Bases anatómicas y fisiológicas del desarrollo de raíces adventicias en *Acca sellowiana* (Myrtaceae)

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Doctora en Ciencias Agrarias

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Tabla de contenido

Página

Resumen

El éxito en el desarrollo de raíces adventicias (RA) es una limitante importante para la propagación de especies leñosas. La habilidad para formar RA está muy afectada por el genotipo. Sin embargo, no se conocen exactamente las causas de esas diferentes respuestas. Un paso importante para dilucidar los mecanismos moleculares que regulan la diferenciación de RA en *Acca sellowiana* es la identificación y estudio de expresión de genes involucrados en la regulación del proceso. Se seleccionaron dos genotipos con capacidad de enraizamiento contrastante (R: alta y NR: baja); se estudió la expresión de ciertos genes, la anatomía y los cambios bioquímicos en respuesta al agregado exógeno de ácido indol butírico y el tipo de estaca. Los nuevos meristemas se desarrollaron por fuera del cámbium a los 14 días, sin formación de callo; las nuevas raíces emergieron a los 28 días. Las estacas se comportaron de manera diferente, tanto su anatomía *in vivo* como la respuesta bioquímica *in vitro.* Se encontraron diferencias anatómicas que pueden explicar la distinta habilidad para desarrollar RA. El genotipo NR presentó un desarrollo más temprano de la peridermis. Este tejido podría usarse como un marcador confiable del cambio de fase para distinguir partes juveniles de otras maduras que han perdido la capacidad de enraizamiento. Identificamos y caracterizamos tres genes que estarían regulando el inicio del desarrollo de las RA: *AsPIN1*, *AsTIR1* y *AsSHR.* El análisis de su expresión mostró que en el genotipo de NR *AsTIR1* aumenta de manera importante en respuesta al AIB exógeno enseguida de su aplicación. La expresión relativa de *AsPIN1* and *AsSHR* también aumenta, pero 24 horas después. Se discute el significado biológico de este patrón de expresión génica. Los resultados obtenidos en el abordaje conjunto de aspectos anatómicos, genéticos y fisiológicos en la formación de raíces adventicias permitirán aumentar la eficiencia de la selección por capacidad de propagación en las etapas iniciales de la domesticación de nuevos cultivos, así como avanzar en el diseño de manejos y tratamientos para mejorar la capacidad de enraizamiento de genotipos de interés recalcitrantes.

Palabras clave: domesticación, juvenilidad, propagación vegetativa, rizógenesis adventicia

Anatomical and physiological basis of adventitious root differentiation in *Acca sellowiana* (Myrtaceae)

Summary

Successful development of adventitious roots (AR) in cuttings imposes an important limitation to the propagation of woody plants. The ability to form AR is strongly affected by genotype. However, we lack an understanding of such different responses. The identification and expression analysis of genes known to be involved in the regulation of the process is an important step to elucidate the molecular mechanisms that regulate AR differentiation in *Acca sellowiana* cuttings. We selected two genotypes with contrasting rooting ability; we studied the expression of certain genes and the anatomical and biochemical effects of exogenous indol-3-butyric acid and type of cutting in rooting experiments. New meristems developed outside the cambial ring, without callus formation by day 14 and new adventitious roots emerged by day 28. Both anatomically *in vivo* and biochemically *in vitro*, cuttings behaved differently. We found anatomical differences that might explain the differences in rooting ability. An earlier development of a periderm was present in the difficult-to-root genotype. This tissue could be used as a reliable phase-change marker to distinguish juvenile from mature plant parts which have lost rooting capacity. We identified and characterized three genes that might regulate the onset of AR development in *A. sellowiana*: *AsPIN1*, *AsTIR1* and *AsSHR.* Their expression analysis showed that in the difficult-to-root genotype *AsTIR1* increases strongly, shortly after IBA induction treatment. Relative expression of *AsPIN1* and *AsSHR* also increases 24 hours later. The biological significance of this gene expression pattern is discussed. The results obtained by an approach that combines anatomical, genetic and physiological aspects will allow a better understanding of these mechanisms and will lead to an improvement of selection efficiency including this trait in the initial steps of the domestication of new cultures and the design of better practices to improve rooting capacity of recalcitrant genotypes of interest.

Keywords: domestication, juvenility, vegetative propagation, adventitious rooting

1. **Introducción**

1.1. Importancia de la propagación vegetativa para la domesticación de especies frutales nativas

La domesticación puede definirse como la producción de nuevas variedades o genotipos con características deseables para el ser humano a través del intercambio de material genético vía sexual o asexual (Harfouche et al., 2012). Es un proceso evolutivo complejo en el cual el uso de especies vegetales y animales por parte de los seres humanos lleva a cambios morfológicos y fisiológicos que distingue a los taxa domesticados de sus ancestros silvestres (Purugganan y Fuller, 2009). Los seres humanos dependemos para nuestra sobrevivencia de unas pocas especies silvestres que han sido domesticadas. Sin embargo, existen muchas otras especies promisorias pasibles de ser domesticadas conociendo cuáles son las dificultades que previamente impidieron o relegaron su domesticación. El modo de reproducción es una característica asociada con la domesticación que ha cambiado en muchas especies y ha evolucionado hacia la autofecundación o la sustitución de la reproducción sexual por la propagación vegetativa de manera de mantener la identidad genética de los individuos cultivados (Gepts, 2004; McKey et al., 2010). Este cambio en el sistema de propagación tiende a asegurar la reproducción aun en condiciones desfavorables y mantiene la identidad genética del material vegetal (Gepts, 2004). Los avances en los conocimientos de biología molecular y genómica son elementos fundamentales para avanzar en la domesticación de esas especies relegadas, usando técnicas modernas para superar esas dificultades (Diamond, 2002).

Las especies leñosas perennes proporcionan un amplio rango de productos comerciales y se encuentran en distintos estados de domesticación (Harfouche et al., 2012). Son predominantemente de polinización abierta, lo que representa una limitante para su domesticación, ya que las ganancias en una característica de interés resultantes de cualquier cruzamiento son, en general, pequeñas, debido al amplio rango de variación intraespecífica en la progenie que resulta de la polinización cruzada. Asimismo, la larga fase juvenil de muchas especies arbóreas implica que se deba esperar varios años antes de poder evaluar, seleccionar y cultivar los frutos (Asaah,

2012). Estas dificultades pueden superarse mediante la clonación de árboles individuales con características superiores. Se estima que la domesticación de las especies frutales comenzó de 3000 a 4000 años a. C.; originalmente cultivadas a partir de semillas, el desarrollo y cultivo de estas especies ha evolucionado mediante la selección de clones élite y la posterior fijación por propagación vegetativa usando injertos o estacas (Fuller et al., 2023; Janick, 2005). Las especies frutales nativas presentan un gran potencial en comparación con otros frutales comúnmente cultivados. Sin embargo, en la mayoría de los casos existen pocos estudios sobre su origen, evolución y uso en su centro de origen (Nodari et al., 2008). El cultivo, en su mayoría, se basa en la propagación vegetativa y subsiguiente mejoramiento por recombinación sexual de genotipos élite. La propagación clonal asegura que los genotipos favorables pasen a la siguiente generación del cultivo y ayuda a preservar genotipos muy heterocigotos que muestren vigor híbrido; además, se pueden identificar y propagar fácilmente mutaciones favorables y fijar interacciones no aditivas (Bisognin, 2011). En las especies leñosas, de crecimiento lento, la propagación clonal asegura un crecimiento inicial más rápido y una mayor tasa de sobrevivencia que la propagación por semillas (McKey et al., 2010). Los cultivos frutales que se pueden propagar vegetativamente con facilidad se consideran preadaptados para su domesticación (Janick, 2005). Entre las primeras especies frutales domesticadas se encuentran la higuera, el olivo, la banana y la vid, debido a la facilidad que presentan para propagarse vegetativamente, entre otras características (Spiegel-Roy, 1986). Muchos trabajos de domesticación de árboles frutales se han enfocado en la selección de características deseables de la fruta y su captura por técnicas de propagación vegetativa (Leakey y Newton, 1994). Por lo tanto, la propagación clonal es un aspecto de creciente importancia para la biología de la domesticación de plantas (McKey et al., 2010). De las diferentes técnicas de propagación vegetativa conocidas, la propagación por estacas es la alternativa más comúnmente empleada en viveros comerciales por su bajo costo y por ser una técnica relativamente sencilla, en comparación, por ejemplo, con los injertos.

1.2. Rizogénesis adventicia

La formación de raíces adventicias en estacas es un proceso crítico que representa una limitante para la propagación de diversos cultivos (Druege et al., 2019; Singh y Tomar, 2023). En especies leñosas, la pérdida de la capacidad de formar raíces adventicias está asociada con la madurez fisiológica y es uno de los principales factores que limitan el éxito de la propagación vegetativa de los árboles adultos seleccionados por su potencial productivo justamente cuando alcanzan la madurez (Pizarro y Díaz-Sala, 2019). En plantas superiores se distinguen tres tipos de raíces: raíces primarias, raíces secundarias y raíces adventicias. La raíz primaria se forma durante las etapas tempranas de la embriogénesis y crece por la actividad del meristema apical de raíz, el cual se divide en dos direcciones y en cuya parte central se ubica el centro quiescente. La parte externa de este meristema da origen a la cofia o caliptra, que actúa como sensor de la gravedad y lubrica a las raíces creciendo a través del sustrato; la parte interior está compuesta por células en activa división, organizadas en cinco capas concéntricas que darán origen a epidermis, corteza, endodermis, periciclo y estela (Dolan et al., 1993). Las raíces laterales son de origen posembrionario y, a diferencia de las ramificaciones del tallo en que los meristemas laterales se forman sobre la superficie del meristema apical, las raíces laterales se inician endógenamente en el periciclo de la raíz primaria (Péret et al., 2009). Las raíces adventicias (RA) cumplen las mismas funciones que las raíces laterales o secundarias, pero se forman a partir de tejidos aéreos (Bellini et al., 2014). En muchas especies se originan durante el desarrollo normal de las plantas y también en respuesta a condiciones de estrés tales como anegamiento o heridas. Las RA inducidas por el corte al extraer una estaca de la planta madre juegan un rol central para la propagación de numerosas especies de importancia, tanto leñosas como herbáceas (Steffens y Rasmussen, 2016). Estas RA emergen a partir de un pequeño grupo de células de los tejidos vasculares o cercanas a ellos (Kidwai et al., 2023), llamadas células iniciales de raíz (Evert, 2006), que se encuentran en diferentes tejidos del tallo, dependiendo de la especie, tales como floema, parénquima u otros tipos celulares (Geiss et al., 2009; Riov et al., 2013; Tarragó et al., 2005). Una característica conservada es que las RA

siempre se desarrollan a partir de células vecinas a los tejidos vasculares (Bellini et al., 2014; De Klerk et al., 1999; Kidwai et al., 2023). Al cortar una estaca se interrumpe el suministro de agua, nutrientes y ciertas hormonas como las citoquininas provenientes de la raíz y, al mismo tiempo, por encima del corte se genera una nueva fosa donde se acumulan sustancias que normalmente se transportan hacia la raíz, como las auxinas; se pone en funcionamiento un nuevo programa de desarrollo en determinadas células de la base de la estaca en respuesta a la herida generada (Druege et al., 2019). El proceso es desencadenado por numerosos factores tanto endógenos como exógenos y los diferentes genotipos varían en sus requisitos y necesidades a lo largo del proceso de reestablecer un nuevo sistema radical (Di Battista et al., 2019). Dada la importancia de la propagación vegetativa en especies leñosas, y, más específicamente, las especies frutales, es de gran relevancia profundizar en el conocimiento del proceso de formación de RA a través de una caracterización anatómica, bioquímica y genética, para poder entender cuáles son los patrones fisiológicos que se asocian con la baja capacidad de enraizamiento de algunos genotipos de interés productivo.

1.3. Etapas del proceso

Generalmente, se reconocen tres etapas en el proceso de desarrollo de raíces adventicias, cada una con requerimientos específicos: inducción, iniciación y expresión (Bellini et al., 2014; De Klerk, 1996; Kevers et al., 1997; Pacurar et al., 2014; Porfirio et al., 2016) (Figura 1). La fase de inducción comprende un período de tiempo durante el cual ocurren cambios bioquímicos previos a las primeras divisiones celulares, sin eventos histológicos visibles. El tipo de célula que participa en la desdiferenciación durante la etapa de inducción es parenquimática, generalmente floema o parénquima cortical, y puede variar según las especies (floema, parénquima u otros tipos celulares) (Riov et al., 2013; Tarragó et al., 2005). Algunos de los cambios que ocurren durante esta fase incluyen un incremento local en los niveles de auxina en la base de la estaca, el establecimiento de una nueva fosa para carbohidratos y un descenso transitorio en la actividad de enzimas peroxidasas. La etapa de iniciación se caracteriza por divisiones celulares que conducen a la formación de un nuevo meristema de raíz, generalmente asociado con un menor contenido de auxina y mayor

actividad peroxidasa. Durante la etapa de expresión, la nueva raíz formada crece a través de la corteza radical, emerge a través de la epidermis y se establecen las conexiones vasculares de la nueva raíz con el sistema vascular de la estaca (Aumond et al., 2017; Bellini et al., 2014; Da Costa et al., 2013; De Almeida et al., 2017; De Klerk et al., 1999; Stuepp et al., 2017). La duración del proceso, desde que se extrae la estaca de la planta madre hasta que se observan los primeros eventos anatómicos, es extremadamente variable entre especies y también se ve afectada por la época del año y el tipo de estaca (Jackson, 1986; Naija et al., 2008). Todas las etapas del proceso de formación de nuevas raíces adventicias ocurren, en última instancia, en función de la expresión diferencial de genes; por lo tanto, una buena estrategia de investigación es la exploración de los programas de expresión génica que afectan el proceso. El conocimiento de dicha regulación será de utilidad para entender las causas de la variabilidad en las respuestas encontradas en diferentes materiales vegetales; por ejemplo, saber si la baja capacidad de enraizamiento es debida a una menor sensibilidad de los tejidos a las auxinas, pérdida de competencia para diferenciar raíces adventicias u otras causas.

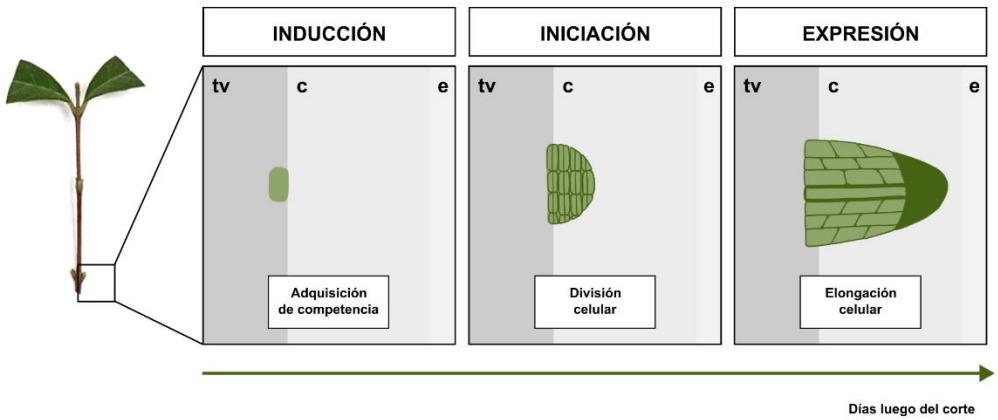

Figura 1

Etapas del proceso de formación de raíces adventicias; tv: tejidos vasculares, c: corteza, e: epidermis (adaptado de Hilo, 2017).

1.4. Factores que afectan la formación de raíces adventicias

La formación de RA es una característica genética cuantitativa regulada por factores tanto endógenos como ambientales, entre los que se incluyen fitohormonas, luz, estado nutricional, respuestas asociadas al estrés provocado por el corte y características genéticas. El conocimiento acerca de cómo los factores endógenos y ambientales interactúan en la regulación del proceso es escaso (Bellini et al., 2014; Garg et al., 2022; Geiss et al., 2009; Kunc et al., 2024; Pop et al., 2011; Porfirio et al., 2016). A nivel molecular, el conocimiento está mayoritariamente basado en especies modelo como *Arabidopsis thaliana*. Sin embargo, la conservación de estos mecanismos en otras especies no está comprobada (Gonin et al., 2019). Varios aspectos genéticos y fisiológicos no pueden ser extrapolados a especies arbóreas (Vilasboa et al., 2018) y las estacas de *A. thaliana* no tienen la estructura típica usada para la propagación de especies leñosas (Ahkami, 2023; Druege et al., 2019; Singh y Tomar, 2023). Para poder mejorar las estrategias de propagación de las especies leñosas, es necesario traducir e interpretar el conocimiento basado en especies modelo.

1.4.1. Factores exógenos en el control del desarrollo de raíces adventicias

La formación de RA es un proceso de desarrollo posembrionario; por lo tanto, no responde estrictamente a un programa genético predefinido, sino que también es muy sensible a los estímulos ambientales (Guan et al., 2015). Las condiciones ambientales afectan el proceso en todas sus etapas, incluyendo desde las condiciones previas de crecimiento de la planta madre hasta el período de establecimiento de las nuevas plantas luego de enraizadas (Jackson, 1986; Leakey, 2024). La irradiancia, temperatura, agua y los nutrientes son los principales factores que inciden en el estado fisiológico de la planta madre de la cual se van a extraer las estacas (Vilasboa et al., 2018). La condición de la planta madre es fundamental para obtener estacas con un adecuado contenido de sustancias como carbohidratos, auxinas y polifenoles, con roles clave en el proceso de formación de RA (Leakey, 2024; Sansberro et al., 2000). Luego de extraídas las estacas y durante las sucesivas etapas, los factores ambientales también afectan el proceso de diferenciación de RA. La nutrición mineral es un factor clave,

con requerimientos específicos que varían según la especie (Geiss et al., 2009; Hilo, 2017; Vilasboa et al., 2022). La luz y la temperatura durante el proceso también inciden en la formación de RA (De Almeida et al., 2017; Ruedell et al., 2013; Vilasboa et al., 2018). Es un proceso de desarrollo con un alto nivel de complejidad y múltiples niveles de regulación.

1.4.2. Factores endógenos en el control del desarrollo de raíces adventicias

Entre los factores endógenos, las hormonas son los moduladores más importantes del desarrollo radical, interactuando entre ellas y con factores ambientales en redes complejas en que las auxinas tienen un rol central (Altamura et al., 2023; Bellini, 2024; Bellini et al., 2014; Pacurar et al., 2014). El hecho de que la auxina ácido indolacético (AIA) esté involucrada en los eventos tempranos de la formación de raíces adventicias está ampliamente aceptado (Aumond et al., 2017; Blakesley, 1994; Della Rovere et al., 2013). La diferente capacidad de desarrollar RA en genotipos de fácil o difícil enraizamiento depende principalmente de la regulación del contenido endógeno de auxina en la base de la estaca, así como de las diferencias en sensibilidad a esta hormona en esas células (Aloni et al., 2006; Aumond et al., 2017; Di et al., 2015; Druege et al., 2016, 2019; Guan et al., 2015; Pizarro y Díaz-Sala, 2019). La concentración de AIA endógeno juega un rol central en el control de la iniciación y desarrollo de raíces adventicias en numerosas especies vegetales (Bellini et al., 2014; Davies, 2010; De Almeida et al., 2020; Druege et al., 2019; Ford et al., 2001; Gonin et al., 2019; Kelen y Ozkan, 2003; Pacurar et al., 2014; Vilasboa et al., 2018; Wendling et al., 2015). Se ha sugerido que la acumulación de AIA en la base de la estaca autorregula su canalización y maximiza su concentración en determinadas células blanco que responden con la inducción del programa de formación de RA (Druege et al., 2019). Los procesos que determinan cuál es la concentración de auxina en cierto momento en un determinado tejido son su biosíntesis, metabolismo y transporte, procesos que son regulados por múltiples mecanismos (Han et al., 2009). El transporte polar de AIA hacia la base de la estaca es determinante para la formación de RA (Druege et al., 2016; Negishi et al., 2014; Pacurar et al., 2014). Esta distribución

diferencial de auxina parece ser señal suficiente para desencadenar o modificar el programa de desarrollo de una célula en plantas modelo (Negishi et al., 2014; Ruedell et al., 2015; Vanneste y Friml, 2009). En genotipos de fácil o difícil enraizamiento, esta acumulación diferencial de AIA puede ser explicada por una expresión diferente de genes que regulan la biosíntesis y el transporte de AIA (De Almeida et al., 2015; Druege et al., 2019), así como una mayor expresión de represores de genes de respuesta a auxina en las especies de difícil enraizamiento (Ruedell et al., 2015).

Otras hormonas como las citoquininas, etileno y brasinoesteroides interactúan con las auxinas y participan en la regulación del proceso con efecto sinérgico o antagónico dependiendo de la especie (Altamura et al., 2023; Bellini et al., 2014; Druege et al., 2019; Geiss et al., 2009; Gonin et al., 2019; Riov et al., 2013).

1.4.3. Variabilidad genética para capacidad de enraizamiento

El desarrollo de raíces adventicias es una característica genética compleja y de gran plasticidad fenotípica, dado los múltiples niveles de regulación que presenta (Gutierrez et al., 2009). Esa plasticidad se logra por una regulación compleja y dinámica que permite integrar señales endógenas y ambientales a través de una intrincada red de regulación génica (Vilasboa et al., 2022). La competencia de plantas adultas para diferenciar raíces adventicias es esencial para obtener éxito en la propagación vegetativa por estacas y existe gran variación entre los distintos genotipos dentro de la misma especie (Bisognin et al., 2018).

El control hormonal es un aspecto clave de la rizogénesis adventicia que difiere entre tallos enteros y estacas de tallo (Vilasboa et al., 2022) y está ligado a la expresión de genes de identidad celular que relacionan el control hormonal con la re-especificación celular (Abarca, 2021). En *Arabidopsis*, la biosíntesis local de auxina y su transporte polar dan lugar a concentraciones máximas de esta hormona en las células que darán origen al nuevo primordio de raíz. Este aumento de la concentración de auxina se ha comprobado también en otras especies estudiadas y se sugiere que es un prerrequisito para el desarrollo de raíces adventicias y una característica conservada en todas las especies vegetales (Garg et al., 2022). Se logra a través de un balance entre las vías metabólicas de biosíntesis, transporte, percepción, señalización e inactivación

de la hormona y se conocen varios de los genes que participan de dichas vías de regulación en diversas especies. *TRYPTOPHAN AMINOTRANSFERASA* (TAA1) y *YUCCA* son dos genes que regulan la biosíntesis de auxina; en el transporte polar participa la familia de proteínas AUX1/LAX, que regula el ingreso de la hormona a la célula y miembros de la familia PIN que regulan su eflujo. Una vez en la célula, la percepción de la hormona es mediada por la proteína F-box TIR1, la cual regula genes de respuesta a auxina. Otra proteína receptora es ABP1, vinculada a respuestas tempranas de crecimiento celular, necesario para el desarrollo del nuevo primordio de raíz (De Almeida et al., 2015).

A su vez, el cambio de fase vegetativa a reproductiva está bajo regulación epigenética y también existen componentes claves en la vía de señalización de auxinas durante la rizogénesis adventicia bajo control epigenético de la expresión génica (Abarca, 2021). La pérdida de capacidad de enraizamiento asociada con la pérdida de juvenilidad en especies leñosas ha sido atribuida, en parte, a un aumento en la expresión de *ARABIDOPSIS RESPONSE REGULATIOR* (*ARR1*), que afecta de manera negativa la homeostasis de auxina (Ayala et al., 2022). En especies forestales, se ha comprobado la participación de miembros de las familias de genes *Gibberellic Acid Insensitive* (GAI), *Repressor of GAI* y *SCR* (GRAS) en la pérdida de capacidad de enraizamiento asociada con la madurez (Abarca et al., 2014; Sánchez et al., 2007; Vielba et al., 2016). En *Arabidopsis* y *Eucalyptus nitens*, el aumento en la señalización de citoquininas mediado por *ABERRANT LATERAL ROOT FORMATION4* (*ALF4*) es otro posible candidato para el control epigenético de la RA en relación con la transición a fase madura (Abarca, 2021; Ayala et al., 2022).

La expresión de estos y otros genes que participan en la regulación del proceso a su vez está afectada por las condiciones ambientales de crecimiento de la planta madre (intensidad y calidad lumínica, temperatura, etc.), además de las condiciones durante el propio proceso de enraizamiento. Esta complejidad de la regulación es lo que determina que puedan existir diferencias muy notorias en capacidad de enraizamiento entre genotipos, incluso dentro de una misma especie.

1.5. Limitantes asociadas a la madurez de la planta

El envejecimiento es un factor limitante para la formación de raíces adventicias. La duración de la fase juvenil se relaciona inversamente con la eficiencia de mejoramiento y la selección de cultivares mejorados (Hackett, 1987). Los cambios fenotípicos que sufren las plantas durante el desarrollo como parte del proceso ontogénico se conocen como cambio de fase o maduración, y cambiar el programa de desarrollo de células adultas para regenerar nuevos órganos como las RA es particularmente difícil en especies leñosas (Díaz-Sala, 2014; Faria et al., 2023). Si bien la transición hacia la floración es un indicador de madurez, otros cambios fenotípicos, como la forma de hoja, filotaxia, etc., ocurren durante la transición de la fase juvenil a madura. La pérdida de competencia para enraizar es uno de los cambios de mayor importancia económica que limita la propagación clonal de genotipos élite en árboles frutales (Bellini et al., 2014; Poethig, 2010; Riov et al., 2013). Durante el cambio de fase ocurren modificaciones celulares y bioquímicas que reconfiguran vías moleculares y conducen a la inhibición de la iniciación de RA en tejidos maduros (Vilasboa et al., 2018). Particularmente en especies leñosas, la pérdida de capacidad para diferenciar raíces adventicias de tipos celulares similares está asociada con la edad y madurez fisiológica de la planta madre, que son barreras para la propagación vegetativa por medio de estacas (Aumond et al., 2017; Pizarro y Díaz-Sala, 2019). Los mecanismos por los cuales células completamente diferenciadas se desdiferencian y dan origen a raíces adventicias se desconocen (Abarca et al., 2014) y se sabe muy poco acerca de las modificaciones celulares y bioquímicas que ocurren durante el cambio de fase y cómo esos eventos reconfiguran algunas vías moleculares que inhiben la formación de raíces adventicias en tejidos maduros (Bellini et al., 2014). El cambio (*switch*) en el destino de las células implica cambios muy importantes en el patrón de expresión génica. Que las células sean o no competentes, en última instancia, es función de la expresión diferencial de genes que afectan la sensibilidad y las distintas fases del desarrollo de un nuevo meristema de raíz; el estudio de la expresión de genes que afectan el enraizamiento es un buen abordaje para entender la diferente regulación en materiales de fácil o difícil enraizamiento (Hutchison et al., 1999). Las redes

génicas que regulan el proceso de diferenciación de raíces adventicias pueden cumplir distintas funciones en diferentes etapas del desarrollo y en estados particulares del desarrollo de una célula; conocer las modificaciones celulares y bioquímicas y la dinámica temporal de estas redes y cómo se modifican durante el cambio de fase ayudará a explicar la diferente capacidad de enraizamiento que presentan distintos materiales vegetales (Bellini et al., 2014; De Lucas y Brady, 2013; Díaz-Sala, 2014; Druege et al., 2019; Kunc et al., 2024).

1.6. Marcadores de enraizamento

Para poder mejorar los procedimientos de propagación, es fundamental conocer los eventos morfológicos, bioquímicos y moleculares asociados con cada una de las etapas del proceso de formación de raíces adventicias. Estas etapas se pueden caracterizar identificando marcadores de enraizamiento, es decir, parámetros que muestren suficiente coincidencia con cierto estado fisiológico, de desarrollo o genético. Estos marcadores pueden resultar útiles para identificar el principio y fin de cada una de las fases, establecer cuál es la fase en que los materiales recalcitrantes se inhiben, predecir el éxito de un tratamiento de enraizamiento y entender los mecanismos de la formación de raíces adventicias (De Klerk, 1996).

1.6.1. Estudios anatómicos

Las raíces adventicias emergen a partir de un pequeño grupo de células denominadas iniciales de raíz, capaces de desdiferenciarse y volverse meristemáticas. Estas células se ubican en distintos tejidos, dependiendo de la especie, aunque siempre son células vecinas o cercanas a los tejidos vasculares (Geiss et al., 2009). En varias especies esto sucede a partir de tejidos indiferenciados como el cambium o células de parénquima asociadas a tejidos diferenciados, como floema o parénquima cortical, que se desdiferencian por efecto de las auxinas (Baltierra et al., 2004) y también se citan como sitio de origen de las RA (Naija et al., 2008; Syros et al., 2004; Tarragó et al., 2005).

1.6.2. Marcadores bioquímicos

En los genotipos de difícil enraizamiento, la iniciación de RA puede ser inducida mediante la aplicación de auxina exógena. Estudios bioquímicos muestran que el ácido indol butírico (AIB) exógeno induce cambios en el metabolismo de enzimas, carbohidratos y proteínas (De Almeida et al., 2020; Elmongy et al., 2018). Luego de la aplicación exógena de auxinas, se ha reportado un aumento en los niveles de carbohidratos solubles en la zona de enraizamiento de estacas de tallo (Agulló-Antón et al., 2014; Elmongy et al., 2018; Goel et al., 2018). Durante la etapa de iniciación, se observan divisiones celulares que conducen a la formación de un nuevo meristema de raíz; durante esta fase, en *Eucalyptus* se ha identificado un aumento en el número de proteínas que refleja estos importantes cambios a nivel celular (De Almeida et al., 2020). El contenido de proteína aumenta de manera significativa a lo largo del proceso de enraizamiento en respuesta al AIB exógeno; los máximos niveles, en general, están relacionados a un aumento en los niveles de síntesis de enzimas durante la iniciación del proceso de regeneración de raíces (Elmongy et al., 2018; Husen y Pal, 2007).

La degradación oxidativa del AIA, entre otros compuestos orgánicos, es catalizada por enzimas peroxidasas (Ljung et al., 2002). Cambios en la actividad de estas enzimas o en algunas de sus isoformas son usados como marcador bioquímico de las sucesivas fases del proceso de enraizamiento (Gaspar et al., 1992; Hatzilazarou et al., 2006; Heloir et al., 1996; Masmoudi et al., 1999; McDonald y Wynne, 2003; Metaxas et al., 2004; Rout et al., 2000; Schwambach et al., 2008). Estas enzimas muestran un mínimo de actividad durante la primera fase de la rizogénesis adventicia (fase de inducción) y un máximo en la segunda fase (fase de iniciación), precediendo los primeros signos visibles de la diferenciación de raíces (Gaspar et al., 1992). Aunque varios autores han observado una correlación positiva entre actividad peroxidasa durante la fase de iniciación y la rizogénesis (Metaxas et al., 2004), en algunas especies como *Populus tremula* (Pythoud y Buchala, 1989), *Castanea sativa* x *C. crenata* (Gonçalves et al., 1998) y *Quercus* sp. (San-José et al., 1992) no se ha podido correlacionar claramente la actividad peroxidasa con el proceso de rizogénesis.

En Arabidopsis se sabe que la enzima DIOXYGENASE FOR AUXIN OXIDATION (DAO1), inducida por Jasmonatos, juega un papel importante en la degradación de algunos conjugados de auxinas durante la RA, actuando como un modulador rápido de la disponibilidad de AIA luego del daño mecánico (Lakehal et al., 2019; Müller, Karel et al., 2021). La interacción entre los jasmonatos y las auxinas es compleja y dependiendo del proceso regulado y del estado de desarrollo de los tejidos pueden tener una interacción sinérgica o antagónica (Lakehal & Bellini, 2019). Estudiar el rol de esta enzima en la regulación de la homeostasis de las auxinas durante el proceso de desarrollo de RA puede aportar información adicional valiosa para la mejor comprensión de esta compleja red de regulación.

1.6.3. Genes involucrados en el control de la formación de RA

Los avances más importantes acerca de los mecanismos genéticos y moleculares que regulan la diferenciación de RA se basan en estudios de plantas mutantes de *A. thaliana* y, en menor grado, otras especies herbáceas como arroz y maíz (Ahkami, 2023; Altamura et al., 2023; Kunc et al., 2024). Varios mutantes afectados en diversas vías de transducción u homeostasis hormonal también se ven afectados en la capacidad de diferenciar RA (Bellini et al., 2014). El genoma secuenciado de *Arabidopsis* constituye una rica fuente de información para estudios comparativos del desarrollo de raíces en otras plantas y los genes identificados pueden servir de base para el estudio de genes candidatos relacionados con la capacidad de enraizamiento en especies leñosas (Benfey et al., 2010; Geiss et al., 2009).

Las auxinas controlan la morfogénesis y el crecimiento direccional a nivel de órganos y tejidos a través de múltiples respuestas y son el principal grupo hormonal involucrado en la diferenciación de RA. Estas respuestas involucran una reprogramación de la expresión génica mediada por el receptor nuclear TRANSPORT INHIBITOR RESPONSE1/AUXIN SIGNALING F-BOX (TIR1/AFB) (Dharmasiri et al., 2005; Kepinski y Leyser, 2005; Pan et al., 2015). Terrile et al. (2012) demostraron que el óxido nítrico (NO) estimula la expresión de genes regulados por auxinas promoviendo la interacción entre TIR1/AFB y AUX/AIA. En *Eucalyptus*

grandis se encontró una correlación positiva entre los niveles de expresión del gen *NIA*, que participa en la biosíntesis de NO, y la diferenciación de RA (Abu-Abied et al., 2014). El transporte activo de auxinas, célula a célula, tiene un fuerte efecto sobre los procesos de desarrollo, incluida la diferenciación de raíces. Este transporte a corta distancia genera un gradiente de auxinas, mediado por proteínas *carrier* de eflujo PIN-FORMED (PIN), *carriers* de influjo AUXIN1 (AUX1)/LIKE AUX1 (LAX) y una subfamilia de transportadores ATP-BINDING CASSETTE, denominado transporte polar de auxinas (Gonin et al., 2019; Vieten et al., 2007).

Las auxinas también regulan el centro quiescente y la actividad del meristema radical a través de genes *PLETHORA* (*PLT*), considerados reguladores máster del desarrollo de la raíz primaria, y genes *SHORT-ROOT* (*SHR*) y *SCARECROW* (*SCR*) que especifican al centro quiescente actuando en paralelo con *PLT*. Las proteínas SCR y SHR son factores de transcripción de la familia GRAS (proteínas específicas de plantas e importantes componentes regulatorios en diferentes procesos celulares), necesarias para la actividad del centro quiescente y el correcto patrón de diferenciación de tejidos a partir del meristema apical de raíz (Helariutta et al., 2000; Lee et al., 2013; Long y Benfey, 2006; Petricka y Benfey, 2008). La expresión de genes *SCARECROW-LIKE* (*SCR-LIKE*) aumenta de manera significativa en respuesta al agregado de auxina exógena en estacas competentes de especies leñosas taxonómicamente muy distantes como *Pinus radiata* y *Castanea sativa* (Sánchez et al., 2007). Este incremento se corresponde con la fase de reorganización celular, previo al reinicio de la mitosis y la formación de un nuevo meristema, sugiriendo que los genes *SCR-LIKE* juegan un papel importante en la adquisición de competencia. En *Arabidopsis*, *SCARECROW* (*SCR*) es un factor de transcripción putativo, requerido para las divisiones celulares asimétricas que dan origen a la endodermis y la corteza de la raíz, así como para mantener la identidad del centro quiescente (Geiss et al., 2009). *SHORT ROOT* (*SHR*) es otro factor de transcripción de la familia GRAS, requerido para la división asimétrica de las células iniciales responsables de la diferenciación de la endodermis y el córtex, participando en una vía de señalización radial e interactuando con SCR (Helariutta et al., 2000). En *Populus*, Xuan et al. (2014) aislaron y caracterizaron tres

genes que codifican proteínas SHR y uno para SCR, implicados en el desarrollo de raíces adventicias.

1.7. *Acca sellowiana*

Acca sellowiana es un arbusto frutal nativo de Uruguay y Brasil en proceso de domesticación, con alto potencial económico y alimenticio (Dos Santos et al., 2009; Ferreira, 2019; Dos Santos et al., 2007). La domesticación de *Acca sellowiana*, una especie frutal cuya polinización es predominantemente cruzada, está limitada por las pequeñas ganancias genéticas resultantes de los cruzamientos para cualquier característica de interés, como proporción de pulpa, color, etc., debido al amplio rango de variación intraespecífica de la progenie. Además, la presencia de períodos juveniles prolongados significa que se debe esperar varios años para poder evaluar, seleccionar y cultivar los frutos. Estas limitantes pueden superarse mediante la multiplicación vegetativa de árboles individuales, para lo cual es imprescindible revertir las limitantes para formar raíces adventicias asociadas con el cambio de fase. El conocimiento de las bases anatómicas y fisiológicas del proceso, así como su regulación a nivel génico son fundamentales para profundizar en el entendimiento de esas limitantes.

El interés despertado por este cultivo en otros países se debe a su adaptabilidad a un amplio rango de condiciones climáticas y cualidades organolépticas de los frutos, aptos para el consumo en fresco y como productos elaborados. La especie presenta una buena resistencia a heladas y precocidad productiva. Sus hojas y frutos son ricos en compuestos con actividad antioxidante, antimicrobiana y propiedades farmacológicas (Bontempo et al., 2007; Mosbah et al., 2018; Raikar et al., 2023; Tortora et al., 2019; Vuotto et al., 2000). El fruto contiene gran proporción de yodo (3 mg/100 g pulpa), vitamina C (851,28 mg/100 g de pulpa) (Fischer, 2020) y calcio (72 mg/100 g de pulpa) (Leterme et al., 2006). Entre los diversos componentes bioactivos presentes en los frutos y su farmacología se destacan la actividad anticancerígena, antimicrobiana, antiinflamatoria y antioxidante. El elevado nivel de compuestos fenólicos en el fruto le otorga excelentes propiedades y un gran potencial comercial como base de compuestos nutracéuticos (Weston, 2010). Su conservación puede durar hasta un mes sin que se pierda su característico sabor y aroma (Azam et al., 1981). Sin embargo, los

métodos de propagación vegetativa son relativamente dificultosos para guayabo del país en comparación con otras especies frutales (Thorp y Bieleski, 2005); la facilidad de propagación normalmente no se incluye en los programas de selección (Citadin et al., 2022; Sánchez-Mora et al., 2020) y esto representa una limitante importante para el desarrollo del cultivo. Estudios anatómicos en estacas de *A. sellowiana* cultivar Unique mostraron diferenciación de meristemas adventicios en las zonas de unión del cambium con los radios parenquimáticos de la médula, a los veinte días de ser cortadas. Asimismo, la ocurrencia de raíces se vio afectada de manera negativa por la presencia de un anillo de esclerénquima rodeando al floema, que podría estar actuando como una barrera física para el crecimiento y emergencia de la nueva raíz (Zhang et al., 2009). La disponibilidad del genoma de *E. grandis* brinda la posibilidad de realizar estudios bioinformáticos usando una especie leñosa taxonómicamente emparentada con *A. sellowiana* (Grattapaglia et al., 2012; Myburg et al., 2014).

1.7.1. Antecedentes internacionales

En su lugar de origen, la especie ha estado históricamente sometida a la influencia antropogénica, con cierto grado de domesticación incipiente de algunas poblaciones *in situ*, lo que ha dado como resultado mayor tamaño de fruto y productividad. Con base en la clasificación propuesta por Clement, puede considerarse como una especie incipientemente domesticada (Clement, 1999). Sin embargo, en otros países como Nueva Zelanda y Colombia se han desarrollado cultivares patentados y se cultiva ampliamente (Bogoni et al., 2018; Moretto et al., 2022; Niella et al., 2018; Thorp y Bieleski, 2005). Los frutos alcanzan un gran tamaño, de hasta 240 g, lo que representa una importante mejora con relación a los materiales silvestres de Uruguay y Brasil, de 25 a 60 g de peso promedio (Cunda Sisto, 2006). Colombia es el mayor exportador de la fruta fresca y Nueva Zelanda, donde la producción alcanza las ochocientas toneladas anuales, es el mayor productor de subproductos elaborados a partir de la especie (Moretto et al., 2022; Zhu, 2018). En Brasil, la investigación acerca de esta especie es realizada en Santa Catarina, por la Empresa de Investigación Agropecuaria y Extensión Rural (EPAGRI) y la Universidad Federal de Santa Catarina (UFSC) y en Río Grande del Sur en EMBRAPA de clima templado (CPAACT).

Actualmente cuentan con cuatro cultivares, SCS 411-Alcántara y SCS 412-Helena, SCS 414-Mattos y SCS 415-Nonante, obtenidos en el programa de mejoramiento de la especie. Estos cultivares son autofértiles, se complementan en términos de maduración y presentan buena resistencia a las principales enfermedades que afectan a la especie en ese país (Epagri, s. f.). Brasil cuenta, además, con un banco de germoplasma de *Acca sellowiana* en la estación experimental de San Joaquín (EPAGRI) en el estado de Santa Catarina.

Las técnicas evaluadas incluyen la realización de injertos, acodos, estacas, micropropagación y estudios de embriogénesis somática. Las plantas de guayabo injertadas presentan el inconveniente de producir numerosos rebrotes del pie, lo cual puede generar problemas de manejo una vez establecido el huerto. Se han evaluado otras *Myrtaceas* como portainjerto, pero no presentaron suficiente afinidad para ser usadas con guayabo (Oltramari et al., 2000). El acodo de cepa, utilizado para la producción comercial de portainjertos de manzana, membrillo y pera, se ha evaluado en plantas de guayabo del país de dos años, con un resultado promedio de 6,6 mudas enraizadas por cepa, pero es una técnica costosa (Fachinello y Nachtigal, 1992). El estaquillado se destaca como método más eficiente, con tasas de prendimiento de entre 4 y 76 % en Nueva Zelanda. Los principales factores que afectan la tasa de enraizamiento son el genotipo y los efectos negativos de la oxidación fenólica. En Brasil reportan porcentajes de enraizamiento muy bajos (entre 0 y 6,2 %) para los cultivares desarrollados en ese país (Cangahuala-Inocente et al., 2004). La embriogénesis somática fue descrita para guayabo en Brasil (Canhoto y Cruz, 1996) y se utiliza como modelo de estudio del proceso de embriogénesis en ANGIOSPERMAS leñosas (Dos Santos et al., 2007; Guerra et al., 2001). Las distintas fases del proceso fueron caracterizadas mediante análisis morfohistológicos e histoquímicos; pero los protocolos desarrollados parten de embriones cigóticos, como explanto, y, por lo tanto, no garantizan la fidelidad clonal (Cangahuala-Inocente et al., 2004). Estos resultados comprometen la posibilidad de multiplicar efectivamente materiales de élite seleccionados, ya sea para su evaluación como para su difusión en el sector productivo.

1.7.2. Antecedentes nacionales

El sector frutícola busca permanentemente alternativas productivas a las especies tradicionales en cultivo, como forma de mejorar la ecuación de rentabilidad. El guayabo del país despierta interés en los productores por el valor económico que pueden alcanzar sus frutos y por la rusticidad de las plantas, lo que permite manejos agronómicos amigables con el ambiente. La existencia en nuestro país de una importante tradición productora y exportadora de fruta fresca constituye una ventaja sobre la que se pueden desarrollar corrientes exportadoras de nuevos frutos. En este sentido, los principales estudios en su región de origen se relacionan con la colecta, caracterización fenotípica, mejoramiento, propagación, uso y conservación (Nodari et al., 2008).

Para la utilización de especies nativas, resulta muy importante el agregado de valor mediante investigaciones sobre su calidad, la promoción de mercados y sobre todo iniciar programas de domesticación que permitan comenzar su cultivo. Se requieren importantes apoyos continuados para la caracterización evaluación, así como estudios de sistemas reproductivos, fisiología de semillas y métodos de propagación, aspectos básicos imprescindibles para su cultivo (Berreta et al., 2010).

El programa de Selección de Frutas Nativas (Facultad de Agronomía-Udelar, INIA y Dirección General Forestal-MGAP) tiene como uno de sus principales objetivos el desarrollo de cultivares de *Acca sellowiana* que permitan alcanzar producción comercial. En este marco, en los últimos años se han desarrollado varios proyectos de investigación que abarcan diferentes aspectos de interés para el desarrollo de este cultivo y que incluyen trabajos de prospección de la diversidad de la especie en estado silvestre y variedades locales, caracterización de la diversidad genética mediante caracteres morfofenológicos y moleculares, evaluación del valor agronómico y el potencial comercial de plantas seleccionadas, técnicas de propagación vegetativa convencional y comportamiento *in vitro* de la especie (Cunda Sisto, 2006; Franklin, 2009; Puppo Mackinnon et al., 2014; Quezada et al., 2014; Rivas et al., 2024; Ross y Grasso, 2010; Silveira et al., 2016; Vignale y Bisio, 2005).

Entre los principales resultados de este programa se conformaron jardines de introducción y evaluación de materiales, se generaron las primeras progenies híbridas por cruzamientos dirigidos entre parentales superiores y se registraron las primeras selecciones en Uruguay (INIA Fagro Isleña, INIA Fagro Cerrillana, INIA Fagro Artillera e INIA Fagro Armonía), que se caracterizan principalmente por su sabor, productividad, estabilidad en la producción y tamaño de fruto; recientemente se comenzó a incorporar la capacidad de propagación vegetativa (Dini y Speroni, 2024; Facultad de Agronomía e INIA, 2024). Los porcentajes de enraizamiento de estacas obtenidos han sido muy variables (entre 3 y 60 %) en función del genotipo y el uso de fitohormonas (Cabrera et al., 2010). En función de las bajas tasas de éxito obtenidas con las técnicas convencionales, se han evaluado alternativas de cultivo *in vitro*, que incluyen la micropropagación y la embriogénesis somática (Guerra et al., 2012; Ross y Grasso, 2010).

Uno de los eslabones en esta cadena de trabajos dirigida a valorizar los frutos nativos es la creación de un vivero con plantas de guayabo en el sur del país. El vivero se desarrolla en un establecimiento particular, enmarcado en las actividades que realiza la Asociación de Fomento y de Defensa Agraria de Juanicó. La iniciativa se concretó a través de un proyecto denominado *Ambiente y sociedad: estudio de las frutas nativas como alternativa para productores familiares*, que fue apoyado financieramente por el Programa de Pequeñas Donaciones del Fondo para el Medio Ambiente Mundial, de Naciones Unidas. El objetivo general del proyecto en el que participan técnicos de INIA y Udelar es «proporcionar elementos para mejorar las condiciones de vida de productores familiares en aspectos sociales, económicos, ambientales y culturales, mediante la utilización de flora autóctona (frutales nativos) como alternativa productiva sostenible» (Uruguay. Conexión tecnológica, 2009). Los métodos de propagación vegetativa de guayabo del país presentan dificultades, ya que existe una gran variabilidad en la capacidad de enraizamiento de los distintos materiales, siendo este uno de los principales factores que impiden la difusión del cultivo de esta especie frutal nativa en nuestro país.

Para poder mejorar las estrategias de propagación de *Acca sellowiana*, es fundamental conocer mejor el proceso de diferenciación de raíces adventicias para

poder identificar elementos que permitan explicar la gran variabilidad que se encuentra entre los distintos genotipos. Este conocimiento es esencial para la domesticación racional de esta especie, de la cual se cuenta con información escasa. Según Zhang et al. (2009), la baja capacidad de enraizamiento en algunos genotipos de *A. sellowiana* está asociada con la presencia de fibras floemáticas, que impiden el crecimiento de los meristemas neoformados. Los bajos niveles de enraizamiento se han relacionado con la presencia de esclerénquima o fibras perifloemáticas en varias especies, incluyendo *Quercus macrocarpa, Juglans nigra* (Amissah et al., 2008) y varias especies de *Eucalyptus* (Bryant y Trueman, 2015; Goulart et al., 2014). Por otro lado, el desarrollo de tejidos que potencialmente pueden representar barreras físicas puede estar asociado al cambio de fase y, por lo tanto, la pérdida de competencia de las células para diferenciar un nuevo meristema de raíz. En muchas especies, tanto de angiospermas como gimnospermas, la capacidad de formar raíces adventicias está fuertemente afectada por el cambio de fase de juvenil a maduro; en las especies leñosas, la anatomía del tallo difiere de manera importante en plantas con diferente estado de madurez fisiológica (Husen y Pal, 2006; Wendling et al., 2014). El cambio de fase se puede observar anatómicamente por la presencia de tejidos tales como el felema hacia el interior de las fibras floemáticas (Beakbane, 1961).

1.7.3. Propagación vegetativa en *Acca sellowiana*

Los métodos de propagación vegetativa son relativamente dificultosos para *Acca sellowiana* en comparación con otras especies frutales. Se han evaluado técnicas de acodo, injerto, estaquillado y micropropagación, con resultados muy variables. La propagación por estacas se destaca como el método más eficiente. Sin embargo, los porcentajes de enraizamiento son sumamente variables en función del genotipo y el uso de fitohormonas, y la capacidad de rizogénesis no es un carácter que generalmente se incluya al momento de la selección de genotipos en el programa de mejoramiento. Debido a las bajas tasas de enraizamiento y la variabilidad que presenta esta característica, se han evaluado múltiples tratamientos para promover la formación de raíces adventicias. Además del uso más habitual de auxinas, se ha evaluado el uso de antioxidantes (Da Silva et al., 2022), bacterias promotoras del crecimiento vegetal

(Chaparro y Pulido, 2024), búsqueda de marcadores moleculares para características genéticas que mejoren la propagación vegetativa (Bini et al., 2024), así como diferentes condiciones de cultivo de la planta madre que inciden en el éxito de la RA (Niella et al., 2018).

Los cultivos comerciales existentes en nuestro país son pocos y provienen todos de semilla. Por ser una especie alógama, las poblaciones naturales presentan gran variabilidad para la mayoría de las características morfológicas. La heterogeneidad de los frutos disminuye la rentabilidad de su producción. Atendiendo a la necesidad de productores y viveristas de trabajar con variedades homogéneas, es necesario contar con un sistema de clonación del material seleccionado. La propagación vegetativa, como única forma de asegurar la reproducción fiel de la planta madre, debería tener un protagonismo mayor al momento de definir los objetivos del mejoramiento y la selección de genotipos (Bisognin et al. 2018). Para poder mejorar las estrategias de propagación de *Acca sellowiana*, es necesario traducir e interpretar el conocimiento basado en especies modelo para el caso de esta especie frutal. Esto permitirá implementar estrategias de manejo que potencien el proceso para alcanzar niveles de enraizamiento compatibles con la producción comercial.

1.8. Hipótesis

La diferenciación de raíces adventicias en materiales de propagación vegetativa de *Acca sellowiana* presenta gran variabilidad entre genotipos, que puede ser consecuencia de la pérdida de juvenilidad en los materiales de difícil enraizamiento; estas diferencias pueden ser detectadas a nivel histológico.

Los materiales de difícil enraizamiento mejoran su capacidad de diferenciar raíces adventicias con la aplicación de AIB exógeno. Este actúa modificando los niveles de expresión de genes involucrados en la percepción y homeostasis de las auxinas durante las primeras etapas del proceso en que algunas células adquieren competencia para diferenciar un nuevo meristema radical.

1.9. Objetivos

1.9.1. Objetivo general

Estudiar las bases anatómicas y fisiológicas del proceso de diferenciación de raíces adventicias en materiales de guayabo del país para generar información que ayude a interpretar el comportamiento de las especies leñosas cuando se propagan vegetativamente y sirvan de base para ajustar metodologías de propagación más eficientes.

1.9.2. Objetivos específicos

Evaluar la variabilidad en la capacidad de enraizamiento de los materiales de guayabo del país preseleccionados en el Programa de Selección de Frutas Nativas con Potencial Comercial, en respuesta a tratamientos hormonales.

Estudiar los cambios anatómicos y bioquímicos a lo largo del proceso de diferenciación de raíces adventicias en materiales contrastantes, en respuesta al agregado exógeno de auxinas que permitan identificar cuáles pueden ser las limitantes para la diferenciación de RA.

Identificar genes de respuesta a auxinas en especies modelo, validarlos en *A. sellowiana* y estudiar su expresión en tiempo real en genotipos contrastantes para poder explicar la diferente capacidad de rizogénesis adventicia.

Enraizamiento *in vitro* de microestacas de *Acca sellowiana*

Resumen

Acca sellowiana, conocida como guayabo del país, es un árbol frutal nativo de la familia Myrtaceae, nativa de Uruguay y el sureste de Brasil. Presenta un excelente potencial agronómico y comercial, alto valor nutricional, y es adecuado tanto para su consumo en fresco como para la elaboración de productos manufacturados. En el programa de mejoramiento de la especie en Uruguay se han seleccionado varios genotipos con características destacadas. Sin embargo, el bajo nivel de éxito logrado hasta ahora con la propagación vegetativa de esos materiales representa un limitante importante para la producción comercial en nuestro país. Se ha considerado la micropropagación como una alternativa para la producción de plantas de buena calidad, libres de patógenos. El enraizamiento de estacas y microestacas de especies leñosas es fuertemente dependiente del genotipo de la planta madre, y la habilidad para enraizar se pierde rápidamente a medida que estas envejecen. El objetivo de este trabajo fue evaluar diferentes alternativas para mejorar la capacidad de enraizamiento *in vitro* de los materiales seleccionados. Las plantas madre fueron cultivadas en invernáculo y usadas como fuente de explantos para la micropropagación. Los explantos se establecieron *in vitro* en Woody Plant Medium (WPM; Lloyd y McCown, 1981) sin reguladores de crecimiento, siguiendo el protocolo desarrollado en nuestro laboratorio para la especie. La máxima tasa de multiplicación se obtuvo con ribósido de zeatina 2,85 µM. Se evaluaron distintas alternativas de enraizamiento con dos materiales vegetales que se diferenciaban en su capacidad de enraizamiento. Se usó medio WPM, suplementado con ácido indol butírico (AIB) 10 µM, nitroprusiato de sodio (SNP) 100 µM, floroglucinol (PG) 1 mM o la combinación de AIB 10 µM con PG 1 mM. El material de bajo enraizamiento respondió al AIB (60 % de enraizamiento). Sin embargo, ni el PG ni el SNP tuvieron un efecto positivo en el enraizamiento de las microestacas.

Palabras clave: *feijoa*, micropropagación, floroglucinol, óxido nítrico, raíces adventicias

In vitro rooting of *Acca sellowiana* microshoots

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Abstract

***Acca sellowiana*, known as 'Guayabo del País' or 'Pineapple guava' is a small evergreen ornamental fruit tree of the family Myrtaceae, native to Uruguay and south-eastern Brazil. It has excellent agronomical and commercial potential, high nutritional value, and is adequate for fresh consumption as well as the elaboration of manufactured products. Several genotypes with outstanding fruit size and flavor have been selected in the Breeding Program for this species in Uruguay. However, the low success achieved so far with conventional vegetative propagation of selected materials is an important limitation for commercial production in our country. Micropropagation has been considered as an alternative for the production of high quality, pathogen-free plant material. Rooting of cuttings and microcuttings of woody species is strongly dependent on the genotype of the donor plant and the ability to root is rapidly lost with ageing of the mother plant. The objective of the present study was to evaluate different alternatives to improve rooting ability of selected materials, in vitro. Selected mother plants were cultivated in a greenhouse and used as source of explants for micropropagation. Explants were established in vitro on Woody Plant Medium (WPM; Lloyd & McCown, 1981) devoid of plant growth regulators, according to a protocol previously developed in our laboratory for this species. Maximum multiplication rate was achieved with 2.85 µM Zeatin riboside. We evaluated different rooting alternatives with two selected plant materials differing in rooting ability. WPM was used as basal medium, supplemented with 10 µM Indole Butiric Acid (IBA), 100 µM Sodium Nitroprusside (SNP), 1 mM Phloroglucinol (PG), or the combination of 10 µM IBA and 1 mM PG. The material that rooted poorly responded to IBA (60% rooting). However, neither PG nor SNP had a positive effect on rooting of microcuttings.**

Keywords: Feijoa, micropropagation, phloroglucinol, nitric oxide, adventitious roots

INTRODUCTION

Several genotypes of *Acca sellowiana* (Berg.) Burret with outstanding fruit size and flavour have been selected in the Breeding Program for this species in Uruguay. Although *A. sellowiana* is relatively easy to propagate from seeds, the quality of fruits obtained from seed-propagated trees is highly variable from one seedling tree to the next (Thorp and Bieleski, 2005). Clonal propagation allows rapid fixation of superior genotypes prior to their introduction into production or breeding programs. Adventitious rooting is an essential step in the vegetative propagation of economically important horticultural and woody species (Geiss et al., 2009). However, the low success achieved so far with conventional vegetative propagation methods of selected materials, such as grafting and cutting, is an important limitation for commercial production of this fruit tree in our country. Therefore, micropropagation has been considered as an alternative for the production of high quality, pathogen-free plant material.

If the basic biology of root formation was better understood, we would be in a better position to more easily manipulate adventitious root formation in commercially important species and genotypes that are currently recalcitrant to efficient artificial induction of adventitious roots (Ernst, 1994). Development of adventitious roots is a complex process, affected by multiple factors including phytohormones, mineral nutrition, light, associated stress responses such as wounding, genetic characteristics, and their interactions. Rooting of cuttings and microcuttings of woody species is strongly dependent on the genotype of the donor plant and the ability to root is rapidly lost with ageing of the mother plant. Auxin is the hormone commonly used to promote adventitious rooting in different species. However, the pattern of auxin action is still poorly understood and interactions of different types and at different levels are suggested. In some species, phenolic compounds such as ferulic acid and phloroglucinol (PG) have been shown to enhance adventitious rooting, but in other cases, they inhibit this developmental process. Their role seems to depend on its nature and the plant species. These compounds might act as antioxidants, inhibiting auxin oxidation and, thus, allowing the formation of adventitious roots (Geiss et al., 2009). Nitric oxide (NO) is known to play a crucial role in root development (Stöhr and Stremlau, 2006). Recent evidence suggests that this molecule mediates several auxin dependent processes during root growth and development, acting downstream in the auxin signalling pathway (Simontacchi et al., 2013). NO released from sodium nitroprusside (SNP) increases the formation of lateral roots during normal development (Correa-Aragunde et al., 2004; Gao and Yang, 2011). In *Eucalyptus grandis*, Abu-Abied et al. (2012) found a positive correlation between NO production and adventitious rooting, suggesting a role for this compound in promoting rooting of both juvenile and mature cuttings. Most plant species require very specific protocols that target specific genotypes, tissues, or organs, and the search for suitable alternatives to current protocols is one of the main driving forces in plant tissue culture research (Teixeira da Silva et al., 2013). One way of improving different aspects of organogenesis would be to include new growth-promoting substances, such as those evaluated in this study; keeping in mind the strong effect that genotype has on the expected developmental response.

In vitro rooting of *A. sellowiana* using IBA and activated charcoal has been reported (Guerra et al. 2013). PG has been shown to reduce hyperhydricity, promote shoot lignification and induce somatic embryogenesis of *A. sellowiana* in vitro (Teixeira da Silva et al., 2013). However, the role of this phenolic compound has not been evaluated in relation to adventitious rooting of this species, or other species of the Myrtaceae family. In the past few years, significant work has been done on NO as a signalling molecule in a variety of plant developmental processes, suggesting a role in adventitious rooting of several species. The present paper reports the results of adding IBA, phloroglucinol (alone or together with IBA), or sodium nitroprusside to the rooting media, and their effect on the differentiation of adventitious roots of selected materials, in vitro.

MATERIALS AND METHODS

Plant material and general procedures

Mother plants supplied by the Breeding Programme for this species (Facultad de Agronomía – INIA) were cultivated in the greenhouse and treated periodically with fungicide (Benlate®, 2g.L^{-1}) in order to obtain vigorous pathogen-free explants to introduce in vitro. Two genotypes, with contrasting ex vitro rooting ability were chosen for this study: C74 (A) and 27-1 (B) with 60% and less than 20% rooting respectively. Sprouts used as

source of explants were thoroughly washed with a commercial detergent under running tap water; nodal segments (1 – 1.5 cm long) were surface disinfected with 2% NaOCl for 15 min and washed three times with distilled water. Citric acid (0.1 $g.L^{-1}$) was added in the third wash to help prevent tissue oxidation. Explants were inoculated in test tubes containing 15 ml of growth regulators-free basal medium and subcultured to fresh medium every 20 days.

Culture media

WPM (Lloyd & Mc. Cown, 1981) basal medium was used in all experiments, supplemented with MS vitamins (Murashige and Skoog, 1962). pH was adjusted to 5.80 before autoclaving at 121º for 20 minutes. Nodal segments were introduced on basal medium supplemented with 0.5 $g.L^{-1}$ polyvinilpirrolidone (PVP), without hormones.

Media used for multiplication of microshoots contained either 1.76 µM bezylamnopurine (BAP), 9.8 µM 2-isopentenyladenosine (2iP) or 2.7 µM Zeatin (Z).

Media for induction of adventitious roots contained either IBA, phloroglucinol (alone or together with IBA), or sodium nitroprusside as NO donor, added to the basal medium. Rooting treatments were as follows:

1. WPM + MS vitamins (used as control)
2. WPM + MS vitamins + IBA (9.8 µM)
3. WPM + MS vitamins + PG (1 mM)
4. WPM + MS vitamins + SNP (100 µM)
5. WPM + MS vitamins + IBA (9.8 µM) + PG (1 mM)

When IBA was added to the rooting media (treatments 1 and 5), explants were subcultured on auxin-free media after seven days in culture.

Growth conditions

All cultures were incubated in a growth chamber, at 25 ± 2ºC, provided with a photon flux of 30 $\mu mol.m^{-2}.s^{-1}$ and a 16-h photoperiod.

Rooting was evaluated as percent rooted microcuttings, number of roots per microcutting and root length, after thirty days on rooting medium.

Experimental design and statistical analysis

Data were analysed statistically by analysis of variance (ANOVA) and means were separated by least significant difference (LSD), with a confidence level of P = 0.05, using Info-Stat® statistical software. The multiplication experiment was a 2 x 4 factorial design with five replicates, where the factors were two genotypes (C74 and 27-1) and four proliferation media (BAP, 2iP, Z, and a control). The rooting experiment was a 2 x 5 factorial design, with three replicates, where the factors were the same two genotypes and five rooting treatments (IBA, PG, SNP, IBA+PG, and a control).

RESULTS AND DISCUSSION

Proliferation rate of both genotypes in response to 1.76 µM BAP, 9.8 µM 2iP or 2.7 µM Z is shown in Figure 1. Both BAP and 2iP were less effective at inducing proliferation of shoots than Z. However, the number of buds per explant obtained when zeatin was added to the medium was inferior to that obtained for this species using liquid medium in bioreactors (Ross and Grasso, 2010). Liquid proliferation medium has several advantages over semi- solid media, such as optimal nutrient and plant growth regulators supply, or loss of apical dominance and better growth of axillary buds. Nevertheless, handling of cultures can be difficult and contamination will quickly develop and disperse in liquid medium and is likely to lead to significant contamination losses (Preil, 2005).

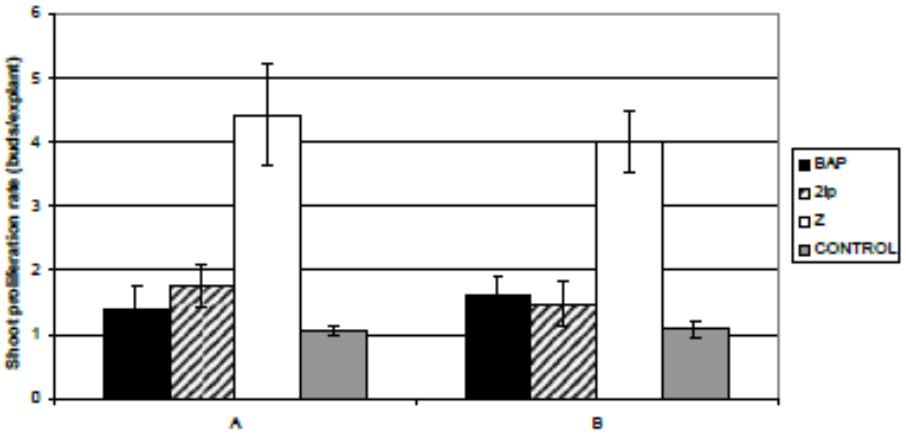

Fig. 1. Effect of cytokinins (BA: benzyladenine, 2iP: 2 isopentenyladenine, Z: zeatin) on shoot proliferation rate of two *A. sellowiana* genotypes (A and B).

In vitro rooting of the two genotypes evaluated was significantly different, similar to rooting of conventional cuttings (Table 1). Genotype C74 had the highest rooting response (65.3%), regardless of rooting treatment, whereas 27-1, which rooted poorly by conventional cuttings, reached 65% rooting in vitro, when IBA was added to the medium (Table 2).

Table 1. Effects of genotype on rooting (%) and number of roots per explant, of *A. sellowiana* microshoots after a month of rooting treatment.

Genotype	Rooting (%)	Nº roots per explant
27-1	29.0 a	1.29 a
C74	65.3 b	2.03 b

LSD means separation (P>0.05)

The addition of 9.8 µM IBA to the culture medium was the only treatment that had a positive effect on rooting of genotype 27-1. Endogenous or exogenously applied auxin plays a central role at each step of the adventitious root formation. IBA is used commercially worldwide to root cuttings from many plant species, but despite its crucial role in this developmental process the pattern of IBA action is still poorly understood (Geiss et al., 2009). Lack of understanding of the modes of action, specifically signal transduction, of identified plant hormones is a serious limitation to manipulate plant organogenesis (Ernst, 1994). A synergistic effect of PG when added to the rooting media together with IBA has been reported. This effect has been especially noted in several apple cultivars (Machakova et al., 2008). The results of our rooting experiment did not show a synergistic effect of PG and IBA for this species at the concentrations evaluated.

Table 2. Effect of rooting treatment on rooting (%) and number of roots per explant of *A. sellowiana* genotypes C74 and 27-1 after a month of rooting treatment.

Rooting treatment	Rooting (%)		Nº roots per explant	
	C74	27-1	C74	27-1
Control	70.0 a	26.7 a	2.68 b	2.13 b
PG	70.0 a	0.0 a	1.40 a	0.00 a
SNP	56.7 a	20.0 a	1.80 ab	1.27 ab
IBA + PG	66.7 a	33.3 ab	2.16 ab	1.47 ab
IBA	63.3 a	65.0 b	2.09 ab	1.57 ab

PG: Phloroglucinol, SNP: sodium nitroprusside, IBA: indol butyric acid

LSD means separation (P>0.05) within column

Neither of the genotypes had a positive response to PG. Genotype 27-1 did not root at all in the presence of PG, and C74 did not differ from the control in the induction of adventitious roots (%). Medium containing PG had a negative effect on the number of roots per rooted explant (Table 2), and roots were shorter (data not shown). According to George (1993), PG has occasionally been found to have inhibitory effects, when explants are exposed to this compound for long periods. The results of the experiment show that when PG was added to the medium together with IBA for seven days and then subcultured to plant growth regulators free medium, root differentiation was not inhibited in genotype 27-1, and number of roots per explant did not differ significantly from the control in neither genotype.

These results confirm that IBA has a significant role in the differentiation of roots of genotypes of *A. sellowiana* otherwise recalcitrant to rooting. However, more efforts are needed to elucidate the pattern of auxin action and possible interactions along the signalling pathway. The identification of anatomical and biochemical markers of adventitious root formation, and rooting associated genes are currently under study with these and other selected genotypes of *A. sellowiana*.

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3. Anatomía de estacas de tallo y respuestas bioquímicas asociadas con la competencia para diferencias raíces adventicias en *Acca sellowiana* (Myrtaceae)

Resumen

Mensaje clave*:* La evidencia anatómica sugiere que las diferencias en capacidad de formar raíces adventicias entre materiales de *Acca sellowiana* se explican por un cambio de fase más temprano en los genotipos de difícil enraizamiento.

El éxito en el desarrollo de raíces adventicias (RA) en estacas impone una limitante importante a la propagación de varias especies leñosas; la habilidad para formar RA está fuertemente afectada por el genotipo. Sin embargo, no se conocen exactamente cuáles son las diferencias entre los genotipos que pueden explicar esas distintas respuestas. En este trabajo, estudiamos el efecto del ácido indolbutírico y el tipo de estaca en la anatomía y respuestas bioquímicas de las estacas en dos genotipos con capacidad de enraizamiento contrastante. Los nuevos meristemas se desarrollaron por fuera del anillo de cámbium, sin formación de callo a los catorce días, y las nuevas RA crecieron a través de la corteza y emergieron a los veintiocho días. Las estacas de los dos genotipos se comportaron de manera diferente tanto *in vivo* como *in vitro*. Encontramos diferencias anatómicas entre los genotipos que podrían explicar las diferencias en habilidad de enraizamiento. En el genotipo de difícil enraizamiento se vio un desarrollo más temprano de la peridermis. Este tejido dérmico secundario podría ser usado como un marcador confiable del cambio de fase para distinguir las porciones de tallo juveniles de las maduras, que perdieron la capacidad de formar RA.

Palabras clave: ácido indolbutírico (AIB), cambio de fase, enraizamiento, anatomía de estacas de tallo, propagación vegetativa

3. Stem-cutting anatomy and biochemical responses associated with competence for adventitious root differentiation in *Acca sellowiana* (Myrtaceae)

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ORIGINAL ARTICLE

Stem-cutting anatomy and biochemical responses associated with competence for adventitious root differentiation in *Acca sellowiana* (Myrtaceae)

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Abstract

Key Message **Anatomical evidences suggest that differences in rooting ability among *Acca sellowiana* materials are explained by earlier phase change in difficult to root genotypes.**

Abstract Successful development of adventitious roots (AR) in cuttings imposes an important limitation to the propagation of woody plants and in some species, the ability to form AR is strongly affected by genotype. However, we lack an understanding of the differences among genotypes underlying such different responses in various species. We examined the anatomical and biochemical effects of exogenous indol-3-butyric acid and type of cutting in rooting experiments of two *Acca sellowiana* genotypes with contrasting rooting ability. New meristems developed outside the cambial ring, without callus formation by day fourteen and new adventitious roots grew through the cortex emerging by day 28. Both anatomically in vivo and biochemically in vitro, cuttings from the different genotypes behaved differently. We found anatomical differences between the genotypes that might explain the differences in rooting ability. An earlier development of a periderm was present in the difficult-to-root genotype. This secondary dermal tissue could be used as a reliable phase-change marker to distinguish juvenile from mature plant parts which have lost rooting capacity.

Keywords Indol-3-butyric acid (IBA) · Phase change · Rooting · Stem-cutting anatomy · Vegetative propagation

Introduction

Propagation by stem cuttings, which relies on adventitious roots (AR) formed in response to wounding is the most widely used method of vegetative propagation around the world and plays a central role in asexual propagation of forest and fruit crops (Steffens and Rasmussen 2016). The success of breeding programmes of woody plants depends on the availability of a cost effective method for vegetative propagation. However, the ability to form AR is variable among genotypes and in some cases it remains the most important limitation for the commercial propagation of elite genotypes. For this reason, a better understanding of the AR differentiation process is needed to explain the great variability found amongst genotypes and to design a strategy to overcome it. This is the case of *Acca sellowiana*, a fruit species native to Uruguay and Brazil with outstanding organoleptic traits (Fischer 2003; Thorp and Bieleski 2005) but little background information available.

In wild plants of *A. sellowiana*, the ability to induce adventitious roots is strongly affected by genotype (Fischer 2003; Cabrera et al. 2010; Ross et al. 2017). Adventitious roots emerge from groups of cells named root initials (Evert 2006). These cells can be found in different tissues of the stem depending on the species (Geiss et al. 2009) including phloem, parenchyma, or other cell types (Tarrago et al. 2005; Riov et al. 2013). The process is triggered by numerous factors both endogenous and exogenous, and different genotypes vary in their requisites and needs along the process of re-establishing a root system (Di Battista et al. 2019).

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Three successive phases are usually recognized in the physiological pathway leading to AR, each with specific requirements: induction, initiation and expression (Pacurar et al. 2014). The induction phase comprises a period of time with no visible histological events during which biochemical changes occur prior to the first cell divisions. During this phase, several events take place at the base of the cutting including a local increase in auxin levels, the establishment of a sink for carbohydrates and transient changes in the activity of several enzymes (de Klerk et al. 1999; Arena et al. 2003; Portirio et al. 2016a). Consequently, although no visible histological events can be observed, biochemical markers can be used to characterize this period in a particular species or genotype and establish its onset and duration.

In difficult to root genotypes, AR initiation may be triggered by the application of exogenous auxins. Biochemical studies indicate that exogenous IBA induces changes in the metabolism of enzymes, carbohydrates and proteins (Elmongy et al. 2018; de Almeida et al. 2020). Increased levels of total soluble carbohydrates in the rooting zone of stem cuttings after exogenous application of auxin have been reported (Agulló-Antón et al. 2014; Elmongy et al. 2018; Goel et al. 2018). Particularly in *A. sellowiana*, IBA has been found to promote in vitro rooting (Guerra et al. 2012; Ross et al. 2017). During the initiation phase, cell divisions leading to the formation of a new root meristem can be observed. A higher number of proteins has been identified in *Eucalyptus* during this phase that reflect these important cellular changes (de Almeida et al. 2020). Protein content increases significantly along the rooting process in response to exogenous IBA, and the maximum levels have generally been related to increased synthesis of enzymatic proteins during the initiation of the root regeneration process (Husen and Pal 2007; Elmongy et al. 2018). The expression phase involves the growth of AR through the cortex and out of the epidermis, and the establishment of vascular connections of the new root with the stem cutting (da Costa et al. 2013). According to Zhang (2009), poor rooting ability of *A. sellowiana* cuttings is associated with the presence of phloem fibres, which prevent growth of the newly formed meristem. Poor rooting has been related to the presence of sclerenchyma or periphloematic fibres in a variety of species including *Quercus macrocarpa* (Amissah et al. 2008), *Juglans nigra* (Stevens and Pijut 2017) and several species of *Eucalyptus* (Goulart et al. 2014; Bryant and Trueman 2015). On the other hand, the development of tissues that may potentially represent physical barriers, may be associated to phase change, and therefore loss of cell competence to form a new AR meristem altogether. In many plant species, both Angiosperms and Gymnosperms the ability to form AR is strongly affected by juvenile to mature phase change; stem anatomy differs markedly in woody plants at different maturational stages (Husen and Pal 2006; Wendling et al. 2014). Phase changes may be observed anatomically by the presence of tissues such as phellem to the inner side of the phloem fibres (Beakbane 1961).

To understand the causes of poor rooting in wild *A. sellowiana* genotypes, we studied temporal changes in carbohydrate and protein levels in response to IBA during AR formation in cuttings and microcuttings of two genotypes with contrasting rooting ability. We examined the stem anatomy at the base of the cuttings in both easy-to-root (R) and difficult-to-root (NR) genotypes using microscopic methods, throughout the AR process and in cuttings with different numbers of nodes to relate rooting ability with maturational stage.

Materials and methods

Plant material

Trials were performed using selected wild genotypes of *A. sellowiana* with contrasting rooting ability, previously identified as R (easy-to-root) and NR (difficult-to-root) (Ross et al. 2017). Four-year-old mother plants were cultured in the greenhouse, under natural light conditions, without heating. Average temperature and humidity during the growing season were 28 °C and 60%, respectively, obtained from data registered with RHT10 datalogger (EXTECH Instruments). Fungicide (Benlate®, 0.2%) and Phostrogen® [NPK (MgO3-SO3): 14-10-27 (2.5–7.5)] were applied periodically to promote pathogen-free vigorous sprout growth. Because all observations were destructive, separate experiments were established to analyze the response to IBA, anatomical evolution in multinodal cuttings and biochemical characterizations.

In vitro culture

An experiment was established to evaluate biochemical differences between R and NR genotypes and their response to exogenous IBA. To facilitate the analysis and obtain the amount of plant material required, this experiment was carried out in vitro. Apical microshoots without leaves were established in vitro on semi-solid Woody Plant basal medium (WPM) (Lloyd and McCown 1980), supplemented with MS vitamins (Murashige and Skoog 1962), 0.44 µM BAP (6-benzylaminopurine; Sigma B3408, Sigma-Aldrich, St. Louis MO, USA) and 0.054 µM NAA (1-naphtalenacetic acid; Sigma N0640, Sigma-Aldrich, St. Louis MO, USA), as previously reported (Ross and Grasso 2010). Multiplication medium was supplemented with 9.8 µM 2iP (6-γ,γ-Dimethylallylamino purine; Sigma D7674, Sigma-Aldrich, St. Louis MO, USA). Two rooting conditions

were compared: IBA 9.8 µM (Sigma I5386, Sigma-Aldrich, St. Louis MO, USA) added to the rooting induction media and media without IBA; explants from both conditions were subcultured on auxin-free media after 7 days. Conditions in the growth chamber were 25 ± 2 °C, 30 µmol photons m^{-2} s^{-1} and 16-h photoperiod. The in vitro rooting experiment was a 2 × 2 factorial design with five replicates, where the factors were two genotypes (R and NR) and two IBA levels (0 and 9.8 µM).

Biochemical tests

Soluble carbohydrates were determined colorimetrically using the anthrone method (Yemm and Willis 1954). Fresh samples (0.10 g) were collected weekly from the basal portion of microcuttings after transfer to auxin-free medium, ground with mortar and pestle and extracted with 1 mL ethanol (80% v/v). After centrifugation for 10 min at 5000*g*, 500 µL of the ethanolic supernatant was diluted in 2.5 mL anthrone (Sigma319899, Sigma-Aldrich, St. Louis MO, USA) and incubated at 100 °C for 10 min. After cooling, 50 µL of sample was diluted in 950 µL anthrone and absorbance was read at 625 nm in a Shimadzu spectrophotometer using as blank ethanol diluted in anthrone at the same concentration. The calibration curve was obtained from dilutions prepared from a glucose solution (0.24 mM). Analyses were carried out in triplicate, with five cuttings per biological replicate, and values expressed as mean ± standard error.

Protein determination was carried out in samples collected weekly after transfer to auxin-free medium from the basal portion (1 cm) of microshoots, and ground with mortar and pestle in liquid nitrogen. Extraction was done with buffer containing 50 mM sodium acetate (Sigma302406, Sigma-Aldrich), 2.0 mM ethylendiamine-tetra-acetic acid (EDTA) (SigmaEDS, Sigma-Aldrich), 1.0 mM mangnesium chloride (SigmaM8266, Sigma-Aldrich) and 1.0 mM phenylmethylsulfonyl fluoride (PMSF) (SigmaP7626, Sigma-Aldrich) at pH 5.5. After centrifugation (20 min at 10,000*g* and 4 °C), the supernatant was used as crude extract for quantification of total protein. Analyses were carried out in triplicate, with five cuttings per biological replicate, and values expressed as mean ± standard error.

Protein concentration was determined according to the method by Bradford (1976), using bovine serum albumin (BSA) (SigmaA2153, Sigma-Aldrich) as a standard. Homogenate (20 µL) was thoroughly mixed with 1 mL Coomassie Brilliant Blue stain reactant and absorbance was read at 595 nm using a Shimadzu spectrophotometer. To obtain the standard curve, absorbance at 595 nm was plotted versus known protein concentration of BSA solution.

Anatomical analysis and response to exogenous IBA

Two different experiments were carried out using semi-hardwood cuttings of the two genotypes under evaluation R and NR. In experiment 1, we emphasized the evaluation of rooting response and the basic anatomical differences between the R and NR genotypes and in response to exogenous IBA application. In experiment 2, we emphasized the evaluation of the anatomical differences between the genotypes along more mature nodes and its response to exogenous IBA. Cuttings for both experiments were collected from the basal branches of greenhouse-grown mother plants once the current season's growth had stopped (February–March, southern hemisphere) following Thorp and Bieleski (2005). Average temperature in the greenhouse at the time of harvest was 28.05 °C and relative humidity 58.97%. To reduce water loss through transpiration, all but the top two leaves half-trimmed were removed (Fig. 1). Cuttings were placed on a propagation bed with bottom heating and overhead mist (Aphos SRL manufacturers), using perlite as rooting substrate, under natural light conditions. Substrate temperature was maintained at 27 ± 1 °C and air relative humidity at 90 ± 5% using an intermittent mist system throughout the rooting experiment.

In experiment 1, only apical uninodal cuttings were used (Fig. 1a) in a 2 × 2 factorial design with three replicates, where the factors were two genotypes (R and NR) and two IBA levels (0 and 12.3 mM). IBA was applied by dipping the base of the each cutting for 5 s in 20 ml of a non-sterile solution of 12.3 mM IBA (Sigma I5386, Sigma-Aldrich) to induce rooting. Ten cuttings were used for each biological replicate. Rooting (%), number of roots per explant and length (cm) of roots were recorded weekly for 5 weeks. Samples for stem anatomy observations were

Fig. 1 Type of apical cuttings of *Acca sellowiana* used for in vivo rooting experiments. **a** Uninodal cutting; **b** two-nodes cutting; **c** three-nodes cutting. Nodes are indicated as N1, N2 and N3. Scale bar in centimetres

collected weekly for 4 weeks, trimming the basal portion (0.5 cm length) of three cuttings per factor combination (genotype × IBA level). Samples were fixed in a formalin-acetic acid–ethanol 70% (FAA, 5:5:90) solution, dehydrated through a series of graded ethanol baths and then infiltrated and embedded in paraffin blocks (D'Ambrogio de Argüeso 1986). Serial sections 10–12 µm thick were cut using a rotary microtome (Slee Medical, Cut 4062), dried, stained with safranin–fast green (Johansen 1940) and finally mounted on microscope slides with Canada balsam mounting medium (Sigma C1795, Sigma-Aldrich). To establish the boundary between the cortex and the stele, we used stem hand sections (12 µm-thick) and employed two different approaches: localization of the starch sheath using Lugol reagent (D'Ambrogio de Argüeso 1986) and detection of callose to identify the phloem, mounting the hand sections in a drop of high-pH solution of aniline blue (Sigma95290, Sigma-Aldrich) (Zarlavsky 2014).

In experiment 2, samples were harvested from cuttings with different sizes: the apical meristem plus one, two or three nodes (Fig. 1a–c) arranged in a 2 × 2 × 3 factorial design with three replicates, where the factors were two genotypes (R and NR), two IBA levels (0 and 12.3 mM) and three sizes of cuttings named N1, N2 and N3 (one, two or three nodes below the apex, respectively). Three cuttings were used for each biological replicate. IBA was applied as in experiment 1.

Samples were collected weekly for stem anatomy observations at each node level and processed in the same way as described above. An exploratory analysis was done in branches of both genotypes, R and NR up to node six. For detection of lignified tissues, histochemical staining of lignin with phloroglucinol/HCl (SigmaP3502, Sigma-Aldrich) was performed on hand sections, 12 µm-thick (D'Ambrogio de Argüeso 1986). Number, width and continuity of periphloematic lignified rings were recorded in photographs of transversal section (Electronic supplementary material 1). The gap distance between fibre strands was used to calculate the percentage gap, later correlated with the percentage of rooting (Amissah et al. 2008), according to the equation:

$$\%\text{Gap} = \frac{\text{Length of fibre free gaps}}{\text{Length of the circumferential arc}} \times 100$$

A Nikon E100 light microscope was used for observation of the samples. Selected cross-sections were photographed with Dino Eye 2.0 digital camera and Dino Capture 1.5.27.A (Electronic Corp.) software. Samples mounted in aniline blue were observed in an epifluorescence microscope (Labotec, exciting 330–380, dichroism 400 and cut-off 420) and photographs were taken using ScopeImage 9.0 software.

Statistical analysis

Data were analysed statistically by analysis of variance (ANOVA) and means were compared by Tukey's test, with a confidence level of $p \leq 0.05$, using Infostat® statistical software. Arcsine transformation was applied to response data before analysis.

Results

Response to IBA

The results of ANOVA from experiment 1 showed a significant effect of exogenous IBA, genotype and interaction genotype x treatment ($p < 0.0001$) (Electronic supplementary material 1). Exogenous IBA (12.3 mM) improved rooting of uninodal cuttings from experiment 1 in both genotypes (R and NR) but the effect was stronger in the NR genotype (Tukey, $p < 0.05$) (Fig. 2). Rooting of R genotype increased from 58.8% (without IBA) to 68.3% (with IBA). When IBA was added to the NR genotype, rooting increased from 1.67 to 56.2%. This value is not different from that of the R genotype without IBA (Tukey, $p < 0.05$) (Fig. 2a). The NR genotype had fewer roots per rooted explant (Fig. 2b), but neither root number nor root length was significantly affected by IBA within each genotype (Fig. 2b, c). Root emergence was not observed earlier than 28 days regardless of genotype, and neither of them formed callus at the base of the cuttings.

Biochemical markers of AR

For biochemichal analysis, materials were multiplied in vitro. Rooting of R and NR genotypes in response to IBA in vitro was not different from uninodal cuttings (Tukey, $p < 0.05$). Rooting of NR genotype increased from less than 5–63% when IBA was added to the rooting medium while R genotype rooting was 71% and 63%, with and without IBA, respectively (Table 1).

We found a significant effect of exogenous IBA on both carbohydrate and protein levels ($p < 0.0001$) but there was no significant effect of genotype and the interaction genotype × treatment was significant only for protein levels (Electronic supplementary material 2). Carbohydrate (CHO) levels increased from day 0 to day 7 in both genotypes and treatments, and decreased gradually from day 7 onwards. Auxin treatment significantly increased carbohydrate levels in both genotypes but no significant differences were found between R and NR genotypes (Tukey, $p < 0.05$) (Fig. 3a). The maximum level of protein was detected at day 14 in both genotypes and treatments. From day 14 onwards protein content at the base of the cuttings decreased gradually. The NR genotype without IBA had the maximum content of

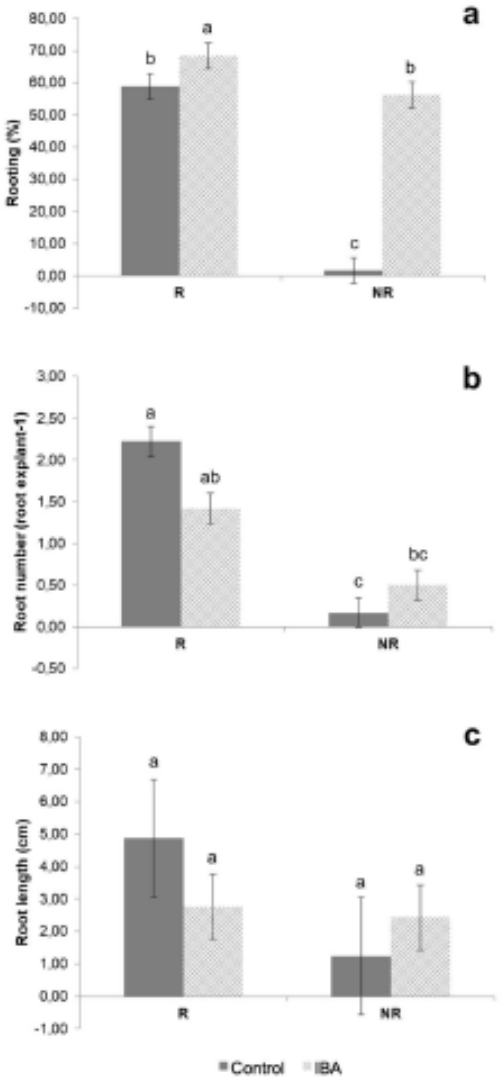


Fig. 2 Effect of IBA (12.3 mM) on: **a** rooting percentage; **b** root number per rooted explant; **c** root length; of two genotypes of *Acca sellowiana* uninodal cuttings with contrasting rooting ability, experiment 1. R (easy-to-root); NR (difficult-to-root). Data are presented as mean ± SE. Different letters indicate significant differences between genotype/treatment combinations (Tukey, $p < 0.05$)

protein and was the only condition (combination genotype × treatment) that differed significantly (Tukey, $p < 0.05$). The addition of exogenous IBA induced a significant decrease in protein content, which reached levels similar to the R genotype. On the other hand, protein content in the R genotype

Table 1 In vitro rooting of two genotypes of *Acca sellowiana* with contrasting rooting ability in response to 9.8 µM IBA

Genotype	IBA (µM)	Rooting (%)
R	0	63.05 ± 3.10
	9.8	71.00 ± 3.50
NR	0	3.65 ± 2.85
	9.8	62.66 ± 3.86

Data are presented as mean ± sd

R easy-to-root, NR difficult-to-root

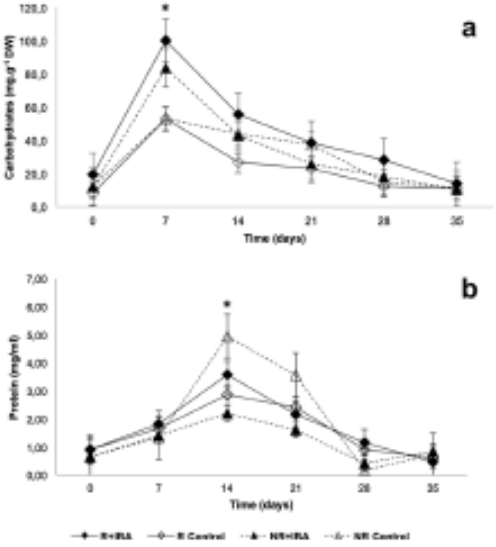


Fig. 3 **a** Soluble carbohydrate content; **b** protein content; throughout adventitious root development in uninodal cuttings of *Acca sellowiana* genotypes (R: easy-to-root and NR: difficult-to-root) with or without IBA (9.8 mM). Asterisks indicate where significant differences between genotypes and treatments were found (Tukey, $p < 0.05$). Data are presented as mean ± SE

was not affected by the presence of exogenous IBA in relation to the control (Fig. 3b).

Anatomical studies of stem cuttings

In the uninodal cuttings, a uniseriate epidermis covers the stem surface of both genotypes and a cortical parenchyma is found below it (Fig. 4a, b). An internal cortical starch sheath, detected by the presence of amyloplasts inside the cells with Lugol staining, surrounds the stele ring (Fig. 4c). Vascular secondary growth is already present at this level of the cuttings. Xylem is continuous with intraxylary phloem and surrounds the central parenchymous pith (Fig. 4a).

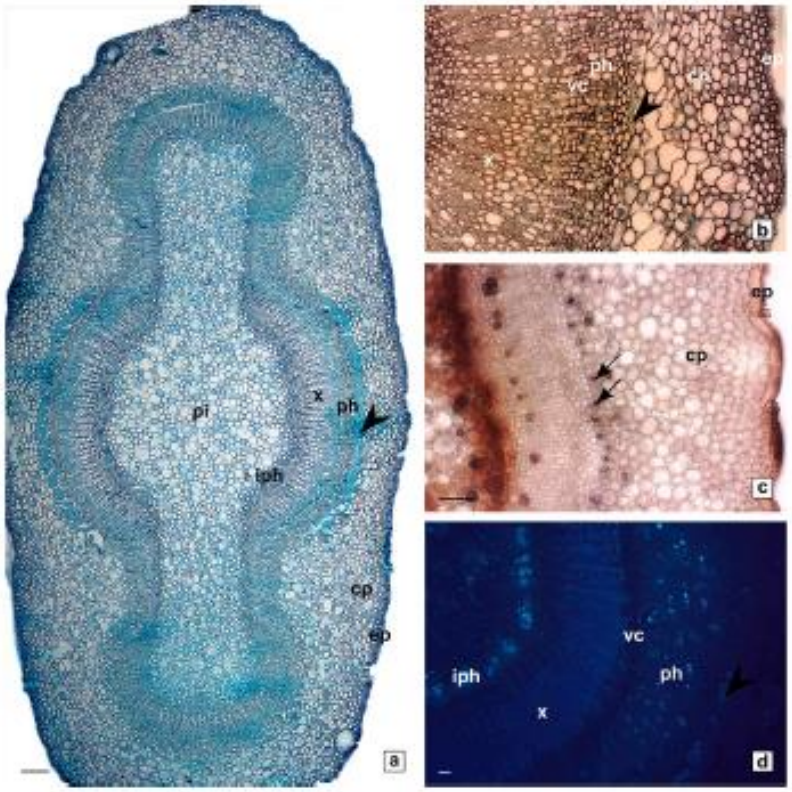


Fig. 4 Micrographs showing stem anatomy at the base of *Acca sellowiana* uninodal stem cuttings (cross sections) from experiment 1 of the R (easy-to-root) genotype on day 0. **a** Panoramic view with safranin-fast green staining; **b** detail of tissue location showing phloem fibres with safranin-fast green staining (black arrowhead); **c** detail showing starch-sheath (black arrows) with lugol staining; **d** micrograph showing epifluorescence of callose staining with aniline blue, black arrowhead points at phloem fibres. *cp* cortical parenchyma, *ep* epidermis, *iph* internal phloem, *ph* phloem, *pi* pith, *vc* vascular cambium, *x* xylem. Scale bars in panel **a** 100 µm; in panels **b**–**d** 50 µm

The internal and external phloem was evidenced by callose detection using aniline blue fluorescence of phloem cellular elements (Fig. 4d). An external discontinuous ring of phloem fibres is present in both materials adjacent to the cortical starch sheath (Fig. 4a, b). No evidence of preformed root primordium was detected and uninodal cuttings of both genotypes had a similar anatomical structure at their bases.

For the first 2 weeks of rooting experiment 1, we found no evidence of anatomical modifications. Although safranin-fast green staining did not show a clear contrast, mitotic figures were first observed in groups of cells within the outer secondary phloem by day 14 (Fig. 5a). Root primordia grew through the cortex (Fig. 5b) and emerged through the epidermis 28 days after cutting establishment (Fig. 5c). AR formation was direct, without callus formation in both genotypes.

Anatomical differences between genotypes were observed in multinodal cuttings below the second node. All cross-sections showed the same basic anatomical structure and rooting behaviour described above, and both genotypes had a discontinuous ring of phloem fibres (Fig. 6a, b). AR initials were also observed as in the previous experiment only in nodes 1 and 2. In the NR genotype, we observed the differentiation of a multistratified tissue with initially thin-walled cells arising by the second node below the apical meristem of the cutting (Fig. 6b, d, white arrowheads). This tissue differentiated from a cell layer located immediately below the phloem fibres. The cells of this layer presented slightly thicker walls than the other phloematic cells and were bigger in the NR genotype than in the R genotype (Fig. 6d). The multistratified tissue formed varied from one to six continuous layers of tangentially flattened cells without intercellular spaces throughout the cross-section. The number of layers increased towards the third node (Fig. 6d, f) and the outermost layers showed thickened cell walls. To explore the progress of this ring towards more basal nodes, we analysed cross-sections at nodes 4–6 (Fig. 6g, h), and found evidence that this multiseriate tissue forms a periderm (internal ring of lignified tissue) which was always present in the NR genotype but seldom appeared in the R genotype.

To compare the position and development of these tissues between R and NR genotypes, we used stem cross sections stained with phloroglucinol/HCl for a better visualization of lignified tissues (Electronic supplementary material 3). Statistical analysis of the data obtained after image processing

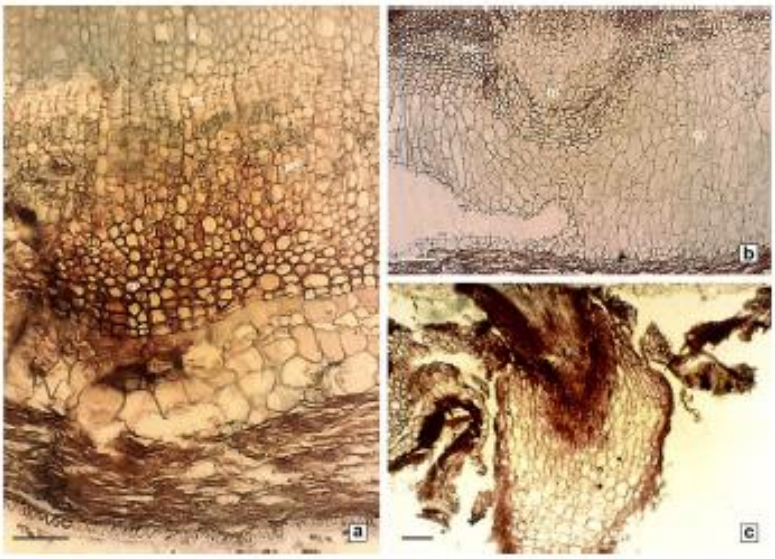


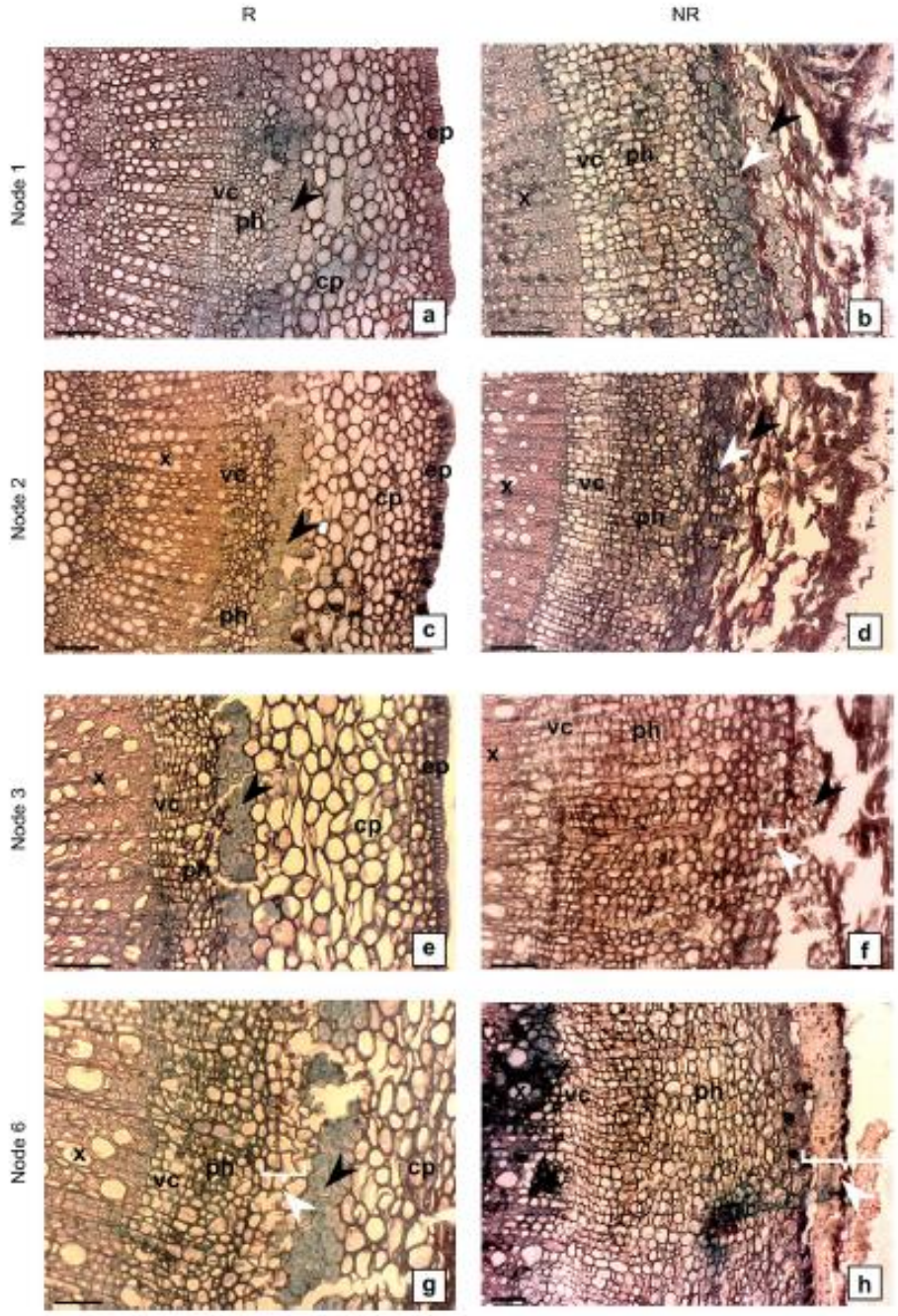
Fig. 5 Light micrographs showing stem anatomy at the base of *Acca sellowiana* uninodal stem cuttings (cross sections) of the R genotype (easy-to-root), at different stages during adventitious root differentiation from experiment 1. **a** Day 14, neoformation of a root meristem outside the cambial ring; **b** day 21, root primordium elongating through the cortex (black arrowheads point at the phloem fibres); **c** day 28, AR emerging. *cp* cortical parenchyma, *ph* phloem, *r* root, *rp* root primordium, *vc* vascular cambium, *x* xylem. Scale bars in panel **a** 50 µm; in panels **b** and **c** 100 µm

showed that the principal effect of genotype was significant on the number of rings of lignified tissues and width of the internal ring ($p < 0.0001$); exogenous IBA affected the width of the internal ring and the proportion of fibre-free gaps of the external ring; width of internal ring was also affected by node. The interaction genotype × node was significant for the three variables. (Electronic supplementary material 4). Depending on the node, the different genotypes (R and NR) had significantly different number of rings with lignified cells (Fig. 7a) and width of the internal ring (Fig. 7b), but no differences were detected on the proportion of fibre-free gaps of the external ring (Fig. 7c). Addition of exogenous IBA affected the internal ring width (Fig. 7b) and the proportion of fibre-free gaps of the external ring in the first two nodes of the R genotype (Fig. 7c) (Tukey, $p < 0.05$). These differences were first detected 2 weeks after IBA treatment.

Discussion

Our results show that the R and NR genotypes of *A. sellowiana* show anatomical differences in vivo and different biochemical responses to IBA in vitro, possibly related to their different rooting ability. In spite of these differences, exogenous IBA was effective to improve the performance of difficult-to-root genotypes which reached levels similar to those of the R genotype without exogenous IBA. Although separate experiments were established to record different characteristics, the differential response to exogenous IBA of R and NR genotypes was maintained both in vitro and in vivo. We had previously obtained a very similar rooting response to IBA for the same R and NR genotypes in a similar in vitro experiment (Ross et al. 2017). The duration of the whole differentiation process did not differ between R and NR genotypes, and no roots were visible in either genotype earlier than 28 days in vivo similar to our previous results in vitro. Zhang et al. (2009) observed new meristem formation by day 20 and reported that it took 30 days for *A. sellowiana* roots to emerge from cuttings. Similar durations of the whole process of AR formation has been reported in several species of *Eucalyptus* (Baltierra et al. 2004; Bryant and Trueman 2015). In *E. grandis*, Abu-Abied (2012) achieved maximum rooting 35 days after IBA treatment.

In response to wounding, a new sink for CHOs is established, providing the structural elements as well as the energy resources needed for development (Kozlowski 1992; Druege et al. 2016). However, the demands for energy and carbon skeletons to support rooting vary between species and depend on the type of cutting (Haissig 1986) and it has been suggested that rooting can be related to individual CHO pools rather than total content in cuttings (Druege 2009). In our experiments, the content of CHOs of both genotypes in vitro evolved and responded similarly to exogenous IBA and reached its maximum levels 7 days after cutting establishment. This increase in CHO levels in *A. sellowiana* cuttings was the first evidence of metabolic changes following wounding that we detected. Exogenous auxin treatments have been found to increase total soluble sugar levels in several species (Steffens and Rasmussen 2016) which in turn modulate gene expression (Rolland et al. 2002; Gibson


R
NR
Node 1
Node 2
Node 3
Node 6
x
vc
ph
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ep
a
b
c
d
e
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◂**Fig. 6** Representative light micrographs of stem-cutting cross-sections on day 0 and at different nodes below the apical bud, of two genotypes of *Acca sellowiana* with contrasting rooting ability from experiment 2: R (easy-to-root) and NR (difficult-to-root). **a** R genotype, Node 1; **b** NR genotype, Node 1; **c** R genotype, Node 2; **d** NR genotype, Node 2; **e** R genotype, Node 3; **f** NR genotype, Node 3; **g** R genotype, Node 6; **h** NR genotype, Node 6. Black arrowheads point at the ring of phloem fibres. White arrowheads point at the initial development of the periderm. *cp* cortical parenchyma, *ep* epidermis, *pe* periderm, *ph* phloem, *vc* vascular cambium, *x* xylem. Scale bars 50 µm

2005; Geiss et al. 2009; Ruedell et al. 2013; De Almeida et al. 2017; Wojtania et al. 2019). In our experiments, cuttings exposed to exogenous IBA showed the highest levels of CHOs, irrespective of genotype. However, the specific role of CHOs in AR formation is controversial and their specific role in the process is not completely clear (Druege 2009). Indeed, even CHO depletion induced by darkness may cause an increase in soluble nitrogen metabolites that can accelerate AR formation (Zerche et al. 2019). Therefore, the content of CHOs per se, cannot explain the different ability to form adventitious roots of the R and NR genotypes. In *E. globulus* cuttings, excised from donor plants before and after they had lost rooting capacity, the concentration of soluble CHOs did not show significant differences (Aumond et al. 2017). Therefore, it is more likely that the increase in CHOs that we observed is a consequence of wounding and physical isolation from the mother plant after excision of the cuttings which leads to the accumulation of substances otherwise transported downwards.

Protein contents showed a very different response suggesting that the nature of the changes induced by IBA were different on both genotypes. The increase in the levels of proteins by day 14 most likely reflects metabolic changes that occur in reponse to wounding which are generally related to the induction phase of AR formation (da Costa et al. 2013). This increase was similar in all the material that was expected to form AR, i.e., cuttings of the R genotype and the NR genotype with IBA. After cutting excision, proteins belonging to several different biological pathways are up or down regulated. In *Eucalyptus*, most of the proteins identified were related to oxidation–reduction processes followed by proteins involved in energy metabolism (de Almeida et al. 2020). Protein content in the NR genotype, on the other hand, was higher to that of the R genotype during the second and third weeks; however, it decreased to levels similar to those in the R genotype with IBA treatment. We did not investigate the identity of the proteins accumulated in each type of cutting in vitro and we cannot confirm whether the same or different genes were responsible for protein accumulation in the R and NR cuttings. However, our results show that internal factors in the untreated cuttings of the NR genotype are responsible for their different biochemical response, and that this further increase in proteins synthesized in response to wounding is then not related to processes associated to rooting at least in vitro.

Cuttings of *A. sellowiana* of the two genotypes with contrasting rooting ability and taken from the same position from mother plants with the same chronological age, differed anatomically. A multistratified tissue that developed between the phloem and the discontinuous ring of phloem fibres was observed in uninodal cuttings of the NR genotype but not in the R genotype. This tissue presented one to several inner continuous layers of thin-walled prismatic cells and several outer thick-walled prismatic cells compactly arranged, lacking intercellular spaces. This pattern corresponds with periderm development during secondary stem growth of several woody species, which may form near the epidermis or deeper into the cortex up to the outer secondary phloem (Metcalfe and Chalk 1950; Evert 2006). A pericycle generally including a sub-continuous ring of fibres is a diagnostic feature of the Myrtaceae stem (Metcalfe and Chalk 1950) and it is coincident with the origin of the periderm found in our anatomical study. Recent investigations in other species of the Myrtaceae family have shown similar results relating reduced rooting capacity and vigour of cuttings with stem anatomy (Abu-Abied et al. 2012; Goulart et al. 2014; Bryant and Trueman 2015; Wendling et al. 2015).

For *A. sellowiana*, poor AR formation has been attributed to the presence of fibre cells in the phloem of stem cuttings that affect the metabolic activity of the new root meristem and mechanically prevent growth of the root primordia (Zhang et al. 2009). Coincidently, we could not find any indication of root meristem formation in any of the cuttings where this periderm was present (data not shown). However, the proportion of fibre-free gaps of the external ring in uninodal cuttings of both genotypes was not significantly different. The main difference was the earlier development of a periderm in the NR genotype and how its width increased from the apex towards the lower nodes and in response to exogenous IBA. Except for this earlier presence of a periderm, both types of cuttings were anatomically similar. According to these results, it is unlikely that this tissue represents a physical barrier to AR development. The lack of AR in this genotype seems to be the absence of meristem formation itself, as observed for several recalcitrant fruit species (Altamura 1996). The development of this tissue by the second node, accompanied by loss of rooting capacity may indicate that the NR genotype actually undergoes an earlier phase change, from juvenile to mature.

The developmental programme of AR differentiation has been inversely linked to the xylogenesis programme in several forest species such as pine (Abarca et al. 2014), peach palm tree (de Almeida et al. 2012) and chestnut (Vielba et al. 2016). It is a consequence of an alteration in auxin homeostasis associated with phase change that negatively affects

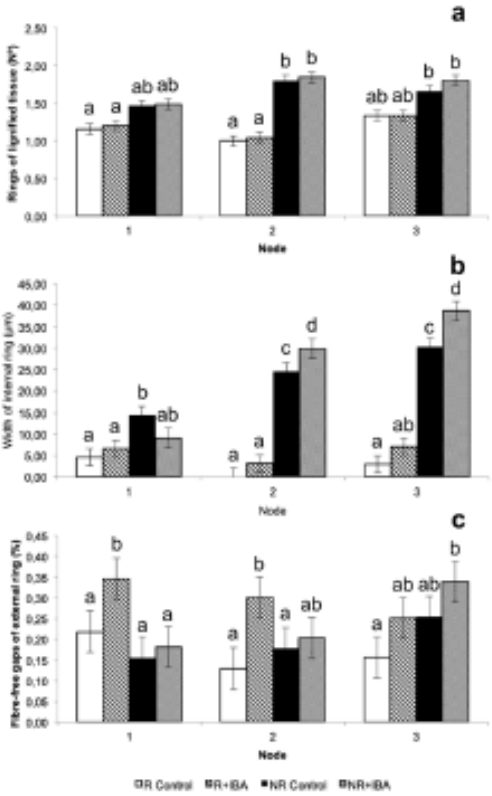

Fig. 7 Characteristics of lignified tissue after 28 days of incubation, at different nodes (1, 2 and 3 from the apex), in two genotypes of *Acca sellowiana* cuttings with contrasting rooting ability: R (easy-to-root) and NR (difficutl-to-root), with or without exogenous IBA (12.3 mM) from experiment 2. **a** Mean number of rings; **b** width of internal ring; **c** fibre-free gaps of external ring. Thirty-six stem cross-sections, corresponding to 12 cuttings, were examined for each combination of genotype-treatment-node. Variables are presented as mean ± SE

AR (Rasmussen et al. 2014). Consequently, high doses of IBA may inhibit rooting of cuttings by promoting lignification and secondary growth (Wendling et al. 2015). In *A. sellowiana*, cuttings of the NR genotype with only one node responded to exogenous IBA improving AR differentiation from less than 10% to more than 50%. However, from the second node downwards AR meristems failed to form, and instead the appearance and width of a periderm tissue was promoted by IBA. In *Eucalyptus*, the difference in rooting capacity among species was related to differences in the metabolism of auxins (Fett-Neto et al. 2001). This apparently contradictory effect of IBA can be understood as a change in sensitivity and response to IBA associated with phase change. Also associated with phase change is the pattern of protein synthesis. Major changes in protein expression accompany phase-change in *E. grandis*; the expression of about 600 up and down regulated genes was significantly different in juvenile and mature cuttings and multiple transcripts related to different regulatory processes, had different expression between juvenile and mature cuttings in response to exogenous auxin (Abu-Abied et al. 2012, 2014). The difference in the pattern of protein expression observed in vitro and lack of AR formation of the untreated NR genotype both in vivo and in vitro is then probably due to its earlier phase change which can be partially reverted by exogenous IBA in the first node when tissues are still responsive.

Conclusions

In our study, we found no evidence of preformed root meristems in *A. sellowiana* cuttings of the wild genotypes used. Instead, AR meristems developed de novo outside the cambial ring. Exogenous application of IBA was effective in promoting rooting rates compatible with commercial propagation in uninodal cuttings. Anatomy of stem cuttings differed between genotypes. However, we found these differences to be more likely a consequence of phase change, from juvenile to mature, and loss of rooting ability of the NR genotype, associated with an earlier development of the periderm.

Author contribution statement Conceptualization, SR and PS; data curation, MS, NÁ and FP; formal analysis, SR; funding acquisition, SR and PS; investigation, SR; project administration, SR; supervision, PS; visualization, SR, MS, GS and AMG; writing—original draft, SR; writing—review and editing, SR, GS, MS, AMG and PS.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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4. Validación y análisis de expresión de genes candidatos para rizogénesis adventicia en microestacas de *Acca sellowiana* (Myrtaceae)

Resumen

Acca sellowiana (Myrtaceae) es una especie multipropósito con frutos comestibles y valor ornamental, nativa de Uruguay y el sureste de Brasil. En Uruguay, la domesticación de la especie es incipiente, aunque en otros países se cultiva ampliamente. Es un arbusto perenne de polinización cruzada por pájaros y abejas. Por este motivo, es necesario desarrollar estrategias de propagación vegetativa como por ejemplo el empleo de ,estacas de tallo, como forma de reproducir los genotipos destacados para su conservación y uso en programas de mejoramiento. La formación de raíces adventicias (RA) en estacas está regulada por factores ambientales y endógenos. Entre las fitohormonas, el ácido indolbutírico (AIB) es la auxina exógena más usada para mejorar el enraizamiento de estacas. La mayor parte de los estudios moleculares acerca de la formación de RA utilizan especies modelo; sin embargo, la conservación de esos mecanismos en otras especies ha sido poco estudiada y los efectos de los distintos factores y sus interacciones en *A. sellowiana* son poco conocidos. La identificación y análisis de la expresión de genes que se sabe que están involucrados en la regulación del proceso es un paso importante para dilucidar los mecanismos moleculares que regulan la diferenciación de RA en estacas de *A. sellowiana*. En este trabajo, comparamos dos genotipos con capacidad de enraizamiento contrastante e identificamos y caracterizamos tres genes que podrían regular el inicio del proceso de desarrollo de RA en *A. sellowiana*: *AsPIN1*, *AsTIR1* y *AsSHR*. El análisis de su expresión mostró que en el genotipo de difícil enraizamiento *AsTIR1* aumenta de manera significativa en respuesta al AIB exógeno, enseguida del tratamiento de inducción. La expresión relativa de *AsPIN1* y *AsSHR* también aumenta, 24 h después. Se discute el significado biológico de este patrón de expresión génica.

Palabras clave: raíces adventicias, ácido indolbutírico, propagación vegetativa, transporte polar

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ORIGINAL ARTICLE

Validation and expression analysis of candidate genes for adventitious rooting, in micro-cuttings of *Acca sellowiana* (Myrtaceae)

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Abstract

Acca sellowiana (Myrtaceae) is a multipurpose species with edible fruits and ornamental value, native to Uruguay and southern Brazil. Domestication of the species in Uruguay is incipient although in other countries, it is widely cultivated. It is an evergreen out-crossing shrub, pollinated by birds and bees. For this reason, it is necessary to develop vegetative propagation strategies such as stem cuttings to reproduce outstanding genotypes for conservation or breeding programs. Adventitious root (AR) formation in cuttings is regulated by environmental and endogenous factors. Among phytohormones, indole-butyric acid (IBA) is the most widely exogenous auxin used to improve rooting of cuttings. Most studies on AR formation at the molecular level use model species; however, the conservation of these mechanisms in non-model plants has been little studied, consequently the effects of different factors and their interactions in *A. sellowiana* are not well understood. The identification and expression analysis of genes known to be involved in the regulation of the process is an important step to elucidate the molecular mechanisms that regulate AR differentiation in *A. sellowiana* cuttings. In this study, we compared two genotypes with contrasting rooting ability, and we identified and characterized three genes that might regulate the onset of AR development in *A. sellowiana*: *AsPIN1*, *AsTIR1* and *AsSHR*. Their expression analysis showed that in the difficult-to-root genotype, *AsTIR1* increases strongly in response to exogenous IBA, shortly after induction treatment. Relative expression of *AsPIN1* and *AsSHR* also increases 24 h later. The biological significance of this gene expression pattern is discussed.

Keywords Adventitious rooting · Indole-butyric acid (IBA) · Vegetative propagation · Polar transport

Introduction

Acca sellowiana (Berg.) Burret is an evergreen shrub of the Myrtaceae family which is cultivated for its fruits and is also valued as an ornamental plant for its flowers and foliage. Extracts taken from fruits and leaves contain antioxidant, antimicrobial, and pharmacological activities (Vuotto et al. 2000; Bontempo et al. 2007; Mosbah et al. 2018; Tortora et al. 2019). Domestication and breeding of this species depend on the ability of elite plant materials to be propagated. However, adventitious root differentiation of cuttings varies between 0 and 80% depending on the genotype set evaluated (Franzon et al. 2004; Guerra et al. 2012; Ross et al. 2017; Niella et al. 2018), and it is difficult to provide nurseries with mother plants of selected materials and particularly difficult-to-root genotypes.

It was hypothesized that these differences in AR formation among *A. sellowiana* genotypes are due to an earlier phase change in the difficult-to-root genotypes which can explain the loss of competence to form adventitious roots (Ross et al. 2021). In woody plants, the ability of cuttings to form adventitious roots declines with the age of donor plants (Wendling et al. 2014a; Aumond et al. 2017) and has been inversely linked with the xylogenesis program (de Almeida et al. 2012; Abarca et al. 2014; Vielba et al. 2016). Some genes of the GRAS family, such as *SCR* and *SHR*, are involved in the maturation-related decline of adventitious rooting (Pizarro and Díaz-Sala 2019). This might be explained by the different functions that the same gene regulatory network may play at different developmental stages

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of a cell type (De Lucas and Brady 2013). The lower rooting capacity associated with ageing of donor plants is little understood in woody plants (Aumond et al. 2017). Anatomy of stem cuttings in difficult-to-root genotypes of *A. sellowiana* shows an earlier development of the periderm associated with loss of competence to form adventitious roots (Ross et al. 2021). It is known that competence to form AR declines with phase change as a result of changes in auxin homeostasis (Rasmussen et al. 2014). The different rooting performance observed in genotypes with different rooting ability is mainly due to the regulation of endogenous active auxin accumulation in the AR source tissues after cutting excision, as well as differences in auxin sensitivity (Guan et al. 2015; Druege et al. 2016, 2019). This differential distribution of auxin seems to be a sufficient signal to trigger or modify the developmental program of a cell in model plants (Vanneste and Friml 2009; Negishi et al. 2011; Ruedell et al. 2015) although the mechanism has not been characterized in *A. sellowiana* specifically.

The fact that IAA is involved in the early events of AR formation has been well established (Blakesley 1994; Della Rovere et al. 2013) and the concentration of endogenous IAA is considered to play a central role in the control of AR initiation and development in various plant species (Ford et al. 2001; Kelen and Ozkan 2003; Davies 2010; Bellini et al. 2014; Pacurar et al. 2014; Wendling et al. 2015; Vilasboa et al. 2018; Gonin et al. 2019; Druege et al. 2019; de Almeida et al. 2020). The concentration of auxin in specific tissues is controlled by its biosynthesis, metabolism, and transport, and these processes are regulated by multiple mechanisms (Han et al. 2009). The differential accumulation of endogenous IAA in easy and difficult-to-root genotypes might be explained by the different expression of IAA biosynthesis and transport genes (de Almeida et al. 2015; Druege et al. 2019) as well as higher expression of repressors of auxin-responsive genes in hard to root species (Ruedell et al. 2015). AR formation depends on the early accumulation of IAA at the base of the cutting, via polar transport (Negishi et al. 2014; Pacurar et al. 2014; Druege et al. 2016). Quantification of indole 3 acetic acid (IAA) is not extensively used in AR studies because of its low concentration in tissues and the interference of other compounds in the analysis (Stuepp et al. 2016).

An alternative strategy to understand AR formation would be to identify and study the expression of genes involved in auxin homeostasis and root meristem patterning in the excised tissues of cuttings. Sensitivity to auxin is determined by the presence and affinity of auxin receptors. The auxin receptor *TRANSPORT INHIBITOR RESPONSE 1 (TIR1)* and its homologous gene *PagFBL1* increased their expression a few hours after auxin treatment (de Almeida et al. 2015; Shu et al. 2019). Once auxin sensitivity is established, other genes in the auxin response network which act downstream in the AR signaling pathway are bound to activate (Teale et al. 2006; Pierre-Jerome et al. 2013; Wachsman et al. 2015). Changes in gene expression are detected during the first hours after cutting excision in response to the stress caused by wounding mainly during the first 24 h (Druege et al. 2016).

Several genes are induced by exogenous auxin during the process of AR formation (Ludwig-Müller 2000; Guan et al. 2015; Druege et al. 2019). Auxin-induced AR formation in cuttings involves transcription factors of the GRAS family, particularly in the context of maturation of woody plants (Druege et al. 2016). Root meristem patterning and stem cell specification in response to auxin require of the GRAS family transcription factors such as *SHORTROOT* (*SHR*) (Blilou et al. 2005). These are also involved in the maturation-related decline of adventitious root formation in distantly related forest species, and the switch between the developmental programs of xylogenesis and AR formation in *A. thaliana* (Xuan et al. 2014; Abarca et al. 2014; Stevens et al. 2018; Pizarro and Díaz-Sala 2019). In other species, these genes are upregulated during AR formation and null mutants exhibit reduced AR formation (Druege et al. 2019). The expression of the auxin efflux carrier gene *PINFORMED 1* (*PIN1*) increases in response to exogenous auxin; short auxin treatments activate the transcription of *PIN* genes in different tissues regulating its own distribution (Vanneste and Friml 2009; Fett-Neto et al. 2011).

AR formation in *Acca sellowiana* cuttings is strongly affected by genotype. Among several treatments used to induce rooting, difficult-to-root genotypes (NR) respond only to exogenous IBA and reach rooting levels similar to the easy-to-root genotypes (R) without exogenous hormone (Ross et al. 2017). Our hypothesis is that exogenous IBA improves AR formation in NR cuttings of *A. sellowiana* by modifying the expression of genes involved in auxin perception and homeostasis during the first stages of the process, leading to the acquisition of competence of some cells to form AR. In order to contribute to the understanding of AR formation and the causes of intraspecific variability specifically in *A. sellowiana*, the purpose of our study was to identify some of those genes in model species, validate them in *A. sellowiana* and study their expression pattern in response to exogenous IBA. In order to provide evidence that supports this interpretation of the differences among genotype, we selected a few but critical genes that have been shown to be involved in auxin homeostasis (*PIN1*; *TIR1*) and root meristem patterning (*SHR*) in *E. grandis* and *A. thaliana*, to study their expression in micro-cuttings of two *A. sellowiana* genotypes with contrasting rooting ability.

Materials and methods

Plant material and culture conditions

Mother plants of *A. sellowiana* were provided by a local breeding program of the species (INIA-Facultad de Agronomía-MGAP; Uruguay). Two selected genotypes with contrasting rooting ability were grown in the greenhouse under controlled conditions, and treated periodically with fungicide (Benlate®, 0.2%) and Phostrogen® [NPK(MgO3-SO3): 14–10-27 (2.5–7.5)]. Genotypes were identified as R (easy-to-root) and NR (difficult-to-root), according to their rooting performance *ex vitro* (more than 60% and less than 20% rooting, respectively). To minimize the phenotypic differences in growth habit that might exist between genotypes, sprouts were collected from the basal branches of 4-year-old, vigorous healthy plants, in the same position in the branch. Apical segments (1.5–2.0 cm long) were surface-disinfected with 2% NaOCl for 15 min, washed three times with distilled water and introduced in vitro on WPM medium (Lloyd and McCown 1980) supplemented with MS vitamins (Murashige and Skoog 1962). Rooting of micro-cuttings was induced by adding IBA (9.8 µM) to the culture medium as previously described (Ross et al. 2017). Micro-shoots without IBA treatment were used as control. Cultures were incubated at 25 ± 2 °C, provided with a photon flux of 30 µmol m^{-2} s^{-1} and 16:8-h photoperiod.

Bioinformatics analysis and primer design of candidate genes and reference genes

Three candidate genes and three reference genes were chosen for further gene expression analysis. Candidate genes were selected based on literature for other species, in which they are known to be involved in auxin transport (*PIN1*), auxin perception (*TIR1*), and root patterning (*SHR*). Reference genes were chosen among genes previously validated for their use during adventitious rooting in *Eucalyptus globulus* (*EF2*, *H2B*, *UBI*) (de Almeida et al. 2010). The primers used for reference genes were those validated for *E. globulus* by the Almeida et al. (2010), shown in Table 1.

For primer design, sequences of the three candidate genes in *A. thaliana* were taken from the GenBank database of the National Center for Biotechnological Information (NCBI) (http://www.ncbi.nlm.nih.gov/genbank/) and Plant Transcription Factor Database (PlantTFDB) http://planttfdb.gao-lab.org/). A sequence similarity search within *E. grandis* genome was performed by BLAST analysis using the Phytozome12 platform (https://phytozome.jgi.doe.gov/pz/portal.html). The predicted amino acid sequences were compared and conserved amino acids are colored by the Jalview multiple alignment editor (Clamp et al. 2004).

Conserved regions were obtained by the alignment of the encoding sequences of both species (*A. thaliana* and *E. grandis*), using BioEdit© Sequence Alignment Editor software (Hall 1999). Primers were designed from these conserved regions using Primer3Plus (https://primer3plus.com/) and sequenced at Macrogen Inc. (Seoul, Korea). If differences in the sequences of both species were observed, the designed primers were biased towards the sequence corresponding to *E. grandis*. Two pairs of specific primers were designed for each candidate gene of interest (*PIN1*, *TIR1*, and *SHR*) based on the sequence alignment of conserved regions of the homologous genes in *A. thaliana* and *E. grandis* (Table 2).

Amplification and sequence analysis of candidate genes in *A. sellowiana*

To confirm whether the primers showed homology with genomic regions of *A. sellowiana*, genomic DNA was extracted from leaves of both genotypes of *A. sellowiana*,

Table 1 Primer sequences for reference genes (de Almeida et al. 2010)

Gene symbol	Forward primer (5′–3′)	Reverse primer (5′–3′)
EF2	GCGTTCCCTCAGTGTGTCTT	GGTCATCTGCTCCTTCAAGC
UBI	AGAAGGAATCGACCCTCCAC	CCTTGACGTTGTCAATGGTG
H2B	GAAGAAGCGGGTGAAGAAGA	GGCGAGTTTCTCGAAGATGT

Table 2 Primer sequences for the genes of interest designed using Primer3Plus software. Two pairs of primers were designed for each gene of interest *PIN1*, *SHR*, and *TIR1*

Gene symbol	Forward primer (5′–3′)	Reverse primer (5′–3′)
1-PIN1	CCTCATGGTCCAGATCGTC	CGAGTATATCTCAGCATTGGTTAGG
2-PIN1	ACGGGGTCGGACTTCTAC	TATACGAGGGCAGAGTAGACG
1-SHR	AAGACTTGCTCCTTCGAGTCCA	TCGGTCTTAACCGTCGGAAA
2-SHR	GCTGGCCTCCTACTTCCTC	GCCTCGCAAATTTCTCCAT
1-TIR1	TCTTTTAAGAATTTTAAGGGTTCTTGT	AGCAATCCCAAAATCCAGA
2-TIR1	ACGCGAGCTGAGAGTGTTTC	GATCGCATTGTCTCCAGCTT

using the cetyl-tri-methyl-ammonium bromide (CTAB) method (Doyle and Doyle 1987). DNA samples of *E. grandis* and *A. thaliana* were included as positive controls, using the same method. DNA quantity and quality were assessed by electrophoresis in agarose gel (0.8%) and spectrophotometry with a NanoDrop ND-1000 (Thermo Scientific©).

PCR amplification was performed in a 20-µl reaction containing: 1 × reaction buffer with 2-mM $MgCl_2$, 1-mM dNTPs, 0.5 µM of each primer, 100-ng template DNA, and 0.5 U Taq DNA polymerase (Thermo Scientific©). The PCR program was as follows: 94 °C for 5 min, followed by 35 cycles of 94°C for 30 s, 52–58 °C for 30 s, and 72 °C for 40 s, using a Gene Touch Thermal Cycler (Bioer Technology ®).

Amplification products were resolved by electrophoresis in an agarose gel (2%), stained with ethidium bromide and visualized under UV illumination. The size of the amplification products was estimated with 1 kb ladder (Thermo Scientific©) as molecular weight marker. Unique amplification products and their respective primers were sequenced at Macrogen Inc. (Seoul, Korea) and edited using FinchTV software (Geospiza Inc.©). Once edited, the final sequences were analyzed using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and the UniProt database (https://www.uniprot.org/). The sequences obtained were deposited to GenBank with accession numbers MZ130946-MZ13095.

Design of specific primers for qRT-PCR

Specific primers for qRT-PCR of target and reference genes were designed using Primer3Plus (Tables 3 and 4). More than one pair of primers for each gene of interest and reference gene was designed in order to obtain combinations with similar amplification efficiency for expression analysis. Target sequence size was confirmed on genomic DNA of *A. sellowiana* by PCR in 20-µl reactions containing: 100-ng genomic DNA, 1 × reaction buffer with 2-mM $MgCl_2$, 1-mM dNTPs, 0.5 µM of each primer, and 0.5 U Taq DNA polymerase (Thermo Scientific©). The PCR program was as follows: 94 °C for 1 min, followed by 35 cycles of 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 40 s, using a Gene Touch Thermal Cycler (Bioer Technology ®). Amplification products were resolved in agarose gels (2%) with 1 × TBE buffer solution and stained with ethidium bromide. The size of amplification products was estimated with 1 kb ladder (Thermo Scientific©) as molecular weight marker and visualized under UV illumination.

Table 3 Primer sequences of target genes used in qPCR analyses in *A. sellowiana*

Gene symbol	Forward primer (5′–3′)	Reverse primer (5′–3′)	Amplicon size (bp)
As1PIN1(a)1	CTCTGATGCTTTTCCTGTTCG	TTGGATGGAGACGATGGAC	94
As1PIN1(b)	CTGATGCTTTTCCTGTTCGAG	GACTTGGATGGAGACGATGG	95
As2PIN1(a)2	CTTCCACTTCATCTCCTCCAAC	AAGAGGGTAATGGACCAGTCG	149
As2PIN1(b)	CTTCCACTTCATCTCCTCCAAC	AGGGTAATGGACCAGTCGAG	146
As2SHR(a)	CAGGGCTTGCTTAGTCGTATG	TGGAACTTGAGCACCATCTTC	115
As2SHR(b)	AAGATGGTGCTCAAGTTCCAG	CAATACGTGTTGCTGATGTCG	130
As2TIR1(a)	CATTGAACCACGTTGTCCTG	CTTGCACTGTTGGACAATGG	78
As2TIR1(b)	CCATTGTCCAACAGTGCAAG	GCCACAGAAAGCATCTCAAG	114

Table 4 Primer sequences of reference genes used in qPCR analyses in *A. sellowiana*

Gene symbol	Forward primer (5′–3′)	Reverse primer (5′–3′)	Amplicon size (bp)
H2B (a)	AAGAAGCGGGTGAAGAAGAG	CGAAGATGTCGTTGATGAAGC	132
H2B (b)	AAGAGCGTGGAGACGTACAAG	CGAAGATGTCGTTGATGAAGC	117
EF2 (a)	TGATGTCCGATCCTTCTGG	TTCAAGCCCTTCCTCTTACG	76
EF2 (b)	CAGTGTGTCTTCGATCACTGG	TTCAAGCCCTTCCTCTTACG	106
UBI (a)	TCCCCTTGTTTCTTCGTCTC	CCTTGACGTTGTCAATGGTG	111
UBI (b)	CCTTCCCCTTGTTTCTTCG	CCTTGACGTTGTCAATGGTG	114

RNA isolation and cDNA synthesis

Samples were collected 12 and 36 h after rooting induction treatment with IBA. Total RNA was extracted from bottom Sects. (10 mm) of three micro-cuttings per replicate, further purified using Qiagen RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and treated with RNase-free DNase (Invitrogen, Carlsbad, USA) to eliminate residual DNA, following the manufacturer's instructions. RNA concentration was determined using a NanoDrop spectrophotometer ND-1000 (Thermo Scientific©). Micro-cuttings used for RNA extraction were discarded, so different repetitions were used for the rooting experiment.

Synthesis of cDNA was performed from 1 µg total RNA to the final 12 µl reaction mixture using MMLV-RT/SS reverse transcriptase according to the manufacturer's instructions (Invitrogen, Carlsbad, USA). We tested different cDNA dilutions (1/5, 1/10, 1/25, 1/50, 1/100) to determine the best concentration to be used. The optimum dilution was chosen with a quantification cycle (Cq) between 18 and 22 cycles for all samples. A standard curve was generated for each gene of interest using a fivefold dilution series, which was used to calculate primer efficiencies.

Gene expression analysis in micro-cuttings

The relative expression levels of *TIR1*, *PIN1*, and *SHR* at the base of micro-cuttings were determined by quantitative reverse transcription-PCR (RT-qPCR). The resulting data were analyzed by the Comparative Ct method (Livak and Schmittgen 2001). Results are presented as fold change in expression according to Eqs. 1 and 2.

$$Foldchange = 2^{-\Delta\Delta Ct} \quad (1)$$

$$\Delta\Delta Ct = [(Ct\ gene\ of\ interest - Ct\ internal\ control)\ treated\ sample - (Ct\ gene\ of\ interest - Ct\ internal\ control)\ untreated\ control] \quad (2)$$

First, the expression of these three genes in both genotypes without addition of exogenous auxin was compared to their expression in the R genotype at the beginning. We then compared the effect of exogenous IBA on the expression of the same genes, in both genotypes (R and NR) 12 and 36 h after the induction treatment.

PCR reactions were carried out using Maxima SYBR Green/ROX qPCR Master Mix (2x) (Thermo Scientific) in a Line-Gene K Fluorescence Quantitative PCR Detection System (Bioer Technology) as follows: 5 min pre-denaturing at 95 °C, followed by 40 cycles of 95 °C for 15 s, 60 °C for 20 s, and 72 °C for 15 s. Three biological and two technical replicates of each sample were done. To confirm the specificity of each PCR reaction, a heat dissociation curve (melting curve) was performed, from 60 °C to 90 °C, following the final PCR cycle. To study changes in gene expression at the onset of AR development, samples were harvested at two time points (12 and 36 h after excision).

The reference genes (RGs) used as internal control were Histone *H2B* and Elongation factor *EF2*, reported as reference genes for qPCR during in vitro adventitious rooting of *Eucalyptus globulus* (de Almeida et al. 2010) and validated for *A. sellowiana*. To check that the expression of these RGs was not influenced by the conditions of the experiment, the effect of genotype, experimental treatment, and time of sampling on their expression was validated by one-way ANOVA ($p = 0.01$) using $2^{-\Delta Ct}$, where ΔCt ($Ctsample - Ctcal$) (Schmittgen and Zakrajsek 2000). Experimental data were analyzed by the Comparative Ct method (Livak and Schmittgen 2001), using the geometric mean of the RGs for normalization of the genes of interest expression (Vandesompele et al. 2002).

Experimental design and statistical analysis

The rooting experiment had a factorial design (2 × 2) with five replicates, where the factors were two genotypes (R and NR) and two levels of IBA (0 and 9.8 µM).

The gene expression experiment had a factorial design (2 × 2 × 2) with two genotypes (R and NR), two levels of IBA (0 and 9.8 µM) and two time points post treatment (12 and 36 h.). For each combination of factors, three biological and two technical replicates were performed.

Data were analyzed statistically by analysis of variance (ANOVA) and means were compared by Tukey's test, with a confidence level of $p \leq 0.05$, using Infostat® statistical software. Arcsine transformation was applied to response data before analysis. Data in figures are given as means ± SE.

Results

Adventitious root differentiation in response to exogenous auxin (IBA)

The effect of exogenous IBA on AR formation was observed after four weeks. In vitro rooting of NR micro-cuttings improved significantly (standard error 2.89, degrees of freedom 3) when exogenous IBA (9.8 µM) was added to the induction media ($p < 0.0001$). Rooting percentage increased 2.4-fold when compared to the control treatment in the absence of auxin (Fig. 1a) although the number of roots per explant was not significantly different ($p = 0.4104$) (standard error 0.25, degrees of freedom 3) (Fig. 1b). No differences were detected in rooting percentage or root number of the R genotype after the addition of exogenous IBA (Fig. 1a

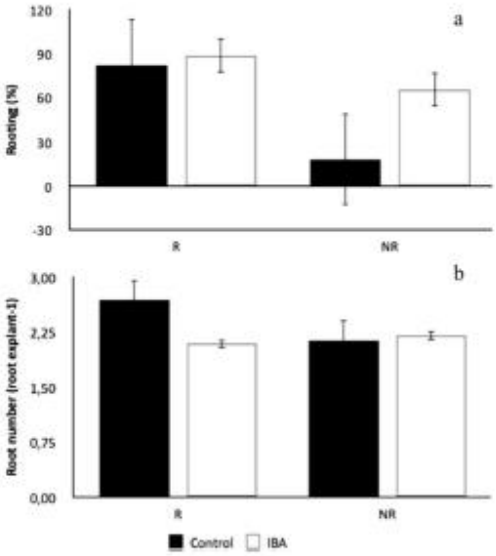

Fig. 1 Rooting performance in vitro of *A. sellowiana* genotypes with contrasting rooting ability, in response to exogenous IBA (9.8 µM). R: easy-to-root genotype; NR: difficult-to-root genotype. **a** Rooting percentage; **b** Root number per rooted explant. Bars represent the mean values ± SE

and b). New adventitious roots developed at the base of the micro-cuttings without callus formation in all cases (Fig. 2).

Amplification of target sequences in *A. sellowiana*

Unique amplification products of the expected size were obtained for the three genes of interest: *TIR1*, *SHR*, and *PIN1*. *SHR* and *TIR1* amplified with only one pair of the specific primers that we designed (Fig. 3a and b). *PIN1* amplified effectively with both pairs of specific primers designed. The resulting sequences (325 bp and 430 bp) partially overlapped and were assembled into a unique sequence (Fig. 3c). The three sequences obtained showed high identity with their homologous genes in *E. grandis* (> 90%) and *A. thaliana* (> 75%), according to NCBI and Uniprot databases respectively (Table 5).

The reference genes chosen for this study resulted in unique amplification products of the expected size in *A. sellowiana*: *EF2*, *H2B*, and *UBI*. The resulting sequences showed high identity with their homologous genes in *E. grandis* (> 90%) and *A. thaliana* (≥ 69%), according to NCBI and Uniprot databases respectively (Table 5, Fig. 4). The predicted amino acidic sequences also showed high

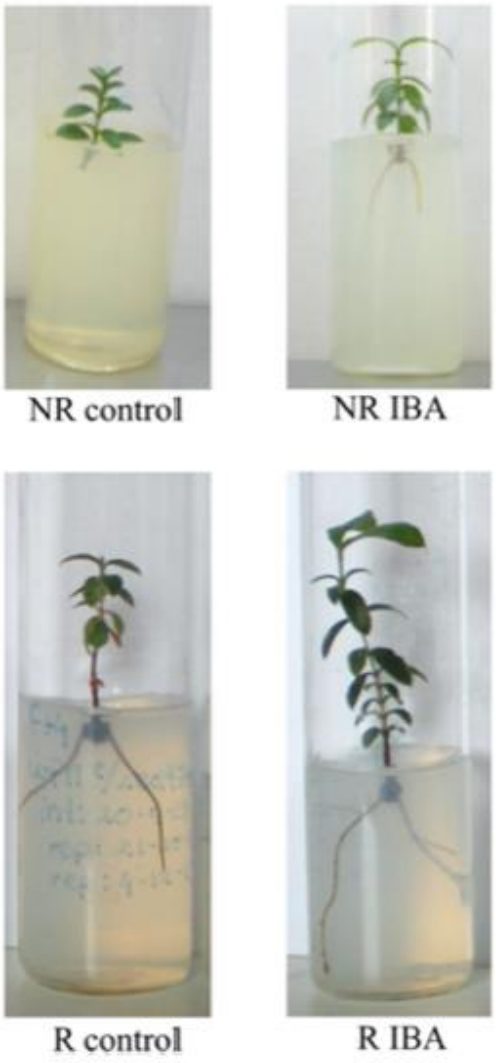

Fig. 2 Examples of root formation in samples of both studied genotypes with and without exogenous IBA. R: easy-to-root genotype; NR: difficult-to-root genotype

identity with the corresponding proteins in *Eucalyptus* and *A. thaliana* (Table 6).

Primers for qPCR

Two pairs of specific primers for qRT-PCR for the genes of interest *TIR1*, *SHR* and the reference gene *H2B* were designed (shown in Fig. 2). However, for the gene of

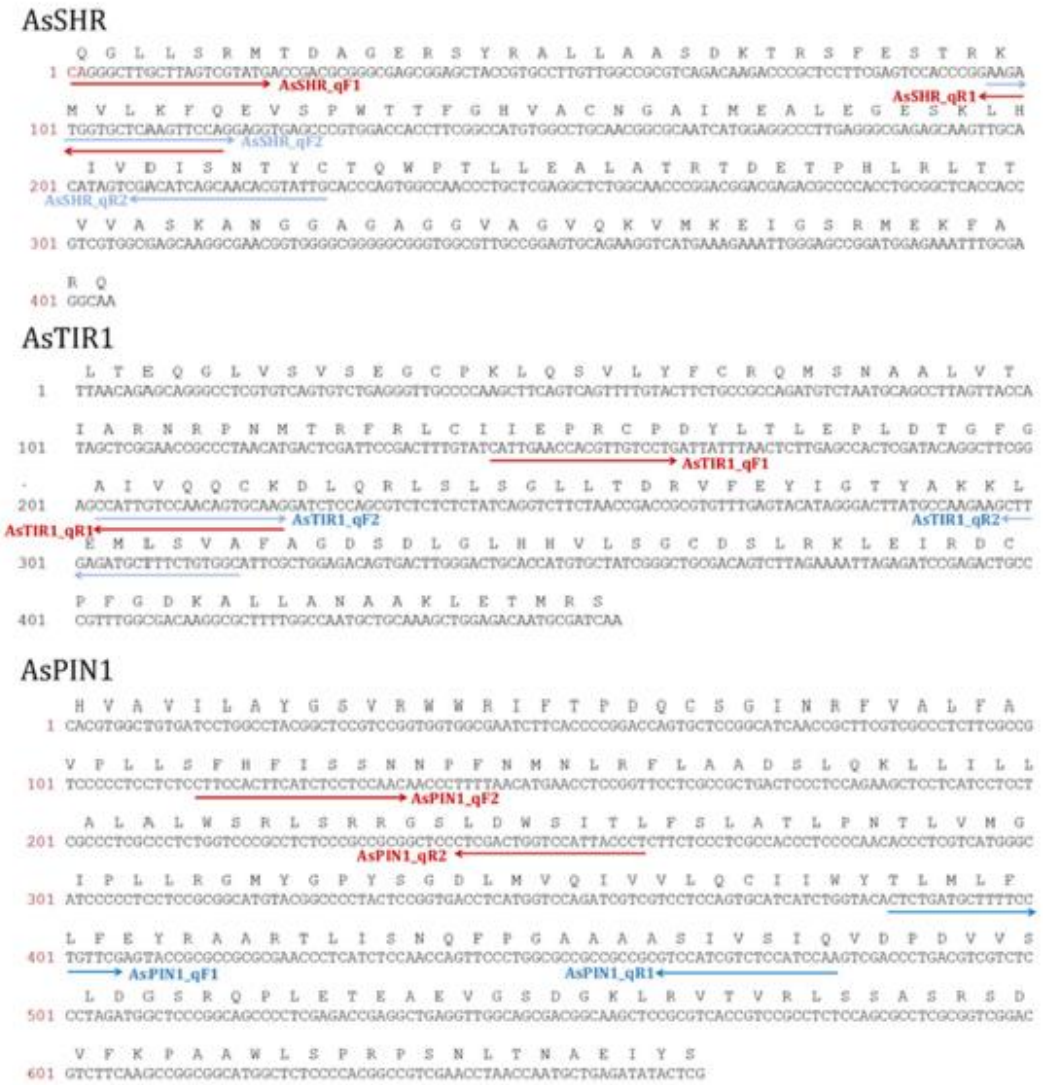

Fig. 3 Nucleotide and deduced amino acid sequences of *AsSHR*, *AsTIR1*, and *AsPIN1* cDNA from *Acca sellowiana*. Two pairs of primers designed on these sequences for qPCR are indicated by horizontal arrows for each gene of interest

interest *PIN1* and the reference genes *EF2* and *UBI*, we were able to design only one forward and two reverse primers or two forward and one reverse primer for each (Tables 3 and 4). These primers were confirmed by conventional PCR using *A. sellowiana* genomic DNA, and amplicons of the expected size for each gene were obtained.

Table 5 Size of the cDNA sequences obtained in *A. sellowiana* for the genes of interest (As*PIN1*, As*SHR*, and As*TIR1*) and reference genes (As*EF2*, As*H2B*, and As*UBI*), and the corresponding identity with their homologous genes in *E. grandis* and *A. thaliana* according to NCBI and Uniprot databases

Gene symbol	Size (bp)	NCBI identity (%) with *E. grandis*	NCBI reference number	Uniprot identity (%) with *A. thaliana*	Uniprot reference number	Domain
AsPIN1	672	94	XM_010035442.1	76.4	Q9C6B8-1	Transmembrane
AsSHR	426	92	XM_010029789.1	75.3	Q9SZF7	GRAS
AsTIR1	521	95	XM_010039868.1	84.8	Q570C0-1	AMN
AsEF2	135	96	XM_010062544.1	69	Q9ASR1	
AsH2B	152	97	XM_010043098.1	94	Q9SI96	
AsUBI	129	98	XR_726060.1	85	Q42009	

Fig. 4 Alignment of the amino acid sequences of **a** *AsPIN1*, **b** *AsTIR1* and **c** *AsSHR* with homologous sequences from *E. grandis* and *A. thaliana*, using Jalview. Most conserved regions are indicated with yellow bars below the alignment. A consensus sequence is shown in the last line

Table 6 Identity of the amino acid sequences obtained in *A. sellowiana* for the genes of interest (As*PIN1*, As*SHR*, and As*TIR1*) and reference genes (As*EF2*, As*H2B*, and As*UBI*), with their homologous genes in *E. grandis* and *A. thaliana* according to Uniprot database

	Eucalyptus (Uniprot reference number)	% identity (aa)	*A. thaliana* (Uniprot reference number)	% identity (aa)
AsPIN1	A0A059D0Y0	95	Q9C6B8	77
AsSHR	A0A059ALM8	91	Q9SZF7	65
AsTIR	A0A059A849	97	Q570C0	91
AsEF2	A0A059BNB6	81	Q9ASR1	70
AsH2B	A0A059AF50	96	Q9SI96	94
AsUBI	A0A059DDH9	77	Q42009	77

Analysis of gene expression in micro-cuttings

Relative expression at the onset of AR induction was measured in micro-cuttings of *A. sellowiana* by RT-qPCR using the geometric mean of two reference genes (*H2B* and *EF2*). *AsTIR1*, *AsPIN1*, and *AsSHR* transcripts were induced in response to exogenous auxin in the difficult-to-root genotype during the early steps of AR formation. First, we examined the expression of these genes in both genotypes (R and NR) without IBA, relative to their expression in the R genotype without IBA 12 h after the onset of the experiment. When no exogenous IBA was added to the medium, the expression of these three genes was lower in the NR genotype than in the R genotype ($p < 0.0001$) throughout the period under study (Fig. 5). Without the addition of exogenous IBA, the R genotype showed an increase in the expression of the three genes 36 h after the beginning of the experiment ($p < 0.0001$).

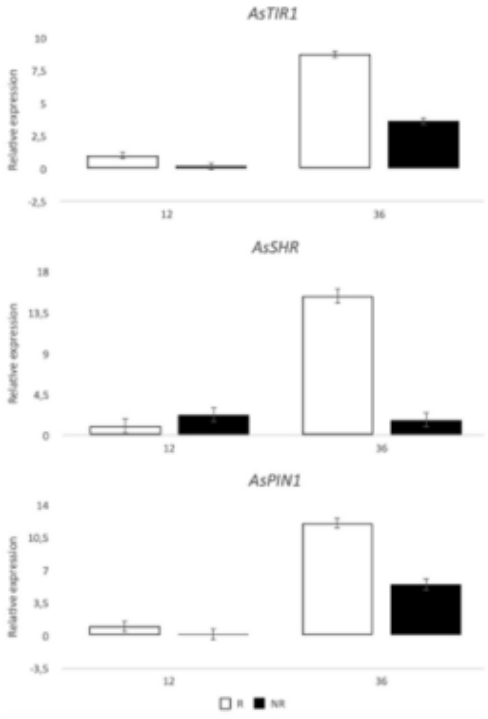

Fig. 5 Expression of *AsTIR1*, *AsSHR* and *AsPIN1* without exogenous IBA in the R and NR genotypes of *A. sellowiana* micro-cuttings relative to their expression 12 h after the beginning of the experiment. R: easy-to-root genotype; NR: difficult-to-root genotype. Data were analyzed by the Comparative Ct method ($2^{-\Delta\Delta Cq}$) using the geometric mean of the reference genes (*H2B* and *EF2*). Bars represent the mean relative expression ± SE of three independent biological replicates and two technical replicates

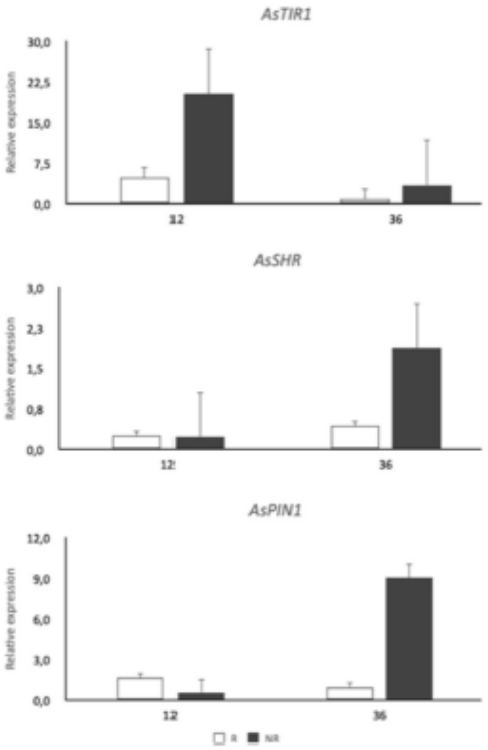

Fig. 6 Relative expression of *AsTIR1*, *AsSHR* and *AsPIN1* in treated (0.98 µM IBA) vs. untreated (0 µm IBA) samples of two genotypes of *A. sellowiana micro-cuttings* with contrasting rooting ability, 12 and 36 h after induction treatment with IBA (9.8 µM). R: easy-to-root genotype, NR: difficult-to-root genotype. Data were analyzed by the Comparative Ct method ($2^{-\Delta\Delta Cq}$) using the geometric mean of the reference genes (*H2B* and *EF2*). Bars represent the mean relative expression ± SE of three independent biological replicates and two technical replicates. $\Delta\Delta Ct = [(Ct\ gene\ of\ interest - Ct\ internal\ control)\ sample\ with\ IBA - (Ct\ gene\ of\ interest - Ct\ internal\ control)\ sample\ without\ IBA]$

In the NR genotype, on the other hand, *AsTIR* and *AsPIN* also increased their expression ($p < 0.0001$) but in a much lower magnitude. Data were analyzed by the Comparative Ct method ($2^{-\Delta\Delta Cq}$) using the geometric mean of the reference genes (*H2B* and *EF2*). The coefficient of variation between technical replicates was < 1 for the three genes of interest. $\Delta\Delta Ct = [(Ct\ gene\ of\ interest - Ct\ internal\ control)\ NR\ genotype - (Ct\ gene\ of\ interest - Ct\ internal\ control)Rgenotype]$

Next, we compared the expression of these genes between treated (0.98 µM IBA) and untreated (0 µM IBA) samples and found that the behavior of the R and NR genotypes was clearly different in response to the exogenous auxin. The relative expression of *TIR1*, *SHR*, and *PIN1* increased in response to IBA, but a much greater response was observed in the NR genotype in the treated vs untreated samples. An increase in the expression of the auxin receptor *TIR1* was induced 12 h after the treatment with exogenous IBA ($p = 0.0111$), while the expression of the auxin efflux carrier *PIN1* and the transcription factor *SHR* increased in the NR genotype 36 h after treatment with exogenous IBA relative to their expression in the untreated sample ($p = 0.0157$ and 0.0189 respectively) (Fig. 6). The relative expression of *TIR1* and *SHR* increased in the R genotype in response to IBA but to a lesser extent ($p = 0.0455$ and 0.0189 respectively), while

changes in relative expression of *PIN1* was not significant ($p=0.1216$).

Discussion

When a well-curated genome of the species under study is not available, the genome of a related species can be used as reference; however, the exact sequence and the genomic location of the genes of interest are unknown. Amplification and sequence comparison can provide evidence to hypothesize that the transcripts that are being quantified are homologous to the genes reported in model species. In this study, we successfully amplified regions of genes which have been shown to be involved at different stages during the onset of AR formation based on published genomes of model species. We showed that the genomic sequences obtained from *A. sellowiana* are consistent with the target genes and observed a pattern of expression that can be interpreted in the light of the current general understanding of AR formation and previously published hypotheses about intraspecific variability in *A. sellowiana* for this trait.

Unique amplification products of the expected size were obtained for three genes of interest: *TIR1*, *SHR*, and *PIN1*. Sequence analysis of the obtained PCR fragments showed that they presented high sequence similarity with the candidate genes *PIN1*, *SHR*, and *TIR1*. For the three genes, identity with *E. grandis* and with *A. thaliana* was high (more than 90% and 70% respectively). Furthermore, the protein sequences resulting from the translation of the isolated gene fragments present, although partially, domains characteristic of the PIN1, SHR, and TIR1 proteins. AsPIN1 sequence includes part of a transmembrane domain, a domain present in PIN1 from *Arabidopsis* (Gälweiler et al., 1998), while AsSHR sequence contains a significant portion of the GRAS domain (Pysh et al. 1999; Helariutta et al. 2000). When analyzing the results in AsTIR1, the isolated fragment presents a partial AMN domain, which corresponds to 4 complete LRRs (Leucine-Rich Repeats). This AMN domain contains 16 LRRs in *A. thaliana*. Although the length of the isolated fragment did not include the FBox domain, which is the other descriptive feature for the *TIR1* gene (Ruegger et al., 1998), the isolated fragment shows 95% and 84.6% identity with the homologous fragments of *E. grandis* and *A. thaliana* respectively. According to these results, *TIR1*, *PIN1*, and *SHR* genes are present in the *A. sellowiana* genome, and their sequences show a similarity of more than 90% with the respective *E. grandis* genes in the region of the gene that was isolated.

Our expression analysis indicates that *AsTIR1*, *AsPIN1*, and *AsSHR* have different levels of expression in R and NR genotypes when there is no hormonal treatment. The level of expression of these three genes in the difficult-to-root genotype 36 h after the beginning of the experiment was always lower than in the easy-to-root genotype. *AsTIR1*, *AsPIN1*, and *AsSHR* transcripts are induced in response to exogenous IBA in stem cuttings of difficult-to-root genotypes of *A. sellowiana*, during the early steps of AR formation. The R genotype already had a high expression without IBA, so the response to exogenous auxin is not as strong as the increase in relative expression of these genes in the NR genotype. This result is in agreement with the rooting behavior itself; the R genotype roots well without the addition of IBA while the NR genotype significantly improves rooting levels when the exogenous auxin is added to the culture media. This may be explained by the presence of an endogenous higher level of auxin in the R genotype. Among various conditions previously evaluated to induce rooting, exogenous IBA was the only treatment that improved the AR capacity of this genotype to levels that are similar to the easy-to-root genotype (Ross et al. 2017). However, the R genotype does not improve rooting in response to exogenous IBA, and the expression of these genes remains stable. Our data show that the expression of these genes was already much higher in the untreated R genotype, and the addition of exogenous IBA had a very small effect on this genotype. In the difficult-to-root genotype, on the other hand, the relative expression of *AsTIR1* increases strongly in response to exogenous IBA, shortly after the induction treatment. In *Populus*, the expression of *PagFBL1* (homolog of *TIR1* in Arabidopsis) was similar to the distribution pattern of auxin during AR formation, with a high expression in the cambium and secondary phloem during the induction and initiation phases that decreased in the emerging primordia (Shu et al. 2019). Although our expression analysis was not focused on specific tissues, neo-formation of adventitious root meristems in *A. sellowiana* takes place outside the cambial ring of the stem, in the secondary phloem (Ross et al. 2021). Thus, it is possible that the increase in the expression of *AsTIR1* that we found is concentrated in this tissue and it would be interesting to explore how the expression varies in different tissues of the stem cutting. The relative expression of *AsPIN1* and *AsSHR* also increases, but 24 h later. The effect of IBA as a rooting agent for difficult-to-root materials of this species can thus be related to the increase in expression of at least these genes. These findings are congruent with our previous results and support our hypothesis of an earlier phase change from juvenile to mature of the NR genotype of *A. sellowiana*. We found that loss of competence to form AR was associated with an earlier phase change, evidenced by the differentiation of a periderm in the NR genotype (Ross et al. 2021).

As plants age, there is a lower expression of the main auxin receptor (TIR1) that explains the loss of sensitivity to auxin (Aumond et al. 2017). The improvement of rooting ability of the NR genotype of *A. sellowiana* when exogenous

IBA was added may have resulted from the increased expression of the auxin receptor *AsTIR1*. This increase in the sensibility to auxin may have led to a further modification in the expression of other genes that act downstream auxin perception and that play essential roles in the rooting of cuttings. Among many other genes involved in AR differentiation, the increase in relative expression of *AsPIN1* and *AsSHR* in NR genotype is congruent with the observed improvement of rooting ability after exogenous IBA treatment. As a consequence of the concerted action of these genes which showed higher expression in response to IBA, the NR genotype improved the rooting performance, reaching levels similar to the R genotype with or without exogenous IBA. Similar changes in gene expression in response to exogenous auxin have been reported in other species of Myrtaceae (Fett-Neto et al. 2011; de Almeida et al. 2015). The relative amount of mRNA of *SHR* and other transcription factors of the GRAS family has been observed to be significantly reduced in adult tissues that have lost the capacity to develop AR. The expression of *SHR* during AR formation is affected by age, auxin level and developmental stage of the cells (Abarca et al. 2014). In *Arabidopsis*, the same cells that are reactivated by auxin to differentiate ARs, are also able to initiate xylogenesis, with SHR among other transcription factors controlling the switch between the programs (Ricci et al. 2016).

The kind of changes in gene expression that we observed in the different genotypes resembles those observed in different stages during AR development in other species of the Myrtaceae family. Studies in other species of the Myrtaceae with poor AR development also show lower expression of auxin receptors. Loss of AR ability in *E. globulus* micro-cuttings has been explained by a combination of lower expression of auxin receptors (*TIR1*, *ABP1*) and higher expression level for auxin repressors (*IAA12*, *TPL*, *ARR1*). Furthermore, the expression of genes related to auxin synthesis (*TAA1*, *YUC3*) and transport (*PIN1*, *AUX1*) was found to diverge between stages of development and auxin treatment (Vilasboa et al. 2018).

Although the regulatory gene network that controls AR formation in *A. sellowiana* or its specific differences from better known species is far from being completely understood, we found evidence that supports the hypothesis that earlier phase change underlies the reduced competence to differentiate new roots in the NR genotypes. Several techniques have been used to delay maturation of juvenile plants or reverse the physiological status of adult plants in other species (Wendling et al. 2014b; Benedini et al. 2015; Stuepp et al. 2016; Bisognin et al. 2017, 2018); among these, epicormic shoots induced by pruning, coppicing, or girdling of adult trees could be evaluated as a source of rejuvenated material for stem cuttings of the NR genotypes of *A. sellowiana*.

Conclusions

We identified and characterized three genes that are induced by IBA and are likely related to AR development in *A. sellowiana* micro-cuttings: *AsPIN1*, *AsTIR1*, and *AsSHR*. The results of the expression analysis showed that in the difficult-to-root genotype, *AsTIR1* increases strongly in response to exogenous IBA, shortly after induction treatment improving sensibility to auxin of the cells. Relative expression of *AsPIN1* and *AsSHR* also increases, but 24 h later.

These results indicate that *AsTIR1*, *AsPIN1*, and *AsSHR* transcripts are induced during the early steps of AR formation in response to exogenous IBA in stem cuttings of the difficult-to-root genotype of *A. sellowiana*, improving AR formation. This behavior is similar to that of mature tissues studied in other species.

Our results show that cloning of *A. sellowiana* by stem cuttings requires physiologically juvenile or rejuvenated material and that different genotypes may require different treatments to produce competent cuttings.

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Data availability The datasets generated and analyzed during the current study are available in the GenBank repository at https://www.ncbi.nlm.nih.gov/genbank/, reference number MZ130946-MZ13095.

Declarations

Conflict of interest The authors declare they have no financial interests. The authors have no competing interests to declare that are relevant to the content of this article.

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5. Discusión

La domesticación de cultivos frutales ha estado acompañada de un aumento en la perennialidad y un incremento en la capacidad de propagación vegetativa de los materiales domesticados con respecto al germoplasma silvestre (Gaut et al., 2015). Esta posibilidad de seleccionar genotipos con alta capacidad de propagación a través del método propio de cada cultivo (estacas, injertos, etc.) debe basarse en la existencia de variabilidad genética para esta característica en el germoplasma. Si bien existe suficiente evidencia de que hay variabilidad para esta característica, la base genética y sus relaciones con la expresión fenotípica de la capacidad para enraizar de los diferentes genotipos no resultan claras. A través de las respuestas diferenciales observadas a varios niveles entre materiales con capacidad de enraizamiento contrastante en *Acca sellowiana*, pusimos en evidencia relaciones entre mecanismos a nivel hormonal, morfológico y genético que permiten establecer hipótesis sobre la base genética de la variabilidad natural (Figura, 2).

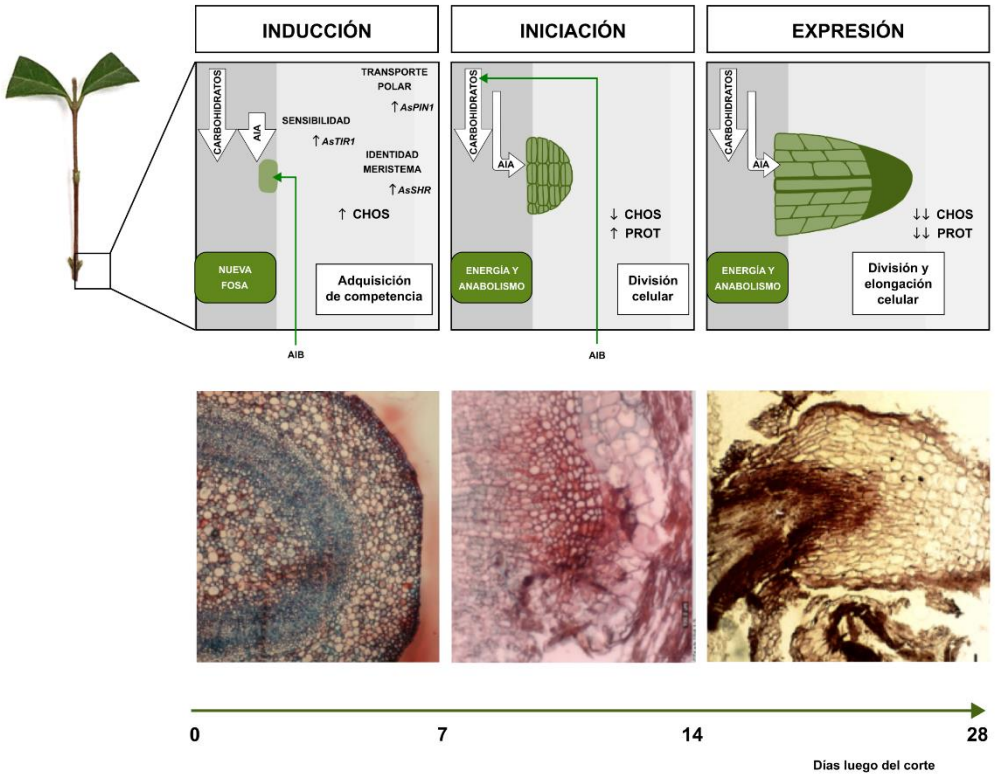

Figura 2. Etapas del proceso de formación de raíces adventicias, indicando la duración de las tres fases (Inducción, Iniciación y Expansión), los principales cambios metabólicos y anatómicos asociados a cada una de ellas y los niveles de expresión de los genes estudiados en la etapa de Inducción. CHO: carbohidratos, PROT: proteínas, AIA: ácido indolacético, AIB: ácido indolbutírico. *AsTIR1*, *AsPIN1* y *AsSHR*: genes estudiados en *Acca sellowiana*.

Las diferencias encontradas en los distintos niveles de estudio abordados permiten interpretar y explicar la diferente capacidad de diferenciar RA de los genotipos estudiados. Si bien se encontró un efecto promotor de RA cuando se aplicó AIB exógeno, esta respuesta parece estar más relacionada con el estado de desarrollo en que se encuentran los tejidos en la base de las estacas. Las estacas del genotipo NR presentaban un grado mayor de madurez fisiológica, evidenciado por la presencia de una peridermis en claro desarrollo a partir del primer nudo, probablemente asociado con la pérdida de competencia de esos tejidos para diferenciar RA. Investigaciones realizadas en otras especies de la familia Myrtaceae muestran resultados similares, relacionando la capacidad de enraizamiento y vigor reducido de las estacas con la anatomía del tallo (Abu-Abied et al., 2012; Bryant y Trueman, 2015; Goulart et al., 2014; Wendling et al., 2015). Los genes que se estudiaron en este trabajo se seleccionaron a partir de genes vinculados al enraizamiento en *Arabidopsis thaliana* (por ser la especie modelo más estudiada) y *Eucalyptus grandis* (por ser una especie leñosa con el genoma secuenciado, de la misma familia que *A. sellowiana*). En este genotipo, el AIB indujo, en primer lugar, la expresión relativa del receptor de auxinas *TIR1* a las 12 h de cortadas las estacas y, *a posteriori*, de otros genes vinculados al metabolismo de las auxinas y formación de raíces. Es decir, el primer efecto de la hormona tiene que ver con la adquisición o aumento de la competencia, para luego poder expresar el programa de desarrollo que conduce a la formación de RA. En el genotipo R, en cambio, que a nivel anatómico mostró características más juveniles, los tejidos en la base de la estaca mostraron competencia desde el inicio. Los genes estudiados tuvieron una alta expresión relativa independientemente del agregado de auxina exógena. Estos tejidos, al encontrarse en una etapa de desarrollo juvenil,

muestran una mayor competencia para responder al estímulo hormonal e iniciar el programa de formación de RA. Los distintos materiales (R y NR) interpretan de manera diferente la señal hormonal porque se encuentran en distintos programas del desarrollo al momento del corte. Esto deja en evidencia que, si bien la regulación del proceso ocurre a nivel genético, estaría más relacionado con la pérdida de juvenilidad del material que con el proceso de RA en sí mismo.

Asociado con esto, el contenido de proteínas también mostró una respuesta muy diferente, lo cual respalda que la naturaleza de los cambios inducidos por el AIB exógeno fue diferente en ambos genotipos. El incremento en proteínas observado a los catorce días probablemente refleja cambios metabólicos que ocurren en respuesta a la herida, los cuales en general se relacionan con la fase de inducción del proceso de formación de RA (Da Costa et al., 2013). Este incremento fue similar en todos los materiales en que se esperaba que se formaran RA, es decir, el genotipo R y el genotipo NR con AIB, el cual, de acuerdo con nuestra hipótesis, adquirió competencia en respuesta a la hormona. La identidad de las proteínas acumuladas no fue estudiada y no podemos confirmar si los mismos o diferentes genes explican la acumulación de proteínas en las estacas de los genotipos estudiados. Sin embargo, nuestros resultados muestran que en las estacas control del genotipo NR hay factores endógenos que son responsables de la diferente respuesta bioquímica y que ese incremento posterior en las proteínas sintetizadas en respuesta a la herida no estaría, entonces, vinculado a procesos relacionados con el enraizamiento, al menos *in vitro*. La diferencia en el patrón de expresión de proteínas observado *in vitro* y la ausencia de formación de RA en el genotipo NR sin auxina exógena tanto *in vivo* como *in vitro* probablemente se deba al cambio de fase más temprano, el cual puede ser parcialmente revertido con AIB exógeno, cuando los tejidos aún responden por encontrarse en un estado más juvenil.

En *Acca sellowiana*, la pobre formación de RA ha sido atribuida a la presencia de fibras floemáticas en las estacas de tallo, que afectan la actividad metabólica del meristema de raíz neoformado e impiden mecánicamente el crecimiento del primordio de raíz (Zhang et al., 2009). En los genotipos evaluados, no pudimos encontrar ninguna indicación de neoformación de meristemas en ninguna de las estacas en que esta

peridermis estaba presente. De acuerdo con estos resultados, es improbable que este tejido represente una barrera física para el desarrollo de RA. Es más probable que la ausencia de RA en el genotipo NR se deba a la no formación del meristema, ya que las células no son competentes. El desarrollo de este tejido, acompañado por la pérdida de capacidad de enraizamiento, podría estar indicando que el genotipo NR sufre un cambio de fase juvenil a maduro más temprano.

La diferenciación de RA como programa de desarrollo se ha relacionado inversamente con el programa de xilogénesis en varias especies forestales como pino (Abarca et al., 2014), palmera datilera (De Almeida et al., 2012) y castaño (Vielba et al., 2016). Es resultado de una alteración de la homeostasis de auxinas asociado con el cambio de fase que afecta negativamente el desarrollo de RA (Rasmussen et al., 2014). En consecuencia, altas dosis de AIB pueden inhibir el enraizamiento de estacas y promover el crecimiento secundario y la lignificación (Wendling et al., 2015). En *Acca sellowiana*, estacas del genotipo NR con un solo nudo respondieron al AIB exógeno y mejoraron la diferenciación de RA. Sin embargo, a partir del segundo nudo hacia los nudos inferiores no se observó neoformación de meristemas de raíz y, en cambio, la presencia y espesor de la peridermis se vio promovido por el AIB. Este efecto aparentemente contradictorio del AIB puede entenderse como un cambio en la sensibilidad y respuesta al AIB asociados con el cambio de fase. El patrón de síntesis de proteínas también se asocia con el cambio de fase.

A medida que las plantas envejecen, hay una menor expresión del principal receptor de auxinas (TIR1) (Aumond et al., 2017). La mejora en la capacidad de enraizamiento del genotipo NR de *A. sellowiana* cuando se agrega AIB exógeno puede ser el resultado de un incremento en la expresión del receptor de auxina *AsTIR1*. Este aumento de la sensibilidad a su vez pudo haber conducido a modificaciones en la expresión de otros genes que actúan corriente debajo de la percepción de auxinas y que juegan roles esenciales en el enraizamiento de estacas. Entre muchos otros genes involucrados en la diferenciación de RA, el aumento en la expresión relativa de *AsPIN1* y *AsSHR* en el genotipo NR es congruente con la mejor capacidad de enraizamiento observada con el tratamiento de AIB exógeno. Como consecuencia de la acción concertada de estos genes que incrementan su expresión en respuesta al AIB,

el genotipo NR mejoró su respuesta en enraizamiento y alcanzó niveles similares al genotipo R con o sin AIB exógeno (Ross et al., 2024). En otras especies de Myrtaceae se han observado cambios similares en la expresión génica en respuesta a las auxinas exógenas (De Almeida et al., 2015; Fett-Neto et al., 2011).

Si bien la red génica que controla la formación de RA en *Acca sellowiana* o las diferencias específicas con especies más conocidas está lejos de ser entendida en su totalidad, encontramos evidencia que apoya nuestra hipótesis de que un cambio de fase más temprano reduce la competencia para diferenciar nuevas RA en los genotipos NR. Estas hipótesis deberán ser generalizadas y los mecanismos subyacentes específicos de la especie que estudiamos y generales para otras especies leñosas podrán ser mejor comprendidos. La comprensión de estos mecanismos permitirá aumentar la eficiencia de la selección por capacidad de propagación en las etapas iniciales de la domesticación de nuevos cultivos, así como avanzar en el diseño de manejos y tratamientos para mejorar la capacidad de enraizamiento de genotipos de interés recalcitrantes. Las condiciones de crecimiento de las plantas madre de manera de mantener la juvenilidad o inducirla como se hace en otras especies leñosas recalcitrantes y al mismo tiempo manejar las condiciones de temperatura, ambiente nutricional y calidad lumínica en que dichas plantas crecen son condiciones determinantes de la respuesta que van a tener las estacas, independientemente de los tratamientos específicos de enraizamiento que puedan aplicarse.

6. Conclusiones

En este estudio no se encontraron evidencias de meristemas de raíz preformados en las estacas de *Acca sellowiana* de los genotipos utilizados. Los meristemas de RA se desarrollaron *de novo* por fuera del anillo cambial. La aplicación de AIB exógeno promovió de manera efectiva tasas de enraizamiento compatibles con la propagación comercial en estacas uninodales. La anatomía de las estacas de tallo difirió entre genotipos. Sin embargo, se encontró que esas diferencias probablemente son consecuencia del cambio de fase de juvenil a maduro y la pérdida de competencia del genotipo NR, asociado con un desarrollo más temprano de la peridermis.

Identificamos y caracterizamos tres genes que son inducidos por AIB y probables reguladores del desarrollo de RA en microestacas de *Acca sellowiana*: *AsPIN1*, *AsTIR1* and *AsSHR*. Los resultados del análisis de su expresión muestran que en el genotipo de difícil enraizamiento *AsTIR1* aumenta fuertemente en respuesta al AIB exógeno poco después del tratamiento de inducción y mejora la sensibilidad de las células a las auxinas. La expresión relativa de *AsPIN1* y *AsSHR* también aumenta, pero 24 h más tarde. Estos resultados indican que los transcriptos de *AsTIR1*, *AsPIN1* y *AsSHR* se inducen durante las etapas tempranas del proceso de formación de RA en respuesta al AIB exógeno en estacas de tallo de los genotipos de *Acca sellowiana* de difícil enraizamiento y aumenta la capacidad de formación de RA. Este comportamiento es similar al de tejidos maduros estudiados en otras especies.

Nuestros resultados muestran que para clonar materiales de *Acca sellowiana* mediante el empleo de estacas de tallo, se requiere material juvenil o rejuvenecido y que diferentes genotipos pueden requerir diferentes tratamientos para proporcionar estacas competentes. Una estrategia posible sería el establecimiento de minijardines emulando las técnicas de manejo de plantas madre en especies forestales para la obtención de miniestacas con características juveniles.

El trabajo realizado permitió desarrollar estrategias para profundizar en el conocimiento del proceso de rizogénesis adventicia a distintos niveles de estudio, extrapolables a otras especies leñosas en las que las técnicas de propagación vegetativa

sean una opción productiva a nivel de viveros, así como en programas de mejoramiento genético, ya que estas permiten obtener una mayor ganancia genética.

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