UNIVERSITAT POLITÈCNICA DE VALÈNCIA
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TESIS DOCTORAL

Desarrollo de materiales de pre-mejora y herramientas biotecnológicas para la adaptación de la berenjena al cambio climático

DOCTORAL THESIS

Development of pre-breeding materials and biotechnological tools for adaptation of eggplant to climate change

TESI DOCTORAL

Desenvolupament de materials de pre-millora i eines biotecnològiques per a l'adaptació de l'albergínia al canvi climàtic

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"Corred insensatos"

(Gandalf el Gris)

Índice

Resumen

La berenjena (*Solanum melongena*) es una hortaliza muy importante en muchas áreas tropicales y subtropicales del mundo. Es la tercera solanácea más producida a nivel mundial, pero a pesar de su importancia los recursos genéticos y las herramientas biotecnológicas para su investigación no han sido desarrollados lo suficiente. Con la actual situación de cambio climático, muchas de las áreas donde se produce este cultivo están sufriendo modificaciones dramáticas en el ambiente y la climatología. Esto está ocasionando una reducción de los rendimientos de este cultivo que cada vez se ven más afectados por la aparición de nuevas enfermedades, plagas, malezas, pérdida en la fertilidad de los suelos, mayor prevalencia de sequía y salinidad, así como el incremento de las temperaturas. La berenjena se encuentra en una situación de vulnerabilidad ante estos cambios debido a los efectos de cuello de botella genético acontecidos durante su domesticación y a la disponibilidad limitada de recursos genéticos accesibles para su mejora genética.

En un primer gran bloque de esta tesis, mediante el uso de especies silvestres relacionadas con la berenjena, se ha iniciado el desarrollo de una colección de líneas de introgresión (ILs). Utilizando tres especies representantes de los tres grupos de germoplasma de la berenjena (*S. insanum* del germoplasma primario, *S. dasyphyllum* del germoplasma secundario y *S. elaeagnifolium* del germoplasma terciario) se ha ampliado el fondo genético de este cultivo. Estas especies, han sido seleccionadas por sus extraordinarias capacidades de adaptación a climas áridos, suelos secos y tolerancia a plagas y enfermedades. Reintroduciendo estos genes en el genoma de la berenjena cultivada hemos desarrollado un conjunto de materiales élite, que ponen a disposición de los investigadores y mejoradores nuevos recursos genéticos para la mejora genética de este cultivo. También hemos desarrollado un modelo experimental (Micro-Mel) a partir de materiales de introgresión con la especie *S. anguivi*. Este modelo consiste en una berenjena de tipo compacto y crecimiento determinado con floración y cuajado múltiple y puede ayudar a desarrollar experimentos rápidos, así como acelerar los ciclos generacionales en los proyectos de mejora.

En otro segundo gran bloque de este trabajo, hemos desarrollado una serie de herramientas biotecnológicas que van a permitir desarrollar otro tipo de investigaciones para la adaptación al cambio climático en berenjena. En primer lugar, frente a la necesidad de un protocolo eficiente de regeneración *in vitro* para poder llevar a cabo experimentos de transformación y edición genética en la berenjena, se ha desarrollado con éxito un protocolo de alto rendimiento basado en el uso del ribósido de zeatina y que presenta una baja dependencia del factor genotipo. Como resultado derivado de este primer desarrollo, diseñamos otro protocolo para la obtención de organismos poliploides en berenjena sin la necesidad de utilizar agentes antimitóticos para la duplicación de su genoma. Empleando los distintos niveles de ploidía presente en algunos tejidos jóvenes (patrón polisomático) conseguimos desarrollar plantas tetraploides *in vitro* a través de la regeneración directa a partir de estas células, suponiendo una nueva vía hacia el desarrollo de plantas triploides sin semillas. Finalmente, la última herramienta de apoyo a la mejora de la berenjena que se ha desarrollado en esta tesis doctoral ha sido una herramienta basada en la inteligencia artificial para la identificación de estadios de desarrollo de las células precursoras del polen en retrocruces avanzados con especies silvestres. Con esto se ha conseguido optimizar los protocolos de androgénesis empleados para la producción de plantas dobles haploides, automatizando y haciendo más eficiente la selección de anteras con estadios inducibles y por tanto incrementando la tasa de plantas dobles haploides producidas. Esto tiene grandes implicaciones en la mejora ya que podemos reducir a una única generación la fijación de caracteres y el desarrollo de líneas parentales.

Resumen

En su conjunto la presente tesis doctoral donde significa un trabajo de gran importancia para la mejora genética de la berenjena estableciendo las bases y proporcionando los materiales y herramientas que permitirán el desarrollo de una nueva generación de variedades adaptadas al cambio climático. A través de estos trabajos se ha ampliación la base genética de este cultivo y se han desarrollan las herramientas biotecnológicas de apoyo a la mejora necesarias para desarrollar investigaciones y proyectos futuros.

Abstract

Eggplant (*Solanum melongena*) is a very important vegetable in many tropical and subtropical areas of the world. It is the third most produced Solanaceae in the world, but despite its importance, genetic resources and biotechnological tools for research have not been sufficiently developed. With the current climate change situation, many of the areas where this crop is produced are undergoing dramatic changes in the environment and the weather. This is causing a reduction in the yields of this crop that are increasingly affected by the appearance of new diseases, pests, weeds, loss of soil fertility, greater prevalence of drought and salinity, as well as the increase in temperatures. The eggplant is in a situation of vulnerability to these changes due to the genetic bottleneck effects that occurred during its domestication and the limited availability of accessible genetic resources for its genetic improvement.

In a first large block of this thesis, using wild species related to eggplant, the development of a collection of introgression lines (ILs) has been started. Using three species representing the three groups of eggplant germplasm (*S. insanum* from primary germplasm, *S. dasyphyllum* from secondary germplasm and *S. elaeagnifolium* from tertiary germplasm) the genetic background of this crop has been expanded. These species have been selected for their extraordinary capacities to adapt to arid climates, dry soils and tolerance to pests and diseases. By reintroducing these genes into the genome of cultivated eggplant, we have developed a set of elite materials that make new genetic resources available to researchers and breeders for the genetic improvement of this crop. We have also developed an experimental model (Micro-Mel) from introgression materials with the species *S. anguivi*. This model consists of an eggplant of compact type and determined growth with multiple flowering and fruit set and can help to develop rapid experiments, as well as accelerate generational cycles in improvement projects.

In another second large block of this work, we have developed a series of biotechnological tools that will allow the development of other types of research for adaptation to climate change in eggplant. In the first place, in view of the need for an efficient in vitro regeneration protocol to be able to carry out transformation and gene editing experiments in eggplant, a high-throughput protocol based on the use of zeatin riboside and which presents a low dependence on the genotype factor was developed. As a result, derived from this first development, we designed another protocol to obtain polyploid organisms in eggplant without the need to use antimitotic agents for the duplication of their genome. Using the different levels of ploidy present in some young tissues (polysomatic pattern) we managed to develop tetraploid plants in vitro through direct regeneration from these cells, assuming a new path towards the development of triploid seedless plants. Finally, the last tool to support the breeding of eggplant that has been developed in this doctoral thesis has been a tool based on artificial intelligence for the identification of stages of development of pollen precursor cells in advanced backcrosses with wild species. With this it has been possible to optimize the androgenesis protocols used to produce double haploid plants, automating, and making the selection of anthers with inducible stages more efficient and therefore increasing the rate of double haploid plants produced. This has great implications for improvement since we can reduce the fixation of characters and the development of parental lines to a single generation.

As a whole, this doctoral thesis means a work of great importance for the genetic improvement of the eggplant, establishing the bases and providing the materials and tools that will allow the development of a new generation of varieties adapted to climate change. Through these works, the genetic base of this crop has been broadened and the biotechnological tools to support the improvement necessary to develop future research and projects have been developed.

Resum

L'albergínia (Solanum melongena) és una hortalissa molt important en moltes zones tropicals i subtropicals del món. És la tercera solanàcia més produïda en l'àmbit mundial, però malgrat la seua importància els recursos genètics i les ferramentes biotecnològiques per a la seua investigació no han sigut desenvolupades el suficient. Amb l'actual situació de canvi climàtic, moltes de les zones on es produeix aquest cultiu estan patint modificacions dramàtiques al seu ambient i climatologia. Açò ocasiona una reducció dels rendiments d'aquest cultiu que cada vegada es veu més afectat per l'aparició de noves malalties, plagues, males herbes, pèrdua de la fertilitat del sol, major prevalença de la sequera i salinitat, així com l'increment de les temperatures. L'albergínia es troba a una situació de vulnerabilitat davant aquests canvis debuts als efectes de l'erosió genètica ocasionats durant la seua domesticació i a la disponibilitat limitada de recursos genètics accessibles per a la seua millora genètica.

En un primer gran bloc d'aquesta tesi, mitjançant l'ús d'espècies silvestres relacionades amb l'albergínia, s'ha iniciat el desenvolupament d'una col·lecció de línies de introgressió (ILs). Utilitzant tres espècies representants dels tres grups de germoplasma de l'albergínia (*S. insanum* del germoplasma primari, *S. dasyphyllum* del germoplasma secundari i *S. elaeagnifolium* del germoplasma terciari) s'ha ampliat el fons genètic d'aquest cultiu. Aquestes espècies, han sigut seleccionades per les seues extraordinàries capacitats d'adaptació a climes àrids, sòls secs i tolerància a plagues i malalties. Reintroduint aquests gens en el genoma de l'albergínia cultivada hem desenvolupat un conjunt de materials elit, que posen a la disposició dels investigadors i milloradors nous recursos genètics per a la millora genètica d'aquest cultiu. També hem desenvolupat un model experimental (Micro-Mel) a partir de materials de introgressió amb l'espècie *S. anguivi*. Aquest model consisteix en una albergínia de tipus compacte i creixement determinat amb floració i quallat múltiple i pot ajudar a desenvolupar experiments ràpids, així com accelerar els cicles generacionals en els projectes de millora.

En un altre segon gran bloc d'aquest treball, hem desenvolupat una sèrie d'eines biotecnològiques que permetran desenvolupar un altre tipus d'investigacions per a l'adaptació al canvi climàtic en albergínia. En primer lloc, enfront de la necessitat d'un protocol eficient de regeneració *in vitro* per a poder dur a terme experiments de transformació i edició genètica en l'albergínia, s'ha desenvolupat amb èxit un protocol d'alt rendiment basat en l'ús del ribòsid de zeatina i que presenta una baixa dependència del factor genotip. Com a resultat derivat d'aquest primer desenvolupament, dissenyem un altre protocol per a l'obtenció d'organismes poliploids en albergínia sense la necessitat d'utilitzar agents antimitòtics per a la duplicació del seu genoma. Emprant els diferents nivells de ploidía present en alguns teixits joves (patró polisomàtic) aconseguim desenvolupar plantes tetraploids *in vitro* a través de la regeneració directa a partir d'aquestes cèl·lules, suposant una nova via cap al desenvolupament de plantes triploids sense llavors. Finalment, l'última eina de suport a la millora de l'albergínia que s'ha desenvolupat en aquesta tesi doctoral ha sigut una eina basada en la intel·ligència artificial per a la identificació d'estadis de desenvolupament de les cèl·lules precursores del pol·len en retrocreuaments avançats amb espècies silvestres. Amb això s'ha aconseguit optimitzar els protocols d'androgènesi emprats per a la producció de plantes dobles haploids, automatitzant i

fent més eficient la selecció d'anteres amb estadis induïbles i per tant incrementant la taxa de plantes dobles haploids produïdes. Això té grans implicacions en la millora ja que podem reduir a una única generació la fixació de caràcters i el desenvolupament de línies parentals.

En el seu conjunt la present tesi doctoral, significa un treball de gran importància per a la millora genètica de l'albergínia establint les bases i proporcionant els materials i eines que permetran el desenvolupament d'una nova generació de varietats adaptades al canvi climàtic. A través d'aquests treballs s'ha ampliat la base genètica d'aquest cultiu i s'han desenvolupen les eines biotecnològiques de suport a la millora necessàries per a desenvolupar investigacions i projectes futurs.

Introducción General

1. La berenjena

1.1. *Taxonomía*

La berenjena (*Solanum melongena* L.) es una planta que pertenece a la familia *Solanaceae,* siendo originaria del sudeste asiático. Es una planta plurianual que se cultiva como anual y siendo angiosperma, su clasificación taxonómica dentro de las angiospermas es la siguiente (Vorontsova y Knapp, 2016) (Tabla1):

Tabla 1: Clasificación botánica de *S. melongena*.

Reino	*Plantae*
División	*Magnoliophyta*
Clase	*Magnoliopsida*
Orden	*Solanales*
Familia	*Solanaceae*
Subfamilia	*Solanoideae*
Tribu	*Solaneae*
Género	*Solanum*
Subgénero	*Leptostemonum*
Sección	*Melongena*
Especie	*Solanum melongena* L.

Dentro de la familia de las Solanáceas, el género *Solanum* es el más numeroso con unas 1800 especies. Se pueden encontrar en él plantas de todo tipo, desde herbáceas, pasando por arbustivas hasta arbóreas y en muchos casos son espinosas y contienen glicoalcaloides venenosos como la solanina, presente en todas las partes de la planta (Barceloux, 2009). *Solanum melongena* pertenece a uno de los grupos no tuberosos del género *Solanum,* tratándose de una especie diploide con un número cromosómico de n=12.

1.2. *Caracterización botánica y especies silvestres relacionadas.*

Las plantas de berenjena presentan un tallo simple, alargado, plano y ramificado. Sus hojas son lobuladas y gruesas miden aproximadamente 10-20 cm de largo 5-10 cm de ancho, son de color verde y presentan una filotaxis alterna. Esta planta produce flores de color púrpura, aunque los tonos pueden ir desde el blanco hasta el morado oscuro pasando por el azul claro; su diámetro oscila entre los 3-5 cm. La berenjena se cultiva por su fruto carnoso comestible, este es carnoso, y por lo general alargado y ovoide que llega a alcanzar los 40 cm de largo, aunque puede presentar muy diversas morfologías, su epidermis es lisa y en su interior alberga una gran cantidad de pequeñas semillas. En este cultivo se diferencian dos estadios de madurez diferentes, la madurez comercial y la fisiológica, por tanto, el fruto es consumido en un estadio de inmadurez fisiológica que dependiendo de la variedad puede presentar distintos colores: blanco, verde, rosa, morado, negro e incluso combinaciones de colores en forma de listado (Figura1). En madurez comercial generalmente el fruto se torna amarillo por la degradación de sus pigmentos. Las temperaturas óptimas para el desarrollo de la berenjena oscilas entre los 26-32 °C diurnos y los 21 °C nocturnos, esta es una planta heliófila y se debe disponer en lugares en los que les dé directamente la luz solar. El pH ideal del suelo se sitúa entre 6,3-6,8 y las plantas deberían de tener un suministro continuo de agua para presentar un desarrollo del fruto óptimo, aunque el sustrato o el terreno de cultivo nunca debería estar seco ni empozado (Maroto, 2008).
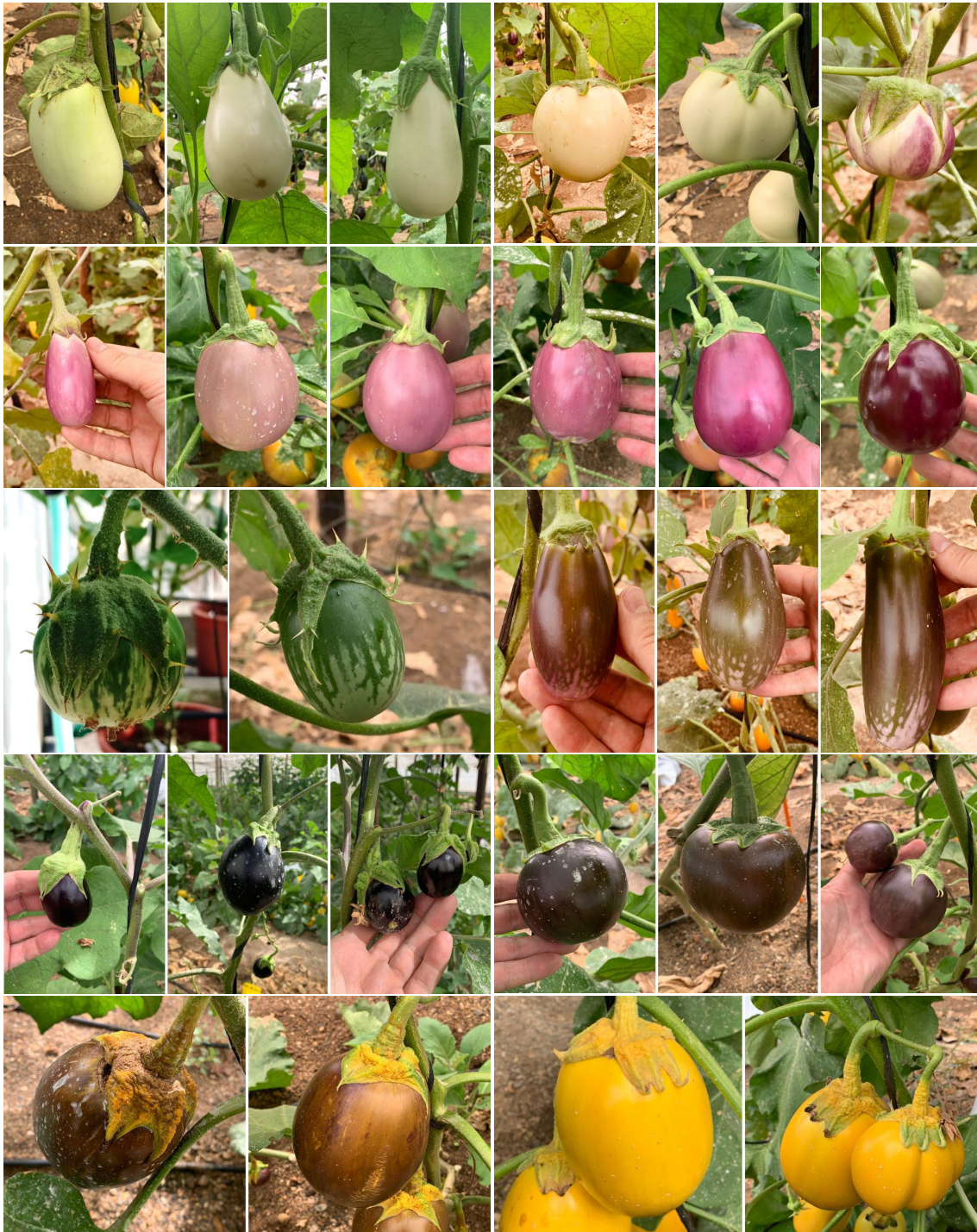
Figura 1: Diversidad en el color, la forma y el grado de madurez de la berenjena. Se pueden observar frutos completamente blancos, otros con diferentes niveles de antocianos, frutos verdes, combinaciones de los antocianos y la clorofila en patrones listados, frutos negros con elevados niveles de ambos pigmentos y finalmente, frutos con diferentes grados de color amarillo, debido a la degradación de los pigmentos típica una vez que se alcanza la madurez fisiológica.

Aparte de las especies cultivadas, otra parte muy importante de los recursos fitogenéticos con los que los mejoradores e investigadores pueden contar son las especies silvestres. Desde los inicios de la humanidad y con la aparición de la Agricultura misma, una serie de largos procesos de domesticación empezaron a llevarse a cabo hasta llegar a lo que hoy conocemos cono las

formas modernas de los cultivos a partir de estas especies, que hoy en día coexisten con nuestros cultivos. En el caso concreto de la berenjena, esta posee muchas especies silvestres relacionadas las cuales crecen en una amplia gama de condiciones ambientales, incluidas áreas desérticas y entornos extremos (Syfert et al. 2016). Se ha descubierto que algunos de estos parientes silvestres son resistentes o tolerantes a algunas enfermedades y plagas que podrían estar aumentando su incidencia debido al incremento de las temperaturas (Brand-Daunay y Hazra, 2012; Namisy et al., 2019). No obstante, la explotación de los recursos genéticos de la berenjena, y en particular de sus especies silvestres relacionadas, ha sido históricamente muy limitado.

En función de su parentesco o distancia filogenética, las especies silvestres se agrupan en tres niveles definidos como grupos de germoplasma primario, secundario y terciario. El criterio principal para realizar la agrupación o clasificar las especies en estos grupos es principalmente la posibilidad de obtener descendencia entre sí. Las especies del germoplasma primario son aquellas que pueden cruzarse entre sí sin ningún tipo problema y generan una descendencia fértil, en el caso de la berenjena aquí encontraríamos a *S. insanum* (Ranil et al., 2017). Las especies dentro del germoplasma secundario son aquellas con las que se puede realizar cruzamientos y obtener una descendencia fértil o semifertil, la trasferencia genética no es tan sencilla como en el caso del germoplasma primario, pero es posible, las especies principales de germoplasma secundario de berenjena son *S. anguivi, S. campylacanthum, S. dasyphyllum, S.incanum, S. lichtenstenii, S. linnaeanum, S. pyracanthum, S. tomentosum y S. violaceum* (Syfert et al. 2016). Por último, tendríamos el germoplasma terciario, donde la dificultad de realizar cruzamientos aumenta más todavía y los cigotos formados en el caso de que se produzca fecundación son anómalos, letales o dan lugar a híbridos completamente estériles. En definitiva, en este nivel, la transferencia genética es posible, pero se requiere de técnicas avanzadas de mejora genética para conseguirlo. Las especies de germoplasma terciario de la berenjena más ampliamente conocidas son *S. elaeagnifolium, S. sisymbriifolium* y *S. torvum*.

En esta tesis doctoral se ha trabajado principalmente con tres especies representantes de los distintos grupos de germoplasma de la berenjena. Como representante del grupo de germoplasma primario está *S. insanum*, se trata de una maleza ampliamente distribuida se caracteriza por crecer en zonas áridas y terrenos infértiles. Esto sugiere que esta especie presenta un gran potencial para la adaptación a la sequía, así como a otros estreses abióticos ambientales (Ranil et al., 2017). Por otra parte, como representante del grupo de germoplasma secundario está *S. dasyphyllum*. Es una especie africana utilizada comúnmente como planta medicinal con un gran potencial fitoquímico (Bukenya and Carrasco 1994; Ajayi et al., 2013; Kidane et al., 2013). Además de esto, varios estudios han demostrado el efecto acaricida de extractos de sus frutos inhibiendo la ovoposición de *Rhipicephalus appendicuhtus* (Van Puyvelde et al., 1985); también presenta ciertos niveles de resistencia a *Fusarium spp*. (Mwanik et al., 2015). Finalmente, como representante del germoplasma terciario está *S. elaeagnifolium*. Esta planta aunque es nativa del norte y el sur de América (Knapp et al. 2017), es una maleza dispersada por todo el mundo con unos niveles de tolerancia al estrés hídrico altísimos (Christodoulakis et al., 2009). Además, esta especie presenta un perfil de compuestos fenólicos muy interesante, lo cual le confiere un potencial enorme para la mejora nutricional de la berenjena (Kaushik et al., 2015). Desde el punto de vista evolutivo y taxonómico, esta selección representa una buena base para la mejora al tener representación de especies de los tres grupos de germoplasma de la berenjena. En próximos apartados de esta introducción se explicará de

qué manera se puede explotar el potencial de estas especies silvestres y cuáles son las limitaciones en función del parentesco que guarden con la berenjena.

1.3. *Origen, domesticación y dispersión*

Solanum insanum es el ancestro silvestre de la berenjena cultivada (*S. melongena)*. La hipótesis histórica de Lester y Hasan (1991) es que *S. insanum* podría haberse desarrollado como una maleza entre los cultivos hortícolas de Asia tropical, derivando también a *S. incanum*. Luego habría sido adoptado progresivamente como un taxón semi-cultivado, y expuesto a una selección progresiva, primero hacia tipos primitivos (como los que se encuentran en Tailandia) y luego hacia tipos avanzados con frutos grandes de varias formas y colores (Lester y Hasan 1991). Page et al. (2019) examinaron más a fondo la cantidad y el reparto de la variación genética en las berenjenas silvestres y domesticadas con muestras amplias y utilizando genotipado por secuenciación. Esto logró respaldar la mayoría del trabajo antes mencionado, es decir: (1) *S. insanum* es el progenitor de la berenjena cultivada y (2) *S. incanum* es hermana del grupo formado por la berenjena y su progenitor silvestre.

Según Vavilov (1926) la berenjena fue domesticada en la región Indo-Birmana debido a evidencias de que ésta ya era cultivada en esta zona en la antigüedad. No obstante, pruebas recientes sugieren que la berenjena tuvo una domesticación múltiple que se distribuye a lo largo de Asia tropical, desde Madagascar hasta Filipinas (Knapp et al., 2013). Muchos estudios han utilizado una gran variedad de marcadores genéticos para abordar aspectos de la taxonomía y la domesticación de las berenjenas. En un intento de proporcionar una mayor resolución, Meyer et al. (2012) utilizó marcadores AFLP altamente polimórficos en una amplia muestra de accesiones, llegando a la conclusión de que existen al menos dos centros de domesticación para la berenjena. Adicionalmente, se realizó una comparación de los usos de las berenjenas, que resume 77 categorías de atributos medicinales, en su mayoría específicos para cada una de estas tres regiones asiáticas (Meyer et al. 2014). Esto permitió reforzar la hipótesis de que existen al menos tres centros de domesticación. Las comparaciones entre accesiones de diferentes países, incluidos Sri Lanka, China, India e Indonesia (Hurtado et al. 2012; Cericola et al. 2013), también han demostrado que la estructuración de los rasgos morfológicos y la diversidad de los marcadores microsatélites son compatibles con la hipótesis de la domesticación multilocal. La superposición parcial de estos resultados también sugiere la difusión e intercambio genético posterior entre los materiales que se originaron en las áreas de domesticación. Esta hipótesis es consistente con las migraciones humanas y el comercio de cultivos en Asia (Meyer et al. 2014).

Desde el Centro Indio de domesticación, considerado como centro primario, la berenjena se extendió a China y la Región Mediterránea, que se consideran centros secundarios de variación (Prohens et al., 2005; Cericola et al. 2013). Desde la zona Indo-Birmana y con ayuda de los árabes, la berenjena se introdujo en la Península Ibérica a través de la ruta de la seda. A partir de los tipos introducidos por los árabes surgieron variedades locales adaptadas a diversas condiciones de la Península como consecuencia de la selección (tanto natural como artificial), la recombinación, la migración, la deriva genética y los cruzamientos espontáneos entre individuos de poblaciones diferentes (Prohens et al., 2003). Finalmente, desde aquí se extendió al resto de Europa, y fue introducida en América tras su descubrimiento.

1.4. *Importancia económica*

La berenjena es uno de los cultivos más importantes del mundo, siendo el tercer vegetal más cultivado dentro de la familia de las solanáceas después de la patata y el tomate; y el sexto cultivo hortícola más cultivado en el mundo (FAOSTAT, 2018) (Figura 2). La producción mundial de berenjena se ha duplicado en los últimos 10 años debido al crecimiento de la producción en los tres principales países productores, que son China, India y Egipto (FAOSTAT 2018). España ocupa el decimoprimer puesto siendo el segundo país europeo más productor, por detrás de Italia. También es una de las hortalizas más cultivadas en España, donde ocupa la decimotercera posición, por delante de las coles o las calabazas. A nivel nacional se ha experimentado un crecimiento tanto en términos de producción como de área cosechada en los últimos años, siendo Andalucía la mayor productora con 207.832 toneladas con una extensión de cultivo de 2.878 hectáreas, seguida por la Comunidad Valenciana con 12.475 toneladas y 170 hectáreas (MAPA, 2018).
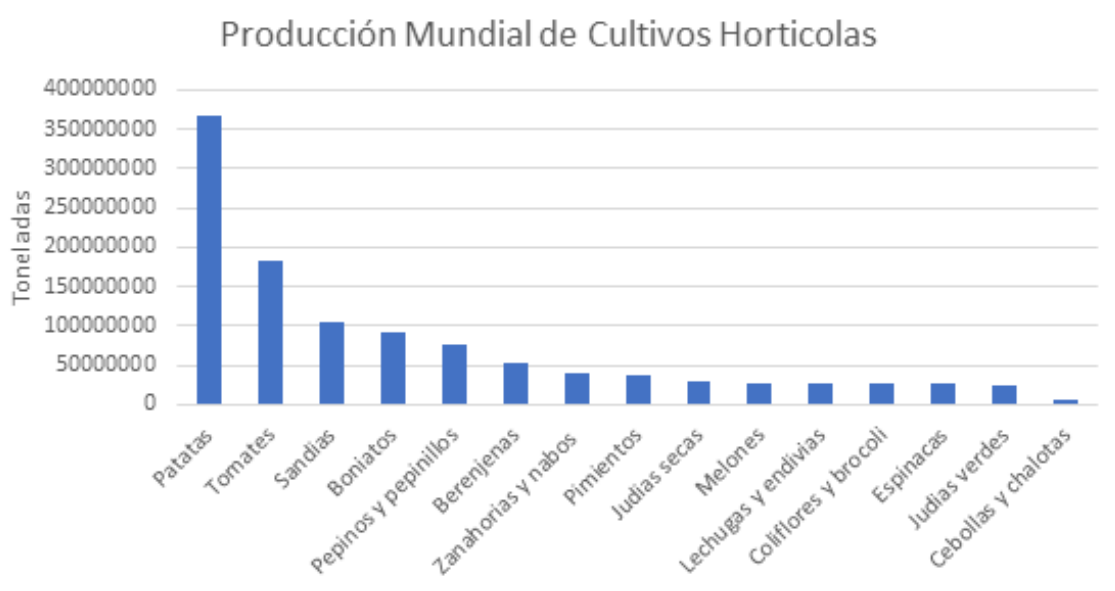
Figura 2: Producción de cultivos hortícolas en toneladas (FAOSTAT 2018).

2. La mejora genética vegetal contra el cambio climático

2.1. *Impacto del cambio climático en los cultivos*

Recientemente Ruiz de Galarreta et al. (2018) han revisado el impacto del cambio climático en la agricultura y las estrategias de mejora genética vegetales disponibles para adaptar los cultivos al mismo. Varios estudios demuestran que la actividad agrícola se va a ver afectada de una forma muy severa por los efectos del cambio climático, sobre todo en lo que se refiere a la productividad (Aggarwal et al. 2019). Los efectos globales del cambio climático como son el incremento de la temperatura media, la variación en los patrones de pluviometría, el incremento de los eventos extremos y la subida en los niveles de ozono van a tener como consecuencia un descenso de la productividad agrícola (Anwar et al. 2013). Por otra parte, el avance del mar como consecuencia del deshielo de los polos, así como nuevos eventos atmosféricos como tornados e inundaciones en zonas donde antes no los había, también supondrán un problema para la agricultura ocasionando la perdida de terrenos cultivables y la salinización de los acuíferos, limitando el agua dulce disponible para regadío (Bebber et al. 2013; Maldonado et al. 2013).

En su conjunto todos estos efectos tendrán un impacto directo sobre los cultivos, ya que se verán sometidos a una serie de estreses abióticos que limitarán su productividad. Por ello los programas de mejora genética para la adaptación al cambio climático implican la búsqueda de las resistencias o tolerancias a dichos factores abióticos; no obstante, este tipo de caracteres suelen ser cuantitativos y están controlados por un alto número de genes (Collins et al. 2008; Habash et al. 2009), algo que dificulta su estudio y posterior uso.

Los principales objetivos de mejora de tolerancia a estreses asociados a las condiciones de cambio climático según Ruiz de Galarreta et al. (2018) estarían relacionados principalmente con la salinidad, la sequía, el aumento de las temperaturas y la aparición de nuevos patógenos. En lo que respecta a la tolerancia a la salinidad, se buscan plantas cuyos mecanismos de respuesta adaptativa a estas nuevas condiciones sean eficientes. En una situación en la que la concentración salina aumenta, la absorción del agua desde un medio más hipertónico se ve dificultada, con lo que también se dificulta la captación de nutrientes esenciales los cuales se encuentran disueltos en una mezcla de sales heterogéneas junto con otros minerales tóxicos (Wani et al., 2020; Khan et al. 2020). Por ello aquellas plantas cuyos mecanismos de respuesta adaptativa son eficientes, regulan la homeostasis iónica del sodio (que es tóxico a altas concentraciones) y del potasio (elemento esencial cuya captación se ve dificultada por los iones de sodio). Por ello se está trabajando en seleccionar cultivos y variedades muy eficientes en la síntesis y uso de las proteínas HKT (*high-affinity potassium transporters*), que retiran el exceso de sodio en el xilema para facilitar la entrada del potasio (Wani et al 2020). Pero debido a la naturaleza poligénica de este carácter, la transferencia de los genes implicados en esta tolerancia no ha sido todo lo eficaz que se espetaba, presentándose resultados inferiores en el campo a lo que se había observado en el laboratorio (Munns et al., 2012). Otro carácter muy relacionado con la salinidad es la tolerancia a la sequía, su objetivo es mantener las funciones de la planta inalteradas ante una situación de déficit hídrico ya que este afecta sistémicamente alterando la anatomía, morfología, fisiología y bioquímica de la planta (Dobra et al. 2010; Shinwari et al. 2020). Como consecuencia la capacidad de germinación, el vigor y el rendimiento de los cultivos se ve mermado. Los mecanismos moleculares para dar una respuesta a este tipo de estreses están muy relacionados con la sobreexpresión de factores de transcripción que activan rutas metabólicas dando respuesta a múltiples estímulos que van desde la presencia de

especies reactivas del oxígeno (ROS), el cierre de los estomas o a la acumulación de solidos solubles para generar citoplasmas más hipertónicos y maximizar la absorción del agua externa (Shinwari et al. 2020; Mahmood et al. 2020). Al igual que en otros tipos de estreses abióticos, muchas de las especies silvestres relacionadas con nuestros cultivos presentan mecanismos más eficientes para su adaptación a los cambios bruscos como una sequía (Figura 3).
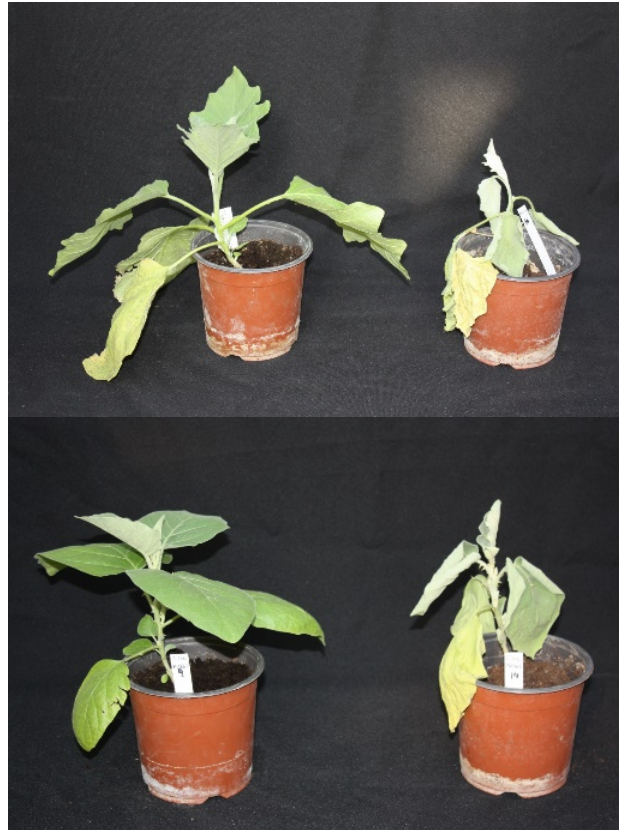
Figura 3: Comparación de los niveles de tolerancia frente al estrés hídrico de la berenjena cultivada (*arriba*) y la especie silvestre relacionada *S. incanum* (*abajo*). Las plantas de la izquierda son los controles irrigados, las plantas de la derecha son las plantas no irrigadas.

Al igual que los dos estreses mencionados anteriormente, las altas temperaturas también afectan a un amplio rango de factores en el desarrollo de las plantas y normalmente vienen acompañadas de situaciones de estrés hídrico (Zandalinas et al. 2017). Los mecanismos de tolerancia frente a este tipo de estrés se desencadenan a partir de una serie de cascadas de señales activadas por el incremento de la fluidez de la membrana citoplasmática como consecuencia del aumento de las temperaturas. Estas cascadas incrementan la concentración de ion Ca^{2+} y la reorganización del citoesqueleto. El final de estas rutas desemboca en el incremento de la producción de osmolitos y antioxidantes como una respuesta al estrés térmico (Bita y Gerats, 2020). Además, el estrés térmico también suele ir acompañado de estrés de tipo biótico (Bita y Gerats, 2020) como se explicará a continuación.

El cambio climático también implicará un incremento en la incidencia de los estreses de tipo biótico, el incremento de la temperatura y la concentración de CO_2 a nivel global tiene como consecuencia la aparición de nuevas razas de patógenos (Juroszek et al. 2020). Estas nuevas condiciones climáticas favorecen los ciclos de fecundidad y de infección de estos organismos gracias a que se presenta un microclima más adecuado (Hunjan y Lore, 2020). Del mismo modo con los cambios de climatología también acontecen cambios en la distribución geográfica de lo

patógenos, apareciendo nuevas enfermedades donde antes no las había (Bebber et al., 2013; Kumar and Khurana 2020).

En definitiva, la mejora genética vegetal ha de dirigirse hacia el desarrollo de variedades más resilientes. Según Darnhofer (2014) la resiliencia se entiende como un indicador de la capacidad de los cultivos para absorber perturbaciones sin alterar significativamente sus características productivas, contemplando tres aspectos fundamentales: la capacidad de los culticos a amortiguar, adaptarse y transformarse (Dutta et al. 2020). El enfoque de una mejora genética que favorezca el desarrollo de nuevas variedades con estas características consiste principalmente en mantener la diversidad genética y ser capaces de adaptar las prácticas de cultivo al nuevo contexto al que nos enfrentamos (Altieri y Nicholls, 2013).

Las aproximaciones pueden ser muchas, pero algunas están limitadas. Un ejemplo sería la combinación de estrategias agroecológicas junto con el uso de organismos genéticamente modificados. Además de acelerar y hacer más eficiente la transferencia de genes involucrados en los mecanismos de tolerancia gracias a la edición genética (CRISPR-Cas9) (Pandey et al. 2011; Dutta et al., 2020), estaríamos aplicando prácticas culturales más respetuosas con el medio ambiente, con lo que esta combinación optimizaría la lucha contra el cambio climático. No obstante, el uso de técnicas de ingeniería genética para desarrollar variedades dirigidas al consumo humano es algo que en la Unión Europea presenta muchas trabas hoy en día. Además, este tipo de técnicas generan un enorme rechazo en la comunidad de agricultores ecológicos, que cada vez tienen más demanda en el mercado europeo. Por lo tanto, es necesario emplear otras alternativas para la búsqueda y desarrollo de variedades adaptadas al cambio climático. Estas estrategias son las que se presentaran en el próximo apartado.

2.2. *Poblaciones de mejora: La introgresiómica*

La erosión genética ha sido una consecuencia inevitable de los propios procesos de mejora, dando lugar a la perdida de diversidad genética existente en las especies (van de Wouw et al., 2010; Carrillo, 2016). La domesticación de las especies silvestres y el posterior reemplazo de las variedades tradicionales por variedades mejoradas representan los dos cuellos de botella principales en los que se produce el evento de erosión (Smýkal et al. 2018). A medida que las especies silvestres van siendo seleccionadas de forma artificial y cultivadas en ambientes cada vez más propicios, la selección natural deja de ejercer su efecto produciéndose un descenso en la variabilidad genética del cultivo y por ende haciéndolo más dependiente de los agricultores y los ambientes propicios que les proporcionan (Tanksley y McCouch, 1997) (Figura 4). En este proceso se produce una perdida irreversible de diversidad que deja de ser aprovechable para los mejoradores. Afortunadamente, se ha hecho un gran esfuerzo en recolectar y preservar gran parte de estas especies silvestres y variedades tradicionales, fuentes de diversidad, en los bancos de germoplasma, habiendo actualmente más de 7 millones de especies cultivadas y silvestres relacionadas con la berenjena a nivel global preservadas (FAO, 2010).
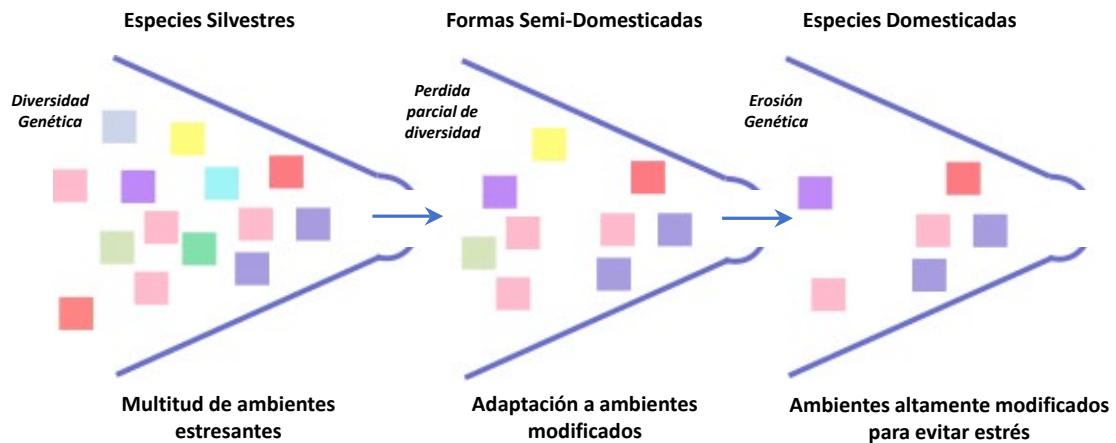
Figura 4: Proceso de domesticación de las especies silvestres donde se ven los dos cuellos de botella principales en los que se produce la erosión genética hasta llegar a las formas domesticadas menos diversas (basada en Tanksley y McCouch, 1997).

Esta erosión que genera una diversidad muy baja en los cultivos es algo preocupante ya que los cambios climáticos se están sucediendo a una velocidad que imposibilita la adaptación de nuestros cultivos a las nuevas condiciones. Es por ello por lo que es imprescindible aplicar métodos de mejora genética que permitan dar una respuesta rápida a los nuevos retos que se presentan (Henry, 2019). Sin embargo, la velocidad de los procesos de mejora va a depender de si se dispone o no de materiales élite con fondo genérico adaptado y esto habitualmente no está disponible o no responde a los objetivos que se están buscando. Por otra parte, las especies silvestres y los materiales no adaptados son excelentes fuentes de variación, siendo muchos de los caracteres que poseen tolerancias o resistencias al cambio climático. Sin embargo, estos son difíciles de reintroducir en los materiales cultivados, dilatándose el proceso a lo largo del tiempo y requiriendo años o incluso décadas para conseguirlo (Zhang y Batley, 2020). Por otra parte, el uso de especies silvestres como fuente de diversidad presenta otros inconvenientes como la introgresión de caracteres indeseables (habitualmente dominantes o poligénicos) que requieren de varias generaciones para su eliminación y que en muchas ocasiones van acompañados de fenómenos de arrastre por ligamiento que hace más difícil todavía su eliminación (Shing et al., 2005; Bradshaw, 2017). Además de los problemas asociados a la incompatibilidad sexual y las barreras reproductivas que habitualmente se encuentran al utilizar especies silvestres relacionadas (Mwangangi et al., 2019; Jayashree et al., 2020; Daunay et al., 2019).

Según Prohens et al., (2017) La "introgresiómica" se presenta como una propuesta dirigida a la incorporación masiva de la diversidad presente en las especies relacionadas con los cultivos de interés y dirigida principalmente a la adaptación al cambio climático. La definieron como "el desarrollo sistemático a escala masiva de materiales y poblaciones portadores de introgresiones de fragmentos genómicos procedentes de especies relacionadas (principalmente silvestres) en un fondo genético de especies cultivadas, de forma que se puedan desarrollar nuevas generaciones de cultivares con propiedades mejoradas". Esta aproximación es un método de mejora preventiva en el que se pretende ampliar la base genética de los cultivos desarrollando poblaciones de mejora con introgresiones de especies silvestres o materiales tradicionales. Con esta estrategia se pueden generar colecciones que ayudarán a hacer frente a los retos presentes y futuros en la mejora ya sean predecibles o no. Disponiendo de este reservorio de genes de origen silvestre en un fondo genético de especies cultivadas, este conjunto de materiales élite permitirán dar una respuesta rápida a cualquier situación ya que estas líneas pueden ser

empleadas e introducidas de una forma muy eficiente en los programas de mejora (Ruiz de Galarreta et al. 2018).

La introgresiómica sigue varios pasos (Prohens et al., 2017). En primer lugar, hay que identificar los materiales silvestres que se van a utilizar para el desarrollo de las poblaciones con introgresiones. En el caso de que estemos hablando de una aproximación de mejora genética "dirigida", es decir, como en el caso que nos concierne en esta tesis doctoral donde queremos adaptar los materiales cultivados a los efectos del cambio climático, las especies silvestres a utilizar se deberían de seleccionar en función de la probabilidad de contengan caracteres de interés para dicho objetivo de mejora. Estos datos pueden obtenerse a través de un fenotipado o de información sobre los ambientes en los que estas especies se desarrollan (Ruiz de Galarreta et al. 2018). Otra cosa muy importante que hay que tener en cuenta a la hora de realizar esta elección es el grupo de germoplasma (*genepool*) al que pertenece la especie ya que esto entraña ciertos riesgos. Según Rizvi y Sarker (2020), las especies de germoplasma primario cruzan fácilmente entre ellas, las de germoplasma secundario pueden cruzarse, pero el porcentaje de éxito disminuye y su descendencia puede ser infértil, finalmente las de germoplasma terciario son difíciles de cruzar y pueden requerir de técnicas especiales para la obtención de su descendencia (que habitualmente es estéril) como por ejemplo el rescate de embriones.

Una vez seleccionadas las accesiones silvestres, el siguiente paso es la hibridación y la obtención de materiales con introgresiones. Como se ha comentado anteriormente, esta hibridación es compleja incluso en el caso de algunas especies del germoplasma primario debido a requerimientos específicos relacionados con el fotoperiodo o la dormancia de las semillas (Prohens et al., 2017). Cuando las especies silvestres pertenecen al germoplasma secundario o terciario aparecen barreras precigóticas (germinación del polen, desarrollo del tubo polínico o fertilización) y postcigóticas (desarrollo del embrión) que dificultan la obtención de los híbridos interespecíficos (Daunay et al., 2020). En caso de que aparezca algunos de estos inconvenientes existen algunas aproximaciones y estrategias para solventarlos, por ejemplo, en el caso de las barreras precigóticas el uso de polen mentor o tratamientos químicos en el estigma, y para el caso de las barreras postcigóticas el rescate de embriones (Prohens et al., 2017).

Una vez obtenidos los híbridos interespecíficos, el desarrollo de los materiales con introgresiones de las especies silvestres suele estar basado en la realización de retrocruzamientos (Kouassi et al. 2016). No obstante, la obtención de estos híbridos interespecíficos no garantiza que vayamos a poder desarrollar poblaciones de retrocruzamientos ya que pueden ser parcial o totalmente estériles (Ju et al. 2019; Wang et al. 2020). Diferentes mecanismos moleculares subyacen a la infertilidad de los híbridos, como las incompatibilidades genéticas (nuclear y citoplasmática) (Maheshwari y Barbash, 2011) o los cambios en la arquitectura del genoma (número de cromosomas o reordenamientos cromosómicos) (Rieseberg, 2001). Existen algunas estrategias cuando la esterilidad no es total, como por ejemplo emplear el híbrido como parental femenino, garantizando así el vigor del polen para que sea capaz de fecundar la célula huevo (Prohens et al., 2017). Otra opción es la duplicación de genoma del híbrido lo que en alguna ocasión da lugar al restablecimiento de la fertilidad del polen (Khush y Brar, 1992; Shivanna y Bahadur, 2015). Esta estrategia entraña algunos pasos adicionales, ya que el híbrido resultante es un individuo tetraploide, por tanto, su ploidía puede no coincidir con el parental recurrente (la especie cultivada), por ello es necesario obtener la versión tetraploide del mismo y tras una o dos generaciones de retrocruzamientos, devolverle a la población la dotación diploide mediante el cultivo de anteras o microsporas (Prohens et al., 2017). No obstante, para que esto sea posible han de estar disponibles las

herramientas biotecnológicas necesarias para llevar a cabo estas técnicas avanzadas de mejora. Afortunadamente, a medida que se avanza en las generaciones de retrocruzamiento, el fondo genético cultivado es mayor y la fertilidad se recupera paulatinamente facilitando el proceso de introgresión (Charron et al. 2019).

El último paso en el desarrollo de poblaciones adaptadas al cambio climático mediante la introgresiómica consiste en la obtención de materiales útiles y fácilmente utilizables para los mejoradores (Prohens et al., 2017). El uso de los marcadores moleculares ha hecho posible la obtención de los siguientes tipos de poblaciones (Figura 5):

> - CSLs: *Chromosome substitution lines* o líneas de sustitución cromosómica. En este tipo de poblaciones cada una de las líneas contiene el genoma de la especie cultivada excepto para una pareja de cromosomas introducida de la especie silvestre. Entre todas ellas representan el genoma silvestre en su totalidad (Cavanagh et al., 2008).

> - ILs: *Introgression lines* o líneas de introgresión. Estas líneas representan la totalidad del genoma silvestre en el conjunto de líneas de introgresión donde la mayoría del fondo genético es el de la especie cultivada exceptuando un pequeño fragmento de la silvestre (Zamir, 2001).

> - MAGIC: *Multi-parental advanced generation inter-cross* o poblaciones multiparentales avanzadas de entrecruzamientos. En estas poblaciones un conjunto de parentales (8 o 16) se entrecruzan entre ellos dando lugar en última instancia a un híbrido cuádruple que al autofecundarse dará lugar a una descendencia ultrasegregante. En este caso alguno de los parentales debería de ser una especie silvestre (Prohens et al. 2017).

Utilizar varias especies silvestres a la vez para obtener este tipo de poblaciones hace aumentar exponencialmente la base genética del cultivo. De esta manera se consigue representar el genoma de dichas especies en un fondo genético cultivado haciéndolo muy accesible en los programas de mejora para la adaptación al cambio climático. La berenjena es un cultivo en el que a pesar de su importancia no hay muchas herramientas de este tipo desarrolladas. A día de hoy, tan solo se dispone de una colección de líneas de introgresión con *S. incanum* (Gramazio et al. 2017) y se está desarrollando una población MAGIC (Arrones et al., 2020), por lo que es muy importante desarrollar más materiales que amplíen el fondo genético de este cultivo y dotar de herramientas tanto a los investigadores como a los mejoradores que trabajan con este cultivo. Es por ello por lo que en el marco de esta tesis doctoral se ha iniciado el desarrollo de tres colecciones de ILs con tres especies silvestres relacionadas con la berenjena, *S. insanum* (germoplasma primario), *S. dasyphyllum* (germoplasma secundario) y *S. elaeagnifolium* (germoplasma terciario).
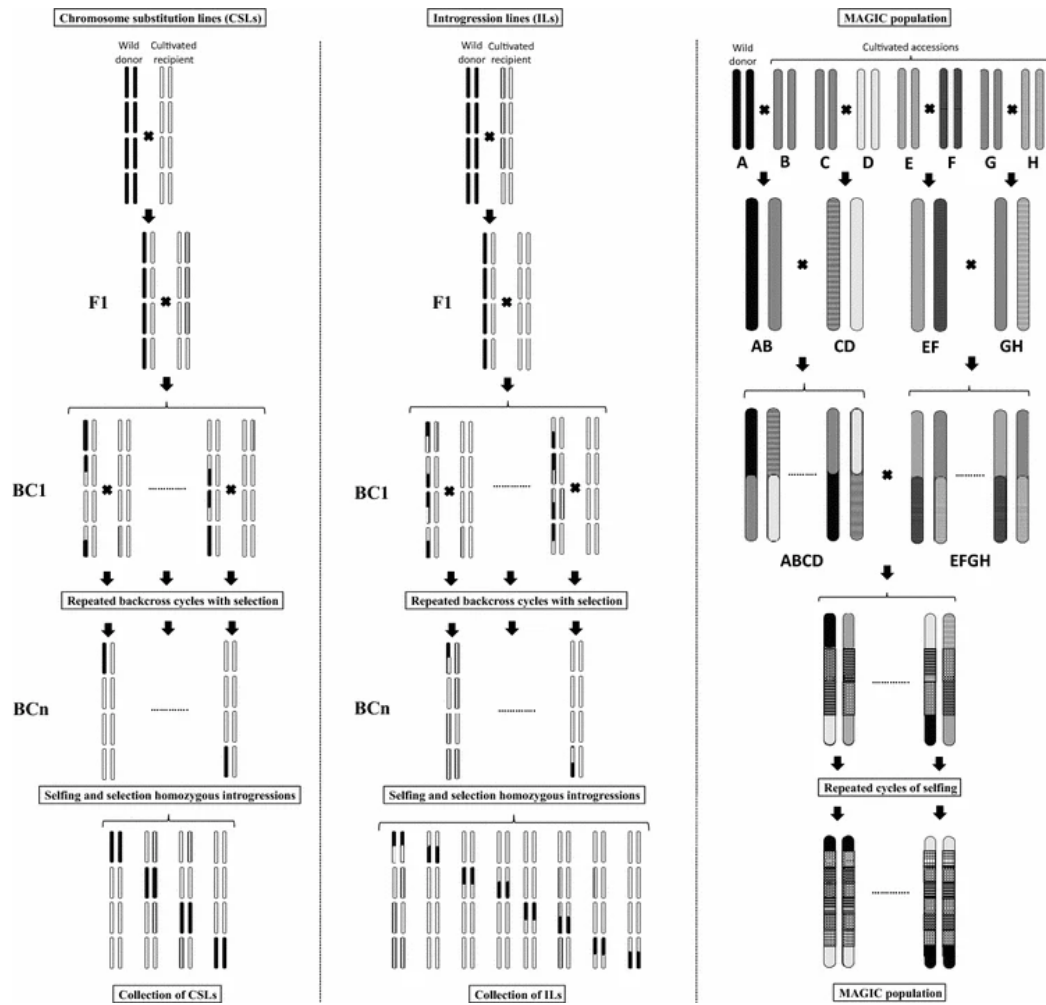
Figura 5: Esquema del desarrollo de algunos tipos de poblaciones de mejora con introgresiones: lineas de sustitución cromosómica (CLS, *izquierda*), líneas de introgresión (ILs, *Centro*) y poblaciones multiparentales avanzadas de entrecruzamientos (MAGIC, *derecha*) (tomada de Prohens et al., 2017).

2.3. *Speed breeding*

Tal y como ha quedado reflejado en los apartados anteriores, la mejora genética vegetal es una carrera contrarreloj en la que se aplica una cantidad enorme de esfuerzo en acortar los tiempos lo máximo posible. En los últimos años se ha acuñado un término que define a la perfección y recoge en una sola palabra todas estas metodologías para acelerar el proceso de mejora; este es el *Speed breeding* (Watson et al., 2018). En definitiva, el *Speed breeding* es el conjunto de técnicas que reduce los tiempos generacionales y acelera los procesos de mejora.

Algunos ejemplos de esto son, modificaciones agronómicas que afectan al fotoperiodo de las plantas cultivadas acelerando la tasa de desarrollo de las plantas así como la cosecha y germinación de semillas inmaduras, reduciendo el tiempo de generación (Sysoeva et al. 2010). La limitación de espacio para el crecimiento en macetas de 1,5 L junto con una reducción del aporte hídrico es otra práctica que estimula la floración y el cuajado de frutos en berenjena tal y como hemos comprobado en nuestro grupo de investigación, acelerando el proceso de avance de líneas.

Existen técnicas que combinan las prácticas de mejora tradicional con las técnicas biotecnológicas avanzadas para acelerar los procesos de mejora genética. Un ejemplo sería la técnica del rescate de embriones, que ha sido fundamental en el desarrollo de esta tesis doctoral para la obtención de los híbridos entre *S. melongena* y *S. elaeagnifolium.* En primer lugar, los cruces interespecíficos se realizan en el invernadero para obtener los frutos inmaduros a partir de los cuales el embrión será rescatado, de lo contrario este no podrá desarrollarse bien debido a las barreras postcigóticas presentes en su desarrollo por incompatibilidades con el endospermo y morirá (Figura 6).
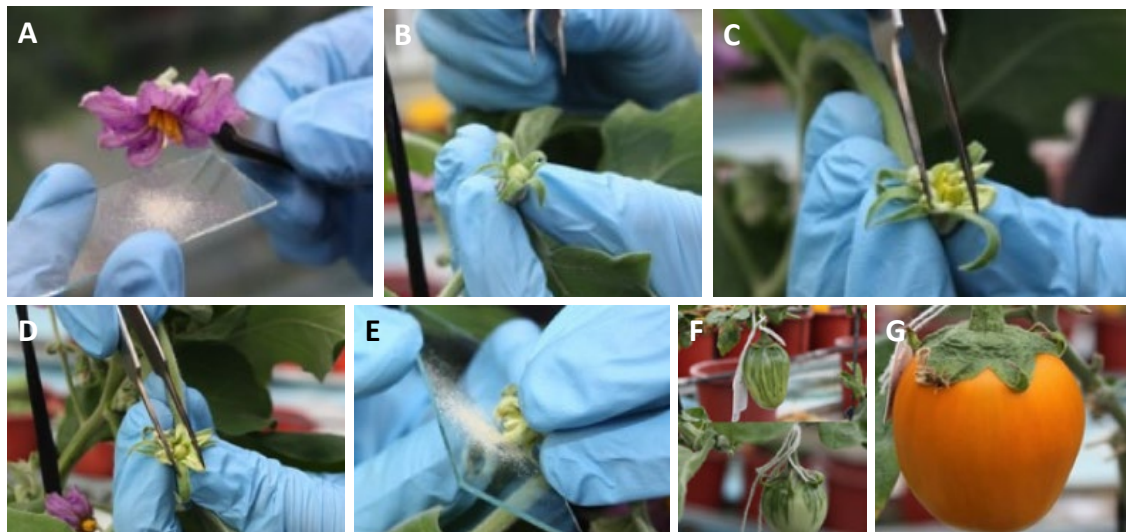
Figura 6: Esquema del proceso para llevar a cabo cruces en berenjena (García-Fortea et al., 2018). Se extrae el polen del parental masculino y se deposita en una superficie plana (A); hay que buscar aquellas flores que todavía no hayan experimentado la antesis pero que empiecen a emerger los pétalos entre los sépalos (B); con las ayuda de unas pinzas las anteras son retiradas (C y D); a continuación el estigma del parental femenino se pone en contacto con el polen previamente obtenido (E); pasadas 2-3 semanas los frutos inmaduros se pueden cosechar para proceder con el rescate de embriones (F); si se deja más tiempo, el fruto madurará y los embriones inviables habrán abortado en el caso de cruces con especies alejadas como en este caso que se utilizó *S. elaeagnifolium*, del germoplasma terciario (G).

A continuación, en condiciones de esterilidad, los frutos se abren y se extraen las semillas inmaduras. Con la ayuda de unas pinzas, en la cabina de flujo laminar los embriones son extraídos del interior de la semilla tal y como se indica en las Figura 7. Finalmente, los embriones rescatados son cultivados en un medio nutritivo y dar lugar a plantas adultas que se aclimatarán y se podrán introducir al programa de mejora (Garcia-Fotea et al.,2018).

Finalmente, existen técnicas biotecnológicas de cultivo *in vitro* que pueden acelerar muchísimo los procesos de mejora genética vegetal. En este caso hablamos de técnicas como la micropropagación, la transformación genética, la obtención de organismos poliploides o la producción de líneas puras mediante la técnica de los dobles haploides. Existen cultivos en los que están ampliamente desarrolladas, sin embargo, en el caso de la berenjena, a pesar de existir algunas aproximaciones, no terminan de presentar un rendimiento elevado. En el siguiente apartado de esta introducción se tratará con profundidad esta serie de técnicas y e presentará el estado actual de cada una de ellas en el caso de la berenjena.
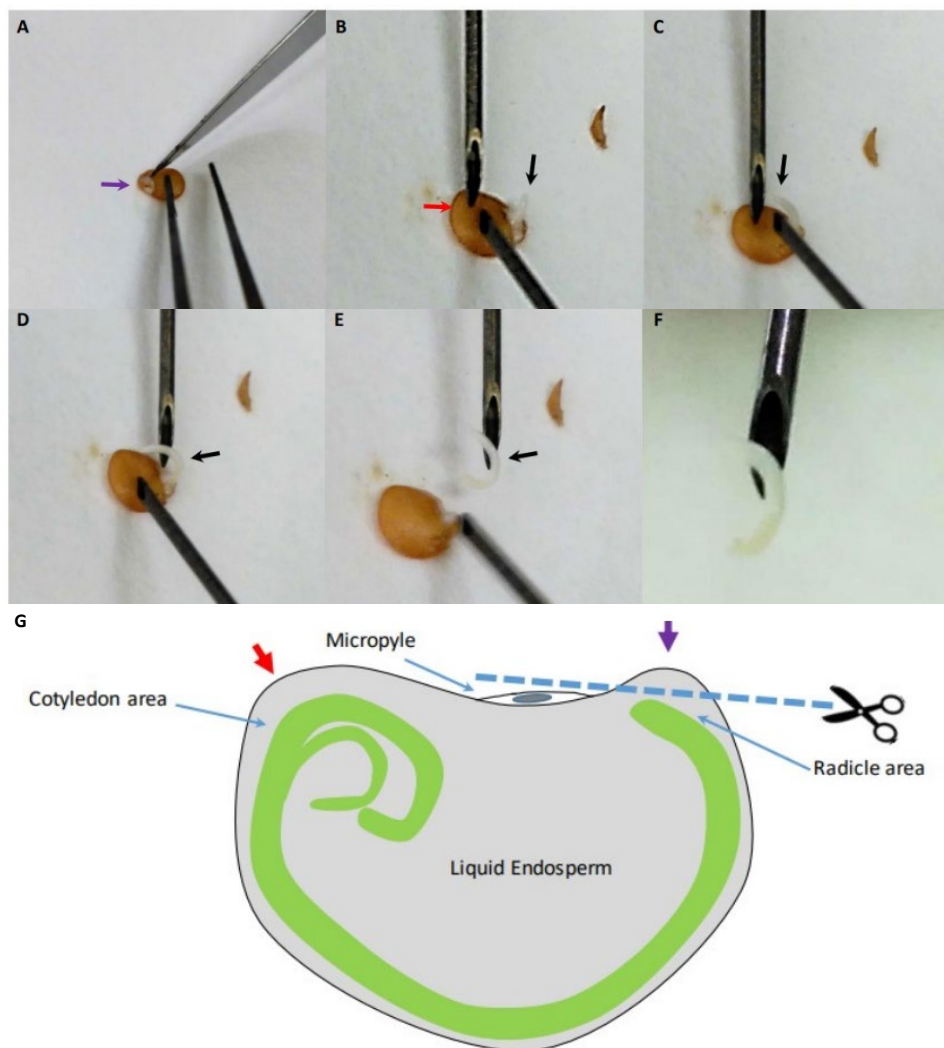
Figura 7: Corte en el área correcta del micropilo para facilitar la salida del embrión, la flecha morada indica el área exacta (A); proceso de extracción del embrión, la flecha roja indica el área en la que hay que ejercer presión, las flechas negras señalan al embrión (BCDEF). Diagrama del interior de la semilla, la línea discontinua indica el área de corte, la flecha morada coincide con la flecha de la figura A. La flecha roja indica la zona aproximada en la que ha que ejercer presión para facilitar la salida del embrión, la flecha roja corresponde con la flecha de la figura B (G).

3. Herramientas biotecnológicas de apoyo a la mejora genética vegetal

3.1. El cultivo in vitro

El cultivo *in vitro* de tejidos vegetales consiste en el crecimiento y multiplicación de células, tejidos y órganos en un medio sólido o líquido con una composición química concreta y en un entono aséptico y controlado. Esta tecnología es una herramienta clave en la biotecnología vegetal, siendo útil para múltiples aplicaciones como la propagación de plantas, aprovechando la naturaleza totipotente de las células vegetales, es decir, su capacidad para desdiferenciarse y diferenciarse de nuevo en cualquier tipo celular (Thorpe, 2007).

El origen del cultivo in vitro se remonta a 1902, cuando Haberlandt formuló el concepto de totipotencia, sentando las bases teóricas para el cultivo de tejidos vegetales (Thorpe, 2007). Ya en la década de 1930, se consiguieron cultivar meristemos radiculares de tomate (White, 1934) y en el período comprendido entre 1940 y 1960 se desarrollaron nuevas técnicas in vitro y medios nutritivos, como el de Murashige y Skoog (Murashige y Skoog, 1962). En las décadas siguientes, estos avances condujeron a la aplicación del cultivo de tejidos en la obtención de plantas libres de patógenos, conservación de germoplasma y propagación clonal (Johri y Bhojwani, 1965; Murashige, 1974). A partir de 1990 asistimos a una expansión creciente en la aplicación de las técnicas in vitro a todo tipo de plantas: cereales (Vasil y Vasil, 1994), legumbres (Davey et al., 1994), cultivos de hortalizas (Reynolds, 1994) o semillas oleaginosas (Palmer y Keller, 1994); en estos trabajos la tecnología fue más allá de la micropropagación, abordando todos los enfoques que pudieran ser relevantes para la especie en particular y los problemas que conllevaba (Thorpe, 2007).

La mejora genética convencional, dirigida a introducir características deseables en una variedad mediante técnicas de hibridación y selección, presenta limitaciones tales como la posibilidad de transmitir rasgos no deseados, la lentitud del proceso y el hecho de que sólo puede usarse como fuente de variación la propia especie u otras compatibles. Las mejoras biotecnológicas superan estos obstáculos mediante el empleo de técnicas de cultivo in vitro que permiten obtener resultados más eficaces en menos tiempo y ampliar las fuentes de variación.

Aplicado al fitomejoramiento, el cultivo *in vitro* facilita la multiplicación rápida de clones superiores mediante micropropagación y, asociado con la biología molecular, ha permitido incorporar rasgos específicos a través de transferencia de genes (Monteiro do Rêgo et al., 2016) y superar barreras de incompatibilidad sexual mediante la hibridación somática (fusión de protoplastos) y el rescate de embriones. También se ha utilizado para la creación de variabilidad genética mediante la producción de haploides, poliploides o variantes somaclonales a partir de los cuales mejorar los cultivos. También se ha aplicado el cultivo *in vitro* en la mejora fitosanitaria mediante el cultivo de meristemos y la conservación de material vegetal (Kothari et al., 2010).

Sin embargo, una de las aplicaciones más importantes y disruptivas de nuestra generación, es la aplicación de las tecnologías de DNA recombinante y de la edición de genes, como CRISPR/Cas9. No obstante, el cultivo in vitro representa la llave que abre o cierra la posibilidad de la aplicación de esas técnicas en la mejora genética vegetal de ciertos cultivos. La regeneración de plantas a menudo presenta dificultades a causa de la ausencia de protocolos de organogénesis eficientes, lo que supone un gran obstáculo para la aplicación eficiente de estas técnicas en muchas especies (Altpeter et al. 2016).

3.1.1. Micropropagación

La micropropagación de genotipos de élite o de plantas que carecen de sistemas de propagación vegetativa natural, permite la multiplicación a gran escala del material vegetal. A partir de un solo individuo se producen in vitro clones de las plantas, genéticamente idénticas (Kothari et al., 2010) o bien regenerar brotes adventicios a partir de explantes. Esta propagación vegetativa asegura que las características deseadas de la planta seleccionada se retengan en todos sus clones (Murashige, 1974). Las ventajas de la micropropagación in vitro sobre la propagación tradicional son evidentes: posibilidad de producción masiva de plantas homogéneas y de alta calidad fitosanitaria en poco tiempo y en espacios reducidos, así como de multiplicar plantas recalcitrantes a las técnicas convencionales.

La técnica se lleva a cabo en cuatro etapas; la primera es el establecimiento del cultivo axénico, para lo cual se selecciona el explante adecuado de la planta madre, se esteriliza y se transfiere a un medio adecuado. La segunda, es la multiplicación, en la que se produce la proliferación de brotes del explante; en esta etapa, los medios y los reguladores de crecimiento juegan un papel crítico y de nuevo la existencia de protocolos de organogénesis eficientes es crucial. En la tercera etapa, se transfieren los brotes al medio de enraizamiento, obteniéndose plántulas completas y, finalmente, las plántulas regeneradas se pasan a macetas para su aclimatación previa a los ensayos de campo (Chung et al. 2020).

3.1.2. Transformación genética

La transformación genética precisa de un método de introducción del DNA foráneo en la célula vegetal y un sistema de cultivo de tejidos y selección *in vitro. Agrobacterium tumefaciens* se ha empleado ampliamente como vector para mediar la transformación. El método requiere un período de co-cultivo en el que la cepa de *Agrobacterium,* portadora de los genes de interés, se pone en contacto con el tejido vegetal herido para que ocurra la transferencia del DNA a las células expuestas a la infección, utilizando un medio que favorezca el proceso. A continuación, el tejido vegetal se transfiere a un medio de cultivo al que se adicionan reguladores de crecimiento combinadas en un balance adecuado que propicie la regeneración para obtener plantas completas y fértiles. Al medio se le añaden agentes de selección (antibióticos como la kanamicina) para garantizar sólo la regeneración de los explantes o células transformadas (Collier et al., 2016).

3.1.3. La organogénesis en la berenjena

Como ha quedado patente en los apartados anteriores, el cultivo *in vitro* es una herramienta fundamental para llevar a cabo un gran número de aplicaciones biotecnológicas que aceleran los procesos de mejora genética vegetal. Pero para poder aplicar estas técnicas de forma eficiente es necesario tener protocolos de organogénesis robustos, estables, reproducibles y en los que el factor genético tenga una baja interferencia. En el caso de la berenjena existen múltiples protocolos para inducir la organogénesis *in vitro,* pero la gran mayoría han obtenido una eficiencia muy baja o las publicaciones presentan rendimientos que al ser evaluados no se ajustan a la realidad. Existiendo fundamentalmente un gran problema con el factor genotipo, condicionando en gran medida el éxito de la organogénesis independientemente del protocolo empleado.

Algunos de los protocolos disponibles han evaluado la respuesta organogénica empleando diversos tejidos como explante inicial (hoja, hipocótilo y cotiledón) y fitohormonas como la bencilaminopurina (BAP) o el ácido indolacético (IAA) (Sharma y Rajam., 1995, Bhat et al., 2013),

el ácido naftalenacético (NAA) (Scoccianti et al. 2000), el thidiazuron (TDZ) (Magioli et al., 1998) incluso irradiación con láser de helio-neón (Swathy et al., 2017). Sin embargo, hasta que Muktadir et al. (2016) emplearon el ribósido de zeatina (ZR) no se vieron resultados estables y prometedores; no obstante, la problemática del factor genotipo seguía sin tener una respuesta (Sharma y Rajam, 1995).

Por ello, ante la ausencia de protocolos estables, la persistencia de la problemática del factor genotipo y la necesidad interna de disponer de un protocolo eficiente de organogénesis para la ejecución de proyectos en el grupo de investigación, dentro del marco de esta tesis doctoral se planteó el desarrollo de un protocolo basado en el ZR estable, para la inducción de la organogénesis en berenjena y la evaluación del factor genotipo.

3.2. Los poliploides

La poliploidía es un evento común en las plantas, que involucra la adición de otra serie completa de cromosomas. A menudo las especies de plantas, que naturalmente son poliploides, muestran una mejora en el vigor durante su crecimiento y una mejor adaptación a ambientes adversos. Estos rasgos son claras ventajas evolutivas que se han incorporado a lo largo de la historia en los programas de mejora genética vegetal en diversos cultivos.

Nuevas herramientas tecnológicas han permitido dilucidar que al menos un 70% de todas las angiospermas han sufrido, al menos, un evento de poliploidía (Masterson, 1994). Las especies con eventos recientes de poliploidía son clasificadas en dos categorías: autopoliploides y alopoliploides. Los autopoliploides son producto de eventos de duplicación completa de genoma dentro de una especie (Vía A, Figura 8) (Ramsey y Schemske, 2002). Por otro lado, los alopoliploides derivan de híbridos interespecíficos, en donde se mantiene las formas duplicadas de dos o más genomas de los parentales del híbrido (Vía B, Figura 8). Los alopoliploide son por tanto el producto de la mezcla de dos genomas completamente diferentes. Los anfidiploides son poliploides formados por la unión de dos juegos cromosómicos distintos y posteriormente su duplicación, siendo por tanto un tipo de alopoliploide (Vía C, Figura 8).

Especie original
Diploide

Especie Diploide
AA

Especie Diploide
BB

Especiación

Especiación

Hibridación con gametos n

Híbrido Diploide
AB

**Duplicación
(Vía A)**

**Hibridación con
gametos 2n
(Vía B)**

**Duplicación
(Vía C)**
Anfidiploidía

Individuo *autotetraploide*
AAAA

Individuo *alotetraploide*
AABB
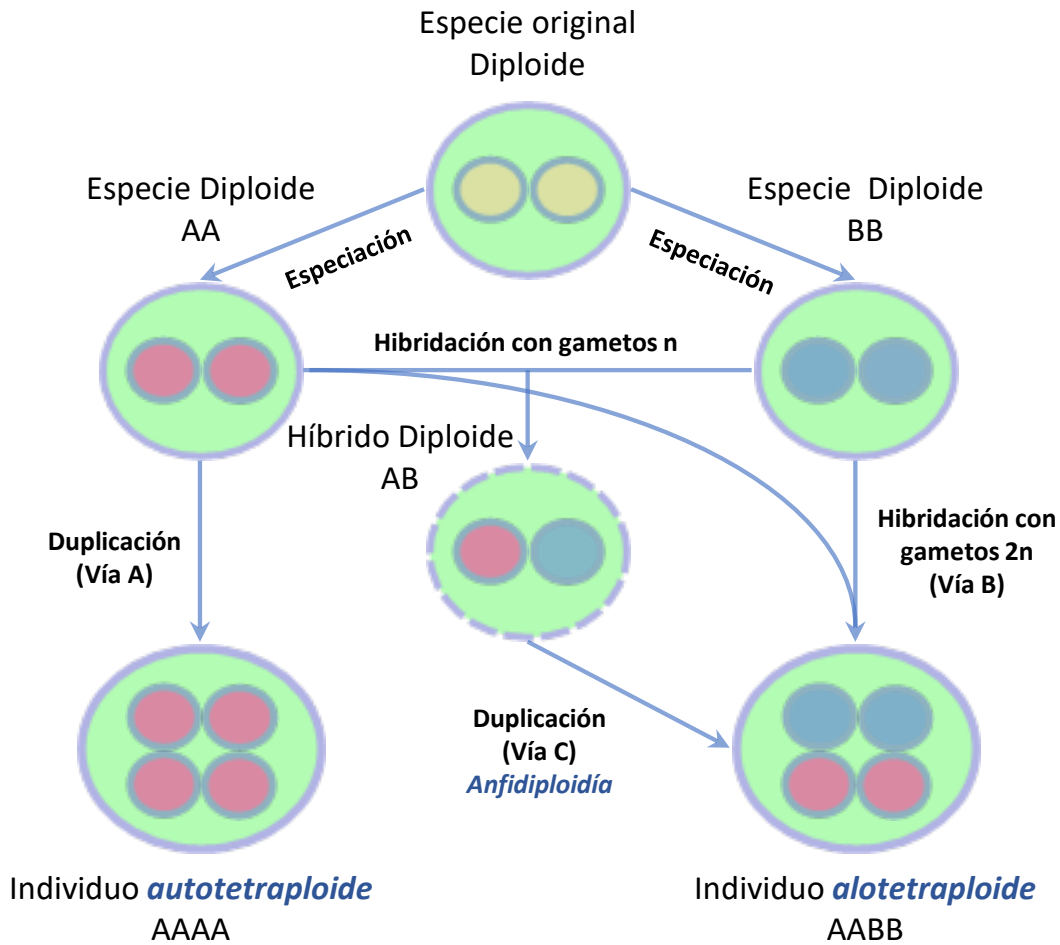
Figura 8: Distintas rutas hacia la formación de un individuo poliploide de manera natural. Autopoliploidía (vía A), alopoliploidía (vía B) y anfidiploidía (vía C).

Los beneficios de la poliploidía, en la evolución del reino vegetal, se atribuyen a factores que aceleran la misma. Como por ejemplo los efectos amortiguadores frente a posibles mutaciones deletéreas, efectos de dosis génica, incremento de la diversidad alélica, incremento de la heterosis y sub- o neofuncionalización de genes duplicados resultando todo esto en variación fenotípica (Te Beest, et al., 2012). La evolución de un genoma poliploide es un proceso altamente dinámico en comparación con el de los diploides. Después del evento de poliploidización, el genoma sufre cambios considerables a nivel genético y epigenético. Algunas de las alteraciones genéticas que ocurren son reestructuraciones genómicas, incluyendo reorganizaciones cromosómicas, amplificación de secuencias repetitivas y pérdida de secuencias de DNA (del Pozo & Ramirez-Parra, 2015). La poliploidización también conlleva cambios funcionales sin alteraciones en la secuencia nucleotídica, como modificaciones epigenéticas; más concretamente ocurre un cambio en el patrón de metilación y el remodelado de la cromatina (Comai, 2005). En conclusión, las alteraciones en el contenido cromosómico conllevan varios tipos de ajustes en el genoma y estos factores usualmente afectan la viabilidad y fertilidad de la planta poliploide (Comai, 2005). Sin embargo, los individuos poliploides nuevos se vuelven genéticamente estables una vez que hayan superado el cuello de botella representado esta inestabilidad genómica (Wang, et al., 2006).

3.2.1. Uso de los poliploides en la mejora genética vegetal

La poliploidía es una importante herramienta en agricultura, ya que confiere ciertas ventajas de adaptabilidad, resistencia o tolerancia a estreses abióticos (del Pozo y Ramírez-Parra, 2015). Suele derivar, en órganos más grandes, un color más verde y un período de floración más largo o tardío; de hecho, los poliploides se emplean en la industria ornamental por el mayor tamaño de las flores (Shao et al., 2003). Esta condición genómica usualmente confiere ventajas de adaptabilidad y tolerancia a estreses abióticos (Tabla 2)

Tabla 2: Ejemplos de especies en donde la poliploidía mejoró la tolerancia a estreses abióticos (del Pozo & Ramirez-Parra, 2015).

Especie	Ploidía	Tolerancia	Referencia
A. thaliana	Autotetraploide	Deficiencia de Boro	(Kasajima, et al., 2010)
A. thaliana	Autotetraploide	Sequía, estrés salino	(del Pozo & Ramirez-Parra, 2014)
A. thaliana	Autotetraploide	Estrés salino	(Chao, et al., 2013)
B. distachyon	Autotetraploide	Sequía (aridez)	(Manzaneda, et al., 2012)
B. rapa L.	Autotetraploide	Estrés salino	(Meng, et al., 2011)
Cenchrus	Alotetraploide/ alohexaploide	Sequia	(Chandra & Dubey, 2010)
C. stoebe	Autotetraploide	Sequia	(Mraz, et al., 2014)
C. limonia	Autotetraploide	Sequía, frio, déficit de nutrientes	(Allario, et al., 2013)
C. limonia	Autotetraploide	Estrés salino en sequía	(Allario, et al., 2011)
D. nankingense	Autotetraploide	Frío, sequia, estrés salino	(Liu, et al., 2011)
D. zingiberensis	Autotetraploide	Calor	(Zhang, et al., 2010)
H. vulgare	Autotetraploide	Sequia	(Chen & Tang, 1945)
N. benthamiana	Octoploide	Sequía, frio, déficit de nutrientes	(Deng, et al., 2012)
P. trifoliata	Autotetraploide	Estrés salino en sequía	(Saleh, et al., 2008)
Triticum	Alotetraploide/ alohexaploide	Estrés salino	(Prazak, 2001)

Otra de las grandes aplicaciones en la mejora genética vegeta que suponen los organismos poliploides es la obtención de frutos sin semillas. Los frutos sin semilla siempre han sido deseados tanto por consumidores como por productores debido a las ventajas que presentan. Los consumidores muestran preferencia por los frutos sin semilla debido a que este carácter hace que el proceso de ingesta del fruto sea una experiencia más placentera a nivel organoléptico, mientras que los productores también prefieren los frutos sin semillas debido a que su ausencia permite prolongar la vida post-cosecha (Maestrelli et al., 2003; Pandolfini, 2009). En casos más concretos, como en la berenjena, la ausencia de semillas también permite conseguir una reducción del pardeamiento de la carne (Maestrelli et al., 2003).

El control sobre este caracter ha sido el objetivo de muchísimos investigadores y mejoradores, aplicando múltiples técnicas para su consecución. Entre ellas se encuentra desde la selección

natural de triploides en la naturaleza, el cultivo *in vitro* de endospermos triploides para inducir la regeneración de plantas, la hibridación sexual entre plantas con distintas ploidías y hasta la fusión de protoplastos para generar parentales alotetraploides (Grosser & Gmitter, 2011; Wang, et al., 2016). A pesar de todas estas aproximaciones, desde un punto de vista aplicado, la técnica que realmente es transferible a los mejoradores y que por tanto puede emplearse rutinariamente es la hibridación sexual entre parentales que difieran en su nivel de ploidía. La hibridación entre un individuo tetraploide y un individuo diploide, da como resultado la formación de un gameto triploide que al crecer conformará un individuo triploide y por lo tanto estéril, que producirá frutos sin semillas. Por ello, los esfuerzos se han focalizado principalmente en la producción de individuos poliploides *in vitro,* de modo que sea posible suministrar a los mejoradores individuos tetraploides para sus trabajos de hibridación. En este método la colchicina es uno de los agentes antimitóticos más utilizados y se ha empleado con buenos resultados en numerosas plantas *ex* o *in vitro* (Väinölä 2000; Blakesley et al. 2002; Kadota y Niimi 2002; Shao et al. 2003). Sin embargo, la mayoría de los agentes antimitóticos son cancerígenos, lo que representa un riesgo para los trabajadores, además el uso de estos químicos es en gran medida ineficiente (Allum et al. 2007). Con frecuencia, las plantas obtenidas son mixoploides o quiméricas, y la mayoría de las veces vuelven al estado diploide original (Lehrer, 2008). También los compuestos como la orizalina y la trifluralina son utilizados para interrumpir la formación de huso y prevenir la división nuclear y celular (Ascough y van Staden 2008; Aleza et al. 2009), no obstante, al igual que la colchicina, la efectividad de estos compuestos depende en gran medida de la concentración aplicada, la duración del tratamiento, el tipo de explante y la penetración del compuesto, lo que conlleva en la mayoría de los casos a una baja tasa de duplicación y una alta tasa de quimerismo (Allum et al. 2007).

Es por estos motivos que en los últimos años se han hecho grandes esfuerzos en buscar alternativas para el desarrollo de poliploides sin emplear agentes antimitóticos. Para ello existen una serie de técnicas que también se beneficia de los protocolos de regeneración *in vitro*, mediante los cuales pueden obtenerse plantas con distinto nivel de ploidía. Esta fuente de variación aprovecha la polisomatía, un fenómeno que ocurre durante la diferenciación de tejidos a lo largo del desarrollo natural de la planta, consistente en la presencia de células de varios niveles de ploidía en un mismo órgano (Langlet., 1927; Galán-Ávila et al., 2020). Se produce como consecuencia de la endorreduplicación, una mitosis en la que el genoma se duplica, pero no llega a suceder la división nuclear o citoplasmática (D'Amato, 1964; Bubner et al., 2006). Este mecanismo se da con el fin de generar suficiente cantidad de DNA en previsión de un rápido aumento de la masa del tejido (Sliwinska y Lukaszewska, 2005). El patrón polisomático de tejidos vegetales se analizó para diversas especies y tejidos en diferentes etapas de desarrollo, por ejemplo, en tomate (Smulders et al., 1994), pepino (Gilissen et al., 1993), remolacha (Sliwinska y Lukaszewska 2005), *Cymodocea nodosa* (Gargiulo et al. 2020) o en *Cannabis sativa* L. (Galán-Ávila et al. 2020).

3.2.2. Poliploides en berenjena

En la berenjena, al igual que cultivos como la sandía, el pepino y la vid, se busca la reducción o ausencia de las semillas. En relación con esto, se han realizado múltiples trabajos para conseguir berenjenas sin semillas, los cuales, van desde el estudio de la partenocarpia (Acciarri, et al., 2002) hasta los tratamientos con fitohormonas. Sin embargo, existe muy poca información sobre trabajos realizados para obtener berenjena sin semilla a través del desarrollo de genotipos triploides. Algunos autores (Ali et al., 1992) consiguieron generar híbridos anfidiploides entre *S. melongena* y *Solanum integrifolium*. El objetivo de esto fue obtener portainjertos fácilmente

propagables y resistentes a enfermedades para la producción de berenjenas y tomates. En otra investigación, Miyoshi (1993) empleando el cultivo *in vitro* de microsporas de berenjena obtuvo algunos callos 3 triploides, aunque este no era el objetivo principal del estudio. En 1998 Hitomi et al., evaluaron la influencia del tipo de auxina en el conjunto de variantes somaclonales generadas a partir de la embriogénesis somática de la berenjena. En este trabajo, obtuvieron algunas plantas con hojas particularmente gruesas que correspondían a plantas tetraploides.

A pesar del interés que suscitan los organismos poliploides en este cultivo, los trabajos en los que se ha conseguido la obtención de plantas tetraploides de berenjena son escasos y su eficiencia muy baja. Por ello dentro del marco de esta tesis doctoral se propone el desarrollo de un protocolo estandarizado para la obtención de plantas poliploides *in vitro* de berenjena aprovechando el potencial polisomático de los tejidos jóvenes de la planta.

3.3. Los dobles haploides

Los individuos dobles haploides se definen como organismos homocigotos para todos sus *loci*, esto es a causa de la duplicación de los cromosomas gaméticos de los individuos haploides, que previamente han sido generados a partir de un único núcleo de origen haploide (Germanà, 2006).

3.3.1. Importancia de los dobles haploides en la mejora genética vegetal

Existen una serie de individuos que presentan una estructura genética muy característica, siendo homocigotos para todos sus *loci,* estos son conocidos como líneas puras. El uso de líneas puras ha sido y sigue siendo la base de la mejora genética vegetal en el desarrollo de poblaciones experimentales, así como en la producción de híbridos comerciales. En lo que respecta a la producción de plantas híbridas, este sigue siendo uno de los principales métodos de mejora debido a su alta eficacia. La semilla híbrida producida posee un altísimo valor añadido (Martín 2002) dando lugar a dos ventajas principales para los agricultores: homogeneidad y vigor híbrido. El hecho de que estas plantas sean genéticamente idénticas entre sí y heterocigotas para todos sus *loci* les otorga una extraordinaria homogeneidad. Por otra parta la heterosis o vigor híbrido les confiere un alto rendimiento y características favorables en general aumentando con la diversidad genética existente entre las líneas puras empleadas para obtener dicho híbrido (Birchler, 2015).

Sin embargo, la producción tradicional de líneas puras requiere de un mínimo de 7-8 ciclos de autofecundación y selección. Este proceso ralentiza y encarece la producción de híbridos nuevos suponiendo un coste mínimo de 3 años hasta su llegada al mercado. La misma problemática la encontramos en el desarrollo de poblaciones experimentales (ILs, MAGIC…) donde tras los 5 ciclos de retrocruzamiento y selección (mediante marcadores moleculares), va a ser necesario fijar los fragmentos genéticos seleccionados con al menos tres ciclos de autofecundación de dichas líneas. Además, el uso de dobles haploides no solo acelera el proceso y lo reduce a una única generación, sino que también facilita la selección de los materiales facilitando la fijación de alelos recesivos beneficiosos, así como la selección de caracteres cuantitativos al eliminar los efectos de dominancia y la segregación intrafamiliar (Snape, 1989). Y es por todos estos motivos que la existencia de técnicas biotecnológicas de cultivo *in vitro* como la de los dobles haploides suponen una gran ventaja a la hora de acelerar estos procesos.

Siendo su uso en mejora genética vegetal el más destacable y con una aplicación práctica más evidente, los organismos dobles haploides también presentan otros usos en investigación básica. Algunos ejemplos son: la asociación de marcadores moleculares a caracteres fenotípicos

mediante el uso de *Bulked Segregant Analysis* (BSA) (Huang et al., 2020), la selección de mutantes recesivos empleando líneas dobles haploides como materiales iniciales en experimentos de tilling evitando los efectos de dominancia (Szarejko and Forster, 2007; Ferrie et al., 2008) aportan precisión en la integración de mapas genéticos y físicos a la hora de identificar genes candidatos y finalmente, son una valiosa herramienta a la hora de realizar un correcto proceso de transformación genética ya que la transformación de células haploides y su posterior inducción de androgénesis evita la aparición de individuos hemicigóticos (Goedeke et al., 2007).

3.3.2. Métodos de obtención

La producción de dobles haploides siempre va a ser a partir de una célula de origen haploide, eso significa que va a poder originarse a partir del gametofito masculino o del femenino (Dunwell, 2010). En la mayoría de las especies dicotiledóneas, la elección de una de estas dos vías se establece en función del rendimiento y la eficiencia (Wedzony et al., 2009) y aunque en este trabajo se profundizará en la ruta androgénica por ser la más efectiva en el caso de la berenjena, a continuación, se hará una breve descripción del resto:

- Método bulbosum: Para llevar a cabo esta técnica es necesario emplear polen de especies sexualmente incompatibles. De este medo al producirse la polinización se formará un embrión que se encontrará con una serie de barreras post-cigóticas que darán lugar a la eliminación progresiva de los cromosomas del parental masculino, generando finalmente un embrión haploide de cuyo genoma provendrá del parental femenino. En el endospermo acontecerá el mismo evento de eliminación cromosómica, pero a una mayor rapidez debido a la su velocidad de división mayor por lo que será necesario realizar un rescate de embriones para evitar el aborto del embrión y obtener la planta haploide. Esta es una técnica muy común en la mejora de cereales (Forster et al., 2007; Dunwell, 2010).

- Hibridación interespecífica: El concepto es similar al anterior, también empleamos polen de especies sexualmente incompatibles, pero en este caso no se produce una fecundación de la célula huevo, en su lugar las dos espermátidas fecundan los núcleos polares (Tai, 2005). En esta ocasión se forma un endospermo que se desarrolla con normalidad e induce el desarrollo de un embrión haploide de origen exclusivamente femenino (Seguí-Simarro, 2010). Esta metodología se ha empleado en la mejora de patata, tabaco, pera y sorgo (Rokka, 2009; Dunwell, 2010).

- Ginogénesis: Esta técnica se fundamenta en la obtención de un individuo haploide a partir de un gametofito femenino no fecundado. Para ello se procede la estimulación de las células haploides que se encuentran dentro del saco embrionario, siendo la célula huevo la que presenta una mayor sensibilidad. Para ello se pueden emplear diferentes estrategias como el cultivo *in vitro* de ovarios u óvulos inmaduros no fecundados. El cultivo de estos en un medio con una composición adecuada y un equilibrio de reguladores del crecimiento correcto estimulará las células del interior del óvulo dando lugar al desarrollo de un embrión haploide. Esta técnica es ampliamente utilizada en la mejora de cebolla, remolacha o melón (Bohanec, 2009; Wei-Ping et al., 2009).

3.3.3. La androgénesis

La androgénesis es un conjunto de vías biológicas que dan lugar a la formación de un individuo cuyo fondo genético proviene únicamente de un gameto de origen masculino. Para ello es necesario reprogramar la ruta de desarrollo del gametofito masculino (microsporogénesis y microgametogénesis) hacia una ruta de desarrollo esporofítica (Seguí-Simarro, 2010).

Cuando se habla de androgénesis habitualmente nos referimos a un proceso que tiene como resultado la embriogénesis de las microsporas, proceso descubierto en el año 1964 por Guha y Maheshwari. Durante este proceso de inducción *in vitro*, la microspora (precursor del polen) se desvía de su ruta de desarrollo natural (ruta azul figura 9) para dar lugar a la formación de un embrión o callo haploide. La producción de individuos haploides o dobles haploides presenta dos pasos principales que se realizan *in vitro*, el primero es la inducción del proceso androgénico, el segundo es la regeneración de las plantas obtenidas ya sea de forma directa (ruta amarilla de la figura 9) o indirecta (ruta verde de la figura 9). Finalmente, estas plantas se aclimatan para poder ser cultivadas en condiciones naturales *ex vitro*.
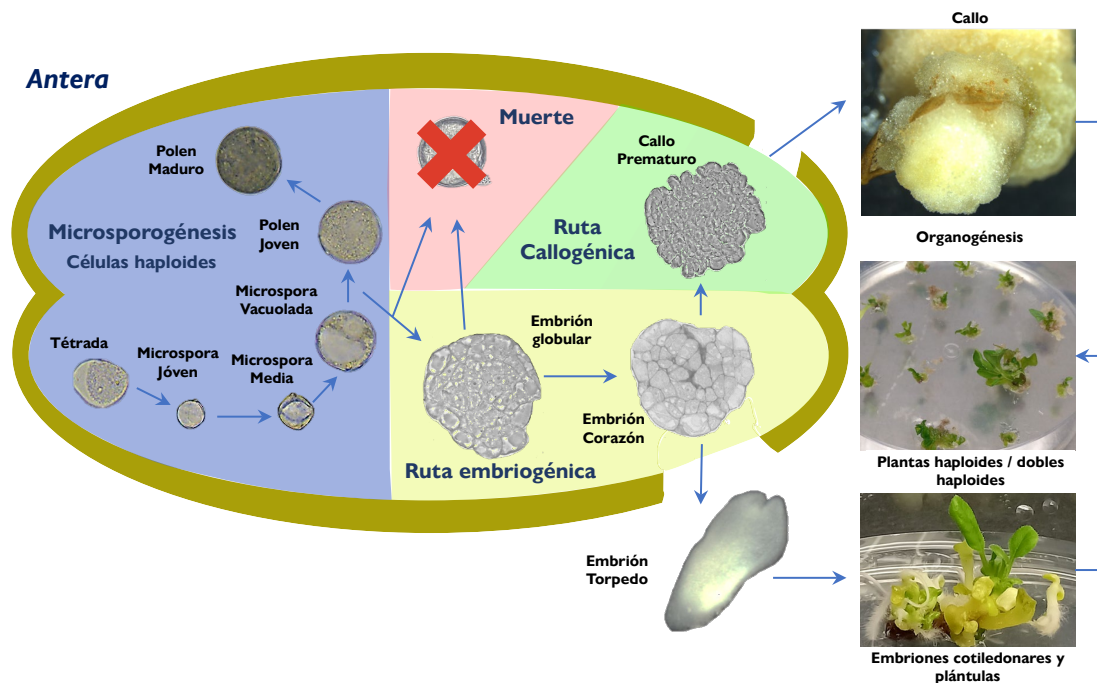
Figura 9: Esquema de la inducción de la androgénesis a partir de microsporas. La ruta azul representa la vía de desarrollo gametofítico natural del polen. Las flechas que sale hacia la zona amarilla indica el estadio de inducción adecuado, normalmente microspora vacuolada o polen bicelular joven. La ruta amarilla representa una ruta de embriogénesis directa, mientras que la ruta verde representa una ruta de callogénesis y su posterior organogénesis indirecta, ambas dando lugar a la formación de una planta haploide. Las rutas rojas son vías muertas. (basado en Seguí-Simarro y Nuez, 2008).

Tanto el proceso de inducción como el proceso de regeneración presentan numerosos cuellos de botella. No obstante, los más preocupantes y numerosos son aquellos que conciernen a la inducción del proceso androgénico, muchos de los cuales son todavía desconocidos (Datta, 2005). Los principales y mejor estudiados son los relacionados con la planta donante (genotipo, condiciones de cultivo y edad de las plantas) y el estadio de desarrollo de la microspora junto con las condiciones de cultivo *in vitro* aplicadas (Seguí-Simarro y Nuez, 2008).

Se sabe que el genotipo es uno de los factores más determinantes a la hora de controlar la respuesta embriogénica de las microsporas. No solo se han observado diferencias entre especies, sino que también se han observado grandes discrepancias en los niveles de respuesta al proceso androgénico dentro de cultivares de una misma especie, así como entre individuos de un mismo cultivar (Seguí-Simarro y Nuez, 2008). Y el genotipo no solo determina la capacidad de inducción de las microsporas, sino que también será fundamental en la capacidad de regeneración de las plantas completas (Gémes-Juhasz et al., 2006). Desafortunadamente este factor no es posible controlarlo y siempre supondrá un cuello de botella inherente a cualquier procedimiento de cultivo *in vitro* como ya se ha comentado en secciones anteriores de esta introducción.

No obstante, hay otro factor importante que sí que es posible estudiar y tener cierto control sobre él. Esté es el referente al estadio de la microspora en el que se apliquen los tratamientos de inducción de la androgénesis. En la mayoría de las especies el estadio óptimo coincide con el momento en el que se da lugar la transición de las microsporas vacuoladas hacia polen biceluar joven, es decir, hacia la primera mitosis del polen (González-Melendi; 1996, Maraschin et al., 2005). Esto es debido a que en este momento la microspora se encuentra en un estado proliferativo y no diferenciado, por ello su nivel de sensibilidad a los tratamientos de inducción es mayor (Dunwell, 2010).

Finalmente, el ultimo parámetro que se puede controlar son los tratamientos de inducción y los medios de cultivo. La aplicación exógena de un estrés será el detonante que permita la reprogramación de las células modificando su patrón de desarrollo hacia la ruta esporofítica. Los tipos de estrés más utilizados son los tratamientos térmicos (altas o bajas temperaturas); aunque también existen otra alternativas como la reducción de la fuente de carbono, la adición la medio de agentes antimitóticos, la irradiación ionizante, la centrifugación, cambios en la composición atmosférica, choques hipertónicos, adición al medio de etanol, acido abscísico o agentes feminizantes, subidas en el pH, cambios en la osmolaridad del medio, adición de metales pesados… (Shariatpanahi et al., 2006). En definitivas, cualquier cambio que modifique y perturbe las condiciones naturales de desarrollo de las microsporas, es susceptible de ser un factor de inducción, no obstante, cada especie tiene sus peculiaridades y suele ser complicado dar con el factor perfecto, convirtiendo esta parte del proceso en un apartado altamente empírico.

Técnicamente existen dos metodologías para la aplicación de estos factores de inducción y la posterior obtención de plantas dobles haploides: el cultivo de anteras y el cultivo de microsporas aisladas.

- Cultivo de anteras: Esta metodología consiste en cultivar las anteras en un medio de cultivo solido o semisólido preservando a las microsporas en el interior del saco polínico en condiciones *in vitro*. Por su sencillez es el método favorito y de carácter universal para la producción de dobles haploides. La presencia del tejido de la antera también facilita el proceso ya que confiere un ambiente propicio para el desarrollo de las microsporas (Seguí-Simarro y Nuez, 2008). Pero a pesar de su sencillez técnica presenta dos inconvenientes principales. El primero es que la presencia de tejido somático puede dar lugar a embriogénesis o callogénesis de origen no haploide dando lugar a regenerantes somáticos (Forster et al., 2007). El segundo inconveniente es que la eficiencia de obtención de haploides obtenidos es menos en comparación con el método de las microsporas aisladas (Forster et al., 2007).

- Cultivo de microsporas aisladas: En esta metodología las microsporas son extraídas del interior de la antera y cultivadas en un medio líquido. Técnicamente este proceso es mucho más complejo y el mayor número de pasos a aplicar junto con el uso de un medio de naturaleza líquida incrementan exponencialmente el riesgo de contaminación de los cultivos. Sin embargo, esta técnica tiene algunas ventajas ya que al aislar las microsporas del tejido de la antera evitamos la formación de estructuras de origen somático (Foster et al. 2007). Por otra parte, tenemos un control absoluto de las condiciones de cultivo y de la composición del medio de cultivo, ya que, en el caso del cultivo de anteras, estas pueden secretar sustancias beneficiosas para las microsporas, pero también perjudiciales bloqueando el proceso de desarrollo de embriones de origen androgénico (Kim et al., 2008).

3.3.4. La androgénesis en berenjena

En berenjena los métodos más estudiados para la obtención de plantas haploides son el cultico de anteras y microsporas.

Los primeros haploides en berenjena se describieron el en año 1979 (Isouard et al., 1979), no obstante, hasta 1982, con el trabajo de Dumas de Vaulx y Chambonnet (1982), no se establecieron las bases para el desarrollo de protocolos de embriones haploides y la regeneración de plantas a partir de cultivos de anteras. Este protocolo consta de dos etapas, una etapa de inducción en la que las anteras se someten a un estrés térmico en un medio de cultivo con una concentración de fitohormonas concreta y una segunda etapa en la que los embriones producidos son subcultivados en un medio de regeneración. Este concepto fue la base para el desarrollo de muchos protocolos de producción de dobles haploides, entre ellos el protocolo en berenjena descrito por Rotino (1996). Este método ha sido empleado ampliamente para el cultivo de anteras de berenjena con algunas modificaciones menores hasta la actualidad. Es también en el año 1996 cuando Miyoshi describe por primera vez la obtención de dobles haploides mediante el cultivo de microsporas. Éste no fue capaz de obtener una embriogénesis directa, sino que obtuvo callos a partir de los cuales regeneró plantas. En este caso las condiciones de inducción se basan en la aplicación de un estrés térmico junto con unas condiciones de ayuno, seguido de una incubación en un medio de cultivo con baja concentración en ácido naftalenacético. Corral-Martinez y Seguí-Simarro (2012) consiguieron mejorar el rendimiento de Miyoshi aplicando una serie de factores externos concretos que modificaron la respuesta obteniéndose por primera vez embriones a partir de las microsporas aisladas.

Como se puede comprobar, hay mucho trabajo desarrollado en lo que se refiere al estudio de los medios de cultivo y las condiciones de inducción en la berenjena, así como en otros cultivos de interés comercial. Pero la determinación de los estadios óptimos de inducción se sigue realizando de forma manual mediante microscopía óptica. Esto sumado a la gran dependencia del genotipo y a las diferencias que existen entre ellos, hacen que este cuello de botella siga todavía siendo un factor limitante. Es por ello por lo que dentro del marco de esta tesis doctoral se propone el desarrollo de una herramienta en berenjena para la identificación automática mediante inteligencia artificial de los estadios óptimos de inducción, así como una nueva formulación para el cultivo de anteras de la berenjena. Con ello buscamos incrementar los niveles de respuesta y mejorar los recursos tecnológicos para la aplicación de las técnicas de cultivo *in vitro* para el desarrollo de organismos dobles haploides.

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5. Declaraciones

En algunas secciones de esta introducción se ha utilizado información basada en los trabajos finales de grado de Lluch-Ruiz A. (2019) y Martínez-López M. (2020) y de los trabajos final de máster de Bracho-Gil JP. (2019) y Sanguedolce-Tala V. (2020) de los cuales el autor de esta tesis (García-Fortea E.) fue director experimental, supervisando la redacción y llevando a cabo la corrección de los documentos escritos.

Objetivos

La siguiente tesis doctoral presenta dos objetivos generales principales:

1. Ampliar el fondo genético de la berenjena mediante el desarrollo de materiales de pre-mejora para la adaptación de este cultivo al cambio climático empleado especies silvestres relacionadas. Para la consecución de este objetivo principal se plantean los siguientes objetivos concretos:

   1.1. Obtener por primera vez generaciones de retrocruzamiento de berenjena con una especie del germoplasma terciario, en este caso *S. elaeagnifolium*.

   1.2. Desarrollar tres colecciones de materiales para el desarrollo de líneas de introgresión de berenjena con las especies de germoplasma primario *S. insanum*, secundario *S. dasyphyllum* y terciario *S. elaeagnifolium*.

2. Desarrollar herramientas biotecnológicas que permitan aplicar técnicas de mejora genética avanzada y aceleren los procesos en la adaptación de la berenjena al cambio climático. Para la consecución de este objetivo principal se plantean los siguientes objetivos concretos:

   2.1. Desarrollar un protocolo de organogénesis *in vitro* de alto rendimiento y baja dependencia del factor genotipo para poder aplicar técnicas de micropropagación y transformación genética en berenjena en el futuro.

   2.2. Desarrollar un protocolo de obtención de organismos poliploides *in vitro* sin utilizar agentes antimitóticos en berenjena.

   2.3. Desarrollar un sistema de reconocimiento y clasificación celular basado en la inteligencia artificial para identificar los estadios óptimos de inducción en las microsporas de la berenjena para mejorar los protocolos de obtención de dobles haploides en berenjena.

Bloque 1

Desarrollo de Poblaciones de

Pre-Mejora

Capítulo I

First successful backcrossing towards eggplant (*Solanum melongena*) of a New World species, the silverleaf nightshade (*S. elaeagnifolium*), and characterization of interspecific hybrids and backcrosses

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1. Abstract

Silverleaf nightshade (*Solanum elaeagnifolium* Cav.) is a drought tolerant invasive weed native to the New World. Despite its interest for common eggplant (*S. melongena* L.) breeding, up to now no success has been obtained in introgression breeding of eggplant with American *Solanum* species. Using an interspecific hybrid between common eggplant and *S. elaeagnifolium* as maternal parent we were able to obtain several fruits with viable seed after pollination with *S. melongena* pollen. Twenty individuals of the first backcross (BC1) generation were crossed again to the *S. melongena* parent and second backcross (BC2) seed was obtained for 17 of them, suggesting that most of the genome of *S. elaeagnifolium* is likely to be represented in the set of BC2 families. Five plants of each of the two parents, interspecific hybrid and BC1 generation were characterized with morphological descriptors and for pollen viability. The interspecific hybrid was intermediate among parents, although in overall morphological characteristics more similar to the *S. elaeagnifolium* parent. However, pollen viability of the hybrid was very low (2.6%). The BC1 generation was intermediate in characteristics between the hybrid and the *S. melongena* parent, with pollen viability increasing to an average of 19.4%. The root system of the interspecific hybrid indicated that it is able to explore larger areas of the soil than the *S. melongena* parent. The phenolics profile of the fruit of the two parents and hybrid revealed a higher diversity in phenolic constituents in *S. elaeagnifolium* compared to *S. melongena*, where the major phenolic compound was chlorogenic acid, while the interspecific hybrid was intermediate. By using flow cytometry it was found that *S. elaeagnifolium*, *S. melongena*, and their interspecific hybrid were diploid, although the genome size of *S. elaeagnifolium* was slightly smaller than that of *S. melongena*. Our results represent the first report of successful development of backcross generations of common eggplant with a New World *Solanum* species.

This makes available a relatively unexplored, phylogenetically distant genepool for eggplant breeding. The backcross materials obtained can make a relevant contribution to developing new eggplant cultivars with new nutritional and environmental properties.

Keywords: Backcrosses*,* Introgression breeding*,* Flow cytometry*,* Phenolics profile*, Solanum elaeagnifolium, Solanum melongena*

2. Introduction

Crop wild relatives can contribute to widening the genetic background of crops and adapting them to new challenges, such as climate change (Dempewolf et al., 2014). The economic impact of the utilization of crop wild relatives in crop breeding has been estimated at the global level in $164.5 \cdot 10^9$ US\$ annually, while the current value of crop wild relatives for breeding in the most important crops could triple in a climate change scenario (Tyack and Dempewolf, 2015). This clearly shows how research in crop wild relatives and its utilization in breeding may have an important economic impact by developing new cultivars with improve characteristics. In this way, a new approach known as "introgressiomics" calling for the systematic development of plant materials containing introgressions from wild species has recently been proposed (Prohens et al., 2017).

One of the vegetable crops in which significant efforts are being done in the last years for introgression breeding from related species for adaptation to climate change is the common eggplant (*Solanum melongena* L.) (Toppino et al., 2008; Liu et al., 2015; Kouassi et al., 2016; Plazas et al., 2016). The common or brinjal eggplant is an Old-World crop domesticated in Southeast Asia (Meyer et al., 2012), and is related to wild species of spiny solanums (Leptostemonum clade) occurring in Asia and Africa (Knapp et al., 2013; Vorontsova et al., 2013; Aubriot et al., 2016; Vorontsova and Knapp, 2016).

Interspecific hybrids and backcrosses of eggplant have been obtained with many related Old World species, and this has included the development of introgression materials with different species and one set of introgression lines with *S. incanum* (Rotino et al., 2014; Kouassi et al., 2016; Plazas et al., 2016; Gramazio et al., 2017; Gramazio et al., 2018). In addition, sexual and somatic hybridization have also been used to develop interspecific hybrids between eggplant and several New World species. In this way, *Solanum aculeatissimum* Jacq. (Zhou et al., 2018), *S. elaeagnifolium* Cav. (Kouassi et al., 2016), *S. sisymbriifolium* Lam. (Gleddie et al., 1986), *S. torvum* Sw. (Jarl et al., 1999; Collonnier et al., 2003), and *S. viarum* Dunal (Prabhu et al., 2009) are of great interest for breeding for its resistance or tolerance to biotic and abiotic stresses (Kashyap et al., 2003; Rotino et al., 2014; Kouassi et al., 2016; Zhou et al., 2018). In fact, some of these New World species, like *S. torvum*, are regularly used as eggplant rootstocks due to their resistance to multiple soil diseases and nematodes (Arao et al., 2008; King et al., 2010; Gisbert et al., 2012; Sabatino et al., 2018). However, interspecific hybrids between brinjal eggplant and New World *Solanum* species have to date been highly sterile (Lester and Kang, 1998; Prohens et al., 2012; Rotino et al., 2014; Liu et al., 2015; Çürük and Dayan, 2017; Afful et al., 2018). Ploidy modification techniques, like the development of tetraploids containing the

full chromosome complements of both parental species allowed fertility restoration in hybrids of common eggplant with the Old World relative *S. aethiopicum* L. (Isshiki and Taura, 2003) but not in hybrids with New World *S. torvum* (Sihachakr et al., 1989). Thus, to our knowledge no backcrosses have been obtained for the introgression of genes or genomic fragments of interest from New World *Solanum* species into the genetic background of eggplant.

One of the New World species of greatest interest in the improvement of eggplant is the silverleaf nightshade (*S. elaeagnifolium*). This distant wild relative of eggplant is native to deserts and dry forests of North and South America and belongs to the sister group of all Old World spiny solanums, the *Elaeagnifolium* clade (Knapp et al., 2017). It is highly tolerant to drought (Christodoulakis et al., 2009) and has spread as an invasive noxious weed in arid and semi-arid regions of the world, where it causes considerable economic damage (Mekki, 2007). In addition, *S. elaeagnifolium* has been barely explored for other traits that may be of interest for eggplant breeding such as the content of nutritionally important bioactive phenolics (Kaushik et al., 2015). Despite its evident interest for eggplant breeding, obtaining interspecific hybrids between common eggplant and *S. elaeagnifolium* has not been described until recently (Kouassi et al., 2016). After multiple crosses between six different accessions of *S. melongena* and one of *S. elaeagnifolium* a few fruit set when using one *S. melongena* accession (MEL3) as female parent, and nine hybrid plants could be obtained after embryo rescue of immature fruits by Kouassi et al. (2016).

Within the Leptostemonum Clade, New World *Solanum* species of the *Elaeagnifolium* clade are those phylogenetically closest to the Old World species (Vorontsova and Knapp, 2016; Knapp et al., 2017). This led us to hypothesize that, compared to other New World species, using interspecific hybrids with *S. elaeagnifolium* would result in higher success in achieving introgression breeding in eggplant. In this way, a New World genepool could be accessible for breeding and for widening the genetic background of eggplant.

In this paper, using the hybrids obtained by Kouassi et al. (2016) we describe the characteristics of interspecific hybrids between *S. melongena* and *S. elaeagnifolium*, and we make a first report of the development and characteristics of backcross generations between eggplant and this New World species. We consider that these results open a way to the use of the characteristics of interest of *S. elaeagnifolium* and its closest relatives for eggplant improvement. Given the high tolerance to drought of *S. elaeagnifolium* (Christodoulakis et al., 2009) these materials may be of great interest for developing a new generation of eggplant varieties adapted to climate change.

3. Material and methods

3.1. Plant material and hybridizations

Parental materials consisted of one accession of *S. melongena* (MEL3) and one accession of *S. elaeagnifolium* (ELE2). *Solanum melongena* MEL3 is an accession from Ivory Coast used in an introgression breeding programme (Kouassi et al., 2016; Plazas et al., 2016) having semi-long fruits. *Solanum elaeagnifolium* ELE2 was collected as a weed in Greece and has small round fruits

(Kouassi et al., 2016). Both parents have green fruits with dark green stripes (Figure 1) that ripen to yellow or orange-brown. Also, materials used included clonal replicates of a plant of the interspecific hybrid *S. melongena* MEL3 × *S. elaeagnifolium* ELE2 obtained after embryo rescue (Kouassi et al., 2016).

In order to obtain backcross generations towards the *S. melongena* parent, the *S. melongena* MEL3 × *S. elaeagnifolium* ELE2 interspecific hybrid, due to its low pollen viability, was always used as female parent in crosses for obtaining the first backcross (BC1) generation. Also, the plants obtained of the BC1 generation were used as female parents for developing the second backcross (BC2) generation. All plants used for hybridizations were grown in an insect-free greenhouse in 15 l pots filled with coconut fiber. Plants were watered and fertilized using a drip irrigation system. Hybridizations were performed early in the morning. Basically, flower buds one or two days before anthesis were opened and emasculated with a forceps and pollen from the male parent was gently deposited on the stigma of the female parent using a glass slide. Flowers were tagged and fruits were harvested when physiologically ripe, with the exception of a first fruit of the backcross between the interspecific hybrid *S. melongena* MEL3 × *S. elaeagnifolium* ELE2 and *S. melongena* MEL3, which was harvested physiologically unripe for embryo rescue using the protocol indicated in Plazas et al. (2016). For fruits left to ripen, seeds were extracted from each individual fruits and left on filter paper for drying at room temperature. Subsequently they were placed in paper bags and stored at 4ºC in hermetic glass jars which contained silica gel for maintaining seed moisture low. Seed germination was performed using the protocol described in Ranil et al. (2015).

Plants used for characterization were transplanted in June 2017 to soil in a screenhouse. Plants were watered and fertilized by drip irrigation and trellised using vertical strings. Weeds were removed manually and phytosanitary treatments against spider mites and whiteflies were performed when necessary. Five plants of each of the parentals, their interspecific hybrid, and of the first backcross (BC1) of the interspecific hybrid towards the *S. melongena* parent were used for the morphological characterization of above-ground parts. Three additional plants of *S. melongena* and of the interspecific hybrid were used for the evaluation of the root system.

3.2. Characterization

Traits used for the characterization of the aerial part included 18 qualitative (Table 1) and 16 quantitative (Table 2) descriptors mostly based on EGGNET and IBPGR descriptors (IBPGR, 1990; van der Weerden and Barendse, 2007; Kaushik et al., 2016). Descriptors used included traits of the habit, leaf, inflorescence, flower, and fruit. Except for plant height and stem diameter, for which only one measurement was taken per plant, at least five measurements were taken from each individual plant in order to obtain individual plant averages for the conventional morphological descriptors (i.e., five measurements per replicate). Pollen viability was evaluated according to Aref (1992) with some modifications. From a cell suspension with a concentration of 500,000 cells/ml, 1 ml of dilution was distributed in a 6 mm diameter Petri dish and stained with 0.001% FDA solution (fluorescein diacetate, 1 µl per ml of suspension) and allowed to incubate for 5 minutes. Fluorescence in FDA was determined by scoring the percentage of fluorescing pollen grains under an ultraviolet (UV) source provided by a mercury lamp. The

principle is based on the uptake of non-fluorescing FDA by the vegetative cells of a viable pollen grain and subsequent hydrolysis by esterase to release fluorescein, which fluoresces under UV (excitation filter = 485 nm and barrier filter = 520 rim). In contrast, nonviable cells are incapable of hydrolyzing FDA and, therefore, do not fluoresce (Heslop-Harrison et al., 1984). Each determination of pollen fertility consisted was performed by counting 300 to 500 pollen grains by examining 10 locations in a series of random areas across the Petri dish that contained the sample under test.

For the characterization of the root traits, four traits were measured. Firstly, the plants were carefully removed from the ground, with the help of a hoe, to reduce root damage; once extracted they were cleaned with water to eliminate the earth or the accumulated mud. Finally, the characters indicated in Table 3 were evaluated manually with the help of a phenotyping scoreboard.

3.3. Phenolics content

Chlorogenic acid (CGA), the main phenolic compound in the eggplant flesh (Stommel and Whitaker, 2003; Whitaker and Stommel, 2003; Prohens et al., 2013), and other hydroxycinnamic acid conjugates were extracted and analyzed using the methodology indicated in Plazas et al. (2014) in order to assess overall phenolic content of the fruit. Extractions were performed with 0.1 g of lyophilized sample homogenized in 1.8 ml of methanol:water (80:20, v/v) plus 0.1% (w/v) of 2,3-tert-butyl-4-hydroxyanisole (BHT). After that the extract was vortexed vigorously, sonicated for 1 h and centrifuged at 2000 rpm for 3 min and the supernatant filtered through 0.2-µm polytetrafluoroethylene (PTFE) membrane filters.

Extracts were analyzed on a HPLC 1220 Infinity LC System (Agilent Technologies, Santa Clara, CA, USA) operated by the OpenLAB CDS ChemStation Edition software package (Agilent Technologies). Aliquots of 10 µL were injected into a ZORBAX Eclipse Plus C18 (3.5 µm; 4.6 mm × 12.5 mm; Agilent Technologies) column protected by a ZORBAX Eclipse Plus C18 guard column (5 µm; 4.6 mm × 12.5 mm; Agilent Technologies). A binary gradient consisting of 0.1% formic acid (Solvent A) and methanol (Solvent B) was used. The mobile phase gradient described in Plazas et al. (2014) was used and absorbance was measured at 325 nm for quantification. CGA concentration in the extracted samples was calculated using calibration curves. The CGA peak area and the total peak area (TPA) of other phenolic acids were determined.

3.4. Determination of ploidy level

Cell nuclei from leaf tissues were isolated mechanically according to Dpooležel et al. (1989) with some modifications. Approximately 0.5 cm^2 of fresh young leaf tissue was chopped with a razor blade in a glass Petri dish containing 0.5 ml lysis buffer LB01 (pH 7.5) containing 15 mM Tris (hydroxymethyl) aminomethane, 2 mM Na$_2$EDTA and 0.5 mM spermine, and was left to incubate for 5 min. Subsequently, the suspensions containing nuclei and cell fragments were passed through a 30µm CellTrics filter (Sysmex). The nuclei in the filtrate were stained by CyStain UV Ploidy (Sysmex) by incubation for 5 min. The fluorescence intensity of the homogenate was

measured using CyFlow ploidy-analyzer (Partec, Münster, Germany), measuring at least 4000 nuclei for each sample.

4. Results

4.1 Backcrossing results

Twenty-two fruits developed after performing over 800 crosses between the interspecific hybrid *S. melongena* MEL3 × *S. elaeagnifolium* ELE2 as a female parent and the recurrent *S. melongena* MEL3 as male parent. The first fruit to set was collected before physiological maturity and it was found to contain developing seeds, from which 10 embryos were rescued (in heart and torpedo stages), which developed well and gave phenotypically normal BC1 plants. Because opening of this first fruit revealed an apparently normal development of the seeds, several subsequent fruits were allowed to develop to physiological maturity; these yielded seeds with a germination rate higher than 50%. We subsequently, therefore, abandoned embryo rescue, and the fruits containing seeds with the BC1 zygotes were allowed to ripen on the plant for extraction of mature seeds. Except for a single fruit that was parthenocarpic, all of the other 21 fruits of the interspecific hybrid after pollination with the recurrent parent *S. melongena* MEL3, presented seeds with a range between 4 and 40 seeds (mean ± SD = 12.32 ± 8.13 seeds/fruit). No fruits were obtained from non-pollinated flowers, although some seedless pseudofruits occasionally formed from mon-pollinated flowers.

We put all 40 seeds from the fruit containing the largest seed number to germinate; this resulted in 50% germination, giving us 20 BC1 plants. These BC1 plants were grown for a next cycle of backcrossing for obtaining the BC2 generation. Multiple crosses (over 3,000) were performed using the BC1 plants as female parent, resulting in at least one fruit obtained in 17 out of the 20 BC1 plants. A total of 92 fruits (between 1 and 9 per individual plant) were obtained and all of them had seeds, with a range between 1 and 150 seeds/fruit (mean ± SD = 62.86 ± 35.99 seeds/fruit). Several BC2 seeds from each BC1 plant were germinated to obtain between 5 and 12 plants per individual BC2 family.

4.2 Characterization of parents, hybrid, and BC1 generations

Important differences were observed between the *S. melongena* MEL3 and *S. elaeagnifolium* ELE2 parents in the morphology of the vegetative part of the plant, leaves, inflorescences and flowers, and fruits. In fact, for 21 out of the 37 characters evaluated, there was no overlap in the ranges of variation (Tables 1 and 2).

Regarding plant habit, *S. melongena* has an upright growth habit and is much taller (more than two-fold) and has a thicker stem than *S. elaeagnifolium* (Table 1 and 2). The hybrids have an upright growth habit and are intermediate for plant height and stem diameter, although the values are closer to those of *S. elaeagnifolium* (Tables 1 and 2; Figures 1A-C). For the BC1 generation, the five plants characterized had an upright growth habit, but a great segregation was observed for plant height and stem diameter, with ranges of variation wider than those of the parents for these characters (Tables 1 and 2; Figure 1D). Amazingly, interspecific hybrids

displayed prickles between nodes, while none of the parents did. Some BC1 plants had prickles between nodes, but their degree of prickliness was much lower than that of the F1 (Table 2).

Leaf morphology also displayed great differences between the parents (Tables 1 and 2; Figures 1E-F). None of the two parents had prickly leaves; however, *S. melongena* leaves had stronger lobing, were more erect, and much larger than those of *S. elaeagnifolium*. The leaves of the hybrid were intermediate for all the observed characters, and again quite variable in the individuals of the BC1, which displayed segregation for the leaf lobing (Tables 1). As occurred with the prickles between nodes, some prickles appeared in the leaves of the hybrids. However, all BC1 plants had non-prickly leaves.

Many differences were observed among parents in inflorescence and flower traits. *Solanum melongena* had flowers with light violet corolla, connivent anther cone, and straight style, while those of *S. elaeagnifolium* had a darker bluish violet corolla, spreading anther cone, and curved style (Table 1; Figures 1G, 2A and 2C). Flowers of *S. melongena* were considerably larger than those of *S. elaeagnifolium*, while pollen viability was very high in *S. melongena* (>90%) and moderate (around 50%) in *S. elaeagnifolium* (Table 2). On the other hand, the number of flowers per inflorescence was similar among both parents and none of the parents displayed anthocyanins in the pistil. Flowers of *S. melongena* were fasciated and displayed higher numbers of petals, sepals and anthers than those of *S. elaeagnifolium*, which were strictly pentamerous (Tables 1 and 2; Figures 1G). Hybrids had light violet corolla and a connivent cone of anthers (like *S. melongena*) and curved style (like *S. elaeagnifolium*) and segregated for the presence of anthocyanins in the pistil (Table 1; Figure 2B). Flower number per inflorescence was transgressive to both parents (Table 2). Flowers of hybrids were pentamerous like those of *S. elaeagnifolium* although smaller than either parent any of them (Table 2; Figure 1G), and pollen viability of the hybrid was very low (<3%). A wide range of diversity was observed for color and size of flowers in the BC1 generation, with wide segregation for these traits (Tables 1 and 2; Figure 1G). Segregation was observed for corolla color, presence of anthocyanins in the pistil, and style curvature, with most plants displaying anthocyanin pigmentation and curved styles. The number of flowers per inflorescence was similar to that of the parents, and like *S. elaeagnifolium* and the interspecific hybrid, flowers of BC1 plants were exclusively pentamerous (Table 2). Corolla diameter was very variable, but average corolla diameter was similar to that of the F1 hybrid (Table 2; Figure 1G). Finally, pollen viability also exhibited a wide range of variation, with a considerable increase (average of around 20%) over pollen fertility levels of the F1 (Table 2).

Few differences existed among parents in fruit firmness and color, except that the predominant fruit color at physiological ripeness was yellow-orange for *S. melongena* and orange for *S. elaeagnifolium*, and the fruit flesh was white and green, respectively (Table 1). However, large differences were observed in fruit size and shape (Table 2). Fruits of *S. melongena* were much larger and more elongated than those of *S. elaeagnifolium*, with fruit length and width on average 15.7-fold and 6.4-fold larger in *S. melongena* than in *S. elaeagnifolium* (Table 2). Also, fruits of *S. melongena* had a thicker peduncle than those of *S. elaeagnifolium*. Regarding the calyx, its relative length in relation to the berry length was shorter in *S. melongena* than in *S. elaeagnifolium*, and it was non-prickly in *S. melongena* and prickly in *S. elaeagnifolium* (Table 2).

The F1 fruits were less firm than those of either of the two parents, probably as a consequence of being parthenocarpic, and in color were similar or intermediate (for those traits that display differences among parents) to the two parents. Fruit size and shape was intermediate to those of the parents, although much more similar to the *S. elaeagnifolium* parent (Table 2). For fruit calyx characteristics, F1 hybrids had a relative calyx length and prickliness similar to the ones observed in *S. elaeagnifolium*. The BC1 plants were also similar to the parents and F1 in color characteristics (Table 1). Although variation was observed for fruit size and shape, fruits from BC1 plants were generally intermediate between those of the F1 and the *S. melongena* parent, although much more similar to the former than to the latter (Table 2). For fruit calyx characteristics, the relative fruit calyx length of BC1 individuals was similar to that of *S. melongena*, while calyx prickliness was variable, with a range from 0 to 10 prickles and an average value slightly lower than that of the F1 hybrid (Table 2).

Table 1: Descriptor states for the qualitative traits evaluated in *S. melongena* MEL3 (P1), *S. elaeagnifolium* ELE2 (P2), the interspecific hybrid *S. melongena* MEL3 × *S. elaeagnifolium* ELE2 (F1), and first backcross (BC1) towards *S. melongena* (BC1) generations. For each generation, five plants (n=5) were evaluated. Where segregation was observed within generation the numbers of plant of each class are indicated.

	Trait	P1	P2	F1	BC1
Vegetative part	Plant growth habit	Upright	Intermediate	Upright	Upright
	Prickle color	-	-	Green	Green
Leaf	Leaf blade lobing	Strong	Weak	Intermediate	1 Intermediate : 3 Strong : 1 Weak
	Leaf surface	Flat	Flat	Flat	Flat
Inflorescence and flower	Corolla color	Light violet	Bluish violet	Light violet	2 Pale violet : 3 Light violet
	Style curvature	Straight	Curved	Curved	4 Curved : 1 Straight
	Presence of anthocyanins in pistil	No	No	3 Yes : 2 No	4 Yes : 1 No
Fruit	Fruit apex shape	Rounded	Rounded	Rounded	Rounded
	Firmness in the wide part	Very firm	Very firm	Firm	Very firm
	Size of the stylar scar	Small	Small	Small	Small
	Fruit predominant color (at commercial ripeness)	Green	Green	Green	Green
	Fruit predominant color (at physiological ripeness)	Yellow-Orange	Orange	Orange	Yellow-Orange
	Fruit predominant color intensity (at commercial ripeness)	Clear	Clear	Dark	Clear
	Fruit additional color (at commercial ripeness)	Dark green	Dark green	Dark green	Dark green
	Fruit additional color distribution	Striped	Striped	Striped	Striped
	Fruit flesh color (cut fruit at commercial ripeness)	White	Green	Intermediate	Intermediate
	Fruit calyx color	Green	Green	Green	Green
	Fruit color intensity under calyx	Medium	Medium	Medium	Medium

59

Table 2: Mean value, standard error and range of the morphological quantitative traits evaluated in *S. melongena* MEL3 (P1), *S. elaeagnifolium* ELE2 (P2), the interspecific hybrid *S. melongena* MEL3 × *S. elaeagnifolium* ELE2 (F1), and first backcross (BC1) towards *S. melongena* (BC1) generations. For each generation, five plants (n=5) were evaluated.

	Trait	P1		P2		F1		BC1	
		Mean	Range	Mean	Range	Mean	Range	Mean	Range
Vegetative part	Plant height (cm)	205 ± 4	190-215	84 ± 10	50-108	109 ± 12	80-140	117 ± 21	87-200
	Stem diameter (cm)	4.5 ± 0.3	4.0-5.5	3.3 ± 0.3	3.0-4.0	2.9 ± 0.1	2.5-3.0	2.7 ± 0.4	1.5-4.0
	Prickles between nodes (n)	0.0 ± 0.0	0-0	0.0 ± 0.0	0-0	2.8 ± 0.4	2-4	0.3 ± 0.2	0-1
Leaf	Leaf prickles[a]	0 ± 0	0-0	0 ± 0	0-0	1 ± 0	1-1	0 ± 0	0-0
	Length of the largest prickle (cm)	-	-	-	-	0.1 ± 0.0	0.1-0.1	-	-
	Leaf pedicel length (cm)	6.4 ± 0.2	6.0-7.0	1.6 ± 0.3	1.0-2.5	3.1 ± 0.2	2.5-3.5	3.2 ± 0.6	2.0-5.5
	Leaf apex angle[b]	3 ± 0	3-3	5 ± 0	5-5	3 ± 0	3-3	5 ± 0	5-5
	Leaf blade length (cm)	15.8 ± 0.4	15.0-17.0	8.5 ± 0.2	8.0-9.0	11.9 ± 1.2	8.5-15.0	13.2 ± 1.0	11.0-17.0
	Leaf blade width (cm)	9.4 ± 0.2	9.0-10.0	2 ± 0	1.8-2.2	6.1 ± 0.4	5.5-7.0	6.9 ± 0.6	6.0-9.0
Inflorescence and flower	Flowers/inflorescence (cm)	4.4 ± 0.2	4-5	4.8 ± 0.2	4-5	7.6 ± 0.4	6-8	4.8 ± 0.6	3-6
	Petals/flower	5.4 ± 0.3	5.2-5.5	5 ± 0	5-5	5 ± 0	5-5	5 ± 0	5-5
	Sepals/flower	5.2 ± 0.1	5.0-5.3	5 ± 0	5-5	5 ± 0	5-5	5 ± 0	5-5
	Stamens/flower	5.5 ± 0.4	5.2-5.6	5 ± 0	5-5	5 ± 0	5-5	5 ± 0	5-5
	Corolla diameter (cm)	4.9 ± 0.1	4.5-5.0	3.7 ± 0.1	3.5-4.0	2.4 ± 0.1	2.0-2.5	2.5 ± 0.5	1.5-4.5
	Pollen viability (%)	91.5 ± 2.4	84.2-97.3	50.7 ± 4.3	41.7-61.4	2.6 ± 0.2	2.3-2.8	19.4 ± 8.7	7.4-53.3
Fruit	Fruit length (cm)	11.8 ± 0.6	11.0-14.0	0.75 ± 0.16	0.5-1.0	1.25 ± 0.13	1.0-1.5	2.75 ± 0.52	1.5-4.0
	Fruit width (cm)	5.4 ± 0.5	4.0-7.0	0.85 ± 0.09	0.7-1.0	1.25 ± 0.13	1.0-1.5	2.0 ± 0.0	2.0-2.0
	Fruit pedicel length (cm)	6.0 ± 1.0	5.0-7.0	2.3 ± 0.2	2.0-2.5	1.8 ± 0.1	1.5-2.0	2.0 ± 0.0	2.0-2.0
	Fruit pedicel thickness (cm)	1.0 ± 0.0	1.0-1.0	0.35 ± 0.03	0.3-0.4	0.3 ± 0.0	0.3-0.3	0.4 ± 0.1	0.3-0.5
	Relative fruit calyx length[c]	1 ± 0	1-1	3 ± 0	3-3	3 ± 0	3-3	1 ± 0	1-1
	Fruit calyx prickles (n)	0 ± 0	0-0	5 ± 0	5-5	5 ± 0	5-5	3.75 ± 2.14	0-10

[a]Measured in a scale (0=Absent; 1= 1 to 2; 3= 3 to 5; 5= 6 to 10; 7: 11 to 20; 9= More than 20).

[b]Measured in a scale (1= Less than 15°; 3=aprox. 45°; 5= aprox. 75°; 7= aprox. 110°; 9= aprox. 160°).

[c]Measured in a scale (0=Less than 10%; 3=aprox. 20%; 5=aprox. 50%; 7=aprox. 70%; 9=More than 75%).
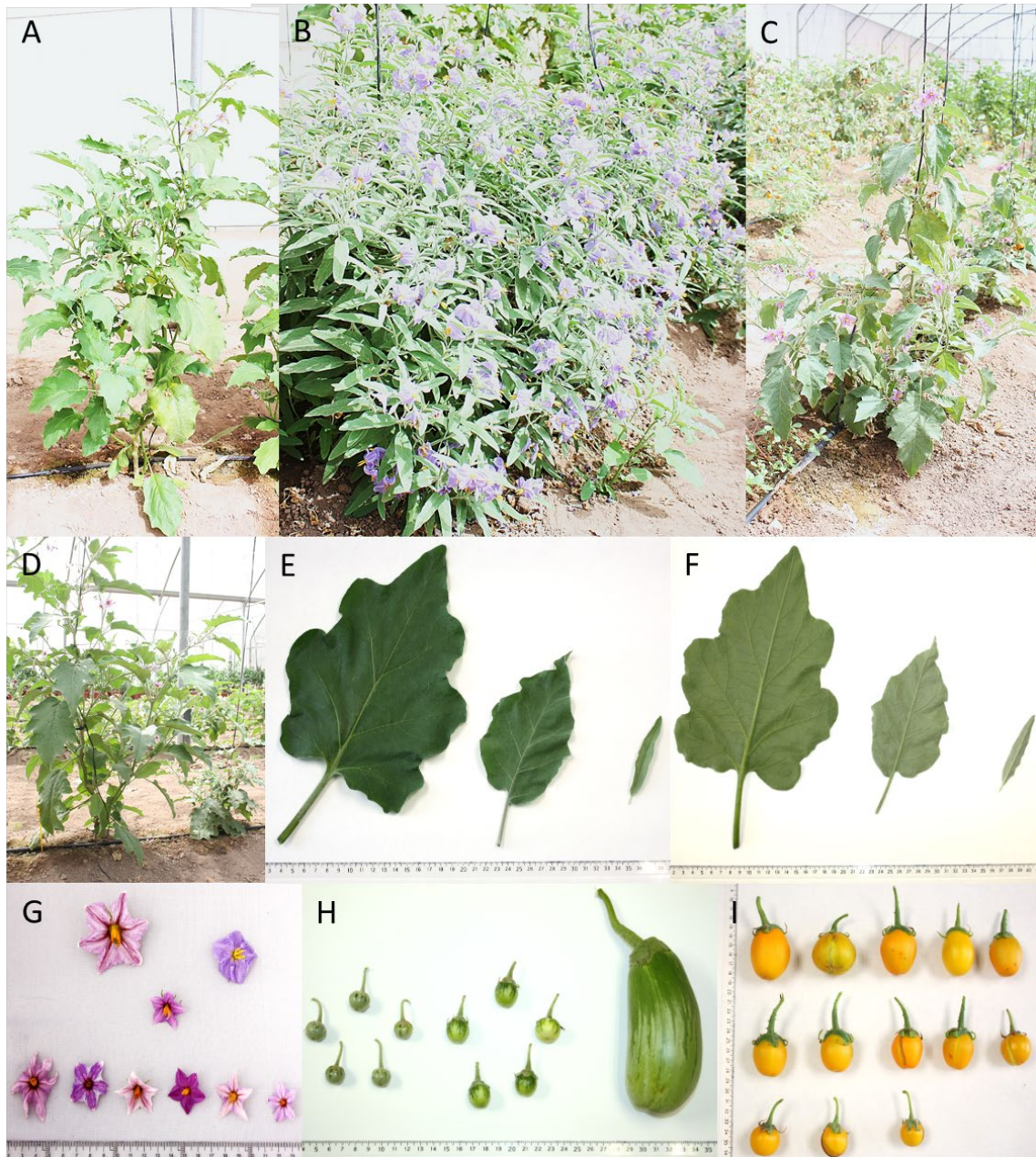
Figure 1: Morphology of plant materials evaluated: *S. melongena* MEL3 plant (A); *S. elaeagnifolium* ELE2 plant (B); *S. melongena* MEL3 *× S. elaeagnifolium* ELE2 interspecific hybrid (F1) plant (C); two plants of the first backcross (BC1) of the F1 interspecific hybrid towards *S. melongena* displaying extreme difference in plant size (D); adaxial part of the leaf of *S. melongena* (left), F1 interspecific hybrid (center) and *S. elaeagnifolium* (right) (E); abaxial part of the leaf of *S. melongena* (left), F1 interspecific hybrid (center) and *S. elaeagnifolium* (right) (F); Flowers of *S. melongena* (left), F1 interspecific hybrid (center), *S. elaeagnifolium* (right) and BC1 individuals (below) (G); Fruits of *S. elaeagnifolium* (left), F1 interspecific hybrid (center) and *S. melongena* (right) (H); Segregation for fruit size and shape in physiologically mature fruits of the first backcross (BC1) of the F1 interspecific hybrid towards *S. melongena* (I). Scale in cm.
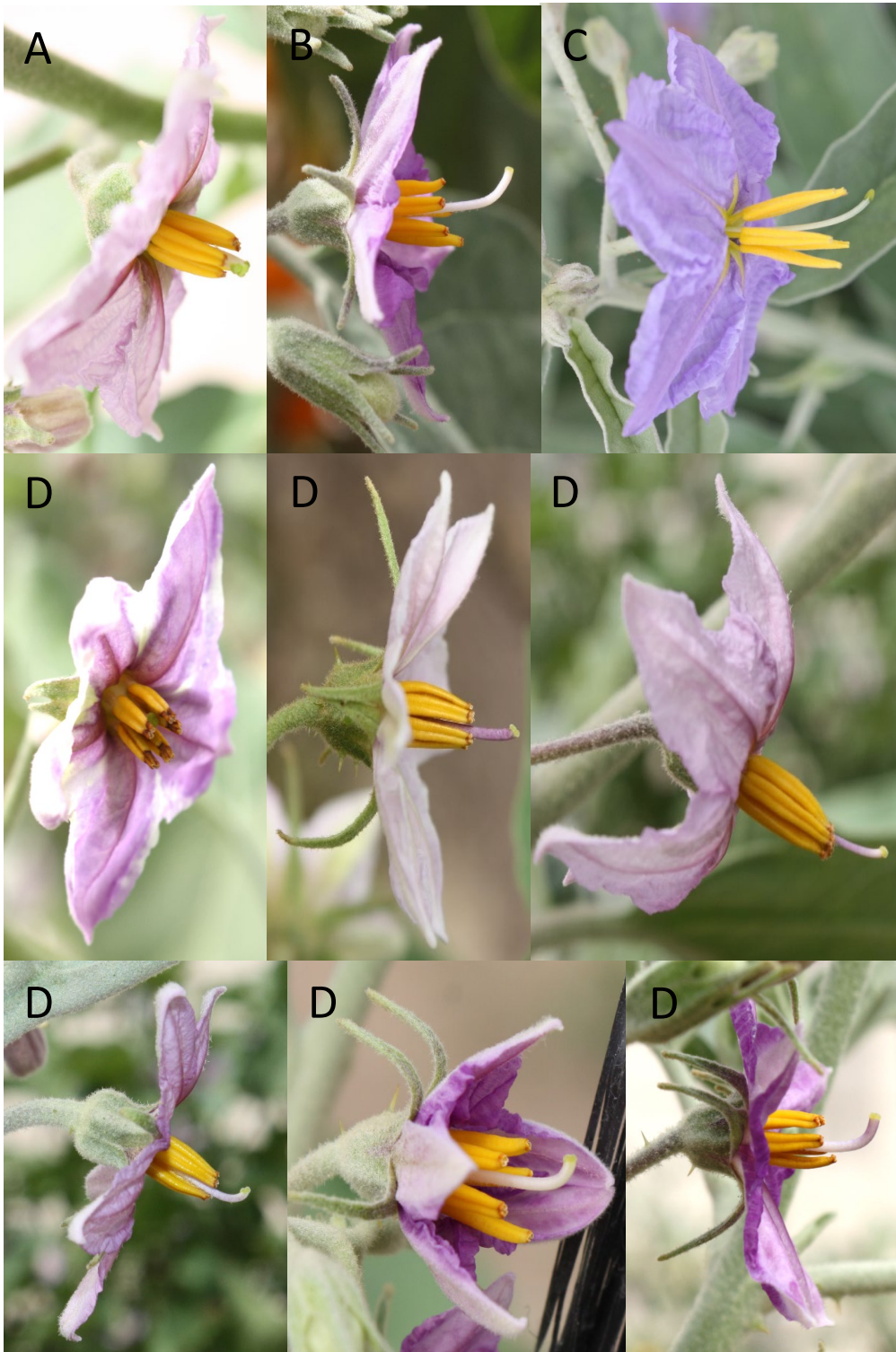
Figure 2: Variation for flower morphology in: *S. melongena* MEL3 (A); *S. melongena* MEL3 × *S. elaeagnifolium* ELE2 interspecific hybrid (B); *S. elaeagnifolium* ELE2 (C); Flowers of different plants of the first backcross (BC1) of the F1 interspecific hybrid towards *S. melongena* (D). Large variation is observed in BC1 plants for flower size, corolla color, style length, curvature and color, anthers length, and opening of the anthers cone.

The root system of developed mature plants of the *S. melongena* parent and of the F1 hybrid *S. melongena* × *S. elaeagnifolium* was characterized and considerable differences were observed (Table 3; Figure 3). Unfortunately the root system of *S. elaeagnifolium* could not be scored, as when the plants were uprooted out plants were already senescent and the root system damaged. The main differences observed were that, compared to *S. melongena*, the F1 had a somewhat longer and thinner main root, a reduced whorl diameter, a higher number of roots of diameter >2 and a lower density of lateral roots in the main root (Table 3). It is also evident from Figure 3 that the F1 hybrid has a root system that explores the soil to longer distances than *S. melongena*, which has a large part of the root system concentrated to a few centimeters around the stem.

Table 3: Mean value, standard error and range of the root morphological traits evaluated in *S. melongena* MEL3 (P1), and interspecific hybrid *S. melongena* MEL3 × *S. elaeagnifolium* ELE2 (F1). For each generation, three plants (n=3) were evaluated.

	P1		F1	
Trait	Mean	Range	Mean	Range
Whorl angle (°)	136.0 ± 3.0	130-140	151.3 ± 5.9	140-160
Main root length (cm)	42.7 ± 0.9	28-68	54.0 ± 2.0	33-66
Main root diameter (mm)	8.7 ± 0.2	8-9	5.0 ± 0.0	5-5
Relative density of laterals in the main root	Intermediate		Low	
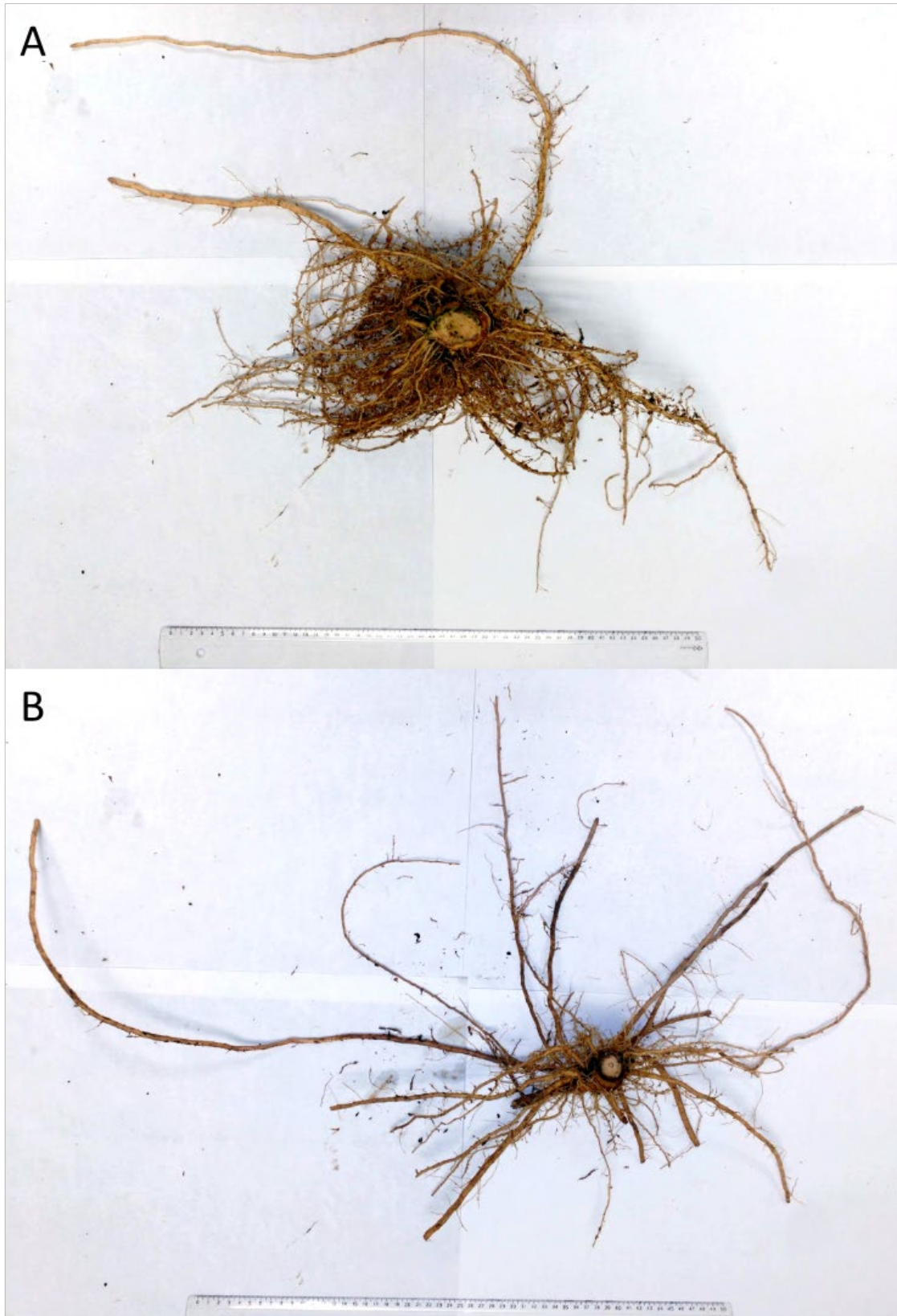
Figure 3: Root morphology in: *S. melongena* MEL3 (A) and F1 interspecific hybrid *S. melongena* MEL3 × *S. elaeagnifolium* ELE2 (B).

4.3 Phenolics profile of parents and hybrids

The analysis of phenolic acids reveals clear differences in the profiles obtained for *S. melongena* and *S. elaeagnifolium* (Figure 4). For *S. melongena*, chlorogenic acid (CGA) was the main compound, representing over 85% of the chromatogram total peak area (TPA) (Figure 4A); also, an unidentified peak very close to the CGA peak and probably representing an isomer or a derivative of CGA (Whitaker and Stommel, 2003) makes a secondary peak. *Solanum elaeagnifolium* also has an important CGA peak, but it represents slightly less than 30% of the TPA, although another peak close to CGA, which probably is also a CGA isomer or derivative, accounts for almost 19% of the TPA. Another important peak corresponding to an unidentified phenolic compound that appears at a retention time of 20.2 minutes in *S. elaeagnifolium*. Also, several other minor peaks appear in the *S. elaeagnifolium* chromatogram that do not appear, or have very low percentage of TPA, in *S. melongena* (Figure 4B). For example, a peak at 15.9 min is detected in *S. elaeagnifolium* but not in *S. melongena*, and a small peak in *S. melongena* at 19.1 min is much higher in *S. elaeagnifolium*. The individuals of the F1 present a chromatogram in which all the major peaks present in the chromatograms of the parents are also present. In this case, the secondary CGA peak disappears, revealing a clear CGA peak representing almost 70% of the TPA (Figure 4C). The unidentified compound from *S. elaeagnifolium* with a peak at 20.2 minutes also appears in the F1 hybrid, although it only represents around 10% of the TPA. In the F1 chromatogram most of the minor peaks observed in the chromatograms of both parents also appear (Figure 4C). Here, the peak at 15.9 min is similar in area to the one found in the *S. elaeagnifolium* parent (Figures 4B and 4C).
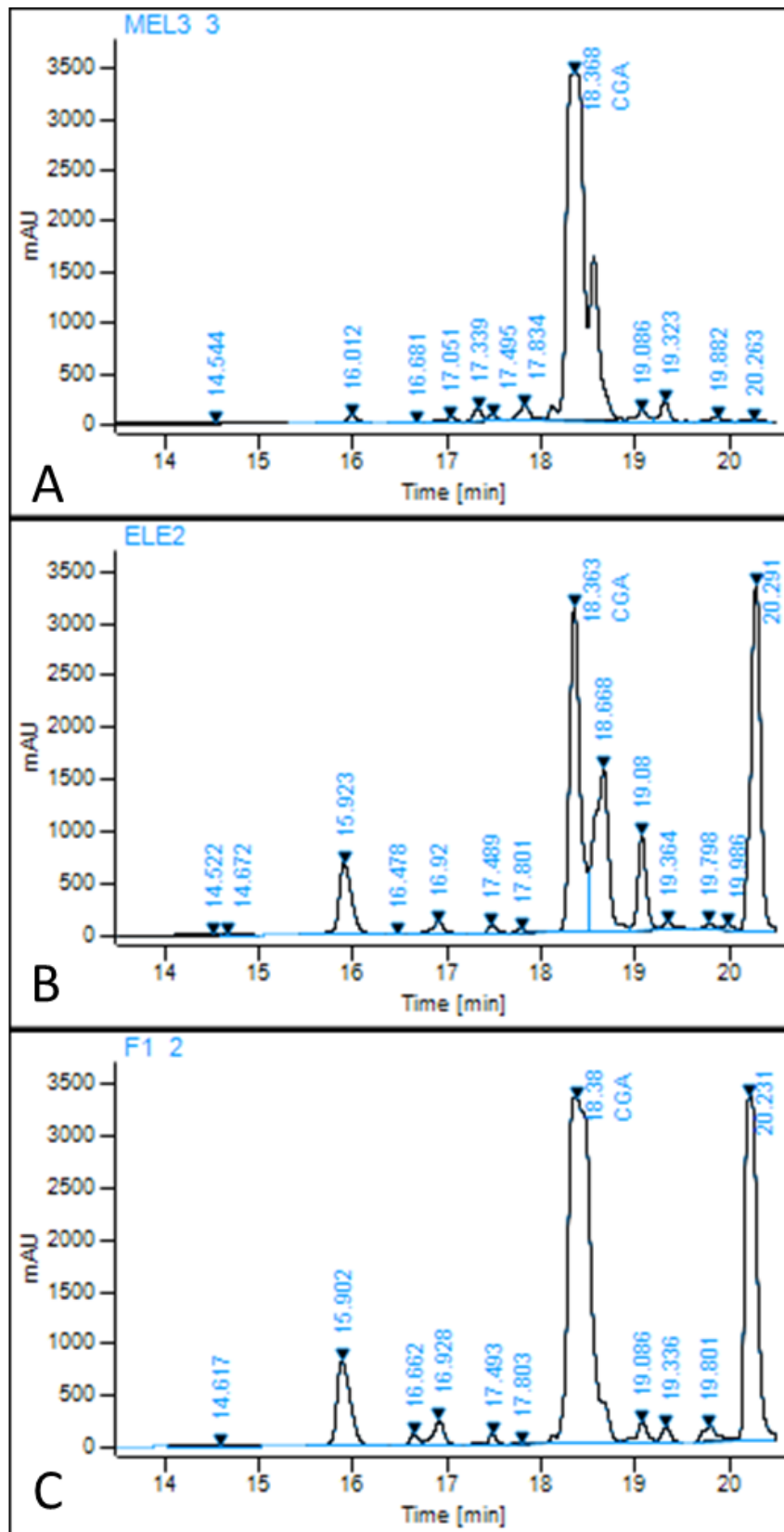
Figure 4: Phenolics profile of fruits of *S. melongena* MEL3 (A), *S. elaeagnifolium* ELE2 (B), and *S. melongena* MEL3 × *S. elaeagnifolium* ELE2 F1 hybrid (C).

4.4 Flow cytometry analysis of parents and hybrids

The analysis with flow cytometry revealed that both parents and the interspecific hybrid were diploid (Figure 5). No large differences were apparent for the genome size among parents and interspecific hybrid, although ELE2 seems to have a slightly smaller genome than *S. melongena*.

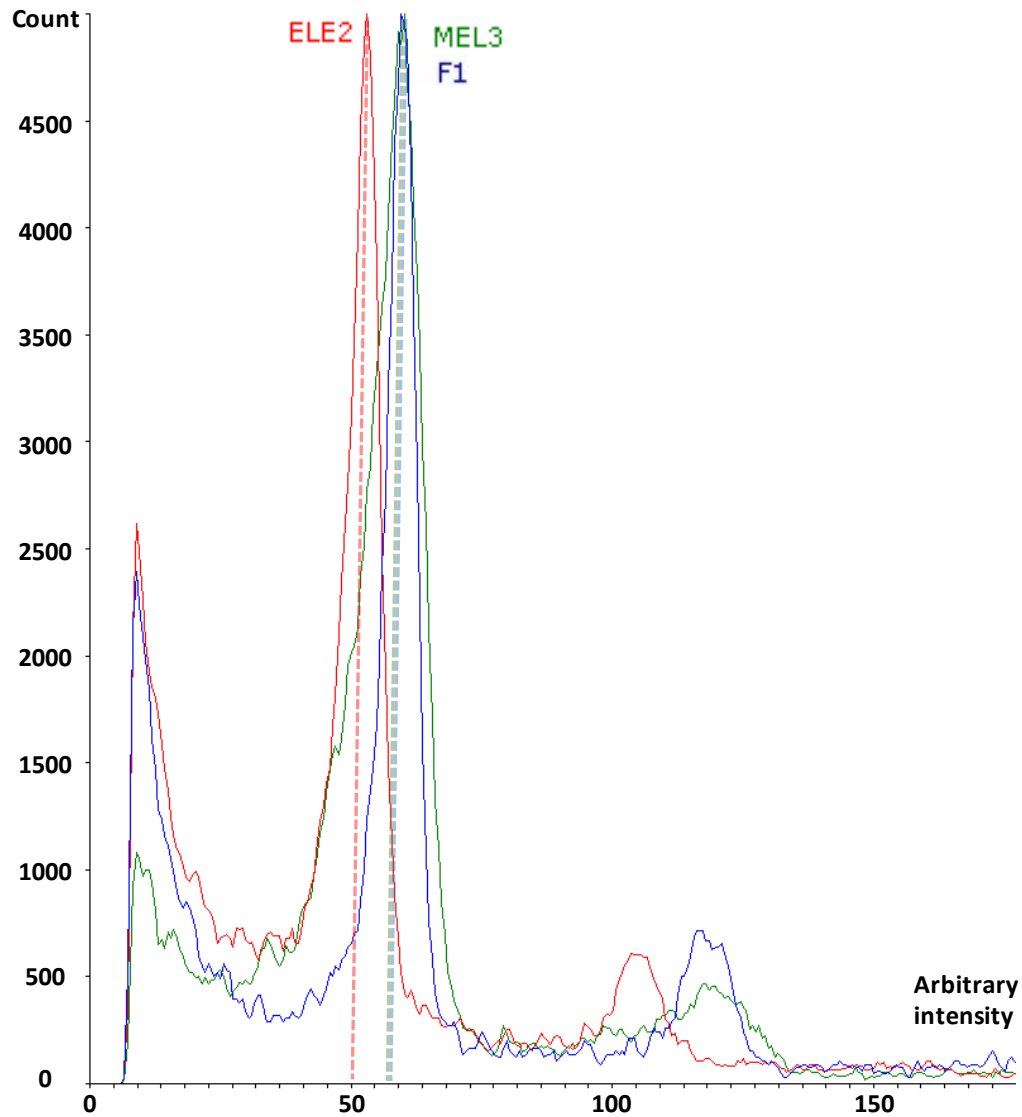**Figure 5:** Flow cytometry histogram of the relative nuclear DNA contents of: *S. melongena* MEL3 (green), *S. elaeagnifolium* ELE2 (red) and F1 interspecific hybrid *S. melongena* MEL3 × *S. elaeagnifolium* ELE2 (blue). The *x*-axis represents the proportional fluorescence intensity level to the nuclear DNA quantity; the position of the main peak reflects the ploidy level. The *y*-axis indicates the number of nuclei analyzed.

5. Discussion

The use of crop wild relatives in breeding has demonstrated in many crops that can make a significant economic impact (Tyack and Dempewolf, 2015). For example, in tomato, introgressions in commercial varieties from a wild relative have contributed to the increase of 2.4% in the soluble solids content of the fruit, which has had an economic impact of 250·10^6 US$ annually only in the US (Hunter and Heywood, 2011). This latter example reveals that although at the global level eggplant has less economic value than tomato (around 6.5-fold less) (FAO, 2018), wild relatives may make an important economic impact in eggplant breeding. However, up to now, to our knowledge, no commercial cultivars of eggplant with introgressions from crop wild relatives are available, and the potential of wild eggplant relatives for the development of commercial cultivars remains untapped. Here we report the successful backcrossing, up to the BC2 generation, of a species native to the New World (*S. elaeagnifolium*) with *S. melongena*, an Old World domesticate (Meyer et al., 2012). According to our knowledge, it is the first time that introgression materials of eggplant with a New World species have been obtained. This has important implications for eggplant breeding, as the introgression materials obtained (up to BC2 generation) indicate that a new distant untapped genepool has become available for eggplant breeding.

By using the interspecific hybrid *S. melongena* × *S. elaeagnifolium* as a female parent and *S. melongena* as a male parent, fruits containing viable seeds were obtained, although the degree of success was lower compared to backcrosses made with other interspecific hybrids of eggplant with Old World species (Kouassi et al., 2016). This is probably due to a greater sterility of the hybrid, as indicated by low pollen fertility. The fact that no seeded fruits appeared in the non-pollinated flowers suggests that pollen sterility in *S. melongena* × *S. elaeagnifolium* hybrids is a more limiting factor than that of the ovules, as has been shown in other crops (Dwivedi et al., 2008; Prohens et al., 2017). The extraction of embryos made in the first fruit of the hybrid pollinated with *S. melongena* pollen revealed a normal appearance in all immature seeds, suggesting that there are no major problems of embryo degeneration and abortion. In this way, mature seeds containing the BC1 zygotes presented a percentage of germination higher than 50%.

Of the 20 BC1 plants we used to develop the BC2 generation, seeds and BC2 offspring were obtained from 17 of them. Assuming a normal segregation and recombination in the F1 hybrid gametes, it would mean that the percentage of *S. elaeagnifolium* genome represented in the BC1 17 plants would be 1-0.5^{17} (>0.99999). Even though it is likely that distortions in the segregation and lack of recombination in some areas of the genome may have occurred (Kreike and Stiekema, 1997; Gramazio et al., 2018), a large part of the genome of *S. elaeagnifolium* is likely represented in the BC1 plants and the BC2 offspring. On the other hand, although variations in the ploidy degree are common in *S. elaeagnifolium* (Moscone E., 1992; Acosta et al., 2005; Powell and Weedin, 2005; Scaldaferro et al., 2012; Knapp et al., 2017) the results of flow cytometry indicate that the accession used of *S. elaeagnifolium* is diploid and presents a genome size similar to that of *S. melongena*, which could have contributed to the success of the backcrosses.

The interspecific hybrids were intermediate in most of the parental characteristics, although generally closer to *S. elaeagnifolium*. This is a common phenomenon in interspecific hybrids in eggplant with wild relatives (Prohens et al., 2013: Kaushik et al., 2016). However, unlike many other interspecific hybrids of eggplant (Kaushik et al., 2016), hybrids between common eggplant and *S. elaeagnifolium* did not display heterosis for vigor, perhaps due to the great phylogenetic distance between the two species (Vorontsova et al., 2013). The hybrid displayed prickles in the leaf, although none of the parents had prickly leaves. This is a common phenomenon in interspecific eggplant hybrids when crossing with a non-prickly species, probably because the mutations that confer lack of leaf prickles are different in the two species (Lester, 1986; Varoquaux et al., 2000; Kouassi et al., 2016; Plazas et al., 2016; Prohens et al., 2012).

In the hybrids the style was curved as in *S. elaeagnifolium*; in addition, some plants of the F1 presented anthocyanins in the style while others did not. Differences among F1 hybrids in style pigmentation could be due to environmental effects or epigenetic modifications (Hahlbrock and Scheel, 1989; Dixon and Harrison, 1990; Shichijo et al., 1993; Noda et al., 2004). Something similar, in addition to segregation, could be taking place in the individuals of the BC1. The fact that the flowers of the hybrid are smaller than those of both parents, but at the same time there are more flowers per inflorescence, has already been observed in hybrids between eggplant and other relatives (Daunay et al., 1993; Kaushik et al., 2016). The fruits of the hybrid have intermediate characteristics between those of both parents, although more similar to those of the wild species, again common in interspecific eggplant hybrids (Prohens et al., 2013; Kaushik et al., 2016).

As in other studies (Prohens et al., 2012, 2013), a regression towards the characteristics of the *S. melongena* parent was observed in the BC1 generation, although an important segregation was observed for all the characters in which the parents differed, except for the number of parts of the flower, which was consistently five, as in the wild parent. Amazingly, the fertility of pollen increased considerably, with an average of 19.4% and a minimum value of 7.4% in one of the five plants characterized, which suggests a rapid recovery of fertility already in this first generation of backcrossing, as described in other crops (Wall, 1970; Prohens et al., 2017).

Although the aerial part of interspecific hybrids is smaller than that of the *S. melongena* parent, the main root of both materials has a similar length. In addition, the fact that the relative density of lateral roots in the main root of the hybrid is smaller than that of *S. melongena* suggests that the hybrid explores other areas of the soil (Chen et al., 2014), whereas the *S. melongena* root system is mostly concentrated in the area where the drip irrigation system supplies water and nutrients. This suggests that an improved eggplant root system can be obtained through introgression from *S. elaeagnifolium* which, apart from being drought tolerant (Christodoulakis et al., 2009), has a rhizomatous root system (Knapp et al., 2017).

Wild species of eggplant generally present a more diverse phenolic profile that that of *S. melongena*, in which chlorogenic acid is the main component (Stommel and Whitaker, 2003; Whitaker and Stommel, 2003; Prohens et al., 2013). In our case, the profile of phenolic compounds shows that *S. elaeagnifolium* and *S. melongena* are also considerably different. In addition, *S. elaeagnifolium* presents a greater total peak area in the chromatogram, while the

hybrid presents an intermediate profile, although more similar to that of *S. elaeagnifolium*. This suggests that *S. elaeagnifolium* can contribute to improving the content of phenolic bioactive compounds of eggplant (Kaushik et al., 2015) without lowering the chlorogenic acid content. In this way, the use of another New World species (*S. viarum*) has been suggested as a potential source of variation to improve the caffeoylquinic acid content and its derivatives in eggplant (Wu et al., 2012).

In conclusion in this study we present for the first time, to our knowledge, the development of backcross generations of a hybrid between eggplant and a wild relative from the New World belonging to its tertiary germplasm pool (Kouassi et al., 2016). Our results suggest that these introgression materials will be of great interest for the genetic improvement of eggplant; they may have an tremendous potential to increase tolerance to abiotic stresses, such as to drought by improving the eggplant root system, as well as by enhancing its bioactive properties by increasing the contents in bioactive phenolics and modifying its profile (Kaushik et al., 2015). In addition, the introgression materials may also contribute to other traits that remain unexplored in *S. elaeagnifolium*, such as tolerance to pests and diseases. Also, because *S. elaeagnifolium* is not phylogenetically closely related to *S. melongena* (Vorontsova et al., 2013) the introgression materials obtained may represent an appropriate model to study epigenetic modifications occurring in the genome following distant hybridization and introgression breeding (Wang et al., 2005; Dong et al., 2006). Finally, we hope that this seminal study opens the way for the incorporation of the *Elaeagnifolium* clade New World genepool (Knapp et al., 2017) for eggplant breeding, ultimately contributing to the development of a new generation of plants adapted to climate change and with improved nutritional and diseases and pest resistance properties.

6. Acknowledgements

7. References

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Capítulo II

Development of Eggplant Pre-Breeding Materials with Introgressions from Wild Species

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Draft to be submitted

1. Abstract

This chapter summarizes the work carried out in the development of three introgression lines (ILs) of eggplant using three wild ancestors within the framework of the Global Crop Diversity Trust eggplant pre-breeding project. The main objective of this project is to develop elite eggplant materials better adapted to climate change conditions that are becoming more and more dramatic in the main cultivation areas of this species. From the interspecific hybrids, developed in the first phase of this project, advanced backcrosses for the development of ILs lines have been obtained that are currently in BC5 generations for the case of ILs with *Solanum insanum* and *S. dasyphyllum* and in a BC4 generation for the ILs with *S. elaeagnifolium*. An experimental model called Micro-Mel has also been developed with compact characteristics suitable as a plant model for eggplant. This genotype was developed from introgression materials with *S. anguivi* and it is currently in a BC2S3 generation. All these materials have been developed by manual backcrosses in isolated greenhouses and the lines have been selected by genotyping based on molecular markers initially with Sequenom MassARRAY, and later with the high throughput mass genotyping SPET technology. Currently, highly-fixed materials are available in which the genetic background is mainly eggplant, thanks to this we have been able to identify some regions related to the genetic control of the presence of prickles and the color of the fruits.

Key Words: *Solanum melongena*, *S. insanum*, *S. dasyphyllum*, *S. elaeagnifolium*, *S. anguivi*, introgression lines, SPET.

2. Background

Eggplant (*Solanum melongena* L.) is an important vegetable in many tropical and subtropical areas of the world. Many of these areas are already suffering from dramatic modifications in the current agricultural environment due to climate change (Rosenzweig et al., 2014). Yield reduction of eggplant results from several factors that will be intensified by climate change, including adverse weather conditions, diseases, insects, nematodes, and weeds (Hulme, 2017). As in other crops, reduction of eggplant yield, fruit quality, shelf-life, and nutritional content results from a number of biotic and abiotic stress, including adverse weather conditions, diseases, insects, pests, nematodes and weeds that will be intensified by climate change.

Eggplant is related to many crop wild relatives (CWRs) growing in a wide range of environmental conditions, including desertic areas and other highly stressful environments (Syfert et al., 2016). Some of these wild relatives have been found to be resistant or tolerant to some prevailing diseases and pests that might have increased incidence because of higher temperatures (Daunay and Hazra, 2012; Namisy et al., 2019). However, exploitation of eggplant genetic resources, particularly CWRs, for adaptation to biotic and abiotic stresses linked to climate change has historically been very limited (Rotino et al., 2014).

Thanks to the initiative "Adapting Agriculture to Climate Change: Collecting, Protecting and Preparing Crop Wild Relatives" (Dempewolf et al., 2014), supported by the Government of Norway, three projects have been undertaken since 2013 to use eggplant wild relatives as primary, secondary and tertiary genepools for the improvement of eggplant landraces from areas vulnerable to climate change, such as Southeast Asia and West Africa (Plazas et al., 2016; Kouassi et al., 2016; Gramazio et al., 2017; García-Fortea et al., 2019).

In the first stage of the Global Crop Diversity Trust eggplant pre-breeding project (started in Dec. 2013), multiple crosses were performed between six eggplant landraces (three from Ivory Coast and three from Sri Lanka) with 35 wild accessions from 15 wild relatives from the primary (one species), secondary (11 species) and tertiary (three species) genepools (Figure 1). The 15 wild species were selected for having one or more known characteristics of interest for adaptation to climate change, such as tolerance to biotic and/or abiotic stresses (Rotino et al., 2014; Syfert et al., 2016). A total of 90 hybrid combinations between the six landraces and 14 wild species (all except the tertiary genepool species *S. sisymbriifolium*) were obtained (Figure 2). For the two other wild species from the tertiary genepool (*S. elaeagnifolium* and *S. torvum*) embryo rescue was used to obtain the hybrids (Plazas et al., 2016; García-Fortea et al., 2019), which were viable, but completely sterile in the case of the hybrids with *S. torvum*.

Figure 1: Fruits of eggplant CWRs and landraces (large fruits in the center) used for introgression breeding.
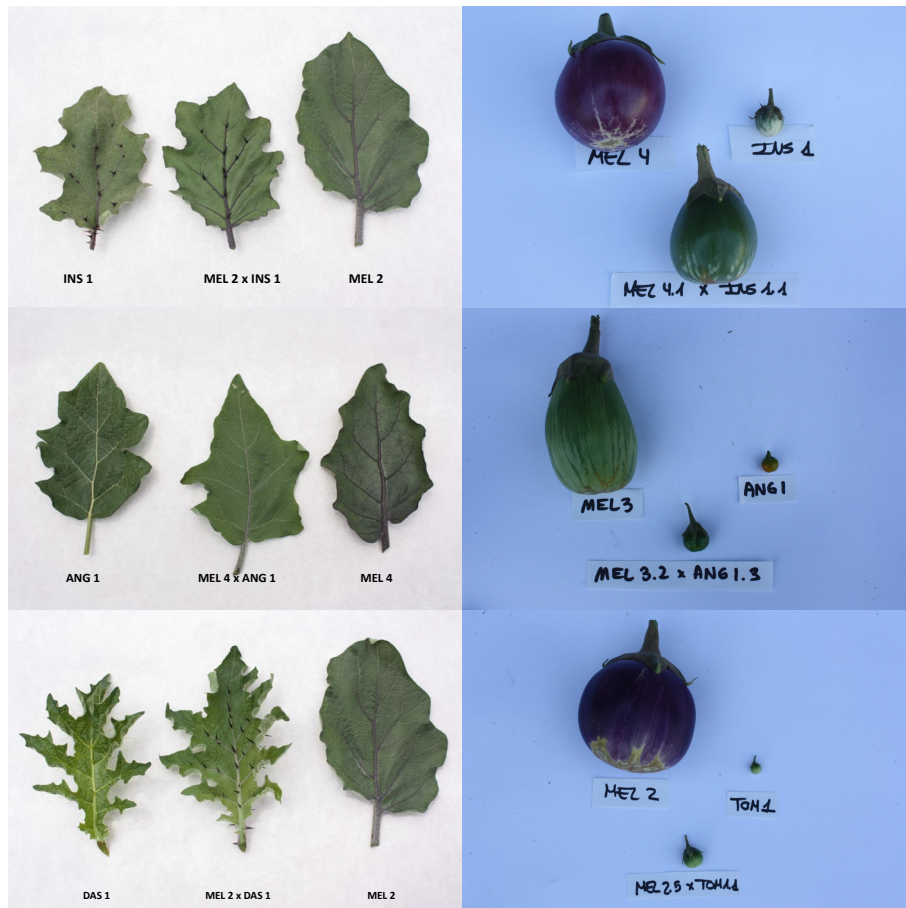
Figure 2: Leaves and fruits of interspecific hybrids between eggplant and CWRs and their respective parents. The left part of the figure displays the leaves of interspecific hybrids between *S. melongena* and *S. insanum* (above), *S. anguivi* (center) and *S. dasyphyllum* (below). The right part of the figure displays the leaves of interspecific hybrids between *S. melongena* and *S. insanum* (above), *S. anguivi* (center) and *S. tomentosum* (below). For both leaves and fruits, the respective parents of interspecific hybrids are included.

As a result of backcrossing of the hybrids to the recurrent cultivated landraces, 48 first backcross (BC1) and 36 second backcross (BC2) generations towards the respective S. melongena parents involving the primary genepool species *S. insanum* and eight secondary genepool species (*S. anguivi, S. dasyphyllum, S. incanum, S. lichtensteinii, S. lidii, S. linnaeanum, S. pyracanthos,* and *S. tomentosum*), as well as with the tertiary genepool species *S. elaeagnifolium*, were developed (Figure 3) (Kouassi et al., 2016; Plazas et al., 2016; García-Fortea et al., 2019).

Figure 3: Fruits of four different plants of the first backcross (BC1) of the interspecific hybrid between *S. melongena* and *S. dasyphyllum,* towards the *S. melongena* parent.

A total of 87 progenies of second generations of selfing obtained by single seed descent from individually selected plants for tolerance to drought coming from 14 BC2 generations involving six wild species (*S. anguivi*, *S. dasyphyllum*, *S. incanum*, *S. insanum*, *S. lichtensteinii* and *S. lidii*) were also obtained. These materials are morphologically highly variable (Figure 4) and are potentially of interest for the development of drought-tolerant materials of eggplant, as they were initially selected for this trait.

Figure 4: Sample of fruits from different BC2S2 progenies between eggplant and accessions of six eggplant CWRs.

In this chapter, we present the most relevant results in the last years of the project regarding the development of three new sets of ILs with the wild species *S. insanum*, *S. dasyphyllum* and *S. elaeagnifolium*. Mainly we explain how the introgression materials have been developed with these three wild species related to eggplant, from the primary (*S. insanum*), secondary (*S. dasyphyllum*), and tertiary (*S. elaeagnifolium*) genepools and at what point are these materials at the date of writing of this document. On the other hand, we also present the development of an experimental model called "Micro-Mel", also derived from the plant material generated in this project, specifically from a BC2 generation with *S. anguivi*.

3. Material and methods

3.1 Starting plant material

For the ILs, we started with BC1 generations towards the *S. melongena* parent of hybrids obtained between three cultivars of *S. melongena* (MEL1, MEL3, and MEL5) and three accessions of *S. insanum* (INS1), *S. dasyphyllum* (DAS1), and *S. elaeagnifolium* (ELE2) (Plazas et al., 2016; García-Fortea et al., 2019). The three hybrid combinations were the following: MEL5 x INS1, MEL1 x DAS1, MEL3 x ELE2.

For the development of the dwarf eggplant material, we started from an individual BC2 plant towards the recurrent *S. melongena* parent of the hybrid between *S. melongena* MEL1 and *S. anguivi* ANG1. BC1 plants of these cross had been selected for drought tolerance and in one of the BC2 generations, a dwarf plant with small fruits was found and represented the starting point of this programme.

Germination and cultivation of plants

A germination protocol in Petri dish developed by our group (Ranil et al., 2015), which breaks the dormancy of fresh seed of eggplant and wild species, was applied. The protocol consists of the immersion of seeds in water for 24 h, followed by immersion in GA_3 500 ppm during 24 h, application of a thermal shock at 37ºC during 24 h, and utilization of KNO_3 1,000 ppm. All plants used for hybridizations were grown in an insect-free greenhouse at Universitat Politècnica de València (GPS coordinates 39.482228, -0.337332) in 15 l pots filled with coconut fiber. Plants were watered and fertilized using a drip irrigation system. Phytosanitary treatments will be carried out when necessary.

3.2 Reproduction control

Self-fertilization and hybridization for obtaining ILs were carried out according to the normal eggplant protocol (Prohens et al., 2010). In the case of self-fertilization, before anthesis, pollen from the same plant was deposited on the stigma and the labeling and bagging were carried out with a paper bag to avoid contamination by exogenous pollen. In the case of hybridizations, before anthesis, the female parent was emasculated with tweezers and pollen from the male parent was deposited on the stigma. Then pollinated flowers were bagged. In all cases, the seeds were extracted once the fruits were physiologically mature. Hybridizations were performed taking special precaution to use the first flowers to obtain ripen fruits as soon as possible. In the case that in some materials the curdling or ripening of the fruit is delayed, embryos were rescued using the methodology of Manzur et al. (2013), which it has proven efficient in eggplant.

3.3 DNA extractions

Genomic DNA was isolated from 3-4 true leaves, according to the SILEX protocol (Vilanova et al., 2020). The extracted DNA was dissolved in Milli-Q water and general quality was confirmed in agarose gel at 0.8%. After a concentration measurement using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, USA), the DNA was diluted at 30 ng/µL for Sequenom MassARRAY (Bradic et al., 2012) or SPET analysis (Barchi et al., 2019) using the Tassel software (Bradbury et al., 2007).

4. Results

4.1 Development of advanced introgression materials for ILs development

The following section summarizes the results derived from the development of the three ILs. Because the data presented dates back to 2016 and each year these data are similar, the following simplified schedule is presented (Figure 5) to facilitate following this section, so that it is understood at what time the germination, backcrossing, DNA extraction and genotyping tasks of each generation in each ILs was done.
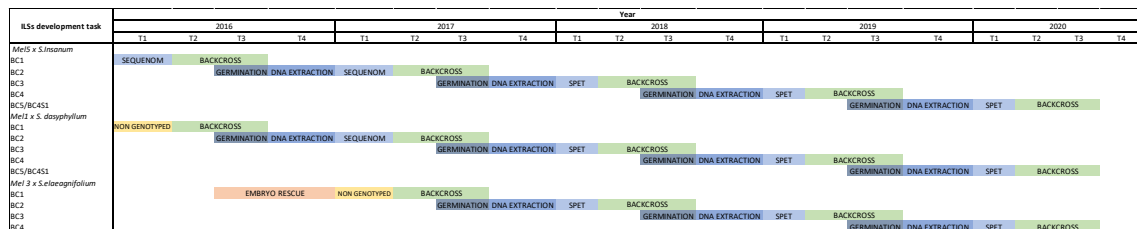
Figure 5: Simplified schedule of the germination, backcrossing, DNA extractions and genotyping tasks during the development of the ILs from 2016 to 2020. The years divided into trimesters are presented where the completion times of each task are indicated

4.1.1 ILs with S. insanum

For the BC1 generation, a total of 181 plants were genotyped using the Sequenom platform by March 2016. Out of these, 30 plants were selected based on the markers profile (Figure 6).
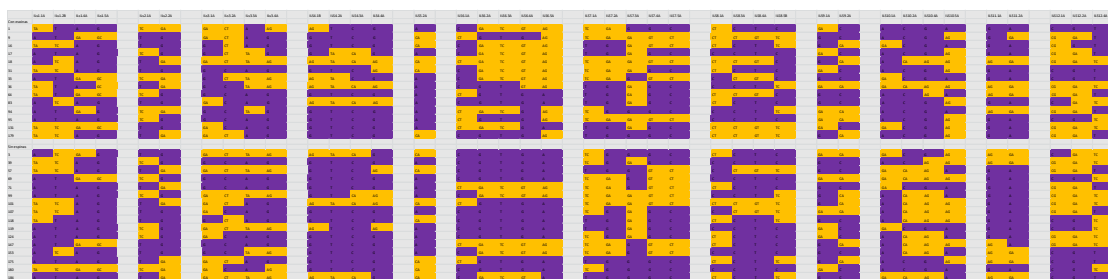
Figure 6: Sequenom genotyping profile for the 30 plants selected (in rows) of the BC1 generation. Purple cells represent the recurrent allele, while yellow cells are heterozygotes containing introgressions from the S. insanum donor parent. Each of the 12 vertical blocks represents one chromosome.

Seeds from 28 BC2 generations previously selected from the cross between *S. melongena* MEL5 with *S. insanum* INS1 were germinated. Several individuals per generation (between 2 and 5) were genotyped, totaling 91 plants, using the Sequenom MassARRAY genotyping platform by March 2017. Out of these, 37 BC2 plants were selected by choosing the best genotypic

combination (i.e., presenting the target fragment from *S. insanum*, and a high proportion of the *S. melongena* background in the rest of the genome). The genotype of these 37 selected plants is displayed in Figure 7. These 37 plants were backcrossed, using the BC2 plants as the female parent, to the *S. melongena* MEL5 parent and BC3 seed was obtained for all the 37 selected BC2 plants.

Figure 7: Sequenom genotyping profile for a subset of pre-selected plants (in rows) of the BC2 generation from which 37 plants were finally selected. Purple cells represent the recurrent allele, while yellow cells are heterozygotes containing introgressions from the *S. insanum* donor parent. Each of the 12 vertical blocks represents one chromosome.

Seeds of the 37 BC3 generations were germinated and DNA was extracted by March 2018 for 3 to 4 plantlets of each generation, for a total of 90 BC3 plants. The extracted DNA was subjected to SPET genotyping, which resulted in a total of 1,323 SNP markers being polymorphic among the two parents (MEL5 and INS1). Based on the SPET genotyping results (Figure 8) a set of 30 BC3 plants was selected.

Figure 8: SPET genotyping profile for a subset of pre-selected plants (in rows) of the BC3 generation from which 30 plants were finally selected. Red cells represent the recurrent allele, while yellow cells are heterozygotes containing introgressions from the *S. insanum* donor parent. Each of the 12 vertical blocks represents one chromosome.

These 30 BC3 plants have been grown and were used as female parents to develop the BC4 generation. Also, selfings were obtained to develop BC3S1 generations. By November 2018, BC4 and BC3S1 seeds were obtained, respectively, for 30 and 16 BC3 plants. Seeds of each BC4 generation were germinated and by December 2018 seedlings had been obtained for 30 BC4 families. SPET genotyping was performed on 105 BC4 seedlings to select those plants that are most appropriate to develop ILs covering most of the genome of the donor *S. insanum* INS1 parent. In this case, 43 BC4 plants were selected (Figure 9).

Figure 9: SPET genotyping profile for a subset of pre-selected plants (in rows) of the BC4 generation from which 43 plants were finally selected. Dark blue cells represent the recurrent allele, while soft blue cells are heterozygotes containing introgressions from the *S. insanum* donor parent. Each of the 12 vertical blocks represents one chromosome.

These 43 BC4 plants were grown and were used as female parents to develop the BC5 generation. Also, selfings were obtained to develop BC4S1 generations. By November 2019, BC5 and BC4S1 seeds were obtained, respectively, for 38 and 28 BC4 plants. Seeds of each BC5 and BC4S1 generation were germinated and by December 2019 seedlings had been obtained for 13 BC5 families and 28 BC4S1. SPET genotyping was performed on 77 BC5 and 202 BC4S1 seedlings to select those plants that are most appropriate to develop ILs covering most of the genome of the donor *S. insanum* INS1 parent. Based on the SPET genotyping results (Figure 10) a set of 46 BC5 and 16 BC4S1 plants were selected. As can be seen in the image, practically the entire wild genome of *S. insanum* is represented in these ILs, not having lost any fragment.

Figure 10: SPET genotyping profile for a subset of pre-selected plants (in rows) of the 46 BC5 and 16 BC4S1 generations from which plants were finally selected. Dark blue cells represent the recurrent allele, while soft blue cells are heterozygotes containing introgressions from the *S. insanum* donor parent, red cells are homozygotes for the *S.insanum* alleles. Each of the 12 vertical blocks represents one chromosome.

4.1.2 ILs with S. dasyphyllum

Seeds from 16 BC2 generations previously selected from the cross between *S. melongena* MEL1 with *S. dasyphyllum* DAS1 were germinated. Several individuals per generation (between 1 and 7) were genotyped, totaling 91 plants, using the Sequenom MassARRAY genotyping platform by March 2017. Out of these, 33 BC2 plants were selected by choosing the best genotypic combination (i.e., presenting the target fragment from *S. dasyphyllum*, and a high proportion of the *S. melongena* background in the rest of the genome). The genotypes of these 33 selected plants are displayed in Figure 11. These 33 plants were backcrossed, using the BC2 plants as the female parent, to the *S. melongena* MEL5 parent and BC3 seed has been obtained for 31 of the BC2 plants.
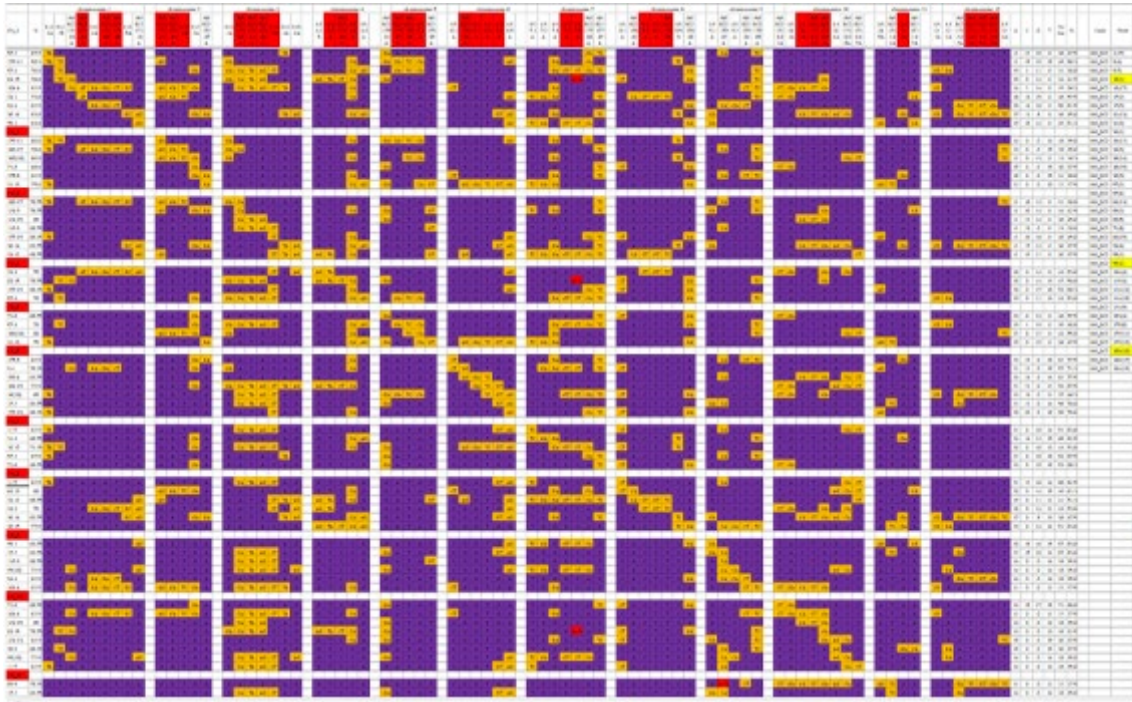
Figure 11: Sequenom genotyping profile for a subset of pre-selected plants(in rows) of the BC2 generation from which 33 plants were finally selected. Purple cells represent the recurrent allele, while yellow cells are heterozygotes containing introgressions from the *S. dasyphyllum* donor parent. Each of the 12 vertical blocks represents one chromosome.

Seeds of the 31 BC3 generations were germinated and DNA was extracted by March 2018 for 3 to 4 plantlets of each generation, for a total of 90 BC3 plants. The extracted DNA was subjected to SPET genotyping, which resulted in a total of 6,840 SNP markers being polymorphic among the two parents (MEL1 and DAS1). Based on the SPET genotyping results, 32 BC3 plants were selected (Figure 12), which cover 99.80% of the *S. dasyphyllum* genome.

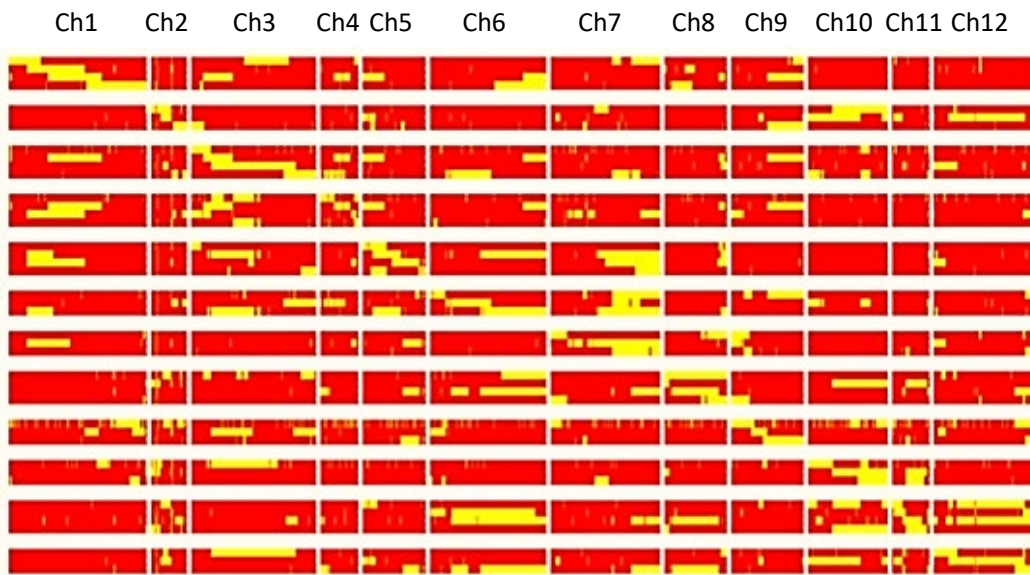| Ch1 | Ch2 | Ch3 | Ch4 | Ch5 | Ch6 | Ch7 | Ch8 | Ch9 | Ch10 | Ch11 | Ch12 |

Figure 12: SPET genotyping profile for a subset of pre-selected plants (in rows) of the BC3 generation from which 32 plants were finally selected. Green cells represent the recurrent allele, while pink cells are

heterozygotes containing introgressions from the *S. insanum* donor parent. Each of the 12 vertical blocks represents one chromosome.

These 32 BC3 plants have been grown and were used as female parents to develop the BC4 generation. Also, selfings were obtained to develop BC3S1 generations. By November 2018, BC4 seed was obtained for 27 plants. Seeds of each BC4 generation have been germinated and by December 2018 seedlings have been obtained for 27 BC4 families. SPET genotyping will be performed on 74 BC4 seedlings to select those plants most appropriate to develop ILs covering most of the genome of the donor *S. dasyphyllum* DAS1 parent. Based on the SPET genotyping results, 37 BC4 plants were selected (Figure 13).
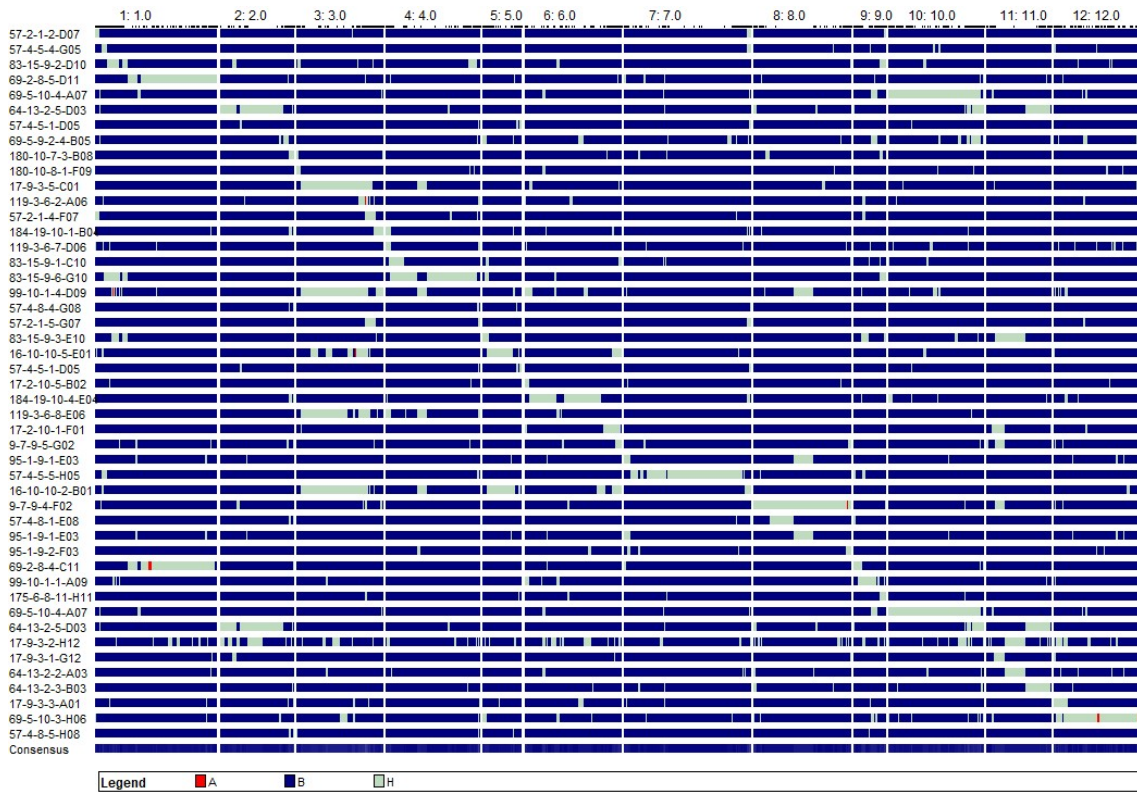

Figure 13: SPET genotyping profile for a subset of pre-selected plants (in rows) of the BC4 generation from which 37 plants were finally selected. Dark blue cells represent the recurrent allele, while soft blue cells are heterozygotes containing introgressions from the *S. dasyphyllum* donor parent. Each of the 12 vertical blocks represents one chromosome.

These 37 BC4 plants were grown and used as female parents to develop the BC5 generation. Also, selfings were obtained to develop BC4S1 generations. By November 2019, BC5 and BC4S1 seeds were obtained, respectively, for 35 and 22 BC4 plants. Seeds of each BC5 and BC4S1 generation have been germinated and by December 2019 seedlings were obtained for 24 BC5 families and 15 BC4S1. SPET genotyping was performed on 144 BC5 and 113 BC4S1 seedlings to select those plants that are most appropriate to develop ILs covering most of the genome of the donor *S. dasyphyllum* DAS1 parent. Based on the SPET genotyping results (Figure 14) a set of 47

BC5 and 4 BC4S1 plants were selected. As can be seen in the image, practically the entire wild genome of *S. dasyphyllum* is represented in these ILs, not having lost any fragment.
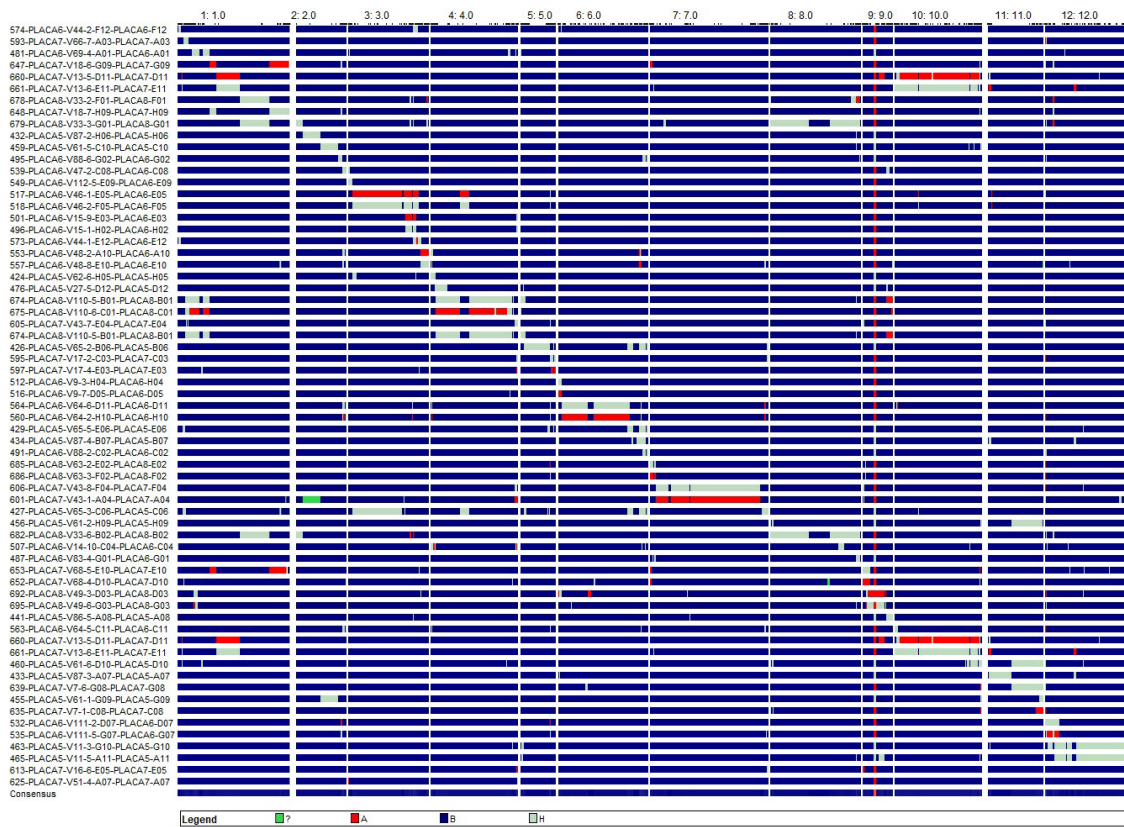

Figure 14: SPET genotyping profile for a subset of pre-selected plants (in rows) of the 47 BC5 and 4 BC4S1 generations from which plants were finally selected. Dark blue cells represent the recurrent allele, while soft blue cells are heterozygotes containing introgressions from the *S. dasyphyllum* donor parent, red cells are homozygotes for the *S. dasyphyllum* alleles. Each of the 12 vertical blocks represents one chromosome

4.1.3 ILs with *S. elaeagnifolium*

Seeds of the BC1 generation (García-Fortea et al., 2019) derived from the hybrid between *S. melongena* accession (MEL3) and the extremely drought tolerant tertiary genepool species *S. elaeagnifolium* accession ELE2 towards *S. melongena* accession (MEL3) were germinated in 2017. Despite the wide genetic distance between the two parents, germination was successful with over 50% of the seeds germinating. A total of 20 BC1 plants were grown and used as female parents for backcrossing with the MEL3 to obtain the second backcross generation. Each BC1 plant was treated individually. By March 2018, BC2 seeds were obtained from 17 individual BC1 plants. Seeds of all these BC2 generations were put to germinate, and plantlets were obtained for 11 of them. DNA was extracted for 5 to 12 plantlets of each BC2 generation, for a total of 105 BC2 plants. DNA was evaluated for quality and concentration and 90 individuals of the BC2 were subjected to Single Primer Enrichment Technology (SPET) genotyping. A total of 10,377 SNP markers were found to be polymorphic among the two parents (MEL1 and ELE2). Based on the genotyping data, a total of 35 BC2 individuals covering 99.6% of the *S. elaeagnifolium* ELE2 donor genome (Figure 15) were selected for developing the BC3 generation.
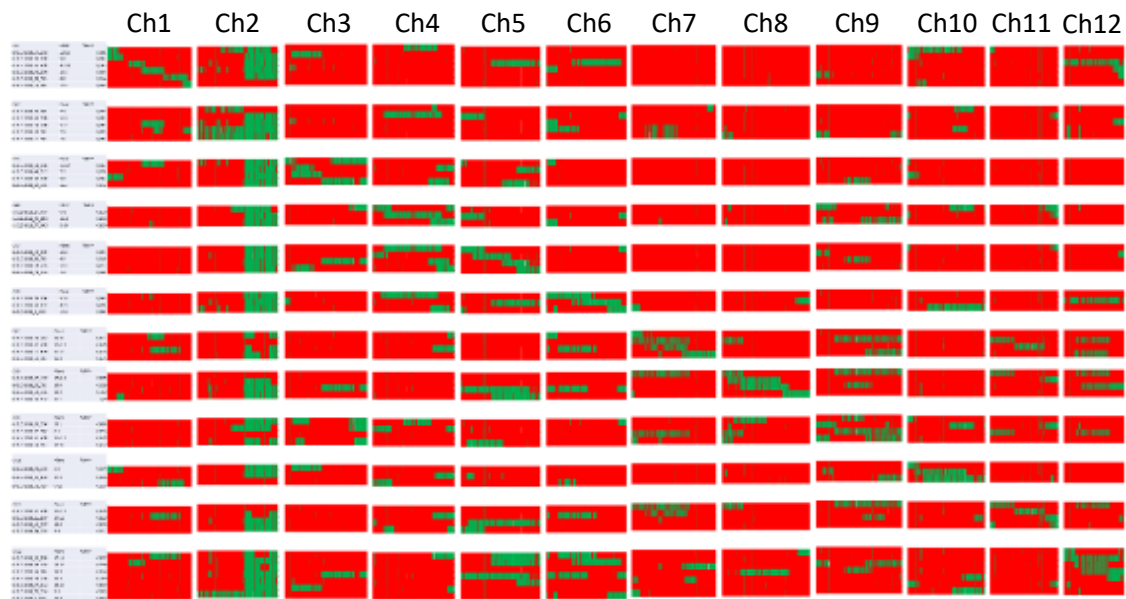
Figure 15: SPET genotyping profile for a subset of pre-selected plants (in rows) of the BC2 generation from which 35 plants were finally selected. Red cells represent the recurrent allele, while green cells are heterozygotes containing introgressions from the *S. elaeagnifolium* donor parent. Each of the 12 vertical blocks represents one chromosome.

These 35 BC2 plants were grown and used as a female parent to obtain the BC3 generation. By November 2018, BC3 seed was obtained for the 35 BC2 plants. Seeds of each BC3 generation were germinated and by December 2018 seedlings had been obtained for 25 BC3 families. SPET genotyping was performed on 74 BC3 seedlings to select those plants that are most appropriate to develop ILs covering most of the genome of the donor *S. elaeagnifolium* ELE2 parent (i.e., presenting the target fragment from S. elaeagnifolium, and a high proportion of the *S. melongena* background in the rest of the genome). Based on the SPET genotyping results (Figure 16) a set of 25 BC3 plants was selected.
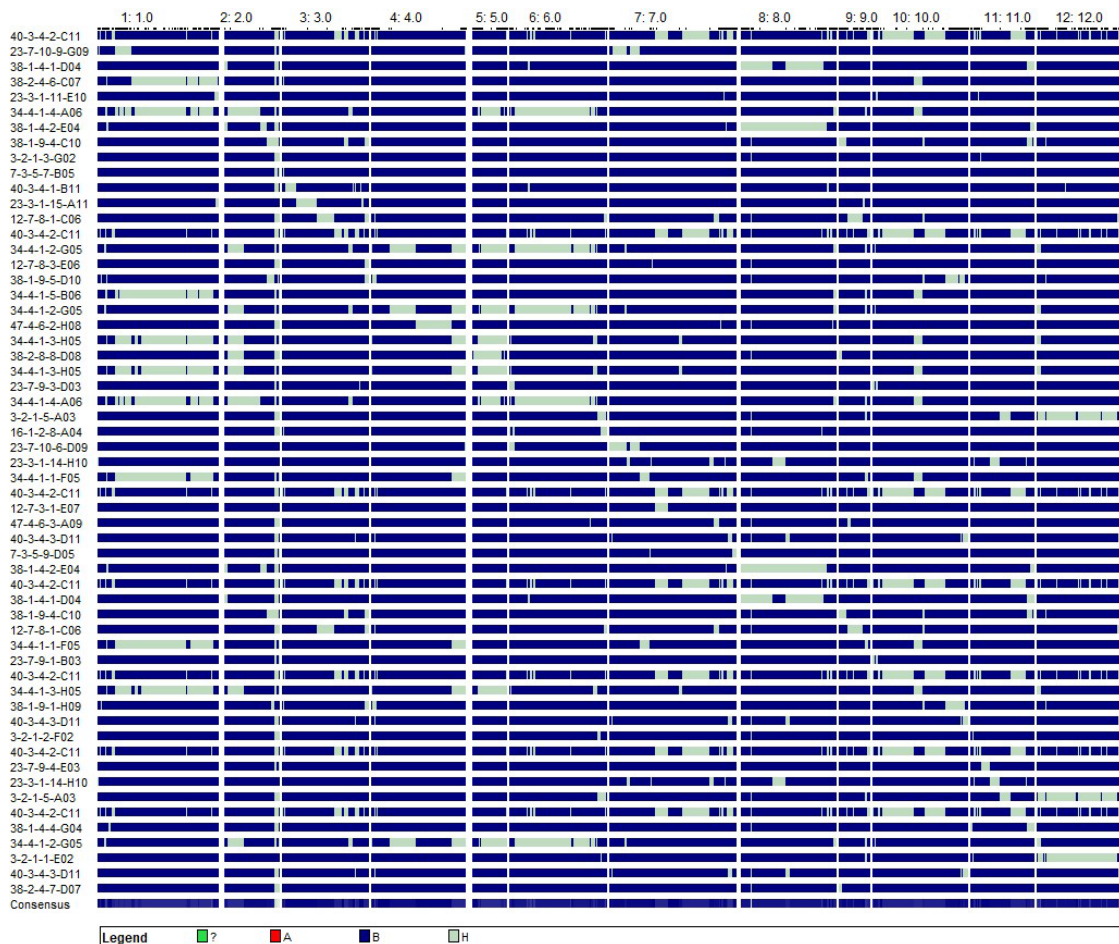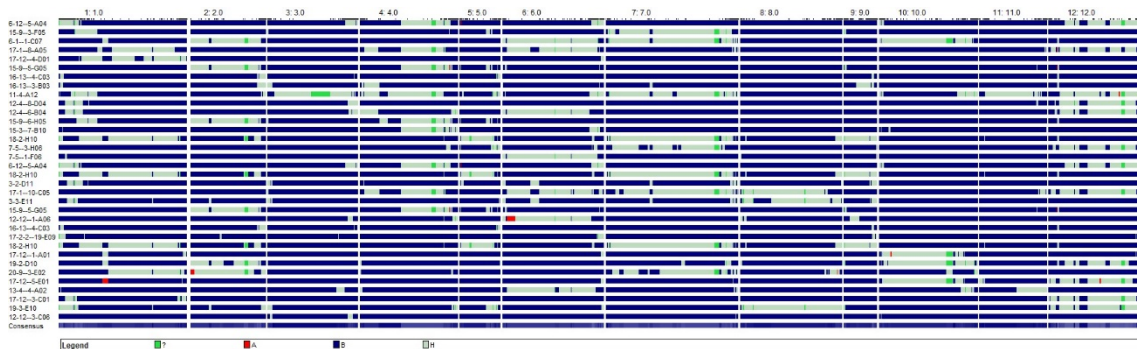

Figure 16: SPET genotyping profile for a subset of pre-selected plants (in rows) of the BC3 generation from which 25 plants were finally selected. Dark blue cells represent the recurrent allele, while soft blue cells are heterozygotes containing introgressions from the *S. elaeagnifolium* donor parent. Each of the 12 vertical blocks represents one chromosome.

The backcrossing of the selected 25 BC3 plants has been continued during 2019 to obtain further seed for the BC4 families. By November 2019, BC4 seeds were obtained for the 23 BC3 plants. Seeds of each BC4 generation were germinated and by December 2019 seedlings have been obtained for 22 BC4 families. SPET genotyping was performed on 170 BC4 seedlings. Based on

the SPET genotyping results (Figure 17) a set of 45 BC4 plants was selected. As can be seen in the image, practically the entire wild genome of *S. elaeagnifolium* is represented in these ILs, not having lost any fragment.

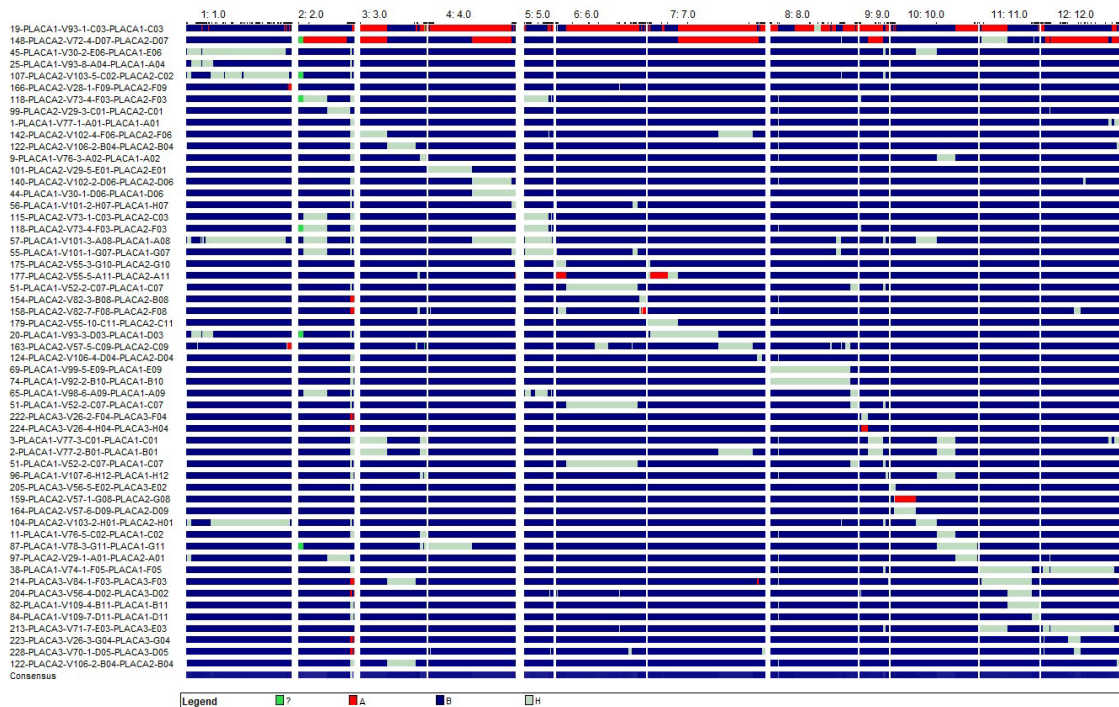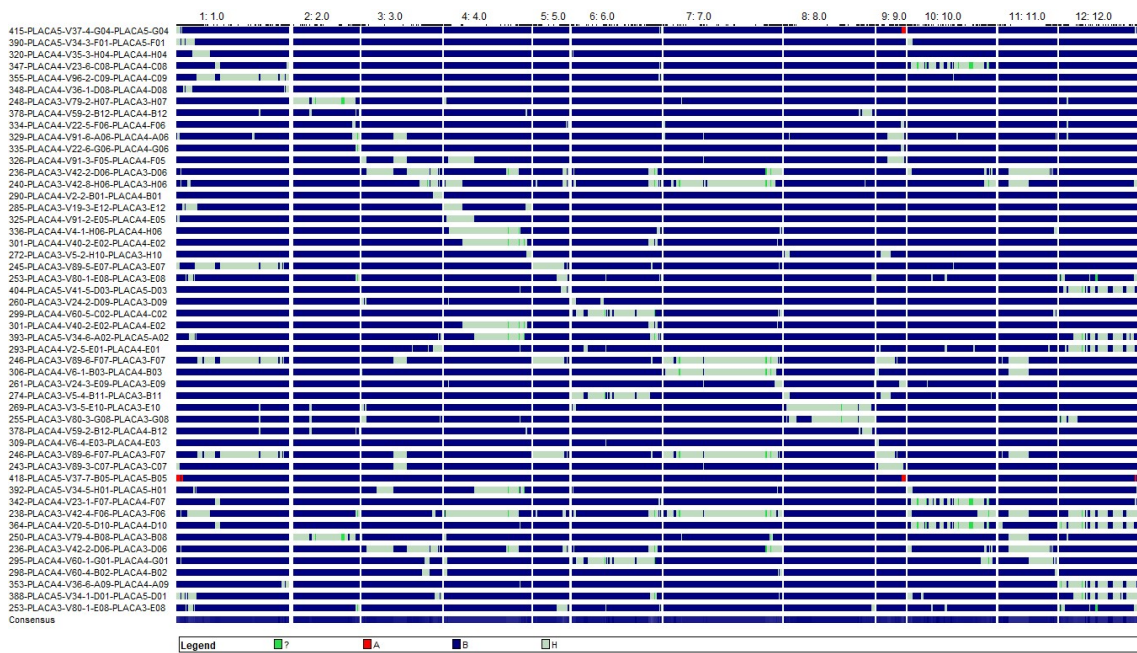**Figure 17:** SPET genotyping profile for a subset of pre-selected plants (in rows) of the 45 BC4 generations from which plants were finally selected. Dark blue cells represent the recurrent allele, while soft blue cells are heterozygotes containing introgressions from the *S. elaeagnifolium* donor parent. Each of the 12 vertical blocks represents one chromosome.

As it has been seen throughout this section, the ILs are currently found in a BC5 generation in the case of *S. insanum* and *S. dasyphyllum* and a BC4 generation in the case of *S. elaeagnifolioum*. Table 1 shows the average heterozygosity percentages for each of the generations at this point. These values indicate the high degree of recovery of the *S. melongena* background in the three cases, being somewhat higher in the case of *S. insanum*, where the marker-assisted selection was performed since BC1 generation, and in the rest of the cases, the genotyping was performed since the BC2 generation. This was already clearly seen in Figures 9, 12 and 16, corresponding to the last genotyped generations of each introgression line, but which is confirmed numerically from the analysis of all the individuals analyzed in the last genotyping performed. The number of alleles analyzed is also presented, this value being much higher in the case of *S. elaeagnifolium* due to its genetic distance.

Table 1: SPET mass genotyping results shown as the percentage of heterozygosity (mean and range) calculated based on the genotyped using SNP markers in the plants for each introgression line (BC5 for *S. insanum* and *S. dasyphillium* and BC4 for *S. elaeagnifolium*). The number of SNPs positions evaluated and those that were not found in the specific accessions are shown.

ILs generation	n plants	Total SNP	Missing SNP	Heterozygosity (%)
BC5 *S. insanum*	275	2906	0.30 (0.00-6.00)	3.47(0.21-14.80)
BC5 *S. dasyphyllum*	229	2690	1.42(0.00-20.00)	7.72(0.00-33.17)
BC4 *S. elaeagnifolium*	203	3823	2.52(0.00-16.00)	7.29 (0.08-39.19)

4.2 Traits of interest discovered in the materials

Among the materials that have been developed, some characters of interest were found. These characters have been phenotyped and mapped with the available genotyping data and for some of them candidate regions have been found.

4.1.2 Dwarf phenotype (towards the development of the Micro-Mel model)

 The dwarf phenotype trait was discovered in a plant of a second backcross (BC2) generation of a white eggplant accession (MEL1) used as a recurrent parent with the wild species *S. anguivi* (accession ANG1) used as donor parent. In the BC2 generation grown in 1.3 L pots, a plant (coded as BC2-9) displaying a small size, semi-determined growth, and with many small white fruits was detected (Figure 18A); this plant was subsequently transplanted in the open field to check if the small size of the plant, profuse fruiting and small fruits maintained, and the initial observations were confirmed (Figure 18B).

The original BC2 plant was self-pollinated, and the fruits gave viable seed from which 52 BC2S1 generation individuals were grown under controlled conditions in 1.3 L pots. In them, evident segregation was observed for the size and shape of the fruits (Figure 18C) in which the range of sizes varied between 2 and 6 cm in length, and between 1 and 3 cm in width. Another feature observed among the plants of this BC2S1 generation was the presence of terminal flowers in some of the plants (Figure 18D), leading to a change in the type of growth of this crop, which is usually indeterminate. Finally, striking segregation was observed for the height of the plant, as well as the distance of the internodes and the type of growth (Figure 18E). In this way, among the plants of the generation BC2S1, we found a wide range of phenotypes, from plants with erect growth, stem internodes with normal length, and standard plant size, to plants with almost rosette growth, extremely short internodes and a plant height that did not exceed 5 cm, validating that the trait is maintained over generations, though as expected segregation exists. Of these 52 BC2S1 plants, only those displaying the reduced size of leaves and flowers, determined growth, short internodes, and setting many seeded fruits were selected. As a result, 19 BC2S1 plants were selected, of which 13 gave fruit with viable seed. A pedigree-based scheme was used and the 13 BC2S2 families were obtained.
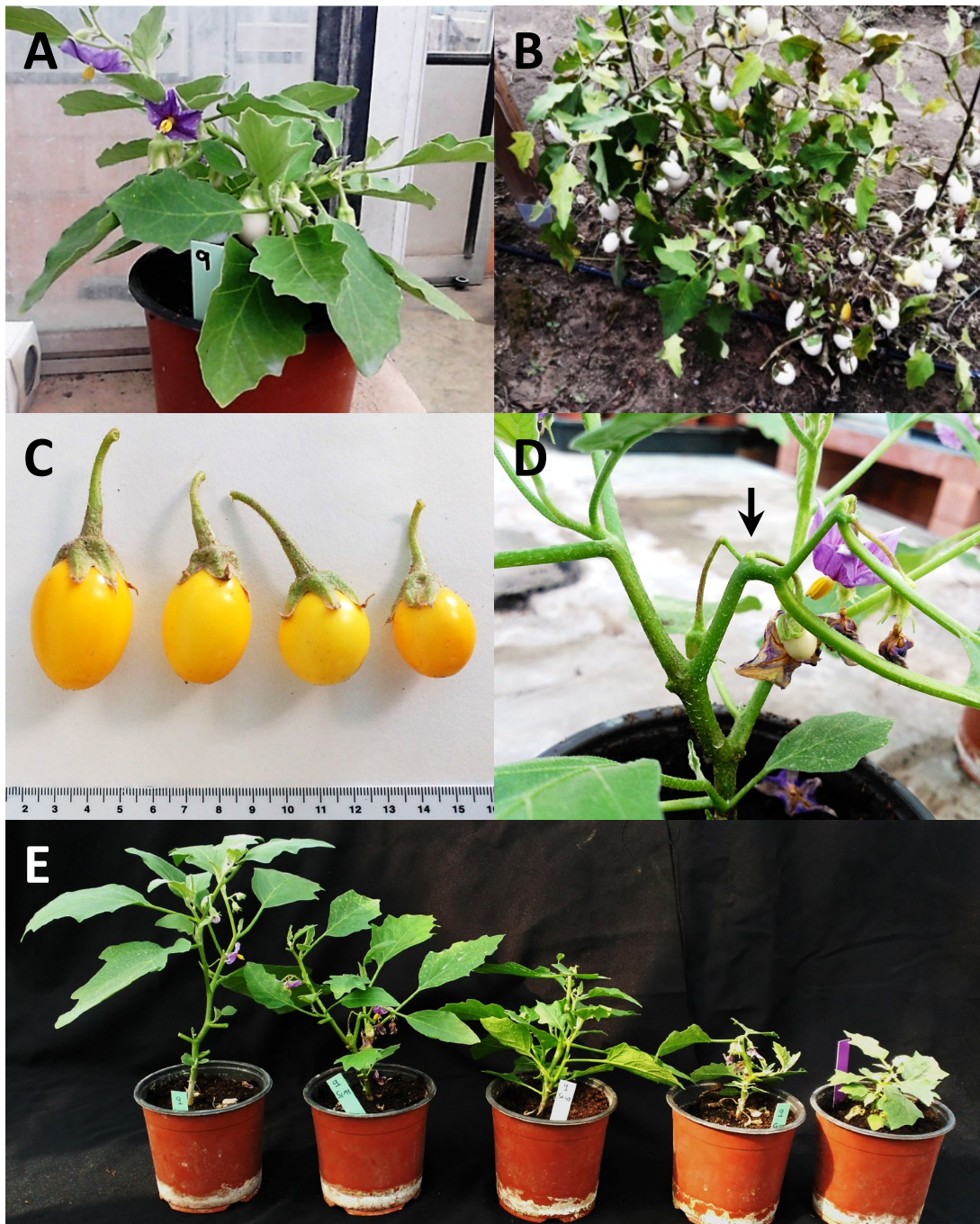
Figure 18: Original BC2 plant of a cross between *S. melongena* MEL1 and *S. anguivi* ANG1 grown in a pot (A) and in the field (B), and segregation observed in the BC2S1 generation for the size and shape of the fruits (C), detail of the terminal flower observed in some plants (D), and segregation for plant size (E).

Of the 13 BC2S1 families, 6 seeds of each were germinated, obtaining BC2S2 plant. In the end, they were obtained with 42 BC2S2 plants, of which 4 individuals were chosen to genotype using SPET technology: 9(57)-3 and 9(33)-1 as representatives of the normal phenotype; 9(72)-1 and 9(34)-1 as representatives of the dwarf phenotype; Parents ANG1 y MEL1. However, the genetic background was not clean enough to find a candidate region that explained the dwarf phenotype.

Nine plants [9(33)-1, 9(33)-2, 9(33)-3, 9(34)-3, 9(45)-1, 9(50)-1, 9(61)-3, y 9(63)-3] of the BC2S2 generation with dwarf phenotypes were selected following the same selection criteria as used so far: short internodes and small size in general (Figure 19A and 19B), determined growth with the presence of terminal flowers, multiple fruit set and seed presence (Figure 19C and 19D). A plant BC2S2 9(33)-3 was also selected as it presented an intermediate phenotype but very interesting fruits from a commercial point of view (Figure 19E), this has been necessary because in some lines the size of the fruits was excessively small and there was generating fruits with unviable seeds (Figure 19F). These 9 plants were self-fertilized to obtain the BC2S3 generation.

Figure 19: BC2S2 generation plants that present the Micro-Mel ideotype (A and B); fruit clusters of a BC2S2 plant (C); fruit with viable seeds of a BC2S2 plant (D); fruit of accession BC2S2 9 (33) 3 which has an intermediate bearing but has been selected for the commercial potential of its fruits as an ornamental plant or for consumption (E); comparison between a BC2S2 fruit and the cultivated parental fruit MEL1 (F).

On the other hand, *in vitro* anther cultures were carried out in some BC2S2 plants that presented an ideal phenotype to fix them with doubled haploid technology. Of all the individuals who tested the plants BC2S2 9(50)-1 (Figure 20A and 20B) and BC2S2 9(33)-3 were the ones that best responded to the protocol. In the case of the BC2S2 9(33)-3 plant, calluses were obtained that are currently forming shoots that will soon be acclimatized (Figure 20C). On the other hand, a tetraploid plant was obtained in the case of the BC2S2 9(50)-1 plant (Figure 20D and 20E).
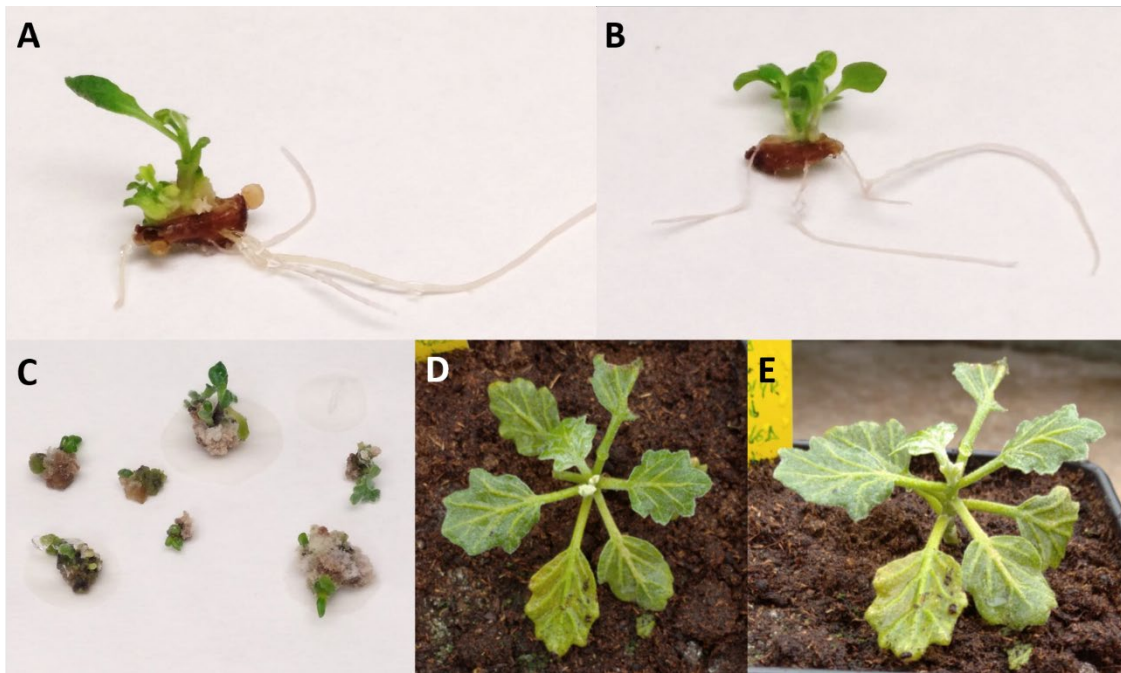
Figure 20: Response to the anther culture of the individual BC2S2 9 (50) 1 (A and B); response to anther culture of individual BC2S2 9 (33) 3 (C); acclimatized tetraploid plant obtained from anther culture of individual BC2S2 9 (50) 1 (D and E).

At the moment of writing this document, there are 15 BC3S3 [5 from each BC2S3 9(33)3, 9(50)1 and 9(63)3 family] plants that are in the greenhouse to continue the self-fertilization process to obtain the BC2S4 generation in parallel to *in vitro* anther culture although, at this time, segregation has hardly been observed between the materials of this BC2S3 generation (Figure 21).

Figure 21: Plants of different families of the BC2S3 of the Micro-Mel. Family BC2S3 9(33)3, has a somewhat more erect bearing than the rest and the fruits are somewhat larger and without multiple fruit set (A); family BC2S3 9(50)1, has an intermediate fruit size and size, presents multiple fruit set (B); family BC2S3 9(63)3, has the least erect bearing, the smallest fruits and the most pronounced multiple fruit set (C).

4.2.2 Identification of genomic regions for three traits of interest: presence of prickles, presence of chlorophyll in the fruit and presence of anthocyanins in the fruit.

In the BC3 plants from *S. insanum* introgression line used for the development of the BC4 and BC3S1 generations, it was possible to classify plants into two clear phenotypes for prickliness, with (Figure 22A) or without prickles (Figure 22B). This character was also observed in BC3 plants from the introgression line with *S. dasyphyllum* (Figure 22C) and in BC3 plants from the introgression line with *S. elaeagnifolium,* where in addition to seeing prickles on the leaves (Figure 22D), numerous prickles were also observed on the stem (Figure 22E) and in the calyx (Figure 22F). Using the genotyping data, it has been possible to delimitate the region containing the gene responsible for prickliness to a 3 Mb genomic section in linkage group 6 (Figure 23).
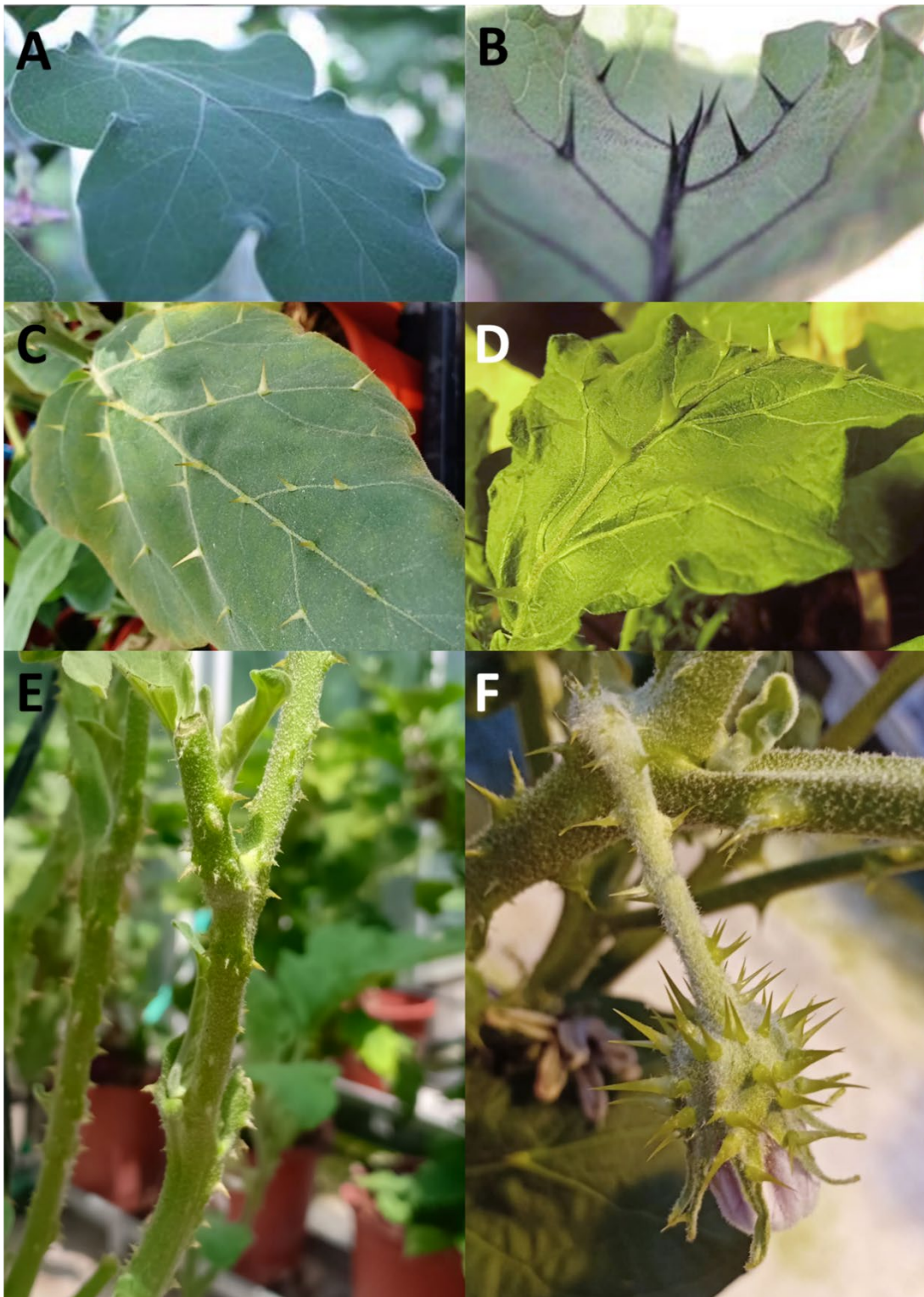
Figure 22: Leaf of a non-prickly BC3 plant from the ILs with *S. insanum* (A); leaf of a prickly BC3 plant from the ILs with *S. insanum* (B); leaf of a prickly BC3 plant from the ILs with *S. dasyphyllum* (C); leaf of a prickly BC3 plant from the ILs with *S. elaeagnifolium* (D); stem of a prickly BC3 plant from the ILs with *S. elaeagnifolium* (E); calyx of a prickly BC3 plant from the ILs with *S. elaeagnifolium* (F).
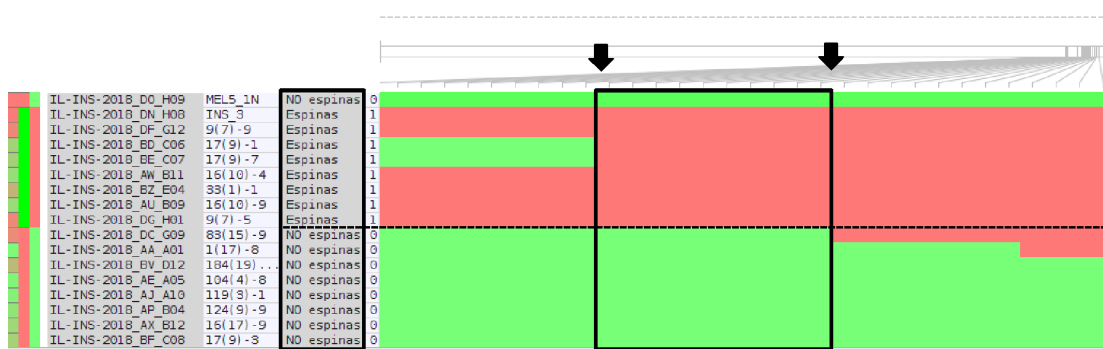
Figure 23: Genetic mapping of the prickliness trait to a region between 104 and 107 Mb regions in linkage group 6 of the eggplant genome. Green areas represent the *S. melongena* (non-prickly) alleles, while red areas are heterozygotes containing the *S. insanum* allele in addition to the *S. melongena* one. Plants marked as "Espinas" are prickly, while those marked with "NO espinas" are non-prickly.

Another of the characters of interest in which segregation was observed both in the introgression line of *S. dasyphyllum* and in *S. insanum* was the color of the fruit. In this case, we are talking about the presence of chlorophyll, anthocyanins or both pigments at the same time in the fruit.

In the case of the BC3 and BC4 fruits from the line of introgression with *S. dasyphyllum*, two very different phenotypes were observed. On the one hand, completely green fruits were seen (Figure 24A), and on the other, completely white fruits (Figure 24B). In no case was intermediate characters observed.

On the other hand, regarding the segregation observed in the fruits of the BC4 plants of the line of introgression with *S. insanum*, the following was observed. Contrary to the previous case, intermediate phenotypes were observed here, this gradation was from white fruits (Figure 24C), pale pink fruits (Figure 24D), deep pink fruits (Figure 24E) until reaching pink fruits with green strips (Figure 24F). It should be noted that in no case were completely green fruits found, these strips always appeared on a pink background.

In these cases, the region responsible for color control in the fruit must still be well defined. After genotypic analysis of individuals from the BC4 generation of these two ILs, a candidate region appears for the control of green color (chlorophylls) in chromosome 8 and another for the control of pink color (anthocyanins) in chromosome 10.

Figure 24: Fruit with the presence of chlorophyll in a BC4 plant of the ILs with *S. dasyphyllum* (A); fruit without chlorophyll or anthocyanins in a BC4 plant from IL with *S. dasyphyllum* (B)*;* fruit without chlorophyll or anthocyanins in a BC4 plant from IL with *S. insanum* (C); fruit with the presence of anthocyanins in a BC4 plant of the ILs with *S. insanum* (D); fruit with an intense presence of anthocyanins in a BC4 plant of the ILs with *S. insanum* (E); fruit with an intense presence of anthocyanins and chlorophyll in a BC4 plant of the ILs with *S. insanum* (F).

5. Discussion

In the present project, advanced introgression materials have been developed, which are already practically ILs. Because these materials have been developed from primary, secondary, and tertiary genepool species, this development has meant a huge widening of the genetic base of *S. melongena*. This means a great advance in the genetic improvement of this crop since the introduction of potential sources of resistance or tolerance to biotic and abiotic stresses has been performed from three wild species using an introgressiomic approach (Prohens et al., 2017). The choice of these three species for the development of ILs was based on their great potential for adaptation to the stress conditions derived from climate change. *Solanum insanum* is a weed that is distributed over a wide range of areas, growing in arid zones and even in infertile soils. This suggests that this species presents great potential for adaptation to drought as well as other environmental abiotic stresses (Ranil et al., 2017). *Solanum dasyphyllum* is an African species widely used in this culture as a medicinal herb and several studies have confirmed the phytochemical potential of this species (Bukenya and Carrasco 1994; Ajayi et al., 2013; Kidane et al., 2013). However, its potential is not only limited to medicinal applications, as a powerful acaricidal effect has also been described in extracts derived from its fruits, inhibiting oviposition in *Rhipicephalus appendicuhtus* (Van Puyvelde et al., 1985), as well as certain levels of resistance to the *Fusarium* spp. (Mwanik et al., 2015). Finally, *S. elaeagnifolium* is a weed native to North and South America (Knapp et al., 2017) and it is highly tolerant to drought and behaves like an invasive herb in arid and semi-arid areas around the world (Christodoulakis et al., 2009). In addition, this species has an interesting profile in phenolic compounds (Kaushik et al., 2015, Garcia-Fortea et al., 2019), thus giving it great potential for the nutritional improvement of the eggplant. All these reasons make these three species the ideal candidates to develop ILs in eggplant to adapt this crop to the biotic and abiotic stresses derived from climate change. In addition, there is an evolutionary and taxonomic interest of working with three representative species of each of the related genepools of the eggplant.

In this work, two different genotyping approaches have been used, the Sequenom technology and the SPET technology. The latter has allowed having a high-resolution in genotyping due to the very high number of SNPs markers evaluated (up to 10,000 positions in the case of *S. elaeagnifolium* program) compared to the 70 positions evaluated with Sequenom technology. This has made it possible to accelerate the introgression process, allowing a much more efficient selection of the lines each year. This is the main reason why it has been possible to achieve a degree of heterozygosity for the non-target part of the genome in generations BC5 in the case of *S. insanum* and *S. dasyphyllum*, and in the BC4 generation of *S. elaeagnifolium*. For this reason, it has been possible to obtain these highly advanced lines, with a high percentage of the *S. melongena* genetic background recovered and a complete and ordered representation in different genome fragments of the respective wild species used. Currently, the steps to follow to finalize the ILs consist of self-fertilization in the BC5 and BC4S1 generations of the ILs with *S. insanum* and *S. dasyphyllum* to fix the fragments. In parallel, anther culture is also being carried out to obtain doubled haploid plants and to try to accelerate the process, since through the self-fertilization process, at least two cycles would still have to be applied. Regarding the introgression line with *S. elaeagnifolium,* because it is a generation behind, it is still necessary to carry out a cycle of backcrosses that is running during this summer of 2020. After this cycle, the lines will most likely be ready to apply the self-fertilization cycles necessary to fix the fragments.

A dwarf phenotype has been detected in the introgression materials with *S. anguivi*. This material has enormous potential as an experimental model for eggplant, as is the case in tomato with the Micro-Tom model (Scott and Harbaugh 1989). The compact structure, the multiple fruit set and its determined growth make this kind of plants an ideal tool to carry out experiments in the laboratory such as transformation and molecular genetics (Dan et al., 2006; Chetty et al., 2013), drought (Barsanti et al., 2019) and salinity (Yin et al., 2010) assays as well as to accelerate the development of experimental populations (Sestari et al., 2014) thanks to the possibility of making several cycles per year in culture chambers. Currently, we have some families in BC2S3 generation in which there is hardly any segregation for growth habit and fruit characters, so it is very close to already having a stable Micro-Mel model. It is likely that in the BC2S4 generation we already have highly fixed materials that we can use as an experimental model. On the other hand, the character presence of prickles has been seen to be determined by a very specific region located in chromosome 6. This character has been seen to segregate in a Mendelian way, and the existence of QTLs associated with it on chromosome 6 as had already been reported in eggplant (Frary et al., 2014; Gramazio et al., 2014; Portis et al., 2015), although the region had not been delimited as much as in our study. A recent study by Miyatake et al. (2020) confirmed that a major gene is present in this region.

In the case of the color of the fruit, in the line of introgression of *S. dasyphyllum* two phenotypes could be clearly differentiated, green (presence of chlorophyll) or white (absence of pigments), being the white character the one that was present in most of the cases. This could be due to the fact that this was the original color of the recurrent parent (MEL1) and that the green color, original in the donor parent (DAS1), has been lost throughout the backcrossing cycles, remaining only in those lines with the fragment that regulates the synthesis of chlorophylls in the fruit. In fact, the green color is dominant because the white color in the fruit means the absence of pigments (Nothmann et al., 1975; Daunay et al., 2004). In other studies of the group in which a MAGIC population is being developed, this character has also been observed by mapping itself in a specific region of chromosome 8 in a position located around 104 MB, results that are coincident with the latest genotyping carried out in the lines of *S. insanum* and *S. dasyphyllum*. Something like the phenotype of green fruits was observed in the case of the pink color in the line of introgression of *S. insanum*. In this case, the pink color is due to the accumulation of anthocyanins (Tigchelaar et al., 1967) and it seems that it is encoded by a region situated on chromosome 10, something that had already been reported by other authors (Barchi et al., 2012; Cericola et al., 2014; Barchi et al., 2019), although we have not been able to delimit the region as well as in the case of the prickles or the green color. When both characters (chlorophylls and anthocyanins) are expressed at the same time in the fruits of this line of introgression, a very characteristic green striatum appears, and when neither of them is expressed, we again find white fruits as in the case of the fruits of the ILs with *S. dasyphyllum*. The control of the color of the fruit is a very interesting characteristic in the case of the eggplant and we hope to be able to better map the genes involved in it thanks to the information that these two ILs are providing us together with the MAGIC population.

6. Conclusion

In this chapter, the most relevant results from the last years of an eggplant pre-breeding project have been presented. Within the framework of this doctoral thesis, a large part of the objectives of this project were developed, being the most important the development of highly advanced backcross materials for developing three ILs in eggplant with the wild species *S. insanum*, *S. dasyphyllum* and *S. elaeagnifolium* and the development of the Micro-Mel experimental model from the species *S. anguivi*. These elite materials are ready to be used by breeders directly since the genetic background is fundamentally eggplant and will allow the analysis of many characters of interest beyond those described in this chapter. Due to the characteristics of all donor parents chosen for the development of ILS, this collection is a reservoir of genes with a high impact on adaptation to climate change. Some of these plants are already being evaluated by different companies, so we hope that they will soon serve to develop a new generation of eggplants varieties adapted to climate change.

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Bloque 2

Desarrollo de Herramientas Biotecnológicas

Capítulo III

A highly efficient organogenesis protocol based on zeatin riboside for *in vitro* regeneration of eggplant

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1. Abstract

Background: The available protocols for organogenesis induction in eggplant (*Solanum melongena* L.) generally present low efficiency, a strong dependence on the genotype, or both. In this work, we aimed at developing a universal protocol for efficient *in vitro* regeneration of eggplant mainly based on the use of zeatin riboside (ZR). We evaluated the use effect of seven combinations of ZR with indoleacetic acid (IAA) for organogenic regeneration in five genetically diverse *S. melongena* and one *S. insanum* L. accessions using two photoperiod conditions. In addition, the effect of six different concentrations of indolebutyric acid (IBA) in order to promote the rooting was assessed. The ploidy level of regenerated plants was studied.

Results: In a first experiment with accessions MEL1 and MEL3, significant differences were observed for the four factors evaluated for organogenesis from cotyledon, hypocotyl and leaf explants, with the best results obtained (9 and 11 shoots for MEL1 and MEL3 respectively) using cotyledon tissue, 16 h light / 8 h dark photoperiod conditions, and medium E6 (E6; 2 mg/L of ZR and 0 mg/L of IAA). The best combination of conditions was tested in the other four accessions and confirmed its high regeneration efficiency per explant when using both cotyledon and hypocotyl tissues. The best rooting media was R2 (1 mg/L IBA). The analysis of ploidy level revealed that between 25% and 50% of the regenerated plantlets were tetraploid.

Conclusions: An efficient protocol for organogenesis of both cultivated and wild accessions of eggplant, based on the use of ZR, is proposed. The universal protocol developed may be useful for fostering *in vitro* culture applications in eggplant requiring regeneration of plants and, in addition, allows developing tetraploid plants without the need of antimitotic chemicals.

Keywords: Zeatin riboside, Regeneration, Somatic organogenesis, *Solanum melongena*, Tetraploids*.*

2. Background

The development of new genome editing technologies in plant breeding has generated a growing interest in *in vitro* culture for regeneration protocols [1, 2]. This is partially due to the difficulties often encountered in plant regeneration, which is a key step in any transformation protocol and a major bottleneck for applying these techniques in many species [1, 3]. On the other hand, other breeding techniques, like the development of polyploids without antimitotic products such as colchicine [4–7], can benefit from regeneration protocols able to induce a certain percentage of plants with changes in ploidy levels. Tetraploids can also be the starting point for obtaining triploids which, in addition to the lack of seeds, can exhibit superior quality parameters in the fruit.

Common eggplant (*Solanum melongena* L.), also known as brinjal eggplant, is one of the most important vegetables, globally ranking fifth in total production among vegetable crops [8]. *In vitro* culture has been of great relevance for the genetic improvement of this crop, including the development of doubled haploids to obtain pure lines [9], or the development of the first commercial transgenic *Bt* eggplant [10]. However, as it occurs in other crops such as onion [11–13] or gerbera [14], available protocols to regenerate eggplants are mostly inefficient or highly dependent on the genotype [15–17]. Thus, more efficient and reproducible protocols suitable to a wide range of genotypes are needed to circumvent the current drawbacks for *in vitro* regeneration in eggplant, mainly those related to the strong effect that the genotype has on regeneration efficiency.

Zeatin riboside (ZR; $C_{15}H_{21}N_5O_5$), is a cytokinin of interest for several plant science applications. Since its discovery by Letham [18] in immature corn kernels, it has revealed as a useful plant growth regulator in a wide range of crops, particularly since the 1990s. One of the earliest applications of ZR for *in vitro* culture was for protoplasts regeneration in several species, such as *Brassica nigra* (L.) W.D.J. Koch [19], *Vigna sublobata* Roxb. [20], or *Solanum lycopersicum* L. [21]. ZR was also used for regeneration from leaf explants, such as in potato (*Solanum tuberosum* L.), where ZR was the cytokinin that resulted in the greater number of shoots per explant [22]. In addition, ZR was used for somatic embryogenesis induction from cotyledon protoplasts, such as in tomato [23], and for shoots induction in axillary buds of bracts in plants from genera *Aloe*, *Gasteria*, and *Haworthia* [24]. More recently, ZR has been used to circumvent the generally low percentage of seed germination in the African baobab (*Adansonia digitate* L.), where the efficient micropropagation from axillary buds in a medium supplemented with ZR has allowed the efficient propagation of plants of this species [25]. In the olive tree (*Olea europaea* L.), ZR has been successfully used for micropropagation from nodal segments, replacing the methodology of hardwood cutting, which is a very time-consuming technique [26]. These reports reveal that ZR has proved to be a very effective plant growth regulator on different *in vitro* culture applications in several species.

Two studies suggested that ZR may improve the organogenic regeneration efficiency in eggplant. Singh [27] proposed a transformation method of plastids in eggplant by using ZR in the culture medium. Muktadir [28] evaluated the capacity of organogenesis induction in five varieties of

eggplant by comparing the effect of different growth regulators, obtaining the best results using an MS medium supplemented with ZR and IAA. More recently, ZR was also used in the production of eggplant doubled haploids from anthers using a modified medium proposed by Rotino [29] by adding 1 mg/L of ZR and 3 mg/L of naphthaleneacetic acid (NAA) [30].

Here, we evaluate the effects of ZR at different concentrations and in combination with IAA on different plant tissues (cotyledon, hypocotyl and leaf) of genetically and phenotypically diverse eggplant accessions under different light conditions (photoperiod and dark). In order to obtain highly reliable estimates of the traits and parameters evaluated, a large number of replicas was used for each combination of conditions. Our study is aimed at providing relevant information on the ZR effects in *in vitro* regeneration in eggplant. As a result, we developed an efficient regeneration protocol that is not only useful for genetically and phenotypically distinct eggplant accessions, but also for a related wild species.

3. Results

3.1 Explant type, induction conditions, accession, and culture media effects in eggplant regeneration

The formation of non-organogenic friable calli was an event that, although observed in all the proven culture media, did not occur in abundance. Nevertheless, meristematic nodes were observed on the surface of the compact organogenic calli of cotyledon tissues (Figure 1A) and at one edge of the hypocotyl tissues (Figure 1B). These structures had an organized appearance, green colour and in most cases presented trichomes on the surface (Figure 1C). On the other hand, non-organogenic friable calli were observed both in cotyledons and at the edge of some hypocotyl explants (Figure 1D). These structures consisted of transparent and disorganized cells that disintegrated easily when touched with tweezers (Figure 1E). A relevant morphological event was the shoots formation after a month in dark culture conditions (Figure 1F). These had an elongated growth habit and presented a pale coloration that turned green shortly after being transferred into the light.

In the experiment 1, significant differences ($P<0.05$) were observed for the main effects of tissue and culture medium in the regeneration of shoots, calli and roots, and for accession in the induction of calli (Table 1). Overall, the tissue potentially more organogenic for shoot regeneration was the cotyledon with an average of 2.55 shoots/explant, followed by the hypocotyl with 1.66 shoots/explant, and the leaf with 0.70 shoots/explant. Although wide ranges of variation were observed for shoot formation, their median and mode values ranged between 0 and 2 and between 0 and 1, respectively, indicating that most explants produced a limited number of shoots. Hypocotyl explants produced more calli, on average almost 1.5-fold than cotyledons or leaves, while for root regeneration cotyledon was the one with the highest average number of adventitious roots generated (0.51 per explant) followed by hypocotyl (0.31) and leaf (0.24). Regarding the accession effect, the only differences observed in regeneration were for the formation of calli, which were higher in MEL1 than in MEL3 (Table 1). The incubation in light conditions after a month favoured the appearance of shoots, with an average of 1.67 shoots/explant, while incubation in the dark gave lower values, of 1.07 explants/shoot. Light had no significant effects on the development of other types of organs. Many significant differences were observed among culture media for shoots regeneration, roots and calli production (Table

1). The media with higher average values for shoot formation were E4 and E5, with over 2.2 shoots/explant, followed by E6 and E3 (2.04 and 1.54 shoots/explant). On the other hand, media E1, E7 and the control E0 had the lowest regeneration rate for shoots, with averages between three and four-fold lower than the best media. All the media tested displayed a formation of calli higher than the control medium (E0) while those without ZR and containing IAA (media E1 and E7) promoted the formation of friable non-organogenic calli (Figure 3D and 3E). Finally, the E1 and E7 media resulted in the highest rate of root formation, much higher than the control medium E0, while the rest of media gave lower ratios than E0 (Table 1).
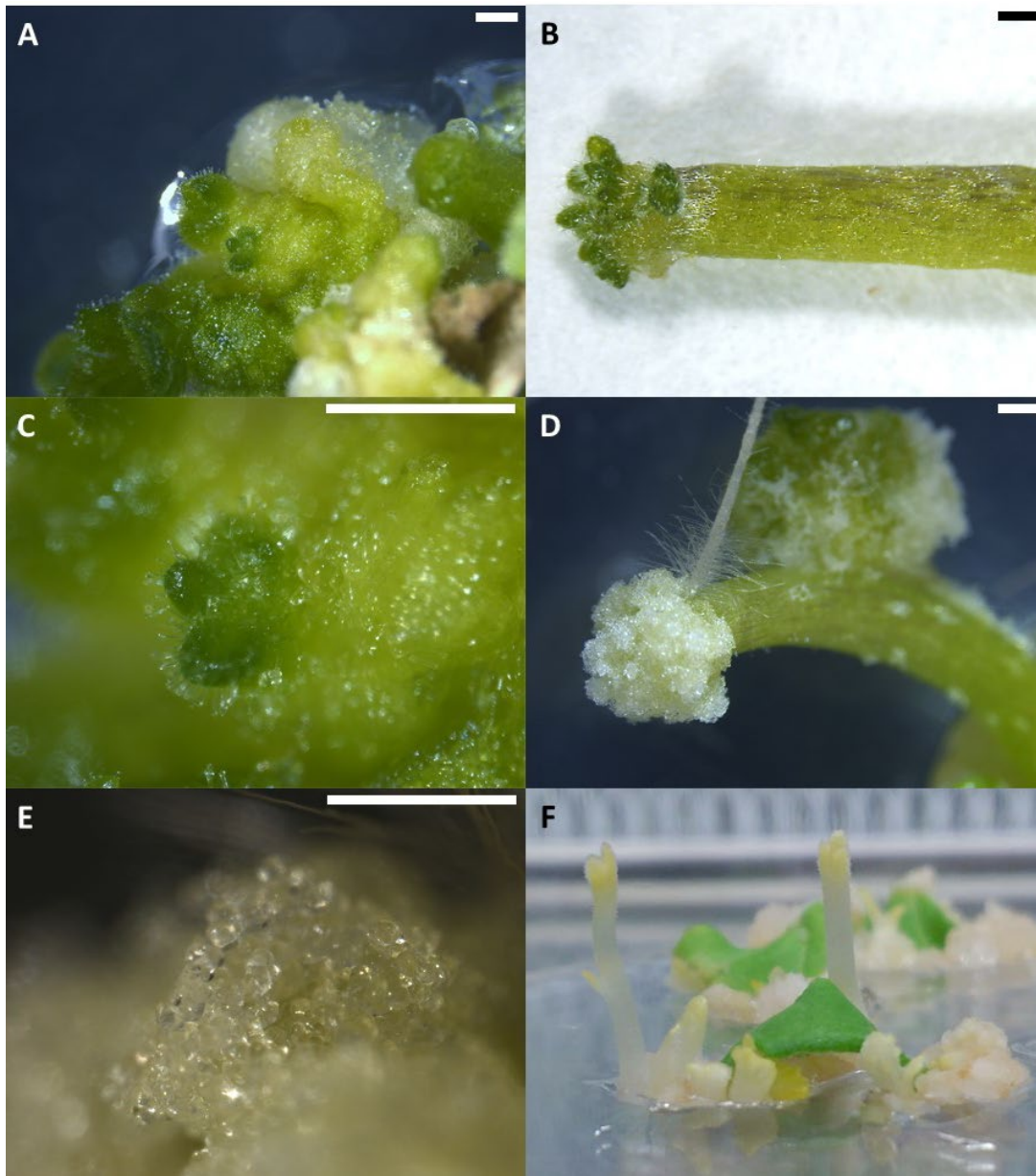
Figure 1: Initiation of bud formation in cotyledonary tissue of eggplant under 16 h light / 8 h dark photoperiod culture conditions (A); formation of shoots in hypocotyl tissue (B); organized structure and the formation of the first trichomes in cotyledonary tissue (C); callus formed in hypocotyl tissue under light culture conditions (D); which, at further magnification can be observed as a disorganized cell structure (E); appearance of the buds formed in cotyledonary tissue in 24 h dark photoperiod culture conditions, with elongated growth and absence of chlorophyll in the apex being observed (F). All the images were taken after a month of culture. The size of the bars is 1 mm.

Table 1: Mean, median, mode, and range for the organs produced during organogenesis in experiment 1 for each of the different levels for the four factors evaluated. Three experimental sessions with three replicates for each combination of factors and five explants per Petri dish were used.

Factors	Shoots				Calli				Roots			
	Mean[a]	Median	Mode	Range	Mean	Median	Mode	Range	Mean	Median	Mode	Range
Tissue												
Cotyledon	2.55 c	0	0	0-26	1.13 a	1	0	0-4	0.51 c	0	0	0-4
Hypocotyl	1.66 b	1	1	0-14	1.52 b	2	2	0-2	0.31 b	0	0	0-2
Leaf	0.70 a	0	0	0-13	1.09 a	1	0	0-4	0.24 a	0	0	0-4
Accession												
MEL1	1.35 a	0	0	0-20	1.16 a	1	2	0-4	0.30 a	0	0	0-4
MEL3	1.41 a	0	0	0-26	1.41 b	2	2	0-4	0.32 a	0	0	0-4
Induction condition												
Light/Dark (12/8 h)	1.67 b	1	0	0-26	1.28 a	1	2	0-4	0.31 a	0	0	0-4
Dark (24 h)	1.07 a	0	0	0-15	1.29 a	1	2	0-4	0.30 a	0	0	0-4
Medium												
E0	0.45 a	0	0	0-3	0.44 a	0	0	0-2	0.24 c	0	0	0-2
E1	0.39 a	0	0	0-5	1.18 c	1	2	0-3	0.80 d	1	0	0-4
E2	1.10 b	0	0	0-13	1.62 e	2	2	0-4	0.14 a	0	0	0-2
E3	1.54 c	1	0	0-14	1.47 e	2	2	0-4	0.23 b	0	0	0-4
E4	2.21 d	1	0	0-22	1.54 e	2	2	0-4	0.15 a	0	0	0-4
E5	2.54 d	2	0	0-21	1.57 e	2	2	0-4	0.09 a	0	0	0-2
E6	2.04 c	1	0	0-26	1.32 d	1	2	0-4	0.12 a	0	0	0-4
E7	0.56 a	0	0	0-5	0.85 b	1	0	0-3	0.75 d	1	0	0-3

[a]For each factor, means separated by different letters are significantly different at $p<0.05$ according to the non-parametric pairwise Wilcoxon test.

As the cotyledon explants and light induction conditions were the two levels of the main factors tissue and induction conditions giving the best average performance, the data subsets corresponding to the combination of these two treatments were studied for the best media (E3 to E6) for the two accessions (MEL1 and MEL3) (Table 2). The analysis of hypocotyl explants data was repeated following the same approach (Table 3). By using these combinations, we observed a clear positive interaction for the number of shoots, with means higher than those expected for the sum of the main effects. In the case of cotyledon explants, the range for shoots formation remained similar, however, the median and the mode increased their values, indicating that in general this combination of factors increased the number of shoots obtained. For hypocotyls, the changes were not as dramatic as in the case of cotyledons. The mean for shoots formation increased slightly while the range decreased considerably, the mode and the median were stable with a value around 2. This shows that hypocotyls under these conditions produce a limited number of shoots, significantly lower than those of cotyledons. However, for both cotyledon and hypocotyl, the values of mean, median, mode and range for the formation of non-organogenic calli and of roots were very low. The mode in the case of hypocotyls had a value of two, showing that callus formation in this type of tissue is more frequent than in cotyledon.

No significant differences for cotyledon (Table 2) or for hypocotyl (Table 3) explants were observed among the four culture media when evaluating all the effects together under these selected conditions. For both genotypes no significant differences were observed among media for cotyledon and hypocotyl, although the average of the E6 medium for the formation of shoots was higher for cotyledon explants in both genotypes. Given that medium E6 presented a higher average in shoots development in the case of cotyledon tissue and being the less expensive medium, it was chosen for the regeneration protocol.

Table 2: Mean, median, mode, and range for the organs produced during organogenesis in experiment 1 for explants grown from cotyledon incubated under 16 light / 8 h dark photoperiod conditions for the levels of media E3 to E6 in accessions MEL1 and MEL3. Three experimental sessions with three replicates for each combination of factors and five explants per Petri dish were used.

Medium	Shoots				Calli				Roots			
	Mean[a]	Median	Mode	Range	Mean[a]	Median	Mode	Range	Mean[a]	Median	Mode	Range
					MEL1							
E3	6.7 a	5	14	0-14	1.4 a	1	3	0-3	0.1 a	0	0	0-1
E4	8.3 a	6	3	0-20	2.1 a	2	1	0-4	0.4 a	0	0	0-1
E5	7.8 a	7	2	2-17	1.8 a	1.5	3	0-3	0.1 a	0	0	0-1
E6	9 a	9	9	2-19	1.4 a	1.5	0	0-3	0 a	0	0	0
					MEL3							
E3	7.6 a	4	4	0-17	1.7 a	1	1	1-3	0.1 a	0	0	0-1
E4	10.1 a	4	3	0-22	1.6 a	1	1	0-3	0.1 a	0	0	0-1
E5	7.8 a	5	4	0-21	2 a	2	3	1-3	0.3 a	0	0	0-1
E6	11 a	4	4	0-26	1.6 a	1	3	0-3	0.2 a	0	0	0-1

[a]For each accession (MEL1 or MEL3), medium means separated by different letters are significantly different at $p<0.05$ according to the non-parametric pairwise Wilcoxon test.

Table 3: Mean, median, mode, and range for the organs produced during organogenesis in experiment 1 for explants grown from hypocotyl incubated under 16 light / 8 h dark photoperiod conditions for the levels of media E3 to E6 in accessions MEL1 and MEL3. Three experimental sessions with three replicates for each combination of factors and five explants per Petri dish were used.

Medium	Shoots				Calli				Roots			
	Mean	Median	Mode	Range	Mean	Median	Mode	Range	Mean	Median	Mode	Range
					MEL1							
E3	2.3 a	2	2	0-8	1.8 a	2	2	1-2	0.1 a	0	0	0-2
E4	2.1 a	2	2	0-6	1.7 a	2	2	1-2	0.1 a	0	0	0-1
E5	2.9 a	2	1	0-9	1.6 a	2	2	1-2	0.2 a	0	0	0-1
E6	2.1 a	2	2	0-7	1.5 a	2	2	0-2	0.2 a	0	0	0-1
					MEL3							
E3	2.7 a	2	2	0-7	1.7 a	2	2	1-2	0.1 a	0	0	0-1
E4	2.5 a	2	2	0-7	1.8 a	2	2	1-2	0.0 a	0	0	0-1
E5	3.5 a	3	2	0-8	1.6 a	2	2	1-2	0.0 a	0	0	0-1
E6	2.5 a	2	2	0-6	1.7 a	2	2	0-2	0.1 a	0	0	0-1

[a]For each accession (MEL1 or MEL3), medium means separated by different letters are significantly different at $p<0.05$ according to the non-parametric pairwise Wilcoxon test.

3.2 Comparison of media for rooting of shoot explants

The rooting performance of MEL1 shoot explants for the six culture media was evaluated in the experiment 2. An increase of IBA concentration resulted in a greater formation of main roots (media R3, R4 and R5), a decrease in the number of secondary roots, as well as a greater thickness and a reduction in the length of the main root (Table 4; Figure 2). Medium E0 (without IBA) presented the lower average of root formation per shoots, while the highest, with 7.6 main roots/shoot, was obtained in medium R5 (4 g/L of IBA). On the other hand, the number of secondary roots reached its maximum value (3.8) in the medium R2 (1 g/L of IBA). For concentrations of 2 g/L of IBA (medium R3) and higher, the number of secondary roots decreased reaching its lowest value in medium R5 with 0.8 secondary roots per explant. The thickness of the main root varied between 1.1 to 2.6 mm, increasing with higher IBA concentrations in the medium, while the length varied between 1.1 to 2.5 cm decreasing when the concentration of IBA increased.
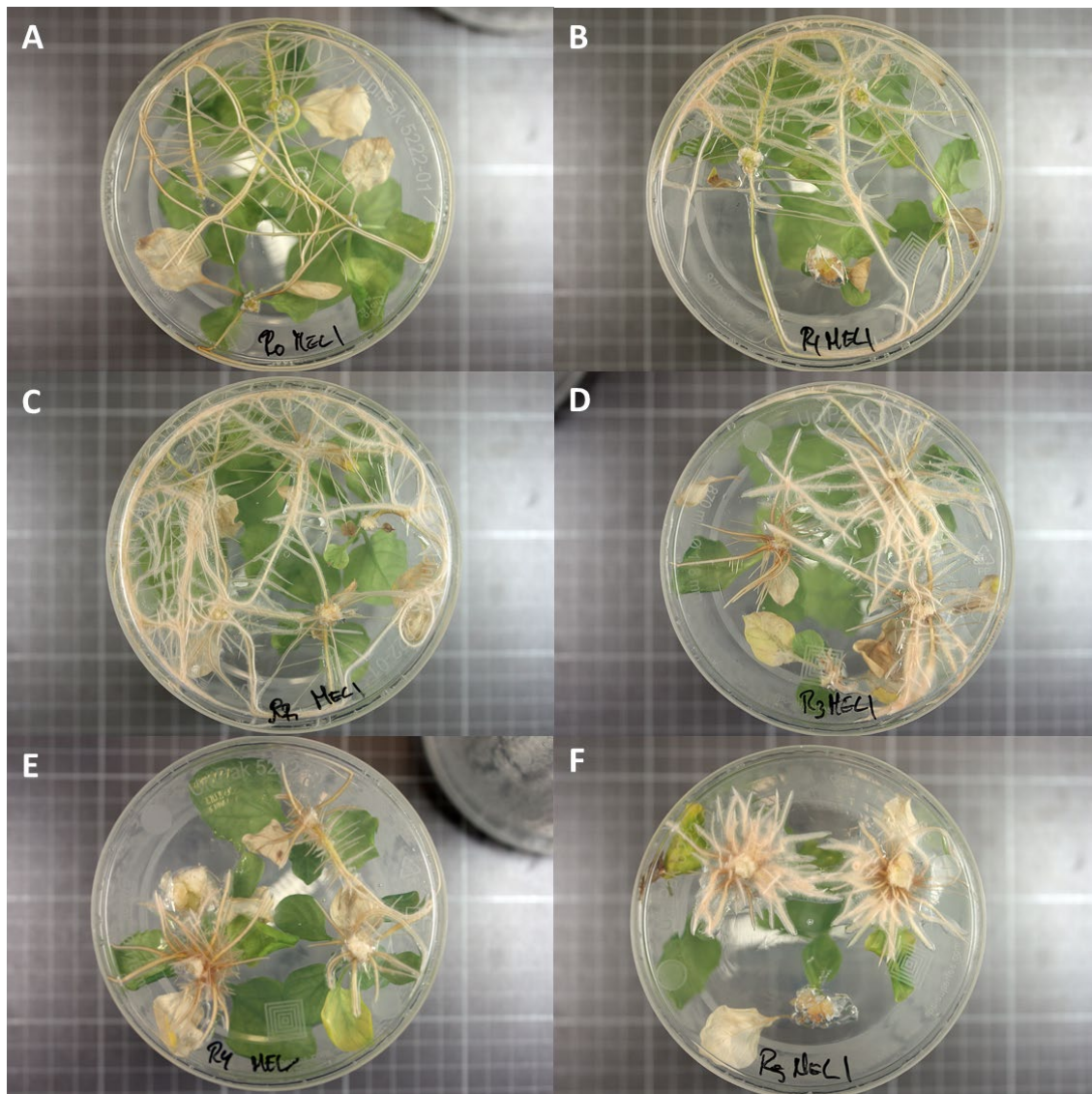
Figure 2: Response in the *in vitro* formation of roots in common eggplant accession MEL1 in response to different concentrations of IBA in media E0 (0 mg/L; A), R1 (0.5 mg/L; B), R2 (1 mg/L; C), R3 (2 mg/L; D), R4 (3 mg/L; E) and R5 (4 mg/L; F).

Table 4: Mean, median, mode, and range for the main roots, secondary roots and thickness and length of the main root per plant produced during rooting of shoot explants in experiment 2 in the accession of common eggplant MEL1. Three experimental sessions with three replicates and five explants per Petri dish for each medium were used.

Medium	Main Roots				Secondary Roots				Main Roots thickness[b]				Main Roots length[c]			
	Mean[a]	Median	Mode	Range	Mean[a]	Median	Mode	Range	Mean[a]	Median	Mode	Range	Mean[a]	Median	Mode	Range
E0	1.3 a	0	1	0-4	3.2 b	0	1	0-10	1.1 a	1	1	1-2	2.5 c	3	3	1-3
R1	3.2 b	3	3	0-7	3.7 c	0	3	0-10	1.2 a	1	1	1-2	2.6 c	3	3	2-3
R2	4.8 bc	2	4	0-10	3.8 c	0	3	0-10	1.4 ab	1	1	1-2	2.5 c	3	3	1-3
R3	5.3 c	10	5.5	0-10	1.6 ab	0	0	0-8	1.8 b	2	2	1-3	1.7 b	2	2	1-2
R4	5.9 c	0	7	0-10	1.4 ab	0	0	0-10	2.0 b	2	2	1-3	1.2 a	1	1	1-2
R5	7.6 c	0	8.5	0-18	0.8 a	0	0	0-10	2.6 c	3	0	1-3	1.1 a	1	1	1-2

[a]Measured in a scale (1= <0.8 mm; 2= 0.8 to 1 mm; 3= >1 mm).

[b]Measured in a scale (1= <3 cm; 2= 3 to 4.5 cm; 3= >4.5 cm).

3.3 Validating the regeneration protocol in different genotypes

After choosing the best conditions and medium, the protocol developed was tested in other genetically diverse materials of common eggplant [IVIA371, black beauty (BB), MM1597] and in INS1. As hypocotyls are also available when germinating the seeds for obtaining cotyledons, their regeneration capacity was also evaluated. The number of shoots regenerated was high for all genotypes, although significant differences among accessions were observed in the production of shoots per explant both for cotyledons and hypocotyls (Table 5). For cotyledons, IVIA371, BB, and INS1 displayed similar means (around seven shoots/explant), while MM1597 had the lowest average (3.1 shoots/explant). In the case of hypocotyls, IVIA371 displayed the highest number of shoots, with an average of 6.5 shoots/explant, while BB presented the lowest shoots number (1.8 shoots/explant).

IVIA371 and MM1597 formed completely compact organogenic calli (Figure 3A-3B and 3E-3F) while for BB and INS1 most of the calli were friable and non-organogenic (Figure 3C-3D and 3G-3H). This occurred for both cotyledon and hypocotyl tissues. For callus formation, INS1 (Figure 3G and 3H) gave the highest number of calli in hypocotyl, with values significantly higher than those of IVIA371 and MM1597 (Table 5). The formation of roots in this medium was very limited and, in most explants, no adventitious roots were formed (Table 5).
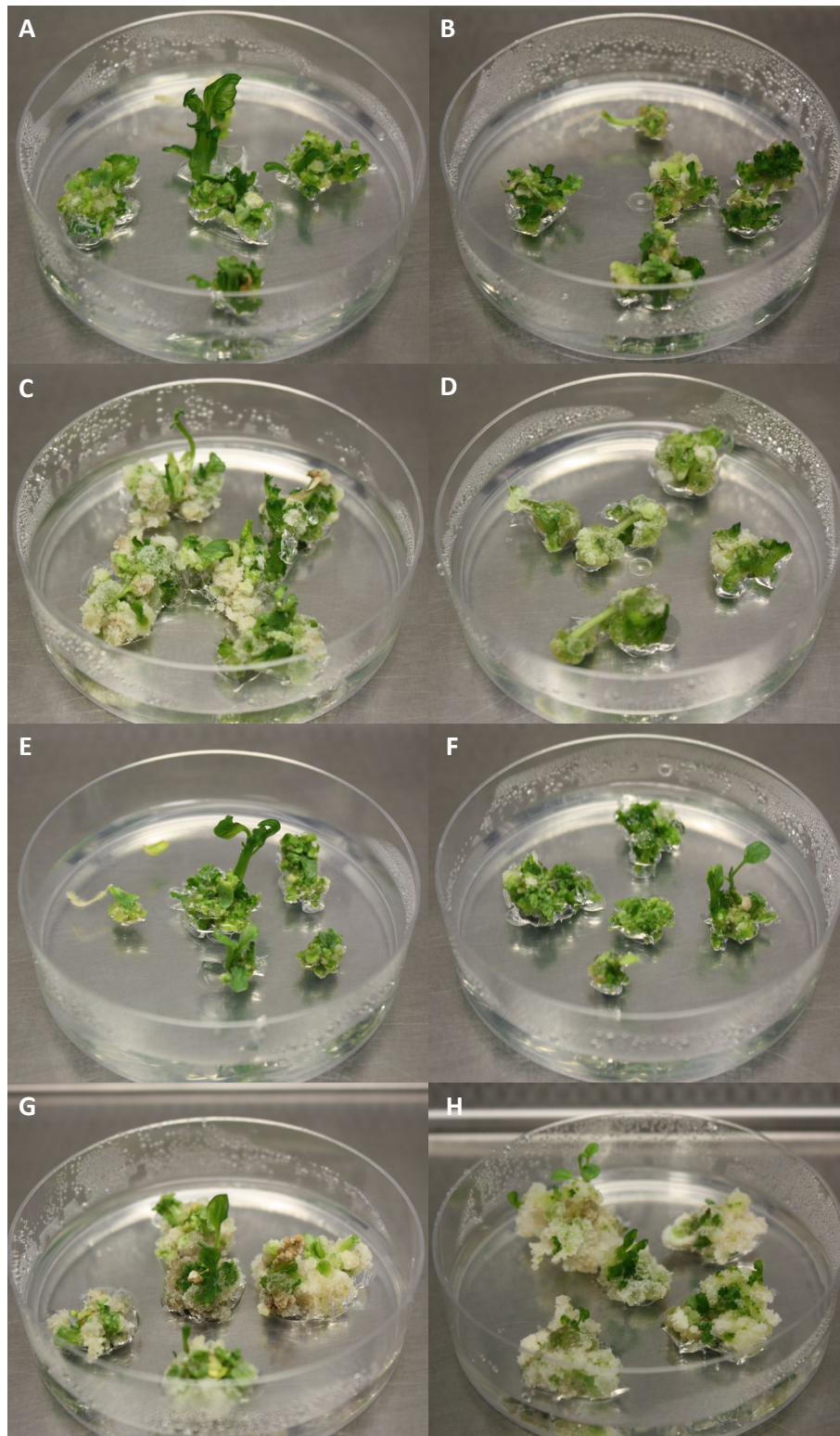
Figure 3: Organogenic response of the different eggplant accessions for the E6 medium cultivated under light conditions for cotyledons (left) and hypocotyls (right) for common eggplant accessions IVIA371 (A and B), BB (C and D), and MM1597 (E and F), and for INS1 (G and H). Differences were observed between the two tissues, being the cotyledon the tissue with the best average results in all the cases, except for MM1597. Small differences between the accessions were also observed, like the larger size and the non-friable aspect of INS1 callus (G and H).

Table 5: Mean, median, mode, and range for the organs produced during organogenesis in experiment 3 for explants grown from cotyledon and hypocotyl incubated under 16 light / 8 h dark photoperiod conditions and using medium E6 in accessions of common eggplant IVIA371, BB, and MM1597 and INS1. Three experimental sessions with three replicates for each combination of factors and five explants per Petri dish were used.

Accessions	Shoots				Calli				Roots			
	Mean	Median	Mode	range	Mean	Median	Mode	Range	Mean	Median	Mode	Range
	Cotyledon											
IVIA371	6.8 b	7	7	0-12	1.8 b	2	2	0-2	0.1 b	0	0	0-1
BB	6.7 b	6	3	2-13	1.9 b	2	2	1-2	0.1 b	0	0	0-1
MM1597	3.1 a	3	0	0-13	1.2 a	1	2	0-2	0.1 b	0	0	0-1
INS1	7.4 b	7.5	5	1-13	1.8 b	2	2	1-2	0 a	0	0	0-0
	Hypocotyl											
IVIA371	6.5 c	7	6	0-16	1.5 a	2	2	0-1	0.2 a	0	0	0-2
BB	1.8 a	1	0	0-6	1.6 ab	2	2	1-2	0.3 a	0	0	0-3
MM1597	4.4 b	4	6	0-11	1.5 a	2	2	0-2	0.6 a	0	0	0-2
INS1	5.8 bc	5.5	9	0-15	1.7 b	2	2	0-2	0.1 a	0	0	0-1

[a]For each accession (IVIA371, BB, MM1597 or INS1), medium means separated by different letters are significantly different at $p < 0.05$ according to the non-parametric pairwise Wilcoxon test.

3.4 Acclimatized plants with the selected protocol for regeneration and rooting

Table 6 summarizes the results of the number of explants with shoots, acclimated plants, and plants per initial explant obtained for the different accessions cultured *in vitro* using the selected protocol for *in vitro* regeneration of eggplant, which consists of using light conditions, the E6 medium for the induction of shoots, and the medium R2 for *in vitro* root induction (Figure 4). By using this universal protocol, all the accessions showed very high yields with percentages of explants with shoots close to or greater than 70% in all cases. The percentage of acclimatizated plants by initial explant ranged between 28.88% and 80.00% for cotyledon explants and between 20.00% and 46.76% for hypocotyl explants.

Seeds germination in medium E0

After two weeks of germination, take the hypocotyl and cotyledon explants and culture it in E6 medium under light conditions

First shoots, 30 days of cultivation

Dissect the shoots and cultivate them in R2 medium

2-3 weeks rooted shoots

During the following weeks, the vessel is drilled to accustom the plant to its new atmosphere. After one month the plant must be completely acclimated

Eggplant plant in process of acclimatization. A plastic cup is placed to avoid dehydration

Eggplant plant after a month and a half of rooting ready for the process of acclimatization

Figure 4: Proposed universal protocol for the regeneration of eggplant plants from cotyledon and hypocotyl. Starting from tissue of seeds sterilized and cultivated *in vitro* in medium E0, the combination of medium E6 and light conditions for both hypocotyl and cotyledon tissue is proposed. Subsequently, after approximately one month of cultivation, the shoots are placed in rooting medium R2 until their root system is ready for the transplant and the process of acclimatization.

3.5 Ploidy level analysis

A similar polysomatic pattern was observed in the different accesions when cotyledon, hypocotyl and leaf tissue explants were analized by flow cytometry (Figure 5). The cotyledon had between three and five times more cells than the leaf at G2 phase peak. In the case of hypocotyl, the number of cells of the G2 peak was between seven and nine times greater than the peak of the leaf sample. Both the cotyledon and hypocotyl tissues showed a peak at the value of 200, corresponding to tetraploid cells in division, while in the case of the leaf, no such peak was observed (Figure 5).

In addition, the ploidy level of regenerated plants was also evaluated. The results revealed that between 25% and 50% of the regenerated plants were tetraploids (Table 6). The percentage of polyploid plants was similar for both cotyledon and hypocotyl as well as among accessions, except for cotyledons of IVIA371 where 50% of the regenerated plants were tetraploid.
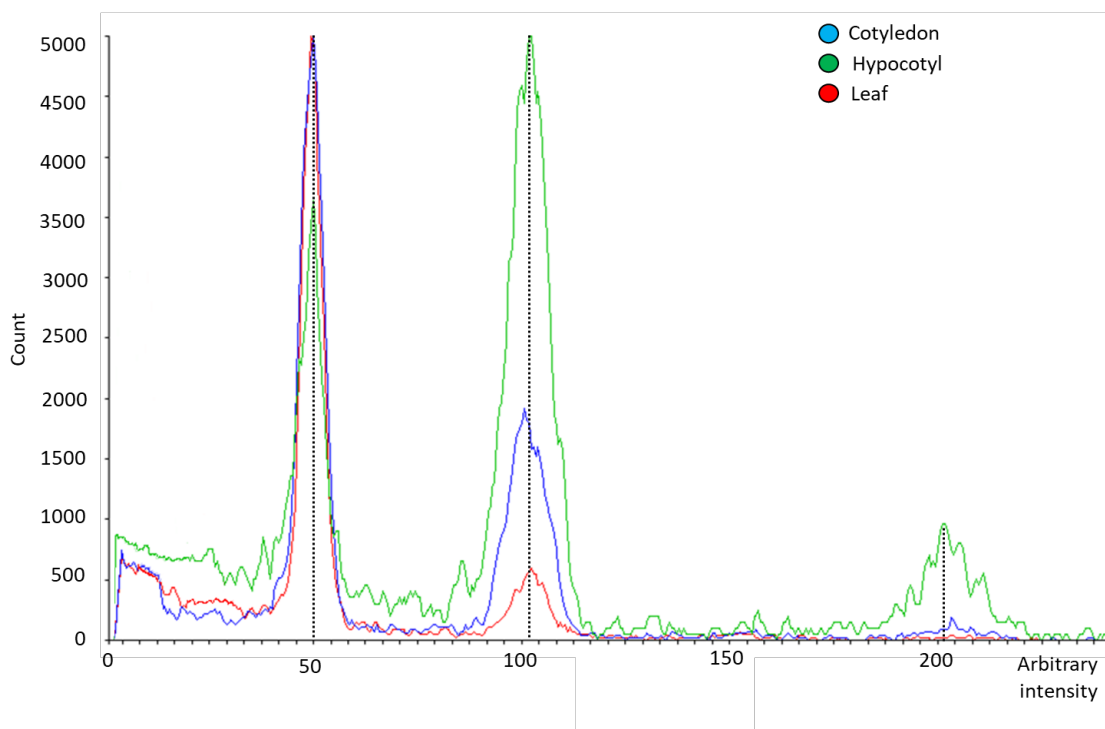
Figure 5: Flow cytometry histogram of the relative nuclear DNA contents of different tissues from common eggplant accessions IVIA371: cotyledon (blue), hypocotyl (green) and leaf (red). The x-axis represents the proportional fluorescence intensity level to the nuclear DNA quantity. The different polysomatic profiles of the different tissues analyzed can be observed. The peak located at the value 50 correspond to the diploid nuclei in phase G1, the peak located at the value 100 corresponds to the sum of the diploid nuclei in phase G2 and the tetraploid nuclei in phase G1, while the one at the value 200 represents tetraploid nuclei in G2 phase. The y-axis indicates the number of nuclei analyzed.

Table 6: Percentage (±SE) of explants with shoots and number of acclimatized plants from the experiment 3 for the accessions of common eggplant IVIA371, BB, and MM1597, and for *S. insanum* INS1 in two different tissues (cotyledon and hypocotyl) using 16 h light / 8 h dark photoperiod conditions and the E6 medium for organogenesis and the medium R2 for root formation induction. The percentage (±SE) of the diploid (2x) and tetraploid (4x) regenerated plants in experiment 4 are also reported. The number of initial explants used for each accession was n = 45.

Accessions	Explants with shoots (%)	Acclimatized plants	Acclimatized plants / initial explants (%)	2x regenerants (%)	4x regenerants (%)
Cotyledon					
IVIA371	96.66 ± 0.03	36	80.00 ± 0.06	50.00 ± 0.08	50.00 ± 0.08
BB	100 .00± 0.00	15	33.33 ± 0.07	61.30 ± 0.13	38.70 ± 0.13
MM1597	69.23 ± 0.07	13	28.88 ± 0.06	64.71 ± 0.13	35.29 ± 0.13
INS1	100 .00± 0.00	24	53.33 ± 0.07	75.00 ± 0.09	25.00 ± 0.09
Hypocotyl					
IVIA371	86.66 ± 0.06	13	28.88 ± 0.06	65.00 ± 0.13	35.00 ± 0.13
BB	70.00 ± 0.08	21	46.76 ± 0.07	66.70 ± 0.10	33.30 ± 0.10
MM1597	86.66 ± 0.06	9	20.00 ± 0.06	76.92 ± 0.14	23.08 ± 0.14
INS1	90.00 ± 0.05	17	37.77 ± 0.07	74.80 ± 0.11	25.20 ± 0.11

4. Discussion

The development of regeneration methods to obtain plants *in vitro* is essential for many applications in micropropagation and plant breeding. To our knowledge, up to now there are no universal methods for the regeneration of eggplant. An example of this is the plethora of works providing different conditions, growth regulators and media for eggplant regeneration, but none of them provides a universal and reproducible protocol in a diverse group of genetically diverse accessions. Some examples are the protocols based on the use of thidiazuron (TDZ) [31], NAA or in combination with benzylaminopurine (BAP) [32, 33], or BAP in combination with IAA [34]. Even explants irradiated with helium-neon laser were evaluated for organogenesis in eggplant [35]. However, all these protocols were highly genotype dependent.

ZR is a plant growth regulator that has been successfully used for many crops and plant species like *Brassica nigra* [19], *Solanum lycopersicum* [21] or *Olea europaea* [26], although it has been much less used than other cytokinins. In this study, we developed a new universal regeneration protocol based on the use of ZR that has proven to be highly efficient in eggplant, which is a genotype dependent recalcitrant species to *in vitro* culture [15, 16, 17]. Due to the good results showed by ZR in some crops including two similar reports on eggplant [27, 28], we aimed at developing a universal protocol for regeneration of eggplant based on the use of ZR. Thus, in our study, we tested genotypes representative of eggplant diversity [36], including an accession of the wild ancestor INS1 [37], using a large number of combinations of factors and replicates.

Cotyledon was the most organogenic tissue for all conditions, giving better results under 16 h light / 8 h dark than in the 24 h dark photoperiod conditions. Similar results were observed in another study in which the organogenic capacity of the cotyledon and hypocotyl tissues in the eggplant was evaluated [34]. This organogenic capacity of the cotyledons has also been reported in other crops such as melon [38], tomato [39], or peanut [40]. Regarding the composition for the culture media, no significant increases in the response were observed for concentrations over 2 mg/L of ZR. In other studies in eggplant, the concentrations determined to be optimal for ZR were 1 mg/L in the case of plastid transformation [27] and anthers [30], and 2 mg/L in the case of hypocotyl culture [28]. These results are in agreement with those obtained in our study. In other crops, the optimum concentration of ZR for the induction of organogenesis were variable, being 0.8 mg/L for potato [22], 1 mg/L for tomato protoplasts [21], 1.89 mg/L in the case of some succulent plants [24], and much higher in woody plants such as olive tree where the optimum concentration was set in 4.77 mg/L [26]. In general, concentrations higher than 2 mg/L resulted in a decrease of the organogenic response, as in hypocotyl protoplasts of *Vigna sublobata* [20] or tomato cotyledons [23], where ZR in combination with gibberellic acid (GA$_3$) and IAA did not show inductive effect on the tissues. Other works confirmed that ZR presented lower regeneration results when it is used in combination with auxins. For example, Rolli [25] reported a decrease in the percentage of regeneration when ZR was used in combination with IBA for African baobab. Similar effects were also observed for brassicas where 2 mg/L of ZR promoted the shoot formation from organogenic calli, but when ZR was combined with 0.2 mg/L of IAA, the shoot induction effect was suppressed, resulting in the formation of somatic embryogenesis [19]. For IAA, negative effects were observed at concentrations higher than 0.5 mg/L [23]. Our results are in agreement with these studies, since the best media found in our study (E6) lacked IAA.

When comparing the results of the E6 medium with the rest of the media we observed a higher average number of outbreaks per explant than the rest of the media. Medium E6 also showed higher percentages of organogenic regeneration compared to the medium proposed by Muktadir [28] where ZR (2mg/L) was used in combination with 0.1 mg/L of IAA. This is further evidence that IAA does not contribute, or even decrease the organogenesis induction in eggplant.

The rooting medium R2 (1 mg/L IBA) displayed the highest yield in the formation of secondary roots, providing a greater number of root nodes for functional roots *ex vitro*. In fact, although the roots formed *in vitro* are not generally functional *ex vitro* [41, 42], they may serve as scaffolds for the new functional secondary roots that can be formed from their nodes. We also observed that an increase in the concentration of IBA higher than 2 mg/L increased the thickness of the roots, reduced their length and promoted the formation of the main roots while reducing the formation of the secondary roots. This is not desirable for the subsequent acclimatization of the plants due to the low number of nodes formed by the absence of secondary root [41].

Despite the genetic divergence between MEL1 and MEL3 [36], we did not detect significant differences in the organogenic response between these two accessions. In the rest of accessions, including one of the wild ancestor of eggplant INS1 [37], the response of cotyledons was very similar, whereas, in the case of hypocotyls, small differences between genotypes were observed. These results indicate that the genotype effect on the regeneration efficiency in this protocol is low and it can applicable to a wide range of genotypes, including eggplant wild relatives. Although many shoots per explant were formed with our protocol, the number of acclimated plants per explant was much lower. This is because not all shoots were large enough to subculture them in the rooting media. This step might be easly improved by incorporating an elongation step prior to the rooting. For example, explants with shoots could be subcultured in an hormone-free E0 medium [17, 43], or in a medium with a concentration of 1.5 mg/L GA$_3$ [9]. In this sense it is worth mentioning that the cultivation of small shoots during one or two weeks in continuous darkness promotes their elongation without the weak etiolation harming the normal future development of the shoots in photoperiod conditions (data not shown).

All accessions had a very similar general polysomatic profile, in which cotyledons and hypocotyls showed higher rates of initial polyploid cells than those of the leaf. This is probably due to the fact that the first endoreplications occurred in the cotyledon and hypocotyl during germination, while the events of polysomatia in the rest of tissues occur at later stage [4, 5]. Although cotyledons had a percentage of polyploid cells lower than the hypocotyl, the proportion of polyploid plants regenerated from both tissues was similar. This may be due to the greater organogenic capacity of the cotyledons that we report in this work. A significant proportion of the regenerated plants were stable tetraploids, which indicated that it is a highly efficient method for the development of tetraploids without the need to use chemical antimitotic agents such as colchicine. Similar results have been observed in different studies such as in in vitro culture of tomato hypocotyls where 42.3% of the plants were tetraploid [44] or in culture of protocorm-like bodies in *Phalaenopsis* species, where an average polyploid production was obtained between 36.0%-74.9% depending on the species [45].

5. Conclusions

We developed a universal protocol for the eggplant regeneration. This universal protocol consists in the cultivation of cotyledons in a medium (E6) with ZR at 2 mg/L under light conditions for one month, and afterwards rooting them in medium R2, which contains IBA at 1 mg/L. Taking advantage of the polysomaty displayed by cotyledon and hypocotyl tissues, this protocol can also be used to obtain stable non-chimeric polyploid plants without the need to use antimitotic agents. This protocol, based on the use of ZR, and the rest of the information provided in this study can foster *in vitro* breeding, transformation and genetic editing of eggplant. Our approach, in which ZR proved of great value for developing a highly efficient regeneration protocol in an otherwise recalcitrant species, might be of interest in other related recalcitrant crops.

6. Methods

6.1 Plant material

Five *S. melongena* accessions, namely MEL1, MEL3, IVIA371, BB, and MM1597, and one of INS1, the wild ancestor of eggplant and the only wild species in the primary genepool of eggplant [37, 46], were used (Figure 6). These materials have been previously morphologically and genetically characterized, and display a wide variation for morphological and agronomic traits, as well as for genetic diversity [36, 47, 48]. MEL1 and MEL3, bearing white and green fruits respectively, are the recipient parents of backcross programmes for the development of introgression lines with wild relatives, and have been used in several breeding programmes [49–51].

Figure 6: Fruits of the common eggplant (*S. melongena*) accessions [MEL 1 (A); MEL3 (B); IVIA371(C); BB (D) MM1597 (E)] and of the wild ancestor *S. insanum* [INS1 (F)] used in the different experiments, revealing the high phenotypic diversity of the materials studied. The size of the grid cells is 1 × 1 cm.

6.2 Experimental layout and workflow

The experimental layout and workflow are presented in Figure 7. The first experiment was aimed at evaluating the effect of different factors in somatic organogenesis in eggplant. Cotyledon, hypocotyl and leaf tissue from MEL1 and MEL3 were tested in eight culture media with different concentrations of ZR and IAA combined with two induction conditions (light and dark). In parallel, for the second experiment, six culture media were evaluated to determine which of them stimulates more efficiently root induction. This step was carried out only with MEL1. After running the first two experiments and having established the best protocols among those tested for the eggplant regeneration, in the third experiment these were tested in the other four accessions (IVIA371, BB, MM1597 and INS1) in order to assess the genotype effect. Finally, in the fourth experiment, the ploidy of the plants regenerated from this protocol was evaluated. Due to the detection of polyploid plants, the polysomatic pattern of cotyledon, hypocotyl and leaf tissues was also checked by flow cytometry.

Experiment 1:
Somatic organogenesis

Accessions: MEL1, MEL3.
Experimental design: 3 experimental sessions with 3 replicas for each factor with 5 explants for each replica.
Objective: Find the best combination of factors to induce organogenesis in eggplant.

Experiment 2:
Root induction

Accessions: MEL1.
Experimental design: 3 experimental sessions with 3 replicas for each factor with 5 explants for each replica.
Objective: Find the best concentration of IBA to induce root formation in eggplant axillary segments.

Experiment 3:
Protocol validation in different accessions

Accessions: IVIA371, BB, MM1597, INS1.
Experimental design: 3 experimental sessions with 3 replicas for each factor with 5 explants for each replica.
Objective: Confirm the best results obtained in experiment 1 and 2 for different eggplant genotypes.

Experiment 4:
Determination of ploidy level

Accessions: IVIA371, BB, MM1597, INS1.
Objective: Check the ploidy level in the initial explants (cotyledon, hypocotyl and leaf). Check the ploidy level of the plants from the experiment 3.

Figure 7: Description of the accessions, the experimental design and the main objectives of the four experiments performed. The flow of the arrows indicates the order in which the experiments were conducted and their relationships.

6.3 Growth conditions of the starting material

For *in vitro* germination, seeds were previously surface-sterilized by immersion in 70% ethanol for 30 s, followed by 10 min in a solution of 7 g/L of NaClO with 0.1% (v/v) of Tween 20, and finally washed three times with sterilized distilled water. After sterilization, seeds were germinated in the darkness on solid medium E0 (Table 7). The medium E0 was distributed in Petri dishes of 9 cm diameter and also in plastic pots of 9.7 cm diameter and 11 cm height fitted with a membrane filter in the lid to allow gaseous exchange (Microbox containers O118/120+OD118/120 #10 (G), SAC02, Nevele, Belgium). The plastic pots were used for growing the plantlets to obtain leaf explants. To synchronize the explants types used in the experiment (cotyledon, hypocotyl and leaf), firstly 50 seeds of each of the MEL1 and MEL3 accessions were sown in plastic pots to obtain leaves. Two weeks later other 50 seeds of each of these two accessions were sown in Petri dishes and kept under dark culture conditions, to obtain the

cotyledons and hypocotyls. The plastic pots were placed in a climatic chamber, at a temperature of 25 °C with a photoperiod of 16 h light / 8 h darkness. At the same time, 50 additional MEL1 seeds were sown in plastic pots with E0 medium for the rooting protocol experiment (Table 7). They were kept in the same climatic chamber for four weeks under the same conditions.

6.4 Experiment 1: Somatic organogenesis

Explants were cultured on different organogenic induction media containing ½ MS basal salts [52], 1,5% (w/v) sucrose, 0,7% (w/v) gelrite, and supplemented with one of seven different hormone combinations (Table 7). From seedlings grown in Petri dishes in dark conditions, the proximal and distal parts of the cotyledons were excised while the hypocotyls were cut into fragments of about 1 cm long. The leaves of the plantlets grown in plastic pots were cut into fragments of one square centimetre. Fifteen explants for each of the three tissues (cotyledon, hypocotyl or leaf) were divided into three plates (five explants per Petri dish). This was triplicated for each of the organogenesis media conditions; thus, 846 Petri dishes and 4,320 explants (3 plant tissues × 2 accessions × 8 inductions media × 2 incubation (light/dark) conditions × 3 plates per plant tissue × 3 experimental replicas) were evaluated.

Half of the plates of each combination of medium and type of explant were kept in light conditions in the culture chamber at 25°C with 16/8h light/dark photoperiod. The other half were kept in dark conditions in the same culture chamber, by wrapping the plates with aluminium foil. After one month, the number of shoots, calli and roots of each explant were counted, and the plates that were in dark conditions were transferred to light conditions.

Table 7: Zeatin riboside (ZR) and indolacetic acid (IAA) concentrations of the different induction media for the *in vitro* germination of seeds (E0) and somatic organogenesis of eggplant explants (E0 to E7).

Growth regulator	Medium							
	E0	E1	E2	E3	E4	E5	E6	E7
ZR (mg/L)	0	0	1	2	3	4	2	0
IAA (mg/L)	0	2	0.5	0.1	0.05	0	0	0.1

6.5 Experiment 2: Root induction

For developing a rooting induction protocol of the explants regenerated with the different combinations of ZR and IAA, different IBA concentrations were used on a medium containing ½ MS basal salts, 1.5% (w/v) sucrose, 0.7% (w/v) gelrite (Table 8). Five explants with axillary buds from MEL1 were planted per plastic box. The plastic pots were taken to the culture chamber and placed at 25ºC and 16 h / 8 h of light/dark photoperiod. After one month, the number of primary and secondary roots was counted and the length and thickness (diameter) of the primary roots were evaluated. This was triplicated for each of the rooting media conditions; thus, 54 Petri dishes and 270 explants (1 accession × 6 inductions mediums × 3 plastic pots × 3 experimental replicas) were evaluated.

Table 8: Indolbutyric acid (IBA) concentrations of different rooting media evaluated in explants from internodal sections from MEL1 eggplant accession.

Growth regulator	Medium					
	E0	R1	R2	R3	R4	R5
IBA (mg/L)	0	0.5	1	2	3	4

6.6 Experiment 3: Protocol validation in different accessions

The common eggplant accessions IVIA371, BB, MM1597 and the INS1 accession were used for this third experiment. The same seed disinfection protocol mentioned above for experiment 1 was used, and 50 seeds of each of the four accessions were sown in Petri dishes of 9 cm diameter with medium E0. The plates were brought to the culture room under the same temperature conditions used in the former experiments (25ºC) in dark conditions for two weeks.

The proximal and distal parts of the cotyledons were excised, and the hypocotyls were cut into fragments of about one centimetre. For each accession, fifteen cotyledons and hypocotyls explants were distributed over three plates (five per plate per type of explant), with E6 medium, in three experimental sessions, totalling 1,440 explants evaluated in this experiment. The plates were maintained into the culture chamber under light conditions, at 25 °C and a photoperiod of 16 h/ 8 h light/dark. After one month, the number of shoots, roots and calli of each individual explant was counted.

6.7 Experiment 4: Determination of ploidy level

Cell nuclei from different tissues (cotyledon, hypocotyl and leaf) were isolated mechanically according to Dpooležel [53]) with modifications. Cotyledon, hypocotyl, and leaf sections of approximately 0.5 cm^2 were chopped with a razor blade in a 6 cm diameter glass Petri dish containing 0.5 ml lysis buffer LB01 (pH 7.5) supplemented with 15 mM Tris (hydroxymethyl) aminomethane, 2 mM Na$_2$EDTA and 0.5 mM spermine, and incubated for 5 min. Subsequently, the suspension containing nuclei and cell fragments was filtered using a 30 μm CellTrics filter (Sysmex, Sant Just Desvern, Spain). The nuclei in the filtrate were stained with CyStain UV Ploidy (Sysmex) and incubated for 5 min. The fluorescence intensity of the homogenate was measured using a CyFlow ploidy-analyzer (Partec, Münster, Germany), measuring at least 4,000 nuclei for each sample. Using young leaves of a diploid eggplant, the diploid control peak was established at 50 points of the arbitrary intensity value of the fluorescence in the histogram. By comparison with this peak, the ploidy of the other tissues evaluated was checked. The ploidy of cotyledons, hypocotyls and leaves was evaluated to verify their polysomatic pattern. For experiment 3, also young leaves from regenerated plants were evaluated.

6.8 Statistical analysis

Independence among variables (distribution-plot test), homoscedasticity (Bartlett's test), and normality (Shapiro-Wilk test) were evaluated for the experiments 1, 2 and 3. Given that for none of these experiments the three criteria were met, the Kruskal-Wallis non-parametric test followed by the pairwise Wilcoxon test at p<0.05 was used to evaluate statistical significance of differences. All these analyses were carried out using R software [54]. The mean, median and the mode of each factors were calculated to complete the information in the data sets. For the

data from the experiment 4, the relative percentage for the ploidy levels each genotype along with its standard error was calculated.

7. Statements

Abbreviations

BAP: Benzylaminopurine; GA_3: Gibberellic acid; IAA: Indoleacetic acid; IBA: Indolebutyric acid; NAA: Naphthalene acid; TDZ: Thidiazuron; ZR: Zeatin riboside

Authors' contributions

Conceived and designed the experiments: EG-F, BJP-C, MP, SV and JP. Performed the *in vitro* culture experiments: EG-F, ALl-R and AG-P. Performed the ploidy analyses: JPB-G and PG. Analyzed the data: EG-P, MP, SV and JP. Contributed reagents and materials: MP, SV, VM and JP. Wrote the manuscript: EG-F and BJP-C. Revised the manuscript: MP, PG, SV, VM and JP. All authors have read and approved the manuscript.

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Availability of data and material

The datasets used and/or analysed and plant materials used in the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Capítulo IV

Ploidy modification for plant breeding using *in vitro* organogenesis: a case in eggplant

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1. Abstract

The use of antimitotic agents such as colchicine has been common to obtain polyploid organisms. However, this approach entails certain problems, from its toxicity to the operators for being carcinogenic compounds to the instability of the individuals obtained, and the consequent reversion to its original ploidy because the individuals obtained in most cases are chimeric. *In vitro* culture allows taking advantage of the full potential offered by the cellular totipotence of plant organisms. Based on this, we present a new *in vitro* culture protocol to obtain polyploid organisms using zeatin riboside (ZR) and eggplant as a model organism. Flow cytometry is used to identify tetraploid regenerants. The regeneration of whole plants from the appropriate tissues using ZR allowed developing polyploid individuals in eggplant, a crop that tends to be recalcitrant to *in vitro* organogenesis. Thanks to the use of the polysomatic pattern of the explants, we have been able to develop a methodology that allows to obtaining stable non-chimeric polyploid individuals from organogenic processes.

Key words plant tissue culture, polysomatic pattern, polyploid, flow cytometry, zeatin riboside, *Solanum melongena*.

2. Introduction

Obtaining polyploids is a strategic objective for many seed and breeding companies. They also have great importance in other sectors, such as ornamental plants, since polyploids tend to have larger and more striking organs [1] and triploid individuals, which are sterile, have more durable flowers [2]. Another sector where polyploids could raise interest is biomedicine and pharmacology, since they may have higher levels of biosynthesis and accumulation of bioactive compounds [3]. The crossing between a tetraploid plant and a diploid allows obtaining triploid offspring, which may be completely or partially sterile. This type of organisms presents agronomic traits of great value, such as the complete or partial absence of seeds, adding a great value as seedless fruits are highly appreciated by the consumer.

One of the most widely used methods to develop polyploids is through the application of antimitotic agents, such as colchicine, to induce genome duplication in a variable proportion of cells from embryos, young plants or adult plant tissues. In addition to posing a risk to the operators, since most of antimitotic agents are carcinogenic, the use of these chemicals for polyploid development is largely inefficient [4]. Frequently, the results obtained are mixoploids or chimeric that frequently revert to the original diploid status [5].

In order to improve the efficiency of polyploid production, we have developed an eggplant protocol to obtain polyploids without using antimitotic agents [4]. For this, we rely on the polysomatic pattern presented by the different tissues of the plant, which can be detected by means of flow cytometry. In this way, in tissues such as hypocotyl or cotyledons there are different cell populations with naturally diverse ploidy levels [6, 7]. This is a mechanism used by plants during the earliest stages of their development to achieve faster and more efficient cell expansion in terms of energy cost, allowing a fast growth and elongation of the seedling in a very short period.

Therefore, if organogenic processes can be induced in these polysomatic tissues, with a high percentage of probability, it will be possible to obtain polyploid plants. Contrarily to polyploidy plants obtained by antimitotic drugs, these polyploidy are generally stable and non-chimeric and thus do not revert to the diploid state. For this, it will be necessary to cultivate hypocotyls or cotyledons, induce the formation of shoots in these tissues, acclimatize them and evaluate their ploidy level [4].

3. Materials

Prepare all solutions and culture media using ultrapure water (prepared by purifying deionized water, to attain a sensitivity of 18 MΩ-cm or lower at 25 °C), or sterile distilled water (autoclaved for 20 minutes at 121 °C) (*see* **Note1**). Prepare all reagents and culture media at room temperature and store them at 4 °C. In the case of hormone stocks freeze at -20 °C.

Solutions

Nuclei extraction buffer: Tris (15 mM), Na_2EDTA (2 mM), spermine (0.5 mM), KCl (80 mM) and NaCl (20 mM). Add approximately 175 mL distilled H_2O. Leave on the magnetic stirrer until all components dissolves completely. Adjust the pH of the mixture to 7.5 with 1M HCl. Now add the 2-mercaptoethanol (15 mM) and the Triton X-100 (0.1%) in the gas extraction hood. Leave it shaking in the hood for at least 30 minutes to fully homogenize the Triton. Once a homogeneous mixture has been obtained, make up to 200 mL with distilled H_2O and store in the refrigerator at 4 °C.

70% ethanol solution: Prepare a volume of 729 mL of 96% ethanol and bring it up to 1000 mL with sterile distilled water using a test tube (*see* **Note 2**).

20% bleach solution: Prepare a volume of 200 mL of commercial bleach (37g/L $HClO_3$) and bring up to 1000 mL with sterile distilled water using a test tube. Add two drops of Tween20 (*see* **Note 3**).

Zeatin Riboside (ZR) stock (1g/L): 20 mg of hormone are dissolved in 2 mL of 1 M NaOH (*see* **Note 8**). Once dissolved, sterile distilled water is added until a total volume of 20 mL is reached. In a laminar flow cabinet, the hormonal stock solution is filtered with a 0.22 µm filter using a plunger syringe and it is distributed in a 2 mL sterile Eppendorf tubes (see Note 9). Store the stock at -20 °C.

Indole Butyric Acid (IBA) stock (1g/L): 20 mg of hormone are dissolved in 2 mL of 1 M NaOH (*see* **Note 8**). Once dissolved, sterile distilled water is added until a total volume of 20 mL is reached. In a laminar flow cabinet, the hormonal stock solution is filtered with a 0.22 µm filter

using a plunger syringe and it is distributed in a 2 mL sterile Eppendorf tubes (see Note 9). Store the stock at -20 °C.

Culture media

E0 medium (germination): To prepare 1 L of culture medium, weigh using a precision balance 2.2 g of MS vitamin salts and 15 g of sucrose, place them in a beaker with 1 L of distilled water and use a magnetic stirrer to homogenize the mixture. Once this is done adjust the pH to a value of 5.8 (*see* **Note 4**) with the help of a pH-meter. Once the pH is adjusted, add 7 g of Gelrite^{TM} (*see* **Note5**) and mix everything well in an autoclavable bottle, close the cap (*see* **Note 6**) and autoclave it for 20 minutes at 121 °C. Once autoclaved, cool to a temperature between 40-50 °C and pour into petri dishes inside a laminar flow cabinet and let it dry (*see* **Note 7**).

E6 medium (organogenesis induction): To prepare this medium the same steps indicated in the previous section (1 L) are followed with the differences explained below. Remove the medium from the autoclave and once it has tempered, add 2 mL of ZR (1 g/L of ZR stock) in the laminar flow cabinet. The mixture is then shaken vigorously. After this the mixture is poured into petri dishes and left to solidify.

R2 medium (root induction): To prepare this medium (Table 2) the same steps indicated for medium E0 (or E2) are followed with the differences explained below. When he medium is removed from the autoclave and tempered, 1 mL of IBA [1 g/L of Indole Butyric Acid (IBA) stock] is added to it in the laminar flow cabinet and the solution is shaken vigorously. After this, the solution is poured into petri dishes and left to solidify.

Plant Material

Seeds of good quality (high germination and pathogens free) need to be sterilized for use in the *in vitro* culture steps. The detailed procedure is indicated in section 3.2 of this document. In our case, we used seeds of one accession of eggplant (MEL3) kindly provided by the germplasm bank of Universitat Politècnica de València (Valencia, Spain; FAO germplasm bank code: ESP026).

4. Methods

The methodology consists of five main stages (Figure 1). First, the polysomatic pattern of the genotypes must be evaluated by flow cytometry in leaves, cotyledon and hypocotyl to verify that there are enough polyploid cells to start the process. The next step is to cultivate the mixoploid explants to induce shoot formation that, after the rooting and acclimatization process, could give rise to polyploid plants. Finally, the ploidy of the regenerated plants must be checked again using flow cytometry.
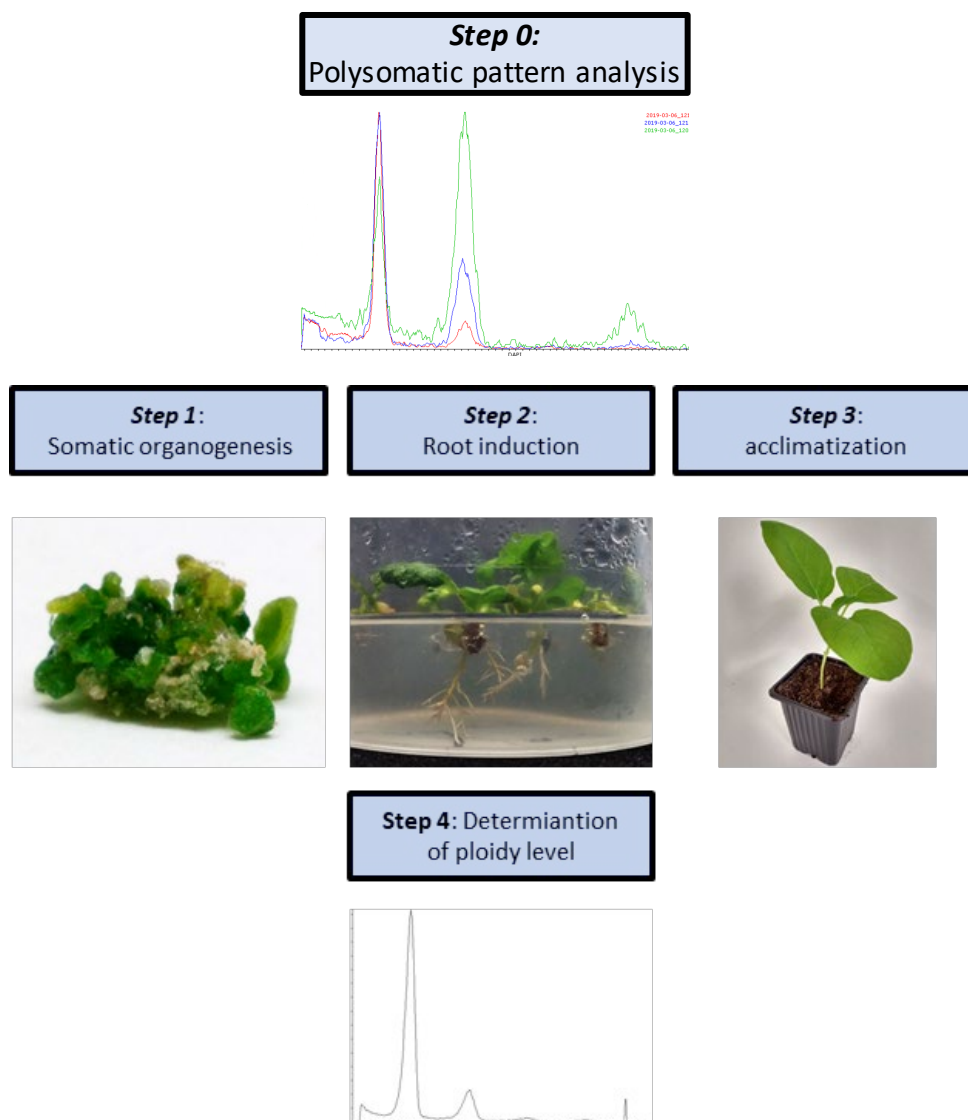
Figure 1: Workflow of the production and identification of polyploid through *in vitro* culture. First the polysomatic pattern is studied. After that, organogenic processes are induced in polysomatic tissues, then root formation is induced, and finally the plants are acclimated. Finally, their level of ploidy is evaluated by flow cytometry.

Polysomatic pattern study

1. Using DAPI staining, the polysomatic pattern of tissues used in *in vitro* culture is evaluated. For this, explants of hypocotyl (1 cm), cotyledon (1 cm^2) and leaf (as a control; 1 cm^2) are processed. Each sample is cut with a scalpel blade in a Petri dish together with 500 µL of nuclei extraction buffer. Several parallel longitudinal cuts are made and then the sample is rotated 90° to repeat the process until the extraction buffer becomes green.

2. Subsequently, the maximum possible volume of the resulting liquid is pipetted and filtered using Celltrics filters in a Sarstedt tube. Finally, 500 µL of the DAPI stain are added to the solution previously filtered.

3. The samples are processed in a cytometer (in our case we used a Cyflow® ploidy analyser; Partec, Münster, Germany) with a gain of 417 (*see* **Note 10**) at a rate of ~ 2 µL / s for about 30 s.

4. The diploid peak of the control (true leaf) is set at an arbitrary fluorescence value of 50 units on the X-axis; consequently, the peak corresponding to the G2 phase is located at the arbitrary value of 100 units of the same axis.

5. After the analysis, the tissues (generally hypocotyl, cotyledon, or both) with higher proportion of tetraploid cells that could potentially undergo the process of organogenic induction and give rise to adult tetraploid plants is chosen. Usually, both cotyledon and hypocotyl tissue are good options. As can be seen in the example of Figure 2, the cotyledon had between 3 and 5-fold more cells than the true leaf at the G2 phase peak. In the case of hypocotyl, the number of cells of the G2 peak was between 7 to 9 times greater than the peak of the true leaf. Both hypocotyl and cotyledon tissue showed a peak in the arbitrary fluorescence value of 200, a non-existent peak in the analysis of the true leaf, which indicated that this excess of cells in the G2 phase peak of cotyledons and hypocotyls are tetraploid cells fully functional that were dividing. This results in a fluorescence peak in the value of 200.
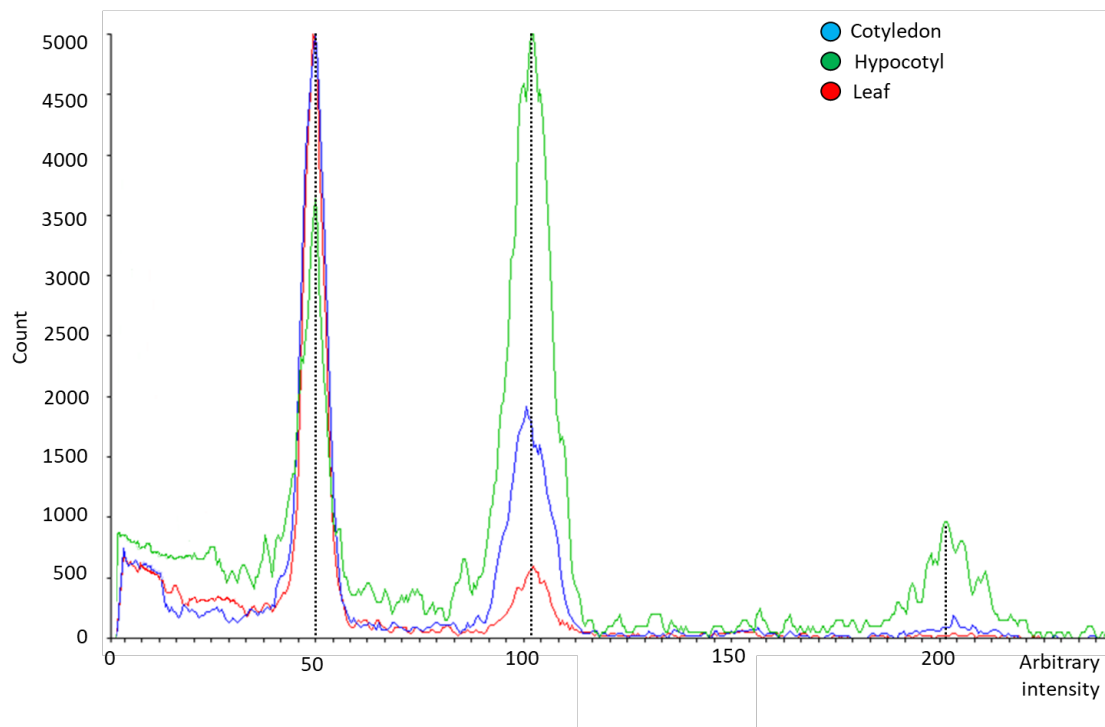

Figure 2: Flow cytometry histogram in which the relative content of nuclear DNA in different tissues of eggplant is represented. The X-axis represents the fluorescence, which is proportional to the amount of DNA. The peak located at the value of 50 corresponds to the diploid nuclei in G1 phase. The Y-axis represents the number of nuclei analysed.

In vitro polyploids production

Once the tissue that is more likely to convert to polyploid is determined (*see* **Note 11**), seeds need to be germinated in sterile conditions. Seeds are sterilized using tea filters or muslin sachets in.

1. In a laminar flow cabinet, a preliminary 70% ethanol wash is performed for 30 seconds.
2. Secondly, seeds are soaked in a 20% commercial bleach (with two drops of Tween20) for 10 minutes.
3. Finally, 3 washes are performed with sterile distilled water for 1 minute each of them. Shake vigorously to wash bleach residues that would have remained in the seeds.
4. Subsequently, the seeds are germinated in Petri dishes with E0 medium under sterile conditions, then the dishes are incubated in the dark. Around 1 month is generally needed until the seedlings showed long hypocotyls and curled cotyledons (*see* **Note 12**).
5. E6 medium is prepared 3-4 days before the experimental session to rule out potential contamination events.
6. Under sterile conditions, five 1 cm long hypocotyl fragments are cultured in each plate with E6 (*see* **Note 13**) medium. Five 1 cm^2 cotyledon explants are also cultivated with two cuts, one in the distal part and another in the proximal part in each of the E6 medium petri dishes. The plates are then kept in a culture chamber at a temperature of 25 °C and photoperiod conditions of 16h light and 8h dark.
7. A minimum of 20 plates with five explants with five explants each is recommended for each tissue in order to obtain good yields in terms of regenerated polyploid plants.
8. When the buds that formed (see Note 14) in the surface of the explants have a size of around 0.5-1 cm or they have the size that allows to make an incision and individualize the plantlet (see Note 15); they are separated from the hypocotyl or cotyledon tissue and subcultured in R2 medium (see Note 16).
9. When the rooted plant has at least two leaves and a root system with at least two main roots and five secondary roots in each of them, the plants can be transferred to the growing substrate (generally any commercial growing substrate for vegetables is appropriate).
10. After transplant and acclimatization, a sample of 1 cm^2 of leaf is taken for the ploidy analysis as explained in the next section.
11. It is highly advisable to place an inverted plastic glass covering the plant (as a kind of mini greenhouse) for two weeks after transplant to prevent it from suffering or dying from dehydration. It is also advisable to humidify it daily with a nebulizer.

Ploidy check with flow cytometry

This analysis is carried out using the same method as indicated in section **3.1.**

After evaluating each of the plants, a cytogram such as the one represents in Figure 3A or Figure 3B is observed. The Figure 3A corresponds to a diploid plant, as it has the same profile than a conventional diploid eggplant plant used as a control. Depending on the equipment, we will adjust the gain with which the control sample is interrogated (in our case it has a value of 417), so that we place the peak in phase G1 at an arbitrary value equal to 50.

All those samples, which display a main peak at the value of 100 and a secondary peak at the value of 200, are tetraploid (Figure 3B). Tetraploid plants peaks appear displaced in the

cytogram because they contain twice as much DNA inside their cell nuclei. At this point *in vitro* tetraploid plants that most likely comes from polyploid cells present in the starting tissue have been generated.

From this point the tetraploid plants will grow, and in the case of eggplant, they are generally fertile and able to produce seeds by selfing, which after germination give tetraploid plants. Therefore, with this method it is possible to generate not only stable non-chimeric tetraploid plants but is also possible to propagate them sexually by selfing and immortalize them as tetraploid materials. One of the added values of this methodology is that antimitotic agents that are very harmful to human health are not used.
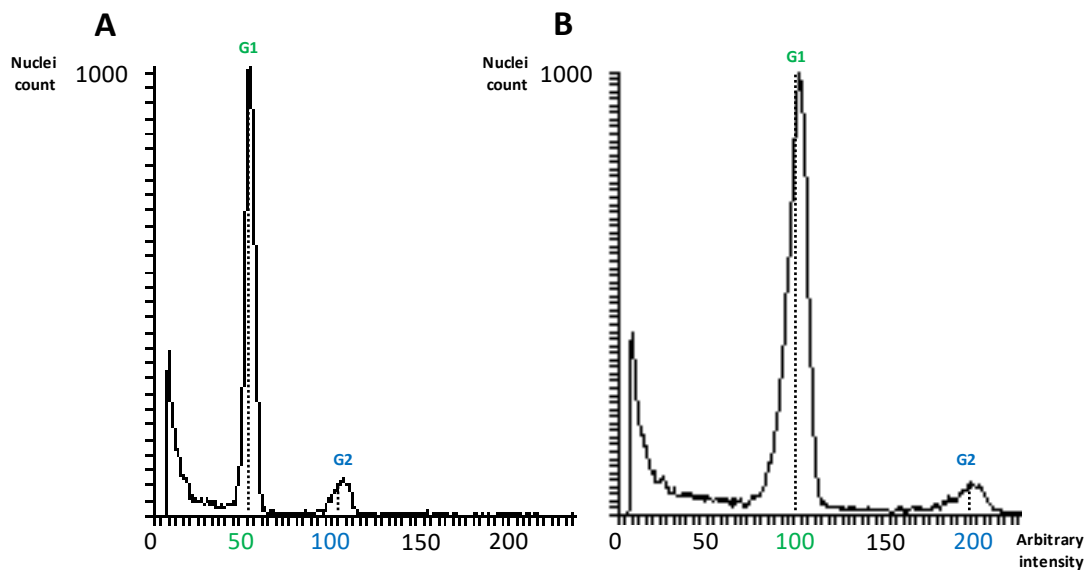
Figure 3: Cytogram of a diploid eggplant plant (A). Cytogram of a tetraploid eggplant plant (B). The corresponding peak with the G1 cell phase is indicated in green, the G2 phase is indicated in blue. The Y-axis indicates the number of nuclei analyzed.

5. Notes

1. The use of ultrapure water is not decisive, although it is recommended, but with sterile distilled water it is possible to perform the protocol.
2. You can work as if it were absolute ethanol, thus measuring a volume of 700 mL to prepare the 70% ethanol solution.
3. Tween20 is a detergent that helps to penetrate the bleach into the tissue and perform a more efficient disinfection. Constant agitation also helps more effective disinfection.
4. It is better to adjust the pH to 5.9, as during the autoclaving process the sugars slightly acidify the culture medium, in this way we correct this effect.
5. The gelling agent should always be added after measuring the pH to prevent the lumps that form from damaging the pH-meterelectrode
6. We should always leave the cap partially unscrewed to prevent the bottle from exploding because of pressure during the autoclaving process.
7. About 20 minutes should be enough for the Gelrite^{TM} to solidify. A good practice is to wait for the drops of condensation on the sides of the petri dishes to disappear to avoid the accumulation of water.

8. In this case, ZR and IBA dissolve very well with 1M NaOH, but this change depending on the growth regulator. It is something that must be checked if other growth regulators are used.

9. It is highly recommended not to fill the Eppendorf to the limit. When freezing, the liquid inside expands and the lid opens, losing sterility and making the stock useless. It is also highly recommended to seal it with parafilm.

10. This gain value is only valid for eggplant and must be calibrated for each species that is analysed as it depends on the size of its genome.

11. The yield on the number of polyploid regenerated plants depends largely on the organogenic competence of its polyploid cells. This means that we can find species in which this yield is much higher than in eggplant and others in which we may not obtain any polyploid regenerant.

12. It is advisable to leave the petri dishes with the germinated seeds in photoperiod conditions (16 h light / 8 h dark) for a day before using the tissues in step 6. This way the cotyledons will have just roll out, and it will be much more comfortable to work with them.

13. This medium is indicated to induce the organogenic processes in eggplant. For other species it will be necessary to use the corresponding medium that does not necessarily have the same composition than E6.

14. In eggplant, this will start happening after a minimum of 30 days after placing the explants in the E6 medium.

15. In case the explants have not elongated enough after a period of 40 days they can be subcultured in E0 medium for a week and then re-subcultured in E0 + Giberelic Acid (GA$_3$) 1 mg/L until the elongation of the shoots is achieved.

16. It is advisable to prepare this medium plastic pots with a membrane filter in the lid to allow gaseous exchange (Microbox containers O118/120+OD118/120 #10 (G), SAC02, Nevele, Belgium), so that the plant can reach a good size that will allow us to take a sample for the subsequent cytometric analysis, as well as facilitate the acclimatization of the plant.

6. Aknowledgement

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Capítulo V

A Deep Learning-Based System (Microscan) for the Identification of Pollen Development Stages and Its Application to Obtaining Doubled Haploid Lines in Eggplant

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Abstract: The development of double haploids (DHs) is a straightforward path for obtaining pure lines but has multiple bottlenecks. Among them is the determination of the optimal stage of pollen induction for androgenesis. In this work, we developed Microscan, a deep learning-based system for the detection and recognition of the stages of pollen development. In a first experiment, the algorithm was developed adapting the RetinaNet predictive model using microspores of different eggplant accessions as samples. A mean average precision of 86.30% was obtained. In a second experiment, the anther range to be cultivated in vitro was determined in three eggplant genotypes by applying the Microscan system. Subsequently, they were cultivated following two different androgenesis protocols (Cb and E6). The response was only observed in the anther size range predicted by Microscan, obtaining the best results with the E6 protocol. The plants obtained were characterized by flow cytometry and with the Single Primer Enrichment Technology high-throughput genotyping platform, obtaining a high rate of confirmed haploid and double haploid plants. Microscan has been revealed as a tool for the high-throughput efficient analysis of microspore samples, as it has been exemplified in eggplant by providing an increase in the yield of DHs production.

1. Introduction

The development of commercial hybrids from elite materials and the introgression of genes of interest from related species that allow expanding the genetic background of crops [1] are basic tools for breeders [2]. Hybrids have multiple advantages, including heterosis for productive traits and they facilitate the accumulation in a single genotype of resistance to multiple diseases conferred by dominant genes [3]. To obtain genetically uniform F1 hybrid plants, the development of pure lines (highly homozygous organisms that breed true by selfing) is one of the main objectives of plant breeders. However, the development of pure lines is not simple and may require a long time [4].

The conventional method to obtain lines has traditionally been the application of successive rounds of self-fertilization and selection, generally requiring 7 to 10 generations for achieving a high degree of homozygosity. By reprogramming the gametophytic development pathways to sporophytic or embryogenic development pathways through the application of different inducing agents in microspores [5], pure lines can be obtained very quickly. This generally requires applying an appropriate stimulus or stress to pollen precursors to induce such reprogramming. These stresses can be very varied, including high or low temperatures, starvation, elicitors, or radiation, and they can be applied both to isolated microspores or to anthers [6–8]. However, this is not a straightforward and simple process and it must also be considered that there are many differences among species and among genotypes within species in the success of the induction. In many cases, small differences in the development stage of pollen translate into great changes in the efficiency of the application of the stresses for a successful induction. This is because not all the stages of the development of pollen are competent for a successful induction in the process of androgenesis. In general, the stages of vacuolated microspore and young pollen are those that will undergo reprogramming towards the embryogenic route in a more efficient way [9,10].

The induction of androgenesis is even more challenging in recalcitrant species, including a number of Solanaceae crops, such as eggplant (*Solanum melongena* L.) [11]. Therefore, it is necessary to develop methodologies that maximize the success in the process of inducing androgenesis and that simplify the process. In eggplant there are protocols for the cultivation of anthers and microspores in which both induction factors and culture media are very well defined [12,13]. However, the selection of the optimal stage for the induction of the androgenesis process is a bottleneck in the process and for which there has not been a practical and efficient solution to date. Thus, prior to the in vitro culture process for androgenesis induction, it is advisable to identify the size of the buds or the anthers with the highest proportion of vacuolated microspores or young pollen [14,15]. This is a fundamental step to ensure that the tissues that are being submitted to the used protocol are in an adequate stage of development and that the efficiency in androgenesis will be maximized. This generally requires the examination of multiple anthers and the manual counting of microspores in appropriate developmental stages [16]. Some general optical systems and software are known to provide an alternative to manual counts [17–19], which continue to be considered as the most reliable technique to date, at least in the case of microspore counting. However, pollen cell stages are partially determined by image details with a high degree of complexity that cannot be discerned with these techniques. In consequence, they have a significant identification error ratio.

The main issue with current automatized systems for the identification of the stages of development of pollen is the approach used. Generally, this is based solely on trying to parameterize, from the expert knowledge, the complexity of the image of a cell [20–22]. This approach has largely failed to date, because the number of variables that must be parameterized for this approach to work successfully is very large. For this reason, we present as an alternative the development of a model based on deep learning. These models are based on extracting statistical patterns from previously characterized pollen cells in different developmental stages [23]. With these patterns, a classifier model is fed to identify the position of the cells in the image and their stage of development. Specifically, the model used in this work consists of a convolutional neural network for image segmentation. These types of computer imaging models have evolved greatly in recent years, reaching for certain types of recognition, such as blood cells [24,25], retina cell nuclei [26], even COVID-19 diagnosis [27,28], with yields superior to those of humans [29].

The main objective of this work was to develop a deep learning-based system, named Microscan, which facilitates the selection of anthers in the appropriate stage of development for obtaining a high yield of doubled haploids through anther culture. Microscan consists of the combination of an automated microscope that scans the samples and an algorithm of artificial intelligence based on machine learning techniques that can make accurate predictions, at least as good as a trained technician, of the proportion of microspore cells in different stages in eggplant. In this way, through the use of Microscan, we were able to make the process more efficient in order to improve the success of androgenesis. For this, we used eggplant introgression materials for testing the efficiency of the approach proposed.

2. Materials and Methods

2.1. Plant Material

Three *S. melongena* accessions, namely MEL1, MEL 3 and MEL5, and two pre-breeding populations, consisting of a fourth backcross (INSBC4) generation of an introgression line between *S. melongena* MEL5 and *S. insanum* INS1, and a second selfing of a second backcross (MBC2S2) generation between *S. melongena* MEL1 and *S. anguivi* ANG1 were used for the algorithm training.

Three genotypes of one of the families of the introgression line between *S. melongena* MEL3 and *S. elaeagnifolium* ELE2 (BC3 17-8, BC3 17-19, BC3 17-4) were used for testing the algorithm efficiency using two different anther culture protocols (E6 and Cb). Finally, BC4 and BC3S1 generations together with the doubled haploid plants derived from accessions BC3 17-19 and BC3 17-4 were used for genotyping. All plants were grown in 10 L pots at Universitat Politècnica de València (UPV) glasshouses (GPS coordinates 39.482228, −0.337332) using coconut fiber substrate and fertigation.

2.2. Experimental Layout and Workflow

The experimental layout and workflow are presented in Figure 1. Phase 1 was carried out to develop an instance segmentation model called RetinaNet [29] based on deep learning that could identify and classify the different stages of pollen development. This model needs to be fed with sample images similar to those that would be classified. For this, concentrated extracts of the different stages from tetrad to mature pollen were obtained from the different accessions of cultivated eggplant and experimental breeding populations. Cells were manually tagged in thousands of digitized images from these extracts. Then, images were split between a training set to feed the model and a validation set to evaluate the accuracy. The model parameters were tuned with the image information during the training phase, until the accuracy was enough to infer tags into new images [30].

The following phase of this experiment consisted of the validation of the model obtained in Phase 1. In Phase 2, the Microscan system was applied to a real case in eggplant. In this experiment, we tried to check if the level of androgenic response improved by selecting the anthers using the prediction model. For this, the anthers recommended by the model were selected and suboptimal ranges were selected as a negative control. These anthers were then cultivated using two different protocols, one of reference (Cb) [12] with some modifications and the another one newly developed in this work (E6). Finally, the plants obtained were analyzed by flow cytometry to ascertain its ploidy level and they were genotyped with the high throughput Single Primer Enrichment Technology eggplant platform [31].
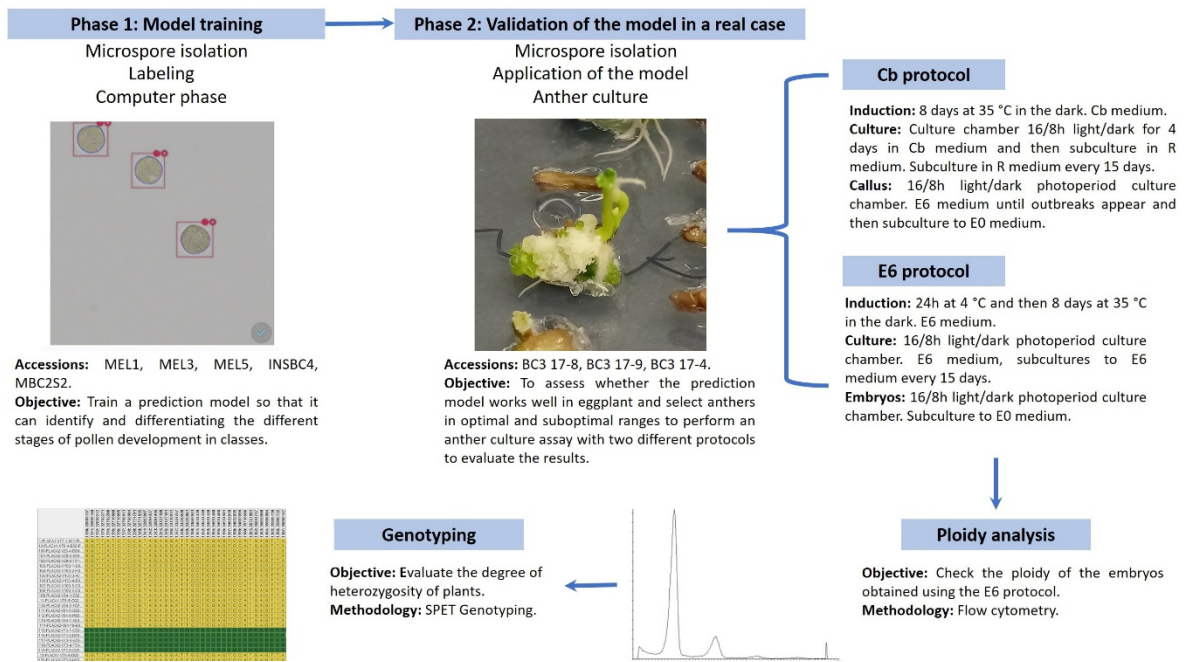
Figure 1. Description of the experimental design and the main objectives. The flow of the arrows indicates the order in which the experiments were conducted and their relationships.

2.3. Phase 1: Model Training

2.3.1. Microspore and Pollen Isolation

In order to have enough cells at the same stage of development to facilitate the tagging task, synthetic samples were prepared by concentrating the cellular content of anthers with a range of similar size. For this, four size ranges of a 1 mm of eggplant anthers were arbitrarily established (<3 mm, 3–4 mm, 4–5 mm, and 5–6 mm) establishing a minimum size of 3 mm since it was not possible to work with smaller anthers and a maximum of more than 6 mm since most of the anthers of this size already contained pollen, and a pool was also prepared with the anthers of different size ranges, with microspores in all their stages, for testing the algorithm. To avoid biases due to possible morphological differences in cells due to differences among genotypes, samples from the eggplant accessions (MEL1, MEL 3, MEL5, INSBC4 and MBC2S2) were mixed. For each of the ranges, 20 flower buds which had at least five anthers each were harvested. That is, each cell concentrate came from a minimum of 100 anthers within the size range. The anthers of each rank were crushed in a beaker with the aid of a syringe plunger, followed by eluting with sterile distilled water. The solution was filtered with a 41 µm nylon membrane, then centrifuged with an ultracentrifuge refrigerated at 4 °C (Centrofriger-BL II, J.P. Selecta) at 850 rpm for 5 min. The supernatant was discarded, and the centrifugation was repeated twice. The microspores already free of tissue debris that could pass through the nylon membrane were resuspended in 5 mL of sterile distilled water, thus leaving the concentrate ready to acquire images with the Microscan system.

2.3.2. Digital Data Image Acquisition

Twenty microliters of concentrated microspore solution was deposited on a slide and a 11 mm^2 coverslip was placed on the top. In this way, a 0.165 mm water column was generated, high enough not to crush the cells and flat enough so that all the cells are at the same depth of field.

The coverslip surface was scanned using an optical motorized digital microscope (MoticEasyScan One, Motic, Barcelona, Spain). It is a non-inverted brightfield optical microscope with a zenithal incoherent white LED illumination source (10W LED (Lifetime: 25,000 h)). To capture the images, an apochromatic l × 10/0.3 optical lens was used together with a zenithal camera with a resolution of 1 Mpx (2× digital zoom), in addition, the equipment incorporates another oblique camera for the detection of the focus plane (autofocus) with the same characteristics as the previous one. This microscope moves in a preselected area at regular steps, acquiring 512 × 512 px images corresponding to regions of a size of 0.26 mm^2. The result is like the mosaic in Figure 2. To correct inaccuracies in the displacements due to the thread pitch, overlapping areas are used to algorithmically merge images by creating a macro image. The 512 × 512 px images were extracted from the generated macro image and used to train the deep learning model. For this, it was necessary to label them by an expert as well as to train the model.
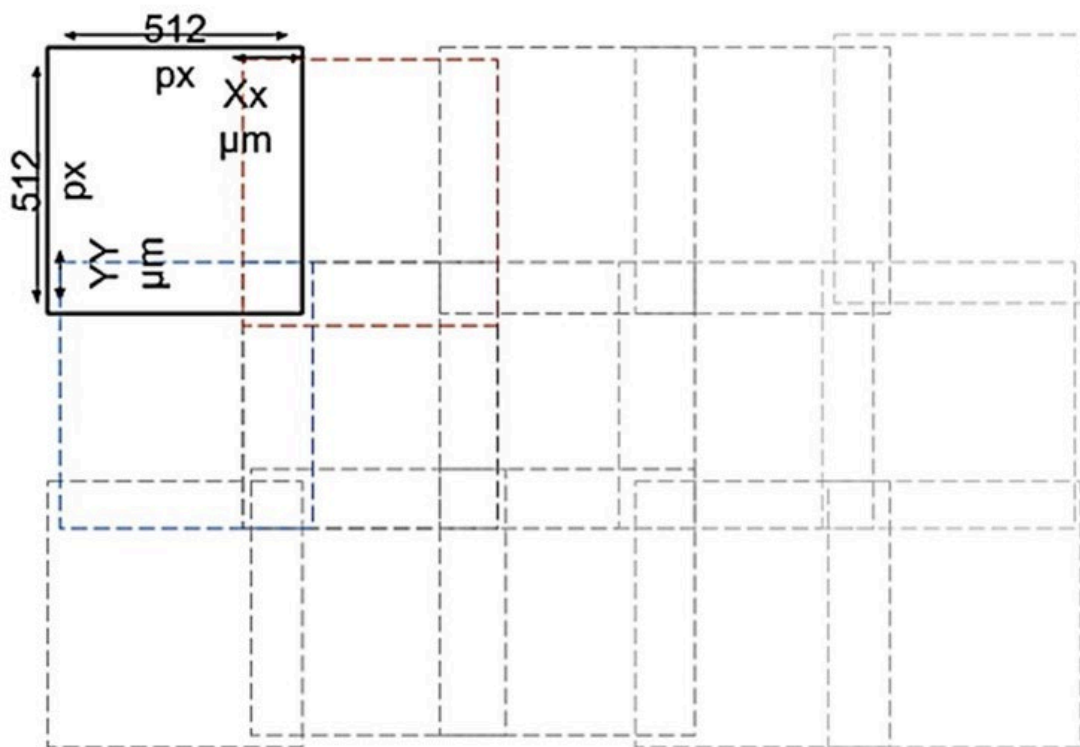

Figure 2. Scheme of how the imaging is performed using the motorized light microscope. The pictures are taken in a 512 × 512 px section, generating overlapping areas to obtain a macro image without blemishes in these areas.

2.3.3. Image Labelling

The chopped images from the previous section were inserted into a labelling software developed by the company SomData Analytics (Valencia, Spain) (Figure 3).
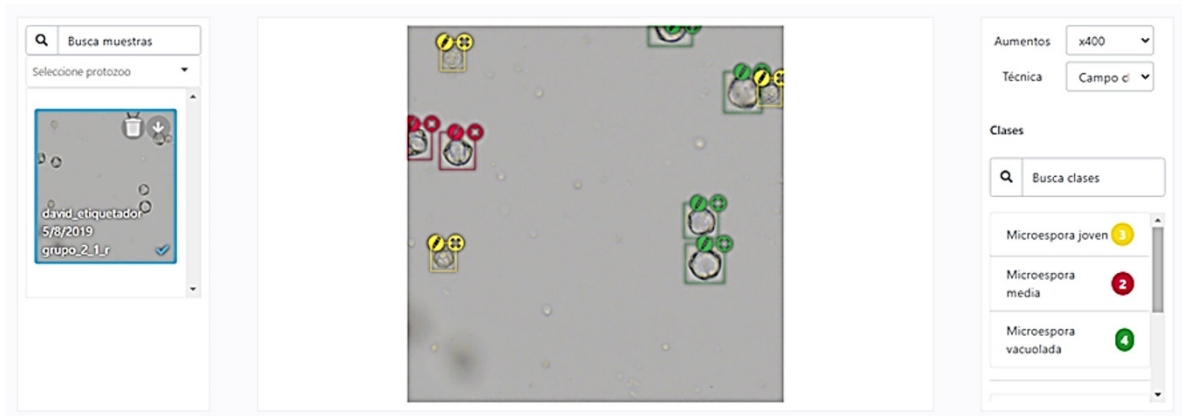

Figure 3. Graphic environment of the manual labelling software used for the classification of the microspores that was used for the Microscan model training. In the central part it can be seen how the cells have been labelled by an expert and classified according to their stage of development.

For the labelling of the images, a bounding box system was chosen which allows a balance to be struck between the quality of the labelling and the effort of the labeler. When a box system is used, all the structures present in the image must be labelled, although sometimes it may be unclear to which class some of the microspores belong to, therefore a stage defined as "doubtful" was added to the pollen development stages in order to revise the structure or to delete this image from the training.

Six classes (tetrad, young microspore, medium microspore, vacuolated microspore, young pollen and mature pollen) were defined as different stages of development of the microspore that can be found inside the anthers depending on their size range (Figure 4A). The criterion used to define them was that they were easily differentiated by using an optical microscope, without the need for any type of histological or fluorescent staining. All these stages have different degrees of maturity and it is the criterion of an expert eye to define them. Even so, the model to be developed will work with a continuous distribution system with a confidence interval instead of a discrete distribution, allowing to assign a probability of belonging in different degrees to one class or another, thus solving the problem of intermediate stages. The eggplant tetrads have a very characteristic rhomboid-shaped morphology (Figure 4B); the young microspores have a low opacity and a reduced size compared to other stages (Figure 4C); the middle microspores (Figure 4D) have an intermediate size between the young and vacuolated microspores and the openings of the exine are clearly appreciated as more thick edges; vacuolated microspores have a more circular perimeter and in most cases a low cytoplasmic opacity where the vacuole itself is seen (Figure 4E); young pollen is larger than the vacuolated microspore and has a higher opacity due to the onset of the accumulation of starch granules (Figure 4F); mature pollen is the most opaque cell type due to the large accumulation of these granules (Figure 4G). Stages such as unicellular and bicellular young pollen could not be defined with this system due to the need to use 4',6-diamidino-2-fenilindol for its classification [32].
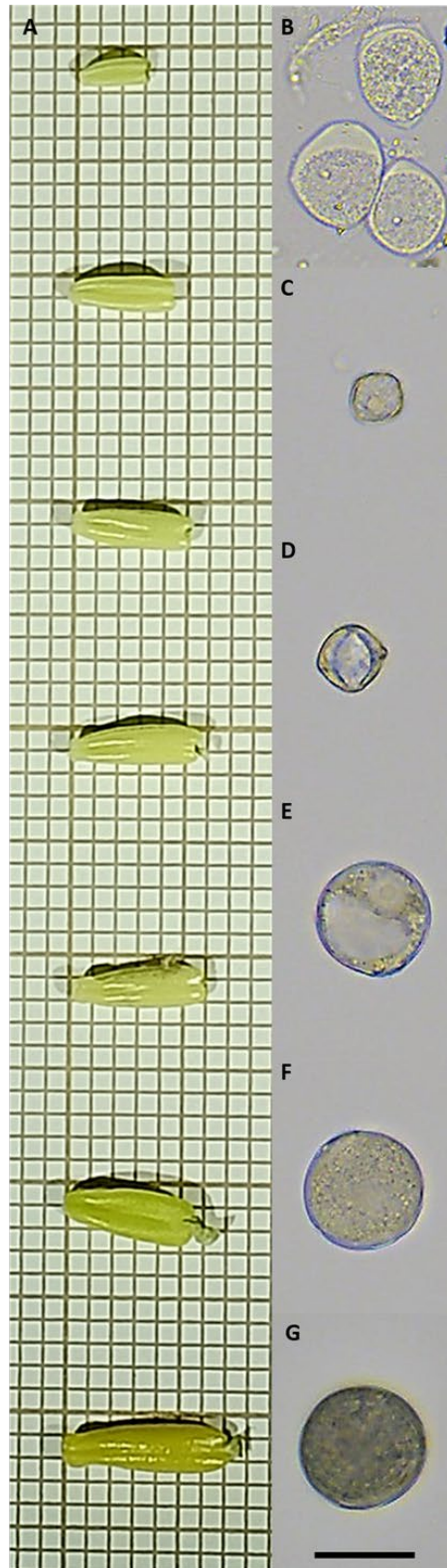
Figure 4. Eggplant anthers at different stages of development (**A**). Different stages of development of the pollen, tetrad (**B**), young microspore (**C**), medium microspore (**D**), vacuolated microspore (**E**), young pollen (**F**) and mature pollen stages (**G**). These have been the stages chosen to define the classes used to train the prediction model algorithm. The predominant stage of pollen development that each contained within each anther inside approximately corresponds to the state indicated in the image to the right of each anther size. The squares in the left images measure 1 mm, while the bar in the images in the right part of the figure measure 20 μm.

A total of 2439 images were labeled and supervised by experts. A total of 290 tetrads, 643 young microspores, 1896 medium microspores, 1191 vacuolated microspores, 1876 young pollen and 2186 mature pollen cells were classified, making a total of 8082 manually classified cellular events.

2.3.4. Preprocessing

The quality of the prediction model depends largely on the quality of the data with which it is fed. For this reason, it is necessary to pre-process the images to equalize the brightness and color so that their levels are similar in all the pictures, as well as to eliminate unfocused images, which are detected with Fourier analysis [33]. Furthermore, all the images in which there were no cellular structures were eliminated.

The evaluation of the model skill on the training dataset would result in a biased score. Therefore, the model was evaluated with the typically called train–test split approach [34]. Specifically, the 2439 images were split into train, test and validation sets in proportions of 80%, 10%, and 10%, respectively. The subsets split maintained approximately the same imbalance ratio between classes than in the original set.

Another factor that must be considered and corrected is the imbalance. Due to the biological nature of the samples, it may occur that a cell group is overrepresented with respect to another, and this would make the prediction model assign by probability to the minority groups the class of the majority group. The way to solve this is to apply a weighted system, where the model pays more attention in its learning when cells of the minority group appear. This reduces the bias in which the model binds by the ruling class in the training set.

Other strategies to increase the quality of the prediction model including the use of image augmentation techniques. This means that the same images that are used to feed the model are modified by slightly rotating them, blurring or subtly altering the sizes. These same images are reintroduced to the model to be used in its training.

2.3.5. Predictive Model

The predictive model used for the development of the Microscan is an adaptation of the RetinaNet instance detection network [29].

This model has been used since, in images where objects are sparse and most of the image is background, as it is one of the most efficient ones [29]. Instances are indicated with bounding boxes. Finally, the training and inference of these models is one of the fastest. In order to carry out this work, it was necessary to have a server with a GPU (graphic processing unit).

2.4. Phase 2: In Vitro Androgenesis Induction Test Using the Anther Selection Software

In this case, 8 anther size ranges were established (<3.5 mm, 3.5–4 mm, 4–4.5 mm, 4.5–5 mm, 5–5.5 mm, 5.5–6 mm, 6–6.5 mm, > 6.5 mm). Following the procedure described in the previous sections, the cell concentrates were prepared and subjected to the analysis of the prediction model.

Two different androgenesis protocols were applied, E6 (based on [35]) and Cb (based on [12]). For each of the three genotypes tested, three experimental replicates were performed with 15 anthers per replicate. Sizes were within the range determined by the algorithm, coinciding with that one which maximized the number of vacuolated microspores (5.5–6 mm), which were used for each of the two protocols. We also added three replicas with 15 anthers each below the recommended range (more specifically in the young microspore stage, 3.5–4 mm) and another three replicas with 15 anthers, each above the recommended range (more specifically, in the

mature pollen stage, >6 mm). In total, 45 anthers were evaluated within the optimal range for each of the genotypes in both protocols (270 anthers) and a total of 45 anthers in suboptimal ranges as a negative control for each of the genotypes in both protocols (540 anthers).

2.4.1. E6 Protocol

Firstly, the flower buds of the young plants were harvested during the autumn season of 2019 and a cold stress pre-treatment was applied, leaving them at 4 °C for 24 h. These buds and the anthers that were obtained were kept at 4 °C throughout the process. This disinfection of the buds, like the culture, was carried out in a laminar flow cabinet. First, the buds were immersed for 30 s in 70% ethanol, followed by 10 min in 20% commercial bleach with a few drops of Tween® 20, and finally, three washings of 3 min each with constant agitation to ensure good sterilization were carried out with sterile distilled water. Once the buds were sterilized, they were left to dry for a few seconds on sterile filter paper. The anthers were extracted from inside the buds, measured, selected, and finally cultivated with their concave side down in medium E6 (Table 1) [35]. The plates were left at 35 °C for 8 days in dark conditions. After this induction period, the plates were taken to the culture chamber which maintained a temperature of 25 °C with a photoperiod of 16/8 h light/dark. A layer of filter paper was placed on the top to simulate diffuse light conditions. Thereafter, the anthers were subcultured in R medium (Table 1) every 15 days until the appearance of embryos, which, upon reaching the cotyledon stage, were subcultured in E0 medium (Table 1).

Table 1. Composition of the different culture media used in the androgenesis protocols of Phase 2. The table collects the information on concentrations of basal salts, carbon source, growth regulators and gelling agent for the preparation of media E0, E6, Cb and R. Preparations C and R are specific formulations previously described [12,36].

Medium	MS + Vitamins (g/L)	Prepared C (g/L)	Prepared R (g/L)	Sucrose (g/L)	Zeatin Riboside (mg/L)	Kinetin (mg/L)	2,4-D (mg/L)	Vitamin B12 (mg/L)	Gelrite (g/L)	Bacto-Agar (g/L)
E0	2.20	-	-	15.00	-	-	-	-	7.00	-
E6	2.20	-	-	15.00	2.00	-	-	-	7.00	-
Cb	-	4.55	-	120.00	-	5.00	5.00	0.20	-	8.00
R	-	-	4.55	30.00	-	0.10	-	-	-	8.00

2.4.2. Cb Protocol

The starting conditions are the same as those of the E6 protocol as regards the cultivation of the plant material and its collection. The protocol procedure is also identical to that described above and the anthers are cultured in the same way except that in this case the induction medium is Cb [12] (based on [36]) instead of E6 (Table 1). When the embryos appeared, they were subcultured in E0 medium (Table 1) (this is a modification of the original protocol in which the embryos are subcultured in V3 medium). In the event of the formation of calli, another modification was applied. These calluses were subcultured in E6 medium. If shoots were obtained, they would be subcultured in E0 medium.

2.4.3. Flow Cytometry

Cell nuclei from leaf tissue were isolated mechanically according to Dpoolezel [37] with modifications. Leaf sections of approximately 0.5 cm^2 were chopped with a razor blade in a 6 cm diameter glass Petri dish containing 0.5 mL lysis buffer LB01 (pH 7.5) supplemented with 15 mM Tris (hydroxymethyl) aminomethane, 2 mM Na$_2$EDTA and 0.5 mM spermine, and incubated for 5 min. Subsequently, the suspension containing nuclei and cell fragments was filtered through a 30 µm CellTrics filter (Sysmex, Sant Just Desvern, Spain). The nuclei in the filtrate were stained

with CyStain UV Ploidy (Sysmex) and incubated for 5 min. The fluorescence intensity of the homogenate was measured using a CyFlow ploidy analyzer (Partec, Münster, Germany), measuring at least 4000 nuclei for each sample. Using young leaves of a diploid eggplant, the diploid control peak was established at 50 points of the arbitrary intensity value of the fluorescence in the histogram (Figure 5). By comparison with this peak, the ploidy of the other tissues evaluated was checked.

2.4.4. Single Primer Enrichment Technology (SPET) Genotyping

Genomic DNA was isolated from 3–4 true leaves, according to the Cetyl Trimethyl Ammonium Bromide protocol [38] with slight modifications. The extracted DNA was dissolved in Milli-Q water and general quality was confirmed in agarose gel at 0.8%. After a concentration measurement using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA), the DNA was diluted at 30 ng/µL for SPET analysis. The Single Primer Enrichment Technology is a mass genotyping approach that is based on the PCR amplification of regions of the genome using primer libraries (previously developed from specific sequencing and resequencing works for the species in question) that are located around a single nucleotide polymorphism [31]. Using the Tassel software [39], a filter was applied in which only those positions for which the donor accessions were heterozygous were selected. Later this filter was applied to the derived materials (BC4, BC3S1 and double haploids (DHs)) to evaluate their percentage of heterozygosity.

3. Results

3.1. Phase 1: Model Training

The precision results of the prediction model developed in this work are presented in Table 2. The precision success percentages were in all cases higher than 80%, the highest being in the case of mature pollen where a value of 92.19% was reached, followed by the vacuolated microspores, which reached 87.60%. The lowest percentage of precision was found for the medium microspores (81.97%). The mean average precision (mAP), a metric for measuring the accuracy of object detectors, in the case of our model, reached an average value of 86.30%. These percentages are based on a very high number of samples analyzed (2439 images and 8071 cells labeled manually), giving great statistical robustness to the model. A simple application has also been developed to generate graphical reports where the analysis of the prediction model can be easily accessed.

Table 2. Results of the precision in the model prediction for the different cell classes defined during the development of the eggplant microspore towards pollen grain. The mean average precision (mAP) of the algorithm is also presented.

Class	Total Cellular Events	Average Precision (%)
Tetrad	290	87.40
Young Microspore	641	86.28
Medium Microspore	1893	81.97
Vacuolated Microspore	1185	87.60
Young Pollen	1876	82.32
Mature Pollen	2186	92.19
mAP		86.30

Finally, the average number of cellular events inferred by the trained prediction model was 2000 every 10 min. This performance ratio was calculated based on an area of 11 mm^2, which is what the coverslip measures, and for a concentrated cell solution. As mentioned in the Materials

and Methods, the performance ratio is subject to the processing capacity of the equipment with which it works, achieving the best performance with equipment that has a GPU.

3.2. Phase 2: In Vitro Androgenesis Induction Test Using the Microscan

The prediction model was applied to three genotypes of interest from three BC3 generations of the introgression lines of eggplant with *S. elaeagnifolium*. These genotypes presented a theoretical degree of heterozygosity of 12.5% and might require several generations of selfing to be fixed in homozygosity. As an alternative to the successive generations of self-fertilization, it was decided to select these individuals for the prediction model validation experiment. Figure 5 shows the results of the Microscan analysis when applied to the eight anther size ranges selected for the execution of this second phase. After applying the prediction model, for these genotypes, the size range of the anther that maximized the number of cells in the vacuolated microspore stage was determined to be of 5.5–6 mm. The mixture of anthers of this range presented a number of vacuolated micropores of 20,115 for genotype BC3 17-8, 14,887 for genotype BC3 17-19 and 18,775 for genotype BC3 17-4, which in the three cases was the highest of all those found in all defined anther ranges. On the other hand, the ranges for the control with young microspores and mature pollen were 3.5–4 mm and >6.5 mm successively, being the ones that maximize these cell stages.

Figure 5. Distribution of the cell types identified by Microscan within each anther range in the three genotypes used for the Phase 2 experiments. The *Y* axis shows the number of cells classified for each type; the *X* axis shows the anther range corresponding to each analysis. The color code in the legend indicates the cell type, tetrad (T), young microspores (YM), medium microspores (MM), vacuolated microspores (VM), young pollen (YP) and mature pollen (MP).

Between 40 and 70% of the anthers of the range with vacuolated microspores (5.5–6 mm) subjected to the E6 protocol presented an embryogenic type response in the three genotypes evaluated (Table 3). On the other hand, the anthers of the negative control ranges did not show any type of response (Table 3); they were necrotic and did not give rise to any type of embryogenic or organogenic structure. After 4 days of induction at 35 °C, structures like globular embryos began to be observed (Figure 6A,B) in the 5.5–6 mm range. After a month of culture, these structures evolved giving rise to torpedo and cotyledon-type embryos (Figure 6C) which

after a month and a half of anther culture already had a seedling appearance (Figure 6D) capable of rooting and being subcultured in an E0 medium to allow its development and subsequent acclimatization. In some cases, abnormal albino embryos also appeared that were completely unviable (Figure 6E,F). The average embryogenic structures per induced anther for the three genotypes had a similar value of around 4.

Table 3. Percentage and type of response in the three eggplant genotypes tested for the two protocols (E6 and Cb) and the three anther ranges selected. The response percentage (±SE) is presented. The type of response has been differentiated depending on whether it was an embryogenic or a callogenic response.

Size Range/Genotype	Protocol E6			Protocol Cb		
	Anthers (n)	Response (%)	Type of Response	Anthers (n)	Response (%)	Type of Response
3.5–4 mm						
BC3 17-8	45	0.00 ± 0.00	-	45	0.00 ± 0.00	-
BC3 17-19	45	0.00 ± 0.00	-	45	0.00 ± 0.00	-
BC3 17-4	45	0.00 ± 0.00	-	45	0.00 ± 0.00	-
5.5–6 mm						
BC3 17-8	45	75.30 ± 0.04	Embryo	45	78.40 ± 0.04	Callus
BC3 17-19	45	60.40 ± 0.05	Embryo	45	80.50 ± 0.04	Callus
BC3 17-4	45	40.30 ± 0.05	Embryo	45	71.20 ± 0.05	Callus
>6 mm						
BC3 17-8	45	0.00 ± 0.00	-	45	3.20 ± 0.02	Somatic callus
BC3 17-19	45	0.00 ± 0.00	-	45	4.00 ± 0.02	Somatic callus
BC3 17-4	45	0.00 ± 0.00	-	45	4.30 ± 0.03	Somatic callus

Table 4 shows the results of the total number of normal embryos generated in the anthers of the range predicted as the most efficient by the model. It also shows the number of plants acclimatized from these embryos and their ploidy. In genotype BC3 17-8, a total number of 42 embryos was obtained; of these, only 12 came to give acclimatized plants that were mostly haploid and some of them were mixoploid. In the case of genotype BC3 17-19, the number of total embryos was 29 while that of acclimatized plants was 12, and in this case the cytometric analysis showed that eight of these plants were haploid, one myxoploid and three diploids. Finally, the genotype BC3 17-4 generated nine embryos, seven of which gave rise to acclimatized plants, of which six were haploid and one was mixoploid.
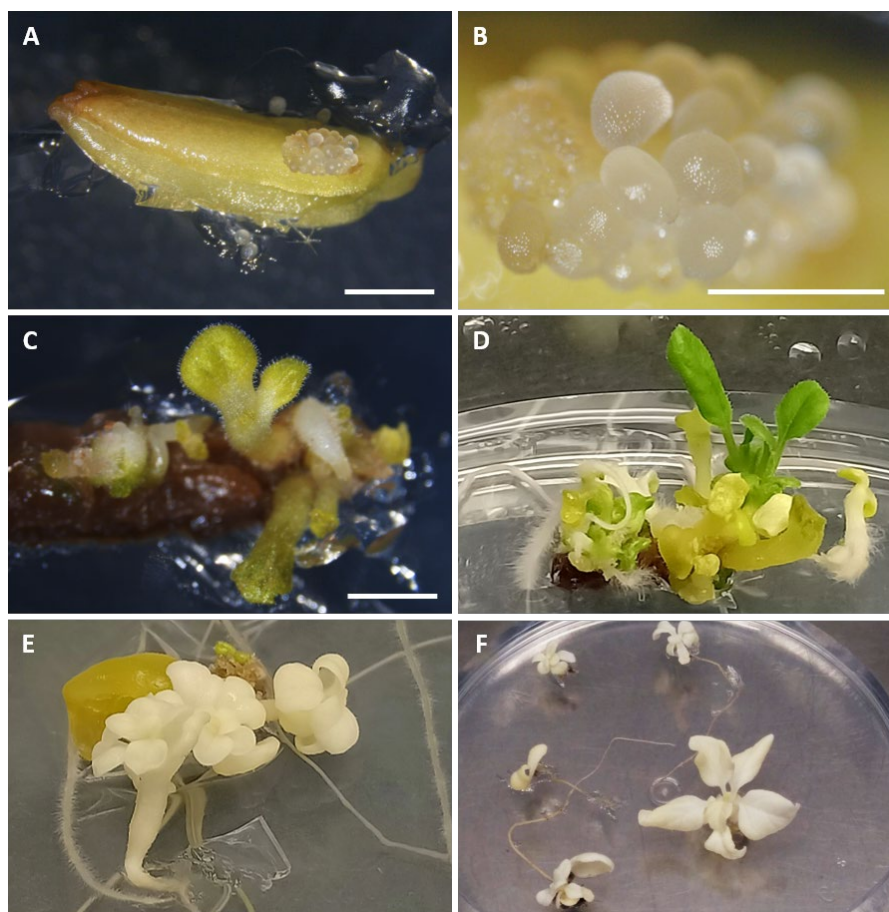
Figure 6. Anther selected through Microscan grown in E6 medium four days after being placed in culture (**A**); detail of the same anther, the formation of globular-type embryogenic structures can be observed (**B**); anther cultivated in E6 medium after one month of culture, it can be seen how embryogenic structures evolve giving rise to torpedo embryo-like structures and in some cases cotyledonal stage (**C**); fully developed seedlings and cotyledonal embryos of eggplant after one and a half months of culture in E6 medium (**D**); albino eggplant embryos from anther culture in E6 medium (**E**); albino embryos isolated from the anthers and subcultured in E0 base medium (**F**). The size of the bars is 1 mm.

Table 4. Total embryos obtained in the three genotypes of the BC3 family 17 from the culture of anthers of the 5.5–6 mm range using the E6 protocol. The total number of acclimatized plants and their ploidy level analyzed by flow cytometry are shown.

Genotype	Embryos (n)	Acclimatized Plants (n)	n	$n + 2n$	$2n$
BC3 17-8	42	12	9	3	0
BC3 17-19	26	12	8	1	3
BC3 17-4	9	7	6	1	0

Anthers within the range recommended by the prediction model (5.5–6 mm) showed androgenic response in Cb protocol in the three genotypes evaluated. As in the previous case, none of the negative control ranges gave rise to androgenic response, generating premature necrosis in both ranges. In the range of >6 mm, a response in around 4% of the anthers was observed too, and some somatic calli were formed in the cutting area where the filament was (Table 3). In the anthers of 5.5–6 mm, the formation of embryogenic structures did not occur, instead the formation of androgenic calluses that came from inside the anther was observed in 70–80% of the cases (Table 3), the calli burst the wall and emerged from within the anther (Figure 7A,B) after approximately two months of culture (i.e., one month after subculturing to

medium R). Plants could be obtained by subculturing these calluses in E6 medium and subcultured every 15 days until the appearance of shoots (Figure 7C), which took place between one month and two months after subculturing the calluses in E6 medium for the first time. Finally, the seedlings obtained were subcultured to E0 medium (Figure 7D).
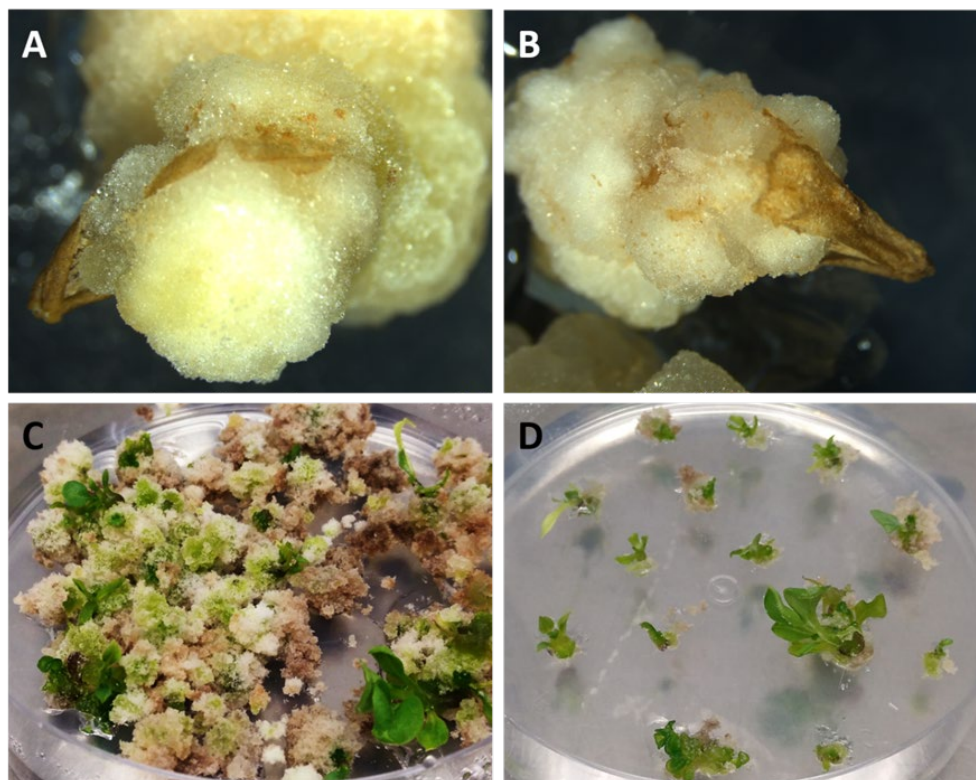
Figure 7. Calluses obtained two months after the culture of anthers in medium R applying the Cb protocol (**A**,**B**). By subculturing these calluses in E6 medium, shoot induction was achieved after approximately one month of induction in this medium (**C**) and finally the seedlings can be isolated (**D**).

The cytometry data was contrasted with a Single Primer Enrichment Technology (SPET) high throughput genotyping, in which 532 single nucleotide polymorphism (SNP) markers were evaluated for which the anther donor plants (BC3 17-19 and BC3 17-4) were heterozygous (Table 5). Apart from genotyping the donor plants, individuals of the BC4 generation obtained by backcrossing between the donor plants and their cultivated parent (MEL3) were genotyped. There were six in the case of plant BC3 17-19 and five in the case of the plant BC3 17-4. It should be noted that during the growth process, many of the haploid plants showed spontaneous duplication events, and it was these that were selected for genotyping together with the accessions that were myxoploid and diploid in the first cytometric analysis. Ten of the 12 acclimatized plants of putative androgenic origin of plant BC3 17-19 were genotyped as well as all acclimatized plants (7) of plant BC3 17-4. The donor plants have a percentage of heterozygosity of 100% since during the analysis of the data, only those positions in which these plants were heterozygous were selected to filter the rest of the SNP positions in the accessions derived from this. In the analysis, for the BC3 17-19 accession, its offspring obtained by backcrossing (BC4) displayed a percentage of heterozygosity with an average of 55.07% (range of 39.06–65.66%). On the other hand, all DH individuals derived from this accession have reduced their heterozygosity with an average of 1.59% (range 0.19–3.95%). Very similar results are found in the case of accession BC3 17-4, where individuals obtained by backcrossing have

high heterozygosity values with an average of 53.87% (range 13.35–92.66%), whereas here DH individuals have an average of 0.67% of heterozygosity (range 0.19–1.70%).

Table 5. SPET mass genotyping results shown as a percentage of heterozygosity (mean and range) calculated based on the genotyping of 532 single nucleotide polymorphism (SNP) positions in the accessions derived from materials BC3 17-19 and BC3 17-4. Among these materials, we find backcrossings with the cultivated parental MEL3 (BC4) and plants obtained from the application of the E6 (DH) protocol. The number of positions SNPs evaluated and those that were not found in the specific accessions are shown.

Accession/Offspring	*n*	Missing SNPs	Heterozygosity (%)
ELE BC3 17-19	1	0.00	100.00
BC4 (ELE BC3 17-19 × MEL3)	6	4.00 (0.00–6.00)	55.07 (39.06–65.66)
DH (ELE BC3 17-19 doubled haploids)	10	5.00 (0.00–12.00)	1.59 (0.19–3.95)
ELE BC3 17-4	1	0.00	100.00
BC4 (ELE BC3 17-4 × MEL3)	5	0.40 (0.00–2.00)	53.87 (13.35–92.66)
DH (ELE BC3 17-4 doubled haploids)	7	1.14 (0.00–4.00)	0.67 (0.19–1.70)

4. Discussion

The determination of the optimal stage for the induction of androgenesis is highly relevant for the development of doubled haploids, especially in recalcitrant crops such as eggplant. For this reason, the development of a tool like Microscan represents a great technical advance around double haploid production and in the study of androgenesis.

The model developed using a deep learning approach has proved highly efficient for the identification of the stages of pollen development. In this way, the mean average precision of the model is within the appropriate values for this type of forecasting system [23,24]. For the specific doubled haploids' development application, a vacuolated microspore detection model would have been sufficient. The vacuolated microspore stage is the factor that will determine which range of anther sizes to select, as this is the most inducible stage in the androgenesis processes [14,15]. The reason for choosing the length of the anther as a morphological marker was that, unlike other species such as pepper where the anthocyanin pigmentation of the anthers is quite common, in our case the genotypes of eggplant that we used do not present this pigmentation, which means that the size of the anther seemed to us a more robust parameter and less dependent on genetic or environmental factors [11]. However, discriminating among stages makes the estimation of the optimal stage more reliable since it is not judged on a single stage, but on a distribution of stages. Just as an expert eye can differentiate different degrees of maturity in the development of a cell type, the algorithm used here is able to assign a confidence interval around each of the predictions for each of the cells. This generates a distribution that allows having a more accurate interpretation of the content of a specific anther. Regarding the specific precision values in some cell types, it should be noted that the lowest values correspond to those intermediate stages that are more difficult to differentiate, such as the case of medium microspores or young pollen, although even in this case, the correct identification values are over 80% [23,24]. On many occasions, the morphology of these cells is intermediate between the young and vacuolated microspore stages in the case of the medium microspore, or vacuolated and mature pollen in the case of young pollen. There are a large number of cellular events that are located halfway between stages that are very well established and recognizable (as is the case of young microspores, vacuolated microspores or mature pollen) and this is what makes the model more hesitant to assign those classes in those cases. Therefore, we decided to establish a continuous distribution system with a confidence interval instead of a discrete distribution. Just the opposite has happened with the tetrads, which despite being the class with

the least number of cellular events tagged, has been one of the classes with the higher percentage of precision (87.40%) due to its unique shape and completely different from the rest of cells. The prediction model has obtained a very satisfactory level of precision that, added to its capacity for cellular analysis per unit of time and the large sample size that it is capable of handling, not only rivals the classification made by an expert technician in cataloging accuracy, but it also vastly outperforms it in volume analysis capabilities [40,41]. It should be remembered that these tasks when performed manually are highly time consuming and visually exhausting [42].

The use of the prediction model has been decisive in selecting those anthers that have shown the best induction results in the genotypes BC3 17-8, BC3 17-19, BC3 17-4, used during Phase 2. The selection of anthers with a high content of vacuolated microspores has been the only one to give an androgenic response, since this stage, together with the stage of young bicellular pollen, is the most inducible [14,15] and in this case, the algorithm maximizes that cellular percentage, adjusting the range of the anthers to be selected very well. Androgenic response has not been observed in those ranges associated with a cellular content of young microspores or mature pollen, non-inducible stages by default and by excess with respect to the optimal stage (vacuolated microspore/young bi-cellular pollen). Some authors suggest that due to the fact that the transcriptional state of the cell is still proliferative and it is not completely differentiated in the case of optimal stages [43], since at the moment in which the grains of pollen begin to accumulate starch, lose their embryogenic capacity, giving rise to the gametophytic development pathway [44,45]. Although this was expected, the fact of being able to compare these results with those obtained in the optimal range gives strength to the usefulness of the prediction model that has been developed. Finally, as we have seen, this result has been consistent and independent of the induction protocol and genotypes used, although the response observed has been different in the two protocols tested.

In the case of the E6 protocol, a direct embryogenic response was obtained. This effect could be associated with the use of ZR as a growth regulator during induction as seen in other works [46]. Furthermore, the appearance of albino embryos was observed, this being the first report in the anther culture of eggplant as far as we know, but which has been associated with androgenic events in other crops such as cereals [47–51], tobacco or arabidopsis [52]. On the other hand, the results observed in the Cb protocol are coincident with some observations by other authors in which, depending on the eggplant genotype used, they obtain embryogenesis or different types of callogenesis in this induction medium [16]. In our case, callus was obtained in all three genotypes using Cb medium, while those same genotypes in the E6 medium gave rise to embryos.

The number of normal final embryos obtained using the E6 protocol was 77 among the three genotypes. Approximately 40% (31) of these embryos successfully acclimatized, giving rise to plants that, after being analyzed with flow cytometry, were mostly haploids (74.2% (23)), 9.7% (3) were diploids, and the remaining 16.1% (5) were mixoploids. The fact that most individuals were haploid ensures the androgenic origin of our materials and therefore the good result of the prediction model when choosing the correct induction stage. However, haploid plants present serious problems since they give rise to small flowers with different degrees of premature abscission, the abnormal formation of buds, anther and other organs within the flower and finally a reduction in the pollen viability [53–56], although in most cases spontaneous genome duplication is observed in this work, except for two individuals derived from accession BC 17-19, the rest of haploid plants ended up duplicating their genome spontaneously. For this reason, in these cases it is crucial to accompany the flow cytometric analysis with a molecular analysis; something that in some works has been done using sequence-related amplified polymorphism, simple sequence repeats or the random amplification of polymorphic DNA markers [57–59], but in many other works this is not done. Due to the dominance problems that some of the

commonly used molecular markers have and the low resolution regarding the percentage of genome explored with them [60], we used high-throughput genotyping using the SPET technology [31] with over 500 single-nucleotide polymorphism (SNP) markers. The genotyping results have been conclusive, showing that in the majority of cases, the percentage of heterozygosity of individuals of supposed androgenic origin did not exceed 1%, although in some cases it was slightly more elevated, but did not exceed 5%. In another eggplant study, in which SNP-type markers were used, similar results were observed in plants from anther culture [61]. The appearance of a residual degree of heterozygosity in doubled haploids may be due to the effect of somaclonal variation during in vitro culture, or most likely as a consequence of the mapping of the SNP markers to paralog genes within the genome [61]. Therefore, our molecular results together with the cytometry results confirm the haploid origin of these materials.

The application of Microscan to a real case in eggplant has shown that it has been able to optimize a procedure to produce doubled haploids in this crop, automating one of the most tedious parts of the process. It has also made it possible to greatly reduce the time needed for the fine-tuning of the protocol in new genotypes. This represents an important saving in resources for determining the size of the anthers that contain the optimal microspore stage for the induction of androgenesis. The implications of Microscan in the field of plant genetic breeding and commercial seed production are therefore very important. In this way, having a more efficient method of producing doubled haploid lines also means having a powerful tool for conducting research in plant genetics and breeding. Thanks to their genetic stability and homogeneity, doubled haploids are excellent materials to carry out research because they do not have dominance or intra-family segregation effects [62]. For this reason, the studies of the association of molecular markers with phenotypic characters through the use of bulked segregant analysis [63], the selection of recessive mutants in Tilling experiments [64,65] or its use in genetic transformation experiments to avoid the appearance of hemizygous individuals [66], are just some examples of the great utility of double haploids, and therefore of how the Microscan indirectly represents an improvement in this area. Another approach that benefits from the use of the Microscan and that has already been presented as an example in this work, is the development of pre-breeding populations such as introgression lines. These populations help to greatly expand the genetic pool of cultivated species by incorporating introgressions from the related wild species [1]. In this case, the doubled haploid technique can accelerate the fixation process of said materials with introgressions, reducing the multiple self-fertilization cycles to a single generation and facilitating the genetic description of traits of interest. On the other hand, commercial hybrids bring great economic benefits to both the seed companies that produce them and the farmers who grow them. Heterosis confers a series of general favorable characteristics such as greater tolerance to biotic and abiotic stresses, phenotypic homogeneity or higher productivity and yield, which provide them with a high added value [67]. However, the production of hybrid seed depends on obtaining pure parental lines, this being the basis of the breeding pipelines of most seed companies. This represents a very important investment in the production of double haploid plants. The integration of Microscan to the double haploid production protocols of these seed companies can lead to a reduction in the production costs of pure parental lines, thus also representing a reduction in the cost of hybrid seed. Finally, the optimization of this system in other crops beyond eggplant would provide these same benefits for their breeding.

5. Conclusions

In this multidisciplinary study, we developed a system (Microscan) based on deep learning to overcome one of the bottlenecks in the production of doubled haploids (DHs) in recalcitrant species. The development of artificial intelligence systems represents a great advance to speed up light microscopy studies and, in this case, it has been demonstrated. The application of

Microscan to eggplant genotypes has allowed selecting the optimal stage of induction which, together with the application of an innovative in vitro culture protocol, has allowed us to obtain DH plants with an androgenic induction rate higher than standard protocols and obtained excellent direct embryogenesis results. It is also the first time that this result has been confirmed with high-throughput genotyping in the case of eggplant. Our challenge now is to expand the detection capacity of Microscan to other species of commercial interest, thus increasing efficiency and breaking the barriers that in many cases are found when applying androgenesis techniques in many crops. Furthermore, this work can serve as a guide to develop similar applications where microscopy is used in agriculture and biology.

6. Statements

7. References

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Discusión General

Bloque 1: Desarrollo de poblaciones de *pre-mejora*

El uso de especies silvestres en la mejora genética vegetal ha mostrado en muchos cultivos un impacto económico positivo (Tyack y Dempewolf, 2015). Por ejemplo, en el tomate el desarrollo de variedades comerciales a partir de especies silvestres ha dado lugar a un incremento en el contenido de solidos solubles en los frutos aumentando su valor en $250 \cdot 10^6$ US$ anuales solo en EE. UU (Hunter y Heywood, 2011). Y al igual que en el tomate, otros cultivos como la berenjena, que aunque tenga una importancia económica menor, sigue siendo un cultivo muy relevante, pueden verse beneficiados del uso de las especies silvestres para aumentar su valor añadido o hacer conseguir cultivos más productivos. Sin embargo, hasta donde sabemos, no existen variedades comerciales de berenjena con introgresiones de especies silvestres disponibles y por tanto el potencial para la mejora genética de la berenjena está por explotar. Hoy en día, tan solo existe una colección de líneas de introgresión, con *S. incanum* (Gramazio et al., 2017), y una población MAGIC actualmente en desarrollo (Arrones et al., 2020), ambas llevadas a cabo por nuestro grupo de investigación, aunque todavía queda un largo camino hasta desarrollar líneas comerciales que aprovechen estos materiales.

Es por estos motivos que el desarrollo de esta tesis doctoral significa un importante avance para la mejora genética de este cultivo, ya que en ella se describe la obtención de una gran cantidad de materiales de introgresión a partir de especies representantes de los germoplasmas primario, secundario y terciario. Esto no solo tiene grandes implicaciones en lo que respecta a la adaptación del cultivo al cambio climático, sino que también significa un incremento enorme en la diversidad genética de la berenjena, lo que contrarresta los efectos de cuello de botella genético asociadoa a su domesticación y supone una estrategia de mejora genética preventiva (Prohens et al., 2017).

Obtención de retrocruzamientos entre S. melongena y S. elaeagnifolium; caracterización de los híbridos interespecíficos y los retrocruzamientos.

En esta tesis hemos obtenido el primer retrocruzamiento exitoso entre una especie nativa del Nuevo Mundo, perteneciente al germoplasma terciario de la berenjena (*S. elaeagnifolium*), y la berenjena cultivada (*S. melongena*), la cual es una especie domesticada del Viejo Mundo (Meyer et al., 2012). Esto tiene importantes implicaciones para la mejora genética de la berenjena, ya que la obtención de generaciones de retrocruzamiento con estos materiales significa que un nuevo acervo genético antes inaccesible, está ahora disponible.

Empleando el híbrido *S. melongena* x *S. elaeagnifolium* como parental femenino y *S. melongena* como parental masculino fue posible obtener frutos con semillas viables, aunque el grado de éxito fue inferior en comparación con retrocruzamientos realizados con otros híbridos interespecíficos entre la berenjena y otras especies del Viejo Mundo (Kouassi et al., 2016). Esto fue probablemente debido a la gran esterilidad del híbrido tal y como indican los análisis de fertilidad del polen. De hecho, no se obtuvieron frutos mediante autopolinización natural en el híbrido, lo cual indica que la esterilidad del polen era un factor mucho más limitante que la de los óvulos, tal y como se ha visto en otros cultivos (Dwivedi et al., 2008; Prohens et al., 2017). Los embriones de los frutos de la generación BC1 mostraron un aspecto y desarrollo normal, presentando tasas de germinación de la semilla superiores al 50%, con lo que en este caso no fue necesario aplicar la técnica del rescate de embriones, algo que sí que fue necesario para la obtención del híbrido interespecífico.

Uno de los factores que consideramos que fue crucial para el éxito en la obtención de la generación BC1 de este cultivo es el nivel de ploidía que presentaba el genotipo de *S. elaeagnifolium* que seleccionamos para este programa. Las variaciones en los niveles de ploidía son algo muy frecuente en *S. elaeagnifolium* existiendo individuos diploides y poliploides con distintas dotaciones (Moscone E., 1992; Acosta et al., 2005; Powell y Weedin, 2005; Scaldaferro et al., 2012; Knapp et al., 2017). Por ello seleccionamos una accesión diploide que presentaba un tamaño de genoma similar al de *S. melongena*, pudiendo ser esto un hecho fundamental para que los retrocruzamientos tuvieran éxito ya que la arquitectura del genoma (número de cromosomas o reordenamientos cromosómicos) puede afectar negativamente a la obtención de descendencia y más en especies tan alejadas filogenéticamente (Rieseberg, 2001). Finalmente, y asumiendo una segregación y recombinación normal en los gametos del híbrido F1, podríamos decir que el porcentaje del genoma de *S. elaeagnifolium,* representado en el conjunto de individuos de la BC1 (17) de la generación BC1 fue superior al 99%.

Fenotípicamente, los híbridos interespecíficos presentaron características intermedias en comparación con los parentales, aunque generalmente más cercanas a *S. elaeagnifolium*, un fenómeno común en las hibridaciones interespecíficas entre berenjena y especies silvestres (Prohens et al., 2013; Kaushik et al., 2016). Sin embargo, a diferencia de otros híbridos interespecíficos (Kaushik et al., 2016), éstos no mostraron vigor híbrido, probablemente debido a la gran distancia filogenética entre las dos especies (Vorontsova et al., 2013). Por otra parte, sí que se observaron eventos de herencia transgresiva al presentarse espinas en los híbridos, carácter que ninguno de los dos parentales presentaba. Este fenómeno es bastante común en los híbridos interespecíficos de berenjena donde al cruzar dos especies sin espinas éstas aparecen en la descendencia debido probablemente a que las mutaciones que confieren la ausencia de espinas son diferentes en las dos especies que se han cruzado (Lester, 1986; Varoquaux et al., 2000; Kouassi et al., 2016; Plazas et al., 2016; Prohens et al., 2012). Aunque sin lugar a duda, uno de los caracteres más importantes que se describieron en el hibrido interespecífico fue la arquitectura de su sistema radicular. Aunque la longitud de su raíz principal fue similar a la de *S. melongena*, el hecho de que la densidad relativa de raíces secundarias en la raíz principal del híbrido fue menor, sugiere que este es capaz de explorar otras áreas del suelo (Chen et al., 2014), mientras que *S. melongena* concentraba su crecimiento en la zona donde el sistema de irrigación por goteo suministraba los nutrientes. Este fue uno de los primeros caracteres de tolerancia a cambio climático que pudimos identificar en estos materiales. Este comportamiento en el crecimiento de las raíces sugiere que sistema radicular de la berenjena podría mejorarse a través del desarrollo de las poblaciones de introgresión y aumentar la tolerancia a la sequía al igual que pasa en *S. elaeagnifolium* (Christodoulakis et al., 2009). Por otra parte, las especies silvestres relacionadas con la berenjena presentan un perfil de compuestos fenólicos muy diverso en los cuales el ácido clorogénico es el componente principal (Stommel y Whitaker, 2003; Whitaker y Stommel, 2003; Prohens et al., 2013). En nuestro caso, *S. elaeagnifolium* mostro un perfil considerablemente distinto al de *S. melongena*, mientras que el hibrido F1 mostro un perfil intermedio entre ambos. Este hecho indica que los materiales de introgresión derivados de *S. elaeagnifolium* también podrían contribuir a mejorar la calidad nutricional de la berenjena (Kaushik et al., 2015)

Con lo que respecta a la caracterización de los individuos de la BC1, al igual que en otros estudios (Pohens et al., 2012, 2013), se observó un retorno hacia las características de *S. melongena* además de una importante segregación para multitud de caracteres en los cuales los parentales diferían como por ejemplo el color de las flores. Sorprendentemente la fertilidad del polen aumentó muy rápido con una media del 19.4% frente a 2.5% del hibrido F1, algo que también

se ha observado previamente en otros cultivos (Wall, 1970; Prohens et al., 2017). Gracias a esto, el desarrollo de las siguientes generaciones se ve facilitado ya que cada vez será más sencillo obtener retrocruzamientos viables y sin problemas de cuajado de frutos o germinación de semillas.

En definitiva, en este primer trabajo se abrió una vía nueva para introducir el fondo genético de *S. elaeagnifolium*, una especie con un importante potencial para la mejora frente al cambio climático en la berenjena. Este desarrollo fue el punto de partida de una de las tres líneas de introgresión desarrolladas dentro del marco de esta tesis doctoral, discutiéndose en el próximo apartado las cuestiones más relevantes al respecto.

Desarrollo de material de pre-mejora en berenjena con introgresiones de especies silvestres

En el apartado anterior se han discutido los resultados más relevantes de lo que fue el inicio del desarrollo de una de las tres líneas de introgresión desarrolladas en esta tesis, concretamente la obtención de los híbridos interespecíficos y la primera generación de retrocruzamientos con la especie de germoplasma terciario *S. elaeagnifolium*. En esta parte de la tesis se describe el resto del desarrollo de esta línea de intrgresión y de dos más, la línea de introgresión con la especie de germoplasma primario *S. insanum* y con la especie de germoplasma secundario *S. dasyphyllum*. Además, también se describe el desarrollo del modelo experimental Micro-Mel a partir de materiales de retrocruzamiento con *S. anguivi*.

Este trabajo de introgresiómica ha supuesto un enorme incremento en diversidad genética de la berenjena ya que en él se han empleado especies representantes de todos sus grupos genéticos (Prohens et al., 2017). La incorporación de potenciales fuentes de resistencia y tolerancia a estreses de tipo biótico y abiótico es aquello que le da valor a este desarrollo significando en sí mismo un proyecto de mejora preventiva. La elección de estas tres especies en concreto se hizo en base a sus características y potencial para adaptarse a situaciones similares a las que se están dando en muchas partes del mundo debido a los efectos del cambio climático. *Solanum insanum* es una maleza distribuida por enormes áreas a lo largo del mundo, ésta se desarrolla incluso en suelos infértiles y por tanto presenta un gran potencial para su adaptación a la sequía y otros tipos de estreses abióticos ambientales (Ranil et al., 2017). Por otra parte *S. dasyphyllum* es una especie africana que se ha utilizado tradicionalmente en África por sus propiedades medicinales (Bukenya and Carrasco 1994; Ajayi et al., 2013; Kidane et al., 2013); pero además presenta tolerancia a ácaros fitófagos, inhibiendo la ovoposición de *Rhipicephalus appendicuhtus* (Van Puyvelde et al., 1985); también presenta ciertos niveles de resistencia a *Fusarium spp.* (Mwanik et al., 2015). Por último, *S. elaeagnifolium* es una mala hierba originaria del continente americano (Knapp et al., 2017) altamente invasiva y tolerante a la sequía (Christodoulakis et al., 2009) que además presenta un interesante perfil en compuestos fenólicos que también podría ser útil en la mejora de las propiedades bioactivas de la berenjena (Kaushik et al., 2015, Garcia-Fortea et al., 2019). Estos motivos hacen de estas tres especies los donantes ideales para el desarrollo de estas tres colecciones de ILs, además del interés evolutivo y taxonómico debido a que entre ellas se encuentran representados los tres grupos de germoplasma de la berenjena.

En este trabajo se han utilizado dos estrategias de genotipado diferentes (Sequenom MassARRAY y SPET). La tecnología SPET (Single Primer Enrichment Technology) ha sido desarrollada recientemente (Barchi et al., 2019) y ha permitido aumentar enormemente la resolución del genotipado debido a la gran cantidad de marcadores analizados (más de 10,000 posiciones en el caso del programa con *S. elaeagnifolium*) en comparación con las 70 posiciones

SNP evaluados con la tecnología Sequenom MassARRAY. Este es el motivo por el cual se ha conseguido acelerar tanto el proceso de desarrollo de las ILs, permitiendo hacer una selección más eficiente y consiguiendo disminuir los niveles de heterocigosidad en las zonas no objetivo de las líneas de forma muy rápida. El resultado de la aplicación de la tecnología SPET ha sido el desarrollo de líneas muy avanzadas con un alto porcentaje del fondo genético de *S. melongena* recuperado y una representación completa y ordenada del genoma de las tres especies silvestres empleadas. Actualmente los pasos a seguir para la finalización de las ILs consisten en fijar los fragmentos realizando autopolinizaciones y/o cultivos de anteras (en paralelo) para las generaciones BC5 y BC4S1 de las ILs con *S. insanum* y *S. dasyphyllum*. Por otra parte, la población IL con *S. elaeagnifolium* necesita una generación más de retrocruzamiento antes de pasar a la fase de fijación de fragmentos, ya que todavía es necesario recuperar algo más del fondo genético de la berenjena.

Otro resultado de gran interés descrito en esta parte de la presente tesis doctoral fue el fenotipo enano encontrado en materiales de introgresión con *S. anguivi*. A partir de la selección de un individuo detectado en una generación BC2 y el posterior trabajo de autopolinización y reselección, ha sido posible desarrollar el modelo experimental Micro-Mel. Este tipo de modelos, al igual que el Micro-Tom (Scott y Harbaugh 1989) son herramientas muy útiles para desarrollar experimentos de genética molecular y transformación (Dan et al., 2006; Chetty et al., 2013), ensayos de sequía y salinidad en interior (Yin et al., 2010; Barsanti et al., 2019), así como para desarrollar proyectos de mejora acelerada gracias a la posibilidad de realizar más de un ciclo por año en cámaras de cultivo (Sestari et al., 2014). Actualmente estos materiales se encuentran en una generación BC2S3 y ya tenemos frutos con semillas BC2S4, en esta descendencia ya tendremos materiales fijados y disponibles para su uso como modelo experimental.

En las ILs también se han observado segregación para caracteres de interés agronómico y de calidad. Uno de ellos es la espinosidad, la cual hemos sido capaces de asociar a una región muy concreta del cromosoma 6, donde otros autores ya habían postulado su implicación con la regulación genética de este carácter (Frary et al., 2014; Gramazio et al., 2014; Portis et al., 2015) y donde recientemente se ha confirmado la presencia de un gen mayor (Miyatake et al. 2020). En el caso del color del fruto también hemos llegado a identificar algunas regiones implicadas en su regulación. Por ejemplo, en las ILs con *S. dasyphyllum* se han encontrado individuos con frutos completamente verdes (como el parental donante), mientras el resto de las plantas de la familia tienen frutos blancos. El color blanco se debe a la ausencia de pigmentos en el fruto (Nothmann et al., 1975; Daunay et al., 2004) y aunque el parental donante (DAS1) los frutos son de color verde, debido a las sucesivas rondas de retrocruzamiento con el parental recurrente (MEL1) cuyos frutos son blancos, los fragmentos que regulan la síntesis de clorofila en el fruto solo están presentes en aquellas líneas seleccionadas para esa región genómica. En otros estudios de nuestro grupo, en los que se está desarrollando una población MAGIC, también se ha observado este carácter pudiendo mapearlo en una región específica del cromosoma 8 localizada aproximadamente a 104 MB. Estos resultados coinciden con las observaciones realizadas en el último genotipado de las ILs con *S. insanum* y *S. dasyphyllum*. Algo similar también se ha observado para el color de fruto morado en las ILs con *S. insanum*. Este color se produce por la acumulación de antocianinas (Tigchelaar et al., 1967), y parece estar regulado por una región en el cromosoma 10, algo que ya ha sido reportado por otros autores (Barchi et al., 2012; Cericola et al., 2014; Barchi et al., 2019b), no obstante, esta región todavía no está tan bien definida como las anteriores.

Los resultados presentados y discutidos en este primer bloque suponen un resumen de aquellas observaciones más relevantes de un proyecto de pre-mejora en berenjena que se inició en el año 2013 y ya está en su etapa final. En su conjunto estos materiales élite, ya están listos para ser usados por los mejoradores e investigadores, suponiendo una herramienta de un valor incalculable para la mejora genética de la berenjena. Son en sí mismos un reservorio de genes de gran utilidad para la mejora frente al cambio climático y la mejora preventiva. Estos materiales ya están siendo evaluados por algunas empresas privadas y esperamos que pronto sirvan para desarrollar una nueva generación de variedades de berenjena mejoradas y adaptadas a las nuevas condiciones climatológicas del planeta.

Bloque 2: Desarrollo de herramientas biotecnológicas

El desarrollo de materiales con un incremento en su diversidad genética tal y como se ha discutido en el bloque anterior, es una herramienta fundamental para la mejora genética de le berenjena. No obstante, esto no es suficiente para poder explotar al máximo sus recursos, ya que una vez se desarrollan las poblaciones, el trabajo no ha hecho nada más que comenzar. Es en este punto cuando a partir de estas plantas se inicia la investigación básica y los programas de mejora, siendo para ambos necesario disponer de las herramientas biotecnológicas adecuadas. El desarrollo de protocolos de cultivo *in vitro* es fundamental como, por ejemplo, para el desarrollo de organismos genéticamente modificados (Shelton et al., 2018), que ayudaran a comprender los mecanismos genéticos gracias a la inactivación de genes mediante la técnica de CRISPR-Cas9 (Zheng et al., 2020). El desarrollo de herramientas de *speed breding* para acelerar la obtención de materiales fijados, también requiere del desarrollo de protocolos de cultivo *in vitro* eficientes y de otras herramientas que incrementen la tasa de éxito en la obtención de plantas dobles haploides (Rivas-Sendra et al., 2015). El desarrollo de variedades nuevas de berenjenas sin semillas, las cuales están siendo demandadas cada vez más por los consumidores (Maestrelli et al., 2003; Pandolfini, 2009), también requiere de este tipo de nuevas soluciones ya que todavía no existen metodologías estables para la manipulación de la ploidía en este cultivo. Por estos motivos, en este bloque se decidió desarrollar una serie de herramientas biotecnológicas, principalmente basadas en el cultivo *in vitro,* que permitiesen superar estas barreras y que en su conjunto representasen una solución completa para explotar al máximo las colecciones de materiales desarrollados en el Bloque I.

Desarrollo de un protocolo basado en el uso del ribósido de zeatina para la regeneración in vitro de la berenjena y su aplicación para la obtención de plantas poliploides.

El desarrollo de métodos de regeneración para obtener plantas *in vitro* es esencial para múltiples aplicaciones en mejora genética vegetal como por ejemplo la micropropagación. Hasta donde nosotros sabemos, no existen métodos universales para la regeneración de la berenjena, considerándose un cultivo recalcitrante a las metodologías de cultivo *in vitro*. Un ejemplo de esto es la enorme cantidad de publicaciones relacionadas con la regeneración de la berenjena donde se proponen múltiples estrategias, pero ninguna de ellas es capaz de evitar el efecto del factor genotipo (Scoccianti et al., 2000; Rahman et al, 2006; Bhat et al., 2013; Swathy et al., 2017). Es por este motivo, y por la propia necesidad interna de disponer de un protocolo de regeneración estable, por lo que se planteó este trabajo.

Tras investigar varias opciones se llegó a la conclusión de que había un regulador de crecimiento que apenas había sido explorado en el caso de la berenjena (muy probablemente por su coste elevado). Esta fitohormona es el ribósido de zeatina (ZR) y ya ha mostrado excelentes resultados en otros cultivos como por ejemplo *Brassica nigra* (Narasimhulu et al., 1993), *S. lycopersicum*

(Hossain et al., 1995) o en *Olea europea* (Farooq et al., 2017). En el caso de la berenjena, dos estudios anteriores habían mostrado resultados muy prometedores en la regeneración empleando el ZR (Singh et al., 2010; Muktadir et al., 2016), no obstante, en ellos empleaban siempre genotipos muy similares y el diseño experimental no permitía extraer información suficiente para desarrollar un protocolo de carácter universal. Por ello, a partir de esta base desarrollamos un experimento en el que evaluamos el efecto del ribósido de zeatina a distintas concentraciones y en combinación con el ácido indolacético (IAA). Estudiamos su efecto en distintos tejidos (hipocótilo, cotiledón y hoja) y bajo diferentes condiciones de inducción (luz y oscuridad) en un total de 6 genotipos que representaban la diversidad genética dentro de la especie *S. melongena* (Acquadro et al., 2017; Muñoz-Falcon et al., 2009; Kaushik et al. 2016), incluyendo su ancestro silvestre *S. insanum* (Ranil et al., 2017).

Tras realizar el experimento observamos que el tejido más organogénico era el cotiledón bajo condiciones de inducción de luz (fotoperiodo 16 h luz / 8 h oscuridad). Estos resultados fueron consistentes con otros estudios en berenjena (Bhat et al., 2013) y en otros cultivos como el melón (Souza et al., 2006), el tomate (Abdalmajid et al., 2011) o el guisante (Matand et al., 2013). Por otra parte, la concentración de 2 mg/L de ZR en ausencia de IAA en un medio con base MS (Medio E6) fue aquella que dio un mejor resultado en cuanto a la regeneración, a diferencia de otros estudios donde la concentración óptima era de 1 mg/L y 0.1 mg/L de IAA (Singh et al., 2010). En general, concentraciones superiores a 2 mg/L de ZR desencadenan una disminución de la respuesta organogénica, por otra parte, al combinarlas con ácido giberélico o con IAA (Bhadra et al., 1994; Chen y Adachi, 1994) disminuye su efecto inductor existiendo estudios en los que se ha reportado un efecto tóxico del IAA en tomate a partir de concentraciones de 0.5 mg/L (Chen y Adachi, 1994). El medio propuesto en este trabajo incrementa la inducción de organogénesis en berenjena simplificando la formulación.

A continuación, era necesario comprobar si el ZR presentaba una tasa de inducción homogénea independientemente del genotipo empleado. Dentro de las accesiones MEL1 y MEL3 que se emplearon en las primeras fases del experimento no se observaron diferencias en cuanto a los niveles de respuesta. En el resto los genotipos, incluida la especie *S. insanum*, tampoco se vieron grandes diferencias en la respuesta al protocolo, obteniendo rendimientos muy similares en producción de brotes por explante en los tejidos de cotiledón. Estos resultados sugieren que el efecto del genotipo en este protocolo es muy bajo siendo eficiente en la regeneración *in vitro* de un amplio rango de variedades de berenjena incluyendo especies silvestres. Además del protocolo de regeneración, también se propuso un protocolo de enraizamiento en el cual el uso del medio R2 (1mg /L de ácido indolbutírico) mostro la formación de la mejor arquitectura radicular con el mayor número de raíces secundarias. Este es un paso muy importante y con grandes implicaciones en la aclimatación de las plántulas, ya que si se maximiza el número de nódulos radiculares se incrementa la posibilidad de formar raíces funcionales una vez sacada la planta a condiciones de cultivo *ex vitro* (Altpeter et al., 2016; Wamann et al., 2015).

Durante el desarrollo de este proyecto, nos encontramos con un curioso resultado y es que un porcentaje importante de las plantas que conseguíamos regenerar a partir de los tejidos de cotiledón e hipocótilo, presentaban niveles de ploidía superiores al normal. Frente a este hecho analizamos los niveles de ploidía de los tejidos iniciales (hipocótilo, hoja y cotiledón) y efectivamente encontramos diferencias para su patrón polisomático, los hipocótilos y los cotiledones presentaban un porcentaje de células poliploides al menos dos veces más alto que las hojas. Esto se debe a que, durante las etapas de desarrollo tempranas de la planta, tienen lugar un elevado número de endorreduplicaciones en estos tejidos para acelerar la tasa de

crecimiento incrementando el tamaño de sus células (Gilissen et al., 1993; Smulders et al., 1994). Debido a la presencia de estas células poliploides en los tejidos iniciales empelados durante el protocolo de regeneración, obteníamos dichas plantas poliploides ya que estas células poseían el suficiente potencial organogénico como para formar brotes. No obstante, en el caso de cotiledón, a pesar de presentar un número menor de celulas poliploides su capacidad organogénica era mayor, por ello fue el tejido con mayor tasa de regenerantes poliploides. Esto fue un resultado muy interesante ya que nos permitió desarrollar un protocolo para obtener plantas poliploides en berenjena sin necesidad de emplear agentes antimitóticos con los beneficios que esto implica. Las técnicas tradicionales para manipular la ploidía de los organismos se basan en el uso de antimitóticos (Ascough y van Staden 2008; Aleza et al. 2009) que no dan lugar a poliploides estables debido a que lo que generan son quimeras que con el tiempo acaban revirtiendo a la ploidía original (Allum et al. 2007).

En este trabajo se desarrolló un protocolo universal para la regeneración de la berenjena basando en el uso del ZR. Esto tiene grandes implicaciones para la mejora genética vegetal de este cultivo ya que abre la posibilidad de aplicar metodologías como la edición genética, algo que antes no era posible en este cultivo o presentaba una dificultad añadida a la hora de regenerar las plantas. Por otra parte, gracias al uso racional de los patrones polisomáticos ha sido posible desarrollar en paralelo un protocolo de obtención de plantas poliploides, algo que también abre una nueva vía hacia el desarrollo de plantas triploides sin semillas.

Desarrollo de un sistema basado en la Inteligencia Artificial (Microscan) para la identificación de los estadios de desarrollo del polen y su aplicación en la obtención de líneas dobles haploides de berenjena.

La obtención de líneas puras es una tarea fundamental tanto en el desarrollo de poblaciones experimentales de pre-mejora como en la obtención de líneas parentales en las empresas de mejora genética para la producción de híbridos comerciales. Tradicionalmente esto se ha realizado mediante múltiples ciclos de autofecundación (entre 7 y 8 generaciones), consumiendo una gran cantidad de tiempo, dinero y espacio de cultivo para conseguirlo. Con el descubrimiento de la androgénesis y la posibilidad de obtener plantas dobles haploides en una sola generación a partir del cultivo de las anteras o las microsporas, el proceso de obtención de líneas puras se aceleró, no obstante, este proceso no está exento de complicaciones. Algunos cultivos como la berenjena son recalcitrantes y presentan varios cuellos de botella que hay que superar.

Es por ello por lo que en esta parte de la tesis desarrollamos una solución basada en la inteligencia artificial para superar uno de estos cuellos de botella. Microscan es una herramienta que permite hacer análisis microscópicos automatizados de modo que la selección de los rangos de antera que maximizan la población celular más susceptible de ser inducida en un proceso androgénico se realiza de forma más eficiente. Es decir, durante los cultivos de antera, no todas contienen en su interior el estadio celular adecuado para la inducción que desencadena el proceso androgénico y la posterior formación de embriones. Este estadio coincide con el de microspora vacuolada o el de polen bicelular joven (Germanà, 2011; Salas et al., 2012) y dicha caracterización y selección siempre se ha hecho manualmente siendo una tarea agotadora visualmente y poco eficiente. Con Microscan podemos automatizar esta parte del trabajo e incrementar el número de análisis por unidad de tiempo que podemos realizar (Akbar et al., 2018; Yan et al., 2019). Gracias a los importantes avances que se han hecho en el campo del *Deep Learning,* siendo el desarrollo del modelo RetinaNet el punto de inflexión (Lin et al., 2017), el desarrollo de este tipo de modelos de predicción ha mejorado mucho. Es por ello por lo que

Microscan ha sido desarrollado a partir de este modelo obteniéndose un valor de precisión media superior al 86%. Para hacer un modelo más realista, Microscan juzga los estadios de forma continua, asignando un valor de confianza a las células que identifica. De esta manera nos informa de que probabilidad tiene cada celula de pertenecer a una clase o a otra, generando distribuciones, al igual que un ojo experto identificaría diferentes grados de madurez en el desarrollo de una microspora. Con esta información podemos saber qué porcentaje de cada tipo celular contiene en su interior una antera, de modo que podemos determinar que rango de tamaño maximiza el contenido en microsporas vacuoladas y seleccionarlas para los experimentos de androgénesis.

Tras el desarrollo del Microscan, este se aplicó a un caso real en el que se seleccionaron tres genotipos de la línea de introgresión con *S. elaeagnifolium* (BC3 17-8, BC3 17-9 y BC3 17-4). El rango de anteras que maximizaba el número de microsporas vacuoladas fue el de 5.5-6 mm mientras que también se seleccionaron dos rangos como control negativo 3.5-4 mm y >6 mm, los cuales maximizaban el número de microsporas jóvenes y polen respectivamente. En todos los genotipos se observó respuesta androgénica en el rango de 5.5-6 mm tal y como se esperaba, mientras que en los rangos control no se observó ningún tipo de respuesta. Por otra parte, se observaron diferencias significativas en cuanto al tipo de respuesta observada en los dos protocolos de androgénesis aplicados (E6 y Cb). En el caso del protocolo E6 la respuesta fue de tipo embriogénesis directa, un efecto que podría asociarse al uso del ZR tal y como se ha visto en otros apartados de esta tesis y en otros trabajos previos (Emrani et al., 2017). Algunos de estos embriones eran albinos, algo que nunca se había descrito en cultivo de anteras de berenjena pero que es característico de los procesos androgénicos especialmente en el caso de los cereales (Immonen y Anttila, 2000; Kiviharju et al., 2000; Liu et al., 2002; Caredda et al., 2004; Makowska y Oleszczuk, 2014), el tabaco o arabidopsis (Kumari et al., 2009). En el caso del protocolo Cb el rango de 5.5-6 mm dio lugar a la formación de callos de origen androgénico algo que ya ha sido descrito por muchos autores y que se asocia al factor genotipo (Salas et al., 2011).

Finalmente, se obtuvieron 77 embriones y aproximadamente el 40% (31) de estos se pudieron aclimatar dando lugar a plantas cuya ploidía fue analizada mediante citometría de flujo. Veintitrés de estos embriones fueron haploides, tres fueron diploides y cinco mixoploides. El hecho de que la mayoría fueran haploides justifica de nuevo que estamos ante un proceso androgénico exitoso, y estos no son de origen somático, demostrando que Microscan predijo bien el rango de tamaño de antera adecuado para el cultivo. La mayoría de estos embriones experimentaron una duplicación espontanea de su genoma y se les extrajo ADN a todas las plantas aclimatadas para secuenciarlas mediante la tecnología de genotipado masivo SPET. Se seleccionaron 500 marcadores SNPs que fueran heterocigotos en las plantas donantes para evaluar qué porcentaje de heterocigosidad que presentaban estas plantas. Los resultados del genotipado confirmaron que estas plantas tenían un porcentaje de heterocigosidad en la mayoría de los casos inferior al 1%, siendo esta la primera vez que se confirma el origen haploide de plantas de berenjena empleando este tipo de aproximación de genotipado masivo.

En su conjunto, este trabajo demuestra como la tecnología Microscan puede adicionarse al conjunto de herramientas biotecnológicas de *speed breeding* permitiendo acelerar y optimizar la producción de plantas dobles haploides. Gracias a esta herramienta la obtención de líneas puras es más simple. Este tipo de individuos presentan unas características excepcionales para para desarrollar experimentos de genética debido a que no presentan efectos de dominancia o segregación intra-familar (Snape, 1989). Por ello los estudios de asociación de marcadores moleculares a caracteres fenotípicos mediante *Bulked Segregant Analysis* (Huang et al., 2020),

la selección de mutantes recesivos en experimentos de *Tilling* (Szarejko y Forster, 2007; Ferrie et al., 2008) o la realización de experimentos de transformación evitando la aparición de individuos hemicigóticos (Ferrie et al., 2008), son solo algunos de los ejemplos de la gran utilidad de los dobles haploides en el área de la genética y como el Microscan de forma indirecta supone un avance en ella. Otra aproximación que se beneficia del uso del Microscan es el desarrollo de poblaciones de pre-mejora, como son por ejemplo las líneas de introgresión. Como ya se ha discutido anteriormente, estas poblaciones ayudan a ampliar en gran medida el acervo genético de las especies cultivadas mediante la incorporación de introgresiones de especies silvestres relacionadas (Prohens et al., 2017). En este caso, la técnica de los haploides dobles puede acelerar el proceso de fijación de dichos materiales con introgresiones, reduciendo los múltiples ciclos de autopolinización necesarios a una sola generación. Finalmente, los híbridos comerciales aportan grandes beneficios económicos tanto a las casas de semillas que los producen como a los agricultores que los cultivan. La heterosis confiere una serie de características generales favorables como es su mayor tolerancia a los estreses bióticos y abióticos, la homogeneidad fenotípica o su mayor productividad y rendimiento, confiriéndoles un gran valor añadido (Birchler, 2015). Sin embargo, la producción de semilla híbrida depende de la obtención de líneas parentales puras, siendo esta la base de los procesos de mejora de la mayoría de las empresas de semillas. Esto representa una inversión anual muy importante en la producción de plantas dobles haploides. La integración de Microscan a los protocolos de producción dobles haploides de estas empresas puede significar una reducción en los costes de producción de las líneas parentales puras, suponiendo así también que representa una reducción en el coste de la semilla híbrida, algo beneficia tanto a los productores como a los propios agricultores. Finalmente, la optimización de Microscan en otros cultivos más allá de la berenjena, supondría un gran avance en la mejora genética vegetal.

Relevancia del trabajo realizado y perspectivas futuras

Los materiales, conocimientos y herramientas desarrolladas en esta tesis doctoral representan un gran avance en la mejora genética y la biotecnología de la berenjena. Así, suponen la base para futuros trabajos, principalmente en berenjena, aunque algunas de las aproximaciones también podrían adaptarse y ser útiles para otras solanáceas e incluso otras especies de interés comercial. En este estudio se inicia el camino hacia el desarrollo de una nueva generación de variedades de berenjena adaptadas al cambio climático, ampliando su base genética, desarrollando nuevos materiales, modelos experimentales y metodologías que tendrán un gran impacto tanto en investigación básica como aplicada. De hecho, estos materiales serán evaluados en futuros trabajos del grupo (alguno de los cuales ya se encuentra en desarrollo) con tal de encontrar tolerancias o resistencias a estreses relacionados con el cambio climático.

El desarrollo de las colecciones de ILs desarrolladas con el enfoque de introgresiómica empleado en este trabajo han ampliado enormemente el fondo genético de un cultivo que presentaba graves problemas de erosión genética. valiéndonos de los recursos fitogenéticos que encontramos en sus grupos de germoplasma primario, secundario y terciario hemos solucionado en gran medida este problema. Por otra parte, los esfuerzos invertidos en el desarrollo de protocolos de cultivo *in vitro* eficientes, así como de sistemas que hacen más fácil la obtención de líneas puras han conseguido dotar a los investigadores de las herramientas necesarias para poder investigar de forma más eficiente una especie que apenas contaba con este tipo de técnicas disponibles. Y en su conjunto todos estos recursos suponen un bien de incalculable valor para la mejora genética de la berenjena, sus métodos de mejora y *speed breeding*.

Finalmente, las perspectivas futuras de este trabajo se centran en acabar aquellos desarrollos que por el tiempo que requiere su desarrollo no han podido finalizarse dentro del marco de esta tesis doctoral. En primer lugar, queda finalizar el desarrollo de las ILs ya que estas todavía presentan fragmentos de la especie silvestre en heterocigosis y es necesario aplicar un par de ciclos de autofecundación y selección más para terminar de fijar las líneas. Por otra parte, el modelo Micro-Mel al igual que las ILs ha de terminar de fijarse y ser evaluado mediante varios experimentos. Es necesario comprobar su capacidad de crecer, florecer y fructificar en cámaras de cultivo, ya que hasta ahora el proyecto se ha realizado en invernadero, pero para que sea un modelo experimental útil también ha de comportarse adecuadamente en cámara de cultivo y bajo condiciones de estrés. Otra de las cosas que también se puede hacer es evaluar la eficiencia del protocolo de regeneración en un experimento real de trasformación mediante CRISPR-Cas9. Hasta ahora el protocolo ha demostrado ser útil para regenerar planta, pero queda ver cómo se comporta al aplicarse en unas condiciones en las que se inocula el tejido con *Agrobacterium* y se añaden agentes de selección al medio de cultivo. Finalmente, la aplicación del Microscan a más genotipos y su implantación como una herramienta de uso estandarizado para la obtención masiva de dobles haploides para fijar materiales en berenjena también puede dar lugar a resultados de gran relevancia. Así mismo, adaptar el algoritmo al reconocimiento de más tipos celulares en otros cultivos de interés como pueden ser el pimiento, el tomate o el melón es un reto futuro.

En definitiva, son muchas las vías que ha abierto esta tesis y muchísimas más las que están por venir y que ni siquiera nos plantemos en este momento. Así, esta tesis doctoral es el principio de un largo camino todavía por recorrer, suponiendo un paso de gran relevancia para la consecución de estos objetivos presentes y futuros.

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Conclusiones

1. Se ha conseguido por primera vez la obtención de una generación de retrocruzamiento entre la berenjena y una especie silvestre relacionada del germoplasma terciario (*Solanum elaeagnifolium*), la cual es originaria del Nuevo Mundo. Los resultados sugieren que estos materiales de introgresión serán de un enorme interés para la mejora genética de la berenjena debido al tremendo potencial para incrementar la tolerancia a estreses del tipo abiótico como es la tolerancia a la sequía, así como también para mejorar las propiedades bioactivas de este cultivo. Estos materiales también podrían contribuir a incrementar la tolerancia a plagas y enfermedades a partir de caracteres de interés todavía inexplorados en el fondo genético de *S. elaeagnifolium*.

2. La utilización de selección asistida por marcadores moleculares ha permitido el desarrollo de tres colecciones de materiales de introgresión con especies representantes del germoplasma primario (*S. insanum*), secundario (*S. dasyphyllum*) y terciario (*S. elaeagnifolium*), que ha ampliado el fondo genético de la especie cultivada *S. melongena*.

3. Dentro de estas colecciones de introgresión se han acotado las regiones genéticas que controlan dos caracteres de gran interés para la mejora, como son la espinosidad y el color del fruto. Esto muestra el gran potencial de este tipo de materiales para el cartografiado e identificación de genes de interés. Por otra parte, en los materiales de retrocruce con *S. anguivi* se ha identificado un fenotipo enano el cual se ha seleccionado y autofecundado para desarrollar el modelo de estudio experimental Micro-Mel.

4. Se ha desarrollado un protocolo universal para la regeneración *in vitro* de la berenjena a través del uso de una fitohormona muy poco utilizada, el ribósido de zeatina. Gracias al uso de este regulador del crecimiento se ha minimizado el efecto genotipo consiguiendo inducir procesos de organogénesis directa en materiales de berenjena genéticamente muy diversos y en su ancestro silvestre *S. insanum,* algo que hasta la fecha significaba el principal cuello de botella a la hora de la regeneración *in vitro* con este cultivo, llegándose a considerar a la berenjena una especie recalcitrante.

5. Aprovechando el patrón polisomático de los cotiledones e hipocótilos de la berenjena, a través de este protocolo de organogénesis hemos sido capaces de obtener plantas poliploides estables sin necesidad de emplear agentes antimitóticos. Esto supone un gran avance ya que se han evitado problemas de quimerismo obteniendo plantas tetraploides estables a lo largo del tiempo y las generaciones, abriendo una posible nueva vía para la producción de frutos sin semillas en este cultivo mediante la producción de híbridos triploides. Finalmente se ha establecido un protocolo estandarizado para la producción de poliploides *in vitro* en berenjena.

6. El desarrollo del sistema Microscan es la primera herramienta para la identificación y caracterización de los estadios de desarrollo del polen en berenjena basada en inteligencia artificial desarrollada hasta la fecha. Su aplicación en un caso real ha permitido seleccionar de forma eficiente aquellos rangos de antera en los que se maximiza la cantidad de microsporas inducibles, incrementando el rendimiento de producción en dobles haploides. Esto supone un gran avance ya que mediante este sistema es posible automatizar una de las

partes más tediosas en los protocolos de androgénesis, reduciendo así sus costes de ejecución. Finalmente, esta metodología ha demostrado ser válida, con lo que su extensión a otras especies de interés es un objetivo futuro plausible que se deberá abordar.

7. El uso conjunto del Microscan y de un nuevo protocolo de androgénesis ha dado como resultado la embriogénesis directa en individuos de la generación BC4 del programa de líneas de introgresión con *S. elaeagnifolium*. Tras el genotipado mediante SPET de las plantas obtenidas a partir de estos embriones se ha demostrado que estas eran dobles haploides. Esto demuestra como este procedimiento reduce a una única generación el fijado genético de las líneas de introgresión imponiéndose como una importante estrategia de *speed breding* a tener en cuenta en los métodos de mejora.

8. El desarrollo de esta tesis doctoral tiene importantes implicaciones en la mejora genética de la berenjena, suponiendo un gran avance en cuanto a los materiales y herramientas biotecnológicas disponibles. El trabajo aquí desarrollado abre nuevas fronteras en la investigación y mejora genética de la berenjena particularmente para la adaptación al cambio climático, permitiendo el desarrollo de nuevos proyectos que permitan modernizar y desarrollar nuevas variedades mejoradas en menos tiempo y así hacer frente a los nuevos retos en el escenario de cambio climático que de forma inevitable la humanidad ha de afrontar.