para el mantenimiento de las dinámicas ecológicas que ocurren en los ecosistemas.

Las interacciones ecológicas en un ecosistema pueden afectarse por diversos factores tanto abióticos como bióticos. Dentro de los factores bióticos se encuentran no sólo efectos provocados por otras especies, sino también características intrínsecas a los organismos; las cuales pueden ser fisiológicas, morfológicas y conductuales, todas en cierta medida, con una base genética. Es sabido que individuos con diferencias genéticas pueden interactuar de formas inesperadas (Hughes 2008), afectando procesos a nivel ecológico. El estudio de cómo la diversidad genética de una población puede tener efectos en la ecología ha sido abordado desde hace años. Por ejemplo, gracias a los estudios en agronomía sabemos que si se utilizan plantas genéticamente diversas dentro de un mismo campo se puede promover la productividad del sistema (Wolfe 1985) y reducir los daños por herbivoría (Zhu et al, 2000). Más recientemente supimos que el impacto de la diversidad genética en procesos ecológicos puede ser detectado incluso a nivel individuo; como es el caso de la variación en la interacción planta-polinizador (Johnson & Agrawal, 2008 y Clare 2013)

Es importante reconocer entonces que la estructura y diversidad genética de los organismos y sus poblaciones tiene consecuencias que van mucho más allá de caracteres morfológicos. Aunque estas consecuencias pueden ser divididas en ecológicas y evolutivas, es relevante mencionar que no son independientes, y que una no puede ser estudiada sin el entendimiento de la otra. Históricamente se pensaba que la evolución ocurría a escalas temporales tan grandes que no podría afectar dinámicas ecológicas. Ahora sabemos que cambios ecológicos pueden llevar a cambios evolutivos y viceversa, en escalas contemporáneas. Este fenómeno se ha reconocido recientemente y se denomina retroalimentación eco-evolutiva (Pelletier et al, 2009 y Bolnick et al, 2011).

4 MODELO DE ESTUDIO: *Gossypium hirsutum* – ALGODÓN MEXICANO

El algodón es una planta de amplia importancia económica y ecológica, y es fundamental en el origen y desarrollo de varias civilizaciones (Fryxel 1979; Stewart et al, 2010; Wegier et al, 2011). El género *Gossypium* se compone de cuatro especies, donde la más cultivada es *G. hirsutum*, también conocida como algodón mexicano. Su importancia radica en ser la fibra natural más utilizada y la tercera fuente de aceite vegetal (FAO 2017). Además de su clara importancia económica, su posible centro de origen y diversidad se ubica en México, por lo que podemos encontrar a las poblaciones silvestres de la especie en las costas del país.

4.1 HISTORIA EVOLUTIVA

La distribución del género *Gossypium* es global, por lo que podemos encontrar plantas de las distintas especies del género alrededor del mundo. Esto ha resultado en que algunas de estas especies hayan pasado por procesos de selección artificial de manera paralela y por culturas completamente diferentes. Este proceso de domesticación paralela se dio en cuatro especies: *G. hirsutum* y *G. barbadense* del continente americano y *G. arboreum* y *G. herbaceum* de África y Asia (Fryxel 1979). Los procesos de domesticación paralelos y aislados han provocado que cada una

de las especies, a pesar de haber sido seleccionadas con el mismo objetivo, presenten variación genética y fenotípica dada por sus historias únicas de diversificación y utilización (Fryxel 1979, Wegier 2011).

4.2 DIVERSIDAD GENÉTICA DE LAS POBLACIONES DE ALGODÓN

Como mencionamos en secciones anteriores, las poblaciones naturales de una especie se caracterizan por presentar altos niveles de diversidad genética. En el caso del algodón, la diversidad originada por procesos evolutivos a lo largo de millones de años ha sido moldeada por manejo y selección artificial, por lo que actualmente en su centro de origen podemos encontrar, conviviendo en el mismo ambiente, a poblaciones silvestres (CWR), cultivos domesticados y más recientemente, cultivos de OGM.

Poblaciones silvestres

Un centro de origen se entiende como el área geográfica donde un grupo de organismos desarrolló sus características distintivas por primera vez (Vavilov 1992 y England 2003). Una de las características de estos sitios es que presentan la mayor cantidad de las poblaciones silvestres de una especie. Bajo este contexto, México es considerado el potencial centro de origen y diversidad de *G. hirsutum*, en parte debido a la presencia de las poblaciones silvestres y a que es el único lugar donde estas se encuentran como vegetación dominante (Stewart et al, 2010).

En 2013, Wegier y colaboradores determinaron que el algodón en México presenta una estructura ***metapoblacional.*** En este trabajo, se propuso la existencia de ocho metapoblaciones diferentes que conforman el algodón silvestre: 1) Metapoblación Baja California sur (Sur de Baja California sur; MBC), 2) Metapoblación Pacífico Norte (Centro y Sur de Sinaloa y Norte de Nayarit; MPN), 3) Metapoblación Golfo Norte (Norte de Veracruz, Este de San Luis Potosí y Sur de Tamaulipas; MGN), 4) Metapoblación Bahía de Banderas (Suroeste de Nayarit y Noroeste de Jalisco; MBB), 5) Metapoblación Golfo Sur (Centro y Sureste de Veracruz; MGS), 6) Metapoblación Pacífico Sur (Sureste de Guerrero, línea costera

de Oaxaca, Centro Oeste, Centro y Sur de Chiapas; MPS), 7) Metapoblación Pacífico Centro (línea costera del Centro y Sur de Jalisco, Colima, Michoacán, Noroeste y Centro de Guerrero; MPC) y 8) Metapoblación Península de Yucatán (Quintana Roo, Yucatán, Campeche, Noreste y Este de Tabasco; MPY) (Figura 3). Debido a esta dinámica metapoblacional, cada una de las sub-poblaciones comparten flujo génico que presenta un patrón a larga distancia, lo que implica que no sólo poblaciones geográficamente cercanas pueden compartir genes, sino que las más alejadas también, lo cual tiene implicaciones ecológicas y evolutivas para la especie (Wegier 2013).

Gran parte de los sitios que abarca la distribución potencial de las metapoblaciones se caracterizan por presentar altos niveles de perturbación humana y poca cobertura vegetal. Bajo este panorama, se ha propuesto que la sobrevivencia de las poblaciones en este tipo de ambientes podría estar dada por su capacidad de crecimiento en alta exposición solar, algunas características de su reproducción y el hecho de presentar poblaciones compuestas por plantas en distintos estadios de desarrollo (Wegier 2013).

La presión selectiva al sobrevivir en zonas altamente perturbadas, sumada a las diferencias ambientales que caracterizan cada una de las regiones geográficas donde crecen las metapoblaciones silvestres de algodón, pueden haber influido fuertemente en la impresionante variación ecológica, genética y fenotípica que estas poblaciones exhiben (Wegier 2013). Debido a esta amplia variación, las poblaciones silvestres de algodón son un buen modelo para probar hipótesis relacionadas a las consecuencias de la variación intra-específica de una especie, o a la adaptación de poblaciones silvestres en ambientes cambiantes.

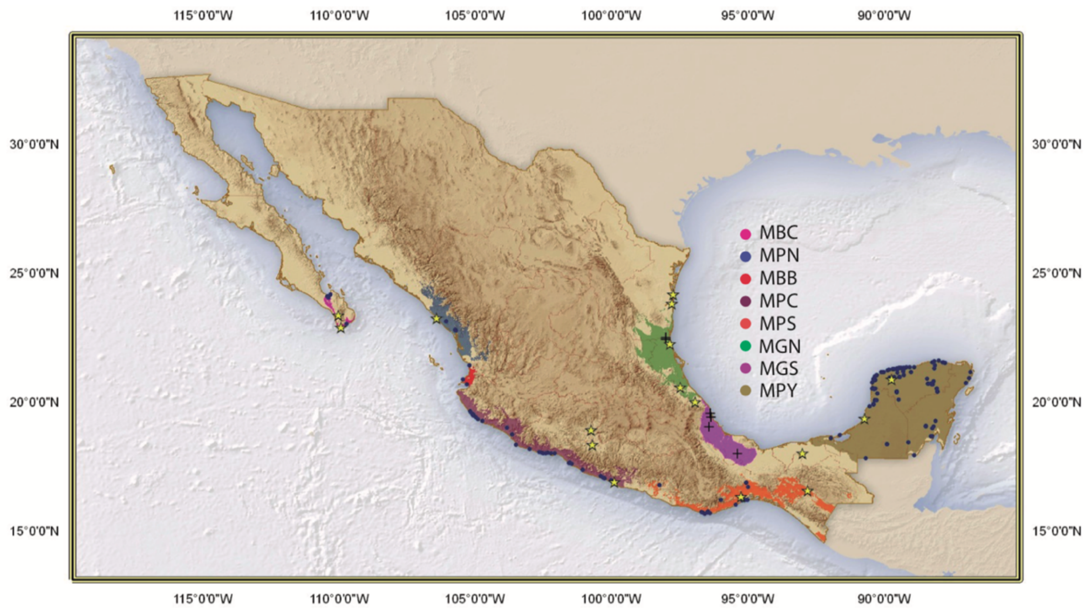

Figura 3. Distribución potencial de las metapoblaciones silvestres de algodón en México. Cada color representa la distribución potencial de cada una de las metapoblaciones. MBC: Metapoblación Baja California Sur; MPN: Metapoblación Pacífico Norte, MBB: Metapoblación Bahía de Banderas, MPC: Metapoblación Pacífico Centro, MPS: Metapoblación Pacífico Sur, MGN: Metapoblación Golfo Norte, MGS: Metapoblación Golfo Sur, Metapoblación Pacífico Sur.

Poblaciones modificadas

Como mencionamos anteriormente, el algodón ha sido domesticado en distintos momentos y lugares del mundo con el objetivo de aprovechar su fibra para la producción de textiles y otros productos. Estos procesos de domesticación se realizaron de manera paralela y actualmente podemos encontrar una gran variedad de plantas mejoradas e híbridos. Además, recientemente se han realizado mejoramientos por ingeniería genética para conferir características novedosas al algodón. Ambos procesos de modificación en la especie se describirán brevemente a continuación.

Algodón domesticado

El algodón domesticado es una de las especies cultivadas más importantes económicamente a nivel mundial. Tiene especial relevancia además debido a que, dentro de los 15 cultivos más importantes del mundo, es el único que no es parte

de la cadena alimenticia (Stewart et al, 2010, Wegier 2013). Esto se debe a que el proceso de selección artificial se originó con la fibra como blanco de selección. Actualmente, la especie *G. hirsutum* es la fibra natural más utilizada y la tercera fuente de aceite vegetal (FAO 2017).

El proceso de domesticación en el algodón, como en muchas otras especies, ha causado consecuencias genéticas y fenotípicas más allá de la selección de la fibra. El síndrome de domesticación en la especie se presenta principalmente en la forma de dominancia apical, alargamiento de tricomas en las hojas, disminución en el número de semillas, entre otros (Velázquez-López et al, 2018) (Figura 4). Además, al igual que en otros cultivos, las poblaciones domesticadas de algodón presentan muy baja variación genética, que podría ser producto de cuellos de botella genéticos ocurridos en el proceso de selección artificial (Wegier 2013).

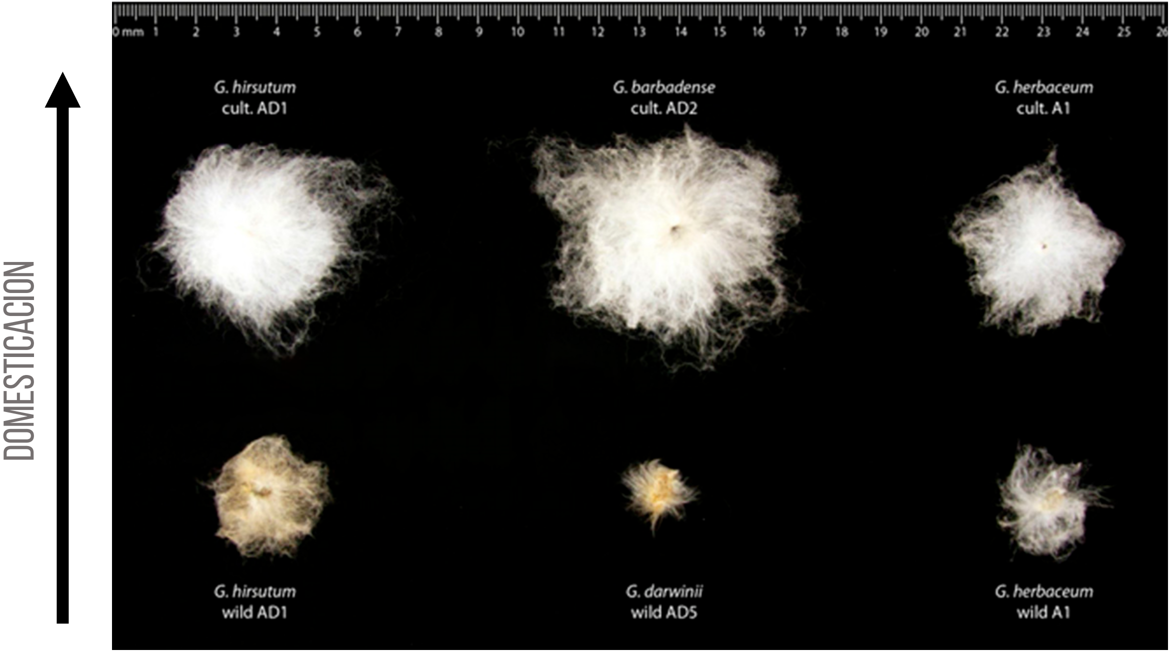

Figura 4. Fibra asociada a versiones silvestres y domesticadas de tres especies de algodón. Especies del género domesticadas de manera paralela para el aprovechamiento de fibra. En la imagen se observa el cambio dramático en el tamaño de la fibra (largo de tricomas) de los individuos silvestres (wild) a domesticados (cult.), además de variación en el color. Imagen modificada de: Bao et al. (2011).

Algodón GM

El primer algodón modificado genéticamente se obtuvo en 1987 por dos grupos de trabajo independientes (Firoozabady et al, 1987 y Umbeck et al, 1987). La transformación en ambos casos, se logró a través de la bacteria *Agrobacterium tumefaciens* (Zhang 2013). A mediados de 1990, productores de distintos países adoptaron cultivares de algodón mejorados a través de ingeniería genética; siendo uno de los primeros cultivos en producirse a campo abierto (Zhang 2013). Actualmente, un total de 65 eventos (Caja 3) de algodón transgénico han sido registrados a nivel mundial (ISAAA 2019). En 1996 fue liberado el primer algodón GM en México y, a la fecha, un total de 35 eventos han sido liberados en el país.

La modificación genética en el cultivo de algodón se ha concentrado en dos rasgos particulares: 1) resistencia a plagas y 2) tolerancia a herbicidas. El primero, también conocido como cultivos "*Bt*", son plantas modificadas para lograr la expresión de una proteína con actividad entomopatógena. Estas proteínas provienen de la bacteria *Bacillus thuringiensis* y pertenecen a la familia proteica "*Cry*", que son expresadas en las hojas de las plantas. El segundo grupo se basa en la expresión de tolerancia al herbicida glifosato, compuesto que representa el ingrediente activo del herbicida Roundup®. La expresión se logra gracias a la inserción de una enzima proveniente de *Agrobacterium sp.* CP4 (EPSPS) que bloquea la síntesis de aminoácidos aromáticos (Zhang 2013).

A partir de la liberación de algodón GM en México, gran parte de los productores adoptaron su uso principalmente en el norte del país. Hoy en día, los cultivares de algodón, domesticados y GM, presentan una distribución geográfica más amplia que la de las poblaciones silvestres; encontrándose a lo largo de la república, mientras que los parientes silvestres se presentan geográficamente restringidos principalmente en zonas periféricas del hábitat natural (Stewart et al, 2010).

La convivencia en el ambiente entre poblaciones silvestres, cultivos domesticados y transgénicos ha provocado eventos de flujo de genes entre estos tres tipos de poblaciones. Gracias a que algunos de estos eventos de flujo se dieron entre cultivos GM y poblaciones silvestres, es posible rastrear los transgenes con técnicas moleculares y corroborar que los eventos de flujo génico sucedieron. En

2011, Wegier y colaboradores encontraron la presencia de transgenes en cinco de las ocho metapoblaciones silvestres de algodón. Actualmente, se sigue encontrando la presencia de transgenes en las poblaciones silvestres, frecuencia que ha aumentado a través de los años, lo que podría significar que gracias al continuo flujo de genes han sucedido eventos de introgresión.

Considerando lo anterior, el algodón en México representa un modelo único para estudiar preguntas relacionadas con los efectos de la diversidad genética en el fenotipo e interacciones ecológicas. Por un lado, contamos con poblaciones silvestres altamente diversas tanto genética como fenotípicamente que nos permiten probar hipótesis relacionadas con la diversidad natural. Por otro lado, tenemos el continuo de plantas domesticadas a mejoradas con ingeniería genética que nos permite responder preguntas acerca de los efectos fenotípicos de la modificación genética más allá de los rasgos buscados. Por último, debido a los procesos de introgresión mencionados anteriormente contamos también con plantas silvestres que han adquirido genes de domesticación y transgenes a través de flujo, lo que nos permite investigar acerca del comportamiento de transgenes en organismos silvestres, sus consecuencias fenotípicas y su impacto en la conservación.

CAJA 3. EVENTO DE TRANSFORMACION GENÉTICA

Un evento de transformación se refiere a una recombinación de ADN única que tuvo lugar en una célula vegetal que se usa posteriormente para la generación de individuos completos (GMO-Compass, 2019).

GLOSARIO

adecuación Habilidad de los organismos, o poblaciones, para sobrevivir y reproducirse en un ambiente determinado. La consecuencia de esta sobrevivencia es que los individuos contribuyen genes a la siguiente generación (Orr et al, 2009; Futuyma 2009). La adecuación media entre dos organismos (genotipos) puede ser medida con la fórmula:

$$W = pW_1 + qW_2$$

Donde:
p= frecuencia del genotipo 1
q= frecuencia del genotipo 2
W_1= adecuación del genotipo 1
W_2= adecuación del genotipo 2

biolística Método de transformación genética que consiste en el bombardeo de copias de fragmentos ADN a alta velocidad hacia una célula.

cuello de botella Evento que drásticamente reduce la variación genética de una población (Doebley et al, 2006).

dominancia apical Fenómeno que ocurre en plantas en donde la rama principal (ápice) domina e inhibe el crecimiento de ramas secundarias (Barbier et al, 2017).

dormancia de semillas Adaptación en semillas que previene la germinación durante condiciones ambientales que podrían no favorecer la sobrevivencia de las plántulas. Una de las funciones más importantes de la dormancia es el retraso de la germinación, lo que permite la dispersión de las semillas y que no todas las semillas germinen al mismo tiempo (Fenner & Thompson, 2005).

entre-cruzamiento Reproducción de organismos que son cercanos genéticamente, que resulta en un incremento en la homocigosis (en el caso de organismos emparentados) y consecuentemente en un aumento en la ocurrencia de rasgos recesivos (Biemont 2010).

flujo génico Migración de genes de una población a otra (Palsboll 2007).

fondo genético Conjunto de genes que componen un genoma (Yoshiki y Moriwaki 2006).

genes modificadores	Genes que afectan el fenotipo y/o la expresión molecular de otros genes. Estos genes pueden afectar la penetrancia, dominancia, expresión y pleiotropía (Nadeu 2001).
genotipo	Repositorio de información heredable que en conjunto con el ambiente genera y mantiene el fenotipo (Fontana 2002).
locus	Localización física de un gen en un cromosoma (Lewin 2001).
metapoblación	Ensamblaje en el que las poblaciones existentes se encuentran en un equilibrio entre extinción, colonización y recolonización (Hanski y Gaggiotti 2004).
mutación	Alteración en la secuencia de nucleótidos del genoma de un organismo (Scitable 2019)
norma de reacción	Función que relaciona los ambientes a los que un genotipo está expuesto con el fenotipo que dicho genotipo produce (Pigliucci 2005).
plasticidad fenotípica adaptativa	variación en el aspecto (fenotipo) que brinda ventajas evolutivas al permitir a los genotipos tener mayor adecuación ante condiciones ambientales adversas o poco habituales (Ghalambor et al, 2007).
transposones	Secuencias de ADN que se mueven de un lugar a otro en el genoma (Pray, 2008).
selección natural	Sobrevivencia y/o reproducción diferencial de organismos que difieren en una o más características (Futuyma 2009).
deriva génica	Cambios al azar en las frecuencias de dos o más alelos dentro de una población (Futuyma 2009).

CAPÍTULO I

META-ANÁLISIS DE LAS CONSECUENCIAS FENOTÍPICAS DE LA MODIFICACIÓN GENÉTICA EN PLANTAS

PREFACIO

Las consecuencias de la diversidad genética en plantas pueden estudiarse desde distintos ángulos. Uno de estos es el estudio de las consecuencias fenotípicas que tiene la modificación de la diversidad genética en plantas de interés. Dado que los procesos de mejoramiento genético tienen una larga historia en la humanidad, además de consecuencias directas en plantas de importancia mundial, resultan ser un buen modelo para investigar preguntas relacionadas con cómo la modificación genética puede tener consecuencias fenotípicas inesperadas.

Como se describió previamente, los procesos de modificación pueden ser tanto por domesticación como por ingeniería genética. Si bien en estos procesos la selección artificial es dirigida hacia rasgos particulares, sabemos que muchas veces, a través de procesos a nivel genético (ver sección 2.1) o en el desarrollo, otros caracteres pueden verse afectados. Entender los mecanismos por los cuales surgen los efectos no intencionados de la modificación y sus consecuencias para las poblaciones que los sufren es fundamental, no sólo para seguir realizando procesos de mejoramiento en el futuro, sino también para entender sus limitaciones.

El estudio de cómo varían los rasgos fenotípicos entre versiones silvestres, domesticadas y genéticamente modificadas (GM) de un mismo cultivo se ha realizado durante décadas. En este tipo de trabajos, se comparan bajo condiciones controladas un número determinado de rasgos (relacionados con la adecuación) entre individuos con distinto nivel de modificación genética. Sin embargo, y aunque la caracterización fenotípica de los organismos en estos estudios es amplia, la mayor parte de los resultados se centran en conocer cómo las modificaciones en rasgos específicos afectan o no la adecuación, dejando de lado otros rasgos que podrían ser comprometidos en el proceso.

En este capítulo, valiéndonos de la amplia información disponible sobre caracteres fenotípicos en cultivos domesticados, GM y sus parientes silvestres; se

presenta un meta-análisis con el objetivo de detectar posibles efectos no intencionados de la modificación en rasgos fenotípicos relacionados con la adecuación. Seleccionamos cinco cultivos de importancia mundial: calabaza, arroz, girasol, maíz y canola, y colectamos toda la información disponible sobre comparaciones de rasgos fenotípicos entre organismos silvestres, domesticados y GM de cada cultivo. Se analizó la información con el objetivo de probar distintas hipótesis: i) conocer si los distintos tipos de modificación (domesticación y transgénesis) pueden alterar el fenotipo de modo que existan diferencias en las características estudiadas entre organismos silvestres, domesticados y transgénicos del mismo cultivo y ii) dado que la transgénesis está diseñada para impactar únicamente una característica, las diferencias fenotípicas entre plantas domesticadas y transgénicas deberían ser menos que al comparar silvestres y domesticadas.

En resumen, encontramos diferencias estadísticas en casi todas las comparaciones de rasgos fenotípicos entre los distintos tipos dentro de un mismo cultivo. Al analizar de manera integral todos los rasgos dentro de un mismo cultivo, encontramos que cada tipo de organismo (silvestre, domesticado y GM) se puede diferenciar claramente en función de su fenotipo. Esto significa que contrario a nuestras hipótesis, los procesos de modificación genética tienen impacto en rasgos fenotípicos más allá de los buscados. Mismos que son detectados a gran escala y en un gran número de cultivos diferentes. Dado que la mayoría de los efectos no intencionados de la modificación fueron detectados en rasgos relacionados a la adecuación de los organismos, estos podrían tener efectos a largo plazo, llevando a los organismos a trayectorias evolutivas inesperadas. Por esto, y considerando que los protocolos de análisis de riesgo de tecnologías de ingeniería genética (IG) se han enfocado históricamente en el estudio de los rasgos blanco, proponemos que, al existir efectos no intencionados de la modificación genética, este tipo de protocolos se ajusten a investigar efectos globales en los organismos. Finalmente, los resultados encontrados en nuestra investigación muestran cómo las intervenciones humanas han causado por años fuertes impactos en las poblaciones de plantas que utilizamos. Aunque históricamente estas estrategias han resuelto los objetivos de producción de alimento, es importante reflexionar acerca de las consecuencias de su impacto en la agro-biodiversidad.

ORIGINAL RESEARCH
published: 05 December 2017
doi: 10.3389/fpls.2017.02030

Domesticated, Genetically Engineered, and Wild Plant Relatives Exhibit Unintended Phenotypic Differences: A Comparative Meta-Analysis Profiling Rice, Canola, Maize, Sunflower, and Pumpkin

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OPEN ACCESS

Edited by:
Charles Roland Clement, National Institute of Amazonian Research, Brazil

Reviewed by:
Rubens Onofre Nodari, Universidade Federal de Santa Catarina, Brazil
Shabir Hussain Wani, Michigan State University, United States

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Specialty section:
This article was submitted to Agroecology and Land Use Systems, a section of the journal Frontiers in Plant Science

Received: *10 August 2017*
Accepted: *14 November 2017*
Published: *05 December 2017*

Citation:
Hernández-Terán A, Wegier A, Benítez M, Lira R and Escalante AE (2017) Domesticated, Genetically Engineered, and Wild Plant Relatives Exhibit Unintended Phenotypic Differences: A Comparative Meta-Analysis Profiling Rice, Canola, Maize, Sunflower, and Pumpkin. Front. Plant Sci. 8:2030. doi: 10.3389/fpls.2017.02030

Agronomic management of plants is a powerful evolutionary force acting on their populations. The management of cultivated plants is carried out by the traditional process of human selection or plant breeding and, more recently, by the technologies used in genetic engineering (GE). Even though crop modification through GE is aimed at specific traits, it is possible that other non-target traits can be affected by genetic modification due to the complex regulatory processes of plant metabolism and development. In this study, we conducted a meta-analysis profiling the phenotypic consequences of plant breeding and GE, and compared modified cultivars with wild relatives in five crops of global economic and cultural importance: rice, maize, canola, sunflower, and pumpkin. For these five species, we analyzed the literature with documentation of phenotypic traits that are potentially related to fitness for the same species in comparable conditions. The information was analyzed to evaluate whether the different processes of modification had influenced the phenotype in such a way as to cause statistical differences in the state of specific phenotypic traits or grouping of the organisms depending on their genetic origin [wild, domesticated with genetic engineering (domGE), and domesticated without genetic engineering (domNGE)]. In addition, we tested the hypothesis that, given that transgenic plants are a construct designed to impact, in many cases, a single trait of the plant (e.g., lepidopteran resistance), the phenotypic differences between domGE and domNGE would be either less (or inexistent) than between the wild and domesticated relatives (either domGE or domNGE). We conclude that (1) genetic modification (either by selective breeding or GE)

can be traced phenotypically when comparing wild relatives with their domesticated relatives (domGE and domNGE) and (2) the existence and the magnitude of the phenotypic differences between domGE and domNGE of the same crop suggest consequences of genetic modification beyond the target trait(s).

Keywords: genotype–phenotype, unintended phenotypic effects, phenotypic profiling, *Oryza sativa*, *Brassica napus*, *Helianthus annuus*, *Zea mays*, *Cucurbita pepo*

INTRODUCTION

Plant domestication and the phenotypic modifications it produces have a long history with humans and have involved practices ranging from traditional management to genetic engineering (GE). The effectiveness of traditional practices, or human selection, is possible because the selected traits have a genetic basis that are phenotypically expressed in particular agroecological and cultural environments (Gepts, 2004; Meyer and Purugganan, 2013). Consequently, domestication processes, either with or without GE, may have important evolutionary effects in cultivated plants (Abbo et al., 2014; Hake and Ross-Ibarra, 2015). Genetically modified crops are also domesticated plants, since the genetic modifications are performed in isogenic lines of the crop of interest (Setlow, 1991). Nonetheless, these domestication processes are qualitatively different. On the one hand, in traditional plant breeding new genetic combinations are, in general, obtained by sexual crosses between individuals of the same species. In GE, on the other hand, DNA sequences (of potentially non-related organisms) are inserted into the crop of interest via bioballistics, *Bacillus thuringiensis* (*Bt* crops) (Agrawal et al., 1999; Nodari and Guerra, 2001) and other novel techniques (e.g., CRISPR, RNA_i) (McManus and Sharp, 2002; Gaj et al., 2013). Thus, the main differences between the two genetic modification techniques involved in domesticated plants are (i) the origin of the novel or foreign DNA that is incorporated in the modified organism, and (ii) the procedure to accomplish such incorporation (Gepts, 2001; Nodari and Guerra, 2001).

Agronomic modification via human selection, domestication without genetic engineering (domNGE), or through genetic engineering (domGE) have phenotypic effects that may not correspond, in magnitude, to the associated genetic changes (Burke et al., 2007). Some of these phenotypic changes are unintended and are usually unrelated to the target traits (Filipecki and Malepszy, 2006). Some studies have attributed these unintended phenotypes to pleiotropic effects in which certain phenotypic traits may be linked and affected by the genetic modification of another trait (Filipecki and Malepszy, 2006), as well as to bottlenecks, selective sweeps, phenotypic plasticity, or gene × environment (G × E) interactions (Remington et al., 2001; Pozzi et al., 2004; Gunasekera et al., 2006; Doust et al., 2014). This phenomena, in which the domesticated organisms show phenotypes that do not correspond to the target traits of domestication, has been documented in many crops, such as potato (*Solanum tuberosum*), soybean (*Glycine max*), and wheat (*Triticum aestivum)* (Dale and McPartlan, 1992; Gepts, 2004; Lenser, 2013). Some of these modified non-target traits have been found to be related to species fitness, which in turn can have a direct impact in the evolution of the plants in potentially unexpected ways (Meyer and Purugganan, 2013).

The unintended phenotypic effects and their evolutionary (and potentially ecological) consequences are of particular relevance if we consider that most of the modifications are done in economically important crops. As such, unintended changes in phenotypes have been observed in crops that are key for global food production, such as rice (*Oryza sativa*), canola (*Brassica napus*), sunflower (*Helianthus annuus*), pumpkin (*Cucurbita pepo*), and maize (*Zea mays*) (Snow et al., 1998; Spencer and Snow, 2001; Halfhill et al., 2005; Guadagnuolo et al., 2007; Cao et al., 2009). Moreover, for cases such as maize, pumpkin, and rice, their cultivation represents important sources of cultural value that involve practices related to their diversification, achieved through the traditional selection of ancestral populations (Purugganan and Fuller, 2009; Chen et al., 2015), and represent an important cultural and genetic repository (Altieri and Merrick, 1987).

Moreover, in the context of food security under climate change and high uncertainty scenarios, *in situ* conservation of agrobiodiversity is of key importance, including not only phenotypic and genetic diversity, but also the accompanying management practices and the environmental context that allows future adaptation (including wild relatives) (Kahane et al., 2013). Therefore, and beyond the merely evolutionary consequences of unintended phenotypic changes, agrobiodiversity studies that look into specific trait changes can help improve protocols of biosafety and risk assessment (Smyth and Mchughen, 2008).

Although the phenomena of the unintended effects of genetic modification have been widely reported, these observations are the product of many individual studies. Thus, we propose a meta-analysis profiling approach in order to perform an unbiased analysis with high statistical power. Meta-analysis profiling allows for the integration of large quantities of data in order to identify patterns among different studies that share a common theoretical framework, but that have been conducted independently (Fiehn et al., 2000). This approach represents a valuable tool that has been used to identify patterns in plant functional genomics (Fiehn et al., 2000) and in phenotypic traits related to growth in plants (Kjemtrup et al., 2003). In the present study, we aimed to profile as many observations as possible into a meta-analysis of the phenotypic consequences of agronomic improvement in five economically and culturally important crops: rice, canola, sunflower, pumpkin, and maize. For the analysis, we included functional phenotypic traits that are potentially related to plant fitness, independently of whether these traits were modified through traditional practices (domNGE) or genetic engineering (domGE), so we could

determine whether there were unintended phenotypic and thus evolutionary consequences. This profile includes 120 scientific publications (110 papers and 10 theses), which cover the period 1990–2017.

MATERIALS AND METHODS

Data Collection

In order to determine whether genetic modifications have unintended phenotypic consequences in plants, we identified suitable studies for our analysis by looking for articles published in agricultural and ecological journals, as well as in the thesis database for the National Autonomous University of Mexico (UNAM) for the case of maize. We focused on five of world's most economically important species: rice, canola, sunflower, pumpkin, and maize. We searched for information in the Scopus®, GoogleScholar®, and UNAM theses databases. For Scopus® and GoogleScholar® databases, we employed Boolean operators for each crop, such as "Cucurbita [AND] wild [OR] domesticated [OR] GMO [AND] fitness."

To be included in the database, all publications had to satisfy three selection criteria: (1) An estimate of plant fitness between wild relatives and domesticated varieties with and without GE must have been measured; (2) Tests must have been performed under conditions in which the agent of selection was absent; and (3) The genetic background must have been controlled to minimize differences affecting the fitness traits being measured. In cases where experiments included extreme biotic and/or abiotic conditions (e.g., soil fertilization, heat, drought), only the data of the controls were used, since we considered these treatments as perturbations and not as natural environmental variation. In the case of maize, we also used information from thesis reports in which a yield comparison between wild relatives and domesticated relatives was performed. All thesis reports had gone through a peer review process [Reglamento General de Estudios de Posgrado (RGEP), UNAM][1] and were obtained from the National Autonomous University of Mexico theses database. We applied these criteria rigorously, rejecting hundreds of comparisons that did not satisfy all of them.

Of all the available information, only 110 articles and 10 theses, representing 990 comparisons of wild relatives and domesticated varieties with and without GE of the five species were incorporated into our dataset. The comparison for each genotype and the number of analyzed publications by crop are shown in **Table 1**. The data reported in the articles were collected for the period 1990–2017, representing the timeframe of the first release of genetically modified organisms to date. Although the available literature sometimes reports more phenotypic traits, only six were chosen in the analysis presented here: height (cm), number of flowers, days to flowering, number of seeds, pollen viability (%), and number of fruits. Those traits were chosen because they are functional traits that have potential impacts in survival and reproduction of the plants (Dafni and Firmage, 2000; Saatkamp et al., 2011; Huang et al., 2016; Williams and Mazer, 2016), besides their availability in most of the published studies. The full dataset can be found in the **Supplementary Data Sheet S1**.

[1] http://www.ddu.unam.mx/index.php/reglamento-general-de-estudios-de-posgrado

Data Analysis

To standardize data from different traits, we followed a procedure based on Song et al. (2004). The method consists in taking all the values of a single trait from low to high, and normalizing between zero and one. Outlier data points were identified using the Viechtbauer and Cheung (2010) approach. In this approach, a multivariate detection method (Cook's distance) is used to calculate the distance among all data points, and then the data points that do not fall into the general model are identified as "influential data points" or outliers. Given the potential biological meaning of outliers (extreme phenotypes), we decided to investigate the experimental origin of each data point before removing it from the database. We considered that the only biologically meaningful outliers would be those which corresponded to common garden experiments of the domGE with their domNGE isogenic lines, in which case, and despite the outlier category of the data point with respect to the general model, we did not remove these data points from the rest of the analysis. This process was performed for all traits and all crops. As we mentioned before, in most cases the genetic modification is performed in domesticated lines, therefore we decided to separate the three categories in all crops with the labels: "wild" for wild relatives, "domNGE" for domesticated organisms that have not gone through a GE process, and "domGE" for those which have been genetically modified to show new traits.

To determine statistical differences among wild, domNGE, and domGE categories within species, we used a Generalized Linear Model (GLM). In the cases where the *p*-value was less than 0.05, we carried out a Glht (Tukey) as a *post hoc* test in the *R* Multcomp package (Hothorn et al., 2008). A graphic representation of the data was constructed as a Spider Chart using *R* Fsmb package (Nakazawa, 2014). In addition, to determine differences between categories (wild, domNGE, and domGE) within species, we conducted a Discriminant Analysis (DA) with the *R* MASS package (Venables and Ripley, 2002) using the genotypes as categories and the values of each trait as predictor variables. To test the significance of differences between categories of the DA per crop, we conducted a follow-up Multivariate Analysis of Variance (MANOVA). Finally, we

TABLE 1 | Comparisons for each category [wild, domesticated without genetic engineering (domNGE), domesticated with genetic engineering (domGE)] and total number of publications analyzed in each crop (*N* = number of reviewed publications).

Crop	Wild	domNGE	domGE	Comparisons	*N*
Rice	64	57	98	219	33
Canola	34	114	52	200	22
Sunflower	27	81	11	119	11
Pumpkin	19	37	33	89	19
Maize	54	254	58	366	35

delimited groupings by drawing 95% confidence interval ellipses around the centroids using the ggplot2 R package (Wickham, 2009). All the analyses were conducted in *R* program (version 1.17.15) (R Core Team, 2013) and all the scripts utilized for the analyses are available online at https://github.com/LANCIS-escalante-lab/plant_phenotype_metaanalysis.

RESULTS

The results of the 990 comparisons show significant phenotypic differences among the three categories (wild, domNGE, and domGE) for almost all of the analyzed crops and the majority of the traits. With regard to outlier management, the number of points that lie outside the normal distribution was significantly less than the total number of comparisons for each crop. In the case of canola, the outliers represent 2% of the total comparisons, for sunflower 1.8%, for rice 4.3%, and for maize 12%. In the case of pumpkin, we did not find any outliers.

The differences between wild relatives and the domesticated categories (either domNGE or domGE) were expected, but unexpected differentiation between the domesticated categories (domNGE and domGE) was also observed in the analyzed traits (which were not the target of selection or genetic modification). Since the proportion of outliers within the dataset is relatively small, this general pattern observed in the results is maintained regardless the outlier treatment, with only some specific differences per crop (**Figure 1** and Supplementary Figure S1).

Phenotypic Variation Can Be Identified As Wild, domNGE, and domGE

Through the DA of the phenotypic traits of all crops (height, days to flowering, number of seeds, pollen viability, number of flowers, and number of fruits), we found a clear distinction of phenotypic variation in three groups, which correspond with the wild, domNGE, and domGE categories [**Figure 1**, all (a) panels]. These three groups are different in size, position, and/or direction along the axes of the DA. In some cases, the overlapping of the groups is larger than in others. For instance, in canola, although the groups can be differentiated, the overlapping of the three groups is the largest compared with the other analyzed crops (MANOVA $F_{(2,52)} = 1.541$, $p = 0.166$), while in maize (MANOVA $F_{(2,116)} = 8.5571$, $p = 1.058e^{-07}$) and rice (MANOVA $F_{(2,100)} = 11.284$, $p = 2.868e^{-11}$) the overlapping is the smallest of all, with pumpkin (MANOVA $F_{(2,46)} = 13.357$, $p = 1.066e^{-08}$) and sunflower (MANOVA $F_{(2,48)} = 4.1348$, $p = 0.00081$) in an intermediate range of overlapping [**Figure 1**, all (a) panels]. Moreover, the percentage of variation explained by the discriminant axes varies considerably among crops, with the most extreme cases being maize and sunflower. For maize, the total phenotypic variation is distributed in the two discriminant axes (LD1 = 74%; LD2 = 25%), while in sunflower and canola, the variation is mainly explained by LD1 (93 and 90%, respectively). A more detailed analysis of the DA results shows that the dispersion of the phenotypic variants within groups is, in most cases, larger in wild groups than in domesticated ones (domNGE and domGE) [**Figure 1**, all (a) panels]. The only case where the phenotypic variation found in the wild group was less than that found in the domGE groups was in sunflower.

Variation in Phenotypic Traits Changes from Wild to Domesticated Populations

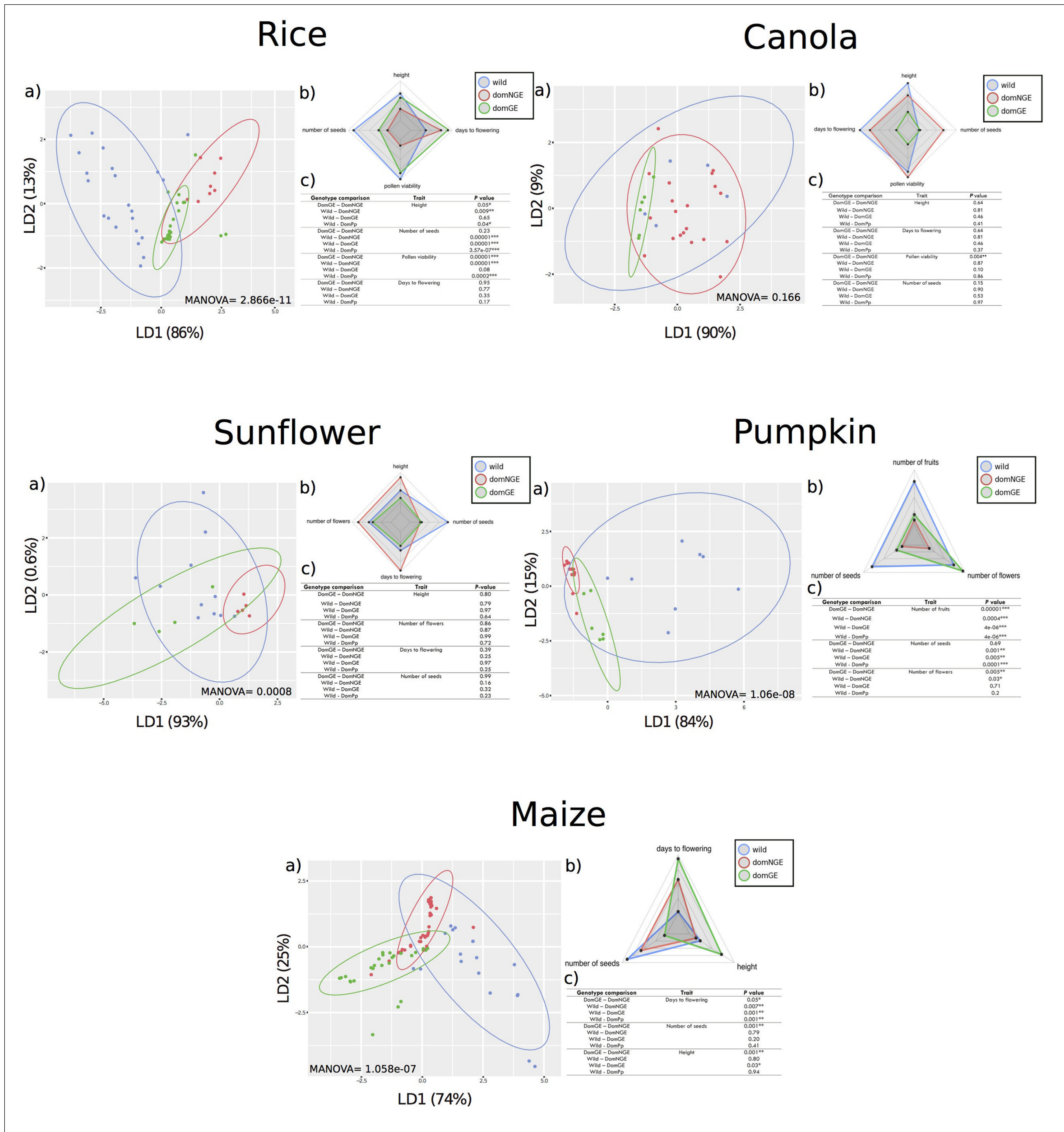
The DA results show a change in the direction of variation between wild and domesticated (domNGE and domGE) categories [**Figure 1**, all (a) panels]. This observation implies that the traits that define the phenotypic variation within each group are different, at least between wild and domesticated categories [**Figure 1**, all (b) panels]. In fact, in almost all the cases, the phenotypic variation of the domesticated groups goes in the same direction, while the wild group is almost orthogonal, and more evenly distributed between the two axes. This observation holds for all of the five analyzed crops.

The weight of the different traits in the resulting grouping per crop is provided by the associated coefficients of each discriminant function (Supplementary Table S1). Thus, it is possible to identify the traits that are statistically more important in the observed differences among groups. For rice, "height" is the trait with the highest coefficient for LD1 and "days to flowering" for LD2. For canola, "number of seeds" is the trait with the highest coefficient for LD1 and "height" for LD2. For sunflower, "days to flowering" has the highest value for LD1 and "number of seeds" for LD2. For pumpkin, "number of fruits" and "number of seeds" were the traits with highest values for LD1 and LD2, respectively. Finally, for maize, "height" is the trait with the highest value for both LDs.

The GLM analysis identifies the traits that explain pairwise differences in phenotypic variation among groups and the results are shown in the (c) panels of **Figure 1**. For instance, for sunflower none of the four analyzed traits show significant differences between wild and domesticated populations. In contrast, for maize, pumpkin, and rice almost all of the analyzed traits show significant differences (days to flowering, number of seeds and height for maize, number of fruits, number of seeds and number of flowers for pumpkin, and height, number of seeds, and pollen viability for rice) [**Figure 1**, (c) panels].

Changes in Phenotypic Variation among Wild, domNGE, and domGE

The normal sequence of reduction of genetic (and potentially phenotypic) variation in the process of domestication and human interventions suggests that wild relative populations represent the largest pool of diversity, which is then reduced during domestication and genetic modification through GE (Flint-garcia, 2013). Moreover, since GE constructs start from isogenic lines (representing the domNGE), and since the modifications are allegedly directed to modify specific phenotypic traits (not included in the present analysis), it was expected that the phenotypic variation of the analyzed populations would be a sequence of subgrouping and reduced phenotypic variation going from wild to domNGE and finally domGE. However, through the DA and GLM analyses [**Figure 1**, panels (a) and (c), respectively], we find evidence that, overall, supports these



Rice (c)

Genotype comparison	Trait	P value
DomGE – DomNGE	Height	0.05*
Wild – DomNGE		0.009**
Wild – DomGE		0.65
Wild - DomPp		0.04*
DomGE – DomNGE	Number of seeds	0.23
Wild – DomNGE		0.00001***
Wild – DomGE		0.00001***
Wild - DomPp		3.57e-07***
DomGE – DomNGE	Pollen viability	0.00001***
Wild – DomNGE		0.00001***
Wild – DomGE		0.08
Wild - DomPp		0.0002***
DomGE – DomNGE	Days to flowering	0.95
Wild – DomNGE		0.77
Wild – DomGE		0.35
Wild - DomPp		0.17

Canola (c)

Genotype comparison	Trait	P value
DomGE – DomNGE	Height	0.64
Wild – DomNGE		0.81
Wild – DomGE		0.46
Wild - DomPp		0.41
DomGE – DomNGE	Days to flowering	0.64
Wild – DomNGE		0.81
Wild – DomGE		0.46
Wild - DomPp		0.37
DomGE – DomNGE	Pollen viability	0.004**
Wild – DomNGE		0.87
Wild – DomGE		0.10
Wild - DomPp		0.86
DomGE – DomNGE	Number of seeds	0.15
Wild – DomNGE		0.90
Wild – DomGE		0.53
Wild - DomPp		0.97

Sunflower (c)

Genotype comparison	Trait	P-value
DomGE – DomNGE	Height	0.80
Wild – DomNGE		0.79
Wild – DomGE		0.97
Wild - DomPp		0.64
DomGE – DomNGE	Number of flowers	0.86
Wild – DomNGE		0.87
Wild – DomGE		0.99
Wild - DomPp		0.72
DomGE – DomNGE	Days to flowering	0.39
Wild – DomNGE		0.25
Wild – DomGE		0.97
Wild - DomPp		0.25
DomGE – DomNGE	Number of seeds	0.99
Wild – DomNGE		0.16
Wild – DomGE		0.32
Wild - DomPp		0.23

Pumpkin (c)

Genotype comparison	Trait	P value
DomGE – DomNGE	Number of fruits	0.00001***
Wild – DomNGE		0.0004***
Wild – DomGE		4e-06***
Wild - DomPp		4e-06***
DomGE – DomNGE	Number of seeds	0.69
Wild – DomNGE		0.001**
Wild – DomGE		0.005**
Wild - DomPp		0.0001***
DomGE – DomNGE	Number of flowers	0.005**
Wild – DomNGE		0.03*
Wild – DomGE		0.71
Wild - DomPp		0.2

Maize (c)

Genotype comparison	Trait	P value
DomGE – DomNGE	Days to flowering	0.05*
Wild – DomNGE		0.007**
Wild – DomGE		0.001**
Wild - DomPp		0.001**
DomGE – DomNGE	Number of seeds	0.001**
Wild – DomNGE		0.79
Wild – DomGE		0.20
Wild - DomPp		0.41
DomGE – DomNGE	Height	0.001**
Wild – DomNGE		0.80
Wild – DomGE		0.03*
Wild - DomPp		0.94

FIGURE 1 | Phenotypic differences between wild and domesticated with and without genetic engineering (GE) in five crops. In all panels: (a) Discriminant analysis (DA), (b) Spider-Chart of the means of each analyzed trait, and (c) results of pairwise comparisons with Generalized Linear Model (GLM). In all (c) "DomPp" = both domesticated populations (GE and NGE). In all panels: "blue" wild relatives, "red" domesticated without GE, and "green" domesticated with GE. $^{*}p < 0.01$; $^{**}p < 0.001$; $^{***}p < 0.0001$.

expectations for the comparison of wild and domesticated categories (domNGE and domGE), but that do not hold for the comparisons between domesticated categories (domNGE vs. domGE). A graphical representation of these results, showing only mean values for all traits and populations, is found on **Figure 1**, (b) panels.

Regarding the comparison between the wild and domesticated (domNGE and domGE) categories, we observe that only canola

fits the expectation of subgrouping. In contrast, regarding the reduced phenotypic variation of domesticated groups compared with their wild relatives, 4/5 analyzed crops fit the expectation (sunflower was the exception). These four crops (rice, canola, pumpkin, and maize) show that, although the phenotypic variation is reduced in the domesticated groups, this is not a subgroup within the wild group. The exceptional case, of sunflower, shows that domGE groups have increased phenotypic variation compared with both domNGE and wild relative groups. The results of the GLM [**Figure 1**, (c) panels], which investigates pairwise differences between wild and domesticated groups (Wild-DomPp), show that rice, pumpkin, and maize have statistically significant differences for almost all traits.

Regarding the comparison of domNGE vs. domGE, we observe that, on the one hand, rice and canola are cases in which the results show some subgrouping of domGE within domNGE populations. On the other hand, maize, sunflower, and pumpkin represent almost the opposite scenario, with almost no overlap, nor subgrouping of the domGE within domNGE populations. Regarding the expectation of reduced phenotypic variation in domNGE, we observe a case of increased phenotypic variation, and specifically we found that domGE groups of sunflower have more variation than their domNGE relatives. Moreover, we also found statistically significant differences in the pairwise comparisons between domNGE and domGE groups in almost all crops and traits [**Figure 1**, (c) panels]. For rice, we found differences between domesticated groups in "pollen viability" and "height;" for canola we found differences in "pollen viability;" for pumpkin the differences were found in "number of fruits" and "flowers;" finally, in maize we found statistical differences in "days to flowering," "height," and "number of seeds." Overall, these results suggest unintended phenotypic effects, and no consistency in the specific traits that change due to human interventions in wild populations, either through domestication or GE modifications.

DISCUSSION

Human interventions in plants of economic, cultural, or nutritional importance via traditional practices (domestication) and, more recently, via GE have a long history in crop management. Despite the major importance of the consequences of these human-driven interventions in crops, no systematic investigation of the actual consequences in plant populations exists. In this study, we conducted a meta-analysis profiling the phenotypic consequences on non-target traits that domestication and GE have had for five global important crops, and here we discuss the potential causes and implications of our observations.

The nature of any meta-analysis implies a large amount of data points or measurements that may correspond to many different individual studies, with different environmental conditions and subject to different sources of error. Given this, it is important to consider carefully both the meaning and treatment of outlier data points, and the implications in the results of the implicit environmental variation. On the one hand, in this study we only removed those outliers that did not correspond to common garden experiments, and thus had biological relevance; in this case the occurrence of extreme phenotypes or big evolutionary leaps [possible "hopeful monsters" (Goldschmidt, 1940; Gould, 1977)]. Nonetheless, of all the comparisons in our analysis, only 3.2% were identified as outliers and, among these, 1% was "true" outliers (not coming from common garden experiments). Moreover, a very limited number of traits of the phenotype were included in the analyses, which precludes us from making major biological or evolutionary inferences about the identified outliers in the different crops, although we recognize the relevance of a more in-depth investigation of those outliers in the evolution of domesticated plants. On the other hand, and regarding the contribution of environmental variation to our overall results, given that different data points correspond to experiments conducted in different environmental conditions, it is not possible to rule out that the observed variation in phenotypes corresponds (in some proportion) to the variation in environmental conditions, and therefore caution should be taken in attributing the observations solely to the genotypic background of populations.

Direction and Magnitude of Phenotypic Variation Changes between Wild and Human-Modified Plant Populations

The differentiation of wild and domesticated populations was expected due to the genetic changes that occur in the evolutionary process of domestication. The genetic changes can be the result of genome level modifications (e.g., genetic bottlenecks), but also can be the result of more localized effects associated with genetic linkage of selected regions (e.g., selective sweeps) (Gepts, 2004, 2014; Pozzi et al., 2004). The phenotypic and genetic variation of wild populations represents the pool from which some variants are selected, thus reducing the original variation via domestication and GE of isogenic domesticated lines (Innan and Kim, 2004). This phenomenon of paired phenotypic variation reduction due to genetic bottlenecks has in fact been described in previous studies with the same crops in this study and others (Miller and Tanksley, 1990; Tenaillon et al., 2004; Stupar, 2010). Nonetheless, we found a notable exception in sunflower, in which variation increases from that observed in the wild relatives. This exceptional case could be explained by the large phenotypic and genetic variation found in the continuum of landraces, hybrids, and genetically modified organisms that increases the phenotypic amplitude in the domesticated populations (McAssey et al., 2016).

Moreover, for most cases, we also observe change in the axis of the variation that could be attributed to the selection of certain variants for the target traits that will then vary in another direction, carrying along linked phenotypic variation in non-target traits. Altogether, the expected reduction of

phenotypic variation and the change in the direction of this variation is in accordance with the concept of the domestication syndrome (Doebley et al., 2006; Meyer et al., 2012). However, we did not find consistency in the specific traits that varied among the three categories (wild, domNGE, and domGE). Potential explanations for this lack of shared traits in the differentiation of populations among crops could be, on the one hand, that although some phenotypic and general traits have been linked to the domestication syndrome, there are many others that are particular to each crop which are associated with specific aspects of their biology. For example, one of the most extreme cases of domestication is maize, where the phenotypic similarities between teosinte (wild ancestor) and contemporary maize are very small. The most important traits that define the domestication syndrome in maize are the change in the number and arrangement of ears and the presence of shorter lateral branches (Wills and Burke, 2007). Nevertheless, in many crops difficulties and ambiguities still exist in defining the domestication syndrome. One good example of such difficulties is Asian rice, in which the high levels of introgression between wild relatives and domesticated populations have caused genetic exchange that makes it difficult to identify the phenotypic traits that distinguish wild from domesticated populations (Vaughan et al., 2008). On the other hand, during the domestication process via selection, the phenotypic targets (or traits) are different for different crops. For instance, while in the case of rice the target of selection is the number of grains (seeds) (Vaughan et al., 2008), in the case of pumpkin, it is size and number of fruits (Meyer et al., 2012). In the same sense, GE of crops has different goals, thus different traits are introduced to different crops. For example, for canola a broad range of traits added through GE exists, such as insect resistance (Lepidopteran), herbicide tolerance (glyphosate/glufosinate), and virus resistance, while in pumpkin, the most frequent genetic transformation is focused on mosaic virus resistance (*ZYMV*) (Supplementary Table S2).

Unexpected Phenotypic Changes in Human-Modified Plant Populations – Changes in Non-target Traits

As mentioned before, given that the GE constructs start from isogenic lines (represented here as domNGE), and that the modifications are allegedly directed to modify specific phenotypic traits not included in the present analysis, it was expected that the phenotypic variation of the domGE would be a subgroup of that in the domNGE group. This expectation is based on the premise that GE works usually with foreign DNA in order to introduce traits that are not present in the species, and this is performed in isogenic hybrid lines; thus, theoretically, the only differences between a Genetically Modified Organism (GMO) and its isogenic line will be the added trait(s) (Cellini et al., 2004). However, we did not find evidence that supports this expectation, suggesting unintended phenotypic effects of GE modifications. Specifically, we identified the most dramatic cases in rice, pumpkin, and maize, where almost all analyzed traits differ statistically between domNGE and domGE categories.

Generally, the intended effects of a genetic modification refer to a specific phenotype. But the transgene may also impart a range of phenotypes that constitute the unintended effects of the transgene. These new (unintended) phenotypes can appear due to the interaction of the transgenes with another genes (pleiotropic effects) (Rijpkema et al., 2007) or by position effects; thus, these unintended phenotypes are usually unpredictable (Miki et al., 2009). The cases in which the transgene, due to genetic interactions, causes unexpected phenotypes have been seen in canola (Légère, 2005), sunflower (Snow et al., 2003), rice (Chen et al., 2006), and maize (Guadagnuolo et al., 2007) among others. Although we intended to control the data for major environmental variation in the comparisons, we cannot rule out phenotypic plasticity due to GxE interactions that may be introducing a confounding effect in the observations. Moreover, these phenotypic differences between closely related (genetically) organisms can also be associated with other factors that depend on the origin and specific context of domestication that may end up in different phenotypic scenarios, causing phenotypic diversity between organisms of the same species (Gepts, 2001).

In addition to pleiotropy, other phenomena related to the genetic modification, such as position effects, that result from non-directed insertions of DNA fragments (i.e., transgenes) in the target genomes can occur (Filipecki and Malepszy, 2006). These position effects can have deleterious consequences on the engineered organisms, but also non-deleterious effects that allow survival of the organisms with no major or apparent phenotypic consequences (Ladics et al., 2015). Nonetheless, in the present study, all the GE crops analyzed were subject to non-directed insertions and we did find significant and unintended phenotypic effects. Currently, GE technology has apparently overcome the problem of position effects through the use of CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats) technologies, which guarantees a more accurate genetic modification through a precise insertion in known locations within the target genomes (Ma et al., 2017). However, the precision of the insertion does not necessarily prevent unintended genetic interactions, such a mutagenesis and pleiotropic effects that could be traced to the phenotypes (Solovieff et al., 2013). The precision of this technology has been recently challenged by research that reveals the presence of high-frequency off-target mutagenesis induced by CRISPR/Cas9 in human and animal cells (Fu et al., 2013; Schaefer et al., 2017).

In maize, phenotypic variation of domGE is reduced and shifted along LD1 compared with domNGE, which is worth noting given the potential implications of introgression (gene flow) with domesticated non-transgenic populations (Quist, 2007) that in turn could decrease the overall variation of domNGE and affect the genetic and cultural repository of diversity that these populations represent. Given the potential of introgression and the risk of affecting the genetic and cultural

repositories of biodiversity, the analysis of these unintended effects is extremely valuable for understanding the destiny of hybrids in natural habitats, particularly in the context of environmental biosafety (Arriola and Ellstrand, 1997; Snow et al., 2003).

Evolutionary Significance of Unintended Phenotypic Changes

In all of the analyzed cases, we can see phenotypic differences among categories (wild, domNGE, and domGE). However, it is worth noting that these differences, although evident through DA and spider charts [**Figure 1**, panels (a) and (b)], are not all statistically significant in the GLM pairwise comparisons between groups [**Figure 1**, (c) panels]. In particular, for sunflower, there is no statistically significant result for the GLM analysis, although differences among populations can be observed in both DA and spider chart analyses. This apparent inconsistency could be rooted in some fundamental reflections about evolutionary processes. For instance, it is known that genetic variation, even if not reflected as statistically significant differences between populations, can be of evolutionary significance (West-Eberhard, 2003). Given that phenotypic variation is directly exposed to natural selection, even small differences that are not statistically significant can have evolutionary consequences for the populations and species. The best example of this is domesticated plants, in which both natural and human selection act on phenotypes, leading to rapid fixation of even rare variants (Zhang et al., 2009; Tang et al., 2010) that statistically could appear as non-significant variation within or among populations. Moreover, this reflection leads to further examination of the finding of these unintended phenotypic effects in the analyzed crops, as it calls attention to the consequences (phenotypic) of genetic introgression events in different economically, culturally, or ecologically relevant crops (domGE→domNGE; domGE→wild). This is particularly important because there is evidence of some of these introgression events [e.g., maize (Quist and Chapela, 2001), rice (Song et al., 2006), and cotton (*Gossypium hirsutum*) (Wegier et al., 2011)]. Although we did not examine consequences of introgressed populations in this study, our results suggest that unintended effects of introgression are possible, and thus need further investigation looking at phenotypic traits that are usually out of scope (such as those associated with fitness), and that can shed more light on the evolution of domesticated (GE and NGE) and wild crop populations.

In addition, the results presented here show how human interventions in plant populations have different consequences in reducing and changing the direction of phenotypic variation to produce food. Historically, these strategies have proven to be effective, but it is worth reflecting on the unintended effects that some interventions can have in these natural resources that might reduce our options to adapt in the future. The reflection on the strategies to follow in this adaptation to future climate change conditions must include a revision of the regulations of crop technologies given the major consequences that this can have in global food security (Smyth and Mchughen, 2008). For example, given the current regulations, it is worth mentioning that the results presented here are in contradiction with the concept of substantial equivalence between NGE and GE crop lines, which argues that given the fact that the lines are isogenic, the resulting lines will only differ in the added trait (Cellini et al., 2004), which can be in fact demonstrated if only the added or target traits are analyzed, but the contrary can happen when looking at non-target traits (Smyth and Mchughen, 2008).

Finally, and in the context of climate change, there is an undeniable urgency to adapt to future uncertain conditions (Wise et al., 2014). However, there is little recognition that some of the current ecosystems (agroecosystems included) may transition to entirely different and unpredictable states, with different goods, services, and natural resources, and that adaptive cycles of decision will be needed in the most ample spectrum of possibilities (Wise et al., 2014). Thus, it is of major importance to preserve options for future decisions, which includes genetic and phenotypic options, in other words biodiversity (Rockstrom et al., 2014).

CONCLUSION

The results presented show how human interventions in plant populations have different consequences in reducing and changing the direction of phenotypic variation to produce food. In particular, we found that (1) genetic modification (either by selective breeding or GE) can be traced phenotypically when comparing wild relatives with their domesticated ones (GE and NGE), and (2) the existence and magnitude of the phenotypic differences between domGE and domNGE of the same crop suggest consequences of genetic modification beyond the target trait(s). Further studies documenting phenotypic changes in human modified crops must include as many traits as possible, preferably non-target traits, to design interventions that do not compromise the decision spectrum in the face of trade-offs for adaptation to current versus future conditions.

AUTHOR CONTRIBUTIONS

AH-T: designed the research, did the analysis, and wrote the manuscript. AW: designed the research and wrote the manuscript. MB: designed the research and revised previous versions of the manuscript. RL: designed the research. AE: designed the research and wrote the manuscript.

FUNDING

This work was financially supported by CONACyT (PN247672) and the Dirección General del Sector Primario y Recursos

Naturales Renovables (DGSPRNR) that belongs to the SEMARNAT and CONABIO.

ACKNOWLEDGMENTS

This work constitutes part of the doctoral research of AH-T, who received a scholarship from the Consejo Nacional de Ciencia y Tecnología (CONACyT, scholarship no. 660255), and extends thanks to the Doctorado en Ciencias Biomédicas, Universidad Nacional Autónoma de México (UNAM). The authors acknowledge technical assistance of M.Sc. Fidel Serrano Candela and M.Sc. I. Karen Carrasco Espinosa.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2017.02030/full#supplementary-material

DATA SHEET S1 | Full dataset of values for all the phenotypic traits included in the meta-analysis profiling rice, canola, maize, sunflower, and pumpkin.

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Conflict of Interest Statement: 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.

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CAPÍTULO II

IMPACTO DE LA VARIACIÓN GENÉTICA EN EL DESEMPEÑO *IN VITRO* DE *Gossypium hirsutum* L.

PREFACIO

Tal como se demostró en el Capítulo I, los procesos de modificación genética en plantas, tanto por domesticación como por ingeniería genética (IG), tienen fuertes impactos fenotípicos que van más allá de los rasgos buscados, muchos de los cuales pueden ocurrir en caracteres relacionados con la adecuación, llevando a los organismos a trayectorias evolutivas inesperadas. Estos efectos han sido detectados en organismos que han sido blanco de transformación genética, sin embargo, ahora sabemos que debido a que en diferentes partes del mundo, cultivos domesticados y genéticamente modificados (GM) conviven en el ambiente con parientes silvestres, han ocurrido eventos de flujo de genes, por lo que actualmente podemos encontrar híbridos entre cultivos y parientes silvetres de los cultivos (CWR).

Estudiar las consecuencias fenotípicas de genes de domesticación e IG en la naturaleza es de especial relevancia sobre todo cuando se encuentran en poblaciones silvestres. La importancia radica en que como mencionamos en secciones anteriores, los parientes silvestres de los cultivos, al representar los repositorios de diversidad genética más grandes a partir de los cuales se pueden lograr futuros mejoramientos, son blanco de conservación desde hace décadas. Actualmente, se ha reportado la hibridación entre al menos 29 cultivos y parientes silvestres alrededor del mundo, sin embargo, seguimos sin conocer cuáles son las consecuencias de esto en términos ecológicos y evolutivos.

Considerando lo anterior, y tomando en cuenta la necesidad de preservar y conservar la diversidad genética de los cultivos para futuros escenarios de incertidumbre ante el cambio climático, resulta relevante investigar qué sucede cuando hibridan poblaciones silvestres y cultivos, y qué consecuencias potenciales podría tener esto en términos de conservación.

En el presente capítulo, presentamos evidencia experimental de las consecuencias fenotípicas que tiene la presencia de genes de domesticación e IG en una de las estrategias de conservación más utilizadas en plantas alrededor del mundo. Escogimos el ambiente *in vitro* para probar los efectos fenotípicos en

términos de adecuación (propagación) de organismos con distinto ***fondo genético.*** Esto porque además de ser una técnica muy utilizada en estrategias de conservación, es un ambiente sumamente controlado, en el que es posible controlar no sólo variables ambientales sino genéticas al usar clonas de los organismos como réplicas. Comparamos entonces el desempeño *in vitro* medido en diferentes rasgos fenotípicos entre individuos de algodón silvestres y domesticados, con y sin transgenes.

Encontramos en general un efecto perjudicial en poblaciones silvestres con la presencia de transgenes en el desempeño *in vitro*, dado por menor crecimiento y tasa de propagación. Hipotetizamos que esta penalización en el crecimiento podría ser causada por procesos a nivel genético como los mencionados en el Capítulo I, pero también por costos ecológicos/energéticos de la expresión de los transgenes. Este hallazgo nos permitió reflexionar sobre las consecuencias fenotípicas que tienen los genes de domesticación e ingeniería genética, y cómo podrían comprometer el crecimiento de los organismos. Finalmente, con base en nuestros resultados, proponemos que al ser el cultivo *in vitro* una de las técnicas más utilizadas en estrategias de conservación, se realicen monitoreos de la presencia de transgenes en los organismos a conservar. Acciones como esta podrían ayudarnos a ahorrar esfuerzos en el establecimiento de organismos que podrían expresar un compromiso en el crecimiento causado por los procesos de hibridación, lo que a su vez, podría permitirnos también enfocar esfuerzos hacia la conservación de la diversidad genética y fenotípica de parientes silvestres que no hayan sufrido procesos de hibridación.

In vitro performance in cotton plants with different genetic backgrounds: the case of *Gossypium hirsutum* in Mexico, and its implications for germplasm conservation

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ABSTRACT

One of the best *ex situ* conservation strategies for wild germplasm is *in vitro* conservation of genetic banks. The success of *in vitro* conservation relies heavily on the micropropagation or performance of the species of interest. In the context of global change, crop production challenges and climate change, we face a reality of intensified crop production strategies, including genetic engineering, which can negatively impact biodiversity conservation. However, the possible consequences of transgene presence for the *in vitro* performance of populations and its implications for biodiversity conservation are poorly documented. In this study we analyzed experimental evidence of the potential effects of transgene presence on the *in vitro* performance of *Gossypium hirsutum* L. populations, representing the Mexican genetic diversity of the species, and reflect on the implications of such presence for *ex situ* genetic conservation of the natural variation of the species. We followed an experimental *in vitro* performance approach, in which we included individuals from different wild cotton populations as well as individuals from domesticated populations, in order to differentiate the effects of domestication traits dragged into the wild germplasm pool via gene flow from the effects of transgene presence. We evaluated the *in vitro* performance of five traits related to plant establishment ($N = 300$): propagation rate, leaf production rate, height increase rate, microbial growth and root development. Then we conducted statistical tests (PERMANOVA, Wilcoxon post-hoc tests, and NMDS multivariate analyses) to evaluate the differences in the *in vitro* performance of the studied populations. Although direct causality of the transgenes to observed phenotypes requires strict control of genotypes, the overall results suggest detrimental consequences for the *in vitro* culture performance of wild cotton populations in the presence of transgenes. This provides experimental, statistically sound evidence to support the implementation of transgene screening of plants to reduce time and economic costs in *in vitro* establishment, thus contributing to the overarching goal of germplasm conservation for future adaptation.

Submitted 30 November 2018
Accepted 24 April 2019
Published 10 June 2019

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Academic editor
Axel Tiessen

Additional Information and Declarations can be found on page 13

DOI 10.7717/peerj.7017

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OPEN ACCESS

How to cite this article Hernández-Terán A, Wegier A, Benítez M, Lira R, Sosa Fuentes TG, Escalante AE. 2019. *In vitro* performance in cotton plants with different genetic backgrounds: the case of *Gossypium hirsutum* in Mexico, and its implications for germplasm conservation. *PeerJ* 7:e7017 http://doi.org/10.7717/peerj.7017

Subjects Biodiversity, Biotechnology, Conservation Biology, Plant Science
Keywords Crop wild relatives, GMOs, *In vitro* germplasm conservation, 13 Aichi Biodiversity Targets, *Ex situ* conservation, Primary genetic pool

INTRODUCTION

Interest in plant germplasm conservation addresses the need to preserve a diverse genetic pool, thus providing options for future decision-making (*Rockstrom et al., 2014*). Such options must include genetic and phenotypic diversity to face current and future challenges in crop production. The conservation of these variants can help in developing or finding solutions to disease, changing environments, and low yields, among others, and is necessary for safeguarding biodiversity and cultural identity (*Hawkes, 1977*; *Plucknett et al., 1983*; *Hajjar & Hodgkin, 2007*). In most crops, the largest genetic variation exists in the Crop Wild Relatives (CWR) found in the centers of origin of the species (*Hawkes, 1977*). Accordingly, research groups in food security identified CWR as a target group for conservation (*Harlan, 1965*; *Hunter & Heywood, 2011*; *Castañeda Álvarez et al., 2016*). Due to the importance of CWR for conservation, international agreements have made the *in situ* and *ex situ* preservation of their genetic diversity one of their goals (Aichi Target 13) (*Leadley et al., 2014*).

Plant tissue culture methods represent a robust approach for many purposes, from being a tool designed to pursue a variety of basic research questions to helping *ex situ* preservation of genetic diversity (*Engelmann, 1991*; *Gosal & Kang, 2012*). The most successful example of tissue culture in commercial and conservation applications is micropropagation: the propagation of plants from small parts under *in vitro* conditions. The success of micropropagation in tissue culture is due to ease of having multiple genetic clones from different geographic locations, thus lowering the risk of loss of such genotypes (*Kumar & Reddy, 2011*; *Rajasekharan & Sahijram, 2015*).

Despite the great advantages of micropropagation for *in vitro* conservation of plant species, different factors can compromise its success, such as culture medium composition, environmental conditions and genotype, among others (*Li et al., 2002*; *Tyagi et al., 2004*; *Kumar & Reddy, 2010*). In fact, it has been shown that different cultivars of the same species can have different *in vitro* performance or success (*Gubis et al., 2003*; *Pathi & Tuteja, 2013*). This reveals the sensitivity of *in vitro* culture to even small genetic variation. In the past few decades, the use of Genetically Modified Organisms (GMO) has become extensive (*ISAAA, 2017*) and a source of new genetic variation even in countries that are considered centers of origin of important crops (*Lu, 2008*). In some cases, the release of GMOs in areas with CWR or with other crops and weeds, has caused gene flow events across populations of different economically important cultivars, such as maize, cotton, papaya, bent grass, alfalfa and canola (*Quist & Chapela, 2001*; *Warwick et al., 2008*; *Piñeyro Nelson et al., 2009*; *Wegier et al., 2011*; *Greene et al., 2015*; *Manshardt et al., 2016*). Thus, given the extensive use of GMO technologies in economically important cultivars, it becomes relevant to analyze all evidence related to the effects of this introduced variation on the *in vitro* culture germplasm conservation efforts.

Mexico is the center of origin for cotton (*Gossypium hirsutum* L.) (*Ulloa et al., 2006*; *Burgeff et al., 2014*), and its metapopulations have been found on the coasts of the country, while extensive cultivars can be observed in the northern states, and backyard/home garden plants and native varieties have been reported in the southeastern states (*Velázquez-López et al., 2018*). Previous studies have described the genetic diversity of the Mexican metapopulations, including the presence of transgenes in some of them (*Wegier et al., 2011*), suggesting gene flow associated with specific transformation events (i.e., transgene introduction) from extensive cultivars to metapopulations. *Ellstrand (2018)* has posed the hypothesis that the majority of Mexican cotton metapopulations do not correspond with wild relatives of cotton (truly wild), but are instead a mix of escaped cultivar individuals that have evolved in wild conditions (weedy-wild). Nonetheless, even if the cotton metapopulations are weedy-wild relatives, they are part of the primary genetic pool (*Heywood et al., 2007*) and are thus of conservation interest due to their genetic diversity (*Ellstrand, 2018*). Phenotypic consequences of genetic flow (including transgene flow) into wild and domesticated lines in other species have been suggested in previous studies (*Hernández-Terán et al., 2017*); therefore, *in vitro* culture performance could also be affected, in principle, by genetic modification. This could have important consequences for the success of germplasm conservation strategies.

In the present study, and given the genetic diversity of *G. hirsutum* in Mexico, we analyzed experimental evidence of the effects of transgene presence on the *in vitro* performance of representative population clones of *G. hirsutum* diversity, and reflect on the implications of such effects for *ex situ* genetic conservation. For these reasons, and given that transgene presence in cotton metapopulations (hereafter wild germplasm) can be directly attributed to gene flow from domesticated populations, we included individuals from domesticated populations in our comparisons in order to differentiate the effect of domestication traits dragged into the wild germplasm pool via gene flow from the mere presence of transgenes. Thus, we hypothesize that (i) given that transgenes are directed to specific traits (e.g., defense and herbicide tolerance), which are not related to *in vitro* performance, we will not find differences in such performance between populations with and without transgenes, and (ii) the only differences to be found in the *in vitro* performance will be those associated with the domestication process, between wild and domesticated populations.

MATERIAL AND METHODS

Experimental design

To evaluate the potential effects of transgene presence on the *in vitro* culture performance of wild cotton plants and its consequences for germplasm conservation efforts, we conducted a systematic analysis of the performance of specific traits of an *in vitro* germplasm collection of cotton plants. We included a representative sample of the genetic diversity of wild population plants with (W_T) and without (W) transgenes (i.e., no isogenic lines), to test the effect of transgenes on the *in vitro* performance of metapopulation variants. Given the interest of this study for conservation strategies, we intentionally look for diversity of the genetic background of the analyzed clones, in other words population level diversity. In

addition, and as a preliminary attempt to distinguish the effects attributable to transgenes from the effects attributable to flow from domesticated or cultivar populations into wild populations, we included domesticated plants with (D_T) and without (D) transgenes in the experiment and analyses.

Germplasm collection

In order to have a germplasm collection that is representative of the genetic diversity of wild cotton populations in Mexico, we collected seeds from individual plants in populations spanning its natural distribution or in metapopulations (*Wegier et al., 2011*). Ten seeds of each individual plant in the collection were germinated in prepared substrate (Peat Moss, agrolite, vermiculite (3:1:1)) and 50 g of slow-release Osmocote fertilizer (14N-14P-14K, [Scott's, Marysville, Ohio]), in a greenhouse under controlled conditions (25 ± 5 °C). Once the seedlings emerged, the apex and the first three axillary buds were explanted to start the *in vitro* culture. We also included germplasm of domesticated plant individuals from farmer's markets in Mexico City as representatives of the domesticated cotton populations.

In vitro culture establishment and propagation

Establishment

All the experimental procedures were done under the license of the Servicio Nacional de Inocuidad y Calidad Agroalimentaria (SENASICA) trough the Aviso de Utilización Confinada de OGM (folio: 007_2016). Once disinfected (Appendix S1), the axillary buds were transferred to culture tubes containing 6 ml of MS basal medium (*Murashige & Skoog, 1962*). Each tube was sealed with Parafilm M (Bemis, USA) to prevent contamination. The culture tubes were incubated in a growth room at 24 °C for a 12-hours photoperiod.

Propagation

Propagation was initiated with individual explants that reached 8 cm height or the height of the culture tube, or when the initial culture exhausted the culture medium. Propagation consisted in removing the explants from the culture tubes, cutting each of the new axillary buds and planting them in a culture tube with fresh medium. The process was performed under sterile conditions in a laminar-flow hood (ThermoFisher, Massachusetts, USA). For more information, see Appendix S1.

Detection of transgenes in the germplasm collection

To characterize the populations under evaluation (i.e., Wild (W) and Domesticated (D)), we looked for two constructions of lepidopteran resistance and one of herbicide tolerance (*Cry1Ab/Ac, Cry2Ab* and CP4EPSPS) in all individuals of the collection. This allowed us to detect 23 of the 33 transgenic cotton events released in Mexico (*ISAAA, 2018*). For the wild cotton populations (W and W_T) we carried out two independent tests to verify the presence of the genetic events: enzyme-linked immunoabsorbent assay (ELISA), and sequencing of Polymerase Chain Reaction (PCR) products. For the domesticated cotton populations (D and D_T), transgenic events were verified only with a PCR-sequencing assay. The ELISA tests were performed in duplicate using the following kits: Bt-Cry1Ab/1Ac ELISA Kit, Bt-Cry2A ELISA Kit, and Roundup Ready ELISA Kit (Agdia, Elkhart, Indiana, USA). The results were

read in a MultiskanFC Microplate Photometer (ThermoFisher Scientific, Massachusetts, USA). We considered a sample to be positive only when its absorbance was equal to or above three standard deviations from the average intensity of all negative controls and blank samples. In all ELISA plates a blank sample (extraction buffer), a negative, and a positive control provided in each detection kit were included. ELISA results are available as supplementary material (ELISA_results.xslx).

For the PCR assays, DNA extraction was performed in duplicate for each individual, following the DNA Miniprep CTAB method reported in *Wegier et al. (2011)*. The quality and concentration of the DNA were analyzed in a NanoDrop 2000 (ThermoFisher Scientific, Massachusetts, USA). The PCR assay was performed with the primers Cry1Ab/Ac (F 5′ACCGGTTACACTCCCATCGA 3′, R 5′CAGCACCTGGCACGAACT 3′), Cry2Ab (F 5′CAGCGGCGCCAACTCTACG 3′, R 5′TGAACGGCGATGCACCAATGTC 3′), and CP4EPSPS (F 5′GCATGCTTCACGGTGCAA 3′, R 5′TGAAGGACCGGTGGGAGAT 3′) from Eurofins Scientific (Brussels, Belgium). The assay was carried out according to the references provided in Appendix S3. Subsequently, the amplicons result of the PCR assay were verified by Sanger sequencing. The sequencing was done in the Laboratorio de Secuenciación Genómica de la Biodiversidad y de la Salud in the Instituto de Biología, UNAM. Raw sequences are available in GenBank platform (accession number MK089921 to MK089930; Appendix S5).

Data collection

To evaluate the potential consequences of transgene presence for the *in vitro* performance of cotton populations, we measured different traits of individuals in our germplasm collection. All data analyzed is included as supplementary material (Supplementary_dataset.xlsx).

During the establishment of the *in vitro* germplasm collection we documented differences in propagation success in a period of two years that included a total of 4,377 axillary buds (corresponding to 74 individual original plants, 27 with transgenes and 47 without transgenes). From this original sample, we randomly selected 20 individuals (five wild with transgenes (W_T), five wild without transgenes (W), five domesticated without transgenes (D) and five domesticated with transgenes (D_T)), and 15 replicates per individual ($N = 300$) to evaluate *in vitro* performance of four phenotypic traits (leaf rate, height rate, microbial growth, and root development). This collection was intended to be a fair representation of the wild cotton metapopulations genetic backgrounds diversity, since it includes five out of the eight populations reported in Mexico (*Wegier et al., 2011*) plus ten individuals from the domesticated genetic background. Data for these traits were collected weekly during a four-month period. As mentioned above, all the experiments were conducted in a growth room at 24 °C under a 12-hours photoperiod. A detailed scheme of the experimental design is available in the Appendix S2.

Specifics for the evaluated traits are:

(i) **propagation rate**, calculated as the number of buds derived from a single individual every two weeks during two years of continual propagation, or the slope of the linear regression model using the *lm* function in *R* (no. buds *Vs.* time) (W_T $N = 1800$, W $N = 2577$, D $N = 490$, D_T $N = 633$);

(ii) **leaf rate**, or leaf production rate, calculated as the number of leaves derived from a single individual every week during four months of *in vitro* culture, or the slope of the linear regression model using the *lm* function in *R* (no. leaves *Vs.* time) (W_T $N = 75$, W $N = 75$, D $N = 75$, $D_T = 75$);

(iii) **height rate**, or height increase rate, calculated as the height (cm) of a single individual measured every week during four months of *in vitro* culture or the slope of the linear regression model using the *lm* function in *R* (cm *Vs.* time) (W_T $N = 75$, W $N = 75$, D $N = 75$, $D_T = 75$);

(iv) **microbial growth**, measured as observable growth of either bacterial or fungal organisms associated to plant tissue, potentially attributable to possible endophyte overgrowth (W_T $N = 75$, W $N = 75$, D $N = 75$, $D_T = 75$). In accordance with *Quambusch & Winkelmann (2018)*, we only considered as possible endophytes those microorganisms growing directly on the explant, not in the culture media;

(v) **root development**, determined by the average number of days that it took for the roots to develop after *in vitro* establishment, multiplied by the number of individuals that developed roots, and divided by the total number of analyzed individuals (W_T $N = 75$, W $N = 75$, D $N = 75$, $D_T = 75$).

No transformation of the dataset was carried out for further analysis.

Data analysis

To determine if there are statistical differences among experimental groups for all the traits simultaneously, we carry out a Permutational Multivariate Analysis of Variance (PERMANOVA) (*Legendre & Anderson, 1999*) based on 1,000 permutations using *adonis* function in vegan *R* package (*Oksanen et al., 2018*). As a post-hoc test we carry out a Wilcoxon rank sums test in order to distinguish differences in the individual traits. This statistical approach was performed based on *Rebollar et al. (2017)*. In addition, to evaluate the potential differences between the analyzed populations, we conducted a Non-Metric Multidimensional Scaling (NMDS) with Manhattan distance in the *vegan R* package (*Oksanen et al., 2018*). We selected the NMDS due to its flexibility, which allowed us to use different types of variables and make few assumptions about the nature of the data (*Legendre & Legendre, 2012*). Given that the number of entries for "propagation rate" was higher than for the rest of the evaluated traits, we subsampled these entries with the "sample" function in *R*. To evaluate if the subsample dataset was representative of the full dataset, we conducted a paired sample *T*-test. All the analyses were conducted using *R* software (version 2.4-6) (*R Core Team, 2013*).

RESULTS

In vitro culture performance is significantly different between W and W_T populations

The analysis for the *in vitro* culture performance traits in wild populations with (W_T) and without (W) transgenes shows statistically significant differences between populations for all traits (PERMANOVA, $F = 7.81$, $p = 0.0009$) and for three out of the five individual traits according to the Wilcoxon test ("height rate" $p = 4.95\text{e}{-12}$; "microbial growth"

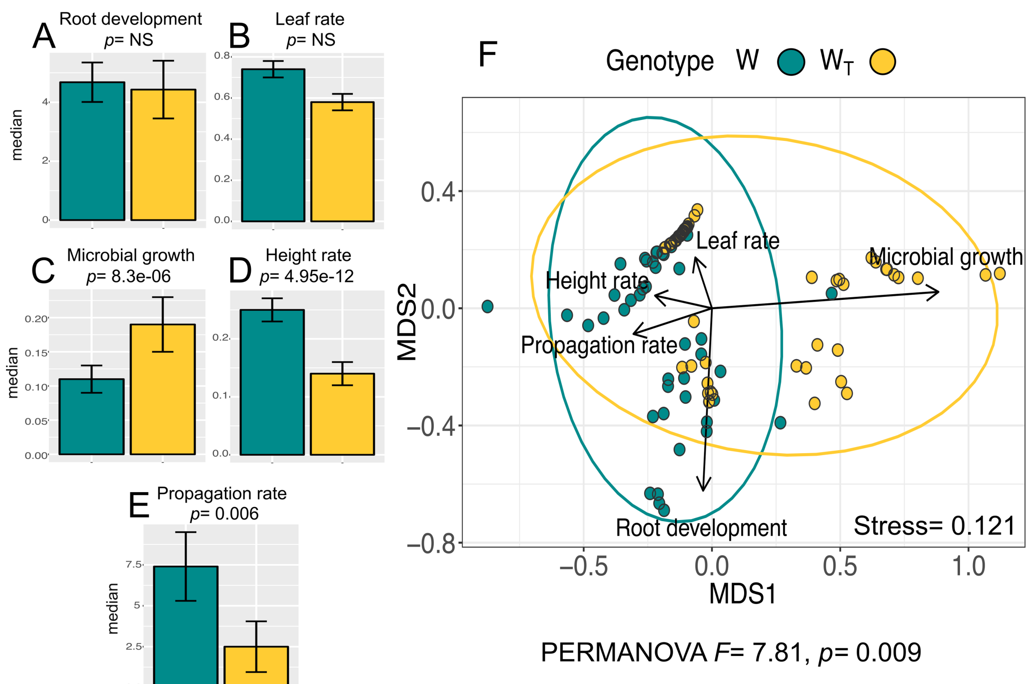

Figure 1 ***In vitro* culture performance traits of W and W_T populations.** W, wild populations without transgenes, and W_T, wild populations with transgenes. (A–E) median and standard error of all analyzed traits in both populations. *P* values were obtained through Wilcoxon test. (F) Non-Metric Multidimensional Scaling (NMDS) that include all the analyzed traits in the two populations. The ellipses represent 95% confidence interval around the centroids.

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$p = 8.3e-06$; "propagation rate"; $p = 0.006$). Figure 1A–1E shows the values for all analyzed traits per population. In particular, we want to emphasize that "height rate" as an *in vitro* performance trait had the largest difference between W and W_T populations.

Multivariate analysis of *in vitro* performance traits (NMDS) in W and W_T populations (Fig. 1F) shows different phenotypic variations attributable to each population (W and W_T). "Propagation rate" is a trait positively related to W population; in contrast, "microbial growth" is positively related to W_T population.

In vitro culture performance differs between W and D populations

The analysis for the *in vitro* culture performance traits in wild (W) and domesticated (D) populations does show statistically significant differences between populations for all traits (PERMANOVA, $F = 9.43$, $p = 0.0009$), and for four out of the five individual traits: "leaf rate" (Wilcoxon, $p = 9.74e-05$), "propagation rate" (Wilcoxon, $p = 0.002$), "root development" (Wilcoxon $p = 0.001$), and "height rate" (Wilcoxon $p = 2.52e-11$) (Figs. 2A–2E).

Moreover, although the graphic representation of multivariate analysis of *in vitro* performance traits (NMDS) in W and D populations shows overlapping of the ellipses representing each population, the statistical analysis of multivariate differences (PERMANOVA) in both populations is statistically significant (Fig. 2F).

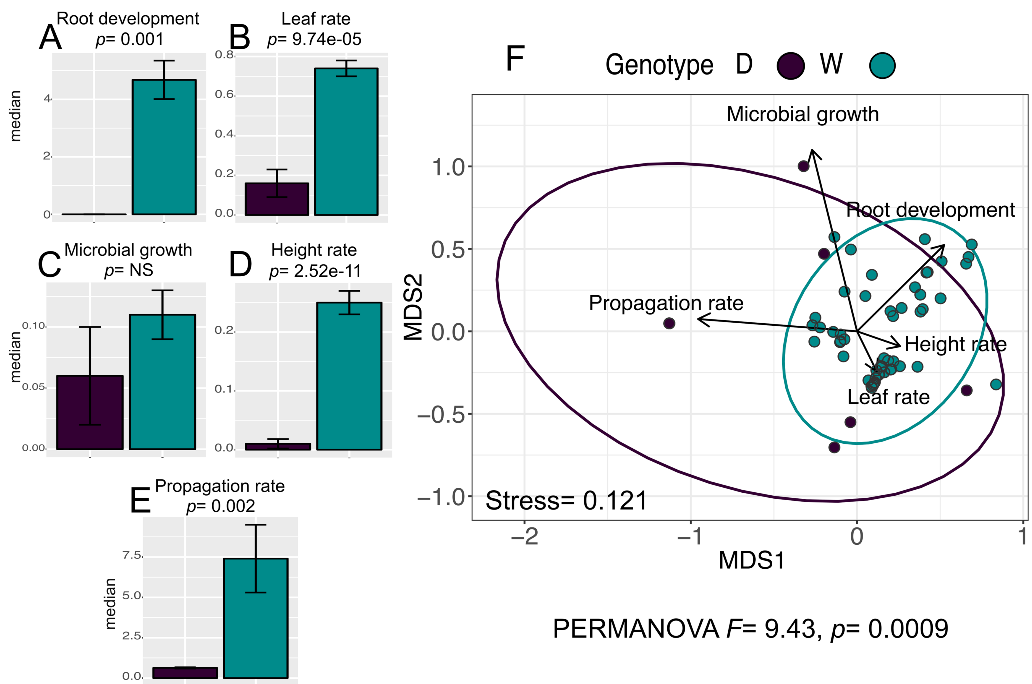

Figure 2 ***In vitro* culture performance traits of W and D populations.** D, domesticated populations without transgenes, and W, wild populations without transgenes. (A–E) median and standard error of all analyzed traits in both populations. *P* values were obtained through Wilcoxon test. (F) Non-Metric Multi-dimensional Scaling that include all the analyzed traits in the two populations. The ellipses represent 95% confidence interval around the centroids.

Full-size DOI: 10.7717/peerj.7017/fig-2

In vitro culture performance differs between W_T and D_T populations and between D and D_T populations

The analysis for *in vitro* culture performance traits in wild (W_T) and domesticated (D_T) populations with transgenes shows statistically significant differences between populations in four out of the five *in vitro* performance traits (Wilcoxon "height rate" $p = 0.008$; "leaf rate" $p = 4.47e{-}05$; "propagation rate" $p = 0.02$; "root development" $p = 0.02$) (PERMANOVA, $F = 5.62$, $p = 0.002$). Figures 3A–3E shows the values for all the analyzed traits per population. In particular, we want to emphasize that although W_T has higher values for "root development", "leaf rate" and "height rate" traits, D_T population has higher "propagation rate".

In the case of the analysis of domesticated populations with (D_T) and without (D) transgenes, we also found statistically significant differences in three out of the five analyzed traits (Wilcoxon "microbial growth" $p = 0.001$; "propagation rate" $p = 0.03$; "root development" $p = 0.04$) (PERMANOVA, $F = 3.86$, $p = 0.0008$) (Figs. 3G–3K), showing that $D_T$in general has a better *in vitro* performance.

The multivariate analysis of *in vitro* performance traits (NMDS) in W_T and D_T populations (Fig. 3F) shows different phenotypic variations attributable to each population (W_T and D_T), which coincides with the same analysis for W and D populations (with no

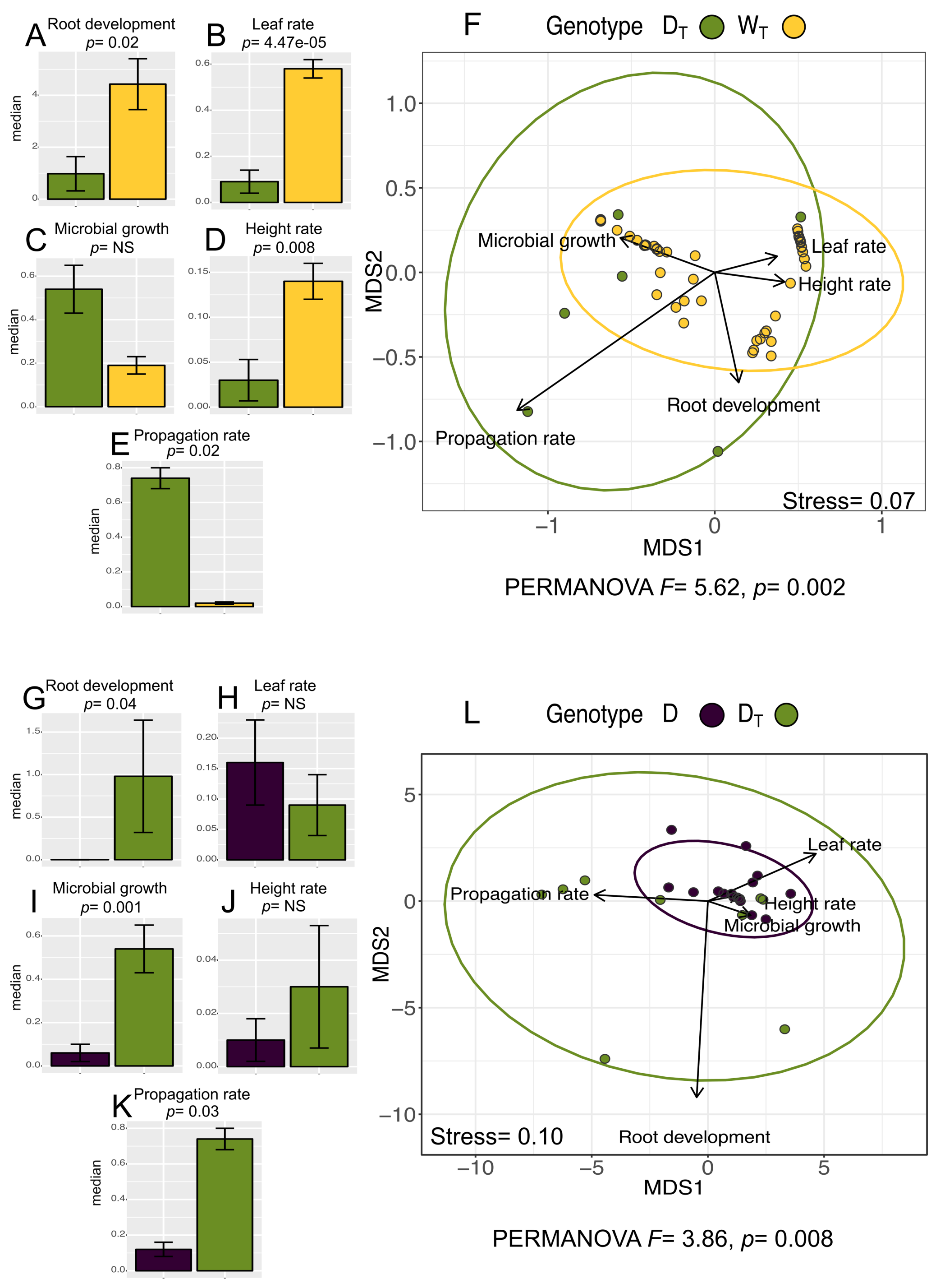

Figure 3 ***In vitro* culture performance traits of D_T and W_T and between D and D_T populations.** In genotype, D_T, domesticated populations with transgenes, W_T, wild populations with transgenes, D: domesticated populations without transgenes. (A–E) (G–K) median and standard error of all analyzed traits in both populations. *P* values were obtained through Wilcoxon test. (F) (L) Non-Metric Multidimensional Scaling that include all the analyzed traits in the analyzed populations. The ellipses represent 95% confidence interval around the centroids.

Full-size DOI: 10.7717/peerj.7017/fig-3

transgenes identified; Fig. 2F). Moreover, "propagation rate" is positively related to D_T population, while the rest of the traits seems positively related to W_T population.

In the analysis of D and $D_T$populations, the NMDS (Fig. 3L) shows overlapping of the ellipses representing the data set distribution of both populations, despite the significance of the statistical analysis (PERMANOVA).

To look at the full data set (W, W_T, D, D_T), and beyond the pairwise hypotheses, we carried out the NMDS and PERMANOVA analyses. The results of the full data set analyses are coherent with the pairwise comparisons, which supports the above mentioned observations (Fig. S5).

DISCUSSION

Conservation of the genetic and phenotypic diversity in the CWR has been acknowledged as key in the preservation of diverse gene pools to secure genetic alternatives for future decisions and interventions regarding crop production. Of the different conservation strategies that exist, *in vitro* gene banks represent a robust approach to preserve genetic diversity without introducing unintended variations into the genetic pool (*Engelmann, 1991*; *Gosal & Kang, 2012*). In recent decades, the use of GMOs has become extensive (*ISAAA, 2017*), and a source of new genetic variation, even in centers of origin of important crops (*Lu, 2008*). This makes it important to evaluate evidence of the effects, if there are any, of this introduced variation on *in vitro* culture germplasm conservation efforts.

In order to analyze evidence of potential effects of transgene presence in cotton metapopulations, we compared *in vitro* performance traits in metapopulations with (W_T) and without (W) transgenes. We found significant differences in *in vitro* culture performance between W and W_T populations for three out of the five analyzed traits (Figs. 1A–1E). Previous studies with different crop populations have shown that even small genotypic changes can have major impact in phenotypes and fitness traits both in field experimental settings (*Hernández-Terán et al., 2017*) and in *in vitro* culture (*Gandonou et al., 2005*; *Landi & Mezzetti, 2006*; *Kumar & Reddy, 2011*). Thus, it can be argued that the observed differences in *in vitro* culture performance could be the result of natural genetic variation within and among populations, however when we look at potential differences among W metapopulations, we found no statistically significant differences (Appendix S4). Nonetheless, the observed differences between W_T and W populations could be attributed, as in other studies, to pleiotropic effects, where certain phenotypic traits may be linked to and affected by the genetic modification of another trait (*Filipecki & Malepszy, 2006*). In this sense, some studies have shown that genetic modification can alter metabolic pathways due to position effects of transgenes or somaclonal variation during tissue culture (*Agapito-Tenfen et al., 2013*; *Mesnage et al., 2016*). In the specific case of cotton (*Wang et al., 2015*), some authors have found overexpression of metabolites related to energy metabolism pathways that could indicate an increased demand for energy, and concomitant changes in resource allocation and development. Pleiotropic effects, have also been attributed to bottlenecks, selective sweeps, phenotypic plasticity or gene x environment (GxE) interactions (*Remington et al., 2001*; *Pozzi et al., 2004*; *Gunasekera et*

al., 2006; *Doust et al., 2014*). In addition, ecological costs in different plant species have been associated with the expression of transgenes (*Chen et al., 2006*). Specifically, some studies show that the physiological production of transgene toxins (e.g., *Cry* proteins) is extremely costly, limiting the energy destined for growth and reproduction. This trade-off caused by the genetic modification has been found in *Brassica* (*Snow, Andersen & Jorgensen, 1999*) and *Arabidopsis* (*Bergelson et al., 1996*). Although in our study we cannot attribute these performance differences only to the presence of transgenes (i.e., no strict control of genotypes), we can say that all individuals identified as W_T were positive for the expression of transgene proteins in the ELISA approach, which is suggestive of a potential cost that gets reflected in the *in vitro* performance of W_T populations. In general, *in vitro* performance differences between W and W_T could be explained, at least with the information here collected, by ecological costs associated to the expression of transgenes and by potential pleiotropic and GxE interactions associated with small genetic differences.

In order to isolate the effect of domestication from the effect of transgenes on the *in vitro* performance of cotton populations, we compared the *in vitro* performance of both wild metapopulations and domesticated populations with and without transgenes. In the case of comparisons of populations without transgenes (W-D) we found significant differences in four out of the five analyzed traits (Figs. 2A–2E). Such phenotypic differentiation, regardless of substantial evolutionary divergence and genetic differentiation (*Fang et al., 2017*), is somehow unexpected, since differentiation between these populations is the result of a selective process focused on traits that are not related to *in vitro* performance, such as length, size and color of the fiber, loss of seed dispersal and germination speed (*Lubbers & Chee, 2009*; *Gross & Strasburg, 2010*; *Velázquez-López et al., 2018*). Nonetheless, strong selective forces associated with domestication and divergence times between populations are together of sufficient strength to show phenotypic differentiation even in an environment to which both W and D populations were naïve to (*in vitro* conditions).

Regarding the comparison of wild (W_T) and domesticated (D_T) populations with transgenes, we found significant differences in four out of five analyzed traits, with W_T populations being better performers than D_T populations in the *in vitro* culture in general (Fig. 3), with the important exception of one trait, "propagation rate". This better performance of D_T populations for "propagation rate" could be the result of a history of selection in *in vitro* culture, which is part of the conventional process of genetic engineering, through which transgenes are introduced in the domesticated plants' genetic backgrounds (*Hooykaas & Schilperoort, 1992*). This suggests that since GMOs have previously gone through an *in vitro* process, it could be possible that GM plants that have been selected for culture are better adapted to these conditions. In contrast, for the rest of traits, W_T populations perform better (higher "height rate", "leaf rate", and "root development"), to which a potential mechanistic explanations or hypotheses are hard to articulate. One possibility is that, given the reduced genetic variation of D populations in comparison with W populations, the general performance of D populations might be expected to be worse in environmentally astringent conditions (*Flint-garcia, 2013*; *Lu, 2013*), such as *in vitro* culture.

Regardless of the trait performance direction of populations (W_T and D_T), we can argue that given the existent differences between D and W genotypes without transgenes (see above and *Velázquez-López et al., 2018*), it is expected that additional genetic changes (due to gene insertion) could contribute to increased phenotypic differentiation. Nonetheless, given that we did not determine the exact location of the inserted transgenes in the genome, it is not possible to give a mechanistic explanation to the specific trait differences. Overall, we can conclude that our results suggest that the presence of transgenes, originally associated with domesticated populations, has a significant impact on the *in vitro* performance of the genotypes, regardless of their wild or domesticated origin.

Implications of transgene presence for *In vitro* wild germplasm conservation

One of the best *ex situ* conservation strategies for wild germplasm are *in vitro* banks (*Gosal & Kang, 2012*). *In vitro* conservation success depends on efficient and reliable micropropagation or *in vitro* performance of the species of interest (*Mycock, Blakeway & Watt, 2004*). Despite the reality of crop intensification, including genetic engineering, the possible consequences of the presence of transgenes for the *in vitro* performance of populations are poorly documented. In this study, we present results that suggest detrimental consequences for the *in vitro* culture performance of wild cotton populations in the presence of transgenes, which calls for monitoring transgenes in the plants to be micropropagated for conservation or future genetic improvement, as has been suggested by other authors, as conservation strategies and protocols (*Bhatia, 2015*). Moreover, it is worth noting that in the present study, with a minimal investment of three primer sets for transgene detection, we were able to identify 23 out of 33 transformation events reported for cotton populations in Mexico (*ISAAA, 2018*). As it stands, our results provide experimental evidence to support the implementation of transgene screening of plants to reduce time and economic costs in *in vitro* establishment, helping the overarching goal of germplasm conservation for future adaptation.

In current scenarios of global change, uncertain future conditions pose the major challenge of securing resources for future adaptations (*Wise et al., 2014*). In this sense, it is of utmost importance to preserve options for future decisions and to guarantee the right to biodiversity and cultural identity for future generations, which includes genetic and phenotypic options (*Rockstrom et al., 2014*). Crop biodiversity preservation is, in other words, part of our life insurance for future adaptation in a changing planet. In this sense, future work on conservation strategies and policies should put effort in expanding the knowledge about the consequences of transgene presence (*Lu, 2013*) beyond the immediate gene pool of wild populations. This means extending the efforts breadth towards other interfertile species; in other words, to the genetic primary pool.

CONCLUSIONS

The results presented show how transgene presence in CWR cotton populations has negative consequences for their *in vitro* culture performance. In particular, reviewing our hypotheses, we found that (1) *in vitro* culture performance is significantly different between

W and W_T populations, and (2) *in vitro* culture performance is different between wild and domesticated populations regardless of transgene presence. Overall, our results suggest that the presence of transgenes in wild populations imposes a cost (e.g., metabolic cost of maintaining the expression of toxins) that is reflected in their *in vitro* performance and that could endanger the success of germplasm conservation efforts. Further studies controlling for specific genotypes and specific transgene constructions would help to better disentangle the costs associated with specific genomic contexts and genetic modifications to improve genetic screenings for *in vitro* banks.

ACKNOWLEDGEMENTS

The authors would like to thank Morena Avitia, Luis Barba Escoto, and Joel Reyna for technical assistance. The authors acknowledge Dra. Florencia García-Campusano for her support in the laboratory work, and to the Laboratorio de Biotecnología CENID-COMEF, INIFAP.

ADDITIONAL INFORMATION AND DECLARATIONS

Funding

This work was financially supported by the project "Program for the conservation of wild populations of Gossypium hirsutum in Mexico" DGAP003/WN003/18 Dirección General del Sector Primario y Recursos Naturales Renovables (DGSPRNR) that belongs to the SEMARNAT and CONABIO and the project UNAM-PAPIIT No. IN214719. Alejandra Hernández-Terán is a doctoral student from Programa de Doctorado en Ciencias Biomédicas, Universidad Nacional Autónoma de México (UNAM) and was supported by CONACYT (scholarship no. 66025). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Grant Disclosures

The following grant information was disclosed by the authors:
Program for the conservation of wild populations of Gossypium hirsutum in Mexico: DGAP003/WN003/18.
UNAM-PAPIIT: IN214719.
CONACYT: 66025.

Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Alejandra Hernández-Terán conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.
- Ana Wegier conceived and designed the experiments, contributed reagents/materials/-analysis tools, authored or reviewed drafts of the paper, approved the final draft.

- Mariana Benítez and Rafael Lira conceived and designed the experiments, authored or reviewed drafts of the paper, approved the final draft.
- Tania Gabriela Sosa Fuentes performed the experiments, approved the final draft.
- Ana E. Escalante conceived and designed the experiments, analyzed the data, contributed reagents/materials/analysis tools, authored or reviewed drafts of the paper, approved the final draft.

DNA Deposition

The following information was supplied regarding the deposition of DNA sequences:

Data is available at GenBank, accession number MK089921 to MK089930, and in the Appendix S5.

Data Availability

The following information was supplied regarding data availability:

The raw data measurements are available in the Supplemental Files.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.7017#supplemental-information.

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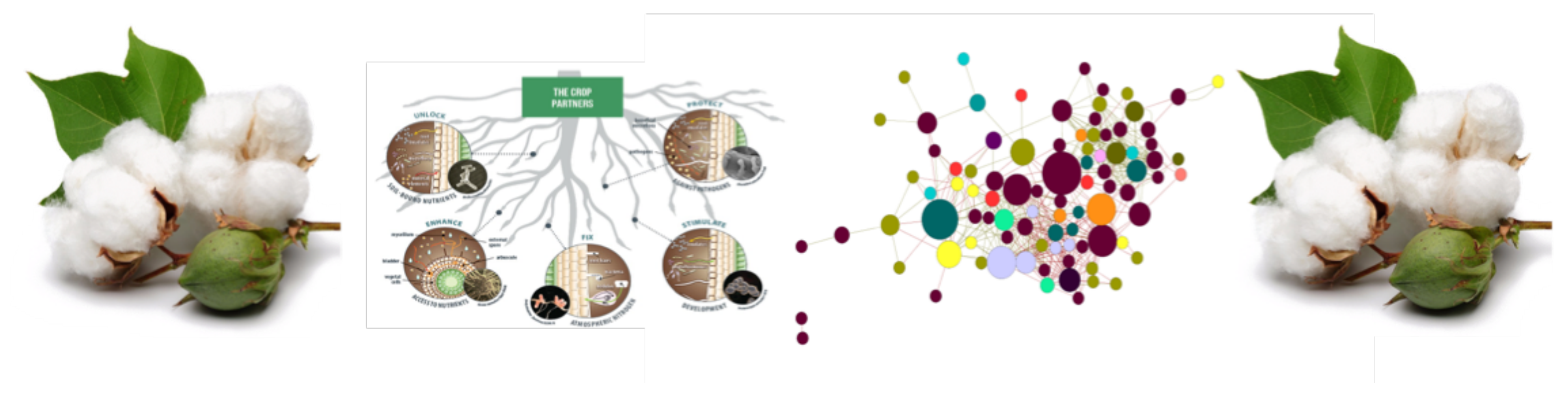
CAPÍTULO III

INTERACCIÓN PLANTA-MICROORGANISMO: IMPACTO DEL GENOTIPO HOSPEDERO EN EL ENSAMBLE DEL MICROBIOMA DE RAÍZ



PREFACIO

Al pensar en las consecuencias fenotípicas de la diversidad genética en un organismo, generalmente imaginamos caracteres morfológicos y cómo estos varían entre individuos genéticamente diversos. Sin embargo, el estudio de las consecuencias de la diversidad genética puede ser abordado desde una perspectiva más ecológica, estudiando por ejemplo cómo los organismos interactúan con otros. Y aunque las interacciones ecológicas no representan un carácter fenotípico específico en un organismo, pueden verse como un "fenotipo extendido" al influir directamente en la sobrevivencia y adaptación de los organismos al ambiente.

El estudio de las interacciones tiene una larga historia en ecología. Como se menciona en la introducción de esta tesis, muchas de las interacciones ecológicas son determinantes para la vida de los organismos y tienen efectos directos en la adecuación. A estos procesos se les ha reconocido recientemente como retroalimentaciones eco-evolutivas, y nos hablan de los efectos que tiene la ecología sobre la evolución y viceversa. Partiendo de esta teoría, consideramos que el estudio de las consecuencias fenotípicas de la diversidad genética tiene sentido si lo hacemos no solamente desde el estudio de caracteres morfológicos sino desde su efecto en las interacciones ecológicas.

En el caso de las plantas, una de las interacciones más interesantes pero también con mayor complejidad es la que ocurre en la zona donde interactúan las raíces de las plantas, el suelo y un gran número de microorganismos. Esta interacción, planta-rizósfera es determinante en la salud y crecimiento de las plantas, al otorgar una serie de beneficios intrínsecos. Es sabido que gran parte de

los beneficios rizósfera-planta son dependientes de la diversidad y composición de las comunidades bacterianas que allí habitan. Sin embargo, los mecanismos detrás del ensamble de las comunidades no se han logrado descifrar del todo. En este sentido, se sabe que a través de la producción diferencial de metabolitos secundarios en la raíz, el genotipo de la planta hospedera puede jugar un papel determinante en la relación planta-rizósfera.

En este capítulo quisimos explorar el efecto de la diversidad genética en la interacción planta-rizósfera. Nuevamente usando el algodón como modelo de estudio, y valiéndonos de la amplia diversidad genética y fenotípica de sus poblaciones silvestres, probamos la hipótesis de si es posible recuperar la diferenciación genética del hospedero en la estructura y diversidad de las comunidades microbianas de raíz. Utilizamos estrategias de jardín común y cultivo de tejidos para controlar variables ambientales y la diversidad genética individual y caracterizamos las comunidades microbianas asociadas a la raíz de cinco de las ocho metapoblaciones silvestres del algodón.

En resumen, encontramos que en efecto la presencia de la planta juega un papel determinante en la estructuración de las comunidades microbianas pero que además, estas son sensibles a la variación genética de las poblaciones, a nivel intra-especie. Este resultado nos permitió indagar en aquellos grupos específicos que resultaron ser más abundantes en la rizósfera de las distintas poblaciones, e hipotetizar sobre su función en este ambiente. Finalmente, abundamos en posibles mecanismos detrás de la especificidad del microbioma asociado a cada población de algodón en México, y discutimos sobre su importancia y relevancia en escenarios de conservación de diversidad genética de poblaciones silvestres.

Host genotype explains rhizospheric microbial community composition: the case of wild cotton metapopulations (*Gossypium hirsutum* L.) in Mexico

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Keywords: wild cotton relatives, plant-microbe interaction, host genetic diversity, rhizosphere microbiome.

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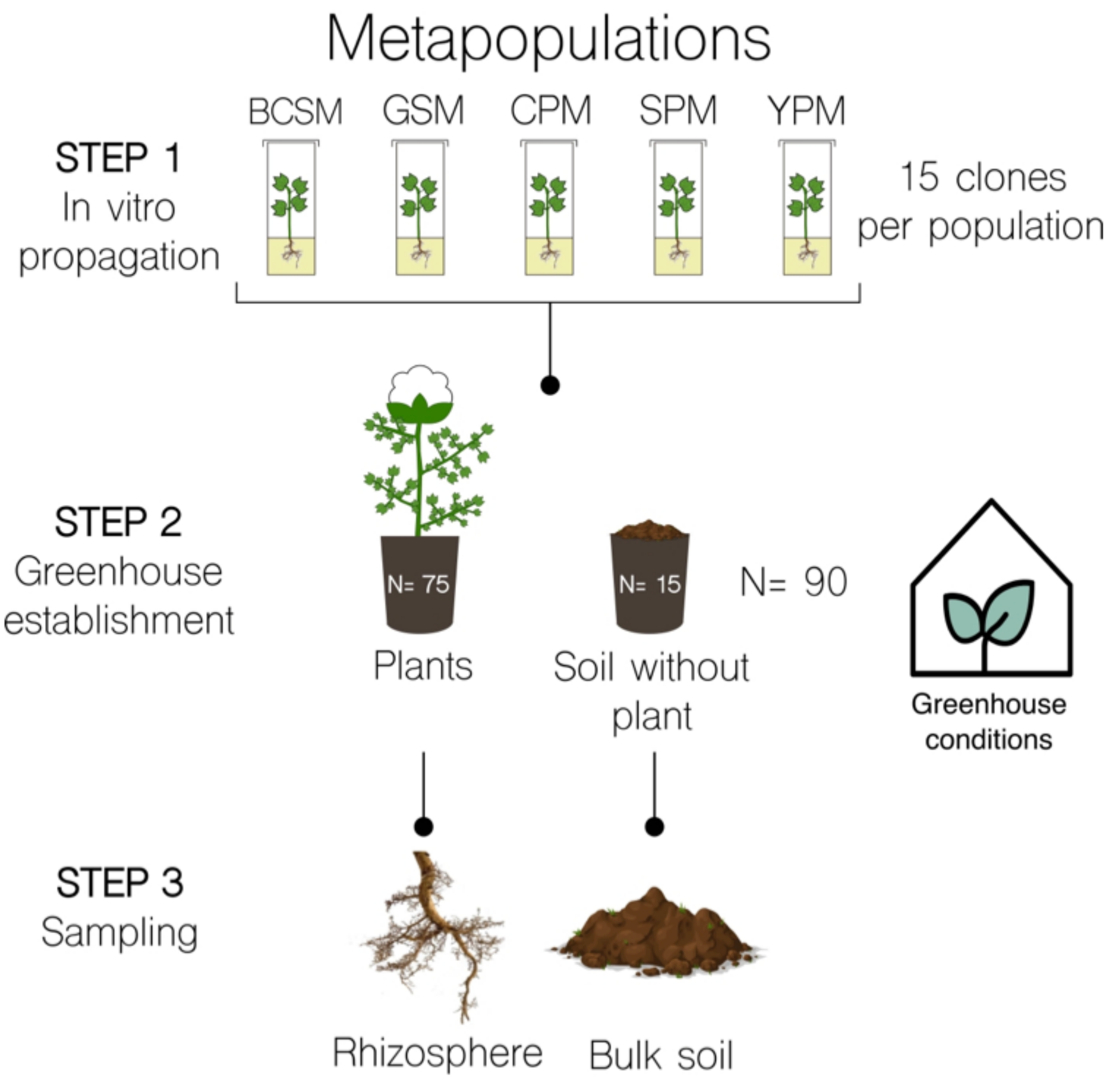
GRAPHICAL ABSTRACT



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ABSTRACT

The rhizosphere, and associated microbes, is an extremely variable environment that provides several benefits to the plant host as a strong determinant for its health, growth and productivity. Nonetheless, the factors behind the assembly of the microbial communities associated with the rhizosphere such as the role of plant genotypes is poorly documented and understood. In the present study we aimed to test the role that intraspecific genetic variation has in the rhizospheric microbial community assemblages, using genetically distinct wild cotton metapopulations as a model of study. We followed a common garden experiment including five wild cotton populations, controlling for plant genotypes, environmental conditions and soil microbial community inoculum, in order to statistically test for differences in the microbial communities associated with genetic variation of the plant hosts. Microbial communities of the treatments were characterized by culture independent 16SrDNA amplicon sequencing with Illumina MiSeq platform. Two lines of evidence were analyzed, one at the level of microbial community diversity (alpha and beta), and another focused in diversity structure of such communities, determined by co-occurrence networks. Overall, the results provide evidence of the role of host genotype in selecting for different rhizospheric microbial community assemblages.

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49 # INTRODUCTION

50 Almost all-known organisms, from the simplest forms to humans, live in close association
51 with microorganisms. Microbial communities associated to host are known as microbiome
52 and it has been shown crucial for growth, immunology, homeostasis, and health of their host
53 (Berendsen, Pieterse and Bakker 2012; Theis *et al.* 2016; Mohajeri *et al.* 2018). It is known
54 that regardless of the host type, two major factors affect the microbial communities in the
55 microbiome: environment, and host-related factors (Haichar *et al.* 2008; Lakshmanan,
56 Selvaraj and Bais 2014). It has been proposed that host factors, specifically the genotype,
57 plays a crucial role in the assembly, diversity, and structure of the microbial communities.
58 Nonetheless, the mechanisms and scope of the host genotype influence on the microbial
59 communities assembly, dynamics, and functional consequences remain as open questions.
60 Thus, given that many of the featured microbe-to-host benefits are dependent on the diversity
61 structure of the microbial communities (Garbeva *et al.* 2008; Mendes *et al.* 2011) it becomes
62 relevant to study the factors affecting them.

63 One of the most diverse and dynamic host-associated microbial communities is in the
64 rhizosphere, defined as the interaction zone of plant roots, soil, and microorganisms (Hiltner
65 1904; Philippot *et al.* 2013). Interactions in the rhizosphere lead to many different types of
66 plant-microorganism symbiotic associations (Philippot *et al.* 2013), along with different
67 chemical feedbacks which strongly affect plant growth, health, development and, in plant
68 crops, productivity (Schnitzer *et al.* 2011; Wagg *et al.* 2011; Qiao *et al.* 2017). Like in other
69 microbiomes, plant genotype has been associated with the assembly and maintenance of the
70 rhizospheric microbial communities (Berendsen, Pieterse and Bakker 2012; Lakshmanan,
71 Selvaraj and Bais 2014). Previous studies have shown that plant species are able to shape

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72 their rhizosphere microbial communities creating a species-specific effect. It has been
73 proposed that this specific selection of the microbial communities is mediated by the
74 rhizodeposition process (Miethling *et al.* 2000; Garbeva *et al.* 2008; Weinert *et al.* 2011),
75 which is the release of carbon compounds from the roots to the soil (Uren 2000; Bais et al.
76 2006). The plant invests 24-30% of the total carbon fixed by photosynthesis in
77 rhizodeposition (Bais *et al.* 2006), and it is believed that the specific composition of these
78 exudates is dependent on the plant genotype (Chaparro, Badri and Vivanco 2014; Ofek *et*
79 *al.* 2014; Rovira 2015). The genotype effect on the composition of the exudates has been
80 well documented in studies looking at different plant species that exhibit variation not only
81 in the microbial communities of the rhizosphere but also in the composition of the root
82 exudates, variation that increases with phylogenetic distance (Wieland, Neumann and
83 Backhaus 2001; Bouffaud *et al.* 2014). Studies looking for host effects in individuals of the
84 same species have been performed in animal populations (e.g. gut and vaginal microbiome
85 in humans (Spor, Koren and Ley 2011; Goodrich *et al.* 2014)) suggesting that even intra-
86 species genetic variation could have consequences for the assembly of microbial
87 communities, including rhizospheric communities. Nonetheless, to date, there is little
88 information about the importance of plant intra-specific variation in the rhizospheric
89 community assembly, thus hindering our understanding of the mechanisms behind the
90 specificity of this association.

91

92 Cotton (*Gossypium hirsutum* L.) is an economically, and culturally important crop
93 that has its center of origin and diversity in Mexico (Ulloa *et al.* 2006; Burgeff *et al.* 2014),
94 where the wild relatives of the species can be found in the coasts. Wild cotton exhibits a
95 meta-population dynamic which is represented by eight populations that are geographically,

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ecologically, and genetically distinguishable (Wegier *et al.* 2011). In particular, the differentiation in the genetic structure among the populations is the result of microevolutionary and demographic processes that act in the populations (Hawkes 1977). Altogether, the fact that *G. hirsutum* have been originated in Mexico and the genetic and phenotypic diversity of its populations, makes the plant cotton a suitable model to study questions related to the impact of the genotype into the rhizosphere microbial communities from an intra-species perspective. Moreover, further understanding in the intraspecific structure of microbiomes may contribute to the assessment and design of conservation strategies of this valuable crop in its center of origin and diversity.

In the present study, we analyzed a representative sample of the cotton wild relatives in order to determine if the genetic differentiation of the populations is traceable into the rhizospheric microbial communities. For this, we used tissue culture techniques to control for the individual variation and obtain clonal replicates of each analyzed population. We grew the populations under controlled conditions in a common garden experimental setting, which included a common microbial community inoculum, allowing the plant genotypes to select for the microbial communities from the same pool of microbial diversity. We hypothesize that since genotype is a determinant factor in the assembly of the rhizosphere microbial communities, each cotton population will host specific diversity structure of microbial communities, with potentially important functional implications for plant growth and conservation of wild plant relatives.

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119 # MATERIAL AND METHODS

120

121 Experimental design

122 To evaluate the effect of the plant host genotype into the rhizosphere microbial communities,
123 we designed a common garden experiment. In the experiment, we compared wild cotton plant
124 individuals from different natural populations in Mexico. Specifically, for each population,
125 we included one plant individual and 15 clonal replicates from five of the eight wild cotton
126 populations to have a good representation of the genetic variation at the intra-species level
127 (N=75). To obtain the clonal replicates per plant individual we used tissue culture techniques
128 (Hernández-Terán *et al.* 2019). Negative controls of the experiment consisted of pots without
129 plant (N=15). All the samples contained a mix of sterilized substrate and a common microbial
130 community inoculum obtained from a coastal dune. Samples of the microbial communities
131 associated with the plant roots were taken at a single time point after nine months of the start
132 of the experiment, associated to fully developed cotton plants. In order to determine the
133 microbial communities diversity structure associated with the different plant genotypes, we
134 took samples of the rhizospheric soil (N=75), bulk soil of the negative controls (N=15), and
135 the microbial community inoculum (N=1) and extracted the DNA to sequence the 16S rRNA
136 gene.

137

138 Plant material

139 The wild cotton populations in Mexico live together with genetically modified (GM) cotton
140 crops and, due to gene flow events, some of the metapopulations show presence of transgenes
141 (Wegier *et al.* 2011). Since the aim of this study was to evaluate the effect of the natural

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142 genetic variation of the host into the rhizosphere microbial communities, we analyzed the
143 presence of transgenes in all wild cotton populations following the methods reported in
144 Hernández-Terán *et al*, 2019. Only five of the eight metapopulations (Wegier *et al.* 2011)
145 were free of the screened transgenes (*Cry1Ab/Ac, Cry2A* and CP4 EPSPS, for more
146 information see Hernández-Terán *et al*, 2019), and thus selected for the present study: Baja
147 California Sur (BCSM), Central Pacific (CPM), South Pacific (SPM), Yucatan Peninsula
148 (YPM), and Gulf South (GSM). From these five populations we collected and germinated
149 seeds according to the protocol reported in Hernández-Terán *et al*, 2019.
150

151 Tissue culture propagation
152 We included one plant individual and 15 clonal replicates from the five selected wild cotton
153 populations (N=75). Clonal replicates were obtained after seed germination, seedling
154 disinfection and establishment. Cuttings of the established seedlings were propagated using
155 tissue culture techniques until 15 clonal replicates per population. Clonal replicates were
156 incubated in a culture room under controlled conditions until roots were evident. All methods
157 related to tissue culture were done according to Hernández-Terán *et al*, 2019.
158

159 *In vitro*-to-soil establishment
160 Once all clonal replicates grew enough to develop roots, we proceeded with soil
161 establishment of plants. We used a sterilized mixture of Peat Moss, agrolite, vermiculite
162 (3:1:1) and 50 g of slow-release Osmocote fertilizer (14N-14P-14K, [Scott´s, Marysville,
163 Ohio]). Given that the substrate was previously sterilized, soil from a coastal dune in
164 Veracruz state was used as common microbial inoculum to all samples by adding 25g of soil
165 per kg of substrate (Robles and Barea 2004). The inoculum served as the pioneer microbial

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166 communities necessary to start the microbial community assembly. Since the *in vitro*-to-soil
167 transfer causes high stress levels in the plant, we standardized an acclimation process for all
168 individuals which is available at Supplementary Material (Figure S1).
169

170 Common garden conditions
171 The common garden experimental design was implemented in a Biosafety greenhouse
172 facility at the Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias
173 (INIFAP). This particular greenhouse facility is in compliance with international biosecurity
174 standards (Adair and Irwin 2001). The environmental conditions for this greenhouse were:
175 temperature at 30 +- 5ºC and automatized sprinkling for four times a week.
176

177 Sampling
178 Root microbial communities were sampled after nine months of soil establishment of plants,
179 which corresponds with the adult developmental stage of the species (Stewart *et al.* 2010).
180 For the rhizosphere samples, we extracted the plants from the pots, and manually shook the
181 roots to remove the excess of soil, subsequently, we sampled 25gr of the adhered soil. For
182 the control soil samples, we took 25gr of the soil contained in the control pots. Both sample
183 types were stored at -80ºC until DNA extraction. In total, we took samples of the rhizospheric
184 soil (N=75), bulk soil of the negative controls (N=15), and the original microbial community
185 inoculum (N=1) and extracted the DNA to sequence the 16S rRNA gene.
186

187 DNA extraction and 16S rDNA sequencing
188 Genomic DNA of each rhizosphere or control soil sample was extracted using PowerSoil
189 DNA Isolation Kit™ (MoBio Laboratories, Solana Beach CA, USA) according to

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manufacturer´s instructions with the following modifications: 0.5 g of sample instead of 0.25 g were used, incubation periods at 4ºC were increased from 5 to 20 minutes, and we added an incubation step at 55ºC for five minutes prior to DNA elution. Inoculum sample was extracted 5 times in order to obtain sequencing replicates. From the genomic DNA per sample, the V4 region of the 16S rRNA gene (515F/806R [Caporaso *et al.* 2011]) was amplified and sequenced using Illumina MiSeq instrument at Research and Testing Laboratory (Lubbock, TX, USA).

16S rRNA sequence data processing

Illumina raw sequences were processed with QIIME2 v. 2017.6.0 (Bolyen *et al.* 2019). Sequences demultiplex, denoising and quimera check (QC) filtering were done with the DADA2 pipeline (Callahan *et al.* 2016). Since the sequences presented low quality at the extremes, we removed 30 bp at the 5´extreme, and 40 bp at the 3´extreme of the forward sequences, and 40 bp at the 5´extreme and 30 bp at the 3´extreme of the reverse sequences. This edition process was done by modifying the parameters *trim_left_f, trun_len_f, trim_left_r,* and *trun_len_r*. All the subsequent steps were done with default arguments. Due to small number of sequences, we decided to remove two samples from two different populations (CPM – rep.1 with 6418 sequences and SPM – rep.1 with 8086 sequences). After this process, we kept 5,674,346 of total reads, and an average of 61,014 sequences for each sample. The amplicon sequence variants (ASVs) were aligned with maft (Katoh *et al.* 2002) and used to construct a phylogeny with fasttree2 (Price et al, 2010). Taxonomy for ASV was assigned with the sklearn-based classifier (Bokulich *et al.* 2018) using the Greengenes 13_8 database (McDonald *et al.* 2012). Rarefaction curves for all analyzed samples are available

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213 as supplementary material (Figure S2). The raw data (paired end files) were deposited in the
214 NCBI sequence read archive (SRA) with the accession number PRJNA590533.
215
216

217 **Diversity analyses**

218 In order to determine the influence of the plant host genotype into the structure and diversity
219 of the root microbiome, we carried out different analysis on the microbial communities
220 diversity based on 16S rDNA sequence data. Analyses were performed at two taxonomic
221 levels: phylum for alpha diversity and first descriptors of beta diversity, and family for
222 biomarkers (beta diversity) and diversity structure (networks) analyses.

223 - Alpha and beta diversity – As first descriptors of diversity and composition of the root
224 microbial communities of the five wild cotton populations we performed alpha and beta
225 diversity analysis. **Alpha diversity.** We calculated the Chao1 index using the function
226 *diversities* in the "microbiome" *R* package (Lahti and Shetty 2017) and ANOVA and post-
227 hoc Tukey tests were conducted in "vegan" *R* package (Oksanen *et al.* 2018). **Beta diversity.**
228 We carried out a Non-Metric Multidimensional Scaling (NMDS) with Bray-Curtis distance
229 at phylum level in the "phyloseq" *R* package (McMurdie and Holmes 2013). To investigate
230 statistical differences across samples we carried out paired Wilcoxon tests of the NMDS
231 scores between all sample types (i.e. controls and plant treatments). In order to detect
232 differentially abundant groups in the populations we performed a Linear Discriminant
233 Analysis (LDA) effect size (LefSe) at phylum level with the web-based tool
234 "MicrobiomeAnalyst" (Dhariwal *et al.* 2017). Subsequently, and in order to analyze more
235 deeply the information of the LefSe, we extracted the information and performed a heatmap
236 of the groups obtained in the LefSe but at the family level. The heatmap was constructed only

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with the statistically significant groups ($p < 0.01$) using the "heatmaply" *R* package (Galili *et al.* 2018).

Diversity structure - co-occurrence networks

In order to determine if the co-occurrence patterns of the microbial groups, as a proxy to community structure, differ between populations we inferred networks in the software CoNet (version 1.1.1) (Faust and Raes 2016) by using ASV tables at family level. We constructed one network per population (N replicates= 15, except for CPM and SPM populations with N= 14). The co-occurrences were tested statistically with Pearson, Spearman, and Kendall tests, considering only correlations >0.85 and with p <0.01. Edges were established only when the co-occurrences/exclusions were supported by the three statistical methods. Besides, we applied a multi-test correction with the Benjamini-Hochberg procedure (Benjamini and Hochberg 1995).

To further our diversity structure characterization, we calculated standard network indexes and inferred the modules of each network. In order to make a visual summary of the networks we inferred the modules and their connections using the ModuLand app (Szalay-Beko *et al.* 2012) for Cytoscape. For each module, we represented the taxa composition as pie charts constructed in the "ggplot2" *R* package (Wickham 2009) with the module information obtained in ModuLand.

Finally, we calculated metrics of the topology of the networks using the NetworkAnalyzer app in Cytoscape. For modularity measures we used the "iGraph" *R* package (Csardi and Nepusz 2006). In order to compare the diversity structure (in terms of network topology) among populations we conducted a Principal Component Analysis (PCA). To visualize potential patterns in the networks structure and reflect on its biological

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significance, we constructed a heatmap with all the network metrics. All procedures related to network inference and metric calculation were done in the software Cytoscape 3.0 (Shannon *et al.* 2003). For statistical analyses we employed the *R* software (version 2.4-6) (R Core Team, 2017).

RESULTS

The root microbial community composition differs between wild cotton populations

Alpha diversity

For all the samples (N=89) we identified 7,874 ASV´s. Relative abundance of phyla among the three types of samples (inoculum, bulk and rhizosphere soil) revealed differences in composition (Figure S3-A). While for alpha diversity analysis calculated as Chao1 diversity index, we found that the lower values were associated to inoculum samples (Figure S3-B). Regarding plant samples and control soil, the relative abundance analysis revealed that all samples are dominated by Proteobacteria, with more variation in the Chloroflexi, Acidobacteria, and Chlorobi phyla (Figure 1A). For alpha diversity (Chao1 index) analysis we identified significant differences among plant populations (Figure 1B). Also, in terms of Chao1, all the populations samples showed significant differences (ANOVA, p <0.01) compared to the control samples, with the sole exception of “CPM”. Moreover, the control showed the lowest values for diversity, while “BCSM” and “YPM” populations showed the highest values.

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284 *Beta diversity*

285 Both beta diversity analyses (Figure 2) showed differences in the bacterial communities
286 associated to the rhizosphere microbial communities and control samples. Specifically, for
287 NMDS analysis (Figure 2A), we observed a distinction of the plant populations and control
288 samples (PERMANOVA, F= 10.29, p= 0.001), but also a statistical distinction across
289 populations (Table S1).

290 LefSe analysis allowed the identification of specific groups that were differentially
291 abundant across samples (plant populations and control samples), this information is
292 presented as supplementary material (Figure S4). The information of differentially abundant
293 groups was visualized through a heatmap representation at the taxonomical level of family
294 (Figure 2B). From this representation we observed that all the populations and control are
295 characterized by different bacterial families that could be "endemic" groups or biomarkers
296 for the populations. For example, the *Pirellulaceae, Rhodothermaceae* and
297 *Rhodobactereacea* were highly abundant in the "BCSM" population. For the "GSM"
298 population the *Rhizobiaceae, Chthoniobacteriaceae* and *Alicyclobacillaceae* families
299 appears to be the biomarkers. In the "CPM" population we observed the *Hyphomicrobiaceae,*
300 *Planctomycetaceae* and *Coxiellaceae* families highly abundant. For "SPM" population, we
301 found *Caulobacteraceae, Burkholderiaceae* and *Erythrobacteraceae* as abundant families.
302 While the "YPM" seemed to be the population with the highest number of biomarkers, being
303 the most abundant groups (*Alteromonadaceae, Streptomycetaceae* and *Rhodobiaceae*) also
304 absent in the rest of the populations. It is worth to mention that the abundant families for
305 control samples (*Xhantomonadaceae, Nocardioidaceae* and *Alcaligenaceae*), were almost
306 completely absent in the plant samples.

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3 308 **The diversity structure of the rhizosphere microbial communities differs among wild**
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6 309 **cotton populations**
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8 310 To analyze the structure of the cotton-associated microbial communities we inferred co-
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10 311 occurrence/exclusion networks (Figure 3 left) for all samples (plant and control treatments).
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12 312 The mathematical analyzes of the inferred networks allowed us to appreciate that the
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15 313 structure of their microbial community is significantly different. According to the PCA and
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17 314 heatmap, we identified that the communities associated with plant samples and control can
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19 315 be separated in two groups and two separated samples according to their structure similarity
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22 316 (Figure 4B). The group comprising "SPM" and "CPM", had the highest *density* and *average*
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24 317 *number of neighbors* measures. The group conformed by "BCSM" and "" showed low values
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27 318 for *network centralization* and high values for *network diameter*. While the separated samples
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29 319 were "YPM" which showed the highest values for *connected components* and *network*
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31 320 *heterogeneity* and control, which had the highest values for *network radius* and also the
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33
34 321 lowest *centralization*. It is worth noticing that when we look at the networks focusing on the
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36 322 topological aspects of their nodes (bacterial families), we do not appreciate a pattern across
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38 323 populations regarding the relative importance of specific phyla for the structure of the
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41 324 network (centrality metric).
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43 325
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45 326 Furthermore, when we look at the centrality of the nodes at the family level, we can
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48 327 see that the most connected nodes are different for all samples (Table S3). For instance, in
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50 328 the control network, the node with higher centrality was represented by a member of the
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52 329 *Moraxellaceae* family (Proteobacteria), for the "BCSM" population was a
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54
55 330 *Desulfovibrionaceae* (Proteobacteria), for "GSM" was a *Microthrixaceae* (Actinobacteria),
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57 331 for "CPM" was a *Methylobacteriaceae* (Proteobacteria), for "SPM" a *Iamiacea*
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(Actinobacteria), and finally, for "YPM" the node with higher centrality was *Legionellaceae* (Proteobacteria). These observations hold when we look at the module enrichment analysis of the networks (Figure 3, right). Overall, the network analyses provide evidence of different diversity structure of the rhizosphere microbial communities across the experimental samples and controls.

DISCUSSION

The rhizosphere is an extremely variable environment that provides several benefits to the plant host (Garbeva *et al.* 2008; Mendes *et al.* 2011). Various studies have established that the plant-microbe interaction in the rhizosphere is determinant for the health, growth and productivity of the host (Wagg *et al.* 2011; Ursell *et al.* 2013). Despite the evidence for the importance of this interaction, the factors behind the assembly of the microbial communities associated with the rhizosphere is still not fully understood. In particular, the role of plant genotypes as a determinant factor in the microbial assemblages is poorly documented. In the present study we aimed to test the role that intraspecific genetic variation has in the rhizospheric microbial community assemblages, using cotton plants as a model of study. We followed a common garden experiment, controlling for plant genotypes, environmental conditions and soil microbial community inoculum, in order to statistically test for differences in the microbial communities associated with genetic variation of the plant hosts. Two groups of evidence were collected, one at the level of microbial community diversity (alpha and beta), and another focused in diversity structure of such communities, determined by co-occurrence networks. Overall, the results provide evidence of the importance of host genotype in producing different rhizospheric microbial community assemblages.

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Different plant genotypes select for different and specific microbial communities from a common inoculum

In order to determine how specific is the influence of the genotype in the assembly of rhizospheric microbial communities, we experimentally tested for differences in the microbial communities associated with different plant genotypes associated to different wild populations of cotton, controlling for environmental conditions. Both alpha and beta diversity analysis showed differences in the microbial communities associated with the different treatments (i.e. plant genotype, and no-plant controls) (Figure 1; Figure 2).

In regard to alpha diversity (Figure 1), we found that composition at the phylum level varies among treatments and populations (Figure 1A), and also diversity index Chao1 (Figure 1B) varies significantly across treatments, being the control (no-plant) samples the ones with lowest diversity values. Low diversity values in no-plant controls coincides with previous studies that associate plant release of nutrients (root exudates) to a higher number of microbial groups in the rhizospheric space (Bulgarelli *et al.* 2013). Moreover, the differences in alpha diversity were also found across different plant genotypes (i.e. populations), meaning that some of these genetically different plants can host higher diversity of microorganisms than other genotypes of the same species. For instance, we can observe that “YPM” and “BCSM” genotypes hosts the highest diversity of microbial communities while “CPM” the lowest (Figure 1B). Different levels of microbial diversity associated with different host-plant genotypes is of special relevance since more microbial diversity has been associated with functional redundancy that, in turn, may provide more stability of the systems in the face of environmental perturbations or external stressors (Allison and Martiny 2008). Hence, the variation in diversity found between cotton populations could potentially have

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consequences in the response and recovery of the host-microbial systems to perturbations such as the extreme environmental conditions (salinity, high temperatures) that cotton populations face in their natural habitats (Stewart *et al.* 2010), which in turn, could have consequences on the adaptation and survival of the host plant itself.

Regarding beta diversity, we found that each of the different treatments (i.e. plant genotype, and no-plant controls) is characterized by a particular microbial composition (Figure 2). Specific microbial composition that differentiate treatments were represented in a multidimensional space, showing spatially distinct groupings according with the identity of the samples, either plant genotype or no-plant control (Figure 2A). Furthermore, we found differentially abundant microbial groups for all samples through a heatmap visualization of the LefSe analysis (Figure 2B, Figure S4), which permits to reflect on the potential mechanisms of host-microbe association and on the possible roles of these microbial groups in such association. For instance, control samples are very distinct from any of the plant genotypes samples (Figure 2A, Figure 2B, Table S1) in terms of the resulting microbial communities composition, which is indicative of the major influence of the plant on the soil microbial communities (Haichar *et al.* 2008; Bulgarelli *et al.* 2013; Lakshmanan 2015).

Regarding sample differentiation in terms of microbial composition, when we look at specific plant genotypes samples, we can also identify different compositions, some of which are less different than others. For instance, “YPM” and “BCSM” genotypes are more similar in their composition but at the same time can be clearly identified as different from other plant genotypes such as “GSM” (Figure 2A, Figure 2B, Table S1). In general, the fact that there are different microbial groups associated with specific genotypes can be taken as evidence of some type of preference (selection) of the host on specific microbial assemblies,

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402 as has been documented in other systems such as legumes and rhizobia associations
403 (Bisseling, Dangl and Schulze-Lefert 2009; Weinert *et al.* 2011; Marques *et al.* 2014).
404 This selection for specific microbial groups allowed us to reflect on the potential roles
405 that those microbes could have for the plant-rhizosphere association. For example, we found
406 that "BCSM" genotype shows high abundance of microbial families that are closely related
407 to N cycling, such as *Rhodobacteraceae* (Buée *et al.* 2009; Edwards *et al.* 2015) and
408 *Plactomycetaceae* (Fuerst and Sagulenko 2011; Nie *et al.* 2015). Besides the enrichment of
409 the *Pirellulaceae* family, which has been associated to cotton rhizosphere specifically
410 (Peiffer *et al.* 2013; Qiao *et al.* 2017). For the "GSM" genotype, we also found microbial
411 groups related to N fixation (*Rhizobiaceae*) (Zarraonaindia *et al.* 2015). Regarding "CPM"
412 genotype, we found high abundance of the *Hyphomicrobiaceae* family, which has been
413 studied for playing roles in improving host ability to sense and respond to drought
414 (Lakshmanan, Selvaraj and Bais 2014). Finally, "SPM" and "YPM" genotypes showed two
415 microbial families (*Burkholderiaceae* and *Streptomycetaceae*) that are known for playing
416 roles in diseases control (Berendsen, Pieterse and Bakker 2012; Lakshmanan, Selvaraj and
417 Bais 2014). In particular, *Spretomycetaceae* family is also recognized for the production of
418 antimicrobial secondary metabolites (Firáková, Šturdíková and Múčková 2007). The
419 preference of the host genotypes to certain microbial groups is in accordance with previous
420 studies which mentioned that this phenomenon could be explained by physiological
421 requirements of the plants such as P and N deficits, which production is linked to microbial
422 communities activity (Weinert *et al.* 2011; Marques *et al.* 2014). Altogether, the preference
423 of the cotton plant genotypes to specific microbial groups that are related with N cycling and
424 fixation, or are involved in disease control processes and response to environmental
425 conditions, could be related to physiological, nutritional needs of the plant host.

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Variation in the structure of the rhizosphere associated to different plant genotypes

To determine if the co-occurrence patterns of the microbial groups, as a proxy of structure, differ between populations we inferred networks and calculated metrics of their topology. The mathematical analysis of ecological networks enables the identification of structural patterns that reflect on variations in the biological properties of the communities of the different populations. In particular, we found that all the microbial communities networks inferred in this work are clearly distinguishable in terms of topological metrics like density, clustering, modularity, and centralization (Figure 4A, Table S2). These properties have been reported in microbial communities networks to be related to robustness and functional aspects of the systems (Albert Reka, Jeong Hawoong 2000; Melián and Bascompte 2002; Bastolla *et al.* 2009; Navarro-Díaz, Valdez-Vazquez and Escalante 2016; Rebollar *et al.* 2017).

According to the graphic representation of the co-ocurrence networks, two types of networks were identified; those with high degree of modularity and highly aggregated such as "SPM" and "CPM" genotypes, and those with less degree of modularity and highly disaggregated such "BCSM" and "GSM" genotypes (Figure 3 left, right, Table S2). We hypothesize that these variations could be the result of the host genotype influencing the microbial communities and promoting the emergence of the co-occurrence patterns between specific microbial groups that we observed in the inferred networks (Figure 3 left, right). In particular, variation on network properties like those found in this study (e.g. modularity, centralization) has been found in other studies modeling microbial communities associated to plant productive systems (Rebollar *et al.* 2017), and have also been proposed to reflect

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habitat heterogeneity and phylogenetic clustering of related species, that could potentially generate non-random patterns of association (Lewinsohn *et al.* 2006).

When we focus on the topological aspects of the network nodes (bacterial families), we do not appreciate a pattern across populations regarding the relative importance of specific phyla for the structure of the network. Furthermore, all the hub nodes (in terms of its betweenness centrality) are different for all samples. These differences in specific groups can lead to the generation of novel and specific hypothesis to be tested. Specifically, regarding to the importance of certain groups in plant growth or interactions between the members of the plant microbiome. For instance, we found the *Methylobacteriacea* and *Rhizobiacea* classes as hubs nodes for the CPM genotype (Table S3), groups that belong to the rhizobiales group, which are diazotrophic bacteria that transform atmospheric N_2 to a form that can be used by plants (Santi, Bogusz and Franche 2013). For the YPM genotype, we found *Hyphomicrobiaceae* and *Legionellacea* classes as hubs nodes for its network. Being the *Hyphomicrobiaceae* family, as we mention before, important in the host response to drought, which could be an important attribute in environments such as coastal dunes, natural habitat of cotton plants (Lakshmanan, Selvaraj and Bais 2014). Moreover, the *Legionellaceae* family have been found as a health indicator by playing roles in disease suppression in species such as *Beta vulgaris* (Mendes *et al.* 2011) and *Musa paradisiaca* (Köberl *et al.* 2017). Finally, “BCSM” genotype presented *Desulfovibrionaceae* family as the hub node, a common microbial group in extreme oligotrophic habitats (Madigan and Martinko 2005) such as the case of coastal dunes. The differences in centrality values for the genotype networks could reflect the relative importance of hubs nodes and the robustness of the networks toward the removal of them (Faust and Raes 2012). This result is indicative of the major influence of plant host into the assembly of the microbial communities in the rhizosphere, and which

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474 could be driven by the rhizodeposition process (Miethling *et al.* 2000; Garbeva *et al.* 2008;
475 Weinert *et al.* 2011). In particular, the differences in diversity structure between rhizosphere
476 and control networks is explained by higher values for some metrics (multi-edge node pairs,
477 characteristic path length, network radius), and lower values for another metrics (connected
478 components, network heterogeneity and centralization) (Figure 4B). We could speculate,
479 though, that differences in values for some of those metrics (i.e. network heterogeneity and
480 centralization) could be related to ecological processes at microbial level that could be driven
481 by the plant presence.

482

483 CONCLUSIONS

484 Despite the evidence of various studies establishing the plant-microbe interaction in the
485 rhizosphere as determinant for the health, growth and productivity of the host, the factors
486 behind the assembly of the microbial communities associated with the rhizosphere is still not
487 fully understood. In particular, the role of plant genotypes as a determinant factor in the
488 microbial assemblages is documented mainly at species level, leaving aside the contribution
489 of intra-specific genetic variation. The results presented in this study show that plant host
490 genotype is determinant in the assembly and diversity structure of the microbial communities
491 in the rhizosphere. In particular, we found that i) host genotype factor is so powerful that
492 even intra-specific genetic variation (population level) can differentiate the microbial
493 communities of the rhizosphere in terms of its composition and diversity structure ii) different
494 plant genotypes are related to the abundance of specific microbial groups which could be
495 driven by physiological and nutritional needs of the plant that, in the case of cotton species,
496 could be related to the survival of the plants in extreme environments. Further studies looking

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for the genotype effect into the root exudates could help us to elucidate the specific mechanisms trough the host can select and modulate the microbial communities in the rhizosphere.

Finally, taking together our previous findings, considering that Mexico is center of origin and diversity for cotton, this study provides a valuable baseline for the microbial diversity associated to wild populations. In turn, this baseline can inform integral strategies for agrobiodiversity conservation and sustainable production that are based on the ecological and evolutionary study of wild and domesticated species complexes (Heywood *et al.* 2007; Maxted *et al.* 2010)(e.g. Maxted et al., 2010; Heywood et al. 2007). Moreover, our results call for precautory and mitigating actions regarding the flow of genes across wild and domesticated populations (potentially including transgenes), since we show that changes in the genotype may affect the ecological interactions with the plants microbiome, with potential functional consequences.

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ACKNOWLEDGEMENTS

We thank Morena Avitia for her technical assistance during DNA extraction, Tania Gabriela Sosa-Fuentes for assistance during transgene detection assays, and Alberto Barrón-Sandoval for his support in the sequence data processing. The authors acknowledge Florencia García-Campusano for her support in the laboratory work, and to the Laboratorio de Biotecnología CENID-COMEF, INIFAP.

FUNDING

This work was financially supported by the project ``Program for the conservation of wild populations of *Gossypium hirsutum* in Mexico" DGAP003/WN003/18 Direccion General del Sector Primario y Recursos Naturales Renovables (DGSPRNR) that belongs to the SEMARNAT and CONABIO, and the project UNAM-PAPIIT No. IN214719. And for Daniel Piñero Dalmau troughout the project "Contribución de la Biodiversidad al Cambio Climático" of the Secretaría de Medio Ambiente y Recursos Naturales (SEMARNAT). Alejandra Hernández-Terán is a doctoral student from Programa de Doctorado en Ciencias Biomédicas, Universidad Nacional Autónoma de México (UNAM) and was supported by CONACyT (scholarship no. 660255).

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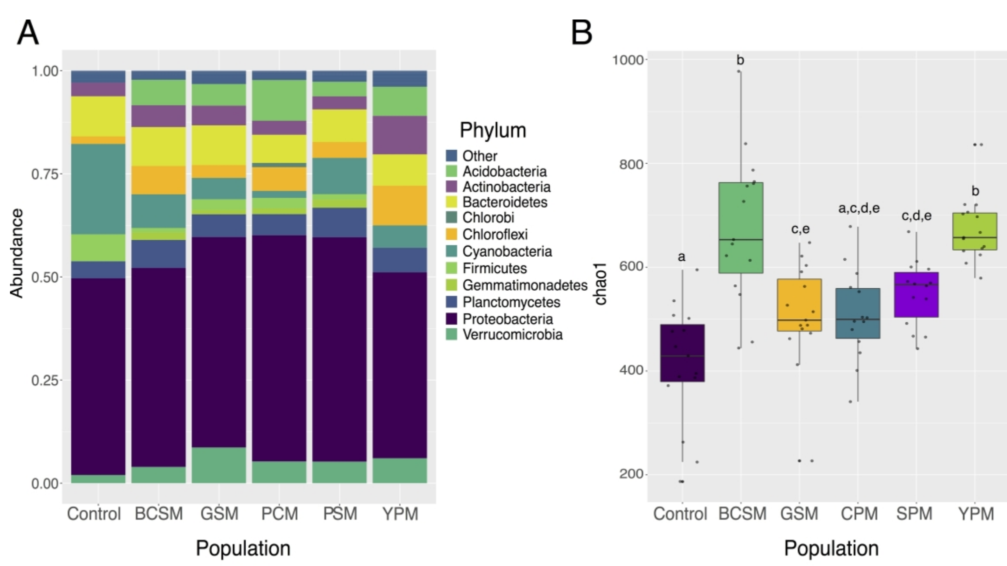

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Figure 1. Alpha diversity of microbial communities of rhizosphere and control samples. A: Mean relative abundance of the most abundant bacterial phyla, each column represents an experimental treatment (i.e. plant population and no-plant control samples). B: Box plot of alpha diversity estimates (Chao1 index) of all analyzed samples (i.e. plant population and no-plant control samples) Different letters indicate statistically significant differences (ANOVA $p < 0.01$). Name codes for plant populations: Baja California Sur (BCSM), Central Pacific (CPM), South Pacific (SPM), Yucatan Peninsula (YPM), and Gulf South (GSM).

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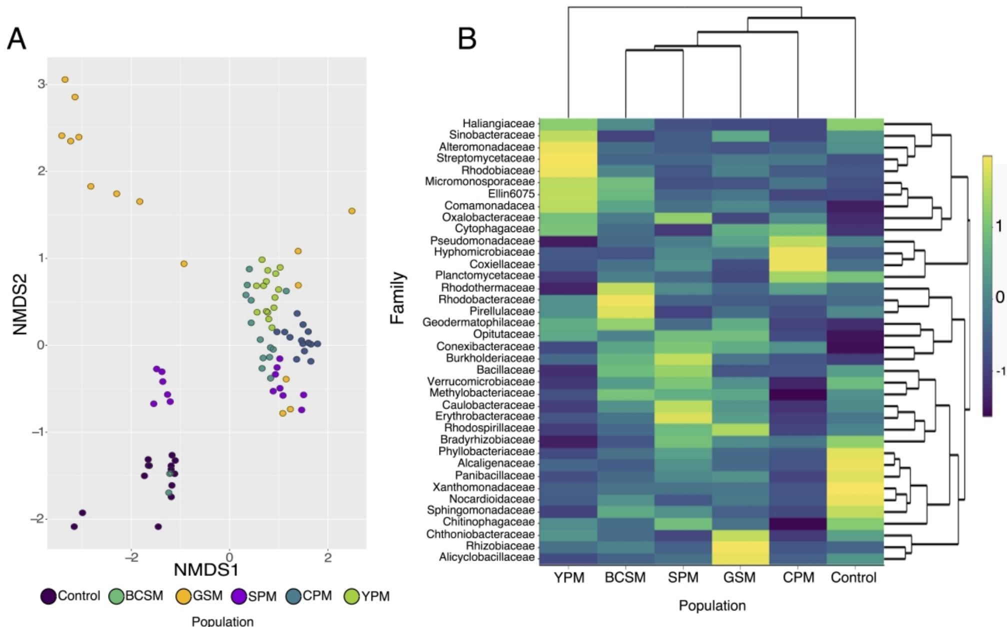

Figure 2. Bacterial community composition dissimilarity across samples. A: Non-Metric Multidimensional Scaling (NMDS) with Bray-Curtis distance at phylum level. B: Heatmap of the most significant groups at family level (LDA scores from LefSe analysis). Temperature scale corresponds to the z-scores of the abundance of each microbial group. Name codes for plant populations: Baja California Sur (BCSM), Central Pacific (CPM), South Pacific (SPM), Yucatan Peninsula (YPM), and Gulf South (GSM).

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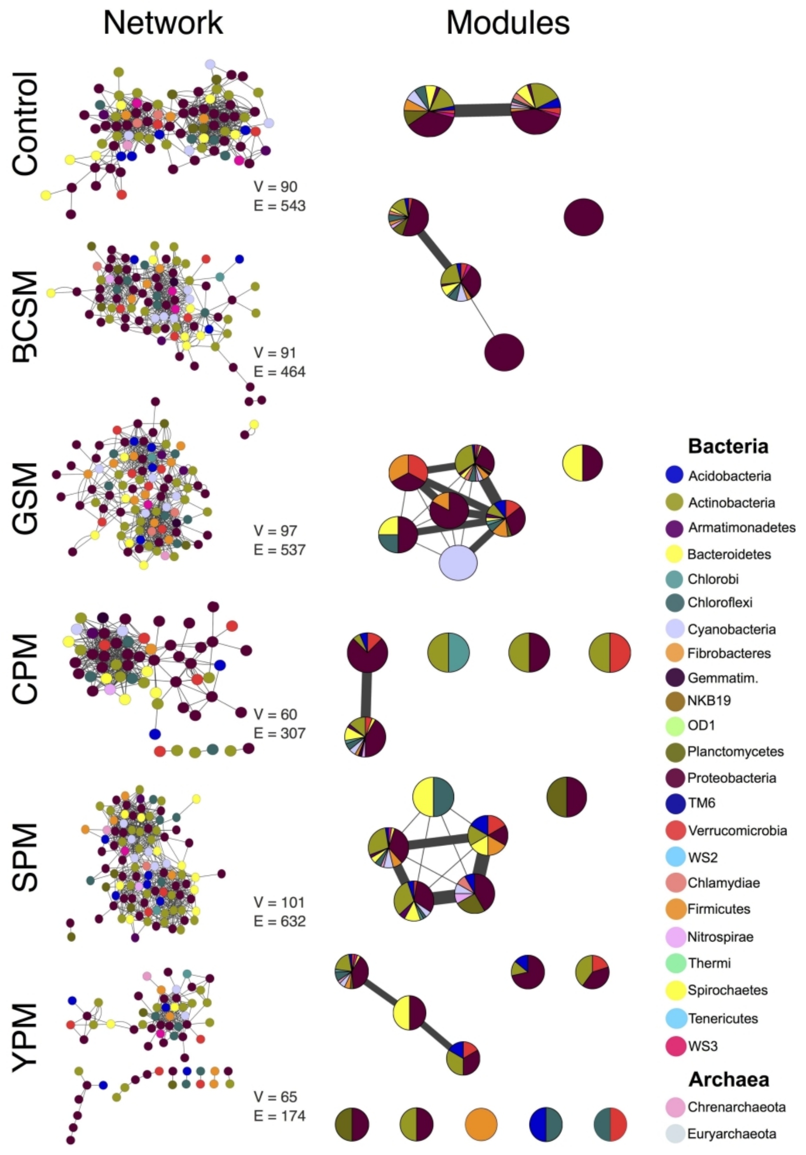

45 Figure 3. Charts at left show the original co-occurrence/exclusion networks inferred at family level and
46 painted by phylum. Charts at right show the module enrichment analysis of each original network. Each
47 circle represents a module and their composition as a pie chart, each color represents a phylum detailed in
48 the Bacteria/Archaea color code. Line thickness indicates the number of edges between the modules.
49 Number of edges (V) and nodes (E) are included next to the corresponding network. Name codes for plant
50 populations: Baja California Sur (BCSM), Central Pacific (CPM), South Pacific (SPM), Yucatan Peninsula
(YPM), and Gulf South (GSM). Networks were inferred with Cytoscape.

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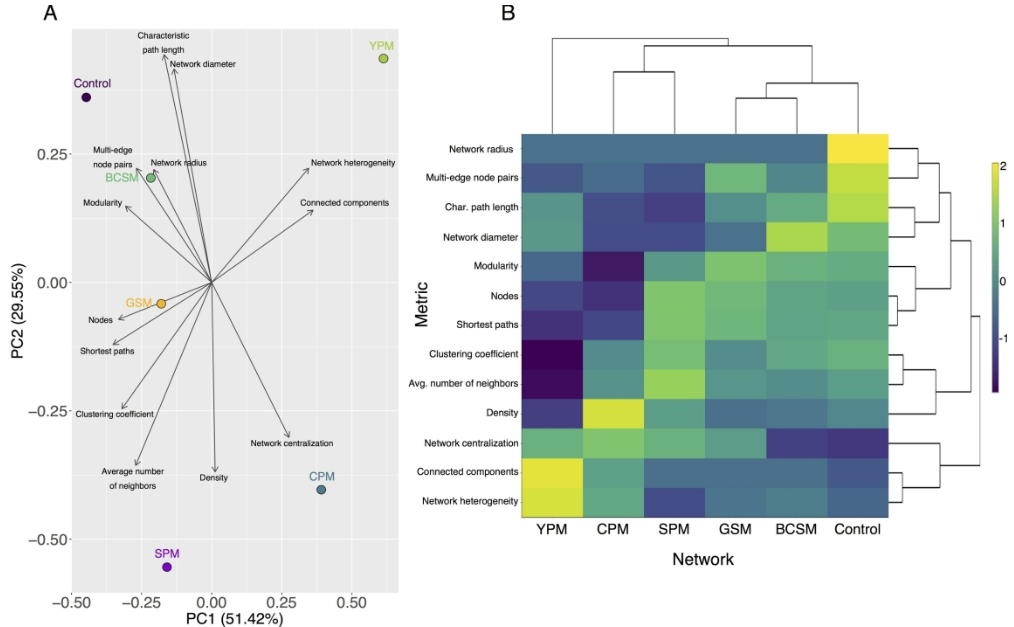

Figure 4. Statistical analysis of microbial communities co-occurrence networks. A: Biplot generated from Principal Component Analysis (PCA) of the standardized (z-score) network metrics for all samples (rhizosphere and controls). Colors represent different samples (five plant populations rhizosphere and control). PC1 accounted for 51.42% of the total variation, and PCA2 accounted for 29.55%. B: Heatmap of the different samples (five plant populations rhizosphere and control) for all the calculated network metrics. Dendrograms were constructed using Bray-Curtis distance. Temperature scale corresponds to the z-scores of the metrics values. Name codes for plant populations: Baja California Sur (BCSM), Central Pacific (CPM), South Pacific (SPM), Yucatan Peninsula (YPM), and Gulf South (GSM).

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CONCLUSIONES GENERALES

A lo largo de este trabajo desarrollamos algunos conceptos clave sobre la importancia de la diversidad genética en procesos de importancia ecológica y evolutiva en plantas. Entendimos que los procesos que dan origen a la diversidad genética y fenotípica son específicos para cada especie y están fuertemente relacionados con las condiciones ambientales donde se desarrollan naturalmente. Brindamos además un panorama amplio sobre el papel que juega la diversidad genética en plantas bajo distintos escenarios, no solamente en términos del fenotipo entendido como características morfológicas de los individuos, sino también como los procesos biológicos relacionados con las interacciones ecológicas, que son fundamentales para la sobrevivencia de los organismos.

El trabajo de revisión e integración de la información nos permitió detectar que en la modificación genética ocurren consecuencias fenotípicas no intencionadas de manera generalizada y en un amplio número de cultivos. Esto es importante ya que aunque teóricamente se creía que estas consecuencias inesperadas podrían ocurrir, además de que se había hablado ampliamente de los mecanismos, no existían trabajos en los que se demostrara formalmente y a gran escala. En el mismo sentido, el trabajo experimental sobre la diversidad genética y su impacto en el rendimiento *in vitro* de poblaciones de algodón, brinda un panorama sobre las consecuencias funcionales de la introgresión de cultivos en un ambiente con relevancia para la conservación. El resultado de la presencia de costos ecológicos por la presencia de transgenes en poblaciones de algodón, nos hace además repensar los pasos a seguir cuando se pretende conservar los recursos genéticos. En conjunto, los resultados de estos esfuerzos son importantes en la toma de decisiones sobre seguridad alimentaria, y abren la discusión respecto a las medidas necesarias para asegurar los recursos genéticos en el futuro. Puntualmente,

este trabajo representa un avance importante en la generación de evidencia que apoya la reestructuración y mejoramiento de los protocolos de análisis de riesgo de tecnologías de modificación genética, además de la implementación de monitoreos de detección de transgenes previo a los esfuerzos de conservación *in vitro*.

Reconociendo también que los procesos ecológicos y evolutivos de las poblaciones de plantas van de la mano, generamos información sobre las consecuencias de la diversidad genética en la estructuración de las comunidades microbianas de raíz. Nuestro hallazgo principal fue que cada genotipo hospedero (metapoblación) puede seleccionar comunidades microbianas diferentes bajo condiciones ambientales idénticas; demostrando por primera vez hasta donde sabemos, que la variación genética intra-especie juega un papel determinante en la selección del microbioma de raíz. Este hallazgo nos permitió reflexionar sobre los procesos adaptativos que ocurren en ambientes naturales, pero también en que la conservación de las poblaciones silvestres *in situ* es necesaria para asegurar los procesos que dan origen y mantienen la diversidad genética.

En conjunto, esta tesis aporta información acerca de cómo la diversidad genética en plantas cultivadas afecta el fenotipo e interacciones ecológicas. Plantea la reestructuración de los protocolos de análisis de riesgo implementados históricamente y la necesidad de estudiar más a fondo y repensar las estrategias de modificación genética que se han usado desde hace décadas. La evidencia generada apunta al fortalecimiento de estrategias de conservación de plantas silvestres como las principales fuentes de variación genética, lo cual a su vez ayudaría a asegurar la posibilidad de nuevos mejoramientos genéticos para generaciones futuras.

CONCLUSIONES PARTICULARES

Capítulo I

1. La modificación genética, ya sea por domesticación o por ingeniería genética, puede ser rastreada fenotípicamente cuando comparamos parientes silvestres contra organismos domesticados
2. La existencia y magnitud de las diferencias fenotípicas entre organismos domesticados con y sin ingeniería genética dentro de un mismo cultivo, sugiere consecuencias fenotípicas de la modificación genética más allá de los rasgos buscados

Capítulo II

1. El rendimiento *in vitro* entre poblaciones silvestres de algodón con y sin transgenes y entre poblaciones silvestres y domesticadas es significativamente diferente
2. La presencia de transgenes en poblaciones silvestres de algodón provoca costos ecológicos que son reflejados en su rendimiento *in vitro*, lo cual potencialmente podría afectar el éxito de estas poblaciones en bancos de conservación de germoplasma

Capítulo III

1. El genotipo hospedero es tan determinante que incluso variación genética a nivel intra-especie puede provocar una diferenciación en las comunidades microbianas de raíz
2. Distintos genotipos de plantas tienen la capacidad de promover el crecimiento de microorganismos específicos que, en el caso de las poblaciones de algodón, podría estar relacionado son la sobrevivencia y adaptación de estas en ambientes extremos

PERSPECTIVAS

En todo trabajo científico se genera información que busca contestar preguntas particulares, aunque muchas veces algunas de estas preguntas se logran responder, muchas más nuevas surgen y permanecen abiertas. En este trabajo integramos y generamos evidencia que nos ayuda en el entendimiento de los procesos por los cuales la diversidad genética de plantas cultivadas afecta el fenotipo e interacciones ecológicas. Sin embargo, abre la puerta a nuevas preguntas y nuevos planteamientos experimentales.

Capítulo I: los resultados de este capítulo resaltan en primer lugar la importancia del estudio sistemático de la información disponible, el cual nos puede dar luz y poder estadístico en el entendimiento de procesos biológicos. Sin embargo, también hace visible la necesidad de realizar experimentos dirigidos a probar hipótesis relacionadas a cómo pueden arrastrarse caracteres fenotípicos en los procesos de modificación. Aunque diversos autores mencionan teóricamente que esto podría ser a través de procesos a nivel genético como pleiotropía o epistasis, estudios que busquen los mecanismos específicos de estos fenómenos son necesarios.

Capítulo II: como se menciona en la introducción de este trabajo, actualmente un total de 29 cultivos de plantas han sufrido procesos de introgresión entre poblaciones silvestres y domesticadas en la vida silvestre. Considerando además los efectos deletéreos encontrados en las poblaciones silvestres de algodón, posiblemente ocasionados por la presencia del transgen, se vuelve relevante probar el comportamiento de estos organismos en la vida silvestre. Esto es determinante para conocer el papel que juegan los genes de domesticación o ingeniería genética en ambientes no controlados (como el caso del ambiente *in vitro*). Lo cual a su vez, nos ayudaría en la toma de decisiones respecto a las tecnologías de modificación genética.

Capítulo III: el trabajo de este capítulo arrojó información novedosa respecto al papel determinante que juega el genotipo de la planta hospedera en la determinación de la estructura de las comunidades microbianas de raíz. Sin embargo, deja muchas más preguntas abiertas. En primer lugar, aunque la clonación *in vitro* para la obtención de réplicas es el primer paso para controlar la diversidad genética, es necesario contar con la genotipificación de los individuos a investigar. En segundo lugar, y con el fin de dilucidar los mecanismos de acción del hospedero sobre la rizósfera, son necesarios futuros esfuerzos en caracterizar la diversidad y composición de los exudados de raíz de plantas genéticamente diferentes, esto debido a que los exudados de raíz parecen ser la señal que media la comunicación hospedero-microbioma. Finalmente, con la evidencia arrojada en los capítulos anteriores sobre el impacto de la diversidad genética de plantas en distintos aspectos del fenotipo, y considerando además el impacto que la variación genética intra-específica del hospedero tuvo para la estructuración de las comunidades microbianas, proponemos un diseño experimental como el realizado en el Capítulo II. Esto con el objetivo de probar si la variación genética de organismos silvestres con y sin transgenes, y domesticados con y sin transgenes, puede ser rastreada en la diversidad y estructura del microbioma de raíz. Esto a su vez con potenciales consecuencias ecológicas y evolutivas.

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APENDICE I

Domesticated, genetically engineered and wild plant relatives exhibit unintended phenotypic differences: a comparative meta-analysis profiling rice, canola, maize, sunflower and pumpkin

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SUPPLEMENTARY MATERIAL

Appendix S1. Coefficients of each trait in the first two Discriminant axes (LD) generated for each crop. The absolute magnitude of the coefficient represents its importance in each Discriminant axis and the sign represents its direction in Discriminant space in Figure 1 (a panels).

Crop	Traits	Coefficients of Discriminants	
		LD1	LD2
Rice	Height	7.04	4.38
	Number of seeds	-3.03	4.30
	Pollen viability	-9.43	-8.23
	Days to flowering	9.31	0.909
Canola	Number of seeds	23.7	-18.6
	Height	0.31	2.47
	Days to flowering	0.62	2.46
	Pollen viability	2.34	-1.14
Sunflower	Height	-17.2	-3.97
	Number of flowers	-1-37	2.36
	Days to flowering	16.01	1.38
	Number of seeds	-0.78	4.44
Pumpkin	Number of fruits	95.9	-30.5
	Number of seeds	-63.6	56.7
	Number of flowers	0.4	-3.47
Maize	Days to flowering	2.5	-23.6
	Height	8.12	15.63
	Number of seeds	-9.43	-3.76

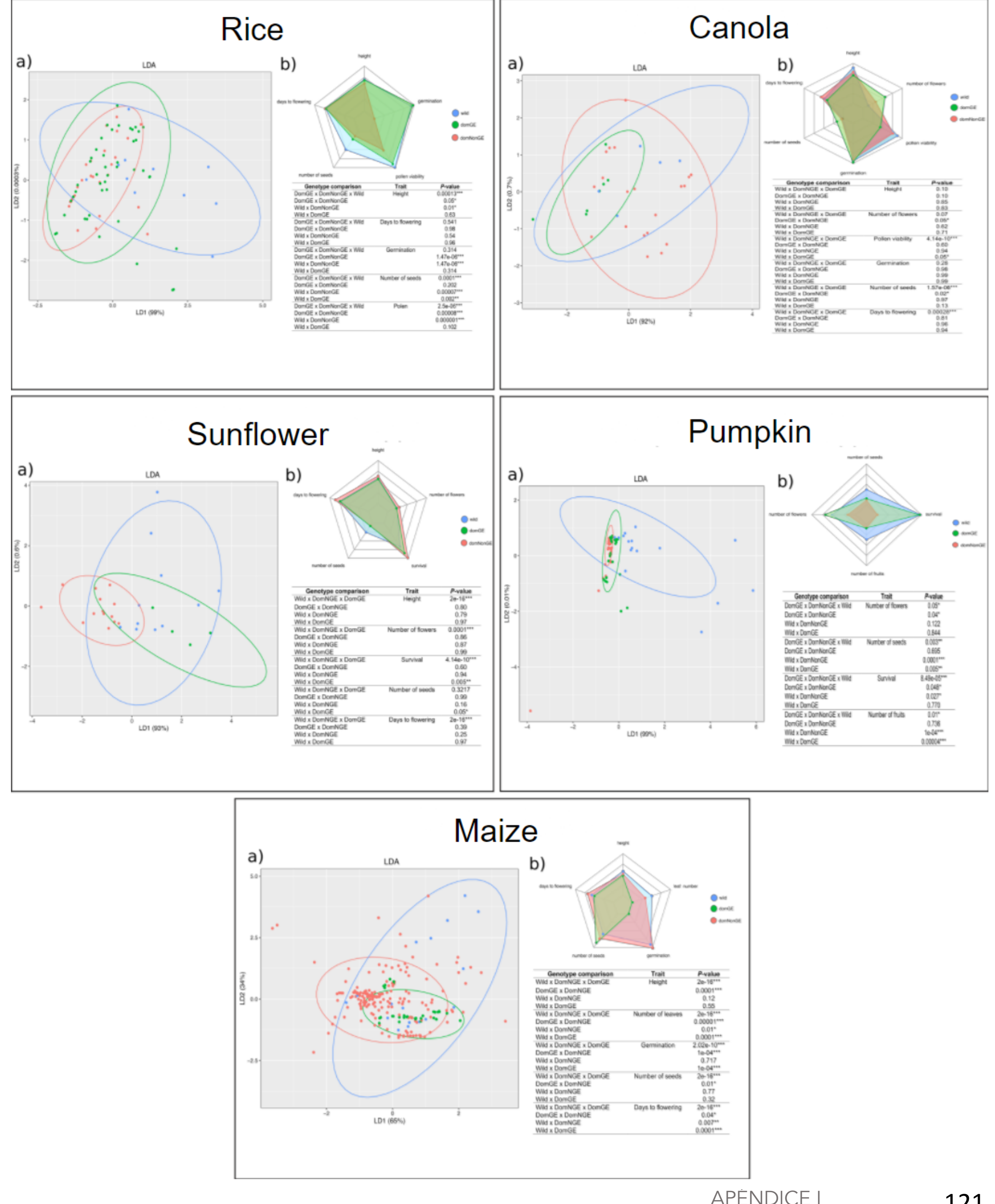
Appendix S2. Genetic transformations included in the dataset for each crop.

Crop	Gene	Product	Phenotype
Rice	Bt	cry delta-endotoxin	Resistance to lepidopteran insects
	CpT1	trypsin inhibitor	Resistance to a wide range of insect pests
	Bt/CpT1	cry delta-endotoxin/trypsin inhibitor	Resistance to a wide range of insect pests
	PPO	double stranded RNA	Block black spot bruise development
	Bar	phosphinothricin N-acetyltransferase (PAT) enzyme	Tolerance to glufosinate herbicide
	IMI	modified acetohydroxyacid synthase large subunit (AtAHASL)	Tolerance to imidazolinone herbicide
	nptII	neomycin phosphotransferase II enzyme	Metabolism of neomycin and kanamycin antibiotics
Canola	Bar	phosphinothricin N-acetyltransferase (PAT) enzyme	Tolerance to glufosinate herbicide
	CP4	5- enolpyruvulshikimate-3-phosphate synthase (EPSPS) enzyme	Tolerance to glyphosate herbicide
	Cry1Ac	cry1Ac delta-endotoxin	Resistance to lepidopteran insects
	Barnase/bastar	barnase ribonuclease (RNAse) enzyme/ barnase ribonuclease inhibitor	Male sterility/ restores fertility
	Bar/nptII	phosphinothricin N-acetyltransferase (PAT) enzyme / neomycin phosphotransferase II enzyme	Tolerance to glufosinate herbicide / Metabolism of neomycin and kanamycin antibiotics
	IMI	modified acetohydroxyacid synthase large subunit (AtAHASL)	Tolerance to imidazolinone herbicide
Sunflower	OxOx	oxalate oxidase	White mold resistance
	Als	herbicide tolerant acetolactate synthase (ALS) enzyme	Tolerance to sulfonylurea and imidazolinone herbicides
	Cry1Ab	cry1Ab delta-endotoxin	Resistance to lepidopteran insects
Pumpkin	ZYMV/WMV	coat protein of zucchini yellow mosaic potyvirus (ZYMV) / coat protein of watermelon mosaic potyvrus 2 (WMV)	Resistance to ZYMV and WMV
	CMV/ZYMV/WMV	coat protein of cucumber mosaic cucumovirus (CMV) / coat protein of zucchini yellow mosaic potyvirus (ZYMV) / coat protein of watermelon mosaic potyvrus 2 (WMV)	Resistance to CMV, ZYMV and WMV
	ZYMV	coat protein of zucchini yellow mosaic potyvirus (ZYMV)	Resistance to ZYMV
Maize	CP4	5- enolpyruvulshikimate-3-phosphate synthase (EPSPS) enzyme	Tolerance to glyphosate herbicide
	ZMVPP1	vacuolar H+ pyrophosphatase	Drought tolerance
	Cry/CP4	cry delta-endotoxin / 5-enolpyruvulshikimate-3-phosphate synthase (EPSPS) enzyme	Resistance to lepidopteran insects / Tolerance to glyphosate herbicide

Cry	cry delta-endotoxin	Resistance to lepidopteran insects
BADH	betaine aldehide deshidrogenase	Drought tolerance

Source: International Service for the Acquisition of Agri-Biotech Applications (ISAAA) (http://www.isaaa.org/gmapprovaldatabase/geneslist/default.asp).

Appendix S3. Phenotypic differences between wild and domesticated with and without genetic engineering in five crops. Results of the analyses performed without management of outliers. In all panels: a) Discriminant Analysis (DA), b) Spider-Chart of the means of each analyzed trait, and c) results of pairwise comparisons through with Generalized Linear Model (GLM). In all c) "DomPp" = both domesticated populations (GE and NGE). In all panels: "blue" wild relatives, "red" domesticated without genetic engineering and "green" domesticated with genetic engineering. P-value: * < 0.01, ** < 0.001, *** < 0.0001.



APENDICE II

In vitro performance in cotton plants with different genetic backgrounds: the case of Gossypium hirsutum in Mexico, and its implications for germplasm conservation

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SUPPLEMENTARY MATERIAL

Appendix S1. Specifics of the *in vitro* culture technique used in the establishment and propagation of the germplasm collection

Disinfection treatment

Steps

1. Remove the petiole and leaves from the stem of each axillary bud
2. Wash with soap and distilled water all the buds
3. Leave 30 sec in 70% alcohol (96%)
4. Wash with distilled water
5. Leave 10 min in 30% chloride (4%)
6. Wash with distilled water
7. Leave in ethil mercaptian (1 g per 1 l of distilled water) as a fungicide agent till establishment

Culture medium: for 1 l of culture medium we used 4.43 g of PhytoTech MS basal medium (PhytoTechnology Laboratories, Shawnee Mission, Kansas, USA solidified with 7 g of Phytagel (Sigma-Aldrich, Darmstadt, Germany) and 30 g of sucrose. Medium pH was adjusted to 5.7 with 0.1 M NaOH prior to addition of the agar. Approximately 6 ml of medium was dispensed into borosilicate culture tubes (25 mm x 95 mm height) and cap-sealed with PhytoTech closures (PhytoTechnology Laboratories, Shawnee Mission, Kansas, USA). Subsequently, each tube was autoclaved at 121°C and 1.5 kg cm^{-2} for 20 min.

Propagation technique: the propagation process was done under sterile conditions in a laminar-flow hood (ThermoFisher, Massachusetts, EUA). To remove the explants from the culture tubes we used forceps and scalpels sterilized into a dry glass bead sterilizer Germinator500 (Stoelting, Illinois, USA). Growth and culture rooms: the propagation process was done in a specialized culture room with controlled access and a double crystal door to avoid air circulation. The environment of the room was controlled with an air purifier that prevent contamination, additionally, UV irradiation was done in the entire room twice a week. The growth room had environmental controlled conditions (humidity and temperature) and was separated from the culture room by another crystal door. The 12h-photoperiod was provided by cool white fluorescent lamps (Philips, Mumbai, India).

Appendix S2. Scheme of experimental design

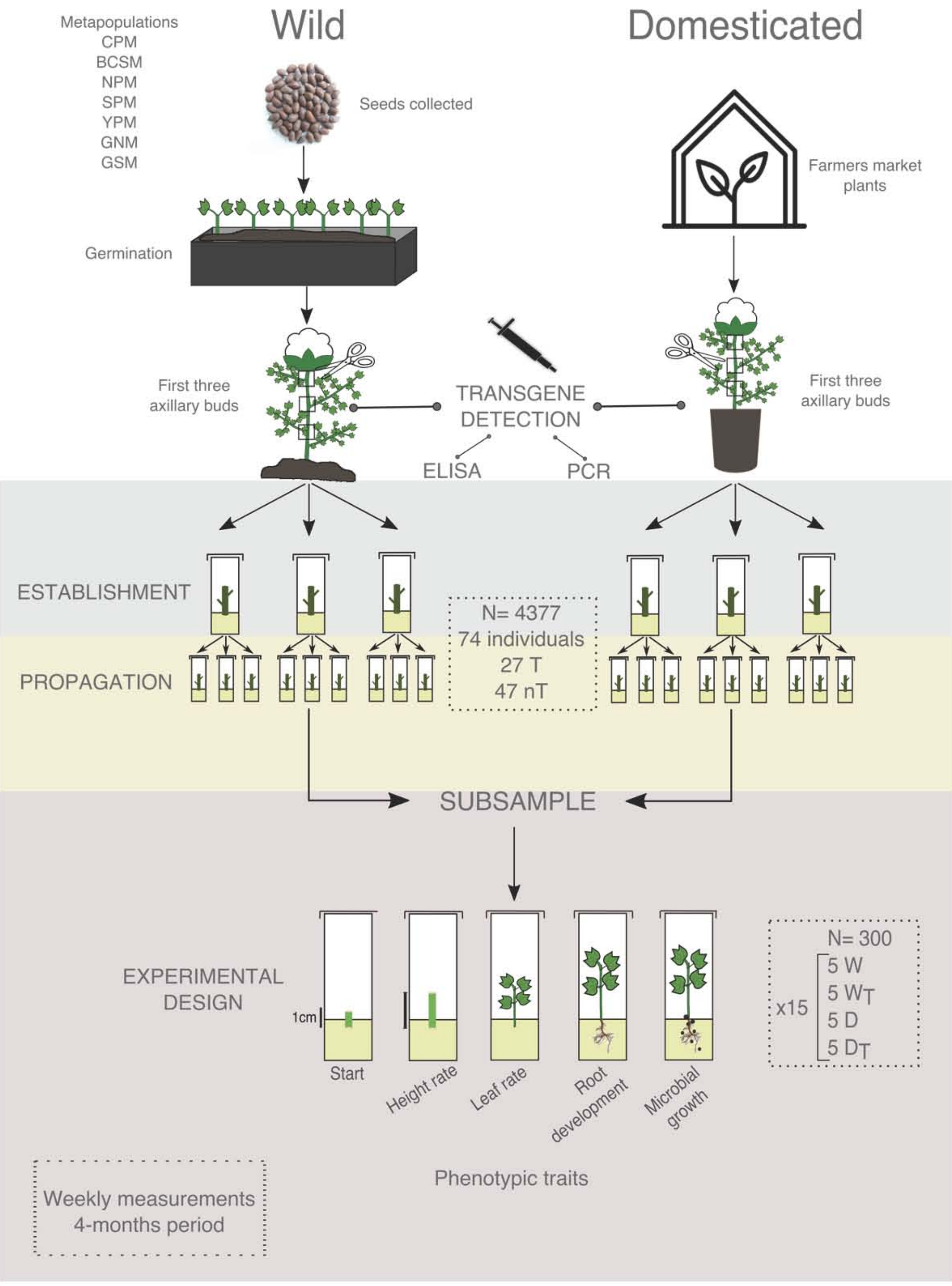

Figure S2. Scheme of experimental design. Origin and treatment of the different genotypes (wild and domesticated) of the germplasm collection. In the wild metapopulations collected; CPM: Center Pacific metapopulation, BCSM: Baja California Sur metapopulation, NPM: North Pacific metapopulation, SPM: South Pacific metapopulation, YPM: Yucatan Peninsula metapopulation, GNM: Gulf North metapopulation, GSM: Gulf South metapopulation. In the "propagation" N square= T: transgene presence, nT: without transgene presence. In the "experimental design" N square= W: wild organisms, D: domesticated organisms, W_T: wild organisms with transgenes and D_T: domesticated organisms with transgenes.

Appendix S3. Transgene detection of *in vitro* germplasm collection

We perform PCR assays for transgene detection in all the individuals from the germplasm collection. We specifically look for some of the transformation events released in Mexico: Cry1Ab/Ac, Cry2Ab, and CP4EPSPS (Table S1). The specific PCR conditions for each primer were applied according to the references show in Table S1.

Table S3. Primers sequence used in the transgene detection protocol.

Primer	Sequence	Amplicon size	Reference
Cry1Ab/Ac	F 5´ACCGGTTACACTCCCATCGA 3´ R 5´CAGCACCTGGCACGAACT 3´	76 bp	Zhang et al (2013)
Cry2Ab	F 5´CAGCGGCGCCAACTCTACG 3´ R 5´TGAACGGCGATGCACCAATGTC 3´	260 bp	Randhawa et al (2010)
CP4 EPSPS	F 5´GCATGCTTCACGGTGCAA 3´ R 5´TGAAGGACCGGTGGGAGAT 3´	108 bp	Barbau-Piednoir et al (2014)

Appendix S4. *In vitro* performance at metapopulation level

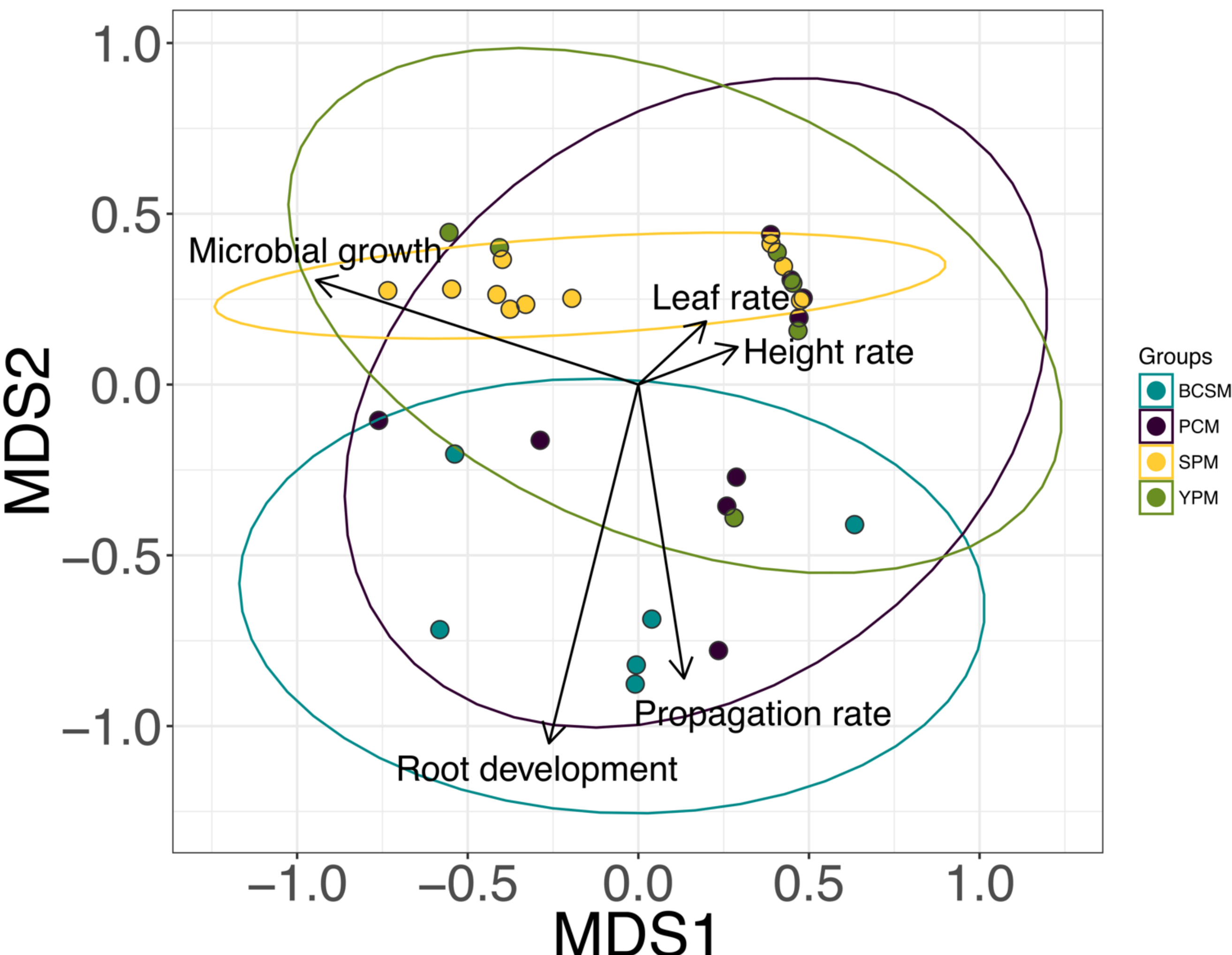

Figure S4. Non-Metric Multidimensional Scaling that include the analyzed traits in all natural populations without transgene presence. The ellipses represent 95% confidence interval around the centroids. Populations; BCSM: Baja California Sur metapopulation, CPM: Center Pacific metapopulation, SPM: South Pacific metapopulation and, YPM: Yucatan Peninsula metapopulation. NMDS Stress: 0.16. PERMANOVA F= 5.91, p= 0.09.

Appendix S5. Integrated analysis of *in vitro* performance between all analyzed genotypes

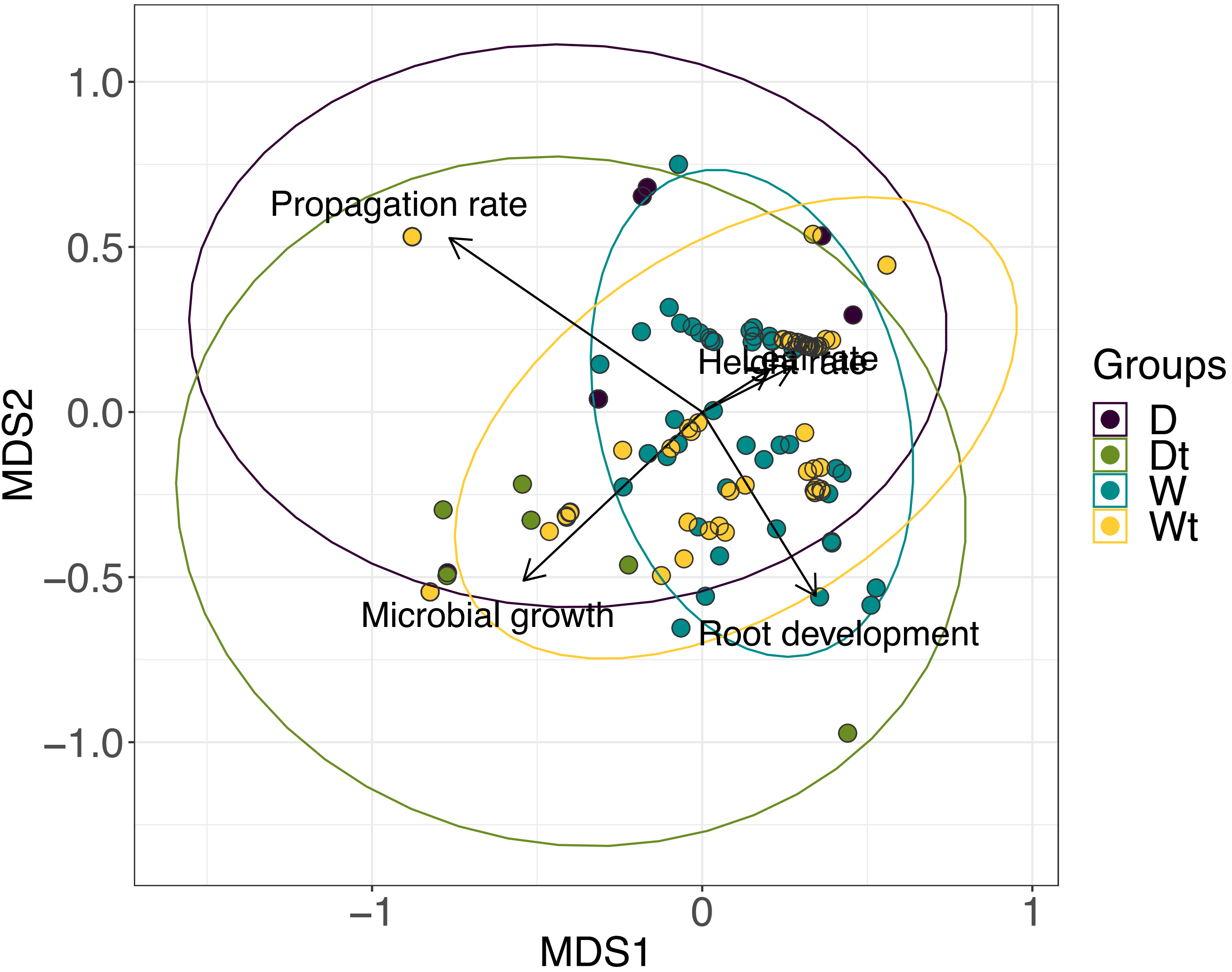

Figure S4. Non-Metric Multidimensional Scaling that include the analyzed traits in all analyzed genotypes. The ellipses represent 95% confidence interval around the centroids. Groups; D= domesticated populations without transgenes, Dt= domesticated populations with transgenes, W= wild populations without transgenes and Wt= wild populations with transgenes. NMDS Stress: 0.151. PERMANOVA F= 7.13, p= 0.0009.

Appendix S6. Raw sequences from Sanger verification of PCR amplicons

>Seq1 Gossypium hirsutum transgenic insert CP4epsps
AATACTACTCGAAGTCCTCTGGTCTTTCTGGAACCGTCCGTATTCCAGGTGACAAGTCT
ATCTCCCACCGGTCCTTCAAGTTTTGTTTCTTGCTGGTTAGCTGCGTGTAATCGAAAAG
GACATCGTGCCAACACATTGTGTTGTAATGTTAAAACCACCCTCCTTCCAGCCCTGCTG
ATCCTCTTCCCTTAATTCAGTCTTGCGCTGGTTCTCCTGCCAAATCCCGTTTCATTTACA
GTAAAAAATTCAATTCACTTATTCATAAAACAACTTGTCTGGGTCTTTGAACAAACCTCT
AAACCAGGATGCAACACTGCCATTATACATGTAAGGGTATACAAGTAGTCTTTCGTTAG
AAGTAGCACAATAACCAATCAAACGAAGCAGATTACGGTGAACAGCCAAGCTGATCAT
CTCCCACCGGTCCTTCAAGATTTTTTTTTC

>Seq2 Gossypium hirsutum transgenic insert CP4epsps
AGAAATCTGACTACGTAGTCCTCTGGTCTTTCTGGAACCGTCCGTATTCCAGGTGACAA
GTCTATCTCCCACCGGTCCTTGACTCGTTTCTTTGGTGTCTCCCACCGGTCCTTCAATC
GATTTAGTGCGGGGGCCTCTTTTATTCCGTTGGCGAGGATAATAAAACCCCCTTTTTGA
CTGATGAGCATATTACCGTACTCTTGTATAACATGGGGCCCTGCCGTGACAATTCCCAT
ACCTTACTATTAAATACGTATGAAATAAACGCACCCACGACCGTTTTGGGTCTCTTAACT
TAGTCTGAGAATAATAAAGGATGCCATGCGGGGGCAGTGTTCACCTACTTCCCAGTGG
TTAGAAGCGGCCTTAAGTGGGGTAATACCTTGCGTTATTTGTCTCAAACCAATTTGACT
ATTCCCCCGGGGCTGCGATTAACTTATTTTTGTGCTGACTAAACTCCAACCCTCACCA

>Seq3 Gossypium hirsutum transgenic insert CP4epsps
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CTATCTCCCACCGGTCCTTCAACGGTGGTTGTTGGTGAGAGGGTTGAATTGTGATTATT
ACCGACGGCCTTTTATTATCATTTTCTTAGCGTTAACACCCTACAATTTGATGGAAGCGG
CTCGACATGACTTATGTACAAGGCCCTCGAGCATACAATGCCCCCCACCACAGGAAAA
TATACGAGATTTTGCCTACTCGTGACCACTTCAAATTATAAAAATTAGATAGAGACGTG
AAAAAAGGGTTTTAATTTTTCCACCCCCCAATTGATTCTAAGAAACAATGGGCGGGCCT
CTGGAGTATAACCCTGAT

>Seq4 Gossypium hirsutum transgenic insert CP4epsps
AAAACTCTCGTAGTCCTCTGGTCTTTCTGGACCGTCCGATATTCCAGGTGACAAGTCTA
TCTCCCACCGGTCCTTCAATCTGTTATTGGTGAACCCATTGTTAAGGAAAATCACCGAG
GTGCCTTACTTCCCTTTATTGTTATTATAAACTCATTCGTCCCTGCTTAGAAACCAAAATA
ATGAATAAACAACGAGCTGCAAAAACCTAATCCATCCAATGCTCTTAAAAGATGGAGA
GTATAACCACCCACTTTTCTCTCCTTAATATAAAACAATAATGAAAAAAGTTGTAAAAGC
ATGTTCTTGTTTGATCCCTTCTTAATGATTATAAGAAAAAAAAAAGTCATCTAAGTATGAAT
ATTACTGTTTC

>Seq5 Gossypium hirsutum transgenic insert Cry1ab
GGGGTGCGTGAATCACGAGATCGAGAACAACACCGACGAGCTTAAGTTCTCCAACTG
CGTCGAGGAAGAAATCTATCCCAACAACACCGTTACTTGCAACGACTACACTGTGAAT
CAGGAAGAGTACGGAGGTGCCTACACTAGCCGTAACAGAGGTTACAACGAAGCTCCT
TCCGGTCCTGCTGACTATGCCTCCGTCCTCTTCCTGATTCACAGTGTAGTCGTTGCAAG
TAACGGTGTTGTTGGGATATAATTTCCTTCCTCGACACAGTTGGAGAACTTAAGCTCGT
CGGTTTTGTTCTCGATGGCGTGGATGGTCACGCAACCCTCATACCCTCCTTGTAGGCG
GTCAAA

>Seq6 Gossypium hirsutum transgenic insert Cry1ab/ac
CTCGCGCTTACTGCCTCAGTCGAGATCGAGAACAACACCGACGAGCTTAAGTTCTCCA
ACTGCGTCGAGGAAGAAATCTATCCCAACAACACCGTTACTTGCAACGACTACACTGT
GAATCAGGAAGAGTACGGAGGTGCCTACACTAGCCGTAACAGAGGTTACAACGAAGC
TCCTTCCGGTCCTGCTGACTATGCCTCCGTCATCTTCCTGATTTCAGTGTGGTCGCTTG
CAAGTAACGGTTTTGTTGGGATAGATTTCTTCCTCGACGCAGTTGGAGAACTTAAGCTC
GTCGGTGTTGTTCTCGATGGGGTGGATGGTCACGCAACCCTCCTACCCTCCTTGTAGG
CGGTCCAA

>Seq7 Gossypium hirsutum transgenic insert Cry1ab/ac
GGATTGCACTCTGAGTAGCGAGATCGAGACGCACCGACGAGCGTTAAGTTCTCCAACT
GCGTCGAGGAAGAAATCTATCCCAACAACACCGTTACTTGCAACGACTACACTGTGAA
TCAGGAAGAGTACGGAGGTGCCTACACTAGCCGTAACAGAGGTTACAACGAAGCTCC
TTCCGGTCCTGCTGACTATGCCTCCGTAAAACATCCTGATCCCCCCTGCACCCCCTGC
AACAACCGGTGTTGTTGGGATAGATTACTTCCTCGACCCAGCCGGATAACTTATACTCG
TACTTGTTGTTTGTGATGACGAGGAATACCTACCTACTTACCACTCCGTGCCTTGAATTA
ACGCTAATGATGAGAGACCATTCGAGCCCATGACAATAACCCTCTCTTGTCTTATCCTT
TTTTTGTTATACCTCTCCCCCCCG

>Seq8 Gossypium hirsutum transgenic insert Cry1ab/ac
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GTCGAGGAAGAAATCTATCCCAACAACACCGTTACTTGCAACGACTACACTGTGAATC
AGGAAGAGTACGGAGGTGCCTACACTAGCCGTAACAGAGGTTACAACGAAGCTCCTT
CCGGTCCTGCTGACTATGCCTCCGTACTCTTCCTGATATTCAGTGTGTACGTTTGCGGT
AACGGTGTTGTTGGGATAGATTTCTTCCTCGACGCACTTGGAGAACTTAAGCTCGTCG
GTTTTGTTCTCGATGTCGTGGATGGTCACGCCCCCCGACCCCCCCGGGGGGGGGGGA
AAAAAAAC

>Seq9 Gossypium hirsutum transgenic insert Cry1ab/ac
GACACTGCGTGAATCACGAGATCGAAAACAACACCGACGAGCTTAAGTTCTCCAACTG
CGTCGAGGAAGAAATCTATCCCAACAACACCGTTACTTGCAACGACTACACTGTGAAT
CAGGAAGAGTACGGAGGTGCCTACACTAGCCGTAACAGAGGTTACAACGAAGCTCCT

TCCGGTCCTGCTGACTATGCCTCCGTCATCTTCCTGCTTGCGGTGTCGTTGGTGGCAC
GGGTTGGTTTGGGTGGATTTTATCCCTCCCTCGATTTGGAGAAGATAAGCTACGCTGGT
TTGTTTTGTTTTCGATGGAGTGGATAGTCACGCATCCCCTGTAACCCTTCCTGTAGGCG
GTCAAAAA

>Seq10 Gossypium hirsutum transgenic insert Cry1ab/ac
GACACTGCGTGAATCACGAGATCGAAAACAACACCGACGAGCTTAAGTTCTCCAACTG
CGTCGAGGAAGAAATCTATCCCAACAACACCGTTACTTGCAACGACTACACTGTGAAT
CAGGAAGAGTACGGAGGTGCCCACACTAGCCGTAACAGAGGTTACAACGAAGCTCCT
TCCGGTCCTGCTGACTATGCCTCCGTCATCTTCCTGCTTACGGTGTCGTTGGTGGCACG
GGTTGGTTTGGGTGGATTTTATCCCTCCCTCGAGTTGGAGAAGATAAGCTACGCTGGTT
TGTTTTGTTTTCGATGGAGTGGATAGTCACGCATCCCCTGTAACCCTTCCTGTAGGCGG
TCAAAAA

>Seq11 Gossypium hirsutum transgenic insert CP4epsps
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GACATCGTGCCAACACATTGGGTTGTAATGTTAAAACCACCCTCCTTCCAGCCCTGCTG
ATCCTCTTCCCTTAATTCAGTCTTGCGCTGGTTCTCCTGCCAAATCCCGTTTCATTTACA
GTAAAAAATTCAATTCACTTATTCATAAAACAACTTGTCTGGGTCTTTGAACAAACCTCT
AAACCAGGATGCAACACTGCCATTATACATGTAAGGGTATACCAGTAGTCTTTCGTTAG
AAGTAGCACAATAACCAATCAAACGAAGCAGATTACGGTGAACAGCCAAGCTGATCAT
CTCCCACCGGTCCTTCAAGATTTTTATTTC

>Seq12 Gossypium hirsutum transgenic insert CP4epsps
AGAAATCTGACTACGTAGTCCTCTGGTCTTTCTGGAACCGTCCGTATTCCAGGTGACAA
GTCTATCTCCCACCGGTCCTTGACTCGTTTCTTTGGTGTCTCCCACCGGTCCTTCAATC
GATTTAGTGCGGGGGCCTCTTTTATTCCGTTGGCGAGGATAATAAAACCCCCTTTTTGA
CTGATGAGCATATTACCGTACTCTTGTATAACATGGGGCCCTGCCGTGACAATTCCCAT
ACCTTACTATTAAATACGTATAAAATAAACGCACCCACGACCGTCTTGGGTCTCTTAACT
TAGTCTGAGAATAATAAAGGATGCCATGCGGGGGCAGTGTTCACCTACTTCCCAGTGG
TTAGAAGCGGCCTTAAGTGGGGTAATACCTTGCGTTATTTGTCTCTAACCAATTTGACT
ATTCCCCCGGGGCTGCGATTACCTTATTTTTGTGCTGACTAAACTCCAACCCTCACCA

>Seq13 Gossypium hirsutum transgenic insert CP4epsps
GCTACCTGACTCGTAGTCCTCTGGTCTTTCTGGAACCGTCCGTATTCCAGGTGACAAGT
CTATCTCCCACCGGTCCTTCAACGGTGGTTGTTGGTGAGAGGGTTGAATTGTGATTATT
ACCGACGGCCTTTTATTATCAATTTCTTAGCGTTAACACCCTACAATTTGATGGAAGCG
GCTCGACATGACTTATGTACAAGGCCCTCGAGCATCCAATGCCCCCCACCACAGGAAA
ATATACGAGATTTTGCCTACTCGTGACCACTCCAAATTATAAAAATTAGATAGAGACGT
GAAAAAAGGGTTTTAATTTTTCCACCCCCCAATTGATTCTAAGAAACAATGGGCGGGCC
TCTGGAGTATAACCCTGATT

>Seq14 Gossypium hirsutum transgenic insert CP4epsps
AAAACTCTCGTAGTCCTCTGGTCTTTCTGGACCGTCCGATATTCCAGGTGACAAGTCTA
TCTCCCACCGGTCCTTCAATCTGTTATTGGTGAACCCATTGTTAAGGAAAATCACCGAG
GTGCCTTACTTCCCTTTATTGTTATTATAAACTCATTCGTCCCTGCTTAGAAACCAAAATA
ATGAATAAACAACGAGCTGCAAAAACCTAATCCATCCAATGCTCTTAAAAGATGGAGA
GTATAACCACCCACTTTTCTCTCCTTAATATAAGACAATAATGAAAAAAGTTGTAAAAGC
ATGTTCTTGTTTGATCCCTTCTTAATGATTATAAGAAAACTAGACAGTCATCTAAGTATG
AATATTACTGTTTC

>Seq15 Gossypium hirsutum transgenic insert Cry1ab/ac
GGGGTGCGTGAATCACGAGATCGAGAACAACACCGACGAGCTTAAGTTCTCCAACTG
CGTCGAGGAAGAAATCTATCCCAACAACACCGTTACTTGCAACGACTACACTGTGAAT
CAGGAAGAGTAGGGAGGTGCCTACACTAGCCGTAACAGAGGTTACAACGAAGCTGCT
TCCGGTCCTGCTGACTATGCCTCCGTCCTCTTCTTGATTCACAGTGTAGTCGTTGCAAG
TAACGGTGTTGTTGGGATATAAATTCCTTCCTCCACACAGTTGGAGAACTTAAGCTCGT
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APENDICE III

Host genotype as determinant for the rhizospheric microbial communities: the case of wild cotton metapopulations (*Gossypium hirsutum* L.) in Mexico

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SUPPLEMENTARY MATERIAL

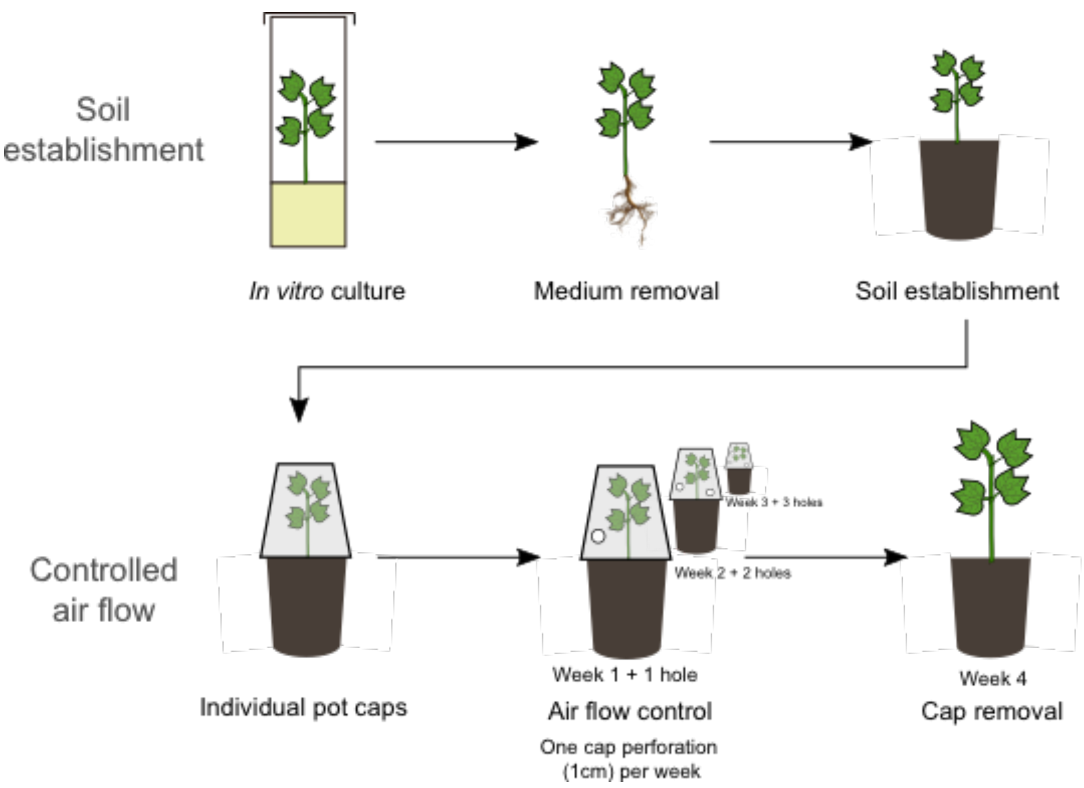


Figure S1. *In vitro*-to-soil acclimation process. Individual plant clones obtained through *in vitro* culture propagation were acclimated to soil pots growth conditions by control of air flow using pot caps as individual "greenhouses". Pot caps were punched (1cm diameter perforation) to create controlled air flow, one perforation per week for three weeks.

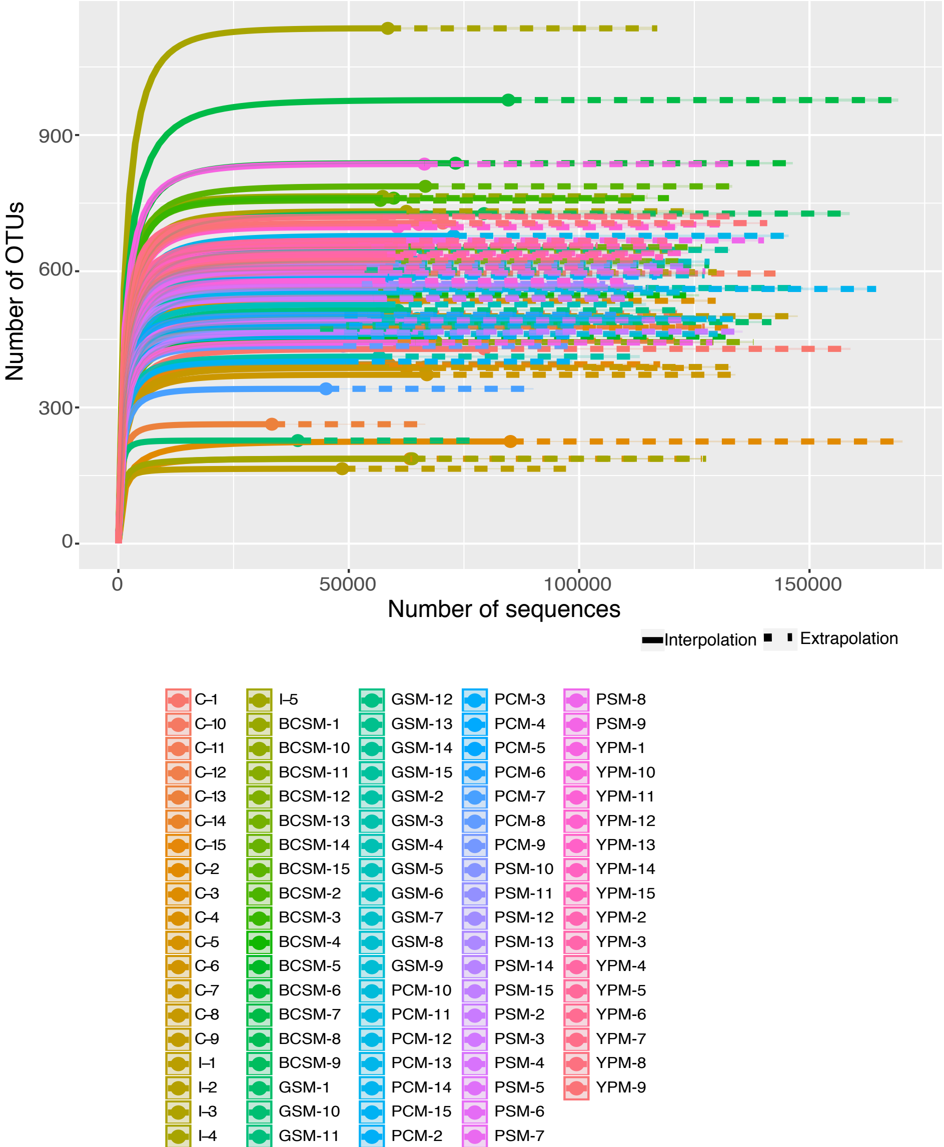


Figure S2. Rarefaction curves of microbial communities of rhizosphere, control samples and inoculum. Rarefaction curves were calculated with the ASVs abundance data using species richness at 95% confidence intervals in the iNEXT R package (REF). Different colors represent different samples, **C-samples** refer to control treatment, **I-samples** to inoculum, **BCSM-samples** to Baja California Sur; **CPM-samples** to Central Pacific, **SPM-samples** to South Pacific, **YPM-samples** to Yucatan Peninsula, and **GSM-samples** to Gulf South.

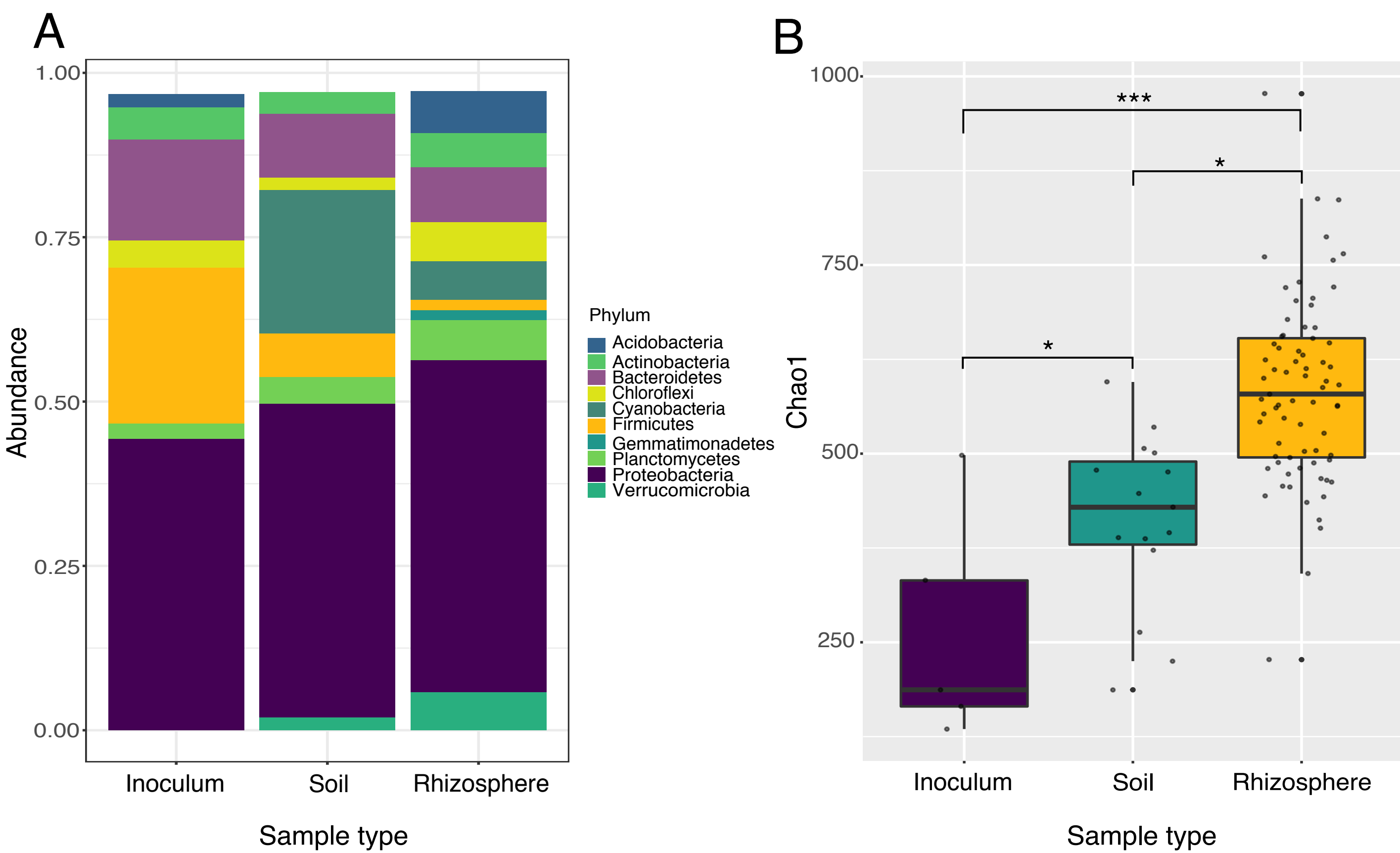


Figure S3. Alpha diversity of microbial communities of rhizosphere, control and inoculum samples. A: Mean relative abundance of the most abundant bacterial phyla per sample type (i.e. inoculum, controls and plant samples). **B:** Box plot of alpha diversity estimates (Chao1 index) of all analyzed samples (i.e. inoculum, controls and plant samples).

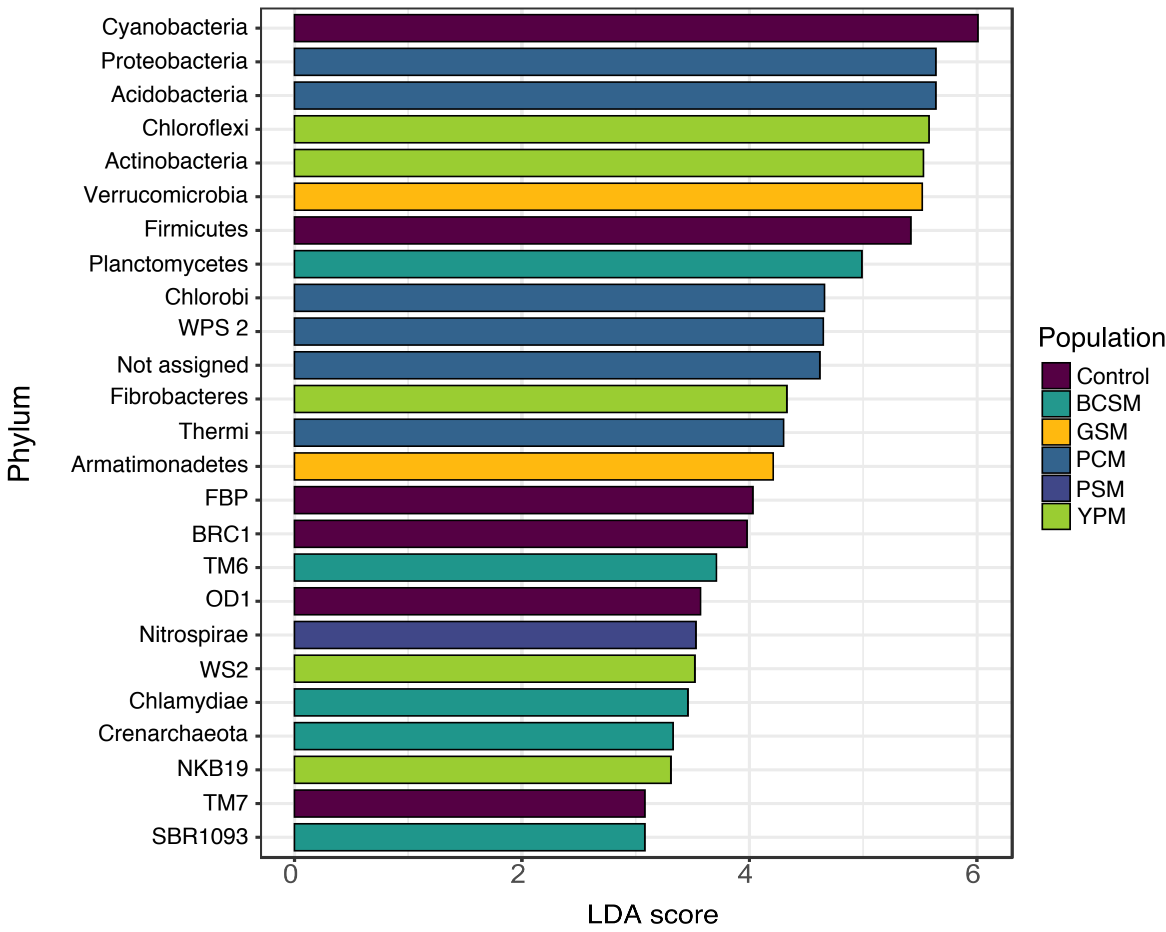


Figure S4. Linear Discriminant Analysis (LDA) effect size (LefSe) analysis. The figure shows differentially abundant groups across rhizosphere and control samples at phylum level. Bars indicate discriminant scores. Name codes for plant populations: Baja California Sur (BCSM), Central Pacific (CPM), South Pacific (SPM), Yucatan Peninsula (YPM), and Gulf South (GSM).

Table S1. Wilcoxon test of NMDS scores for all pairwise sample comparisons. Significant differences are indicated with asterisks and bold font. Name codes for plant populations: Baja California Sur (BCSM), Central Pacific (CPM), South Pacific (SPM), Yucatan Peninsula (YPM), and Gulf South (GSM).

	NMDS1		NMDS2	
Comparison	**W**	***p***	**W**	***p***
Control vs BCSM	16	**1.177e-05***	19	**2.655e-05***
Control vs GSM	112	1	0	**1.289e-08***
Control vs CPM	0	**2.579e-08***	0	**2.579e-08***
Control vs SPM	50	**0.015***	0	**2.579e-08***
Control vs YPM	0	**1.289e-08***	0	**1.289e-08***
BCSM vs GSM	135	0.3669	41	**0.0023***
BCSM vs CPM	2	**1.031e-07***	101	0.8805
BCSM vs SPM	95	0.683	175	**0.0016***
BCSM vs YPM	60	**0.0295***	44	**0.0036***
GSM vs CPM	30	**0.00062***	168	**0.005***
GSM vs SPM	79	0.2703	178	**0.0009***
GSM vs YPM	90	0.3669	174	**0.0098***
CPM vs SPM	178	**7.822e-07***	194	**1.994e-07***
CPM vs YPM	208	**1.031e-07***	3	**1.805e-07***
SPM vs YPM	108	0.9145	0	**2.579e-08***

Table S2. Co-occurrence networks metrics. Metrics values for the inferred co-occurrence networks of microbial communities of all samples (five plant populations rhizosphere and control), calculated with Cytoscape 3.0 (Cline et al., 2007) except for modularity metric which was calculated with R software. Name codes for plant populations: Baja California Sur (BCSM), Central Pacific (CPM), South Pacific (SPM), Yucatan Peninsula (YPM), and Gulf South (GSM).

Metric / Network	Control	BCSM	GSM	CPM	SPM	YPM
Shortest paths	8010	7834	8932	2868	9704	1878
Network radius	4	1	1	1	1	1
Network heterogeneity	0.708	0.768	0.749	0.876	0.648	1.078
Network diameter	8	9	6	5	5	7
Network centralization	0.193	0.198	0.256	0.284	0.271	0.27
Multi-edge node pairs	79	27	52	13	2	4
Density	0.115	0.107	0.104	0.166	0.125	0.082
Connected components	1	2	2	4	2	8
Clustering coefficient	0.553	0.539	0.504	0.502	0.571	0.348
Characteristic path length	3.152	2.913	2.807	2.557	2.508	2.83
Average number of neighbors	10.2	9.604	9.938	9.8	12.475	5.231
Modularity	0.51	0.52	0.55	0.29	0.48	0.4

Table S3. Taxonomic identity of co-occurrence networks hub nodes. Taxonomic identity at the Family/Class level, based on ASV taxonomic assignment on Greengenes database for the more central nodes or hubs (in terms of its betweeness centrality) of each co-occurrence network (Figure 3 left). Co-occurrence networks correspond to five plant populations rhizosphere and experimental controls. Name codes for plant populations: Baja California Sur (BCSM), Central Pacific (CPM), South Pacific (SPM), Yucatan Peninsula (YPM), and Gulf South (GSM).

Network	Node	Betweenness centrality
Control	Moraxellaceae / Gammaproteobacteria	0.177
	Brucellaceae / Proteobacteria	0.157
	Sphingomonadeaceae / Proteobacteria	0.129
BCSM	Desulfovibrionaceae / Deltaproteobacteria	0.178
	Ardenscatenaceae / Anaerolinae	0.159
	Phormidiaceae / Oscillatoriophycidaea	0.114
GSM	Microthrixaceae / Acidiimicrobia	0.143
	[Mogibacteriaceae] / Clostridia	0.143
	Rhodobiaceae / Alphaproteobacteria	0.099
CPM	Methylobacteriaceae / Alphaproteobacteria	0.266
	Rhizobiaceae / Alphaproteobacteria	0.197
	Chloroflexaceae / Chloroflexi	0.140
SPM	Iamiaceae / Acidiimicrobia	0.168
	Gordoniaceae / Actinobacteria	0.136
	Promicromonosporaceae / Actinobacteria	0.133
YPM	Legionellaceae / Gammaproteobacteria	0.8
	Alteromonadaceae / Gammaproteobacteria	0.666
	Hyphomicrobiaceae / Alphaproteobacteria	0.533

Table S4. Principal Component Analysis (PCA) Eigen values for co-occurrence networks metrics. Eigen values of the first two components of the PCA analysis (PC1; PC2; Figure 4A) for all the calculated metrics of the inferred co-occurrence networks. These values were used to select non-redundant metrics for the heatmap representation (see Figure 4B).

Metric	PC1	PC2
Shortest paths	-0.3529	-0.1216
Nodes	-0.3334	-0.7224
Network radius	-0.2075	0.2206
Network heterogeneity	0.34715	0.2231
Network diameter	-0.1350	0.4117
Network centralization	0.2767	-0.302
Multi-edge node pairs	-0.2694	-0.2230
Density	0.0122	-0.3702
Connected components	0.3627	0.1412
Clustering coefficient	-0.3213	-0.2468
Characteristic path length	-0.1710	0.4450
Average number of neighbors	-0.2720	-0.3576
Modularity	-0.3084	0.1490

APENDICE IV

Intervenciones humanas: desde la domesticación hasta la ingeniería genetica y su impacto en la agro-biodiversidad

Alejandra Hernández Terán y Ana E. Escalante
Oikos= 22. 2018.

Intervenciones humanas: desde la domesticación hasta la ingeniería genética y su impacto en la agro-biodiversidad

Oikos= 22
Alejandra Hernández-Terán y Ana E. Escalante

La domesticación en los tiempos de la prehistoria

Uno de los cambios más importantes en las poblaciones humanas sucedió cuando éstas pasaron de ser nómadas a ser poblaciones sedentarias. Este proceso que cambió el curso de la humanidad coincidió con el inicio de la agricultura, cuando nuestros ancestros comenzaron a cultivar y seleccionar características de interés en las plantas silvestres con las que convivían. Esta selección de características, cuyo fin era el de asegurar su sobrevivencia y/o aumentar la productividad, es un proceso evolutivo hecho por y para el hombre que se conoce como domesticación. La constante selección sobre las especies durante este proceso dio lugar a los cultivos que conocemos actualmente, muchos de los cuales tienen muy poco en común con sus parientes silvestres (a partir de los cuales se mejoraron. Ver: Relatos breves sobre evolución y regulación genética). Con el paso del tiempo, la domesticación fue volviéndose cada vez más certera y sofisticada gracias, en parte, a un mayor conocimiento de la biología de las especies, pero también a la introducción de otras tecnologías. Uno de los ejemplos más modernos es la ingeniería genética.

La domesticación en el siglo XX

Actualmente, las nuevas tecnologías permiten la obtención de cultivos con mayor productividad, que incluso presentan características que antes no existían en la especie. Estas tecnologías mediadas por la ingeniería genética se han utilizado para transformar una gran cantidad de cultivos que forman parte de la alimentación básica mundial, tales como el maíz, el arroz, la calabaza, la papaya, la soya, el trigo y algunos otros muy importantes en la industria, como el algodón. Las plantas producto de estas tecnologías son mundialmente conocidas como Organismos Genéticamente Modificados (OGM) o transgénicos. Generalmente, la modificación genética se realiza sobre organismos que ya pasaron un proceso de selección artificial, de modo que un cultivo transgénico es también un cultivo domesticado. Sin embargo, los procesos que nos permiten llegar a cada uno de los tipos de modificación (domesticado y transgénico), son muy distintos. Por un lado, la domesticación tradicional utiliza como materia prima la variabilidad de las especies, es decir, la diversidad de formas y tamaños que ya se encuentran en la naturaleza, y las características buscadas se obtienen al cruzar distintos organismos. En la transgénesis, en cambio, se inserta ADN de otras especies que se sabe que puede conferir características agronómicas importantes a los organismos.

Comparación del cultivo de maíz modern (a) con su pariente silvestre: el teosintle (b). En la imagen se puede observar cómo el proceso de domesticación ha ocasionado cambios fenotípicos extremos, en la apariencia de los cultivos, además del tamaño de la mazorca y el número de granos, la altura de la planta cambió al igual que la forma de las hojas y el número de ramas. Imagen: Carmen Loyola

Consecuencias de la modificación

Como ya mencionamos antes, estos procesos de modificación se centran en sobre-expresar o introducir en el genoma características específicas de interés. Por ejemplo, en el caso del maíz lo que interesó a los primeros agricultores fue aumentar el número de mazorcas por planta y el número de granos en cada mazorca (ver: De la milpa a la mesa: maíz, esquites y más). Sin embargo, en el camino para lograr la manifestación de estas características, otras más fueron alteradas, pues el desarrollo de los organismos vivos es un proceso complejo en el que todo está conectado. Como consecuencia, muchas veces observamos cambios en otras características de las plantas modificadas que no son los que se buscaban. A estos cambios se les conoce en biología como efectos no intencionados de la modificación, y los podemos observar en el fenotipo de la gran mayoría de los cultivos actuales. Estos efectos no intencionados ocurren a través de complejos procesos a nivel genético dentro de los organismos, que son ocasionados tanto por las múltiples cruzas como por la introducción de nuevo material genético. Debido a que estos efectos se han observado en la mayoría de los cultivos que son la base de la alimentación mundial, sus consecuencias en la ecología y evolución de las especies son de especial importancia para las poblaciones humanas. Por otra parte, la agro-biodiversidad que constituyen las poblaciones silvestres y domesticadas es de suma importancia, ya que es la herramienta que le permite a los organismos adaptarse a condiciones ambientales cambiantes como las que se prevén en los escenarios de cambio climático.

¿Qué hicimos?

Los efectos no intencionados de la modificación son un fenómeno ampliamente reportado en laliteratura científica; por esto nos propusimos a compilar toda esa información para poder realizar un análisis integral de estos fenómenos en plantas que tienen gran importancia alimenticia y social. Recopilamos cientos de estudios que analizan características fenotípicas que pueden resultar afectadas en los procesos de modificación por ingeniería genética. Escogimos un total de cinco cultivos representativos: arroz, maíz, canola, girasol y calabaza. Todos ellos tienen una gran relevancia para la alimentación e industria a escala mundial. Seleccionamos todas las investigaciones en las que se hicieron experimentos controlados, donde se comparan características fenotípicas que son importantes para la reproducción y supervivencia de plantas silvestres, domesticadas y transgénicas en un mismo cultivo.

¿Cómo lo hicimos?

Estudiamos los datos de un total de 120 investigaciones publicados en revistas científicas utilizando análisis estadísticos. Dentro de las características fenotípicas analizadas están la altura de la planta, el número de semillas, el número de frutos, los días a la floración y la viabilidad del polen. Realizamos análisis individuales por cada especie, comparando, por ejemplo, el número de semillas del maíz silvestre (teosintle) con el número de semillas del maíz domesticado y transgénico. Además, llevamos a cabo análisis estadísticos que permiten integrar toda la información disponible de un mismo cultivo, para así poder observar, por ejemplo, si podíamos distinguir a las plantas silvestres de las domesticadas y las transgénicas.

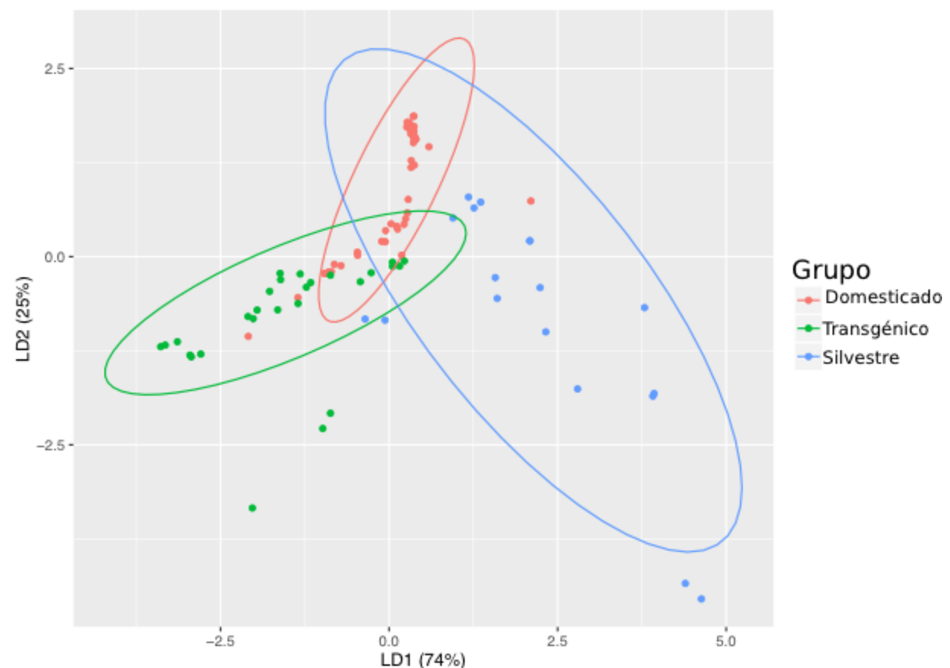
¿Qué esperábamos encontrar?

Buscábamos conocer si los distintos tipos de modificación humana (domesticación y transgénesis) pueden alterar el fenotipo de las plantas de tal manera que fuera posible diferenciar organismos silvestres, domesticados y transgénicos en una misma especie. Adicionalmente, dado que la transgénesis está diseñada para impactar únicamente una característica, sería de esperarse que hubiera menos diferencias fenotípicas entre plantas domesticadas y transgénicas que al comparar silvestres y domesticadas.

¿Qué encontramos?

Luego de analizar toda la información recopilada, encontramos diferencias estadísticas en casi todas las comparaciones de características fenotípicas entre los distintos tipos de plantas (silvestres, domesticadas y transgénicas) dentro de un mismo cultivo. Al analizar de manera integral todas las características dentro del mismo cultivo, encontramos que cada tipo de organismo se puede distinguir claramente, ya que se parecen más entre sí, que con otro tipo de organismos. Por ejemplo, un maíz silvestre comparte más características con otro maíz silvestre, que con un maíz domesticado.

El caso del maíz



Análisis estadístico integral de las características fenotípicas, apariencia, en el cultivo de Maíz. Cada eje de la gráfica se compone por una combinación de las características fenotípicas que ocasionan la posición (ordenamiento) espacial de los distintos grupos de maíz. Los números en los ehes representan el porcentaje que las combinaciones de características explican en la variación encontrada. Los tres grupos están representados por colores: los puntos rojos son las plantas domesticadas, los puntos verdes las plantas transgénicas y los azules las plantas silvestres. Un óvalo más grande representa una mayor variación fenotípica encontrada en las características analizadas. Cuando los óvalos se sobreponen puede significar que comparten similitudes en sus características fenotípicas. Imagen: Hernández-Terán et al, 2017.

El caso del maíz es un buen ejemplo para ilustrar nuestras observaciones ya que presenta casi todos los efectos que encontramos en los cinco cultivos. En la imagen observamos el gráfico correspondiente al análisis integral de los datos del maíz, en el cual se distinguen claramente los tres tipos analizados. Los organismos se agrupan, por su parecido fenotípico, en silvestre, domesticado o transgénico. Observamos también que el óvalo correspondiente a los maíces silvestres es más grande que el de los domesticados, lo que corresponde a una mayor diversidad fenotípica. Esto se debe a que en los procesos de domesticación únicamente se seleccionan algunas características de interés agronómico, dejando de lado gran parte de la diversidad fenotípica, por lo cual los organismos domesticados tienen menor diversidad fenotípica que sus parientes silvestres.

Finalmente, observamos también que, contrario a lo que esperábamos, existe variación biológica entre los organismos domesticados y los transgénicos. Esto contradice nuestras expectativas, pues uno de los supuestos de los OGM es que, al trabajar con características específicas, únicamente éstas se ven afectadas y las demás permanecen sin variaciones. Esto implicaría que al comparar un organismo transgénico con su pariente domesticado más cercano, solamente encontraríamos diferencias en la característica específica que se modificó. Por ejemplo, en el caso del maíz transgénico resistente a plagas, si lo comparáramos con su pariente domesticado encontraríamos que, en un ambiente en presencia de plagas que dañan el maíz, el pariente domesticado sufriría mayor daño que el modificado genéticamente, pues este último cuenta con una nueva característica que le permite defenderse mejor. Sin embargo, nuestros resultados muestran que esto no es una regla, ya que en muchos casos podemos encontrar diferencias, otras características que no son las de interés pero que fueron provocadas por el proceso de modificación, es decir, surgen efectos no intencionados de la modificación.

Nuestra conclusión

Los resultados encontrados en nuestra investigación muestran cómo las intervenciones humanas han causado, en algunos casos a lo largo de miles de años, importantes cambios en las poblaciones de plantas que utilizamos. Aunque históricamente estas estrategias han satisfecho las necesidades de producción de alimento de nuestra especie, es importante reflexionar acerca de las consecuencias de su impacto en la agro-biodiversidad de las poblaciones de plantas. En muchos casos, podemos observar

consecuencias de estas intervenciones humanas en diversas características que no son el objetivo principal de la modificación, es decir, efectos no deseados de la modificación. Sin embargo, las consecuencias ecológicas y evolutivas de estos efectos no deseados siguen sin conocerse. Esto se debe, principalmente, a que en los esfuerzos de regulación y determinación de impacto ambiental de los OGM se analizan únicamente las características en las que se esperan los cambios ocasionados por la modificación, dejando de lado otras que podrían verse afectadas, tales como las presentadas en este estudio.

Con el fin de contribuir a entender mejor este impacto, en el futuro es necesario que los estudios que documenten cambios en cultivos mejorados por el hombre integren el mayor número de características posibles, de modo que permitan la adecuada detección de efectos no deseados. Esto con el fin de diseñar estrategias de mejoramiento de nuevos cultivos, o de conservación, que no comprometan las posibles decisiones de nuevas generaciones humanas en materia de agrobiodiversidad.

Este trabajo se puede obtener gratuitamente en la página de la revista científica Frontiers in Plant Sciencie:

Referencia: Hernández-Terán, A., A. Wegier, M. Benítez, R. Lira y A.E. Escalante. 2017. Domesticated, Genetically Engineered, and Wild Plant Relatives Exhibit Unintended Phenotypic Differences: A Comparative Meta-Analysis Profiling Rice, Canola, Maize, Sunflower, and Pumpkin. Front. Plant Sci. 8:2030. doi: 10.3389/fpls.2017.02030

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APENDICE V

Laboratory biases hinder Eco-Evo integration: hints from the microbial world

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JEZ-B Molecular and Developmental Evolution, 2019.

Received: 4 July 2019 | Revised: 9 September 2019 | Accepted: 2 October 2019
DOI: 10.1002/jez.b.22917

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PERSPECTIVE AND HYPOTHESIS

Laboratory biases hinder Eco-Evo-Devo integration: Hints from the microbial world

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The peer review history for this article is available at https://publons.com/publon/10.1002/jez.b.22917

Abstract

How specific environmental contexts contribute to the robustness and variation of developmental trajectories and evolutionary transitions is a central point in Ecological Evolutionary Developmental Biology ("Eco-Evo-Devo"). However, the articulation of ecological, evolutionary and developmental processes into integrative frameworks has been elusive, partly because standard experimental designs neglect or oversimplify ecologically meaningful contexts. Microbial models are useful to expose and discuss two possible sources of bias associated with conventional gene-centered experimental designs: the use of laboratory strains and standard laboratory environmental conditions. We illustrate our point by showing how contrasting developmental phenotypes in *Myxococcus xanthus* depend on the joint variation of temperature and substrate stiffness. Microorganismal development can provide key information for better understanding the role of environmental conditions in the evolution of developmental variation, and to overcome some of the limitations associated with current experimental approaches.

KEYWORDS
developmental plasticity, gene-centrism, laboratory biases, multicellularity, *Myxococcus xanthus*

1 | ECO-EVO-DEVO AT THE MICROSCALE

Understanding the multi-causational origins of biological variation constitutes a longstanding question. The interest in the variation generated by developmental processes occurring in different environmental conditions, as well as its evolutionary significance, is not new (Gupta & Lewontin, 1982; Sarkar, 2004; Scheiner & Goodnight, 1984; Schmalhausen, 1949). However, since genetic variation has been considered the major cause of phenotypic variation, the organismal interaction with varying environments has been often reduced to noise or to deviations from a norm. From this perspective, a univocal genotype-phenotype relation has been naturally assumed (Lewontin, 2001; Robert, 2004; Sultan, 2017). As such, the goal of much research in developmental biology has been to describe the effects of genetic differences on phenotype.

To reveal the phenotypic effects of genetic change, the dominant experimental approach has included two key elements. First, experimental designs have relied on a limited set of model organisms, sometimes restricted to particular laboratory lines or strains of those species (Ashburner et al., 2000; Kaletta & Hengartner, 2006; Robert, 2004). Second, these studies have deliberately excluded realistic environmental variation, instead rearing organisms in controlled, constant conditions that may be both very different from and much more stable than those in natural environments (Gilbert, 2001). Although these approaches have generated a wealth of valuable results, they have also limited biological understanding to the extent that (a) model organisms do not capture key aspects of biological diversity, and (b) laboratory conditions intentionally restrict potential effects of natural environments (Bolker, 1995; Gasch, Payseur, & Pool, 2016; Gilbert, 2009; Minelli & Baedke, 2014). Thus, some important questions about the developmental and evolutionary

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processes occurring within specific ecological contexts have remained unsolved, or even unaddressed. For instance, how do both plastic and robust processes arise during development under varying natural contexts? What are the mechanisms by which phenotypic plasticity itself shapes ecological interactions? How often and how strongly does plasticity contribute to evolutionary processes such as phenotypic innovation in natural populations?

Considering these questions, there has been a recent increase in attention to phenotypic variation beyond single genetic sources and the role of the environmental context of phenotype expression and evolution (Bateson & Gluckman, 2012; Levis & Pfennig, 2016; Moczek et al., 2011). This has given rise to the "Eco-Evo-Devo" approach, which emphasizes the relevance of the reciprocal interactions between ecology, evolution and development and focuses on multicausal development occurring in the "real-world" (Gilbert, 2001; Sultan, 2003). Despite the great efforts and progress on the conceptual approaches of Eco-Evo-Devo, the integration of the three fields that conform it is not yet complete due, at least in part, to the very nature of traditional experimental designs.

Microorganisms have been invaluable laboratory models in biotechnology and genetic research because they have short generation times and small size, are relatively easy to manipulate genetically and control experimentally, and are resistant to long-term storage (Jessup et al., 2004; O'Malley, Travisano, Velicer, & Bolker, 2015). However, they are largely missing from Eco-Evo-Devo efforts, which have focused mainly on plants and animals, perhaps mostly for practical reasons such as ease of evaluating phenotypic outcomes (Gilbert & Epel, 2015; Sultan, 2015). Microorganismal growth and development offer remarkable examples of the restrictions implied by studies based on model organisms and standard laboratory conditions, which tend to cancel, minimize or underestimate the causal role of environmental variation in development. In fact, microorganisms represent huge biological diversity in terms of their metabolic capabilities and the wide range of ecological contexts they habit (Johri et al., 2005), especially when the environment can be as patchy as a mammalian gut or a soil matrix, containing many physical habitats and numerous other species. Nevertheless, only approximately 1% of the microbial diversity has been cultured using these experimental design strategies, illustrating our limited understanding of the organism–environment interaction required to reproduce microbial species (Nai & Meyer, 2017; Pande & Kost, 2017; Pham & Kim, 2012).

Furthermore, in light of their ubiquity across environments and their diverse uni- and multicellular lifestyles, microorganisms can provide invaluable insights to further understanding organism–environment interactions and the processes generating the variation that enables evolution. Moreover, multicellular microbial groups can yield information about the organism–environment interactions during the evolution of multicellularity since they develop in a scale and environment similar to those in which multicellularity presumably emerged (Arias Del Angel, Escalante, Martínez-Castilla, & Benítez, 2017; Bonner, 2009; Rivera-Yoshida, Del Angel, & Benítez, 2018). Focusing on microorganisms also leads to the study of environmental variables that are less evident or relevant at the macroscale, such as the mechanical properties of cell-to-cell and cell-to-medium interactions (Persat et al., 2015).

Overall, microbial models can help to unmask biases in experimental designs implemented mostly in plants and animals, contributing to a better integration and experimental planning within the Eco-Evo-Devo field. Here, we focus on microbial models to illustrate how gene-centered experimental designs harbor two possible sources of bias: (a) the use of laboratory strains, and (b) standard laboratory environmental conditions. In particular, we gather evidence from different microorganisms and use our own results from *Myxococcus xanthus* development under different environmental conditions to exemplify and comment on these biases. In our opinion, such biases should be explicitly considered when interpreting results and extrapolating them to natural contexts and, ideally, should be overcome in novel empirical approaches.

2 | LABORATORY STANDARD STRAINS VERSUS NATURAL POPULATIONS

Choosing a model organism is often limited to some well-established options. Among microorganisms, *Escherichia coli*, *Bacillus subtilis* and *Saccharomyces cerevisiae* are widely used models (Blount, 2015; Love & Travisano, 2013). The use of laboratory standard strains has undeniable practical advantages that, in turn, reinforce the use of particular strains and species. These advantages include, for instance: (a) existing important technological investment including complete genome sequencing, protein and metabolite quantification methods, and mutant construction; (b) pure genetic lines and robust phenotypes that have been domesticated to grow under simple and standardized laboratory conditions; (c) minimal variation, which leads to tractable, systematic and reproducible results, and thus reliable comparisons; (d) data and techniques that can be shared among research groups, since they correspond with standardized conditions, including strains; and (e) popularity and facilitation of acquiring funding (Ankeny & Leonelli, 2011; Gasch et al., 2016; Leonelli & Ankeny, 2013). These features are particularly useful in exploring genetic mechanisms, since they help control the influence of nongenetic factors.

Nonetheless, while using standard strains can be of great value in microbiology, molecular biology, and some evolutionary studies, it becomes a limitation for other scientific purposes, such as those related with the Eco-Evo-Devo framework. Since biological questions should match the model organism and experimental decisions, standard strains are not well-suited to questions about phenotypic plasticity and its mechanisms, since plasticity has been intentionally or indirectly suppressed through invariant environmental conditions in already relatively unplastic organisms (Love, 2010; Travis, 2006). Indeed, model organisms often exhibit rapid development and developmental canalization (expression of a specific developmental outcome regardless of minor variations in environmental conditions;

Waddington, 1942), a well-known phenomenon in animal models (Bolker, 1995; Gilbert, 2001).

"Domestication" is commonly used to refer to the adaptation of wild strains to new, human-created habitats. When laboratories are the new habitats, domestication occurs in long-term, stable cultures or during repeated passaging (Branda, González-Pastor, Ben-Yehuda, Losick, & Kolter, 2001; Eydallin, Ryall, Maharjan, & Ferenci, 2014; Kuthan et al., 2003; Palková, 2004). For microorganisms, laboratory-domesticated strains express robust phenotypic traits and apparently decreased phenotypic plasticity compared to strains that have been manipulated in the short term (Eydallin et al., 2014). However, whether these traits are actually canalized or not remains to be explored as reaction norm experiments are just starting to become available for microbial systems (Rivera-Yoshida et al., 2019). Thus, while domesticated strains enable important scientific and technical advances, relevant variation possibly occurring naturally at ecological complex scenarios, and the causes behind it, could be encrypted in these strains (Branda et al., 2001; Eydallin et al., 2014; Kuthan et al., 2003; Palková, 2004; Steensels, Gallone, & Voordeckers, 2019).

Laboratory domestication has been reported for several microbial species. Interestingly, several species have been observed to develop common phenotypic traits during domestication when exposed to similar experimental contexts (Table 1). For instance, in laboratory conditions, standard *E. coli*, *B. subtilis* and *S. cerevisiae* strains present a smooth biofilm phenotype compared with the rough one observed in wild type strains (Branda et al., 2001; Eydallin et al., 2014; Kuthan et al., 2003; Palková, 2004). Moreover, in these three cases the smooth phenotype is related to the loss of complexity in the extracellular matrix structure (Table 1). Also, pathogenic laboratory strains present lower virulence compared to the newly isolated strains (Heddleston, 1964; Barak, Gorski, Naraghi-Arani, & Charkowski, 2005; White & Surette, 2006; Sommerville et al., 2011). The phenotypic convergence shared between species that have undergone independent domestication processes is reminiscent of the well-known domestication syndrome observed in crops (Burke, Burger, & Chapman, 2007; Gepts & Papa, 2002).

It should also be considered that domestication and genetic modification processes also involve the unintended selection of nontarget traits (e.g. Hernández-Terán, Wegier, Benítez, Lira, & Escalante, 2017). Laboratory strains for the study of microbial multicellularity are a clear example. Wild *Myxococcus xanthus* and *Bacillus subtilis* strains can develop complex resistance structures in response to adverse environmental conditions. For these multicellular structures to occur, social behavior is needed. However, experimental setups tend to select easily dispersed cells or colonies, and then grow them in unstructured liquid mediums, which is reported to be associated to a reduction in social behavior (Aguilar et al., 2007; Velicer et al., 1998). This domestication pathway may thus hinder collective organization and actually makes them suboptimal for the study of multicellular development (Aguilar et al., 2007).

While there can be some convergences or similarities, it is overall difficult to generalize about domestication processes and outcomes, since different dynamics underlie each specific case. For instance, populations or ecotypes of the same microbial species can be widespread in completely dissimilar environments, and can exhibit different domestication trajectories (Eydallin et al., 2014). Laboratory domestication processes, phenotypes, and metabolic changes depend on the ancestral strains, on physical and chemical properties of the culture medium (e.g. liquid medium vs. hard agar plate), and how long they have been exposed to the culture medium (Eydallin et al., 2014). Finally, studying systems with standardized strains and environmental conditions has the objective of supporting reliable comparisons across different research groups. However, due to the sensitivity of microbial strains to small variations on experimental treatments and also due to their long laboratory life history, sublines of the same laboratory standard strain could present phenotypic and genetic differences (Bradley, Neu, Bahar, & Welch, 2016).

Comparisons between laboratory strains and generalization to wild strains should be done with caution, since domestication processes occurring in association to widespread experimental approaches could impose important biases. The rapid domestication of microorganisms to laboratory conditions highlights the importance of working with recently isolated wild strains, at least for some research questions. In some cases, we do not even know if phenotypes that are commonly observed in laboratory strains actually exist in nature and are ecologically and evolutionarily relevant. For example, while in laboratory conditions *Myxococcus xanthus* forms well-known multicellular structures called fruiting bodies, we are not sure about what fruiting bodies look like when they develop in their natural soil environments. Further studies considering repeated, well-documented and already ongoing lab-domestication processes could also contribute to a better understanding of organism–environment interactions, phenotypic variation and robustness in a wide phylogenetic context (Bradley et al., 2016).

3 | LABORATORY SETTINGS VERSUS NATURAL ENVIRONMENTS

Laboratory strains are good proxies of their wild ancestors if comparisons of their phenotypes and genotypes are not biased due to their history of experimental manipulation. However, that can only occur if (a) the phenotypic outcomes of these strains were invariant with respect to the environment, or (b) if laboratory conditions mimic natural conditions. The latter is clearly an unrealistic assumption, because as soon as an organism is isolated in laboratory culture media, natural environmental variables are modified. Moreover, as explained above, experimental designs have focused not on re-creating natural environments, but on generating "controlled environments" in which selected variables (often genetic variables) can be modified within a constant background (Robert, 2004). In this approach, controlled environments are assumed to be "neutral," but they are actually conformed by several biotic and abiotic components

TABLE 1 Microbial strains commonly used in laboratory conditions

Species	Natural habitat	Laboratory strain phenotype	Wild strain phenotype	Research focus	Laboratory strain limitations	References
Bacillus subtilis	Plant roots	Simple macroscopic architecture	Structurally complex	Molecular mechanisms of colony morphogenesis	Lack of surfactin production: no spreading behavior	McLoon, Guttenplan, Kearns, Kolter, and Losick (2011)
	Soil	Thin, fragile, smooth biofilm				Hong, Fakhry, Baccigalupi, Ricca, and Cutting (2009)
	Animal intestinal tracts		Thick and rough biofilm	DNA mediated transformation	Inability to form resistant spore structures: multicellularity cannot be fully studied	Aguilar, Vlamakis, Losick, and Kolter (2007)
					Loss of genes or mutations	Branda et al. (2001)
Escherichia coli	Soil	Smooth biofilm	Structurally complex	Pharmaceutical production	Changes in biofilm structure: loss against predators	DePas et al. (2014)
	Water					
	Plant tissues		Rough phenotype	Genetic engineering		van Elsas, Semenov, Costa, and Trevors (2011)
	Animal gut			Biotechnology industry	Changes in metabolic properties	Blount, Borland, and Lenskiet (2008)
						Tao, Bausch, Richmond, Blattner, and Conway (1999)
						Mikkola and Kurland (1992)
Bartonella henselae	Animal blood and endothelium	*	Fimbriae presence	Medical research	Mutations and genomic rearrangement during laboratory passaging	Arvand, Schubert, and Viezens (2006)
			Population genetic variation		Decreased genetic variability	Velho, Moraes, Uthida-Tanaka, Cintra, and Gigliogi (2002)
					Loss of fimbriae	
					Lower virulence	
Staphylococcus aureus	Human skin, bones, blood and mucous membrane	*	High growth yield	Medical research	Mutations and genomic rearrangement during lab passaging	Periasamy et al. (2012)
			High ROS production		Lower virulence	Somerville et al. (2002)
			High fitness		Alteration in cell density sensing	
					Alteration of surfactant production	
Pasteurella multocida	Animal lungs, bloodstream and mucous membrane	*	Loss of capsule production	Medical research	Loss of capsule production: critical for its virulence and antibiotics resistance	Steen et al. (2010)
						Harper, Boyce, and Adler (2006)
						Heddleston, Watko, and Rebers (1964)

(Continues)

TABLE 1 (Continued)

Species	Natural habitat	Laboratory strain phenotype	Wild strain phenotype	Research focus	Laboratory strain limitations	References
Salmonella enterica	Birds eggs	Smooth biofilm	Structurally complex biofilm	Infectious disease studies	Diversification of genotypes	Davidson, White, and Surette (2008)
	Plant tissues		Dry and rough biofilm		Altered biosynthesis of cellulose and polysaccharides: loss of spatial phenotype morphology	White and Surette (2006)
	Water					
	Animals intestinal tract				Resistant to desiccation	Barak et al. (2005)
Saccharomyces cerevisiae	Oak bark and other trees and plants	Smooth colonies	Structurally complex biofilm	Genetic engineering	Loss of extracellular matrix	Šťovíček, Váchová, Begany, Wilkinson, and Palková (2014)
	Human microbiota					
	Insects	Change in cells shape	Highly glycosylated extracellular matrix	Biotechnological industry	Gene expression reprogramming	Piccirillo and Honigberg (2010)
	Soil					Diezmann and Dietrich (2009)
	Bread		Resistant to antioxidative stress			Palková (2004)
	Beer and wine		Phenotypic heterogeneity			Kuthan et al. (2003)
			Sporulates on a wider range of carbon sources			
Myxococcus xanthus	Soil	Smooth colonies	Large genetic heterogeneity	Multicellularity	Loss of social behavior: multicellularity cannot be fully studied	Kraemer, Toups, and Velicer (2010)
			Large variation in phenotypic and developmental traits	Cell differentiation		Velicer, Kroos, and Lenski (1998)
				Cell motility		

*No information found.

contributing to the organism–environment interaction, which in turn may give rise to particular phenotypes (Lewontin, 2001; Sultan, 2017).

Within this controlled-environment setting, development -and its plastic nature– cannot be fully understood since it represents only one specific set of a wide possible repertoire of environmental conditions. Furthermore, beyond the constant background, experimental settings where a single variable is selected for modification can also be misleading in several ways. First, selected variables may not be ecologically meaningful for the studied organisms and developmental moment. Second, these variables could be ecologically meaningful but tested in non-significative ranges. Third, selected variables and unconsidered ones could be dynamically interacting and modifying the whole developmental system (see, for instance, Box 1). Also, studies in constant environments may underestimate the effects of cryptic or neutral variation in development and evolution (Kimura, 1983; Payne & Wagner, 2018). Indeed, meaningful environmental features could be the result of additive effects and complex interactions among variables, but it has been usually considered convenient to test only a few "key" variables, mostly in independent experimental sets (Rivera-Yoshida et al., 2019). This approach, commonly associated with reaction norm studies, leads to interpreting the environment as a sum of major variables and, consequently, to limited conclusions.

Microorganisms have been considered important experimental models partially due to their ease of manipulation (Jessup et al., 2004; Love & Travisano, 2013). However, the natural history of most species, even cultivable ones, is unknown. Thus, their successful growth in the laboratory is informative about their ability to adapt to laboratory conditions but not necessarily about their growth and development in ecologically meaningful ones. For instance, the design of culture media is specially focused on chemical components for nutrient supply, while other physical or ecological factors are often overlooked. Remarkably, choosing the correct media chemical properties is not an easy task and may itself uncover interesting environmental dependencies (Uphoff, Felske, Fehr, & Wagner-Döbler, 2001).

The uncultivability phenomenon can provide clues about meaningful variables and ranges of natural settings neglected in current experimental designs, for example, by contrasting experimental properties with natural ones. Here we identify some key experimental conditions that differ from natural contexts. Growth media are restricted to either solid agar plates or liquid cultures commonly kept at constant agitation, which in turn, is known to favor loss of social behavior after just a few generations (Velicer et al., 1998). For agar plates, stiffness is standardized by fixing the agar concentration, but phenotypic plasticity has been described for microbial development and growth at different substrate stiffness (Be'er et al., 2009; Guégan et al., 2014; Rivera-Yoshida et al., 2019; Box 1). Also, agar plates represent flat and unstructured surfaces, determining properties of microbial aggregates and films such as movement, size and surface tension (Persat et al., 2015; Rivera-Yoshida et al., 2018). Nutrient supply is constant at optimum concentration rates or at complete scarcity. Genome reduction –also known as genome streamlining– occurs in natural populations when interacting species are metabolically complementary but also in long-term laboratory conditions (Koskiniemi, Sun, Berg, & Andersson, 2012; Lee & Marx, 2012; Pande & Kost, 2017). Constant and high nutrient supply are often part of experimental conditions, which could partially explain genome streamlining. Environmental settings are also restricted to small and homogeneous areas, limited by instrument walls or growing space. In contrast, natural environments are heterogeneous at different scales, which could in turn drive or constrain collective phenomena. For instance, the multicellular phenotype of *M. xanthus* can be characterized at the single fruiting body structure, but also at the population level (Rivera-Yoshida et al., 2019; Box 1). The population spatial distribution is determined by the experimental conditions (i.e. size of the culture plate or flask), however, it is unknown whether phenotypic expression at the individual or population scales are expressed in large and complex media like soil.

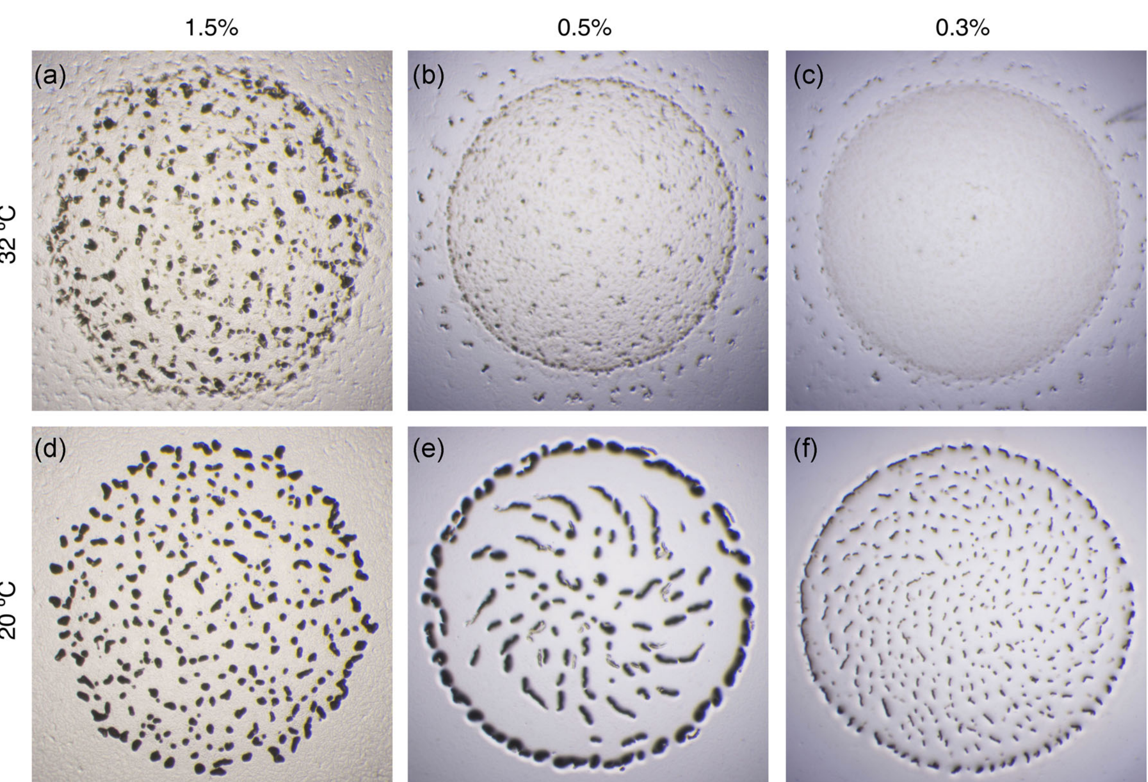
Abiotic physicochemical factors such as temperature, humidity, pH, pressure, salinity, and oxygen concentration, among others, are usually kept constant. However, these are important parameters determining microbial interactions and metabolism (Pham & Kim, 2012). Some of our own unexpected results with *M. xanthus* show how variations in temperature and medium stiffness can strongly affect bacterial multicellular development (Box 1). Indeed, *M. xanthus* fruiting bodies exhibit contrasting phenotypes when developed at different substrate stiffness (Box Figure 1), so much so that at very low stiffness and standard temperature, no fruiting bodies are formed (Box Figure 1). At standard stiffness, temperature modification yields little phenotypic variation, which could easily lead to the immediate conclusion that temperature does not affect development in a significant way. However, temperature variation reveals drastically different phenotypes at non-standard stiffness conditions, widening the spectrum of phenotypic variation associated with stiffness change (Box Figure 1). The joint modification of these two factors renders a phenotypic diversity that could not have been expected from *M. xanthus* being grown at standard conditions, nor from reaction norm experiments considering a single environmental factor (Rivera-Yoshida et al., 2019).

Besides the experimental substrate, other differences between experimental and natural settings can be associated with the management of biological material. Development or growth rates may be different among species, requiring longer or shorter periods to become visible to the experimenter. However, given the high nutrient supply, no more than a few days are given to cultures for their density to increase. Also, population densities are probably much higher than on natural substrates (Pande & Kost, 2017). In *M. xanthus*, multicellular development of fruiting bodies under conditions of nutrient scarcity is known to happen at high cell densities, around 1×10^4 cells per fruiting body (Velicer et al., 1998), but actual cell density in natural substrates remains unknown. In the likely case that natural densities are much lower than experimental ones, what is known about developmental and quorum sensing mechanisms might be substantially different in natural populations. Additionally, axenic cultures are promoted in experimental

Box 1 Organism–environment interactions shaping multicellular development in *Myxococcus xanthus*

M. xanthus is a widespread soil bacterium with a multicellular developmental stage. It moves by gliding over semi-solid surfaces in the direction of the cell's long axis. In nutrient-rich substrates, cells are in a vegetative stage and swarm, expanding the colony outwards. When nutrients are depleted, they glide inwards, developing multicellular structures called fruiting bodies (FBs), where some cells eventually differentiate into resistant spores (Yang & Higgs, 2014). The standard protocol for multicellular development consists of depositing a drop of a liquid culture medium onto a nutrient depleted agar plate, commonly prepared with 1.5% agar. After the drop dries, the plate is stored at 32°C avoiding light for about 96 hr until FBs have developed.

M. xanthus cells sense and respond to the structural and mechanical properties of the substrate over which they move. For example, they realign perpendicularly to mechanical compression applied to the agar mesh (Fontes & Kaiser, 1999; Lemon, Yang, Srivastava, Luk, & Garza, 2017). Additionally, *M. xanthus* development has revealed two scales of phenotypic expression: the single FB scale and the population scale (the collection of FBs within a drop), both of which present phenotypic plasticity when substrate stiffness is modified (Rivera-Yoshida et al., 2019). The effect of other variables such as temperature, has not been widely or systematically tested. Furthermore, substrate stiffness is modified by varying the agar concentration. However, substrate mechanical properties might be the result of the interaction of more than this single variable. For instance, substrates with the same agar concentration but different temperatures, could differ in stiffness.



Box Figure 1. Phenotypic plasticity of *Myxococcus xanthus* multicellular structures. Micrographs of completely matured fruiting bodies (FBs) populations developed over TPM agar plates. Black structures are FBs. The DZF1 standard laboratory strain was tested modifying temperature and agar concentration: (a) standard protocol condition: 32°C and 1.5% agar concentration. (b) 32°C, 0.5%; (c) 32°C, 0.3%; (d) 20°C, 1.5%; (e) 20°C, 0.5%; and (f) 20°C, 0.3%. Micrographs of each drop were taken at 370.8 pixels/mm using a LEICA m50 stereomicroscope with an ACHRO ×0.63 objective lens and a Canon-EOS Rebel T3i camera. Apart from variation in temperature and agar percentage, *M. xanthus* were grown and developed as described in Yang and Higgs (2014).

designs so that species are intentionally isolated from interspecific interactions. Yet, the importance of dependence, predation and cooperation, among other interactions, for microbial growth and development are largely known (Jacobi, Reichenbach, Tindall, & Stackebrandt, 1996; Pande & Kost, 2017). Finally, laboratory populations are mainly composed of clonal populations so that their genetic background lacks the heterogeneity observed in natural populations (Eydallin et al., 2014; Gasch et al., 2016).

The organism–environment interaction is a constantly changing bidirectional process, which also changes with spatiotemporal scale. In both natural and experimental settings, organisms contribute to the reconstruction of the interspecies niche (Miner, Sultan, Morgan, Padilla, & Relyea, 2005; Ryan, Powers, & Watson, 2016). For instance, bacterial extracellular matrix secretion is altered by the medium mechanical properties, which in turn are altered by the extracellular matrix secretion (Be'er et al., 2009; Fauvart et al., 2012; Rivera-Yoshida et al., 2018; Trinschek, John, Lecuyer, & Thiele, 2017). Thus, dynamics associated with natural and experimental settings cannot be fully compared as they follow their own evolutionary tempos and paths. Complex ecological interactions are still far from laboratory proxies and efforts to improve protocols in the field or alternative experimental designs that consider environmental complexity are thus necessary.

4 | FINAL REMARKS

The microbial world has provided new insights and approaches in the study of organism–environment interactions at both the evolutionary and ecological levels (Jessup et al., 2004; Love & Travisano, 2013; O'Malley et al., 2015; Rivera-Yoshida et al., 2018). However, developmental mechanisms have been only partially understood since they have been studied through the establishment of experimental designs using domesticated strains and invariable conditions. This approach is only informative about a specific and simplified condition from the wide repertoire of environmental settings occurring in nature, in which phenotypic plasticity mechanisms may be obscured. Nevertheless, observations of microbial development highlight the importance of commonly overlooked, yet meaningful properties of the environment at the microscale. For instance, mechanical factors affecting living and nonliving matter play a key role determining substrate properties, which in turn, modify organisms' dynamics, such as spread, movement and development (Persat et al., 2015; Rivera-Yoshida et al., 2018). Microbes' plastic responses to other ecological factors such the presence of predators, interspecies interactions or environment fluctuation remain largely unknown.

The use of laboratory models and conditions like the ones described above respond, at least in part, to the pressure on science to be efficient in terms of time and costs, which in turn favors certain experimental setups and approaches, including standardized organisms and limited experimental conditions (Ankeny & Leonelli, 2011; Leonelli & Ankeny, 2013; Levins & Lewontin, 1985). Compelled by these pressures, microbial ecological and evolutionary processes are probably forced into tempos and conditions that do not match those of natural environments, leaving many open questions. For example, what are the ecologically relevant spatiotemporal scales and variables for microbial development? Is the strength and expression of phenotypic plasticity scale-specific? How plastic are interspecific interactions? How do different environmental variables interact with each other to affect microbial development?

Furthermore, the role of phenotypic plasticity as a driver of and constraint on evolutionary mechanisms, considered in several "plasticity-first" hypotheses, is probably underestimated since it cannot be easily tested in current experimental designs nor compared with phenomena occurring in natural populations (Levis & Pfennig, 2016). For instance, to the best of our knowledge, environmentally-triggered phenotypic novelties, canalization, and complex interactions among environmental variables have not been explored in microbial systems, not even in paradigmatic long-term evolutionary studies. Additionally, due to their high mutation rate and short generation time, microbial groups could be suitable to the comparison of adaptations driven by plasticity versus mutation.

To tackle some of the questions regarding plasticity, canalization and other processes derived from the organism–environment interaction it is worth noting that current experimental designs have mostly focused on paired relationships between single traits and specific environmental factors (e.g. the reaction norm approach) (Rivera-Yoshida et al., 2019; Sultan, 2015). Moreover, this punctual relationship is studied for a given moment and not for the whole developmental trajectory, nor for transgenerational processes (Sultan, 2015). It would thus be important to reinvestigate conventional protocols and their assumptions and to propose new approaches considering the dynamical interaction among numerous phenotypic traits and ecologically meaningful environmental variables.

Overall, further investigating microbial multicellular development and considering the practical biases underlying its current study can provide invaluable insights for the integration of Eco-Evo-Devo and understanding of major transitions in evolution.

ACKNOWLEDGEMENTS

Natsuko Rivera-Yoshida is a doctoral student in the Programa de Doctorado en Ciencias Biomédicas, Universidad Nacional Autónoma de México (UNAM) and received fellowship 580236 from CONACYT. The authors thank Sonia Sultan for encouraging us in pursuing this study and for her valuable comments on a previous version of this opinion piece. The authors thank members of LANCIS, Marcelo Navarro-Díaz, Alejandro V. Arzola, Alessio Franci and Juan A. Arias Del Angel for their feedback and Morena Avitia for her technical assistance. DZF1 strain was kindly provided by J. Muñoz-Dorado. Lynna Kiere's proofreading was extremely helpful. Authors thank two anonymous reviewers for valuable comments and suggestions.

CONFLICTS OF INTEREST

The authors declare there are no competing interests.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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How to cite this article: Rivera-Yoshida N, Hernández-Terán A, Escalante AE, Benítez M. Laboratory biases hinder Eco-Evo-Devo integration: Hints from the microbial world. *J Exp Zool (Mol Dev Evol)*. 2019;1–11. https://doi.org/10.1002/jez.b.22917

APENDICE VI

Enfrentando el reto de evaluar los daños ambientales ocasionados por organismos genéticamente modificados

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En: Antropización: Primer Análisis Integral, 2019.

Enfrentando el reto de evaluar los daños ambientales ocasionados por organismos genéticamente modificados

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Resumen

Esta propuesta surge a partir de la necesidad de evaluar y comparar los daños ambientales que los organismos genéticamente modificados pudieran ocasionar. Estos cambios ambientales son, a su vez, procesos de atropización complejos, que se originan en distintos niveles ecosistémicos, consecuencia de cuatro diferentes tipos de adversidad (el flujo de genes, los cambios ocasionados por los organismos genéticamente modificados (OGM) en sí mismos, efectos en organismos que no son el objetivo de desarrollo de los OGM y la evolución de la resistencia de los organismos que se pretende controlar con el OGM). Esto a su vez se relaciona con el tiempo que duran los impactos en el ambiente y las acciones que se pueden realizar para mitigarlos. Poner en práctica esta metodología debe integrar la mayor cantidad de información disponible sobre los organismos que se estén evaluando y a su ves apoyará a identificar la información faltante. Con este modelo esperamos contribuir a una mejor aplicación de las leyes y proporcionar una herramienta útil para los tomadores de decisiones, investigadores y sociedad civil interesada, así como propiciar las discusiones científicas sobre el tema.

Introducción

Las poblaciones humanas manejan, con diferente intensidad, los ecosistemas, sus componentes, procesos y funciones, con el propósito de adaptar los ambientes para hacerlos más habitables y asegurar la disponibilidad y continuidad de los recursos (Morales *et al.*, 2017). La domesticación es un proceso evolutivo continuo derivado de dicha interacción con el ambiente y es quizás la manifestación humana más importante de la transformación del planeta (Kareiva *et al.*, 2007). Generalmente, el proceso de domesticación se estudia a nivel de poblaciones, pero también puede ser entendido desde una perspectiva del paisaje (Clements *et al.*, 2014) por lo que, como consecuencia de la complejidad socioambiental del espacio y el tiempo, se analiza a partir de diversas disciplinas; sin embargo, independientemente del enfoque de estudio, sus causas y

efectos son componentes de los procesos de antropización (Szujecki, 1987; August *et al*., 2002; Bogaert *et al*., 2011).

Los procesos de domesticación pueden implicar distintos manejos, prácticas o técnicas, que dependen de la especie de interés y su entorno. En Mesoamérica, por ejemplo, los paisajes son transformados a causa de prácticas conocidas dentro de los procesos *in situ* de domesticación de plantas, pero que son aplicables a cualquier especie: recolección sistemática (los productos o individuos completos son seleccionados, extraídos y transportados) (Casas *et al*., 2007); individuos tolerados para su aprovechamiento en paisajes transformados; estimulación del crecimiento con base en estrategias dirigidas a incrementar la densidad de algunas especies útiles; o especies protegidas mediante la eliminación de competidores y depredadores de la especie de interés, protección contra adversidades climáticas e incluso adición de insumos al sistema (Diamond, 2002; Larson y Fuller, 2014). Estos procesos impactan en cada población que conforma la comunidad y en las comunidades que forman el ecosistema o agroecosistema.

En la actualidad existe una nueva tecnología directamente relacionada con el manejo y domesticación de plantas, conocida como ingeniería genética moderna. Esta técnica, a partir de un conjunto de métodos biotecnológicos, permite la transferencia de ADN entre organismos muy distantes taxonómicamente o su modificación y edición de la misma especie. Dichas modificaciones, generalmente motivadas por intereses económicos externos a los paisajes transformados, pueden conducir a impactos ambientales muchas veces inesperados, dentro o fuera del área de liberación, que adquieren gran relevancia a causa de la creciente demanda mundial de la producción de OGM; (Hails, 2000; Hilbeck *et al*., 2011; Brookes y Barfoot, 2012; Arpaia *et al*., 2017).

La utilización a gran escala de la ingeniería genética moderna ha generado la necesidad del desarrollo de leyes locales y tratados internacionales para regular su uso seguro. Debido a lo anterior y a la naturaleza de las transformaciones genéticas, los efectos no esperados más documentados o previstos teóricamente son aquellos que afectan directa o indirectamente a la fauna (Tabashnik *et al*., 2008; Tabashnik *et al*., 2013; Zeilinger *et al*., 2015), sin embargo, a partir de una visión integral del entorno, pueden esperarse consecuencias en otros componentes del ecosistema, tales como alteraciones en las cadenas tróficas a las que pertenece la fauna afectada, pero también otras propiedades emergentes de los niveles ecológicos superiores. Por lo tanto, pro-

ponemos un método práctico que podría utilizarse cuando exista sospecha de un impacto ambiental -en cualquiera de sus niveles- causado por OGM, empleando cualquier información disponible o sirviendo de guía para generar una línea de base apropiada para estudios adicionales y esfuerzos de monitoreo. Así, pretendemos entender a los componentes del ecosistema dentro de una metodología que permita cuantificar y comparar los daños ocasionados por los OGM (figura 1).

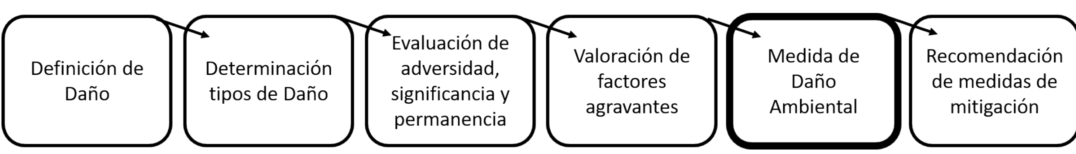

Figura 1. Representación esquemática de la metodología para la evaluación de daño ambiental causado por OGM.

Definición de daño ambiental producido por OGM

Desde 1992, el Convenio sobre la Diversidad Biológica (CDB) y, posteriormente, el Protocolo de Cartagena sobre Seguridad de la Biotecnología (2000), mencionaron la posibilidad de que se produzcan "efectos secundarios", "riesgos potenciales" e, incluso, "impactos o daños ambientales" causados por OGM, sin proponer una definición formal de dichos conceptos. En México, a raíz de estas y otras preocupaciones regulatorias, surge la Ley de Bioseguridad de Organismos Genéticamente Modificados (LBOGM; 2005) que a su vez menciona de manera recurrente términos como: "posibles riesgos", "efectos adversos", "daños graves, irreversibles, irreparables, al medio ambiente y a la diversidad biológica", sin definirlos, ni mencionar las implicaciones de los mismos (DOF 08/03/2005). Con el fin de llenar ese vacío conceptual y con la creciente necesidad de regular las actividades vinculadas a los OGM en el mundo, el Protocolo Suplementario de Nagoya-Kuala Lumpur sobre Responsabilidad y Compensación del Protocolo de Cartagena (2010), definió como daño: *un efecto adverso en la conservación y el uso sostenible de la diversidad biológica teniendo en cuenta los*

riesgos para la salud humana. Además, el protocolo establece claramente que el daño a la biodiversidad debe ser medible, o por lo menos observable, con base en evidencia científica sólida. En consecuencia, provee elementos a evaluar para determinar si el daño es significativo: a) que el cambio sea a largo plazo o permanente (no será reparado por recuperación natural en un periodo de tiempo razonable); b) el alcance de los cambios cualitativos o cuantitativos que afectan negativamente a los componentes de la diversidad biológica; c) la reducción de la capacidad de los componentes de la diversidad biológica para proporcionar bienes y servicios; y d) la amplitud de los efectos adversos sobre la salud humana en el contexto del Protocolo.

La definición que plantea el Protocolo de Nagoya-Kuala Lumpur, establece el punto de partida hacia la evaluación del daño ambiental, sin embargo, la falta de una metodología para cuantificarlo impide la aplicación legal y práctica del documento (Bartz *et al*., 2010; Sanvido *et al*., 2011; 2012). El CDB, discute que la introducción de OGM puede incluir impactos adversos y significativos sobre la diversidad biológica, la cual se entiende como: la variabilidad de organismos vivos de cualquier fuente, incluyendo ecosistemas terrestres, marinos y otros ecosistemas acuáticos y los complejos ecológicos de los que forman parte. Esto incluye diversidad dentro de especies, entre especies y de ecosistemas (CDB, 1993). Por lo tanto, la determinación del daño requiere que los recursos naturales sean considerados en su totalidad y en términos de sus componentes. Estos componentes van desde los genes hasta los niveles ecológicos superiores, tomando en cuenta las propiedades emergentes de cada uno (*e.g.* composición, estructura, funciones; Bartz *et al*., 2010) e incluyendo los factores abióticos. Lo anterior sienta un marco teórico sobre el cual se establecen los elementos y parámetros que deben ser considerados en las metodologías que persiguen evaluar el daño ambiental.

Tipos de daño ambiental ocasionado por OGM

Para poner en práctica la metodología que plantearemos, primero es necesario definir cuatro categorías de daños ambientales que se han relacionado con los OGM (NRC, 2002; Ervin *et al*., 2003; Ellstrand, 2006; Lu y Yang, 2009). Las evaluaciones se deben realizar, cuando exista sospecha de un impacto ambiental causado por OGM o simplemente para monitorear su ausencia. Se recomienda conformar un Comité Técnico-Científico (CTC) conformado por expertos en áreas teóricas y apli-

cadas, que pueda delegar a especialistas los estudios necesarios para emitir las resoluciones finales.

Tipo 1: Los daños asociados con el movimiento de los genes y su subsiguiente expresión en diferentes organismos y especies

Los daños asociados con el movimiento de genes pueden suceder por varias vías de dispersión. Los más estudiados son la transferencia horizontal y el flujo génico, el cual puede ocurrir después de la migración de semillas, propágulos GM, cigotos, huevos y larvas, entre otros (Ellstrand, 2003).

La dispersión de polen o gametas da una oportunidad para la transferencia sexual de transgenes a organismos relacionados, incluyendo otras especies, variedades del cultivo de la misma o parientes silvestres de la especie y parientes cercanos (Ellstrand, 1999; 2003). El vector específico depende de las características de cada organismo, siendo el viento, agua, insectos y mamíferos, los principales transportes (Lu y Yang, 2009). El flujo génico además podría ocurrir sistemáticamente con diferente intensidad, por lo que sus características y causas se deben incorporar. Una vez que el flujo génico o la introgresión ha ocurrido se desconoce el nuevo contexto genético, por lo tanto, es generalmente indeterminado su riesgo por las evaluaciones previas a la liberación del OGM.

El flujo de genes entre los cultivos GM y las poblaciones asilvestradas de los cultivos o parientes silvestres, puede generar maleza que contenga adaptaciones derivadas de sus progenitores, tales como mayor tiempo de latencia de las semillas o reducción de asistencia humana para la reproducción, que son suficientes para incrementar la persistencia e invasividad de estas plantas como malezas (Goodman y Newell, 1985; Ellstrand, 1988; NCR, 1989; Fitter *et al.*, 1990; Boudry *et al.*, 1993; Keeler *et al.*, 1996; Snow y Moran Palma, 1997; Ammann *et al.*, 2000; NCR, 2000; Lu y Yang, 2009). Esto tiene un efecto en la composición del paisaje, las comunidades y ecosistemas, además de un impacto directo en los agroecosistemas (NRC, 2002).

Otro de los daños asociados a la dispersión de polen y gametas, es la sustitución de las especies comunes y locales por las que tienen transgenes, incrementando los riesgos de extinción por hibridación (Ellstrand y Elam, 1993; Johnson *et al.*, 2006). Los modelos teóricos han demostrado que la extinción por hibridación puede proceder rápidamente, resultando en una extinción local de poblaciones. Un ejemplo de

esto es la hibridación entre cultivos y parientes silvestres implicados en un incremento de riesgo de extinción, como la desaparición de los cocos silvestres (Harries, 1995), el flujo genético de especies cultivadas de nogal hacia las poblaciones silvestres en California (Skinner y Pavlik, 1994; Potter *et al*., 2002), o bien, el impacto en el salmón del Atlántico (*Salmon salar*) por el escape e invasión de salmón cultivado en granjas (Fleming *et al*., 2000).

Tipo 2: Daños asociados causados directa o indirectamente por los OGM

Este daño se identifica cuando es provocado por el OGM y las subsiguientes generaciones, ya sea directamente en los ecosistemas o en los servicios ecosistémicos asociados. Los *efectos directos* pueden llevarse a cabo por derrames accidentales de semillas durante el proceso de transporte (Crawley y Brown, 1995; Johnson *et al*., 2006; Von Der Lippe y Kowarik, 2007) o bien, puede ocurrir directamente desde los cultivos GM hacia los ambientes que se encuentran en los alrededores (Arnaud *et al*., 2003; Dyer *et al*., 2009) o incluso dentro del mismo agroecosistema donde fueron legal o ilegalmente sembrados. Las consecuencias usualmente asociadas con la dispersión de semillas es un incremento de malezas y plantas invasoras, por lo tanto, ocurre un cambio de las interacciones dentro de las comunidades, entre otras (Dale *et al*., 2002; Ervin *et al*., 2003; Gepts y Papa, 2003; Johnson *et al*., 2006; Lu y Yang, 2009).

Los OGM pueden convertirse en un riesgo ambiental debido a los rasgos que reciben para mejorar su aptitud y el rendimiento ecológico. Dependiendo de su ubicación, algunos cultivos (por ejemplo, los tomates) evolucionan hacia un fenotipo de tipo salvaje muy rápidamente y se podrían convertir en poblaciones asilvestradas viables en la generación F_2. La existencia de estas poblaciones demuestra que los transgenes que confieren adaptación a importantes factores limitantes pueden crear riesgos significativos relacionados con la planta completa, especialmente si los efectos ecológicos de los cultivos GM son evaluados en forma global (NRC, 2002; Hancock, 2003). La frecuencia de las poblaciones asilvestradas de los cultivos también revela la dificultad de distinguir entre los daños provocados por el flujo de genes y aquellos provocados por toda la planta. Se ha propuesto que este tipo de daño podría, de igual manera, ocurrir posterior a la liberación de otros organismos, tal es el caso de los mosquitos GM (*Anopheles gambiae*), siendo los principales riesgos la disminución en la aptitud de los organismos GM y la aparición de cepas resistentes (Riehle *et al*., 2003).

Los *efectos asociados indirectamente a OGM* dependen de la expresión de los transgenes. Los OGM podrían causar un daño ambiental en los factores abióticos asociados a los mismos, como por ejemplo, agua, suelo o aire, reduciendo la calidad del medio ambiente y su sustentabilidad (Stotzky, 2000; US Environmental Protection Agency, 2000; Dale *et al.*, 2002; Dunfield y Germida, 2004), o con repercusiones sobre los servicios ecosistémicos. Un ejemplo de efectos sobre el servicio ecosistémico de abastecimiento, es el caso de la miel de abeja en la Península de Yucatán, que comenzó a ser rechazada por los mercados internacionales en 2014 debido a la presencia de polen GM en la misma (Vera, 2012; Villanueva-Gutiérrez *et al.*, 2014).

Tipo 3: Los daños a los organismos no blanco

Los organismos *no blanco* son todos aquellos individuos que el diseño y uso del OGM no pretende afectar. A la fecha, la gran mayoría de los estudios publicados que examinan esta problemática se han centrado en los transgenes con propiedades insecticidas, fuera y dentro de los agroecosistemas. La lista de las posibles especies no blanco es muy extensa, algunos OGM ni siquiera fueron creados con un blanco específico, si no que se desarrollan, por ejemplo, para sobre expresar o inhibir alguna característica intrínseca, tales como disminución de la oxidación en manzanas (Bulley *et al.*, 2007) y maduración tardía en tomates (Wang *et al.*, 2005). Estos organismos pueden ser convenientemente agrupados en cinco categorías que no son mutuamente excluyentes: 1) especies benéficas, incluyendo los enemigos naturales de las plagas (crisopas, catarinas, avispas parásitas, microbios y parásitos) y los polinizadores (abejas, moscas, escarabajos, mariposas, aves y murciélagos); 2) plagas no blanco; 3) los organismos del suelo; 4) las especies que no entraron en las categorías anteriores y fueron afectadas por los OGM; y 5) daños en especies presentes en otros sistemas productivos (Hilbeck *et al.*, 1998a; Hilbeck *et al.*, 1998b; NRC, 2002).

Un claro ejemplo es la alteración en la tasa de sobrevivencia y fecundidad de los pulgones *Aphis gossypii* y *Myzus persicae* cuando son afectados por Cry1A+CpTI y nptII (Alla *et al.*, 2003) respectivamente, que fueron diseñados para controlar plagas específicas de lepidópteros (Liu *et al.*, 2005) y aunque aún no está reportado un efecto adverso, está demostrado que el transgén puede estar presente en los depredadores de los organismos blanco (Harwood *et al.*, 2005; Obrist *et al.*, 2006). Por otro lado, a nivel comunidad, se ha observado la modificación de las interacciones y diversidad

de la artropofauna debido a la alteración que tienen las poblaciones de organismos blanco (Sosa y Almada, 2014 ; Pálinkás *et al*., 2017). Otro reporte de alteración a nivel comunidad en nematodos es el de Liu y colaboradores (2015).

Tipo 4: Evolución en organismos blanco

Los organismos blanco son las especies que se pretende controlar al diseñar y usar un OGM. En muchos de los casos el *Tipo 4* no se aplicará porque todas las especies relacionadas con el OGM serán analizadas en el *Tipo 3*, por ejemplo, cuando la evaluación se realice en un ecosistema donde no se pretendía la liberación del OGM. Aunque exista el pariente silvestre de la especie plaga, será tomado como organismo no blanco, ya que no era el uso previsto de la tecnología.

La evolución de la resistencia puede ocurrir en los organismos que son objeto de control del OGM debido a la presión de selección constante (Gould, 2000; Moyes *et al*., 2002; Senior *et al*., 2002). Su daño potencial es esperado cuando los controles alternativos tienen más riesgos ambientales que el uso del OGM. En insectos, malezas y patógenos microbianos, se ha demostrado el potencial para contrarrestar las tácticas de control utilizadas en su contra (Barrett, 1983; Georghiou, 1986; Georghiou y Lagunes, 1988; NRC, 2000; Green, 2014). La resistencia de los insectos a los cultivos Bt se considera inevitable y se están haciendo esfuerzos por la *US Environmental Protection Agency* (EPA) para controlar la evolución de resistencia de estos OGM, además de generalizar el uso de refugios. OGM resistentes a virus, hongos y bacterias no han sido utilizados ampliamente, sin embargo, se han documentado casos de su rápida evolución en controles convencionales (menos de 5 años, Delp 1988). La evolución de las malezas tolerantes a herbicidas es un riesgo ambiental indirecto (Ramachandran *et al*., 2000; VanGessel, 2001). Los OGMs tolerantes a los herbicidas están diseñados de tal forma que herbicidas específicos puedan ser utilizados para controlar las malezas, sin embargo, generalmente después de que el cultivo ha emergido, el riesgo es tener que utilizar herbicidas cada vez más dañinos para el ambiente: activos por más tiempo, con mayor cantidad de ingredientes activos, mezclados con otros agentes que dañen otros recursos naturales y a la salud o que provoquen el cambio en el uso de la tierra exclusivamente por cultivos GM, o el abandono total o parcial de tierras (NRC, 2002; Bejarano *et al*., 2017).

Componentes de la evaluación de daño ambiental causado por OGM

Para establecer ciertos parámetros que formarán parte de nuestra medida compuesta de "daño ambiental", consideramos pertinente definir tres elementos inherentes al daño: *adversidad*, *significancia* y *permanencia*. Estos aspectos serán relevantes para: 1) determinar la magnitud del daño; 2) permitir que los daños sean comparables en el tiempo y el espacio; y 3) proporcionar información para realizar una evaluación de los costos de remediación.

> *Adversidad*: Se refiere al tipo de daño relacionado al efecto que el OGM tiene en el medio ambiente (ver sección, Tipos de daño ambiental ocasionado por OGM).
>
> *Significancia*: El nivel ecosistémico donde el daño impacta. La significancia se determinará con base en efectos detectados en las propiedades emergentes de cada nivel (*e.g.* Población: tamaño, densidad, tasas de natalidad, mortalidad o crecimiento, diversidad y estructura genética, adecuación, etcétera; Comunidad: riqueza, abundancia, composición u otros; Ecosistema: productividad primaria, estructura trófica, servicios ecosistémicos de abastecimiento, regulación, apoyo, culturales y evolutivos, entre otros).
>
> *Permanencia*: El tiempo en que un efecto puede ser revertido.

Finalmente, la medida de daño ambiental puede agravarse si especies y/o áreas legalmente protegidas se ven afectadas.

Propuesta metodológica para la evaluación de daño ambiental causado por OGM

El método puede iniciarse analizando si se observan cambios cualitativos en las diferentes áreas o directamente con el método cuantitativo mostrado abajo (por razones de espacio, omitiremos la primera aproximación).

A. Evaluación de adversidad, significancia y permanencia

Es necesario responder a las preguntas A1, A2 y A3, que se presentan a continuación, para evaluar la existencia y magnitud de los efectos ambientales adversos causados por OGM. La información colectada se empleará para completar la tabla 1, que se introduce abajo. En ella, los diferentes impactos serán ponderados de acuerdo a su adversidad y significancia en el ambiente. Para llenar esta tabla, el valor numérico asociado a las categorías de permanencia de la pregunta A3 debe ser reemplazado en las funciones T_P, T_C y T_E, como se explicará con detalle más adelante. Después de realizar las operaciones pertinentes, se obtendrá un valor preliminar de daño ambiental *(Z)*.

A1. *Adversidad. ¿Cuál de los 4 tipos de impacto se ha identificado?*

A2. *Significancia.* ¿En qué niveles ecosistémicos el impacto ha tenido consecuencias y cuántas poblaciones, comunidades y/o ecosistemas han sido afectados?

A3. *Permanencia.* ¿En cuánto tiempo el efecto podría ser revertido? Este tiempo se denota por las funciones T_P, T_C y T_E, que dependen de cada población, comunidad, ecosistema y su tipo de daño, respectivamente. Denotamos por P al conjunto de poblaciones, C al conjunto de comunidades, E al conjunto de ecosistemas y por $X=\{1,2,3,4\}$ al conjunto de tipos de daño. Los elementos de P (las diferentes poblaciones) se describen por la variable p, elementos de C (las distintas comunidades) se describen por la variable c, elementos de E (los diferentes ecosistemas) se describen por la variable e y los elementos de X se denotan por la variable x, de manera que: $T_P(p,x)$ es el tiempo de permanencia del daño x en X correspondiente a la población p en P; $T_C(c,x)$ es el tiempo de permanencia del daño x en X correspondiente a la comunidad c en C; y $T_E(e,x)$ es el tiempo de permanencia del daño x en X correspondiente al ecosistema e en E. Dada una población p en P y un daño x en X, se asignan los siguientes valores a $T_P(p,x)$ de acuerdo al tiempo en que puede revertirse el daño observado: *sin efecto* $(T(p,x)=0)$; *antes dos años* $(T(p,x)=1)$; *entre dos a cinco años* $(T(p,x)=2)$; *entre cinco a ocho años* $(T(p,x)=3)$; *entre ocho y 10 años* $(T(p,x)=4)$; *perma-*

necerá más de 10 años o es irreversible (T(p,x)=5). Las mismas categorías aplican para $T_C(c,x)$ y $T_E(e,x)$.

Tabla 1. Evaluación de adversidad, significancia y permanencia.

	Tipo 1	Tipo 2	Tipo 3	Tipo 4
Población	$\sum_{p\in P} T_P(p,1)$	$\sum_{p\in P} T_P(p,2)$	$\sum_{p\in P} T_P(p,3)$	$\sum_{p\in P} T_P(p,4)$
Comunidad	$\lvert S\rvert * \sum_{c\in C} T_C(c,1)$	$\lvert S\rvert * \sum_{c\in C} T_C(c,2)$	$\lvert S\rvert * \sum_{c\in C} T_C(c,3)$	$\lvert S\rvert * \sum_{c\in C} T_C(c,4)$
Ecosistema	$\lvert S\rvert * \sum_{e\in E} T_E(e,1)$	$\lvert S\rvert * \sum_{e\in E} T_E(e,2)$	$\lvert S\rvert * \sum_{e\in E} T_E(e,3)$	$\lvert S\rvert * \sum_{e\in E} T_E(e,4)$
Total por tipo de daño Z_X	$Z_1 = 3 * \left(\sum_{p\in P} T_P(p,1) + \left(\lvert S\rvert * \sum_{c\in C} T_C(c,1)\right) + \left(\lvert S\rvert * \sum_{e\in E} T_E(e,1)\right)\right)$	$Z_2 = 2 * \left(\sum_{p\in P} T_P(p,2) + \left(\lvert S\rvert * \sum_{c\in C} T_C(c,2)\right) + \left(\lvert S\rvert * \sum_{e\in E} T_E(e,2)\right)\right)$	$Z_3 = 3 * \left(\sum_{p\in P} T_P(p,3) + \left(\lvert S\rvert * \sum_{c\in C} T_C(c,3)\right) + \left(\lvert S\rvert * \sum_{e\in E} T_E(e,3)\right)\right)$	$Z_4 = \sum_{p\in P} T_P(p,4) + \left(\lvert S\rvert * \sum_{c\in C} T_C(c,4)\right) + \left(\lvert S\rvert * \sum_{e\in E} T_E(e,4)\right)$
Daño parcial Z	$\boldsymbol{z} = \sum_{x\in X} Z_X$			

En la tabla 1, la significancia en los niveles de comunidad y ecosistema se ponderarán por el número de especies afectadas, cuyo conjunto se denota por S; los elementos de S (las diferentes especies) se describen por la variable s, de manera que $|S|$denota el número de elementos de S. La adversidad, por otra parte, se ponderará por un factor numérico relacionado a los efectos del daño a largo plazo sobre el uso de la diversidad (ver total por tipo de daño en esta tabla).

B. Agravantes

A continuación, se evaluarán dos factores agravantes a la medida de daño de acuerdo a especies y áreas identificadas con protección especial. A partir de las preguntas B1 y B2, mostradas abajo, se obtendrán dos valores que se sumarán al valor de daño ambiental (Z) obtenido anteriormente. Este análisis se realizará con base en los acuerdos internacionales pertinentes y al marco legal local del lugar donde se esté evaluando el daño.

> B1. ¿Las especies analizadas están dentro de una categoría de protección especial? Por ejemplo: especies prioritarias, NOM-059-SEMARNAT, lista roja de especies amenazadas de la Unión Internacional para la Conservación de la Naturaleza (IUCN) o cualquier otra.

El primer factor agravante se denota por la función S_P, que depende de cada especie y su categoría de protección especial. Como mencionamos arriba, S es el conjunto de especies afectadas, y denotamos por $Y= \{1,2,3,...,n\}$ al conjunto de categorías de protección especial. Los elementos de S se describen por la variable s y los elementos de Y se denotan por la variable y, de manera que: $S_P(s,y)$ es el agravante de y en Y correspondiente a la especie s en S. Dada una especie s en S y una categoría de protección especial y en Y, se asignan los siguientes valores a $S_P(s,y)$: *sin protección especial* (S_P(s,y)=1); *dentro de cualquier categoría de protección especial* (S_P(s,y)=2); *otros criterios relevantes para expertos* (S_P(s,y)=2). Así, el primer factor agravante, dependiente de la protección legal con la que cuentan las especies evaluadas, se obtiene de la siguiente manera:

$$\boldsymbol{Sp} = \sum_{s \in S} S_P\,(s, y)$$

> B2. ¿Las áreas identificadas en el análisis pertenecen a una categoría de protección legal? Tales como: categorías de áreas protegidas de la IUCN, áreas relevantes para el Protocolo de Cartagena sobre bioseguridad (centros de origen, y centros de diversidad genética), entre otras.

El segundo factor agravante de denota por la función *As*, que depende de cada área y su categoría de protección especial. Denotamos por *A* al conjunto de áreas afectadas y por *Y*= {1,2,3,...,n} al conjunto de categorías de proetección especial. Los elementos de *A* (las diferentes áreas) se describen por la variable *a* y los elementos de *Y* se denotan por la variable *y*, de manera que: *As(a,y)* es el agravante de *y* en *Y* correspondiente al área *a* en *A*. Dada un área *a* en *A* y una categoría de protección especial *y* en *Y*, se asignan los siguientes valores a *As(a,y)*: *sin protección especial* (*As(a,y)*=1); *áreas protegidas* IUCN (*As(a,y)*=2); *centros de origen o diversidad* (*As(a,y)*=2); *otras áreas relevantes para expertos* (*As(a,y)*=2). Así, el segundo factor agravante, dependiente de la protección legal con la que cuentan las áreas evaluadas, se obtiene de la siguiente manera:

$$\boldsymbol{As} = \sum_{a \in A} A_S\,(a, y)$$

C. Medida de daño ambiental. Los valores resultantes (*Sp*) y (*As*) se sumarán al valor de daño ambiental (*Z*) calculado arriba para obtener una medida compuesta de daño ambiental (*D*):

$$\boldsymbol{D = Z + Sp + As}$$

D. Interpretación de resultados. Cuando el valor *(D)* sea igual a cero, la evaluación reflejará que no hay información disponible para realizar el análisis o que el daño ambiental ocasionado por OGM no fue detectado por esta metodología. Sin embargo, cuando *(D)* sea mayor de cero, se ha detectado daño ambiental y será necesario acompañar el resultado con alguna de las siguientes recomendaciones de mitigación, dependiendo de la reversibilidad del impacto:

a. Acciones severas para restaurar
b. Acciones intermedias para restaurar
c. Sin procedimientos especiales para restaurar

Consideraciones

Integrar los componentes que deben ser analizados para la evaluación cuantitativa de daños ambientales ocasionados por OGM es el primer paso para lograr tener resultados objetivos en el mediano plazo, a partir de una herramienta metodológica adecuada. La propuesta, después de ser probada y discutida por expertos, seguramente será mejorada, sin embargo, en materia de evaluación de daños para proyectos, peritajes y consultorías ya puede ser considerada. Como mencionamos al inicio, la supervisión de un CTC puede conducir a mejores resultados y recomendamos que exista entre ellos un mecanismo establecido previamente para permitir solucionar discrepancias por mayoría de votos.

Los daños ambientales ocasionados por procesos de antropización en general, pueden cuantificarse con modificaciones a la metodología aquí propuesta. En este capítulo nos hemos enfocado a explicarla considerando los efectos adversos ocasionados por OGM, pero su utilización en otros ámbitos dependerá de las aplicaciones que se le quiera dar, además de la delimitación y cantidad de los tipos de daño que se analicen. Los resultados, en todos los casos, sirven para comprender y comparar la magnitud de los daños; para determinar los costos económicos, sociales y culturales se requieren evaluaciones paralelas, posteriores y a largo plazo.

Conclusiones

Con este modelo esperamos contribuir a una mejor aplicación de las leyes y proporcionar una herramienta útil para los tomadores de decisiones, investigadores y sociedad civil interesada, que pretenda desarrollar una investigación en caso de sospechar daños por la presencia de OGM o simplemente querer monitorear su ausencia. También esperamos fomentar una discusión científica muy necesaria en torno a este tema.

Agradecimientos

Agradecemos el apoyo financiero a los proyectos PAPIIT IN214719, PAPIIT IV200117, CONACYT-PN 247672 y CONABIO DGAP003/WN003/18. Así como a los Posgrados en Ciencias Biomédicas y Ciencias Biológicas a los que pertenecen Melania Vega y Alejandra Hernández-Terán, así como al CONACYT por las becas de posgrado 435586 y 660255. También queremos agradecer a todos los colegas que contribuyeron en las discusiones sobre este tema, especialmente a Daniel Piñero, Francisca Acevedo, Alejandra Barrios y Oswaldo Oliveros, así como a Miguel Ballesteros y Haven López por apoyarnos en la expresión matemática.

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