Universitat de Lleida

Efecto de la inducción de resistencia con curlano y silicio sobre la respuesta de defensa en plantas de aguacate Hass inoculadas con *Phytophthora cinnamomi*

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TESI DOCTORAL

EFECTO DE LA INDUCCIÓN DE RESISTENCIA CON CURLANO Y SILICIO SOBRE LA RESPUESTA DE DEFENSA EN PLANTAS DE AGUACATE HASS INOCULADAS CON *Phytophthora cinnamomi*

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En memoria de mi madre **Blanca Mery Puentes Coy**.

Todo lo que soy se lo debo a ella.

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ABSTRACT

In agriculture, problems caused by pathogens represent great economic and environmental losses. The costs of phytosanitary management of pathogens such as *Phytophthora cinnamomi* in a crop such as avocado can represent 25% of annual production, in addition to the traceability and environmental problems associated with the products applied. Therefore, the current objective is sustainable agriculture, which reduces the use of chemical synthesis products without affecting crop yields or the profits obtained from it.

This doctoral thesis focuses on the induced resistant with *curdlan* and silicon in Hass avocado plants inoculated with *Phytophthora cinnamomi*. Because this is one of the most important pathogens in this crop and commercial products for its control are currently scarce. The inducers used, a β-glucan and a mineral, have been reported to work in other pathosystems, so we want to test their effect and determine how avocado plants respond at the enzymatic and metabolic level.

First, the inducers were applied. In the case of *curdlan*, foliar spraying was carried out one day before pathogen inoculation, while silicon was applied to the soil 10 days before. Measurements of lesion progress and destructive sampling were performed at different times (0, 3, 24, 144 and 312 hai). The plant material sampled was always kept cold. From this material, extractions with different buffer solutions or solvents were performed to evaluate enzymatic activities (SOD, PPO, POD, PAL), phenol concentration, free radical scavenging activity and chlorophyll and carotenoid content. In both cases, the inducers used favored the plant response to the pathogen by modulating the expression of enzymes such as POD and PPO, suggesting that their production may be associated with the transformation of phenols into quinones for the production of antimicrobial compounds, regulation of ROS and a possible activation of the jasmonic acid pathway.

A second part sought to deepen the response of Hass avocado plants that were treated with *curdlan*. Extractions and GC-MS analysis were carried out to study the metabolic profile of the plants at different sampling times in order to contrast their response to the pathogen, the inducer and the combination of the two. Phenolic compounds (caffeic, ferulic and p-coumaric acid) and phytohormones (SA and JA) were also quantified. The results show that plants in the presence of the inducer, the pathogen with the inducer or only the pathogen, change their metabolic profile. Defense-related metabolites such as phenolic compounds, thymol, vitamin E, ploroglucinol, among many others, are produced in all cases, but the times or concentration at which they are

generated are not the same. If the above is related to the visual monitoring of the plants, which when they had the inducer and the pathogen, showed minimal or no signs of wilting, compared to those inoculated only with the pathogen, it could be suggested that the inducer has a positive effect on the plants by regulating the production of metabolites, which must be generated at a certain concentration or time, for adequate control of the pathogen. In the same way, the inducer helped to regulate the concentration of phytohormones in the plants. SA remained at a stable concentration, while JA increased after 24 h, a behavior that favors pathogen control and has been related to avocado genotypes resistant to *P. cinnamomi*.

In conclusion, this doctoral thesis provides valuable information on the use of inducers in Hass avocado plants, showing that both *curdlan* and silicon have positive effects on the defense against *P. cinnamomi* and in the future could be included in integrated pest management plans, thus impacting the safety of the product.

RESUMEN

En la agricultura, los problemas ocasionados por patógenos, representan grandes pérdidas tanto económicas como ambientales. Los costos del manejo fitosanitario de patógenos como *Phytophthora cinnamomi* en un cultivo como el de aguacate, pueden representar el 25% de la producción anual, sumado a la trazabilidad y problemas ambientales que tienen los productos que se aplican. Por tanto, en la actualidad el objetivo es una agricultura sostenible, que reduzca el uso de productos de síntesis química sin que esto afecte los rendimientos del cultivo ni las ganancias que se obtienen de él.

Esta tesis doctoral se centra en la inducción de resistencia con curlano y silicio, en plantas de aguacate Hass inoculadas con *Phytophthora cinnamomi*. Debido a que este es uno de los patógenos más importantes en este cultivo y los productos comerciales para su control son escasos actualmente. Los inductores empleados, un β-glucano y un mineral, tienen reportes de funcionar en otros patosistemas, por lo cual se quiere probar su efecto y determinar la forma cómo responden las plantas de aguacate a nivel enzimático y metabólico.

En primer lugar, se realizó la aplicación de los inductores. Para el caso del curlano se realizó aspersión foliar un día antes de la inoculación del patógeno, mientras que el silicio se aplicó al suelo 10 días antes. Se realizaron mediciones del progreso de la lesión y muestreos destructivos en diferentes tiempos (0, 3, 24, 144 y 312 hai). El material vegetal que se tomó como muestra se mantuvo siempre en frío. De este material se realizaron extracciones con diferentes soluciones tampón o solventes para evaluar las actividades enzimáticas (SOD, PPO, POD, PAL), la concentración de fenoles, la actividad captadora de radicales libres y contenido de clorofilas y carotenoides. En ambos casos, los inductores empleados favorecieron la respuesta de la planta frente al patógeno modulando la expresión de enzimas como POD y PPO sugiriendo que su producción se puede asociar con transformación de fenoles en quinonas para producción de compuestos antimicrobianos, regulación de ROS y una posible activación de la ruta del ácido jasmónico.

Una segunda parte buscó profundizar en la respuesta de las plantas de aguacate Hass que fueron tratadas con curlano. Se realizaron extracciones y análisis por GC-MS, para estudiar el perfil metabólico de las plantas en los diferentes tiempos de muestreo y así poder contrastar la respuesta de estas al patógeno, al inductor y a la combinación de los dos. Asimismo, se cuantificaron

compuestos fenólicos (ácido cafeico, ferúlico y *p*-cumárico) y fitohormonas (SA y JA). Los resultados muestran que las plantas en presencia del inductor, del patógeno con el inductor o solo del patógeno, cambian su perfil metabólico. Se producen en todos los casos metabolitos relacionados con la defensa como compuestos fenólicos, timol, vitamina E, floroglucinol, entre muchos otros, pero los tiempos o la concentración en que se generan no son las mismas. Si se relaciona lo anterior, con el seguimiento visual de las plantas, que cuando tenían el inductor y el patógeno, presentaban mínimos signos de marchitez o ninguno, respecto a las inoculadas solo con el patógeno, podría sugerirse que el inductor tiene un efecto positivo en las plantas regulando la producción de metabolitos, los cuales deben generarse en determinada concentración o tiempo, para que se de un control adecuado del patógeno. En el mismo sentido, el inductor ayudó a regular la concentración de fitohormonas en las plantas. El SA se mantuvo en una concentración estable, mientras que el JA fue en aumento después de las 24 h, comportamiento que favorece el control del patógeno y que se ha relacionado con genotipos resistentes de aguacate frente a *P. cinnamomi*.

En conclusión, la presente tesis Doctoral proporciona información valiosa sobre el uso de inductores en plantas de aguacate Hass, mostrando que tanto el curlano como el silicio, tienen efectos positivos en la defensa contra *P. cinnamomi* y podrían a futuro ser incluidos en planes de manejo integrado de la enfermedad, impactando con ello la inocuidad del producto.

RESUM

A l'agricultura, els problemes ocasionats per patògens, representen grans pèrdues tant econòmiques com ambientals. Els costos de la gestió fitosanitari ade patògens com Phytophthora cinnamomi en un cultiu com el d'alvocat poden representar el 25% de la producció anual, sumat a la traçabilitat i problemes ambientals que tenen els productes que s'apliquen. Per tant, actualment l'objectiu és una agricultura sostenible, que redueixi l'ús de productes de síntesi química sense que això afecti els rendiments del cultiu ni els guanys que se n'obtenen.

Aquesta tesi doctoral se centra en la inducció de resistència amb curlà i silici, en plantes d'alvocat Hass inoculades amb Phytophthora cinnamomi. Aquest és un dels patògens més importants en aquest cultiu i els productes comercials per al seu control actualment són escassos. Els inductors emprats, un β-glucà i un mineral, tenen referències d'haver funcionat en altres patosistemes, per la qual cosa se'n vol provar l'efecte i determinar la manera com responen les plantes d'alvocat a nivell enzimàtic i metabòlic.

En primer lloc, es va fer l'aplicació dels inductors. Per al cas del curlà es va realitzar per aspersió foliar un dia abans de la inoculació del patogen, mentre que el silici es va aplicar al sol 10 dies abans. Es van dur a terme mesuraments del progrés de la lesió i mostrejos destructius en diferents temps (0, 3, 24, 144 i 312 hai). El material vegetal que es va prendre com a mostra es va mantenir sempre en fred. D'aquest material se'n van fer extraccions amb diferents solucions tampó o solvents per avaluar les activitats enzimàtiques (SOD, PPO, POD, PAL), la concentració de fenols, l'activitat captadora de radicals lliures i contingut de clorofil·les i carotenoides. En tots dos casos, els inductors empleats van afavorir la resposta de la planta davant del patogen modulant l'expressió d'enzims com POD i PPO suggerint que la seva producció es pot associar amb transformació de fenols en quinones per a producció de compostos antimicrobians, regulació de ROS i una possible activació de la ruta de l'àcid jasmònic.

En una segona part es va buscar aprofundir en la resposta de les plantes d'alvocat Hass que van ser tractades amb curlà. Es van realitzar extraccions i anàlisis per GC-MS, per estudiar el perfil

metabòlic de les plantes en els diferents temps de mostreig i així poder contrastar-ne la resposta al patogen, a l'inductor i a la combinació de tots dos. Així mateix, es van quantificar compostos fenòlics (àcid cafèic, ferúlic i p-cumàric) i fitohormones (àcid salicílic i àcid jasmònic). Els resultats mostren que les plantes en presència de l'inductor, del patogen amb l'inductor o només del patogen, en canvien el perfil metabòlic. Es produeixen en tots els casos metabòlits relacionats amb la defensa com a compostos fenòlics, timol, vitamina E, floroglucinol, entre molts altres, però els temps o les concentracions en què es generen no són les mateixes. Si es relacionen aquest canvis amb el seguiment visual de les plantes, que quan tenien l'inductor i el patogen, presentaven mínims signes de pansiment o cap, respecte a les inoculades només amb el patogen. Així es podria suggerir que l'inductor té un efecte positiu en les plantes regulant la producció de metabòlits, els quals s'han de generar en determinada concentració o temps, perquè es doni un control adequat del patogen. En el mateix sentit, l'inductor va ajudar a regular la concentració de fitohormones a les plantes. El ‘acid salicílic (SA) es va mantenir en una concentració estable, mentre que el àcid jasmònic (JA) va anar augmentant després de les 24 h, comportament que afavoreix el control del patogen i que s'ha relacionat amb genotips resistents d'alvocat davant P. cinnamomi.

En conclusió, aquesta tesi Doctoral proporciona informació valuosa sobre l'ús d'inductors en plantes d'alvocat Hass, mostrant que tant el curlà com el silici, tenen efectes positius en la defensa contra P. cinnamomi. Aquest podrien ser inclosos en plans de gestió en un futur integrat de la malaltia, impactant amb això en la innocuïtat del producte consumit.

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INTRODUCCIÓN

El crecimiento exponencial de la población plantea grandes retos para la agricultura, que debe aumentar los rendimientos por hectárea frente a la creciente demanda. Se estima que para el 2050 la población habrá aumentado en unos 2.2 billones de personas y por tanto, se requeriría un extra de 200.000 billones de calorías por año [1]. Además, dentro de los objetivos de desarrollo sostenible de la ONU, se plantea el de hambre cero, que busca "poner fin al hambre, lograr la seguridad alimentaria, la mejora de la nutrición y promover la agricultura sostenible" [2]. Para satisfacer esa demanda que aumenta anualmente, la agricultura ha recurrido a nuevas tecnologías, expansión agrícola, uso de fertilizantes y diferentes estrategias de control de plagas y enfermedades que han resultado efectivas de cierto modo, trayendo consigo mayores niveles de producción [3]. Aunque mediante estas estrategias se ha permitido cumplir con los requerimientos de alimentos para la mayor parte de la población, el costo ambiental y ocupacional por el uso frecuente de sustancias de síntesis, ha ido en detrimento del ambiente y la salud, debido a que muchos cultivos requieren grandes cantidades de pesticidas y un uso frecuente para mantener sanas las plantas [4].

A pesar del uso de productos químicos, la agricultura, desde que inició hace más o menos 10000 años, ha tenido que lidiar con diferentes organismos nocivos que causan disminución de la producción y que aún hoy son un problema. De hecho, alrededor del mundo, los patógenos de plantas ocasionan el 13% de la disminución del rendimiento de los cultivos, que junto a las producidas por insectos y malezas llegan a causar una pérdida de 2 billones de dólares [5,6]. Está claro que si no se empleara el control químico y no químico los daños llegarían a ser de entre un 40 a un 70%. En ese sentido, la aplicación de pesticidas es necesaria pues de esta depende en gran medida la producción, aunque actualmente existe presión por parte de algunos gobiernos, mercados y consumidores para reducir el uso de los mismos. En virtud de ello, se ha generado en Europa un reglamento, el (EC) 1107/209 complementado por la directriz 2009/128/EC, que fuerza a los miembros del estado a generar políticas que contemplen planes basados en un manejo integrado de la enfermedad con menor dependencia de químicos [7]. Algo que incluso la ONU, se ha planteado, buscando no solo duplicar la productividad, sino asegurar la sostenibilidad de los sistemas de producción de alimentos. No obstante, estas iniciativas de agricultura sustentable no se han implementado en otros lugares como Norteamérica, ni tampoco en Colombia, donde para exportar sólo se exige el registro del predio, pero nada al respecto de cultivos con menor uso de

agroquímicos, pero que seguramente pronto será exigido, debido precisamente a la contaminación que existe tanto de la tierra como de los productos.

En consecuencia, esta legislación que surgió en Europa, plantea un nuevo escenario donde se hace importante buscar estrategias que puedan emplearse junto con las tradicionales en un plan de manejo integrado de la enfermedad (IPM). Por lo cual, la estimulación de defensas de las plantas es una de las que ha vuelto a tenerse en cuenta porque está basada en el uso de productos de origen biológico. Al respecto, ha ido creciendo el interés por el uso de elicitores en plantas, prueba de ello es el aumento en los últimos 10 años de la producción científica al respecto, la cual se ha duplicado, con al menos unos 500 artículos científicos por año para 2021, siendo las áreas de agricultura (37,2%) y bioquímica (33,6%) en las que más se ha publicado, según lo registrado en la base de datos Scopus®.

Inicialmente el termino elicitor, se empleó para moléculas capaces de inducir la producción de fitoalexinas, sin embargo, hoy se reconoce a los elicitores como componentes que inducen una señal para activar o estimular los mecanismos químicos de defensa en las plantas. Varían entre sí en función de su fuente, naturaleza y estructura molecular, es decir, no son un único tipo de metabolito, están formados por moléculas como oligosacáridos, péptidos, lípidos y proteínas [8,9] (Figura 1).

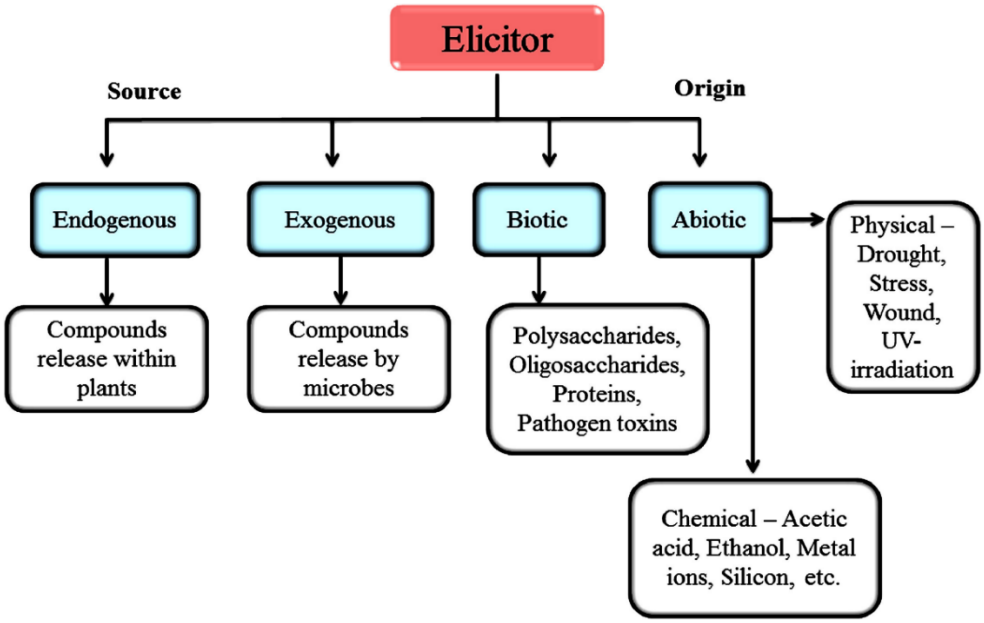

Figura 1. Clasificación de los elicitores basado en su naturaleza y origen [8].

Los elicitores en los patógenos pueden clasificarse en dos categorías: elicitores generales y elicitores específicos de la raza. Los generales están relacionados con las vías de señalización de resistencia, mientas que los específicos de la raza están asociados con la señalización mediada por genes R. Comúnmente, a los elicitores que son capaces de desencadenar la defensa tanto en plantas no hospederas como en hospederas, a través de percepción como patógenos potenciales se conocen como elicitores generales. Así, la mayoría de elicitores generales están esencialmente presentes en las paredes celulares de los patógenos como componentes estructurales, por ejemplo, glucano, quitina, flagelina y lipopolisacáridos [10,11] (Figura 2.).

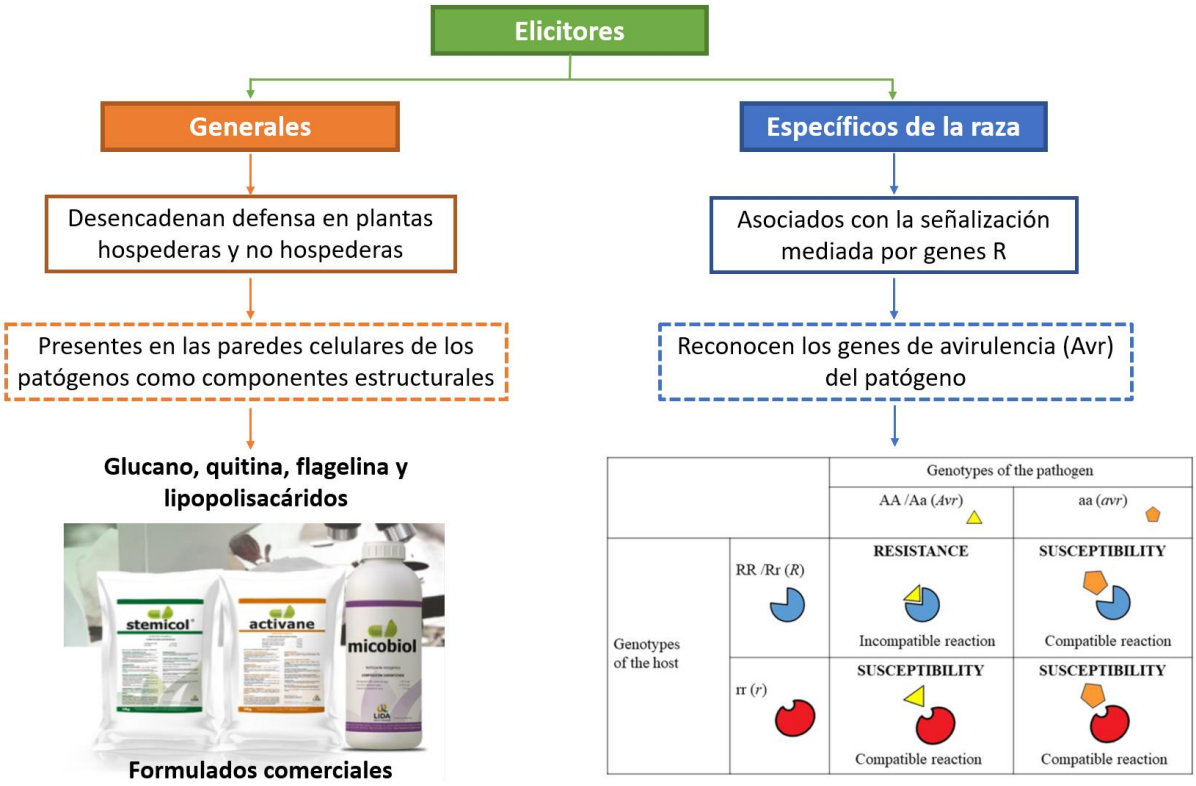

Figura 2. Clasificación de elicitores de acuerdo a su reconocimiento en plantas. Figura realizada por la autora con imágenes tomadas de LIDA plant research y Kistner, *et al.* [12].

Algunos elicitores sólo pueden ser reconocidos en un limitado número de plantas y son activos sólo en huéspedes específicos, por ejemplo algunos elicitores oligosacáridos provenientes de *Magnaporthe grisea* son reconocidos en arroz pero no en soya, mientras β-glucanos de *Phytophthora sojae* activan la respuesta en ambas plantas e incluso en otras no hospedero [13,14]. En ese sentido, se conoce que proteínas asociadas a la membrana pueden reconocer elicitores β-

glucanos con una alta afinidad, pero no cuenta con un dominio de señalización, por lo que se sugiere que esas proteínas interactúan con otros componentes para transducir la señal [9].

En relación con lo mencionado, queda claro que, el uso de oligosacáridos como moléculas que pueden generar inducción de resistencia, podría ser una alternativa ambientalmente sostenible [15]. La actividad de estos compuestos ha llevado a la generación de nuevos productos comerciales con alta eficiencia, estabilidad y menor impacto ambiental [16]. Estos productos incrementan la productividad por reducción no sólo de pérdidas sino también por aumentar el crecimiento vegetal [17], aspectos que serán ampliados con detalle en el capítulo 1.

De igual manera, es importante resaltar que diferentes tipos de moléculas elicitoras provenientes de disímil origen (bacterias, hongos, insectos y químicas) han mostrado efectos en una gran variedad de plantas como tomate, arroz, fríjol, maíz, uva, tabaco, arabidopsis, pimienta o en familias de plantas como solanáceas o brasicaceas [9]. Siendo un tema que no se ha quedado solo en investigaciones individuales que evalúan el efecto del elicitor, sino que esta información trascendió y hoy por hoy es posible encontrar diversos productos comerciales, como el Fytosave®, Stemicol® o Activane®, que se emplean en la agricultura con buenos resultados. Se ha demostrado así que los elicitores pueden ser incluidos en planes de manejo integrado y que la investigación con ellos puede ser la base para futuros desarrollos que conduzcan a productos con menores trazas de químicos sintéticos.

Atendiendo a estas consideraciones, esta tesis doctoral se desarrolla en el marco de la agricultura sustentable y tiene como objetivo evaluar el efecto de la inducción de resistencia con el elicitor de tipo oligosacárido curlano sobre la respuesta bioquímica de plantas de aguacate Hass inoculadas con *Phytophthora cinnamomi*. Este oligosacárido es un aditivo alimentario seguro y aprobado para consumo humano [18,19], característica que lo hace un candidato interesante dentro del marco de IPM, por lo cual, esta tesis doctoral se enfoca en los retos asociados al uso de oligosacáridos en protección de plantas de interés comercial.

1. El aguacate (*Persea Americana* Mill.)

Taxonomía

Reino:	Vegetal
División:	Spermatophyta
Subdivisión:	Angiospermae
Clase:	Dicotyledoneae
Subclase	Dialypetalae
Orden:	Ranales
Familia:	Lauraceae
Género:	*Persea*
Subgénero:	*Persea*
Especie:	*Persea americana* Mill.
Subespecies:	*P. americana* var. *americana* Mill. (Antillano) *P. americana* var. *drymifolia* (Mexicano) *P. americana* var. *guatemalensis* Williams (Guatemalteco)

La clasificación botánica del aguacate se ha prestado para que se presente una controversia, reconociéndose una, dos o tres especies, según los taxónomos. Actualmente es aceptado por la mayoría, que el aguacate puede ser agrupado bajo una sola especie: *Persea americana* Mill.

El aguacate pertenece a la familia *Lauraceae*, una de las más antiguas en nuestro planeta. Comprende poco más de 50 géneros y unas 2,200 especies. De esta familia se deriva el género *Persea*, el cual tiene dos subgéneros: *Persea* y *Eriodaphne*. El aguacate se clasifica dentro del género *Persea* y subgénero *Persea.* En el subgénero *Persea* se reconocen tres especies: *P. americana* Mill., *P. schiedeana* Nees, y *P. parvifolia* Williams [20-22].

La especie *P. americana* Mill. es poliforme y está constituida por varios taxones separados, que pueden ser considerados como variedades botánicas o subespecies. En literatura técnica estas subespceies son descritas como razas "hortícolas". Dentro de este grupo están tres subespecies ecológicamente separadas, que se comercializan actualmente *P. americana* var. *americana* Mill.

que corresponde al aguacate Antillano o de tierras bajas, *P. americana* var. *drymifolia* que corresponde al aguacate mexicano o de tierras altas y *P. americana* var. *guatemalensis* Williams, perteneciente a los aguacates guatemaltecos. Como resultado de la amplia distribución del germoplasma de aguacate hacia regiones distantes de sus sitios de origen, ocurrió un cruzamiento interracial, a tal grado que en la actualidad los cultivares económicamente importantes tanto en áreas tropicales como subtropicales donde se cultivan, son resultado de la hibridación entre razas [23].

Las tres razas de *P. americana* no están lo suficientemente diferenciadas como para ser consideradas especies diferentes, pero si pueden considerarse como subespecies o variedades botánicas [20,24]. Ya que las tres razas tienen un genoma similar (2n=24), la hibridación entre ellas ocurre con facilidad y sus híbridos obtienen ventajas de adaptación climática, así como características agronómicas mejoradas [21,22].

Características morfológicas

Figura 3. Esquema de la morfología de la planta de aguacate. Imágenes tomadas de diferentes fuentes de internet. Composición realizada por la autora.

2. Variedad Hass

Es una variedad cultivada en climas tropicales y subtropicales [25]. Es un cultivar predominantemente Guatemalteco, proviene de dos razas, cuenta con un 10 a 15% de la raza Mexicana y el resto, 80 a 90% de la raza Guatemalteca [26]. Por su arquitectura, el árbol se asemeja al naranjo, pero con mayor tamaño, es de buena producción, con frutos de calidad, que permiten ser almacenados. Los frutos son de tamaño mediano, con un peso que va de 150 a 400 g [27] y de 8 a10 cm de largo [28]; de forma ovoide a piriforme; la cascara es de mediana a gruesa, coriácea, de textura rugosa y suberosa, de superficie áspera y granulosa; los granos desaparecen cuando es sembrado a gran altitud; es de color verde que se oscurece al madurar, tornándose morada a negra [27].

Es una variedad distribuida alrededor del mundo. En Colombia parece presentar buenas características organolépticas y en el país se han registrado rendimientos de producción muy

elevados, ha mostrado en suelos colombianos rendimientos por hectárea superiores a los que presentan los principales países exportadores, que van de 12,4 a 18,8 t/ha en arboles de 8 y 9 años [25].

3. Comercialización

El aguacate (*Persea americana* Mill.) es la quinta fruta tropical más importante en el mundo, medida en términos de volumen y área cultivada [29]. Su consumo se ha incrementado a nivel mundial en tres puntos porcentuales anuales en Europa, América del Norte y Asia, pero su producción no ha crecido al mismo ritmo [30].

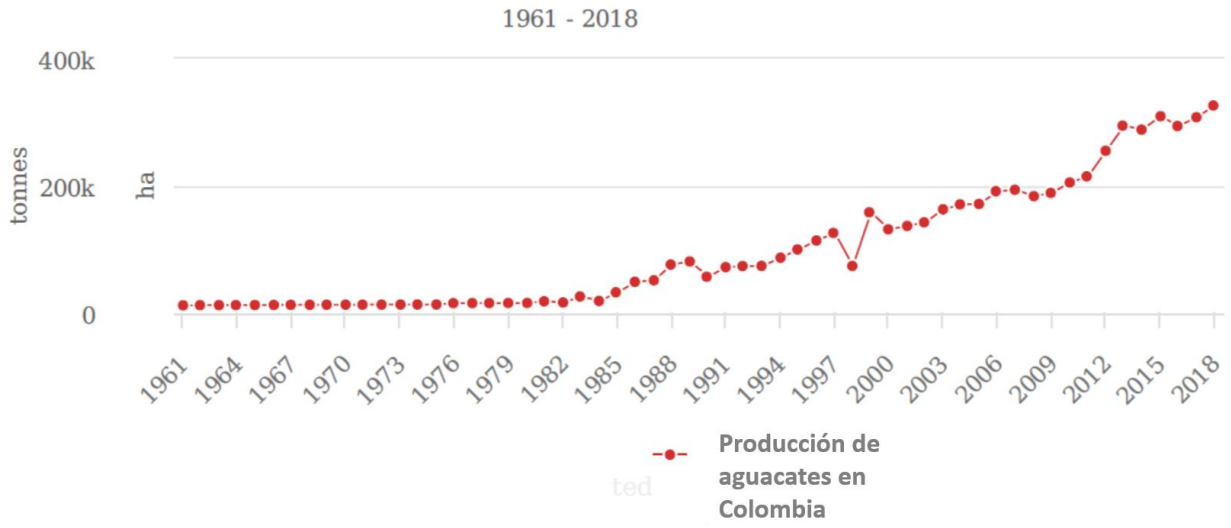

Figura 4. Producción de aguacate en Colombia entre 1961 – 2018. Fuente: FAOSTAT [31].

Teniendo en cuenta la oportunidad de mercado, Colombia ha visto en el cultivo de aguacate una oportunidad económica importante, es por esto que, la producción de aguacate se ha incrementado notablemente (Figura 4), para el 2016 el área sembrada se estima alcanzó las 60000 hectáreas, con una producción de 343.000 toneladas. Por su parte la variedad Hass presenta gran protagonismo a nivel nacional porque mientras la producción de los aguacates criollos creció por el orden de 38% en los últimos cinco años, el aguacate Hass creció un 126%.

Respecto a la producción mundial, Colombia se ha mantenido por varios años entre el cuarto y quinto mayor productor a nivel mundial según datos FAOSTAT [32]. No obstante, no se encuentra entre los primeros siete exportadores de aguacate [33]. Aun así, las cifras son muy positivas para

el país, según datos de ProColombia, en el 2019 las exportaciones en el país sumaron US$ 89 millones, con un crecimiento del 42% frente al 2018. Dichas exportaciones están representadas en el país en un 90% por el aguacate Hass, lo que implica que los demás cultivares (pieles verdes) representan menos del 10% [34].

La principal competencia en el mercado internacional para el país son Egipto y Arabia Saudita, quienes con menores costos de producción dominan el mercado europeo, el principal destino para la exportación colombiana durante los meses de más alta producción [31]. Allí cuatro países concentran el 89% de las exportaciones de aguacate Hass colombiano, en su orden: Países Bajos, Reino Unido, España, Bélgica.

Por su parte, Estados Unidos, ha sido tradicionalmente el principal mercado para aguacate Hass. Sin embargo, es un mercado nuevo para Colombia, se hizo la apertura apenas en agosto de 2017 y a día de hoy ya representa el 5% de las exportaciones de la fruta y tiene un alto potencial de crecimiento a corto plazo, debido a que las normas exigidas, se flexibilizaron para facilitar su cumplimiento en el segundo semestre del año pasado [33].

Finalmente, entre los retos más importantes para el país está el cumplir con los protocolos fitosanitarios que exige el mercado japonés y chino. Este último, ya dio vía libre a la exportación el 13 de diciembre de 2019 y el primer cargamento arribó a ese país en el mes de Julio de 2020, año en el cual las exportaciones de aguacate aumentaron más de 25 % en volumen y valor, con ventas que superaron los US$100 millones [35].

4. Enfermedades del Aguacate

El aguacate presenta enfermedades severas que en casos extremos provocan la muerte del árbol y en general, una disminución en la producción que varía del 10 al 40% y una reducción en la calidad entre un 15 y 30% [36].

La Corporación Colombiana de Investigación Agropecuaria - Corpoica, realizó un inventario de los principales limitantes fitopatológicos de este frutal en Colombia, entre las enfermedades de mayor importancia por su frecuencia y severidad, se nombran a continuación en orden por nombre común y microorganismo que la causa: 1) pudrición de raíces, *Phytophthora cinnamomi* var.

Cinnamomi, 2) marchitez, *Verticillum sp.*, 3) llaga radical, *Armillaria mellea* y *Rosellinia sp.*, 4) roña, *Sphaceloma perseae*, 5) antracnosis del fruto causada por *Glomerella cingulata* (anamorfo *Colletotrichum gloeosporioides*), 6) la mancha de la hoja y la mancha negra del fruto por *Pseudocercospora purpurea* y 7) otras enfermedades en poscocecha como *Rhizopus stolonifer*, *Lasiodiplodia theobromae* y *Dothiorella sp.* [37].

Es pertinente resaltar que *Phytophthora cinnamomi* Rands es el patógeno más importante, se manifiesta generando un declinamiento progresivo del árbol, hojas marchitas, llegando a secarse por completo ocasionando así baja fructificación o en algunos casos fructificación excesiva con frutos que no llegan a engordar [38]. Las pérdidas ocasionadas por este patógeno pueden llegar hasta un 90% [39].

El control de esta enfermedad puede realizarse mediante un manejo genético, usando patrones comerciales que han mostrado resistencia moderada o tolerancia a *P. cinnamomi* como Duke 6, Duke 7 y Thomas, o mediante manejo químico que puede hacerse en diferentes etapas con hipoclorito de calcio y fungicidas tipo vitavax 300, Basamid, Metalaxil – Mancozeb, Fosetil Aluminio y Captan, entre otros [37].

5. Aspectos bioquímicos de la interacción planta - patógeno

Los animales y las plantas deben hacer frente al ataque de patógenos, los cuales representan el estrés biótico más importante [40]. En el caso de las plantas, las enfermedades bióticas son ocasionadas por organismos patogénicos vivos (hongos biótrofos y hemibiótrofos, bacterias, oomycetes y nematodos) y patógenos no celulares (virus y virioides) [41], como estas son sésiles, están bajo constante ataque de microorganismos. Para defenderse, como las plantas carecen de un sistema circulatorio y células inmunes móviles, no hay circulación tampoco de receptores que detecten señales no propias [42]. Sin embargo, la primer barrera que existe es el sistema complejo de pared celular que los microorganismos deben pasar [43], en esta misma pared celular y en el citoplasma, existen receptores, que son altamente específicos, con una autorreactividad restringida y que a menudo genera una “memoria” de por vida de los patógenos encontrados [42].

En el capítulo uno se mencionará con más detalle la información sobre los receptores, el proceso de reconocimiento y como eso ayuda en la búsqueda de nuevas estrategias en la defensa de plantas.

Durante el curso de la evolución, ambos, plantas y patógenos han desarrollado habilidades que les permiten combatirse entre ellos. Para cada respuesta de defensa de la planta el patógeno desarrolla una nueva estrategia para atacar, esa interacción ha sido descrita como el "modelo zig-zag" [44], el cual plantea el reconocimiento del patógeno en dos vías.

La primera línea de defensa es el reconocimiento de moléculas características conservadas, estas moléculas llamadas elicitores son conocidas como patrones moleculares asociados a microbio- o patógenos (MAMPs/PAMPs), patrones moleculares asociados a daño (DAMPs) y patrones moleculares asociados a herbivoría (HAMPs) potencialmente capaces de iniciar una respuesta de defensa por parte de la planta [45]. Esas moléculas antes mencionadas son reconocidas por receptores de patrones de reconocimiento (PRRs) por lo que al ser detectadas generan una inducción de defensa llamada MAMP/PAMP desencadenante de inmunidad (M/PTI). Estos PRRs están localizados en la superficie de las células de las plantas, específicamente en la membrana plasmática y son cinasas tipo receptor (RLKs) o proteínas tipo receptor (RLPs).

Por su parte, la inmunidad desencadenada por efectores o (ETI) es la segunda y robusta vía de respuesta de inmunidad de las plantas, es activada por genes de resistencia (R) cuando los efectores son liberados dentro de la planta de acuerdo a la teoría gen por gen [41,46,47] (Figura 5). Entendiendo efector como la molécula que manipula la estructura y función de la célula huésped, facilitando la infección (factores de virulencia o toxinas) y/o desencadenando respuestas de defensa (factores de avirulencia o elicitores [48].

La inmunidad desencadenada por PAMPs (PTI) provee resistencia a una infección, como respuesta general a clases enteras de microrganismos patógenos, a través de un conjunto de respuestas celulares tempranas que comprende: flujo de iones a través de la membrana, producción de especies reactivas de oxígeno (ROS), cascadas de fosforilación de proteína quinasas activadas por mitógeno (MAPK), producción de especies reactivas de nitrógeno como el óxido nítrico (NO), alteraciones de la pared celular, inducción de compuestos antimicrobianos (fitoalexinas) y respuestas de largo término que incluyen la deposición de callosa y la sobreexpresión de los genes relacionados con la defensa (R) [41,45,47].

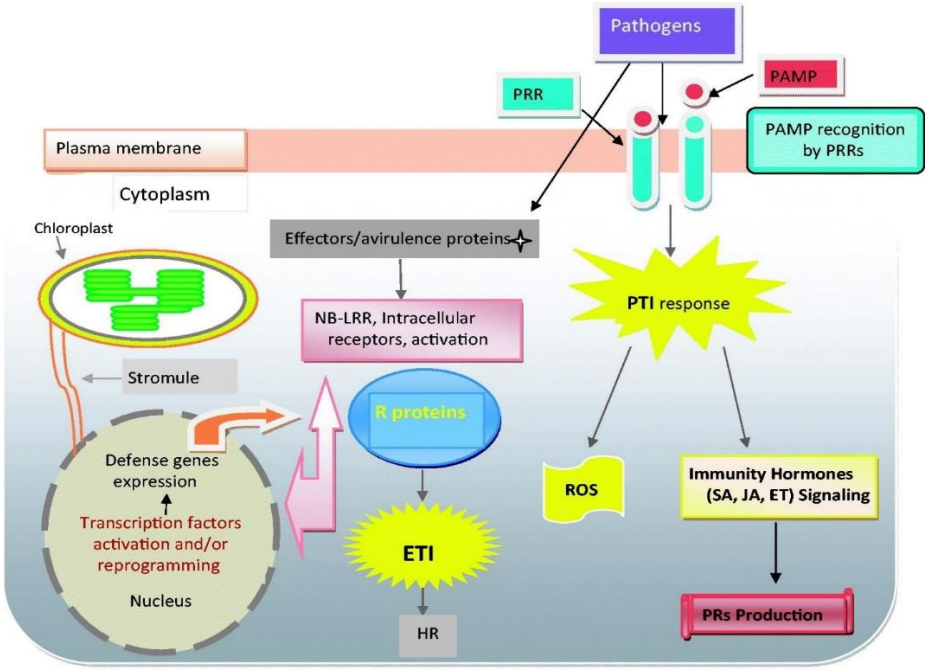

Figura 5. Modelo hipotético de los mecanismos de defensa en una planta infectada [41].

En cuanto a la inmunidad desencadenada por efectores (ETI), esta es considerada como una respuesta PTI acelerada y amplificada, que resulta en resistencia a la enfermedad y respuesta hipersensible (HR) en el sitio de la infección [46]. Asimismo, las fitohormonas relacionadas a defensa como ácido salicílico (SA), ácido jasmónico (JA) y etileno (ET) son componentes de señalización fundamentales y son producidos en ambas respuestas PTI y ETI [41].

5.1. Papel de las especies reactivas de oxígeno en la interacción planta - patógeno

Las plantas responden a ROS de forma dosis – dependiente. Altas concentraciones de ROS resultan en daño celular e incluso muerte celular hipersensible, mientras bajas concentraciones de ROS funcionan como señal evolutiva, controlando varios aspectos de la biología de las plantas [49-51].

Las ROS actúan en pasos anteriores o posteriores de varias cascadas de señalización y el grado de acumulación determina su papel en la célula. Podría actuar como una molécula importante en transducción de señales (a bajos niveles) o como moléculas tóxicas (en niveles altos) siendo entonces, como su nombre lo indica extremadamente radiactivas u oxidantes [52] en la naturaleza. Pueden interactuar con un número grande de otras moléculas y metabolitos tales como DNA,

pigmentos, proteínas, lípidos y otras moléculas esenciales para la célula lo que conduce a una serie de procesos destructivos [53].

Siendo las ROS altamente reactivas, la mayoría de ellas puede causar daño de membrana, inhibir actividades enzimáticas y cuando se acumulan no son compatibles con la función celular y son consideradas deletéreas y perjudiciales. Así llega a ser claro que la expresión de genes asociada con respuestas a estrés en gran medida es sensible al estado redox de la célula [51].

Adicionalmente, previo a la exposición de estrés abiótico o biótico que induce una "explosión oxidativa" puede desencadenarse una función protectiva o inmunizar la planta contra estrés ambiental, así jugando un rol en la tolerancia al estrés [50,51,54].

Se han estudiado los mecanismos mediante los cuales actúan las ROS en los procesos de estrés, encontrando que se da por activación de genes y otras que no requieren de dicha activación, sino que son radicales generados como resultado de un daño oxidativo de la membrana (radicales alcoxi y peroxi) que generan muerte celular programada, reacción hipersensitiva entre otros [51]. Así pues, las proteínas que están involucradas en el ajuste redox del estatus de las células de la planta pueden desencadenar reacciones de defensa o superar los efectos deletéreos del estrés oxidativo [55].

Es importante señalar que en la mayoría de respuestas a estrés biótico, la principal fuente de ROS parece ser generada por peroxidasas localizadas en la pared celular que generan peróxido de hidrógeno o por NADPH/NADH oxidasas localizadas en la membrana plasmática que generan superóxido o incluso puede ser por la actuación de los dos sistemas a la vez actuando en tándem[56].

En cuanto al rol que desempeñan las ROS como antimicrobiano depende de la sensibilidad que tenga el patógeno a las ROS. La mayoría de los patógenos biótrofos son altamente sensibles a H_2O_2 (incluso concentraciones micromolares). Los necrótrofos pueden frecuentemente tolerar altas concentraciones de ROS y pueden incluso contribuir a un incremento de la acumulación de ROS durante la interacción patógeno – hospedero [57].

5.2. Mecanismos antioxidantes enzimáticos en plantas

Las plantas tienen la habilidad de desintoxicarse de las ROS o mantenerlas en una concentración determinada gracias a la producción de diferentes tipos de antioxidantes, que pueden ser categorizados generalmente en dos diferentes tipos que contienen antioxidantes enzimáticos y no enzimáticos. Los antioxidantes enzimáticos incluyen superóxido dismutasa, catalasa, peroxidasa y algunos otros antioxidantes enzimáticos que están a cargo en el ciclo glutatión – ascorbato como la ascorbato peroxidasa (APX), monohidroascorbato reductasa (MDHAR), dehidroascorbato reductasa (DHAR) y glutatión reductasa (GR). Por otra parte los comúnmente conocidos antioxidantes no enzimáticos son glutatión (GSH), ascorbato (AsA), carotenoides, tocoferoles, flavonas y antocianinas [53].

La metaloenzima superóxido dismutasa (SOD) es el más efectivo antioxidante enzimático intracelular el cual es ubicuo en todos los organismos y en todos los compartimentos celulares propensos a estrés oxidativo mediado por ROS. Está bien establecido que varios tipos de estrés ambiental frecuentemente conducen a un aumento de la generación de ROS, donde, la SOD ha sido considerada importante en tolerancia al estrés en plantas y provee la primera línea de defensa contra los efectos tóxicos de niveles elevados de ROS. La SOD elimina O_2^- catalizando su dismutación, un O_2^- es reducido a H_2O_2 y otro oxidado a O_2. Se elimina O_2^- y por lo tanto disminuye el riesgo de formación de $OH^{\cdot}$ [49,58].

Por su parte, la glutatión reductasa es una flavo proteína oxidorreductasa encontrada en procariotas y eucariotas [59]. La GR cataliza la reducción de glutatión (GSH), una molécula involucrada en muchos procesos metabólicos regulatorios y antioxidantes en plantas donde la GR cataliza la reacción dependiente de NADPH del enlace disulfuro del glutatión oxidado (GSSG) y es así importante para mantener la reserva de GSH. De hecho, el GSSG consiste en dos GSH unidos por un puente disulfuro el cual puede ser convertido de nuevo a GSH por la GR. La GR está involucrada en la defensa contra el estrés oxidativo mientras que el GSH juega un papel importante dentro del sistema celular que incluye participación en el ciclo ASH-GSH (ácido ascórbico-glutatión), mantenimiento del grupo sulfhidrilo (-SH) y un sustrato para las glutatión S-transferasas. GR y GSH juegan un papel crucial en la determinación de la tolerancia de una planta a varios tipos de estrés [49,60,61].

Otra enzima importante es la ascorbato peroxidasa se cree que juega el rol más esencial en el secuestro de ROS y protección de células en plantas vasculares, algas, euglena y otros organismos. La APX está involucrada en la depuración de H_2O_2 en los ciclos agua-agua y ASC-GSH y utiliza el ácido ascórbico como donador de electrones. [49,62]. Las APX tiene alta afinidad por H_2O_2 (rango μM) que las catalasas o peroxidasas (rango mM) y puede tener un rol más crucial en el manejo de ROS durante el estrés [49].

5.3. Mecanismos antioxidantes no enzimáticos: Compuestos fenólicos

Los compuestos fenólicos en plantas están fuertemente involucrados en la interacción planta-patógeno [63]. Tienen efecto antimicrobial [64] y a su vez desencadenan procesos de defensa [65]. Entre los diferentes ácidos fenólicos, los fenoles constitutivos son conocidos por proveer resistencia directa exhibiendo actividad bactericida o fungicida o indirecta a través de la activación de respuestas de defensa en el hospedero [66].

Aunque algunos compuestos fenólicos individuales pueden llegar a concentraciones tóxicas para los patógenos, varios de estos compuestos a menudo se acumulan simultáneamente en el tejido infectado y es posible que su efecto tóxico combinado, más que el efecto fungitóxico de cada uno por separado serían responsables de la inhibición de la infección de cultivares resistentes [63,67].

En particular, muchos estudios apuntan a la eficacia antimicrobiana de ciertas clases de compuestos fenólicos, tales como ácido hidrobenzoico derivatizado, ácido cafeico y cumárico derivatizados, ácido clorogénico, ácido ferúlico, flavonoides y cumarinas [66,68,69]. No obstante, fenoles sin efecto contra patógenos como el ácido gálico, la planta los puede transformar en galotaninos que si tienen actividad antifúngica [66,70].

5.4. Ácido jasmónico y salicílico en la interacción planta - patógeno

El ácido jasmónico (JA) es una molécula de señalización derivada de los lípidos involucrada en varios procesos de desarrollo y defensa en plantas. La aplicación exógena de JA resulta en sobreproducción de proteínas relacionadas con la defensa como defensinas y tioninas [71].

Estudios sobre el tema sugieren que JA induce resistencia contra patógenos necrotrofos, algunos insectos que se alimentan del floema y herbívoros, mientras que el ácido salicílico (SA) induce resistencia contra patógenos biotrofos e insectos que se alimentan del floema. En la naturaleza, las plantas son atacadas simultánea o subsecuentemente por patógenos biótrofos y necrótrofos o por insectos. Consecuentemente, la intercomunicación entre varios componentes de señalización llega a incrementarse de forma importante.

Las plantas sobreviven en respuesta a múltiples interacciones entre atacantes priorizando una vía de señalización específica y estableciendo una red de señalización hormonal [72]. Por eso, durante la inmunidad frente a patógenos biótrofos, el JA es conocido por su acción antagónica con la vía del SA. Así, la interacción de esas dos hormonas es considerada la columna vertebral de las respuestas inmunes de la planta frente a patógenos, cambiando las respuestas de defensa a la vía JA o SA dependiendo del estilo de vida del patógeno particular que invade [73].

Por su parte, el ácido salicílico es un compuesto fenólico con actividad hormonal en plantas que es reconocido como una molécula de señalización endógena importante en la inmunidad de plantas. La vía de respuesta del SA es típicamente (pero no exclusivamente) efectiva contra patógenos microbiales biotróficos. Una vez la vía del SA es activada en el sitio de infección, una respuesta similar es frecuentemente desencadenada en partes distales de la planta para proteger tejido no dañado contra la posible invasión de patógenos. Esta resistencia inducida y de amplio espectro se conoce como resistencia sistémica adquirida (SAR) [74].

Aunque, las ideas expuestas anteriormente muestran que clásicamente, se ha asociado la presencia de ácido salicílico (SA) con la resistencia a patógenos que se alimentan de tejido vivo o de tejido vivo y muerto (biótrofos y hemibiótrofos, respectivamente); y el ácido jasmónico (JA) e incluso, el etileno, con la resistencia a patógenos necrótrofos o que se alimentan de tejido muerto. Estudios más recientes demuestran que una sola hormona no controla la inmunidad, sino que tienden a trabajar interdependiente a través de interacciones complejas de sinergismo o antagonismo.

Estas interacciones resultan en cambios de la fisiología de la planta que culmina en una respuesta de defensa apropiada contra el ataque de patógenos, es una reacción precisa frente a determinado patógeno, pero particularmente relevante contra los de tipo hemibiótrofo. Al respecto, se ha demostrado la participación de más de una fitohormona (JA y SA) en el desarrollo de resistencia sistémica adquirida, es decir en la resistencia frente a un patógeno específico, por lo cual, una

correcta expresión de estas fitohormonas en esa interacción planta - patógeno es crucial para una eventual patología, situación que se verá afectada directamente por el estilo de vida de ambos, patógeno y planta [71,73].

5.5. Enzimas de la ruta fenilpropanoide en la interacción planta - patógeno

Los procesos de señalización de las ROS y fitohormonas, actúan sobre la activación de genes de defensa, esto hace que se inicien otras rutas metabólicas como la que genera fenilpropanoides. Estas constituyen un amplio rango de sustancias cíclicas estructurales que poseen numerosas funciones fisiológicas y en su formación intervienen varias enzimas, se mencionarán algunas que tradicionalmente se tienen como referencia cuando se habla de mecanismos de defensa.

En primer lugar, la fenilalanina amonio liasa (PAL) induce la polimerización de monolignoles en la etapa temprana del proceso de lignificación. La desaminación del aminoácido fenilalanina por esta enzima genera compuestos de tipo fenilpropanoide (ácido trans-cinámico) que es muy importante en numerosas funciones fisiológicas, precursor de la lignanos, flavonoides y cumarinas (Figura 6).

Después de la acción de la PAL, una segunda enzima, la peroxidasa (POD), actúa en el último paso de la vía fenilpropanoide formando unidades de lignina H (*p*-hidroxifenil propano), G (guaiacil) y S (siringil). La sobre expresión de estas enzimas, sus productos y de otras enzimas en plantas inoculadas con diferentes patógenos deja ver su rol como respuesta de defensa [75-78]. Luego de la acción de la PAL, se genera cinnamato o ácido cinámico que, tras reacciones de hidroxilación, metilación y deshidrogenación, se generan sus derivados fenólicos como ácidos: *p*-cumárico, cafeico, ferúlico y sinápico) y también cumarinas simples. Todos los compuestos antes mencionados son inducidos por estrés biótico o abiótico, así como las enzimas que participan en su generación.

Finalmente, las polifenol oxidasas (PPOs) son enzimas codificadas en el núcleo, que contienen cobre y tienen una distribución filogenética diversa entre plantas, animales, hongos y bacterias. Curiosamente, se han reportado familias multigénicas para varias PPO de plantas, pero no se sabe mucho acerca de sus funciones biológicas específicas. Las PPO conocidas están constituidas por tirosinasa, fenolasa, catalasa, cresolasa, polifenolasa y catecol oxidasa. Las polifenol oxidasas

catalizan la oxidación de monofenoles/ o-difenoles a o-quinonas y las reacciones secundarias de o-quinonas produce un oscurecimiento indeseable que ocurre como resultado de la senescencia, heridas o infección del patógeno [79].

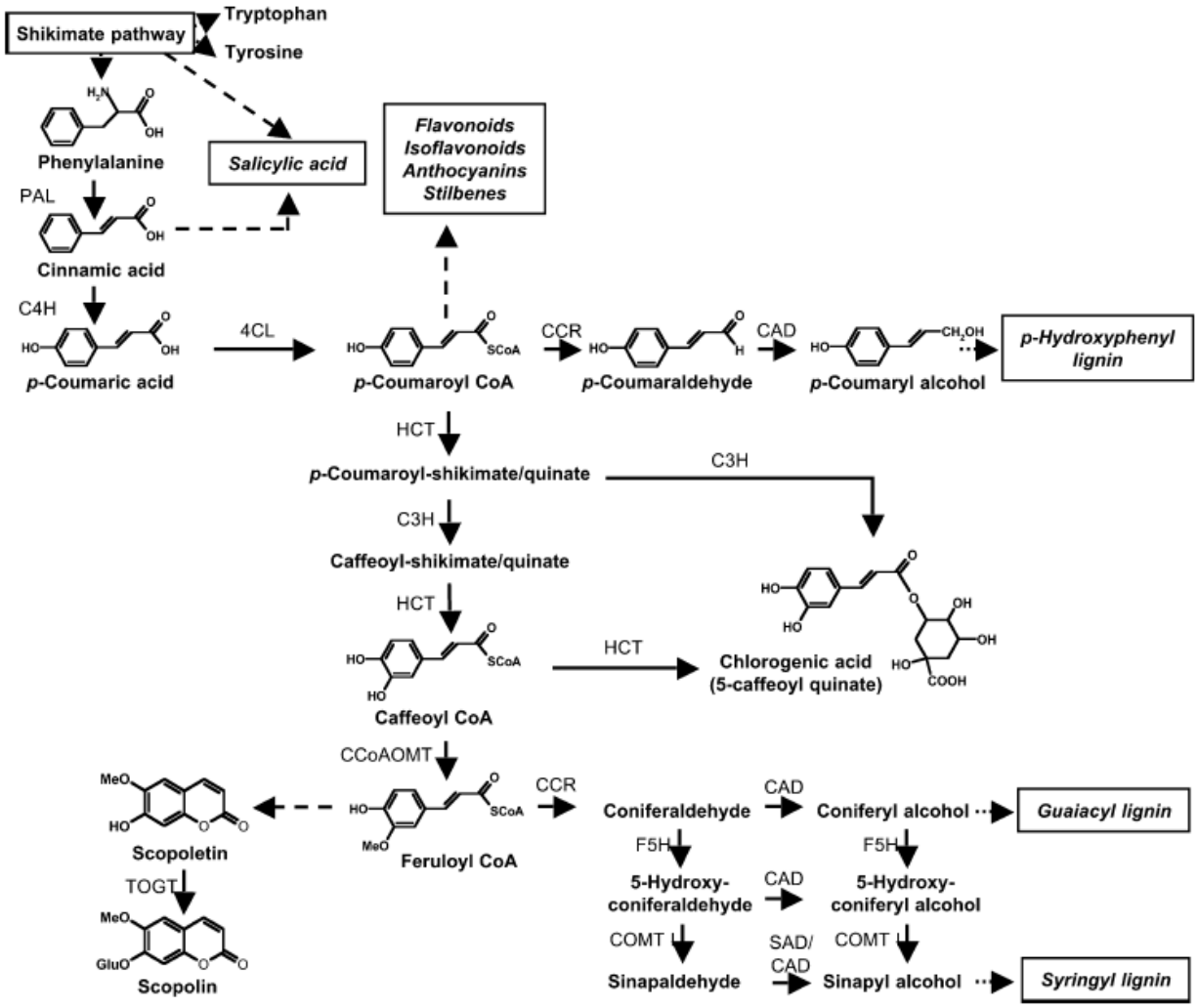

Figura 6. Principales rutas biosintéticas de los compuestos fenilpropanoides. Las flechas punteadas indican vías con enzimas no caracterizadas o múltiples enzimas [80].

Las PPOs han sido implicadas por su rol potencial en la vía fenil propanoide, regulación del oxígeno, defensa de plantas, entre otros procesos biosintéticos [81]. Entre otras funciones, se han relacionado con la defensa de las plantas principalmente debido a la aparición de sus productos de reacción ante el ataque de patógenos o insectos, heridas e inducibilidad en respuesta a diferentes estreses [81].

Por otra parte, peroxidasa y polifenoloxidasas están disponibles para oxidar compuestos fenólicos produciendo quinonas altamente tóxicas las cuales pueden potencialmente prevenir germinación de hongos [63,67].

Otras enzimas también participan en la ruta fenilpropanoide como: 4CL, 4-cumarato CoA ligasa; C3H, *p*-cumarato hidroxilasa; C4H, cinamato 4-hidroxilasa; CAD, alcohol cinamílico deshidrogenasa; CCoAOMT, cafeoil-CoA O-metiltranferasa; CCR, cinamoil-CoA reductasa; COMT I, ácido caféico / 5 ácido hidroxiferúlico O-metiltransferasa; F5H, ferulato 5 hidroxilasa; HCT, hidroxicinamoil transferasa; SAD, sinapil alcohol deshidrogenasa; TOGT, tabaco glucosil transferasa.

6. Elicitores o Inductores

Los elicitores son componentes que inducen una señal para activar los mecanismos de defensa químicos en las plantas. Varían entre sí en función de su origen, naturaleza y estructura molecular. Pueden ser clasificados como exógenos, producidos por los patógeno, o endógenos que son moléculas liberadas por a planta en respuesta al ataque de un patógeno. Aunque, moléculas de composición similar a estos dos tipos son empleadas con éxito [8].

Comúnmente se ha establecido que los elicitores son químicos, pero estos también pueden ser físicos, es decir, factores bióticos o abióticos. De esta manera, es posible decir que no cuentan con una estructura química en particular. Se conocen de diferentes tipos de moléculas que incluyen oligosacáridos, oligonucleótidos, péptidos, proteínas, glicoproteínas y lípidos. Además de compuestos a base de silicio o fósforo [82,83].

Dentro de los ampliamente estudiados están los elicitores químicos como ácido salicílico, metil salicilato, quitosano, ácido benzoico, entre otros. De los cuales, se sabe que tienen un efecto sobre la producción de varios compuestos fenólicos y activación de enzimas [84].

Por su parte, algunos de más reciente interés, como fragmentos de glucano o quitina, hacen parte de los componentes estructurales de la pared celular de patógenos. En particular, los glucanos se encuentran en la pared celular de oomycetos como un componente estructural en forma insoluble. Sin embargo, ese fragmento activo del elicitor debe liberarse primero antes de que se produzca el reconocimiento. Por lo cual, es probable que la β-1,3-glucanasa de las plantas, además de tener función directa como enzima antifúngica, también actúa en la liberación de elicitores [85].

Información más detallada sobre los elicitores empleados en esta tesis, se podrá encontrar en el inciso 1.7 y en el capítulo 1.

7. El silicio en la inducción de la defensa en plantas

El silicio (Si) es el segundo elemento más abundante en la corteza terrestre y en los suelos (28%). Algunos grupos de organismos como juncos, diatomeas, algas amarillas y doradas requieren silicio como nutriente esencial. Mientras que en plantas superiores los efectos benéficos de este elemento se evidencian más cuando las plantas están sometidas a estrés, que en aquellas que crecen en condiciones óptimas [83].

Desde hace un tiempo se ha asociado la aplicación de silicio al suelo, en su forma soluble (silicato de potasio), con la inducción de resistencia a enfermedades en plantas [86-88]. Son muchos los ejemplos registrados en la literatura donde se observaron efectos positivos sobre la barrera física, respuesta bioquímica o cambios moleculares que favorecieron a las plantas, en diferentes interacciones compatibles como algodón – *Fusarium oxysporum* f s. *vasinfectum,* café – *Hemileia vastatrix*, soja – *Phytophthora sojae*, tomate – *Pseudomonas syringae*, entre otros [89-92]. Se conoce también, que puede ayudar en el estrés abiótico como en la toxicidad por metales pesados, salinidad, sequía, entre otros [93].

Los mecanismos a través de los cuales el silicio puede mitigar las enfermedades en plantas, impidiendo la penetración de patógenos, son variados e incluyen, refuerzo estructural, producción de compuestos antimicrobianos, activación de múltiples vías de señalización y resistencia sistémica [94-96].

Con respecto al papel favorable generado por el silicio durante el estrés biótico, se ha evidenciado su rol en la modificación de las propiedades de la pared celular de las plantas [97]. Mientras que al mismo tiempo, la deposición de sílice biogénico, aumenta el componente estructural de la planta y crea una capa externa dura [98]. De este modo la mayoría de los beneficios reportados en la calidad del cultivo y el rendimiento después de la fertilización con silicio resultaron de la resistencia mecánica general mejorada producto de una capa externa de protección para la planta [83].

Por otro lado, para establecer una infección exitosa, los fitopatógenos deben tener acceso al tejido del huésped superando las barreras físicas conferidas principalmente por la cera, la cutícula y una

pared celular gruesa [99]. Por tanto, la primera hipótesis de cómo actúa el silicio, fue precisamente la formación de una barrera física, al encontrar en las plantas células bulliformes silicificadas en la epidermis de las hojas [83]. Dichas barreras físicas inhiben la penetración de patógenos y hacen que las células vegetales sean menos susceptibles a la degradación causada por la invasión de hongos patógenos [100,101]. El silicio se acumula y, cuando se deposita bajo la cutícula puede formar una doble capa cutícula unida a Si, que impide la penetración de patógenos, reduciendo así la incidencia de la enfermedad [102] (Figura 7).

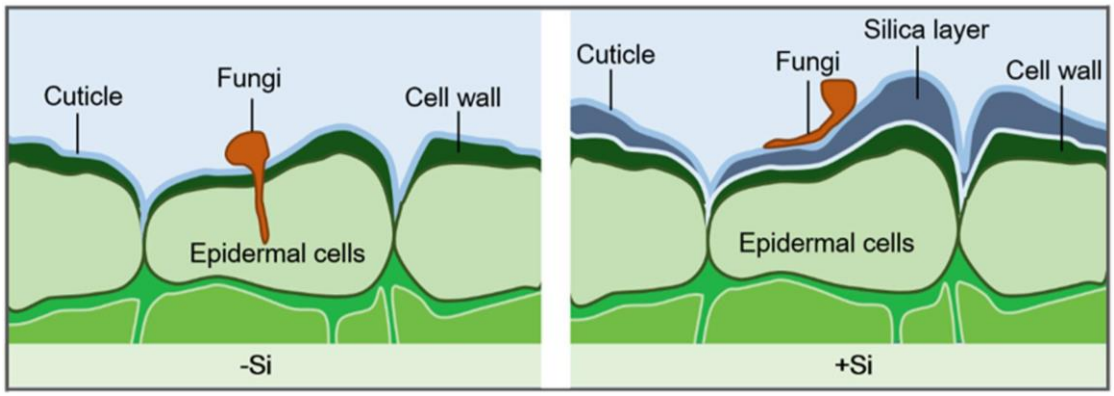

Figura 7. Formación de una capa de silicio en la pared celular de plantas tratadas con Si y mejoramiento de la resistencia de la planta a la infección por hongos mediante barreras físicas Wang, *et al.* [103].

En referencia al refuerzo estructural, esto ocurre una vez se absorbe el silicio, el cual se transporta a través del xilema depositándose en la epidermis de la hoja, en las cuales se condensa en un gel de sílice polimerizado duro que es conocido como fitolito. Estos fitolitos se encuentran en las células de sílice en los haces vasculares y en cuerpos de sílice en células bulliformes, fusiformes o pelos espinosos [83]. Si el silicio es suministrado a través de las raíces se moverá para localizarse en la endodermis, la membrana celular del haz vascular o células de la epidermis debajo de la cutícula. Mientras que si el modo de aplicación es foliar, la deposición es en la superficie de la hoja y por tanto es fácilmente removido por el agua de riego o lluvia [104].

Adicionalmente, se ha comprobado a través de diferentes investigaciones que la aplicación de silicio mejora la respuesta de defensa en plantas a través de diversos mecanismos bioquímicos. Se ha sugerido que las fitoalexinas y compuestos de tipo fenólico tienen un rol principal en plantas a las que se les ha aplicado silicio [105,106]. Igualmente, la aplicación de Si ha incrementado la

actividad de enzimas como quitinasa, β-glucanasa [107], peroxidasas, polifenoloxidasas [93,108] y la concentración de compuestos antimicrobianos como flavonoides [109].

Por otra parte, varios estudios han reportado la relación entre la suplementación de silicio y el mejoramiento del metabolismo antioxidante en plantas, cuando son infectadas por un patógeno. Una rápida producción de especies reactivas de oxígeno (ROS) se ha relacionado con fortalecimiento de la pared celular a través de la reticulación de glicoproteínas para retrasar la colonización del tejido del huésped [110]. Su disminución en cambio ha impedido daños por la peroxidación de lípidos [96,111], esta reducción se puede atribuir al aumento de enzimas que eliminan ROS como ascorbato peroxidasa, glutatión reductasa, superóxido dismutasa y catalasas [111-113].

De lo anterior, es posible colegir que más que aumentar o disminuir una actividad, lo que hace el silicio es una regulación del metabolismo de la planta. En relación a lo mencionado, se ha propuesto un modelo del mecanismo de acción del silicio cuando se aplica a las raíces. En él, es posible ver los dos mecanismos de acción del silicio mencionados antes. En el primero se observa cómo la presencia de cuerpos de sílice dentro de las células epidérmicas inhibe la penetración del hongo. Mientras que en el segundo hay una región sin cuerpos de sílice, por tanto, la defensa del huésped se ve potenciada por la presencia de silicio soluble en el lugar de la infección fúngica (Figura 8).

Existe también evidencia de señalización sistémica, varios estudios han sugerido que el Si puede regular las respuestas de la planta al estrés, modulando la homeostasis de las fitohormonas y las vías de señalización [114-116]. Las fitohormonas vegetales se acumulan en las plantas tratadas con Si en respuesta a la invasión de patógenos, las heridas o la herbivoría [117,118].

El Si induce genes relacionados a defensa (PRs) y transcritos pertenecientes a la vía dependiente del SA, lo cual va acompañado de un incremento de los niveles endógenos de SA y la subsiguiente expresión de PRs [119]. El silicio puede estimular también la expresión de un amplio espectro de respuestas de defensa inducibles y amplifica la respuesta de defensa inducida mediada por JA, sirviendo como un agente de cebado para la vía del JA. Por ejemplo, la inducción mejorada de enzimas y proteínas relacionadas con la defensa, así como la inducción mejorada de transcritos que codifican proteínas implicadas en la señalización de JA, mientras que el JA promueve la

silicificación general de la hoja y la maduración de las células de sílice portadoras de fitolitos mediante el aumento de la acumulación de Si [117,118].

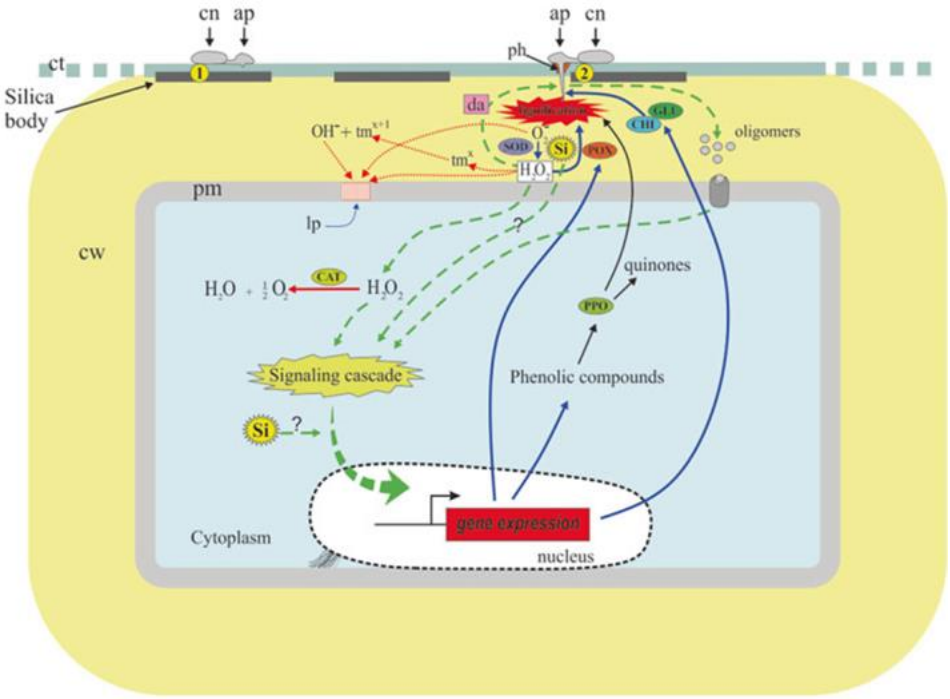

Figura 8. Modelo propuesto de la acción del silicato de potasio cuando se aplica a través de las raíces. Las líneas azules continuas indican la ruta estimulada; Las líneas verdes discontinuas indican la probable ruta estimulada; las líneas rojas continuas indican la ruta reprimida; las líneas rojas discontinuas indican la ruta probablemente reprimida y las líneas grises indican las rutas que no se ven directamente afectadas. Apresorio (ap), catalasa (CAT), quitinasa (CHI), conidio (cn), cutícula (ct), pared celular (cw), acción directa sobre el patógeno (da), β -1,3-glucanasas (GLU), peróxido de hidrógeno (H_2O_2), peroxidación lipídica (lp), anión superóxido (O_2^-), radical hidroxilo (OH^-), penetración de hifas (ph), membrana plasmática (pm), peroxidasas (POX), polifenoloxidasas (PPO), silicato potásico polimerizado (Psp), silicio soluble (Si), superóxido dismutasa (SOD) y metal de transición (tm) [83].

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OBJETIVOS

El **objetivo general** de esta tesis doctoral es evaluar el efecto de la inducción de resistencia con curlano y silicio sobre la respuesta de defensa en plantas de aguacate Hass inoculadas con *Phytophthora cinnamomi*.

Para alcanzar el objetivo general, se han considerado cuatro objetivos específicos:

El **primero** es evaluar la respuesta de sistemas enzimáticos y no enzimáticos asociados al estrés oxidativo y la ruta fenilpropanoide en plantas de aguacate Hass inducidas con curlano e inoculadas con *P. cinnamomi*.

El **segundo** objetivo es establecer la respuesta que genera la inducción de resistencia con curlano sobre el balance de hormonas asociadas a la defensa en plantas de aguacate Hass inoculadas con *P. cinnamomi*.

El **tercero** es comparar las variaciones metabólicas que se generan por la inducción de resistencia con curlano en plantas de aguacate Hass inoculadas con *P. cinnamomi*.

El **cuarto** es determinar la respuesta de sistemas enzimáticos asociados al fortalecimiento de la pared celular, así como no enzimáticos asociados al estrés oxidativo en plantas de aguacate Hass inducidas con silicio e inoculadas con *P. cinnamomi*.

METODOLOGÍA

1. Plantas, inducción de resistencia, inoculación del patógeno y conservación del material vegetal

Las plantas de aguacate Hass fueron obtenidas de un vivero de plantas registrado y con calidad fitosanitaria, localizado en el Líbano, Tolima, Colombia. Plantas jóvenes de seis meses de edad fueron empleadas para el análisis experimental. Estas fueron mantenidas con un fotoperiodo de 12 horas luz y 12 horas de oscuridad.

El curlano se adquirió en Sigma (Israel) y se disolvió en una solución de DMSO al 1% y NaOH 0,05mM a una concentración de 2658 mg/L. La solución se almacenó a 4°C y no se observó ninguna precipitación del compuesto. La aplicación del curlano se realizó según la metodología propuesta por Li, Zhu et al. (2014) con algunas modificaciones. Las plantas de aguacate Hass fueron tratadas de cuatro maneras diferentes, incluyendo 1) Plantas asperjadas con 5mL de la solución, lo que representa 26,58 mg de curlano, 1 día antes del tratamiento con *P. cinnamomi* (Curd+Phy), 2) Plantas rociadas con 5mL de la solución, lo que representa 26,58 mg de curlano, 1 día antes sin patógeno para establecer un control de elicitores (Curd), 3) Plantas con el patógeno (Phy), 4) Plantas control (Control). Un total de 120 plántulas de aguacate Hass fueron utilizadas al azar en cada repetición del experimento, 30 en cada grupo. La unidad experimental estaba compuesta por 2 plántulas y tres repeticiones.

El patógeno del suelo de plantas de aguacate Hass, *P. cinnamomi*, fue suministrado por la Corporación de Investigaciones Biológicas (CIB), localizada en Medellín (Colombia). El cultivo del patógeno fue mantenido en agar papa dextrosa (PDA por sus siglas en inglés) a 25°C. Se empleó un cultivo de 15 días como micelio activo.

Las plantas de aguacate Hass fueron inoculadas con *P. cinnamomi* según la metodología de Dinis*, et al.* [1], se realizó una incisión en forma de "T" en las plantas y se incorporó un disco de PDA con el oomiceto. Después de la inoculación, la incisión se cubrió con un trozo de algodón para asegurar un nivel de humedad adecuado para la infección y el desarrollo de la enfermedad. Todo

el ensayo se repitió en tres ocasiones, llevadas a cabo en noviembre de 2018, marzo y abril de 2019. Las hojas se recolectaron entre las 10:00 y las 13:00 horas a las 0, 3, 24, 144 y 312 horas después de la inoculación con el patógeno (hai). Un total de 120 plantas de aguacate Hass fueren usadas al azar en cada repetición del experimento.

El muestreo se realizó tomando las hojas enteras de dos plantas para hacer una muestra. Se estableció un total de tres muestras (de seis plantas) en cada interacción entre tiempo y tratamiento para tener en cuenta las variaciones biológicas. Las muestras se congelaron rápidamente con nitrógeno líquido (congelación de choque), luego se pulverizaron en las mismas condiciones y se almacenaron posteriormente a -80°C [2].

2. Medición de las actividades enzimáticas relacionadas a defensa, contenido fenólico total y actividad captadora de radicales libres.

El proceso de extracción de las enzimas fue llevado a cabo de acuerdo a la metodología propuesta por Sellamuthu, *et al.* [3]. Las muestras de hojas (800 mg) trituradas fueron homogenizadas con soluciones tampón específicas y centrifugadas a 15.000 rpm por 30 min a 4 °C y el sobrenadante fue usado para determinar las actividades enzimáticas. El buffer fosfato de sodio (100 mM, pH 7) fue usado para las enzimas SOD, APX, GR, POD and PPO. El buffer borato (100 mM, pH 8.8) que contenía 2 mM EDTA fue usado para la enzima PAL.

La actividad enzimática SOD se evaluó midiendo su capacidad de inhibir la reducción fotoquímica del azul nitro-tetrazolio (NBT) a 560nm [4]. La mezcla de reacción consistió en buffer fosfato 40mM (pH 7.8), metionina 13mM, NBT 75µM, EDTA·Na_2 0.66mM y riboflavina 0.0033mM. La riboflavina se añadió al final. Los tubos de ensayo que contenían la mezcla fueron agitados y luego dejados en reposo por 10 min bajo 300µmol $m^{-2}s$-1 de irradiancia a temperatura ambiente. La reacción sin enzima desarrolló el color máximo debido a la máxima reducción del NBT. Los tubos que contenían la enzima y se mantuvieron en oscuridad sirvieron como blanco. La actividad

enzimática se expresó como unidad mg^{-1} de proteína. Una unidad fue definida como la cantidad de enzima que causaba el 50% de inhibición de NBT.

La actividad APX fue ensayada de acuerdo a la metodología propuesta por Nakano and Asada [5]. La mezcla de reacción contenía buffer fosfato 50mM (pH 7.0), ascorbato 0.5mM, peróxido de hidrógeno 0.1mM y EDTA 0.1mM en un volumen total de 1mL. La reacción se inició añadiendo la enzima o el peróxido de hidrógeno y se registró la disminución de la absorbancia 30 seg después de esa adición. La actividad enzimática se midió a 290 nm y se calculó empleando el coeficiente de 2.8 mM^{-1} cm^{-1}. La actividad específica de la enzima se expresó como U mg^{-1} de proteína.

La actividad GR fue determinada según lo descrito por Smith, *et al.* [6] con algunas modificaciones. La mezcla de reacción estándar contenía buffer fosfato de potasio 0.2M (pH 7.5), EDTA 1mM, DTNB 3mM, NADPH 2mM y GSSG 20mM. La actividad específica de la enzima se expresó como U mg^{-1} de proteína.

La actividad PAL se analizó de la siguiente manera: se incubó 1.0 mL del homogenizado con 1.0mL de buffer borato 50 mM (pH 8.8) y 1.0 mL de L-fenilalanina 20 mM por 60 min a 37°C. La reacción fue detenida adicionando 0.1 mL of HCl 6.0 M. La actividad PAL se determinó con base a la producción de trans-cinamato, medida por el cambio de aborbancia a 290 nm. El blanco fue la preparación enzimática mezclada con L-fenilalanina con tiempo de incubación cero. Una unidad de actividad enzimática representa la cantidad de enzima que produce 1.0 μmol de ácido cinámico por hora [7].

La actividad de la POD se determinó según el método de [3]. 36 µL de enzima fueron adicionados en 144 µL de buffer fosfato de sodio (100 mM, pH 7 and 20 mM de guayacol) e incubados por 5 min at 30 °C. A continuación, se añadieron 72 µL of H_2O_2 (100 mM) y se midió el aumento de la absorbancia a 460 nm por 6 min (Thermo Scientific™ Multiskan™ GO Microplate Spectrophotometer). La actividad específica de la enzima se expresó como U mg^{-1} proteína.

La actividad de la enzima PPO se ensayó espectrofotométricamente según el método de Xie, Chai, Xu, Liu, Yang, Deng, Jin and He [7] con una ligera modificación. La mezcla de reacción consistió en 20 µL de homogenizado y 200 µL de solución de sustrato que contenía catecol 0.1 M como sustrato y 0.1 M de buffer fosfato de sodio (pH 7.4). La celda de referencia solo contenía el sustrato. La tasa de oxidación del catecol se monitorizó a 410 nm a 25°C por 1 min. La actividad de la PPO se presenta como el cambio en la densidad óptica a 410 nm. La actividad específica de la enzima se expresó como U mg^{-1}protein.

El contenido de proteína en cada extracto se estimó empleando el método de Bradford [8] con albúmina de suero bovino (Sigma, USA) como estándar.

Los compuestos fenólicos totales se extrajeron según el método de [9] con algunas modificaciones. Se tomaron 50 mg de material vegetal en un tubo con 750 µL de solución de agua acetona 70% y se pusieron en agitación durante dos horas a temperatura ambiente. A continuación, los tubos se sometieron a centrifugación a 3000 rpm por 10 min a 4°C. Se recolectó el sobrenadante y se mantuvo en frío, se suspendió una alícuota de 80 µL en 920 µL de metanol. Se empleó el método de Folin-Ciocalteu para la determinación de fenoles totales mediante el método propuesto por Cheplick, *et al.* [10] con algunas modificaciones. Se añadieron 150µL of agua, 20 µL de muestra y 10 µL de reactivo de Folin-Ciocalteu a cada pozo y se dejó reposar durante 6 min. Después se adicionaron 20 µL de solución de carbonato de sodio (7.5%) a cada pozo y se incubó a 22°C por 1 h en oscuridad y se midió la absorbancia a 760 nm (Thermo Scientific™ Multiskan™ GO Microplate Spectrophotometer). Se realizaron estándar empleando concentraciones crecientes de ácido gálico en etanol al 95%. Los valores de absorbancia se convirtieron a contenido fenólico soluble total y se expresaron como µg equivalentes de ácido gálico por gramo de peso fresco (FW) de material vegetal.

La actividad de captación de radicales libres de los extractos se determinó con base en el DPPH [11], con algunas modificaciones. Se añadió una alícuota de 50 µL de extracto (obtenido en la sección 2.8) en 100 µL de una solución diluida de DPPH en etanol (0.0236 mg/mL), se agitó y se

incubó por 30 min en la oscuridad, y se midió la absorbancia a 517 nm. La curva estándar se realizó con Trolox (5-80 µmol/L en etanol). Los resultados se expresaron en mM de Trolox/mg de extracto.

3. Análisis metabolómicos y cuantificación de compuestos fenólicos y fitohormonas

A 250 mg de hojas pulverizadas se les adicionó 5mL de hexano + 0.1% de butilhidroxitolueno (BHT). Se llevó la muestra a baño de ultrasonido por 30 min, posteriormente se centrifugó a 1400×g durante 10 min (Hettich Eppendorf Centrifuge MIKRO 22 R; Alemania). El sobrenadante se trasladó a un vial previamente tarado y se llevó a sequedad bajo nitrógeno. El residuo sólido se extrajo con 5 mL de metanol + 0.1% de ácido ascórbico. Se dejó en baño de ultrasonido por 30 min, posteriormente se centrifugó a 1400×g durante 10 min (Hettich Eppendorf Centrifuge MIKRO 22 R; Alemania). El sobrenadante se trasladó a un vial previamente tarado y se llevó a sequedad bajo nitrógeno.

Compuestos fenólicos enlazados

Hidrólisis básica de los ácidos fenólicos enlazados: El material sólido liofilizado obtenido de las extracciones anteriores se hidrolizó directamente siguiendo un método descrito previamente por Nardini*, et al.* [12] pero con algunas modificaciones Una parte del material restante (50 mg) se mezcló con 1,5 mL de NaOH 2 M que contenía 10 mM de EDTA y 1% de ácido ascórbico y se agitó (Eppendorf© Thermomixer Comfort, Hamburgo, Alemania) durante 30 min a 45 °C. La mezcla de reacción se acidificó (c.a. pH 3) añadiendo 0,285 mL de HCl 7,2 M y se centrifugó a 1400×g durante 5 min (Hettich Eppendorf Centrifuge MIKRO 22 R; Alemania) [13].

Análisis metabolómico por GC-MS y procesamiento de datos

Para la derivatización, se suspendieron 10 mg del extracto liofilizado en 300 µL de clorhidrato de metoxilamina (MEOX, Sigma-Aldrich) en piridina (20 mg/mL). La muestra se cerró con un tapón, se agitó con un vórtex hasta que el residuo se disolvió completamente y se colocó en un ThermoMixer para su incubación a 40 ºC. Después de una hora, se retiró la muestra y se añadieron

80 µl de N-metil-N-(trimetilsilil)trifluoroacetamida (MSTFA, Sigma-Aldrich). Finalmente, la muestra se agitó en vórtex y se incubó a 40ºC durante 1 hora [14].

Los análisis cuantitativos de las muestras se llevaron a cabo empleando un cromatógrafo de gases (Agilent GC7890N, Agilent Technologies España) acoplado a un espectrómetro de masas de impacto-simple cuadrupolo (5973N MSD, Agilent Technologies España). La columna analítica fue una DB-35 MS UI de Agilent con capilar de sílice fundida de 30 m×0,25 mm recubierta con una película de 0,25 µm equivalente a (35%-fenil)-metilpolisiloxano. La temperatura del horno comenzó a 80°C durante 2 minutos y luego aumentó a 310°C a 6°C/min durante 10 minutos. Se utilizó helio como gas acarreador a un flujo constante de 2 mL/min. Se empleó una inyección dividida con una proporción de 5:1. El volumen de inyección fue de 1 µL de la muestra derivatizada. El sistema de inyección se mantuvo a 250°C. La fuente de ionización del espectrómetro de masas fue un impacto electrónico (EI). El cuadrupolo único funciona en modo de barrido entre 35 y 700 amu, con un retardo de disolvente de 4 minutos. Los picos obtenidos se identificaron comparando su espectro de masas con la base de datos del National Institute of Standards and Technology 17 (NIST, EE.UU.) NIST MS Search 2.3 y WYLEY275.

Cuantificación de compuestos fenólicos por UPLC-MS

El extracto seco, proveniente de la extracción de compuestos fenólicos ligados, se disolvió en la fase móvil antes de los análisis, los cuales se llevaron a cabo empleando el equipo Waters Acquity™ Ultra Performance LC. Se inyectaron alícuotas (5 µl) en una columna ACQUITY UPLC HSS T3 de fase inversa (100 × 2,1 mm 1,8 µm) utilizando un sistema de gradiente compuesto por el disolvente A, agua:metanol (98:2 v/v)+1% de ácido fórmico y el disolvente B, metanol+1% de ácido fórmico 80:20 (v/v). El gradiente lineal fue el siguiente: 0-0.6 min, 80% A, 0.6-6 min, 30% A, 6.10-8.5 min, 100% B, y entre 8.55-10 min se restablecieron las condiciones iniciales. El flujo se mantuvo constante a 0,3 mL/min, la columna se mantuvo a 40°C. Se empleó el modo ESI negativo y los efluentes de la HPLC se introdujeron en un espectrómetro de masas de triple cuadrupolo (Xevo TQS). El control del instrumento y la adquisición y el procesamiento de los datos se realizaron con el software MassLynx™ (versión 4.1; Waters, EE.UU.).

Cuantificación de fitohormonas por UPLC-MS

El material fresco se congeló en nitrógeno líquido y se liofilizó. Antes de la extracción, se añadió una mezcla de estándares internos que contenía 100 ng de [$^2H_6$] JA y 100 ng de [$^2H_4$] SA. El tejido seco (0,05 g) se homogeneizó inmediatamente en 2,5 mL de agua ultrapura. Tras la centrifugación (5000 *g*, 40 min), se recuperó el sobrenadante y se ajustó a un pH de 2,8 con ácido acético al 6%, y posteriormente se extrajo dos veces en un volumen igual de éter dietílico. La fase acuosa se descartó y la fracción orgánica se evaporó al vacío a temperatura ambiente y el residuo sólido se resuspendió en 1 mL de una solución de agua/metanol (90:10) que se filtró a través de un filtro de acetato de celulosa de 0,22 µm. Una alícuota de 20 µl de esta solución se inyectó directamente en el sistema de HPLC. Los análisis se llevaron a cabo empleando el equipo Waters Acquity™ Ultra Performance LC. Se inyectaron alícuotas (20 µl) en una columna ACQUITY UPLC HSS T3 de fase inversa (100 × 2,1 mm 1,8 µm). Las fitohormonas se eluyeron con un gradiente de Acetonitrilo+0,1% de ácido fórmico y agua+Acetonitrilo+0,1% de ácido fórmico 10:90 (v/v) y se alcanzó linealmente 90:10 en 4 min. Se restablecieron las condiciones iniciales y se dejó que se equilibraran durante 1 minuto, lo que supuso un tiempo total de 5 minutos por muestra. El flujo de disolvente fue de 0,4 mL/min. Se empleó el modo ESI negativo, los efluentes del HPLC se introdujeron en un espectrómetro de masas de triple cuadrupolo (Xevo TQS) (Durgbanshi et al., 2005; Flors et al., 2008). El control del instrumento y la adquisición y el procesamiento de los datos se realizaron con el software MassLynx™ (versión 4.1; Waters, EE.UU.).

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CAPÍTULO 1

Oligosaccharides: defense inducers, their recognition in plants, commercial uses and perspectives

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El presente capítulo es una revisión de literatura en aspectos importantes para el desarrollo de la tesis. El tema principal es el uso de oligosacáridos como elicitores, debido al reciente interés que ha generado la inducción de resistencia en la agricultura, que busca controlar los fitopatógenos de una manera amigable con el ambiente y disminuyendo los daños sobre la salud.

Asimismo, hay información general sobre la respuesta de defensa y la inducción de resistencia, el concepto de elicitor, su función en la planta, así como sus ventajas y desventajas. Por otra parte, se hace énfasis en los oligosacáridos, por qué actúan como inductores de resistencia y cómo las plantas los reconocen y responde a ellos.

Finalmente, se mencionan los usos comerciales de los oligosacáridos como estrategia de gestión de enfermedades, buscando resaltar la importancia que tienen en la actualidad y mostrar también las perspectivas.

Oligosaccharides: defense inducers, their recognition in plants, commercial uses and perspectives

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Abstract: Plants have an innate immune system or defense mechanisms that respond to the attack of pathogenic microorganisms. Unlike mammals, they lack mobile defense cells, so defense processes depend on autonomous cellular events with a broad repertoire of recognition to detect pathogens that compensates for the lack of an adaptive immune system. These defense mechanisms remain inactive or latent until they are activated after exposure or contact with inducing agents, or after the application of the inductor, they remain inactive only until they are affected by a pathogen or challenged by an elicitor from the same. Resistance induction represents a focus of interest as it is an alternative that promotes the activation of plant defense mechanisms, reducing the use of chemical synthesis pesticides, an alternative that has even led to the generation of new commercial products with high efficiency, stability and lower environmental impact that increases productivity by reducing not only losses but also by increasing plant growth. Considering the above, the objective of this review is to address the issue of resistance induction with a focus on the potential of the use of oligosaccharides in agriculture, how they are recognized by plants, products of commercial use in topicality and perspectives.

Keywords: oligosaccharides, plant defense elicitor, resistance induction.

1. Introduction

The phenomenon of resistance induction as a strategy that seeks to improve the natural defenses of plants was described for the first time in 1901 in the work of Beauverie, "Essais d'immunisation des vegetaux contre les maladies cryptogamiques" [1], followed by several studies in the early 1900s, as mentioned by Alexandersson, *et al.* [2], and many more recently developed,

showing that it is an alternative strategy of interest [3-9]. However, in agriculture, its use is not common, as is genetic improvement where plant varieties have been domesticated based on fruit quality and yield, but this has resulted in reduced resistance to pathogens compared with wild varieties, so most crops are susceptible to numerous diseases [10]. In this sense, diseases continue to be responsible for considerable losses in crop production around the world [11], which is traditionally controlled by the use of chemical pesticides that today have been rejected by society for the damage it generates in crops, the environment and even on the health of farmers and consumers, thus generating an agriculture that seeks sustainable and environmentally friendly practices [10,12]. Therefore, a regulation has been generated in Europe, the (EC) 1107/209 complemented by directive 2009/128/EC, which forces members of the state to generate policies that contemplate plans based on an integrated management of the disease with less dependence on chemicals.

As a result of the aforementioned, resistance induction represents a focus of interest as it is an alternative that promotes the activation of plant defense mechanisms, reducing the use of chemical synthesis pesticides, [13], an alternative that has even led to the generation of new commercial products with high efficiency, stability and lower environmental impact [14] that increases productivity by reducing not only losses but also by increasing plant growth [15].

Considering the above, the objective of this review is to address the issue of resistance induction with a focus on the potential of the use of oligosaccharides in agriculture, how they are recognized by plants, products of commercial use in topicality and perspectives.

2. Plant defense responses and resistance induction

Plants have an innate immune system or defense mechanisms that respond to the attack of pathogenic microorganisms [16,17]. Unlike mammals, they lack mobile defense cells, so defense processes depend on autonomous cellular events with a broad repertoire of recognition to detect pathogens that compensates for the lack of an adaptive immune system [18]. These defense mechanisms remain inactive or latent until they are activated after exposure or contact with inducing agents [19], or after the application of the inductor. The defense mechanisms remain inactive only until they are affected by a pathogen or challenged by an elicitor from the same, in

which case a response has been observed, or faster, or stronger with respect to the plants that did not have contact with the inductor [20].

Among the varied defense responses that the plant has, there are changes in the ion flow that lead to depolarization of the plasma membrane, production of reactive oxygen species (ROS), nitric oxide (NO), activation of calcium-dependent protein kinases or mitogen-activated protein kinases (CDPKs and MAPKs) [13,21-23] and activation of the octadecanoid pathway, one of the best known defense mechanisms, whose end product, jasmonic acid (JA), induces the expression of several genes [24]. In fact, together, these signaling events modulate the expression of transcription factors (TFs), activities that lead to massive transcriptional reprogramming related to defense, which in turn results in the activation of various genes that modulate various antioxidant enzymes [25,26] and metabolites specifically related to stress. These include, for example, pathogenesis-related (PR) proteins that include β-1,3-glucanases and chitinases, compounds with antimicrobial activity such as phytoalexins, and deposition of callose and lignin for cell wall strengthening [10,27,28].

All of the aforementioned defense mechanisms are generated because the plant recognizes "nonnative" signals related microbe-associated molecular patterns or pathogen-associated molecular patterns (MAMPs/PAMPs), which are constituents of the cell wall of bacteria, such as flagellin, of fungi, chitin or fragments of pathogens oomycetes (*Phytophthora*) in the case of β-(1,3) - (1,6) -glucan. Many MAMPs have been described and can be (glyco) type proteins, carbohydrates or lipids. In addition to the signals that come from microorganisms, there are also those associated with the plant, from the host itself, the damage-associated molecular patterns (DAMPs), which are very structurally diverse and include, for example, pectin-derived oligogalacturonides. Once MAMPs/PAMPs are recognized by pattern-recognition receptors (PRRs), located in the plasma membrane or in the cytoplasm, a wide variety of responses related to MAMP/PAMP-triggered immunity (MTI/PTI) are triggered [13,18,29,30].

In addition to the innate immune system, plants can also acquire immunity against the perception of specific biotic and abiotic stimuli, a process mediated by the priming of inducible defenses, which causes plants to show an activation of various rapid or strong cellular defense

responses or both [20]. In this sense, two types of induced resistance have been identified and widely studied: systemic acquired resistance (SAR) and induced systemic resistance (ISR). The first is a state of defense induced by a local infection with pathogens or by the application of a chemical substance that confers resistance to a broad spectrum of pathogens and is dependent on salicylic acid (SA) [31] Therefore, when this process occurs, greater resistance is observed in the distal parts as a consequence of mobile signals in the plant belonging to the SA, which is biologically active in tissues far from the point of origin from where the response began [32], which is related to localized necrosis, can present as a hypersensitive response or local necrotic lesion caused by a virulent pathogen [33,34]. The induction of SAR requires the accumulation of endogenous salicylic acid signals, which mediate the activation of a wide set of pathogenesis-related (PR) genes. It has also been known that SA can have a dual role in signaling: activation of PR genes or low doses of SA that do not activate defense genes directly, can prepare the tissue for an enhanced expression of defense genes in a subsequent infection of the pathogen. In fact, in some cases, it has been found that there is no response to induction with SA until the plant is inoculated with the pathogen or its components [35].

Regarding induced systemic resistance (ISR), this appears when there is a beneficial interaction between plants and microorganisms, the latter helping plant nutrition but also overcoming biotic or abiotic stress in addition to improving defense capacity of the plant and effectively prevent a broad spectrum of pathogens [36]. This type of induced resistance is considered independent of SA and is not associated with major changes in the expression of defense genes, probably because this would lead to a strong investment in resources and reduction of host fitness, as has been shown in previous research [20,37,38]. In this sense, it is widely known that ISR is governed by jasmonic acid (JA) and ethylene (ET) [34], although it may not alter the production of JA or ET, as mentioned Pieterse, *et al.* [39], in which case the effect could be more related to improved sensitivity to these plant hormones.

So far, the defense mechanisms of plants and the types of resistance induction have been mentioned, but what inducers are has not been conceptualized. Plant resistance inducers (PRIs) of exogenous application are interesting because they can be incorporated into integrated disease management programs. These inducers are also called elicitors, although sometimes the terms are

confused. Elicitors, in a broad sense, are chemicals or biofactors from various sources that can induce physiological and morphological changes in the target organism. These include abiotic elicitors, such as metal ions and inorganic compounds, and biotic elicitors from fungi, bacteria, viruses (or fragments of these), components of the plant cell wall, as well as chemicals that are released at the site of attack by plants after the emergence of pathogens or herbivores [40]. In the same way, other authors define that elicitor or inducer is a molecule, or molecules present in an organism or produced by the plant itself and whose function includes the generation of defense responses [19]. β-1,3-glucan of *Septoria tritici* triggered wheat defense responses related to the production of β-1,3-glucanases and callose deposition to strengthen the cell wall [41] or the effect that the extract from *Phaseolus vulgaris* leaves had on the same plant, generating a strong defense response related to the production of reactive oxygen species [42]. However, it is important to note that in the field of crop protection, they offer many advantages because they do not attack the pathogen as such (they are not directly toxic to pathogens, which is the basis of pesticides, so they do not generate resistance); however, they are recognized by the receptors of the plant membrane and induce innate immunity [13]. They can potentially protect against multiple pathogens [43] mainly because they do not have a specific mechanism of action [14].

In general, it should be noted that the results of resistance induction will depend on the genotypes of both pathogens and hosts, which results in variable responses where the relationship between the level of resistance and the level of resistance induction is not clear, although in most cases, it has a direct relationship [44,45]. Likewise, an important issue regarding the induction of resistance is the influence of the environment and genotype on the plant's response capacity to defense inducers, which was shown by Bruce [46], where the attack of herbivores could promote the phenotypes of defense induction through generations and epigenetic change may be the basis of its lasting effect.

3. Oligosaccharides as defense inducers

Some molecules that are used as elicitors are therefore PAMPs/MAMPs that, as mentioned above, can be of different types, such as oligosaccharides (OGAs), on which research has focused in recent times, given that a large amount of these mediate the pathogenesis [47]. In this context, the knowledge of different glucans that are part of fungi, oomycetes or bacteria presents a new

perspective to not only analyze and understand plant-pathogen interactions but also to determine the potential of these as defense-inducing agents for the protection of plants.

In this sense, it has been shown that some oligosaccharides can act as elicitors and consequently activate the defense responses of plants. This has been studied in different plants, and as a reference for this review, studies that have used oligosaccharides, as well as the use of some chemical elicitors, are referenced (Table 1), all in order to show evidence that the responses shown by the plants with any of the elicitors are similar; therefore, it is possible to consider the oligosaccharides as elicitors that can be used in agriculture.

Regarding the origin, the oligosaccharides involved in plant-pathogen interactions are produced by enzymatic degradation of polysaccharides that are structural constituents of the cell wall of fungi or plants or pathogenicity factors of pathogens [48]. The activity of these compounds is dependent on the dose but also on the degree of polymerization (DP) [49,50] without consensus in this regard. In the case of tobacco cells, laminarin (MW: 504.4 g/mol), a storage β-glucan composed of glucose and mannitol (fig. 1. A), from brown algae, which has an average of 25- 33 DP and up to three β-glucose branches in position 6, induces defense responses slower than other β-1,3-glucans with low DP (2-10) [5]. Given that other studies showed contradiction with these results, as in the case of chitin (MW: 203.1925 g/mol), a linear polymer of N-acetylglucosamine (fig. 1. B), with higher activity reported for DP 7 -8 and low or none for DP <5 [51] or curdlan a linear polymer of glucose (fig. 1. C), which showed a marked effect with DP 5-7 with respect to those of DP3 [52], seeks to have greater clarity in this regard, finding that it is important to also take into account other factors such as the type of polymer used and the pathosystem (on which the activity of one molecule or another depends highly), that is, for lower OGA size, a better response of the plant is generated, while with chitosan, DP between 7-10 is usually more active [50].

Table 1. Inductors of chemical synthesis and oligosaccharides that generate resistance in different cultures against their respective pathogens

Inductor	Plant	Disease	Pathogen	Application	Response	Reference
Chemical Synthesis						
Acibenzolar-S-methyl (ASM)	Cape gooseberry	Vascular wilt	*Fusarium oxysporum*	Foliar	Greater accumulation of defense proteins and signal molecules in the root.	[53]
Acibenzolar-S-methyl (ASM)	Jimson weed *Nicotiana benthamiana*	Yellow spot	Iris yellow spot virus (IYSV)	Sprayed	Expression of PR proteins, fewer lesions and reduction in virus levels quantified by PCR.	[54]
B-aminobutyric acid (BABA)	Tomato	Gray mold disease	*Botrytis cinerea*	Application to the soil of the compound	Upregulation of defense-associated compounds such as phenylpropanoids.	[9]
Hexanoic acid	Orange	Canker	*Xanthomonas citri* subsp. *citri*	Sprayed and soaked soil	Reduction of lesions, improved expression of PR proteins and callus deposition.	[55]
Salicylic acid	Green soybeans	Virosis	Mungbean yellow mosaic India virus (MYMIV)	Sprayed	Stimulation of SOD and GPX enzymes. Activation of defense-related proteins, increased phenolic and H_2O_2 content.	[6]
Salicylic acid	Rice	Blight of rice	*Magnaporthe oryzae*	Sprayed	Activation of proteins involved in functions such as defense, antioxidant enzymes and signal transduction. Increase in the levels of reactive oxygen species.	[7]
Salicylic acid	Avocado	Root rot	*Phytophthora cinnamomi*	Submerged root	Production of the phenol-2,4-bis (1,1-dimethylethyl) compound, with antifungal activity and against *P. cinnamomi* .	[56]
Salicylic acid (SA) and Methyl jasmonate (MeJA)	Tomato	Wilt	*Fusarium oxysporum* f. sp. *lycopersici*	Daily spray for three days	Increased activity of antioxidant enzymes, decreased lipid peroxidation.	[8]
Riboflavin	Rice	Rice pod blight	*Rhizoctonia solani*	Sprayed	Priming of the expression of lipoxygenase, key in the octadecanoid pathway, upregulation of the PAL enzyme, and therefore, an improved lignification.	[57]
Silicon (Yes)	Paprika		*Phytophthora capsici*	Soil	It reduced the severity and improved the development of the plant.	[58]
Copper sulfate pentahydrate (Phy)	Cotton		*Fusarium oxysporum* sp. *Vasinfectum*	Foliar and seeds	Low severity index.	[59]
Oligosaccharides						
Oligosaccharides isolated from *Ulva lactuca*	Tomato	Wilt	*Fusarium oxysporum* f. sp. *lycopersici*	Internodal injection in the middle part of seedlings	Stimulation of PAL activity accompanied by increased phenolic compounds and the induction of salicylic acid.	[4]
Curdlan oligosaccharide (CurdO)	Potato	Late blight	*Phytophthora infestans*	Infiltration	Expression of defense-related proteins, reduction of leaf lesions, higher concentrations of salicylic acid, higher PAL, GLU and CTN activity and better yields.	[52]
Oligogalacturonides	Arabidopsis		*Pectobacterium carotovorum* sp. *Carotovorum* SCC1	Foliar	Activation of defense-related genes among which are groups associated with phytohormones, oxylipin biosynthesis, programmed cell death and other signaling.	[60]
Laminarin (β-1,3-glucan)	Tobacco	Bacteriosis	*Erwinia carotovora* subsp. *carotovora*	Addition of elicitor to cell culture	Accumulation of PR proteins, release of H_2 stimulation of PAL and LOX activity and accumulation of SA.	[61]
Chito-oligosaccharides (COS)	Blackberry	Biotic and abiotic stress		Foliar spray	Higher phenolic content and greater free radical trapping capacity (ABTS). As well as increased succinate dehydrogenase activity.	[62]
Curdlan-oligosaccharides (β-1,3-glucan (CRDO)) and Laminarin	Tobacco	Tobacco mosaic	Tobacco mosaic virus (TMV)	Addition of elicitor to cell culture	Production of nitric oxide, greater movement of stomata with CRDO and greater protection against the virus in the case of laminarin.	[5]
Tramesan	Durum wheat	Nodorum blotch	*Parastagonospora nodorum*	Sprayed	pro antioxidant molecule, induced the expression of oxidative stress defence-related genes.	[63]
Tramesan	Durum wheat	Septoria Leaf Blotch complex (SLBC)	*Parastagonospora nodorum* *Zymoseptoria tritici*	Sprayed	Increased the levels oj jasmonic acid and the early expression of plant defense genes.	[64]

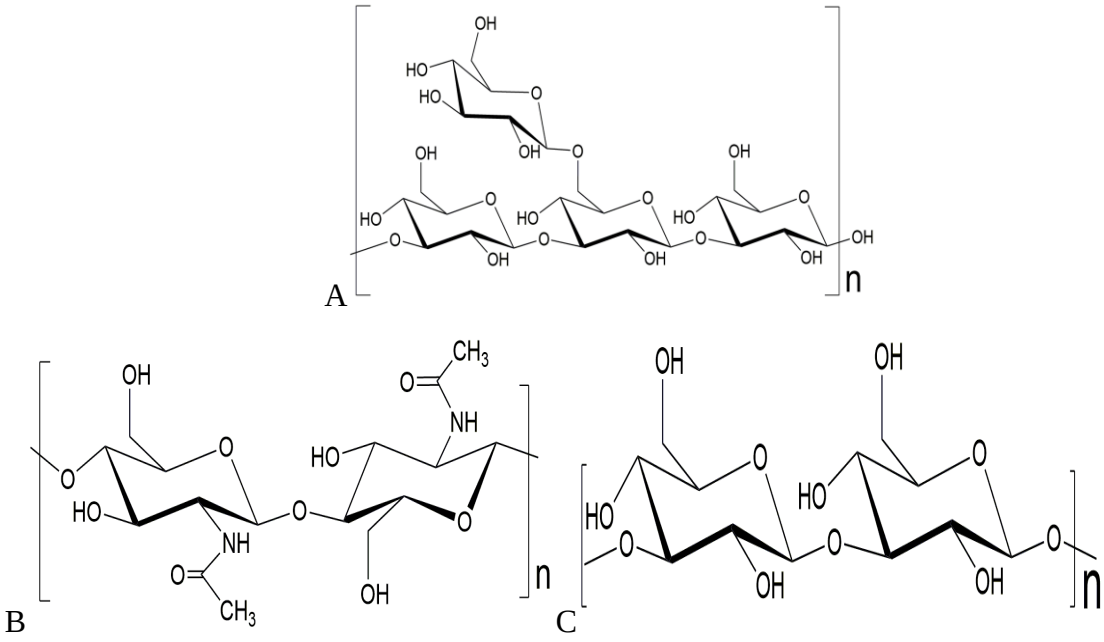

Fig. 1. Oligosaccharides used in resistance induction, (A) Laminarin, (B) Chitin, (C) Curdlan.

Regarding the aforementioned pathosystem towards which the process of induction of resistance with oligosaccharides is directed, it is precisely this that seems to determine the way in which the plant responds to structurally different β-glucans, a response that must have evolved in these plants and that makes them react against one or another molecule [50]. In the case of rice, the recognized β-glucans are branched [48], while tobacco reacts to linear β-1,3-glucans [5,61], and soybeans that were initially thought to only recognize branched oligosaccharides have actually been shown to also recognize DP 3 linearly [60,65,66].

In addition, acetylations, methylations and degrees of sulphation influence the biological activity of these biomolecules. For example, the CERK1 receptor, which is a chitin receptor [29] and triggers defense responses, recognizes partially deacetylated chitin but not fully deacetylated chitin [67]. Regarding the degree of methyl esterification of oligosaccharides modulated by the enzyme pectin methyl esterase (PME), this is crucial for the activity, that is, the overexpression of the enzyme reduces the degree of methyl esterification of OGA and with it, a reduction of symptoms caused by *Botrytis cinerea* in Arabidopsis and strawberries [68,69]. For sulfate groups, when they are substituents in biomolecules, generally implies greater biological functionality, these substituents modify the three-dimensional structure of the molecule, making it more resistant

to degradation by β-1,3-endoglucanases and exoglucanases [50]. Its ability to improve defense responses has been evaluated in vine plants [49] and tobacco [61] to cite some examples.

4. Recognition of polysaccharides in plants

Protein glycosylation is generally recognized as one of the major co- and post-translational modifications. This interaction between carbohydrates and their associated protein mediates important biological events. These proteins of nonimmune origin, which bind to specific carbohydrate structures, are classified as lectins. The location of these abundant proteins in the extracellular space or vacuoles suggests a role in plant defense [70]. They are found at a basal level but are inducible; that is, they are overregulated in response to biotic or abiotic stress [71].

In this sense, the first inducible line of defense common to plants and animals is MAMP-triggered immunity and PAMP-triggered immunity (MTI/PTI) [16]. MTI or PTI is based on the recognition of highly conserved structures for microorganisms that are not present in the host organism. In plants, these MAMPs/PAMPs are detected on the surface by pattern recognition receptors (PRRs) and as many carbohydrate structures, such as peptidoglycans (PGN), have been established as MAMPs/PAMPs [18,72,73] Thus, lectins seem to play an important role as PRRs. These PRR lectins are highly variable in structure and may be soluble but also associated with the membrane [70] (Fig. 2). It seems that they recognize long fragments of the PGN sugar skeleton, although the specific size that this fragment must have to trigger immunity has yet to be established [73], what has been evidenced is its effect on primers of defenses, for example, in tomato plants that acquired a greater resistance to the infection caused by *P. syringae* pv. *tomato* [74]. Regarding soluble receptors, it is important to clarify that these receptors recognize the presence of cytoplasmic effectors; these receptors are named for their conserved nucleotide-binding and leucine-rich repeats (NB-LRR) domains [75] (fig 2).

Many of the lectin-type receptors described so far in plants, belong to the lysine motif protein family (LysM) (Fig 2); among the first identified are the LysM domain protein (LYM 1) in Arabidopsis and AtLYM3, two PGN-binding proteins bound to glycosylphosphatidylinositol (GPI) and the chitin receptor (AtCERK1) that does not bind to PGN but is required for signal transduction in Arabidopsis [76,77]. In the same way, many other families have been identified,

such as Hevein (*Hevea brasilensis*), Jacalin (jackfruit) and from legumes that recognize chitin, mannose/galactose and mannose, respectively [70].

PRRs translate one or more extracellular lectin domains that are frequently coupled to an intracellular Ser/Thr kinase domain. Therefore, lectin receptor kinases (LecRK) can be classified into four types: G-, C-, L- and LysM. The G-type LecRKs have a lectin domain that resembles agglutinin, the C-type LecRKs are calcium-dependent, the L-type LecRKs (legumes) are the most numerous group of kinase receptors, and LysM is the most studied, one of its domains, contains a lysine that binds to several bacterial peptidoglycans and fungal chitin, as previously mentioned [78].

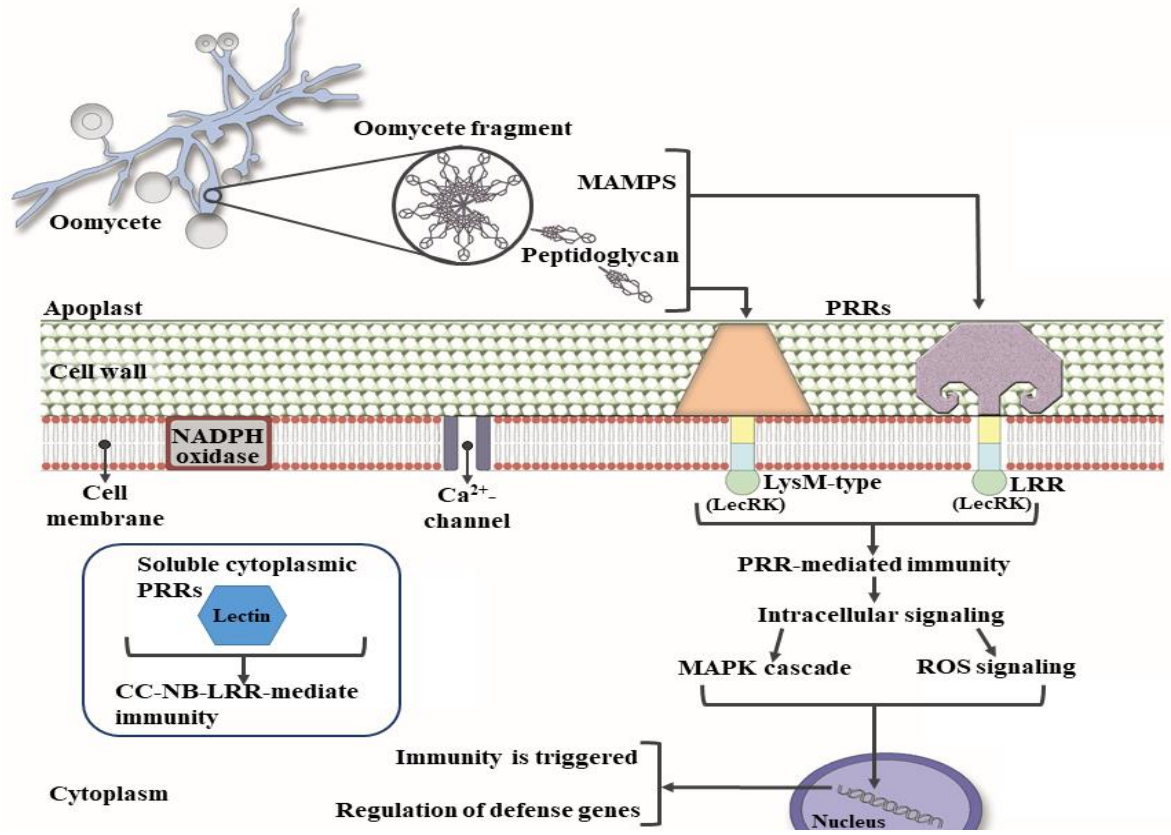

Fig. 2. Recognition of effectors in plants by PRR lectins. Once the plant perceives the peptidoglycans (MAMPs) through pattern recognition receptors (PRRs) with lectin domain (LecRK and lectin-type lectin-kinase receptor), an intracellular signaling cascade is generated downstream that leads to protein phosphorylation, generation of reactive oxygen species (ROS), regulation of phytohormones, activation of transcription factors due to what is known as microorganism-/pathogen-triggered immunity (M/PTI) or, when soluble receptors recognize effectors from the pathogen, effector-triggered immunity (ETI) is generated.

Similarly, for the specific case of oomycete pathogens, there are effectors (Avr avirulence factors) that activate R genes according to the gene-by-gene model; this effector is recognized by specific receptors (R proteins), a process that leads to a hypersensitive response (HR) [16]. To delve a little further, reference will be made to what has been identified for *Phytophthora infestans* because it is one of the most studied pathogens in reference to this recognition process. In this way, R genes typically encode immune receptor proteins called, supercoiled helix proteins of the coiled coil, nucleotide binding, leucine rich repeat (CC-NB-LRR) [79,80], these proteins have important biological functions, such as the regulation of gene expression. In this case, as a molecular trap, it detects effectors directly or by manipulation of target proteins performed by the effector that are monitored by the receptor as such [81,82]. In contrast, all the oomycete effector proteins known so far, contain an RXLR motif (Arg-Xaa-Leu-Arg) that is believed to mediate the delivery of the effector to the host cell, although the mechanism by which this occurs continues being a matter of discussion [83]. These effectors perform various functions, mainly promoting the virulence of the pathogen by suppressing plant immunity [75,84].

5. Oligosaccharides as a disease management strategy: Commercial use

Alternatives to commercial fungicides have emerged for some years. In 2014, a group of scientists with knowledge on the possible advantages of working with oligosaccharides as resistance inducers set out to create the company FytoFend SA, which works with two main approaches. The first is the optimization of the production of elicitors, and the other is the study of their mechanisms of action. Their efforts today can be seen in various products that are successfully marketed for crop protection, the first product FytoSave®, composed of a complex of oligochitosans and oligopectates as an active substance, which was tested in a growing crop against downy mildew of grapes and cucumber, finding that when applied by spraying in an interval between 7 - 14 days, it reduced the severity of the disease, which also had a persistence of the product and a cumulative effect of the same. On the other hand, it could be perfectly combined with traditional chemical management without problems and with some interesting advantages, such as not having a preharvest interval (for some chemicals, such as bitertanol applied in cucumber, it can be three days and three-week for the triadimenol), reducing application risks for the operator and the environment, increasing their market valuation and, most importantly, reducing the risk of increasing fungicide resistance [85].

In later developments, COS-OGA was tested against potato late blight caused by *Phytophthora infestans*, resulting in leaf spray increased resistance to the disease, which could be corroborated with the analysis of gene expression, where an increase of two pathogenesis-related proteins PR-1 and PR-2 was observed, the first, a protein known to be linked to partial resistance to *P. infestans* in species of the genus *Solanum* and the second, to encode a β-glucosidase with the ability to hydrolyze β-1,3-glucan, the largest component of the cell wall of oomycetes but also a protein that appears to be linked to the pathway of salicylic acid (SA), a compound required in the defense of plants [3]. In fact, in a previous research, the same authors reported that the induction of immunity in plants due to the COS-OGA elicitor is a cumulative process involving SA. The plant remains without modification of JA and ET but with upregulation of PR proteins, proteases and increased peroxidase activity, suggesting a mechanism of action of systemic acquired resistance (SAR) of the elicitor [86].

On the other hand, the Spanish Lida Plant Research SL sells what they have called "phytovaccines", among which is FytoSave® and Stemicol®, of the first some things have been mentioned previously, while of the second, it should be noted that it is a chito-oligosaccharide that has an effect on the reduction of fruits with rot, in the case of tomato, strawberries, grapes and benefits such as increased production of paprika, onion and potato [87]. In general, Lida defines phytovaccines as "substances capable of optimizing the expression potential of genes related to defense to the maximum", and they have a blog where they record different reports from many farmers who have had good results in the control of powdery mildew in zucchini, pepper and mildew in table grapes. Recently, an alliance between Lida Plant Research and FytoFend SA managed to make the FytoSave® product the first plant phytovaccine with phytosanitary registration admitted by the European Commission for use in organic agriculture in 2018, and its active component COS-OGA, is listed by the same commission as low risk [88], all of which allows us to see that the interest in oligosaccharides for resistance induction is a topic of interest that has a promising future since the current world is moving towards ecological agriculture that uses fewer chemicals in its production.

6. Perspectives

The induction of resistance is therefore an alternative that seeks to restrict or eliminate chemical synthesis pesticides, which currently requires a considerable investment per hectare with respect to the total production cost of each crop. In addition, it is clear that the trend of consumers today is inclined towards foods that are produced in a way that is sustainable with the environment, using fewer pesticides in a way that balances the economic, environmental and quality of life benefits not only for farmers but also for consumers [89]. This type of agriculture is therefore a differential factor in the market that is well valued in economic terms, so that a product that in its chain has required a lower use of pesticides will have a higher price, which ultimately translates into a more competitive producer, with a product with less traceability and better acceptance in international markets.

On the other hand, as mentioned, plants have various ways of recognizing a pathogen, structural changes caused by it or effectors of the pathogen that disturb or modify proteins of the plant. This information has become relevant and could also be a very useful tool in crop protection in two ways for the construction of new molecules or assays with B-glucans or other oligosaccharides from different sources that, when recognized by the plant, improve their defense responses, as well as the possibility to isolate and transfer receptors between species, paths that have a great potential to generate durable resistance and with good results.

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CAPÍTULO 2

Elicitor Activity of Curdlan and Its Potential Application in Protection of Hass Avocado Plants against *Phytophthora cinnamomi* Rands

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Este capítulo se centra en la evaluación de la respuesta de sistemas enzimáticos y no enzimáticos asociados al estrés oxidativo y la ruta fenilpropanoide en plantas de aguacate Hass inducidas con curlano e inoculadas con *P. cinnamomi*, así se aborda el primer objetivo planteado en la tesis.

La actividad antioxidante, medida a través de sistemas enzimáticos y no enzimáticos, es relevante en el metabolismo de la planta, debido a que estos son los mecanismos que emplea para, por un lado, eliminar los compuestos tóxicos constituidos por las especies reactivas de oxígeno, que se generan en un ambiente de estrés por la presencia de patógenos. Por otro lado, regular la concentración de las mismas para controlar la activación de otras rutas metabólicas.

De igual forma, en este estudio se midió el contenido de clorofilas y carotenoides con el fin de conocer el impacto sobre el proceso de fotosíntesis y determinar cambios en pigmentos protectores de la radiación. Además, se realizó la evaluación de las enzimas relacionadas con la ruta fenilpropanoide, a través de las cuales se generan compuestos con actividad antimicrobiana y para el reforzamiento de la pared vegetal, procesos fundamentales en la defensa de las plantas.

Elicitor Activity of Curdlan and Its Potential Application in Protection of Hass Avocado Plants against *Phytophthora cinnamomi* Rands

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Abstract: *Phytophthora cinnamomi* causes one of the most important diseases in avocado crop and its chemical management represents 25% of the production cost per year. Induction of plant defense responses by elicitors is a promising strategy that is compatible with sustainable agriculture. This study aimed to evaluate the effect of curdlan application on the induction of defense responses in avocado plants against *P. cinnamomi*. The trials were conducted under greenhouse conditions, and curdlan leaf spraying was performed one day before the inoculation of the pathogen. The results showed that the application of elicitor significantly increased the protection of avocado plants against *P. cinnamomi*, decreasing the injury and wilting. The Curd + Phy treatment improved the defenses of plants by increasing the enzymes peroxidase (POD) and polyphenol oxidase (PPO) in the first 3 h after inoculation and increasing the enzymes superoxide dismutase (SOD) and phenylalanine ammonium lyase (PAL) 144 h after inoculation ($p < 0.05$). Also, chlorophyll and carotenoid content increased or remained stable in Curd + Phy treatment. Therefore, these results suggest that curdlan increases the protection against *P. cinnamomi* and its protection could be due to an increase in the activity of the enzymes related to the phenylpropanoid pathway as well as the effect on chlorophyll and carotenoids.

Keywords: avocado; *Phytophthora cinnamomi*; induced resistance; curdlan; defense-related enzymes

1. Introduction

The avocado (*Persea americana*) is an important fruit appreciated for its taste and for its high nutritional value and bioactive compounds with health benefits, such as antioxidants, phenolic compounds, fiber, vitamins, and low sugar content [1,2]. Recently, it has been a highly profitable crop on the national and international market, which increased the areas for its cultivation worldwide [3]. The "Hass" variety is the most common worldwide [4]. It has been suggested that organoleptic characteristics and better post-harvest conservation of Hass avocado fruit has influenced its increased production worldwide [5]. However, there are phytosanitary problems caused by the oomycete *Phytophthora cinammomi* Rands, the main avocado Hass crop pathogen, causing losses of up to 90% [6-9]. *P. cinnamomi* is also the species with the widest host range within its genus and is considered an invasive species and one of the most destructive phytopathogens, known by some as the "biological bulldozer" [8,10].

The cost for chemical management of root rot disease caused by *Phytophthora* is important; the fungicide market represents over 25% of production costs per year. However, there are phylogenetical differences between *P. cinnamomi* and true fungi, implying an inefficient action of fungicides in general against this pathogen [11]; for that reason, many broad-spectrum fungicides are not effective against these organisms and few alternatives are available to manage this pathogen (e.g., metalaxyl, mefenoxam and fosetyl-Al). Nevertheless, the rapid regeneration times and exceptional adaptability of *Phytophthora* spp. have resulted in the development of fungicide resistance within specific pathogen populations [12].

On the other hand, new strategies are needed urgently to minimize the quantities of pesticides in the soil and their residues in food products. The stimulation of natural plants' defenses by the use of elicitor molecules is considered one of the most promising alternatives that can be included in integrated pest management (IPM) [13].

These important molecules offer an interesting possibility that activates the defense genes, leading to a systemic acquired resistance [12,13]. Microbe- or pathogen-associated molecular patterns (MAMPs or PAMPs) can induce plant innate immunity to protect plants from microbial pathogens [14-16]. The recognition of a pathogen, after eliciting the plant, generates an improved

defense response to prevent the development of the pathogen. One of the common responses is the accelerated generation of reactive oxygen species (ROS), which are second messengers in a variety of cellular processes in response to stress caused by pathogens. Their production regulates processes such as the upregulation of enzymes of the phenylpropanoid pathway that generate phenolic compounds with antioxidant activity that regulate ROS, production of antimicrobial compounds to attack the pathogen directly and cell wall strengthening to avoid the entry of the pathogen [17,18].

These elicitors include glycoproteins, carbohydrates (like β-1,3-glucan), chitin, fatty acids, proteins, glycosphingolipids and peptides [19-21]. Curdlan (Curd), a linear water-insoluble β-1,3-glucan, was first detected in *Alcaligenes faecalis* var. *myxogenes* and belongs to microbial exopolysaccharides and also is produced by fermentation of *Agrobacterium* sp.; it is also approved as a safe food additive [22,23]. Curd activates the defense responses in tobacco (*Nicotiana tabacum* L.) cells [23], and it also elicits production of another defense response, such as capsaicin in *Capsicum frutescens* [24]. More recently, it has exhibited an activation effect on the early- and late-defense responses in potato leaves [22].

This study aimed to investigate the role of curd as a defense inducer in Hass avocado plants against *P. cinnamomi* under greenhouse conditions. To determine the effect of the inducer, differences in defense-related enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR), phenylalanine ammonylase (PAL), peroxidase (POD), polyphenoloxidase (PPO), phenolic content and antioxidant activity were evaluated. The authors hope that this work can be of great value and can afford a theoretical basis for further studies on the possibility to include β-glucans in many integrated management programs in the avocado crop.

2. Materials and Methods

2.1. Plant Material

Hass avocado plants were obtained from a plant nursery that has a registered and phytosanitary status, located in Líbano, Tolima, Colombia. Young (six-month-old) plants were used for experimental analysis. The plants were kept in a greenhouse at ambient humidity and temperature

conditions in the facilities of the University of Tolima (4.428242, −75.213478) at 1285 masl. They were maintained with a photoperiod of 12 h light and 12 h of darkness.

2.2. Pathogen

The Hass avocado soil pathogen *P. cinnamomi* was supplied by Corporación de Investigaciones Biológicas (CIB), located in Medellín (Colombia). The culture was maintained on Potato Dextrose Agar (PDA) at 25 °C. Fifteen-day-old culture was used as an active mycelium.

2.3. Elicitor Preparation and Application

Curd was purchased from Sigma (Israel) and was dissolved in a solution of 1% DMSO and 0.05 mM NaOH at a concentration of 2658 mg/L. The solution was stored at 4 °C and no precipitation of the compound was observed. The application of the curd was made according to the methodology proposed by Li et al. [22][22], with some modifications. Hass avocado plants were treated in four different ways, including: (1) Plants sprayed with 5 mL of the solution, representing 13.29 mg of Curd 1 day before *P. cinnamomi* treatment (Curd + Phy); (2) Plants sprayed with 5 mL of the solution, representing 13.29 mg of Curd 1 day before without pathogen to establish an elicitor control (Curd); (3) Plants with the pathogen (Phy) and (4) Control plants (Control). A total of 120 Hass avocado plants were randomly used in each repetition of the experiment, 30 in each group. The experimental unit was composed of 2 plants, and 3 replicates were assayed.

Hass avocado plants were inoculated with *P. cinnamomi* according to the methodology of Dinis, et al. [25]. A "T" form incision was made in the plants, and a PDA plug with oomycete was incorporated. Inoculation was done directly in the stem. After inoculation, the incision was covered with a piece of cotton wool to ensure an adequate moisture level for infection and disease development. The whole assay was repeated three times, carried out in November 2018, March, and April 2019, and the leaves were collected between 10:00 and 13:00 at 0, 3, 24, 144 and 312 h after inoculation (hai).

2.4. Observation of Lesions Caused by Phytophthora Cinnamomi on Hass Avocado Plants under Greenhouse Conditions

To observe the development of lesions, the inoculated Hass avocado plant were inspected every three days for three times. The lesion was measured with a vernier caliper to see its progress. This process was done until day nine after inoculation.

2.5. Sample Preparation

The harvest was made by taking the whole leaves of two plants to make one sample. A total of three samples (from six plants) were established in each interaction between time and treatment to account for biological variations. Samples were quickly frozen with liquid nitrogen (shock freezing), then powdered under the same condition and subsequently stored at −80 °C [26].

2.6. Measurements of Active Defense Response-Related Enzymes, Antioxidant Enzymes Activities and Total Phenolic Content in Avocado Fruit

The enzyme extraction process was carried out as proposed by Sellamuthu, et al. [27]. SOD activity was assessed according to the methodology proposed by Beauchamp and Fridovich [28], with the modifications made by Demiral and Türkan [29]. APX activity was assayed according to Nakano and Asada [30]. POD activity was determined according to the Sellamuthu, et al. [27] method.

GR activity was determined as described by Smith, et al. [31], with some modifications. The standard reaction mixture contained 0.2 M potassium phosphate (pH 7.5), containing 1 mM EDTA, 3 mM DTNB, 2 mM NADPH and 20 mM GSSG. The specific activity of the enzyme was expressed as U mg^{-1} protein.

PAL activity was assayed as follows: 1.0 mL homogenate was incubated with 1.0 mL of 50 mM borate buffer (pH 8.8) and 1.0 mL of 20 mM L-phenylalanine for 60 min at 37 °C. The reaction was terminated by the addition of 0.1 mL of 6.0 M HCl. PAL activity was determined based on the production of trans-cinnamate, measured by the absorbance change at 290 nm. The blank was the crude enzyme preparation mixed with L-phenylalanine with zero-time incubation. One unit of enzyme activity represents the amount of enzyme that produces 1.0 μmol of cinnamic acid per hour [32].

PPO activity was assayed spectrophotometrically according to the method of Xie, et al. [32] with a slight modification. The reaction mixture consisted of 20 µL homogenate and 200 µL substrate solution, containing 0.1 M catechol as the substrate and 0.1 M sodium phosphate buffer (pH 7.4). The reference cuvette contained only the substrate. The rate of oxidation of catechol was monitored at 410 nm at 25 °C for 1 min.

2.7. Protein Assay

The protein content in the extract was estimated using the protein-dye binding method of Bradford [33] with Bovine Serum Albumin (Sigma, St. Louis, MO, USA) as a standard.

2.8. Total Phenolic Content (TPC)

Total phenolic compounds were extracted according to the method of Makkar [34], with some modifications. A total of 50 mg of plant material was taken in a tube with 750 µL of 70% aqueous acetone and put on a shaker for two hours at room temperature. Then, the tubes were subjected to centrifugation at 3000 rpm for 10 min at 4 °C. The supernatant was collected and kept cool, and an aliquot of 80 µL was suspended in 920 µL of methanol. The Folin–Ciocalteu method was used for the determination of total phenols by some modification of the method of Cheplick, et al. [14]. A total of 150 µL of water, 20 µL of sample and 10 µL of Folin–Ciocalteu reagent were added to the well and allowed to stand for 6 min. Afterward, 20 µL of sodium carbonate (7.5%) solution was added to each well and incubated at 22 °C for 1 h in the dark, and the absorbance was measured at 760 nm (Thermo Scientific™ Multiskan™ GO Microplate Spectrophotometer, Waltham, MA, USA). Standard curves were made using increasing concentrations of gallic acid in 95% ethanol. Absorbance values were converted to total soluble phenolic content and expressed as µg equivalents of gallic acid per gram fresh weight (FW) of shoot sample.

2.9. DPPH Free Radical Scavenging Activity

The activity of elimination of the free radicals of the extracts was determined based on the DPPH [35], with some modification. A 50 µL aliquot of the extract (obtained in Section 2.8) was added into 100 µL of a diluted DPPH solution in ethanol (0.0236 mg/mL), stirred, and incubated for 30 min in the dark, and the absorbance was measured at 517 nm. The standard curve was

obtained with Trolox (5–80 µmol/L in ethanol). The results were expressed in mM Trolox/mg extract.

2.10. Determination of Chlorophyll and Carotenoid Content

Chlorophyll a (Chl a), chlorophyll b (Chl b) and total carotenoids were determined spectrophotometrically [36] and the results were calculated using the equations described in [37].

2.11. Statistical Analysis

Data from at least three replicates consisting of a pool of six plants were analyzed. All statistical analyses were performed with R software. All data underwent a one-way ANOVA, and differences between treatments and control data were assessed using the Tukey post-hoc test at a significance level of 0.05.

3. Results

3.1. Observation of Lesions Caused by Phytophthora Cinnamomi on Hass Avocado Plants under Greenhouse Conditions

Minimum length of the developed lesion was observed when the plants were sprayed with curdlan one day before the inoculation of *P.cinnamomi*, unlike in the plants in which only the pathogen was inoculated (Table 1). The plants treated with Curd were found to have dark brown lesions significantly smaller than those in untreated plants, and no sign of wilt was observed until the end of the trial (Curd + Phy). Otherwise, it was also possible to observe a wilting in the leaves and branches of the plants inoculated with *P. cinnamomi* but without curdlan spray (Phy).

Table 1. Lesion length caused by *P. cinnamomi* on avocado plants sprayed with curdlan and on untreated plants inoculated with the pathogen.

Treatment	Average Length of the Lesion (cm)		
	3 dai	6 dai	9 dai
Curd + Phy	2.32 ± 0.32 a	3.39 ± 0.34 a	3.90 ± 0.24 a
Phy	3.56 ± 0.24 b	4.53 ± 0.28 b	5.69 ± 0.31 b

Curd + Phy: Plants treated with the elicitor and inoculated with the pathogen. Phy: plants inoculated with the pathogen. Values are the mean ± standard deviation (n = 9). Means with same letters within the same column are not significantly different ($p < 0.05$).

3.2. Effect of Curdlan on the Activities of Disease-Resistant Enzymes

In plants, SOD catalyzes the dismutation of the superoxide radical into oxygen and hydrogen peroxide, which is closely related to disease resistance because of its antioxidant activity. The activity of SOD showed an increase in the plants inoculated with *P. cinnamomi84*. The enzyme in Phy was significantly higher during the early stages of inoculation. After 24 h after inoculation (hai), the enzyme activity of Curd + Phy increased (Figure 1). The first peak in SOD activity occurred at 3 hai for Phy and 312 hai for Curd + Phy, which is statistically significant concerning Curd and Control groups ($p < 0.05$). Therefore, the curdlan treatment (Curd + Phy) appears to increase SOD activity at certain stages following inoculation with *P. cinnamomi*.

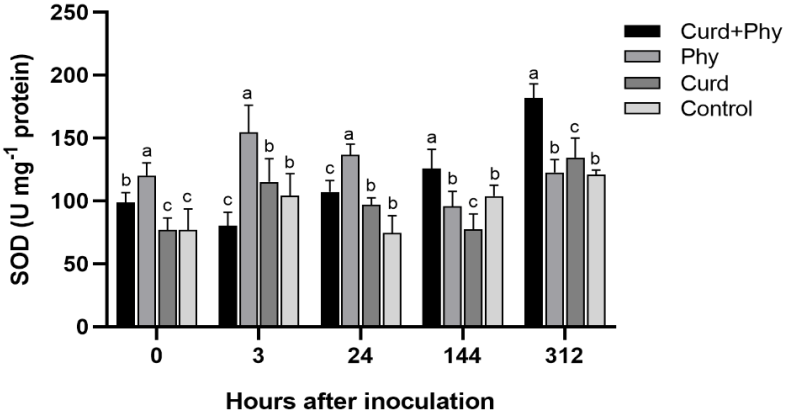

Figure 1. Effect of curdlan treatment on SOD activity from avocado plant leaves inoculated with *P. cinnamomi*. Curd+ Phy, Phy, Curd and Control represent the application of Curd one day before inoculation of *P. cinnamomi*, inoculation of *P. cinnamomi*, application of Curd without inoculation of *P. cinnamomi* and the control (distilled water), respectively. Data are presented as the mean ± standard deviation for nine replicates. Means with the same letter are not significantly different (ANOVA followed by Tukey's test at $p < 0.05$).

APX and GR enzymes were not detected. All relevant modifications were made to detect the activity but could not be measured.

PAL enzyme is important in the first step of phenylpropanoid pathway for the production of some metabolites including lignin (as part of cell wall strengthening). PAL activity was significantly higher ($p < 0.05$) in the pathogen at the early stage, until 24 hai. After 3 hai, Curd treatment (Curd + Phy) was slowly increased, and the enzyme activity peaked at 144 hai ($p < 0.05$) (Figure 2a).

POD is an enzyme known to be involved in increasing defenses against pathogens, because it catalyzes the final lignin synthesis. The most significant increase in POD activity was achieved by Curd + Phy at 3 hai with respect to control plants (Figure 2b), whereas the Curd control had a significant increase at 24 and 144 hai ($p < 0.05$). Meanwhile, at other times, the trend was similar among all treatments. However, at the last time, 312 hai, the activity of POD was the same between Curd + Phy and between Curd and Control. After the inoculation with *P. cinnamomi*, plants treated with curdlan increased POD activity at the early stage.

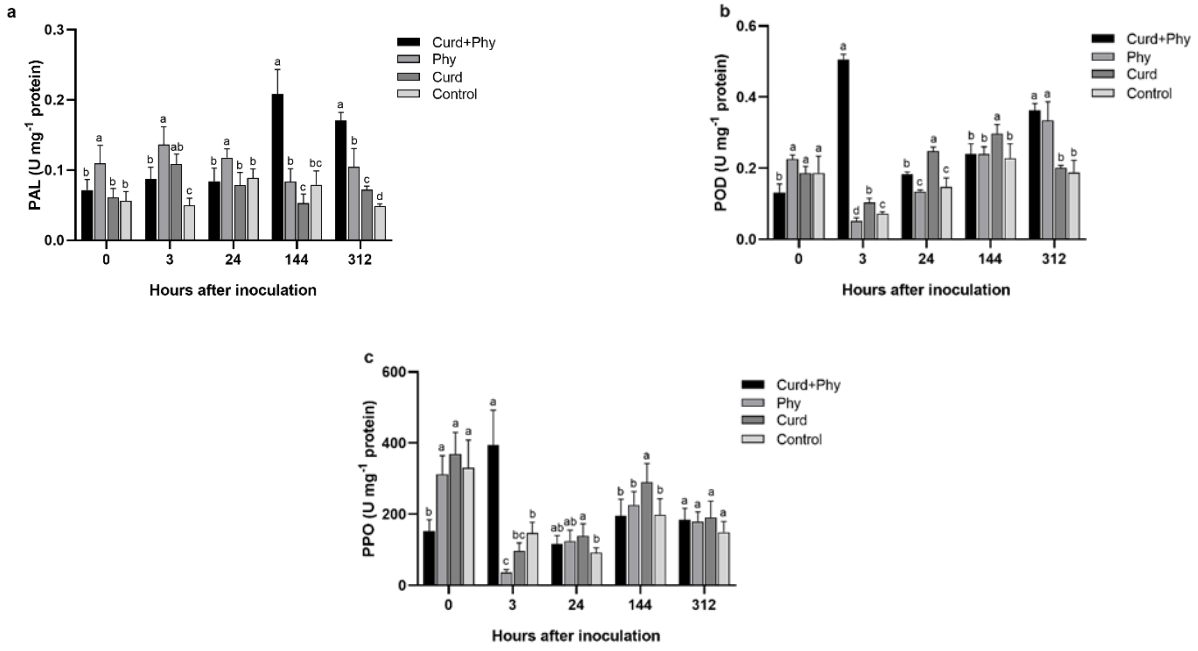

Figure 2. Effect of curdlan treatment on (**a**) PAL activity, (**b**) POD activity and (**c**) PPO activity from avocado plant leaves inoculated with *P. cinnamomi*. Curd+ Phy, Phy, Curd and Control represent application of curdlan one day before inoculation of *P. cinnamomi*, inoculation of *P. cinnamomi*, application of Curd without inoculation of *P. cinnamomi* and the control (distilled water), respectively.

Data are presented as the mean ± standard deviation for nine replicates. Means with the same letter are not significantly different (ANOVA followed by Tukey's test at $p < 0.05$).

On the other hand, the enzyme PPO has been implicated in plant defense. The oxidation of polyphenols into quinones could have an antimicrobial effect and help avoid the spread of pathogens. In the initial stage, the PPO activity was low in Curd + Phy. After 3 hai, there was a significant increase in PPO activity in the same treatment to enhance toxicity ($p < 0.05$) (Figure 2c), with a maximum value at 3 hai. After that, the enzyme activity decreased. Moreover, the activity for the pathogen treatment was similar to the control group without any peak in the hours evaluated. Thus, curdlan applied before the inoculation with *P. cinnamomi* increased the activity of PPO, providing an increase in activity at the early stage.

3.3. Effects of Curdlan Treatment on Total Phenol Content and Antioxidant Activity

Plants inoculated with *P. cinnamomi* significantly increased the total phenolic content ($p < 0.05$). The content in Phy accumulated rapidly during the early stages (3 hai). Other treatments did not cause many changes in the hours after inoculation (Figure 3a).

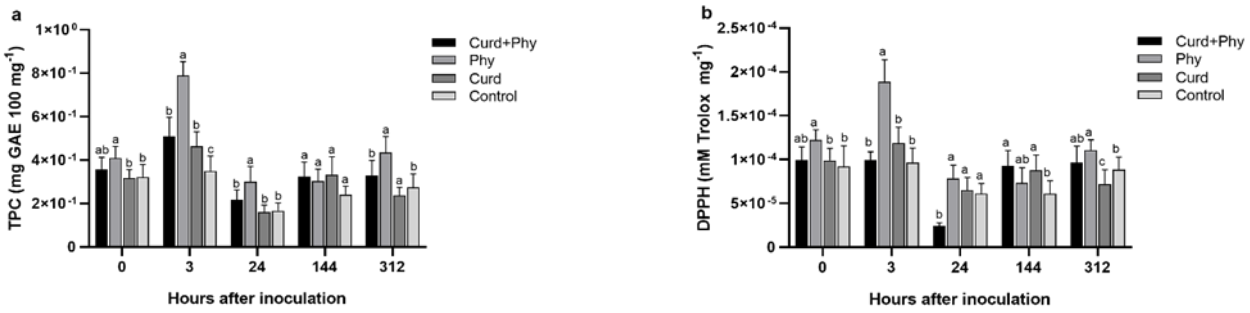

Figure 3. Effect of curdlan treatment on (**a**) TPC and (**b**) DPPH activity from avocado plant leaves inoculated with *P. cinnamomi*. Curd+ Phy, Phy, Curd and Control represent application of Curd one day before inoculation of *P. cinnamomi*, inoculation of *P. cinnamomi*, application of Curd without inoculation of *P. cinnamomi* and the control (distilled water), respectively. Data are presented as the mean ± standard deviation for nine replicates. Means with the same letter are not significantly different (ANOVA followed by Tukey's test at $p < 0.05$).

The antioxidant scavenging activity was found to be higher in plants inoculated with the pathogen (Phy) ($p < 0.05$). In general, the activity showed a similar trend to total phenolic compounds, so the treatment is similar except for Curd + Phy at 24 hai; at this time the activity is lower than that of other treatments (Figure 3b).

3.4. Effect of Curdlan on Chlorophyll and Carotenoid Content

A significant decrease in Chl a, Chl b and in carotenoid content was observed in the *P. cinnamomi*-infected plants at 3 hai. Treatment with Curd prior to oomycete inoculation elevated chlorophyll and carotenoid contents at 3 hai. Before and after 3 hai, there were no statistically significant differences between the plants treated with the elicitor and those that were not (Figure 4a–c).

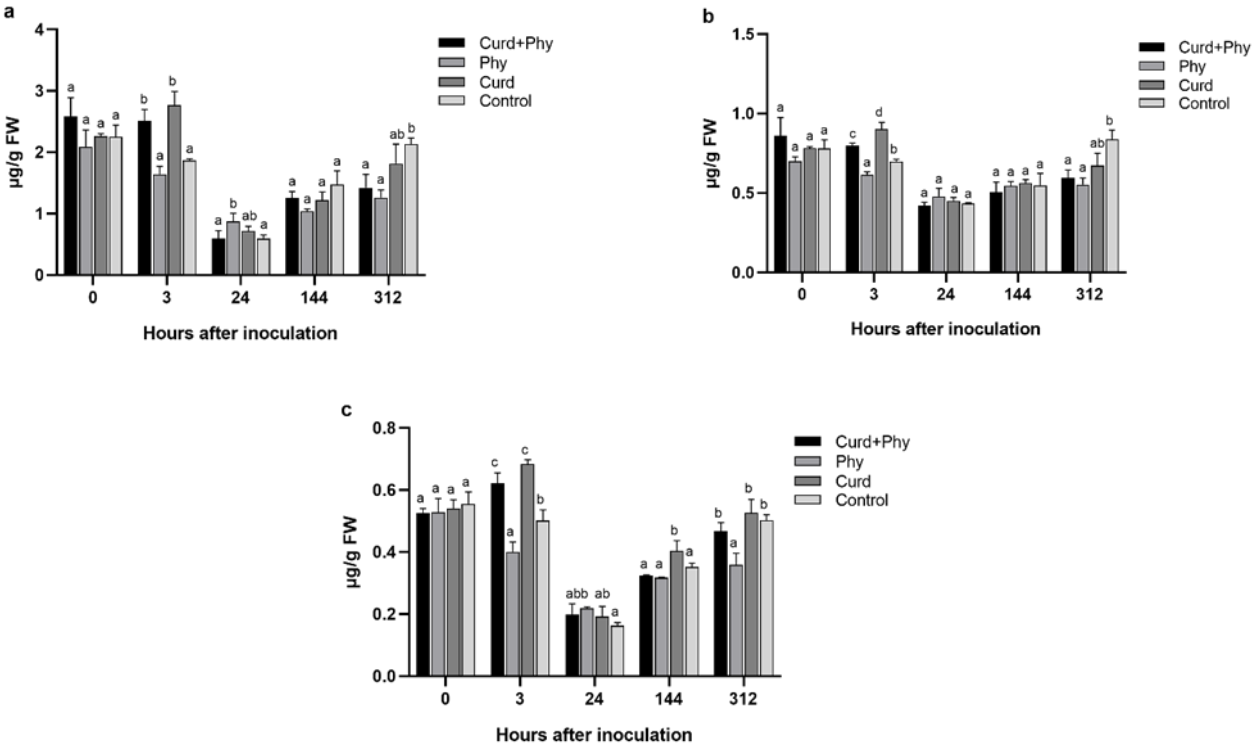

Figure 4. Effect of Curd treatment on (**a**) chlorophyll a, (**b**) chlorophyll b and (**c**) carotenoids from avocado plant leaves inoculated with *P. cinnamomi*. Curd+ Phy, Phy, Curd, and Control represent the application of Curd one day before inoculation of *P. cinnamomi*, inoculation of *P. cinnamomi*, application of Curd without inoculation of *P. cinnamomi* and the control (distilled water), respectively. Data are presented as the mean ± standard deviation for nine replicates. Means with the same letter are not significantly different (ANOVA followed by Tukey's test at $p < 0.05$).

4. Discussion

Modern agriculture seeks to be environmentally friendly; therefore, resistance induction is an effective strategy that could be included in integrated disease management plans. As curdlan is an oligosaccharide used as a food additive and is not a potential environmental pollutant, we suggest it could be used in organic agriculture.

The induction of SOD in plant cells in response to different stressful environments reflects its important role in the defense mechanism of plants [38]. In the present study, the SOD enzyme was increased in plants inoculated with the pathogen (Phy) at early stages but decreased in the later stages, whereas plants inoculated with a previous application of Curd (Curd + Phy) had a sustained increase, maybe associated with increased resistance to the pathogen. In the same way, many authors have associated increased production of the SOD enzyme with resistance. For melon against *Colletotrichum lagenarium*, the resistant cultivar has a higher SOD activity than the susceptible cultivar [39]. Likewise, a study conducted with four cotton genotypes showed, after 90 days of inoculation of the pathogen, that resistant genotypes have a high superoxide dismutase activity compared to susceptible ones [40]. Furthermore, an investigation with salicylic acid as a resistance inducer in beans showed that the SOD enzyme increases in response to that induction, which was associated with a reduction in the susceptibility of this plant [41].

The enzymes APX and GR detoxify the hydrogen peroxide generated by the SOD enzyme. Measuring their activity would have been useful to know how much peroxide was transformed and how much was not. However, it was not possible to measure its activity. In this case, we could suppose that the avocado defense response under the treatment conditions is not associated with an increase of this enzymatic pathways, at least under the detection threshold of the applied techniques. All possible modifications suggested by Bisswanger [42] were performed, but the activity could not be measured. However, in previous works carried out in the research group, these two enzymatic activities have been measured in avocado, rice and tomato leaves (unpublished data), without problems.

Phenylpropanoid metabolism pathway in plants generates phenolic compounds, which have antimicrobial, anti-oxidative, and free radical elimination activity. They also act as phytoalexins,

modulators of pathogenicity-signaling molecules [43] and are markers for resistance to pathogens [44] as well as the enzymes involved (PAL, POD and PPO). Consequently, the induction of resistance has been also measured with other defense-related enzymes that are activated when a pathogen is inoculated. PAL was expressed in the treatment with the pathogen (Phy) until 24 hai; after that, it is possible to see an effect of the application of curdlan (Curd + Phy) at 144 and 312 hai. This is an interesting result because many authors report the relation among a high production of PAL enzyme both in resistant genotypes [39] and plants with prior application of an inductor [13,22,45-47].

In this report, the treatment with Curd before the inoculation of *P. cinnamomi* increased PPO and POD enzymes with very similar behavior, and the highest activity of the enzyme was observed at 3 hai. These enzymes, PPO and POD, are involved in the production of quinones, H_2O_2 removal, oxidation and lignification [48]. Some studies show that the POD enzyme is associated with resistance to a specific pathogen [49,50]. The increase in POD activity indicates that it has a key role in local and systemic resistance; hence, an increase might be related to lignin biosynthesis, which prevents the entry of pathogenic [51] cells such as *P. cinnamomic*. It does so through mechanical pressure. The use of elicitors has also been shown to increase POD activity, as the study conducted with amino acids from milk to enhance resistance against *Sclerospora graminicola* [47] showed. Another research study showed that another way of using oligosaccharides is the application of algino-oligosaccharides as elicitors in rice plants against *Magnaporthe grisea* [45].

The PPO activity increased in the treatment with curdlan. Previously, studies have shown the relationship between increased PPO enzyme and resistance to disease [40,52]. The use of elicitors has also been shown to increase PPO activity, as the study conducted with exogenous caffeic acid and epicatechin to enhance resistance against *Botrytis cinerea* in apples [53] showed. Interestingly, another study using fungi as resistance inducers in tomato plants found an increase in the resistance-related enzyme PPO and showed that these fungi, which are known to have the ability to produce β-glucan, can be used as inducers [54]. Furthermore, a specific study using β-glucan, Salecan, triggered the defense response of *Arabidopsis thaliana* Colo against *Botrytis cinerea* infection, related to an increase in different enzyme activities including PPO [55].

Phenolic compounds are a plant defense against pathogens either because there is an oxidation of them when there is a production of reactive oxygen species or because they have an antimicrobial function. In addition, they are precursors of structural polymers or serve as signaling molecules. Thus, when there is a decrease in phenols in plant–pathogen interaction, it is attributed to their oxidation [43]. The present study indicated that Curd treatment did not affect phenol production or antioxidant activity, contrary to the treatment that had only the inoculation of the pathogen. Similar results have been found in tomato against the pathogen *Oidium neolycopersici*, in which no increase in phenolic content was evident in the medium-resistant tomato variety. However, the resistant variety did increase phenolic content, but it was much higher in the susceptible variety. The authors concluded that this phenomenon is difficult to explain and could be related to the enzymatic transformation of phenols by peroxidase [56] or polyphenol oxidase (PPO) enzymes, which means that they are related to other defense mechanisms. It is also important to note that the total phenolic content only reflects some phenols that are quantified, those that the extraction methodology allows; many may not be observed because they are bound, as found in a study of fibers [57], or because they are generated in low quantities in the plant tissue and therefore are not detectable.

In plants, chlorophyll content is an essential factor in determining the growth capacity. Under normal physiological conditions, the major part of light absorbed by the photosynthetic pigments was used for photosynthetic quantum conversion. Thus, photosynthetic pigment content directly influenced the light absorption, transmission, distribution between the PSI and PSII, and energy conversion [58]. The curdlan foliar sprays noticeably enhanced the chlorophyll (a and b) accumulation under biotic stress at 3 hai in comparison to the plants that were only inoculated with the pathogen (Phy). However, in the other measurement times, there were no statistically significant differences, although at 312 hai, it is observed that the plants to which only curdlan was applied have a chlorophyll content similar to the control plants.

It was noticed that Curd showed significant superior carotenoids accumulation at 3 hai in the treatment Curd + Phy over the Phy. The possible reason is that carotenoids possess dual functions, such as the harvesting of light pigment and scavenging of free oxygen radicals at abnormal irradiance levels [59]. It is important to consider that the pathogen, when entering, covers the

vascular beams and induces not only a high production of reactive oxygen species but also damage in the transport of water and nutrients, which in turn affects the process of photosynthesis.

The results found in this research correspond with those reported in the literature: when a foliar elicitor of oligosaccharide type has been applied to different stresses, the contents of chlorophyll and carotenoids have increased or remained stable [58,60-63].

The reactive oxygen species exert various effects on plant defense responses. In this case, the SOD activity remained high after several hours after inoculation of the pathogen in Curd treatments, suggesting that this compound can make the defense response persist over time. The production of ROS, in this case of H_2O_2 produced by SOD, may have several functions. It has been determined that biotrophic pathogens can be sensitive to H_2O_2 at micromolar concentrations or lower [64], whereas necrotrophs can be favored from that production [12]. In the case of *P. cinnamomi*, H_2O_2 seems not to be involved in avocado resistance to this pathogen [64]; however, this was proved in vitro and not directly in the plant. Nevertheless, this result is somewhat contradictory because in other investigations the effect of this ROS against *Phytophthora infestans* [65] has been proved as well as its participation in the resistance of *Lomandra longifolia* to *P. cinnamomi91* [66]. In addition, that H_2O_2 generated can also be used by the POD enzyme as an electron acceptor to catalyze the final step in the synthesis of lignin from the oxidation of cinnamyl alcohols, which as mentioned is an important defense response, acting as a physical barrier that blocks the entry of the pathogen [67].

Since this research seeks to understand the effect of Curd as a resistance inducer, it is important to mention that what has been observed suggests that it may confer systemic acquired resistance (SAR). This is because, on the one hand, the defense response was found in a different place than the point of inoculation; on the other hand, the increase of PAL in the final sampling time could indicate the participation of salicylic acid as a signaling agent [67]. It is possible to think that plant defenses were primed by curdlan because there is a rapid and intense response to the pathogen in the first 3 hai of POD and PPO enzymes [68]. Other enzymes such as SOD and PAL increased over time, which is similar to what has been observed in other investigations with inducers, such as that conducted by Eshraghi, et al. [69]. Furthermore, these results add to the

shorter length of the lesion and the healthy appearance of the plants at the end of the trial. However, this requires other experiments using inhibitors of enzymes and analyzing the behavior of the plants.

Finally, although Curd had a positive effect on treated avocado plants, it may not be the β-glucan that best triggers the defense response in avocado plants. Despite the fact that a commercial one with a high cost was used, knowing that it works may be the basis for including it in integrated disease management to reduce the use of pesticides. Even other similar molecules can be sought in the framework of sustainable agriculture. Obtaining β -glucans from plant residues would provide an alternative for integral use in crops that can even be degraded by macromycetes fungi. In this process, both the transformed biomass and the fungus itself can be used, since they are recognized for producing β -glucans and it has been seen that they can trigger defense responses, such as those mentioned above.

Even in a specialized search on this topic, some molecule that further potentiates the response against this pathogen could be found. It has been observed that different modifications in the molecule can have a better response; they are like a key in a lock: many can be recognized, but the one with a precise shape generates a more effective defense response. This is something that turns out to be very specific in each pathosystem and that will be dependent on conditions such as adequate nutrition, stage of development, host genotype and pathogen, as well as abiotic stress [70].

5. Conclusions

The application of the elicitor curdlan on Hass avocado plants reduced the symptoms of the disease caused by *P. cinnamomi*. The response between plants inoculated with the pathogen with (Curd + Phy) and without the inducer (Phy) were mostly contrasting. Curdlan modulated the expression of different enzymes such as SOD, PAL, POD, PPO, total phenolic content and chlorophyll, having contrasting effects in these two treatments for almost all sampling times. Moreover, the plants with the pathogen died or wilted considerably, whereas those with the previous application of the inducer had no or minimal signs of wilting. All these results lead to the conclusion that there was a protective action of curdlan and that possibly what is required is that

the expression of the metabolites occur at an adequate time and concentration, so that it effectively leads to a defense against the pathogen. However, further research is needed to establish when the application of curdlan should be repeated to maintain its effect, if it behaves similarly in the field or if it is affected by climatic conditions, and what its impact on crop productivity is. Likewise, it would be appropriate to know which other inducers work or which ones generate a better response than the one found in this research.

Author Contributions: All authors have contributed significantly to the research article. All authors contributed to article conceptualization; N.G. wrote the original article; N.G., A.Á., D.O. performed the experiments, analyzed the data; O.B. contributed the methodology for the analysis of enzymatic activity; J.E.J., M.B.B.-C., W.M.-A. revised the article critically for important intellectual content. All authors have read and agreed to the published version of the manuscript.

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CAPÍTULO 3

Metabolomic analysis of Hass avocado plants inoculated with *Phytophthora cinnamomi* responses to curdlan elicitor

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Este capítulo se centra en el segundo y tercer objetivo de la tesis mediante el estudio comparativo de las variaciones metabólicas que se generan por la inducción de resistencia con curlano en plantas de aguacate Hass inoculadas con *P. cinnamomi*. En ese sentido, los análisis por GC-MS han demostrado ser una herramienta robusta que permite obtener un perfil de metabolitos de las diferentes muestras evaluadas, con el fin de observar los cambios en concentración o tiempos de producción de los diferentes compuestos y la relación que tiene esto con la respuesta de defensa de las plantas y el posible efecto protector del curlano.

Igualmente, para que se genere una adecuada respuesta de defensa en las plantas, estas deben producir una concentración específica de fitohormonas. En ese sentido, es fundamental establecer la respuesta que genera la inducción de resistencia con curlano sobre el balance de hormonas en plantas de aguacate Hass inoculadas con *P. cinnamomi*. Concretamente, se han medido mediante UPLC-MS-tO, las concentraciones de ácido salicílico y jasmónico en extractos de hojas. Un efecto protector de este inductor debería ocasionar en las plantas un comportamiento de las fitohormonas similar a lo que sucede en un genotipo resistente.

Metabolomic analysis of Hass avocado plants inoculated with *Phytophthora cinnamomi* responses to curdlan elicitor

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Abstract

Elicitors trigger plant defense responses, including the production of phenylpropanoid pathway compounds and phytohormones. In this context, we proposed to evaluate the effect of curdlan (B-glucan type elicitor) on the defense response of Hass avocado plants inoculated with *Phytophthora cinnamomi*. To answer this question, Hass avocado plants were sprayed with curdlan and subsequently inoculated with *P. cinnamomi*. Next, GC-MS analyses were performed to determine the differences in metabolomic profiles and specific LC-MS analyses for the quantification of caffeic acid, ferulic acid, *p*-coumaric acid and phytohormones from plants with the different treatments. The results showed that curdlan generated changes in metabolic profiles. Plants with curdlan application prior to pathogen inoculation (Curd+Phy) generated the same compounds as plants inoculated only with the pathogen (Phy), but at different times and concentrations. The curdlan-induced response also impacted the production of phenolic compounds, generating a lower production of phenolic compounds at most of the times evaluated in Curd+Phy compared to Phy. Only at 144 hai was there an increase in p-coumaric and caffeic acid in Curd+Phy compared to Phy. Phytohormones were differentially produced in the treatments, salicylic acid (SA) was produced in higher amount for Phy at all times. While jasmonic acid (JA)

remained stable, except for 144 hai where it was produced in higher quantity in Curd+Phy compared to Phy.

Keywords: avocado; *Phytophthora cinnamomi*; induced resistance; curdlan; defense-related enzymes, metabolomic analysis.

Introduction

Hass avocado is the leading cultivar marketed worldwide; its commercialization has doubled in the span of 6 years [1]. Driven largely by its taste, high quality and health-related attributes. The dense nutritional and phytochemical composition of avocados is attracting more and more consumers [2]. However, there are phytosanitary problems caused by the oomycete *Phytophthora cinnamomi* Rands, the main pathogen of the Hass avocado crop, which causes losses of up to 90% [3-6].

P. cinnamomi is one of the most devastating plant pathogens known. It has a worldwide distribution and host range approaching 5000 species, such as avocado and macadamia, and has caused significant economic losses in agriculture [7,8]. As a result of the phylogenetic distance between *Phytophthora* and true fungi, most fungicides are ineffective and the choice of chemicals available for management of this pathogen is limited. The most commonly used chemicals for the control of *P. cinnamomi* are etridiazole, metalaxyl, mefenoxam and fosetyl-Al. However, their prolonged use has led to decreased sensitivity and development of resistance [7,9,10].

Recent advances in the knowledge of plant defense mechanisms have opened a new approach to manage plant diseases in an economical and sustainable way. Among these new alternatives that can be included in integrated pest management (IPM), the stimulation of natural plant defenses is considered one of the most promising [11], one of them being the use of elicitor molecules.

These important molecules offer an interesting possibility for defense genes to be activated, leading to systemic acquired resistance [9,11]. Common microbial components, such as bacterial or fungal components (known as pathogen- or microbe-associated molecular patterns, PAMPs or MAMPs, can induce innate immunity in plants to protect them from microbial pathogens [12-14]. Recognition of these molecules by a host membrane receptor induces a primary non-host immune

resistance in the plant that can reduce infection by the pathogen. This response is termed PAMP-triggered immunity (PTI) [15]. Recognition of a pathogen after eliciting the plant, generates an enhanced defense response to prevent pathogen development. A cascade of signaling events including the production of reactive oxygen species (ROS), phytohormones and nitrogen monoxide (NO), increased intracellular calcium concentration and activation of MAPKs then allows the activation of defense genes. This leads to accumulation of pathogenesis-related proteins (PR), biosynthesis of phytoalexins, reinforcement of plant cell walls and, in some cases, a hypersensitive response [16].

Elicitors include glycoproteins, carbohydrates (such as β-1,3-glucan), chitin, fatty acids, proteins, glycosphingolipids and peptides [17-19]. Curdlan, a water-insoluble linear β-1,3-glucan, belongs to the microbial exopolysaccharides and was first detected in *Alcaligenes faecalis* var. Myxogenes, is also produced by fermentation of Agrobacterium sp. and is approved as a safe food additive [20,21]. Curdlan activates defense responses in tobacco (*Nicotiana tabacum* L.) cells [20], also, it generates the production of another defense response such as capsaicin in *Capsicum frutescens* [22] and more recently exhibits an activation effect on early and late defense responses in potato plant leaves [21].

Meanwhile, metabolomics is the third most widely used omics technology in the post-genomic era, after genomics and proteomics. It is an emerging strategy to systematically investigate metabolic variations in the biosystem (e.g., cell, tissue, and organism) in the face of genetic or environmental stress [23,24]. Metabolomics studies often generate insights that can provide an approach to unravel complex mechanisms by measuring many metabolites involved in diverse biochemical processes and in many biological systems [25,26]. For plants, applications of metabolomics have mainly focused on abiotic and biotic stress responses. A wide range of metabolites (including mainly sugars, aromatic amino acids and flavonoids), which could play critical roles in plant defense, have been studied [23,27]. Among these platforms, GC-MS has proven to be a powerful and reproducible tool that allows broad coverage of the primary metabolome, such as sugars, amino acids, and organic acids [28]. While, LC-MS has greatly benefited from high sensitivity and selectivity [29] to identify different metabolites such as phytohormones and phenolic compounds.

The objective of this study was to investigate the role of curdlan as a defense inducer in Hass avocado plants against *P. cinnamomi* under greenhouse conditions to determine differences in metabolic profiles and expression of phenolic-compounds (caffeic, ferulic and p-coumaric acid) and phytohormones (jasmonic and salicylic acid).

2. Material and methods

2.1. Reagents, solvents and standards

Curdlan, was supplied by Sigma-Aldrich (Israel) and NaOH by Panreac (Barcelona, Spain). The antioxidants were 2,6-di-tert-butyl-4methylphenol (BHT) and ascorbic acid provided by Sigma-Aldrich (Madrid, Spain). The reagents methoxyamine hydrochloride (MEOX) and N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) were also provided by Sigma-Aldrich. The solvents used in the extract, hexane and methanol, were purchased from Scharlab (Barcelona, Spain). Dimethyl sulfoxide (DMSO), ethylenediamine tetra acetic acid disodium salt dihydrate (EDTA-Na2), formic acid and hydrochloric acid (HCl) 37% were obtained from Merck. Pyridine was obtained by J.T. Baker (Deventer, Netherlands. The chromatographic solvents were supplied acetonitrile and methanol, also from J.T.Baker and water was purified in a Milli-Q system from Millipore (Bedford, MA, USA).

Standards of phenolic compounds, caffeic acid, p-coumaric acid and ferulic acid, were supplied by Sigma-Aldrich Chemie (Steinheim, Germany). All stock standard solutions of phenolic compounds were prepared in MeOH and stored at -80 °C.

The phytohormone standard [2H4] SA was purchased from Sigma- Aldrich.

2.2. Plant material

Hass avocado plants were obtained from a plant nursery that has a registered and phytosanitary status, located in Líbano, Tolima, Colombia. Young (six-month-old) plants were used for experimental analysis. The plants were kept in a greenhouse at ambient humidity and temperature conditions in the facilities of the University of Tolima (4.428242, −75.213478) at 1285 masl. They were maintained with a photoperiod of 12 h light and 12 h of darkness.

2.3. Pathogen

The Hass avocado soil pathogen *P. cinnamomi* was supplied by Corporación de Investigaciones Biológicas (CIB), located in Medellín (Colombia). The culture was maintained on Potato Dextrose Agar (PDA) at 25 °C. Fifteen-day-old culture was used as an active mycelium.

2.4. Elicitor Preparation and Application

Curd was purchased from Sigma (Israel) and was dissolved in a solution of 1% DMSO and 0.05 mM NaOH at a concentration of 2658 mg/L. The solution was stored at 4 °C and no precipitation of the compound was observed. The application of the curd was made according to the methodology proposed by Li et al. [21], with some modifications. Hass avocado plants were treated in four different ways, including: (1) Plants sprayed with 5 mL of the solution, representing 13.29 mg of Curd 1 day before *P. cinnamomi* treatment (Curd + Phy); (2) Plants sprayed with 5 mL of the solution, representing 13.29 mg of Curd 1 day before without pathogen to establish an elicitor control (Curd); (3) Plants with the pathogen (Phy) and (4) Control plants (Control). A total of 120 Hass avocado plants were randomly used in each repetition of the experiment, 30 in each group. The experimental unit was composed of 2 plants, and 3 replicates were assayed.

Hass avocado plants were inoculated with *P. cinnamomi* according to the methodology of Dinis, et al. [30]. A "T" form incision was made in the plants, and a PDA plug with oomycete was incorporated. Inoculation was done directly in the stem. After inoculation, the incision was covered with a piece of cotton wool to ensure an adequate moisture level for infection and disease development. The whole assay was repeated three times, carried out in November 2018, March, and April 2019, and the leaves were collected between 10:00 and 13:00 at 0, 3, 24, 144 and 312 h after inoculation (hai).

2.5. Observation of Lesions Caused by *Phytophthora cinnamomi* on Hass Avocado Plants under Greenhouse Conditions

To observe the development of lesions, the inoculated Hass avocado plant were inspected every three days for three times. The lesion was measured with a vernier caliper to see its progress. This process was done until day nine after inoculation.

2.6. Sample Preparation

The harvest was made by taking the whole leaves of two plants to make one sample. A total of three samples (from six plants) were established in each interaction between time and treatment to account for biological variations. Samples were quickly frozen with liquid nitrogen (shock freezing), then powdered under the same condition and subsequently stored at −80 °C [31].

2.7. Extraction process

5mL of hexane + 0.1% of 2,6-di-tert-butyl-4methylphenol (BHT) was added to 250 mg of pulverized leaves. The sample was placed in an ultrasonic bath for 30 min, then centrifuged at 1400×g for 10 min (Hettich Eppendorf Centrifuge MIKRO 22 R; Germany). The supernatant was transferred to a previously tared vial and brought to dryness under nitrogen. The solid residue was extracted with 5 mL methanol + 0.1% ascorbic acid. It was left in ultrasonic bath for 30 min, subsequently centrifuged at 1400×g for 10 min (Hettich Eppendorf Centrifuge MIKRO 22 R; Germany). The supernatant was transferred to a previously tared vial and brought to dryness under nitrogen.

2.7.1. Bound phenolic compounds

Basic hydrolysis of bound phenolic acids: The lyophilized solid material obtained from the previous extractions was hydrolysed directly following a method previously described but with some modifications [32] Part of the remaining material (50 mg) was mixed with 1.5 mL of 2 M NaOH containing 10 mM EDTA and 1% ascorbic acid and then shaken (Eppendorf©Thermomixer Comfort, Hamburg, Germany) for 30 min at 45 °C. The reaction mixture was acidified (*c.a.* pH 3) by adding 0.285 mL of 7.2 M HCl and centrifuged at 1400 x *g* for 5 min (Hettich Eppendorf Centrifuge MIKRO 22 R; Germany).

2.8. Metabolomics analysis by GC-MS and data processing

10 mg of the lyophilized extract was suspended in 300 µL of methoxylamine hydrochloride (MEOX, Sigma-Aldrich) in pyridine (20 mg/mL). The sample was sealed with a cap, agitated with a vortex until the residue is completely solved and placed on a ThermoMixer for incubation at 40 ºC. After one hour, the sample was removed and 80 µL of *N*-methyl-*N*-

(trimethylsilyl)trifluoroacetamide (MSTFA, Sigma-Aldrich) were added. Finally, the sample was vortex agitated and incubated at 40 ºC for 1 hour.

Quantitative analyses of the simples were carried out using a gas chromatograph (Agilent GC7890N, Agilent Technologies España, S.L., Las Rozas, Spain) coupled to an electronic impact single quadrupole mass spectrometer (5973N MSD, Agilent Technologies España, S.L., Las Rozas, Spain). The analytical column was a DB-35 MS UI from Agilent with a 30 m × 0.25 mm fused silica capillary coated with a 0.25 µm film equivalent to (35%-phenyl)-methylpolysiloxane. The oven temperature started at 80°C for 2 minutes and then increased to 310°C at 6°C/min for 10 minutes. Helium was used as the carrier gas at constant flow rate of 2 mL/min. A split injection with a 5:1 ratio was used. The injection volume was 1 µL. The injection system was held at 250°C. The ionization source of the mass spectrometer was an electronic impact (EI). The single quadrupole operates in scan mode between 35 and 700 amu, with a solvent delay of 4 min. The peak obtained were identified by comparing their mass spectra with the National Institute of Standards and Technology 17 (NIST, EE.UU.) NIST MS Search 2.3 and WYLEY275.

2.9. Quantification of phenolic compounds by UPLC-MS

Dry extract, from the extraction of bound phenolic compounds, was dissolved in the mobile phase prior to analysis, which was carried out using Waters Acquity™ Ultra Performance LC equipment. Aliquots (5 µl) were injected onto a reversed-phase column ACQUITY UPLC HSS T3 (100 × 2.1 mm 1.8 µm) using a gradient system consisting of solvent A, water:methanol (98:2 v/v)+1% formic acid and solvent B, methanol+1% formic acid 80:20 (v/v). The linear gradient was as follows: 0-0.6 min, 80% A, 0.6-6 min, 30% A, 6.10-8.5 min, 100% B, and between 8.55-10 min the initial conditions were restored. The flow rate was kept constant at 0.3 mL/min, the column was maintained at 40°C. Negative ESI mode was employed and HPLC effluent was fed into a triple quadrupole mass spectrometer (Xevo TQS). Instrument control and data acquisition and processing were performed with MassLynx™ software (version 4.1; Waters, USA).

2.10. Quantification of phytohormones by UPLC-MS

Fresh material was frozen in liquid nitrogen and lyophilized. Prior to extraction, an internal standard mixture containing 100 ng of [$^2H_6$] JA and 100 ng of [$^2H_4$] SA was added. The dried

tissue (0.05 g) was immediately homogenized in 2.5 mL of ultrapure water. After centrifugation (5000 *g*, 40 min), the supernatant was recovered and adjusted to pH 2.8 with 6% acetic acid, and subsequently extracted twice in an equal volume of diethyl ether. The aqueous phase was discarded and the organic fraction was evaporated under vacuum at room temperature and the solid residue was resuspended in 1 mL of a water/methanol solution (90:10) which was filtered through a 0.22 µm cellulose acetate filter. A 20 µL aliquot of this solution was injected directly into the HPLC system. The analyses were carried out using Waters Acquity™ Ultra Performance LC equipment. Aliquots (20 µl) were injected onto an ACQUITY UPLC HSS T3 column (100 × 2.1 mm 1.8 µm). Phytohormones were eluted with a gradient of Acetonitrile+0.1% formic acid and water+Acetonitrile+0.1% formic acid 10:90 (v/v) and linearly reached 90:10 in 4 min. Initial conditions were restored and allowed to equilibrate for 1 min, which was a total time of 5 min per sample. The solvent flow rate was 0.4 mL/min. Negative ESI mode was employed, the HPLC effluents were fed into a triple quadrupole mass spectrometer (Xevo TQS) [33,34]. Instrument control and data acquisition and processing were performed with MassLynx™ 4.1 software (Waters, USA).

3. Results

3.1. Observation of the lesion caused by *Phytophthora cinnamomi* on Hass avocado plants under greenhouse conditions

A smaller lesion was observed when plants were sprayed with curdlan one day before *P.cinnamomi* inoculation, compared to plants inoculated only with the pathogen (Table. 1). Plants to which curdlan was applied showed a small dark brown lesion, but no signs of wilting at the end of the trial (Curd+Phy). Wilt could also be observed on leaves and branches of seedlings inoculated with *P. cinnamomi*, but without curdlan spray (Phy) (Fig. 1.).

Other parameters such as severity or incidence could not be measured due to the way the pathogen is inoculated, which means that the symptoms of the disease are not expressed in the same way as when the pathogen enters naturally.

Table. 1. Lesion length in avocado plants caused by *P. cinnamomi* after spraying with curdlan and plants inoculated with *P. cinnamomi*

Treatment	Average length of the lesion (cm)		
	3 dai	6 dai	9 dai
Spraying with curdlan+inoculation of *P.cinnamomi*	2.32±0.32a	3.39±0.34a	3.90±0.24a
Inoculation of *P. cinnamomi*	3.56±0.24b	4.53±0.28b	5.69±0.31b

Values are the mean ± standard deviation (n=9). Means with same letters within the same column are no significantly different ($p<0.05$).

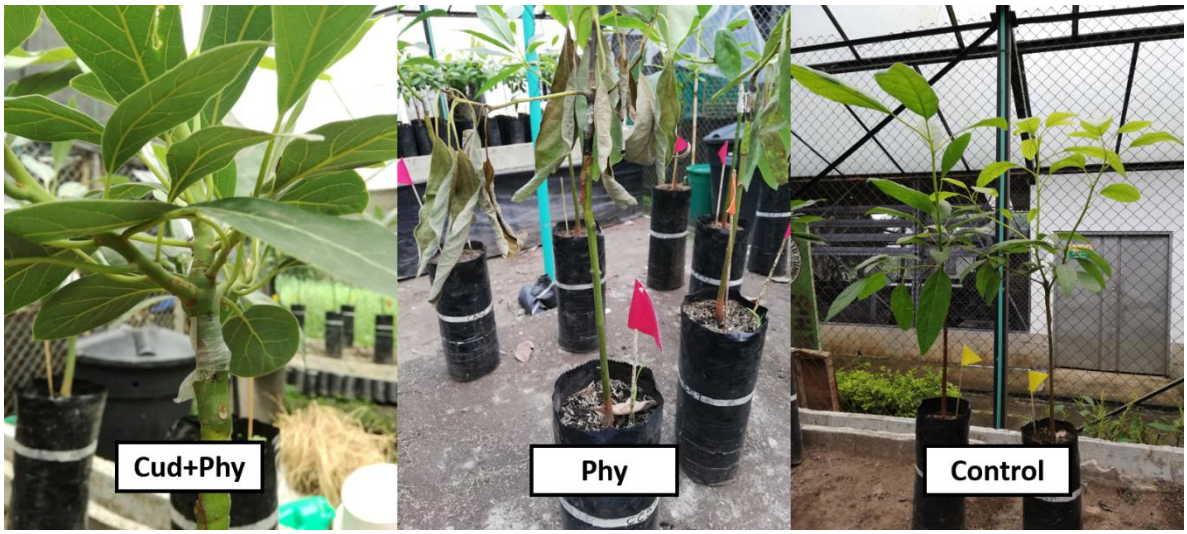

Figure 1. Appearance of avocado plant inoculated with *P. cinnamomi* 312 hai. Curd+ Phy, Phy and Control represent application of curdlan one day before inoculation of *P. cinnamomi*, inoculation of *P. cinnamomi* and the control (distilled water), respectively.

3.2. Metabolic profiles of Hass avocado plants treated with curdlan and inoculated with *P. cinnamomi.*

The differences between the metabolite profiles of plants with inducer and pathogen (Curd+Phy) and those with only pathogen inoculation (Phy), identification and statistical comparison was performed. A total of 60 metabolites were detected in the leaves of Hass avocado plants by gas chromatography coupled to mass (GC-MS) (Table. 2). Subsequent hierarchical clustering analysis distinguished a clear metabolite profile dependent on the treatment applied, with the chromatographic relative area, expressed through the vertical dendrogram of the compounds in each sampling time being different (Fig. 2.).

Table 2. Identification of metabolites by GC-MS in the leaf extracts of Hass avocado plants.

Signal	T.R (min)	Identification	Molecular mass
1	4.957	Lactic acid (2-OH-C3:0)	234.11
2	5.500	Acetic acid	220.10
3	5.675	Phenol	166.08
4	6.120	2-OH-C4:0	248.13
5	6.800	3-OH-C3:0	234.11
6	7.511	Oxalic acid	234.07
7	7.768	3-Me-3-OH-C4:0	262.14
8	7.899	Phenylmethanol	180.10
9	8.712	Malonic acid	248.09
10	9.264	2-OH-C6:0	276.16
11	10.325	2,3-diOH-C3:0	322.14
12	10.409	Benzoic acid	194.08
13	11.096	Succinic acid	262.11
14	11.312	Fumaric acid	260.09
15	11.773	Tetradec-2-ene	196.22
16	12.018	Thymol	222.14
17	12.460	2-hydroxymalonic acid	333.12
18	12.531	Salicilyc acid	210.07
19	12.916	3,4-dihydroxydihydrofuran-2(3H)-one	262.11
20	14.056	Malic acid	350.14
21	14.763	2,3,4-triOH-C4:0	424.19
22	15.935	Hexadec-1-ene	224.25
23	16.260	2,6-di-tert-butyl-4-methylphenol	220.18
24	16.844	2-hydroxy-3-phenylpropanoic acid	310.14
25	17.368	Phloroglucinol	342.15
26	17.747	4-hydroxybenzoic acid	282.11
27	19.723	Octadec-1-ene	252.28
28	19.851	3,4,5-trihydroxycyclohex-1-enecarboxylic acid	462.21
29	20.127	Citric acid	480.19
30	20.863	Protocatechuic acid	370.14
31	21.028	C14:0	300.25
32	21.224	2-(3,4-dihydroxyphenyl) acetic acid	384.16
33	21.452	p-coumaric acid	308.13
34	22.000	3,4-Dihydroxymandelic acid	326.32
35	22.176	4-Hydroxyphenyllactic acid	398.18
36	23.167	C16:0	270.26
37	23.360	9-C16:1	268.24
38	24.101	3,4-Dihydroxycinnamic acid	396.16
39	24.370	3-(3,4-dihydroxyphenyl)-2-hydroxypropanoic acid	486.21
40	24.767	C17:0	284.27
41	25.826	Phytol	368.35
42	26.660	Ferulic acid	338.14

43	26.762	9,12,15-C18:3	292.24
44	27.196	11-C:18:1	354.30
45	27.335	Linoleic acid (9,12-C18:2)	280.24
46	29.183	Sinapic acid	368.15
47	29.637	11,14,17-Eicosatrienoic acid, methyl ester	278.23
48	31.973	C22:0	354.35
49	34.540	Methyl tetracosanoate	382.38
50	35.845	Catechin	650.28
51	36.830	13-Docosenamida	337.33
52	36.910	Methyl hexacosanoico	410.41
53	38.115	Octacosan-1-ol	482.49
54	39.128	C28:0	438.44
55	39.446	Vitamin E	502.42
56	40.191	Triacontan-1-ol	510.52
57	41.163	Stigmasterol	484.41
58	41.285	C30:0	466.48
59	41.879	Sitosterol	486.43
60	42.454	Dotriacontan-1-ol	538.55

[1] Compounds with carboxylic groups have been identified in GC-MS as the corresponding methyl esters. Compounds with hydroxyl groups have been identified as the corresponding trimethylsilyl derivatives (O-TMS ether derivatives).

Similarly, the principal component analyses show that from 3 h there is no great similarity in the variability of the data originated by the metabolites determined through GC-MS and that contribute to the formation of each component. The contribution of metabolites is not equal and because of this, there is difference in the way the different treatments Curd+Phy, Curd, Phy and Control are grouped. Although, the treatments that are grouped to a certain extent are Curd+Phy, Curd and Phy, which would be demonstrating that the plant in effect recognizes the inducer and has a response to it. Thus, it can be observed that the interaction as such with the inducer definitely causes metabolic changes in the plants reflected in the metabolite profiles produced, which are for most of the times evaluated, different from those produced in the control plants (Fig. 3).

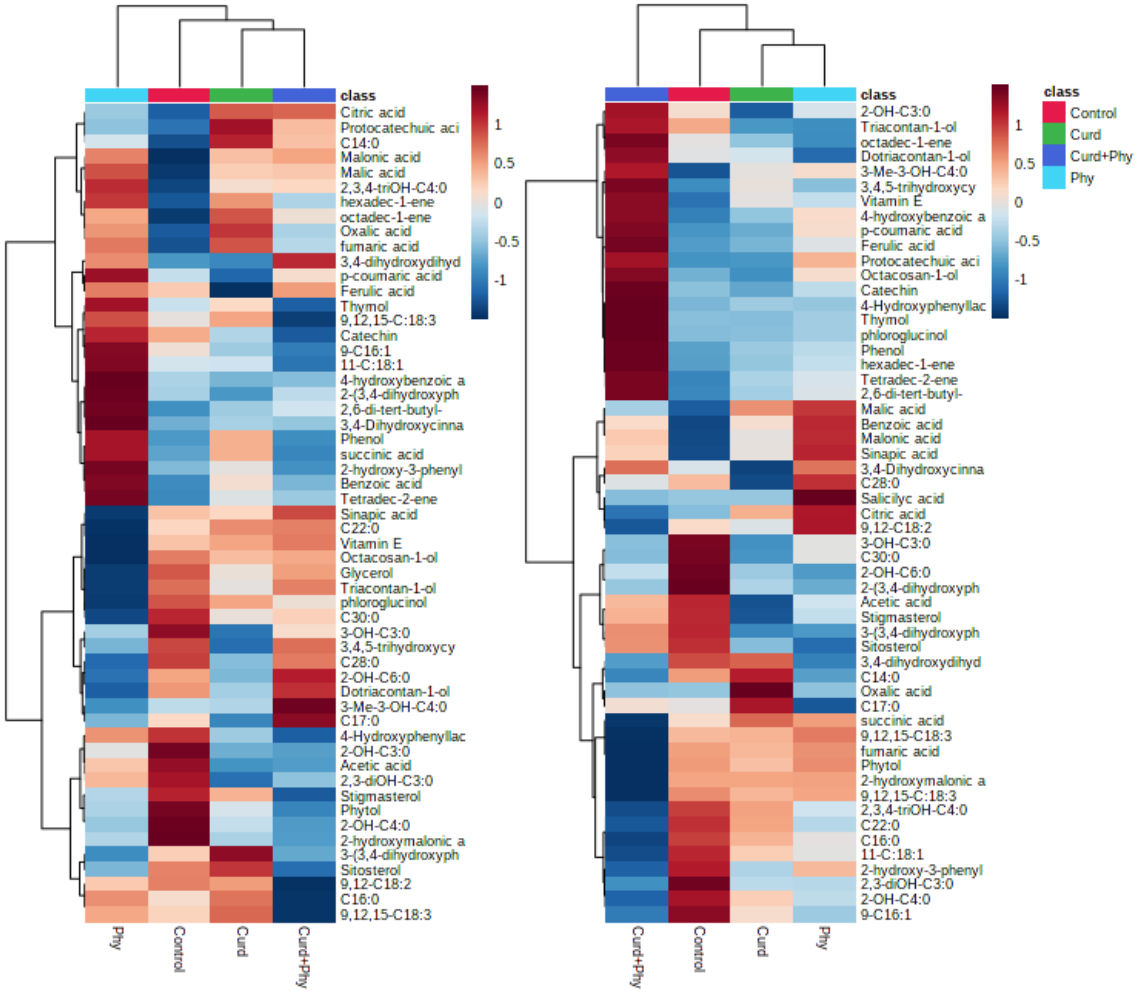

Figure. 2. Heatmap obtained after hierarchical cluster analysis of the metabolite profiles of Hass avocado plants at 24 and 144 hai. The names on the right indicate the metabolite identified.

In addition to these results, those obtained by liquid chromatography coupled to mass (LC-MS) were added. By means of this technique it was possible to quantify phenolic compounds such as caffeic, ferulic and *p*-coumaric acid, as well as the phytohormones jasmonic acid and salicylic acid. In the case of phenolic compounds, the results correspond to a large extent with those found by GC-MS. However, detection by liquid chromatography is more sensitive in this case, since the metabolite standards were used as a reference and their quantification was carried out. Indeed, it is possible to see that ferulic acid is higher in the Phy treatment at the first sampling times, while for Curd+Phy, it is in higher concentration at 144 h after inoculation (Fig. 4 c).

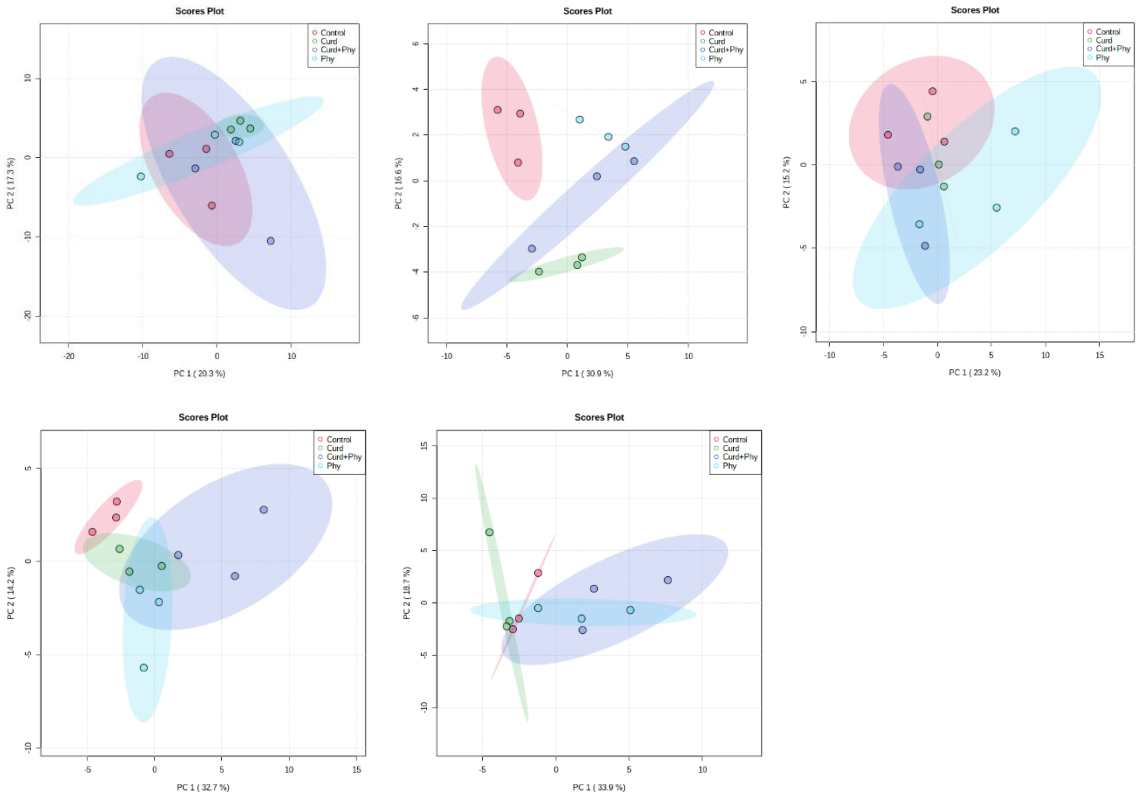

Figure 3. Principal component analysis (PCA) of the metabolome of Hass avocado plants inoculated with *P. cinnamomi* at (a) 0, (b) 3, (c) 24, (d) 144 and (e) 312 hai. Curd+ Phy, Phy, Curd and Control represent curdlan application one day before *P. cinnamomi* inoculation (purple), *P. cinnamomi* inoculation (blue), curdlan application without *P. cinnamomi* inoculation (green) and control (red), respectively.

In respect of *p*-coumaric acid, no major differences are found between the Phy and Curd+Phy treatments, only for the 144 h time, where for the latter treatment there is a higher concentration of this acid (Fig. 4a). In the case of caffeic acid, the trend is similar to that of ferulic acid, there is a higher concentration for the Phy treatment at 3 h but the situation changes at 144 h where the increase of this metabolite is found in Curd+Phy (Fig. 4 b).

On the other hand, the behavior of the phytohormones was inverse, for the plants inoculated with the pathogen (Phy) the concentration of salicylic acid increased as time went by, while jasmonic acid increased in the early stages and decreased at the end. The opposite case was observed in the plants to which the inducer (Cur+Phy) was applied prior to pathogen inoculation, in which salicylic acid remained stable, while jasmonic acid increased in the final stages (Fig. 5 a and b).

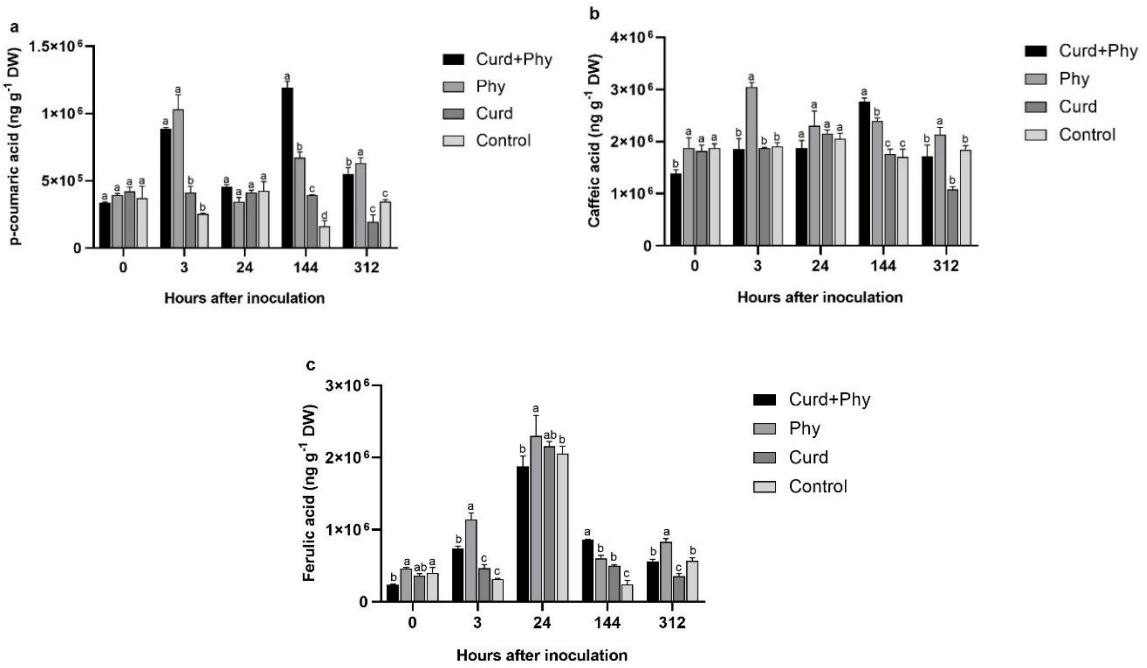

Figure 4. Effect of curdlan treatment on (a) p-coumaric acid, (b) caffeic acid and (c) ferulic acid in Hass avocado plants inoculated with *P.cinnamomi*. Curd+ Phy, Phy, Curd and Control represent curdlan application one day before *P. cinnamomi* inoculation, *P. cinnamomi* inoculation, curdlan application without *P. cinnamomi* inoculation and control (distilled water), respectively. Data are presented as the mean ± standard deviation of nine replicates. Means with the same letter are not significantly different (ANOVA followed by Tukey's test with $p<0.05$).

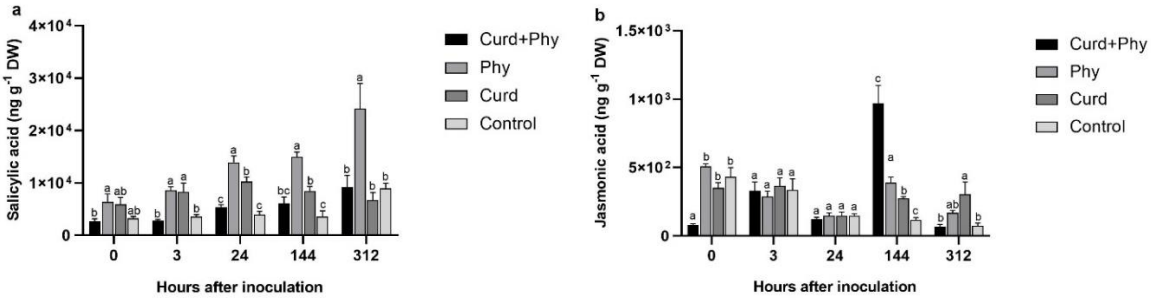

Figure 5. Effect of curdlan treatment on (a) salicylic acid and (b) jasmonic acid in Hass avocado plants inoculated with *P. cinnamomi*. Curd+ Phy, Phy, Curd and Control represent curdlan application one day before *P. cinnamomi* inoculation, *P. cinnamomi* inoculation, curdlan application without *P. cinnamomi* inoculation and control (distilled water), respectively. Data are presented as the mean ± standard deviation of nine replicates. Means with the same letter are not significantly different (ANOVA followed by Tukey's test with $p<0.05$).

4. Discussion

Phytocompounds are produced by a specific biochemical pathway, a process that occurs inside the cells, these compounds include secondary metabolites, many of which are synthesized for plant defense against a specific pathogen.

In the first 24 h, plants inoculated with *Phytophthora* showed an increase in metabolites related to the primary metabolism of the Krebs cycle, such as malic, oxalic, fumaric and succinic acids (Fig. 2). This is in response to the biotic stress that generates an increased demand for carbon, which is provided by the intermediates of the tricarboxylic acid cycle through different pathways, since these compounds (such as fumarate or succinyl-CoA) are critical for energy generation in plants [35]. Likewise, a higher concentration of phenolic metabolites such as caffeic, ferulic and p-coumaric acid is present. These secondary metabolites from the phenylpropanoid pathway have antimicrobial and antioxidant properties, which play an important role in plant defense. However, their increase at this time seems to be a precipitous response to a hemibiotrophic pathogen, since the literature reports that a rapid production of these metabolites occurs as an inducible defense response to necrotrophic pathogens [36], although this will depend greatly on the pathosystem evaluated.

In addition, other compounds also appear in high concentrations, such as malonic acid, a precursor for the formation of flavonoids, compounds with antioxidant activity, protection against ultraviolet light and defense against phytopathogens [37]. Within this type of compounds, catechin, a flanvan-3-ol with antifungal activity synthesized in response to pathogen infection, was found [38]. Benzoic acid and its hydroxylated derivative, hydroxybenzoic acid, which have antimicrobial activity [39] and are precursors in the formation of salicylic acid, as well as overproduction of thymol, a compound from the mevalonate pathway, recently employed as a natural agrochemical for its antimicrobial activity [40]. Other compounds from this same metabolic pathway, such as stigmasterol and sitosterol, were synthesized but not differentially in the treatments, although it is important to highlight that in general, terpenes from this pathway are necessary for many vital plant functions such as metabolism of phytohormones, photosynthetic pigments and membrane components, as well as the fundamental role they play in secondary metabolism, performing ecological functions as a response to biotic and abiotic stresses [41].

In contrast, at the final sampling times 144 hai, plants inoculated with the pathogen changed their metabolite profile, retaining a higher concentration of malic, malonic, benzoic and caffeic acids and increasing the concentrations of others such as salicylic, sinapic, citric and linoleic acids, many of which, as previously mentioned, are related to defense.

On the other hand, plants inoculated with the pathogen that had the previous application of the inducer (Curd+Phy) showed at 24 hai some compounds similar to the plants that were only inoculated with the pathogen, such as ferulic acid and p-coumaric acid. However, other compounds appear such as synapic acid, a phenylpropanoid that is generated in response to biotic stress and has antimicrobial activity [36]. Protocatechuic acid, which has antioxidant and antimicrobial activity, and phloroglucinol, a polyketide with reported antimicrobial and insecticidal activity [39,42]. In addition to some alcohols and acids that occur as part of the waxes of the leaves and participate in the metabolism of lipids [43]. Likewise, there is an overproduction of citric acid which is presumed to play an important role in gene expression and metabolite signaling in various organisms[44]. A furan-like compound, 3,4-Dihydroxyhydroxyhydrofuran-2(3H)-one, very relevant in plants for its antioxidant, insecticidal and antifungal activity and vitamin E, which inhibits the propagation of lipid peroxidation, protects photosystem II from photoinhibition and exerts control over defense-related transcriptional reprogramming and hormone modulation, are also produced [45,46](Fig. 2).

At 144 hai, the Curd+Phy treatment retained the high production of the metabolites it had generated at 24 hai but also produced others of interest such as catechin and thymol which were found in the plants inoculated with the pathogen (phy) at 24 hai and which have relevant activities for plant defense as an antifungal and natural agrochemical, respectively.

Due to the above, it is possible to identify some similarities in the metabolic profiles of the plants inoculated with the pathogen with (Curd+Phy) and without (Phy) after inducer application, but also to observe differences in many metabolites generated. This is a clear sign that, in both treatments, the plants were facing the pathogen, because most of the compounds produced that increased their concentration have biological activities that protect them against the stress

represented by the attack of a microorganism (Fig. 2.). However, it is interesting how the application of curdlan, prior to inoculation of the pathogen, causes the generation of different metabolites or the same ones, but at opposite times to the plants inoculated only with the pathogen. This could possibly be what causes a differential response in favor of the plants that had the inducer applied, so that they are able to survive the disease generated by the entry of *P. cinnamomi*.

In general, many of the metabolites produced in the Hass avocado plant *P. cinnamomi*, with or without inducer, have been previously identified in avocado plants recognizing the functions mentioned above [47]. However, what is disturbing if compared with the results of the visual observation made to the plants under study, is that by the final times, the plants inoculated only with the pathogen, were in a considerable wilting and were even beginning to die, while those that received the previous application of the inducer, were in good condition, with no or minimal signs of wilting in the leaves.

All these results together with transcriptomic studies of the species where genes associated with defense against oomycetes have been found [48], lead to the conclusion that Hass avocado plants, which are susceptible to the pathogen *P. cinnamomi*, have the capacity to generate all the metabolites necessary to defend themselves against it. However, they do not, due to an inadequate biosynthesis of these metabolites in time or incorrect concentrations. This is similar to what has been observed in other plants such as banana, where they compared a susceptible variety with one resistant to *Fusarium oxysporum* f. sp. *cubense* tropical race 4. In this transcriptomic study, they found that the resistant variety expresses genes related to defense metabolites at different times, even before the pathogen arrives, that is, it has an effective constitutive defense. While the susceptible variety has a late expression [49]. In the same sense, another research comparing two varieties (susceptible and resistant), also of banana, suggested that the defense mechanism incorporates various combinations of antioxidant compounds that may be specific to each cultivar [50].

Based on the results of the phenolic compounds quantified, it could be established that the rapid production of phenolic compounds in plants inoculated with the pathogen (Phy) is due to an effective response that seeks to counteract its effect, as has been reported in other studies where

an early increase of these compounds is associated with resistance [51]. However, there are also reports of increased phenolic compounds in susceptible varieties or at a more advanced stage of the disease, as has also been reported in the literature [52,53].

Likewise, it is interesting to note that the early increase of phenolic compounds in the Phy treatment could be adequate. However, it should not be forgotten that the pathogen has the possibility of continuously sensing the plant response and secreting effector proteins that act on the host, disarming its defenses and promoting colonization [54]. Therefore, it could also be possible that plants with previous application of the inducer, synthesize phenolic compounds at more advanced times seeking to better regulate the defense response.

Other compounds evaluated were phytohormones. In the Phy treatment, the SA increased as time passed, while JA had an inverse response to SA. This behavior for the Phy treatment may be generating a scenario that favors the pathogen in its necrotrophic stage. Since it has been seen that pathogens of this type, such as *Alternaria solani* or *Botrytis cinerea*, enhance the SA signaling pathway to antagonize with jasmonic acid and promote the development of the disease in other plants such as tomato [55].

In addition to the above, the production of phytohormones is the inverse of what plants should do to respond correctly to *P. cinnamomi*. This correct response is widely reported in the literature, where the role of salicylic acid in the defense against biotrophic pathogens is mentioned. This compound is a signaling compound that activates certain immune responses and establishes disease resistance, while JA activates defense against necrotrophic pathogens [56].

Similarly, it has been found that plants with silenced susceptibility-associated genes have increased resistance to the pathogen, which is related to an early onset of SA- and ethylene (ET)-mediated signaling pathways [57]. In the same way, van den Berg, Mahomed, Olivier, Swart and Crampton [48] reported a transcriptomic analysis of an avocado variety resistant to *P. cinnamomi*. That research showed that the successful defense response was due to the expression of SA during the first hours of interaction with the pathogen, passing to a decrease at 24 h, time in which JA increased. According to this information, the application of the inducer curdlan, failed to modulate

correctly the phytohormone response, but it did keep SA levels low in the first hours and increased JA levels by the final hours. This, as mentioned before, is important, because hormone interaction adjusts the defense response of plants against specific attackers, so it is known that the interaction between SA and JA is often antagonistic [58].

Based on the above ideas, it is clear that there is a differential behavior of the metabolic profiles between the plants inoculated with the pathogen (Phy), those that prior to inoculation were applied the inducer (Curd+Phy), those that were only treated with inducer (Curd) and the control, all of which leads to the conclusion that the inducer was recognized by the plant and there is a response to it (Fig. 2 and 3). As it has happened in different investigations that have studied the role of saccharide-type inducers in different plants [21,59,60].

This protective effect in these studies has been due to the modulation of the biochemical response of plants, causing the expression of enzymes such as phenylalanine ammonium lyase or peroxidase (previous studies). Even though accumulation of phenolic compounds, changes in the profiles of metabolites involved in the construction of leaf waxes, precursors of oxylipin biosynthesis, among others. These responses were very similar to those found in this research. However, the scope of this research is limited and further studies are required to determine whether curdlan or a similar molecule can function effectively as a resistance inducer in Hass avocado plants and be included in an integrated disease management plan.

5. Conclusion

The results of all the analyses carried out allow us to conclude that curdlan inducer has a positive protective effect on Hass avocado plants, but its response is not the most effective. The regulation of metabolites or phytohormones could be better adjusted in terms of time and concentration. This deficiency may be due to the fact that it is necessary for the inducing molecule to have specific modifications that optimize the response, because many molecules can be recognized by plants, but the precise one is the one that generates the most effective response. This is something that is specific to each pathosystem and depends largely on adequate nutrition, developmental stage, host and pathogen genotype, and abiotic stress.

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CAPÍTULO 4

Resistance induction with silicon in Hass avocado plants inoculated with *Phytophthora cinnamomi* Rands

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Resistance induction with silicon in Hass avocado plants inoculated with *Phytophthora cinnamomi* Rands. Álvarez, A., Oliveros, D., Ávila, Y. C., Sabogal-Palma, A.C., Murillo-Arango, W., Eras J., Bermúdez-Cardona, M. B., & Guarnizo, N. Sometido en *Plant Signaling & Behavior*.

El cuarto capítulo busca determinar el efecto protector del silicio como inductor de resistencia en plantas de aguacate Hass inoculadas con *P. cinnamomi*, en consecuencia, se aborda el cuarto objetivo específico. Estudios previos han demostrado que la aplicación de silicato de potasio al suelo, genera en las plantas un aumento de la resistencia a patógenos, mediante la modulación de la expresión de sistemas enzimáticos asociados al fortalecimiento de la pared celular. Por tanto, medir actividades enzimáticas como PAL, POD y PPO es un indicativo de esto, pues son enzimas que brindan información sobre cómo se están transformando los compuestos fenólicos en otros productos relevantes en la defensa.

De la misma manera, el contenido fenólico total y la capacidad captadora de radicales libres son actividades que están relacionadas con la respuesta de las plantas a una inducción de resistencia. Se ha generalizado tradicionalmente un aumento de ambas actividades con un mayor nivel de defensa. Sin embargo, deben relacionarse siempre su respuesta con otras mediciones, para explicar lo que está sucediendo a nivel metabólico. En ese sentido, es posible que, por ejemplo, los compuestos fenólicos no aumenten porque están sirviendo como sustrato de diferentes enzimas como las mencionadas anteriormente. Consecuencia de ello, se pueden producir compuestos que funcionan como refuerzo de la pared celular o para la generación de diferentes compuestos con actividad antimicrobiana, por lo cual siempre es necesario integrar los diferentes resultados para lograr una conclusión sólida, sobre el funcionamiento del inductor.

Resistance induction with silicon in Hass avocado plants inoculated with *Phytophthora cinnamomi* Rands

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Abstract

The Hass avocado is recognized for being one of the most relevant Colombian export products, which has generated the need to optimize the technological management packages to maintain the competitiveness and sustainability of the crop. Currently, root rot caused by *Phytophthora cinnamomi* Rands, is one of the main factors that limits avocado production worldwide; silicon as a defense inducer seems to be a viable strategy to integrate into the management of this disease. In this way, the present study evaluated the induction of resistance with silicon in Hass avocado plants inoculated with *P. cinnamomi*, as a possible alternative to conventional agrochemical management. Hass avocado plants established under greenhouse conditions were used, to which a potassium silicate solution (10 mL, 0.2 M expressed as SiO_2) was applied by irrigation, for ten days before inoculation with *P. cinnamomi*. Leaf samples were taken at 3, 24, 144, and 312 h after inoculation with the pathogen. The application of the inducer influenced the biochemical pathways related to the defense response of the studied plants, favoring their resistance to *P. cinnamomi*.

Peroxidase (POD) and polyphenol oxidase (PPO) enzymes had their highest activity 3 h after pathogen inoculation ($p<0.05$). On the other hand, there was evidence of a decrease in the activity of the enzyme phenylalanine ammonia-lyase (PAL), in the content of total phenols, and the inhibition capacity of the DPPH$^{\bullet}$ radical, between 3 h and 24 h in the plants with the inducer and inoculated with *P. cinnamomi* ($p<0.05$). The results suggest a beneficial effect of silicon as a defense inducer in Hass avocado plants, manifested in the activation of enzymatic pathways related to the regulation of oxidative stress and the synthesis of structural components, such as POD and PPO. Therefore, the application of silicon as a defense inducer emerges as a strategy to include in the integrated management of the disease caused by *P. cinnamomi* in Hass avocado.

Keywords: Hass avocado, *Phytophthora cinnamomi*, silicon, defense induction, elicitor.

1. Introduction

The Hass avocado is recognized for being one of the main Colombian export products, going from 1,760 tons exported in 2014 to 28,487 tons in 2017 [1]. Due to its favorable agroclimatic conditions, Colombia has reported the highest fruit yields (up to 9.85 t/ha in 2007) [2]. For the year 2021, a production of 214,618 tons was estimated, distributed in approximately 20,446 ha in Colombian territory [3]. This increase in the national statistics for Hass avocado generates the need to optimize the technological management packages, to maintain the competitiveness and sustainability of the crop.

In this aspect, one of the main factors that limit the production of avocado cultivation worldwide is root rot caused by *Phytophthora cinnamomi* Rands; This disease generates losses in commercial crops between 45 and 90% [4]. In Colombia, between 30% and 50% of affected trees in the nursery stage have been reported, during the first two years of the establishment of the crop [5]. The disease causes a progressive decline that eventually leads to the death of severely attacked trees, which is evidenced by a general wilting of the leaf area, also known as "sadness" [2]. Initially, the plant presents mild to moderate partial defoliation and chlorosis, vegetative growth stops, and therefore, fruit production [6].

In addition to the above, there is currently no consensus in the studies carried out for management against this pathogen. That is necessary to allow the design of effective control and

prevention strategies for the disease, added to the little knowledge about the plant-pathogen interaction in avocado cultivation [4]. For the same reason, it has been seeking to diversify the strategies used for the management of a crop. The induction of resistance seems to be an effective alternative in attenuating the effect of the disease on avocado production [7,8].

In this sense, different studies have focused on evaluating the role of silicon in plant/pathogen interactions, finding that its application can induce the structural reinforcement of the plant cell, the production of antimicrobial compounds, as well as increase the resistance of the plant. This response is associated with the activation of multiple signaling pathways and modulating the expression of defense-related genes, which translates into stimulation of acquired systemic resistance [9,10].

Additionally, the ability of silicon to induce a decrease in the incidence of fungal diseases and enhance resistance in monocots and dicots has been described [11]. Moreover, the application of silicon in crops has been considered safe, since it requires a minimum concentration to attenuate the disease, can be as effective as a fungicide, and manages to increase partial resistance to almost the same level as complete genetic resistance [11,12]. Therefore, this study evaluated the effect of silicon as an inducer of the defense response in Hass Avocado plants inoculated with *P. cinnamomi* Rands, by measuring enzyme systems associated with cell wall strengthening and phenolic compounds.

2. Materials and methods

2.1. Treatments

Six-month-old Hass avocado plants (plants grafted with Hass cup and Hass rootstock), established under greenhouse conditions in individual pots, were used. The treatments consisted of: Plants irrigated with Si and inoculated with *P. cinnamomi* (SiPc), plants irrigated with Si, without inoculating (Si), plants inoculated with *P. cinnamomi* without Si (Pc), plants without application of Si and not inoculated (C). For each treatment, different times were evaluated from the inoculation with the pathogen in the corresponding treatments [3, 24, 144, and 312 hours post inoculation (hpi)], to verify the biochemical changes related to the induction of resistance. Three samples per treatment were arranged, each sample equivalent to two plants (six plants sampled in

each treatment, for each time). The distribution of the plants in the greenhouse was random; Finally, the complete experiment was repeated 3 times in different periods: in November 2018, March 2019, and in April 2019; a total of 96 Hass avocado plants were used in each repetition, and a total of 288 in the entire experiment.

2.2. Silicon application

The application of silicon was done by direct irrigation in the soil for the SiPc and Si treatments. 10 mL of soluble potassium silicate, equivalent to 0.12 g of silicon dioxide (0.2 M SiO_2) were applied daily for 10 consecutive days, for a total of 1.2 g of SiO_2 per plant [13,14]. On day 11, the plants of the corresponding treatments (SiPc and Pc) were inoculated with *P. cinnamomi* (Rodríguez, 2015).

2.3. Inoculation of Hass avocado plants with *P. cinnamomi*

The strain of *P. cinnamomi* used for the inoculation of the plants was supplied by the Corporation for Biological Research (CIB); the microorganism was replicated two weeks before in a culture medium for fungi, PDA (potato dextrose agar), taking discs of mycelium from the original culture to guarantee an optimal state at the time of inoculation. A 1 cm transverse cut was made in the stem of the Hass avocado plant with sterile blades, 5 cm above the grafting point; Subsequently, an agar disc with mycelium of the pathogen with a diameter of 5 mm was inserted into the plant wound and the wound was sealed with Parafilm [15].

2.4. Sampling for biochemical analysis

Leaves of the different treatments were taken at the corresponding times. Sampling was carried out between 10:00 a.m. and 1:00 p.m. The sampled leaves were immediately frozen with liquid nitrogen; the plant material was placed in labeled aluminum foil bags and stored at -80 °C. Subsequently, the plant material was crushed using ceramic mortars with liquid nitrogen to maintain the biochemical integrity of the material. The leaves were stored in 50 mL conical tubes until used for biochemical tests [16].

2.5. Pigment quantification.

The determination of the content of chlorophylls a, b, and carotenoids was carried out based on what was reported by Lichtenthaler and Buschmann [17], from an 80% acetone extract of the tissue sampled in each treatment.

2.6. Quantification of phenols and inhibition of the DPPH● radical

The Hass avocado leaf extract for the quantification of phenols was obtained with acetone (60%) to which the quantification of phenolic compounds was carried out by the Folin-Ciocalteu method [18], using gallic acid as a reference standard. From the same extract, the inhibition capacity of the 1,1-diphenyl-2-picrylhydrazyl (DPPH•) radical was measured, using the method reported by Alcântara et al. [19] with some modifications: Trolox was used as a standard at concentrations between 4.99 and 79.99 µM and was evaluated against the radical; DPPH• absorbance values were measured at 517 nm after 10 minutes of reaction using a 96-well UV/VIS microplate reader (Multiskan® GO Thermo scientific); each reaction consisted of 200 µL of the radical and 50 µL of the extract to be evaluated.

2.7. Phenylalanine ammonia-lyase (PAL) activity

The measurement of the PAL activity of the samples obtained was determined based on what was reported by Abhayashree et al. [20], with some modifications: extraction buffer (50 mM borate buffer, pH 8.8) and reaction buffer (50 mM borate buffer + 20 mM Phenylalanine, pH 8.8) were prepared. In a 1:20 ratio, the extraction buffer was applied to the sample. It was centrifuged at 11,000 rpm, 4 °C, 10 min. The supernatant (enzymatic extract) was taken. 200 µL of reaction buffer were added to 20 µL of enzyme extract. The blank consisted of 20 µL of extraction buffer and 200 µL of reaction buffer. After 4 min of reaction, it was read at 290 nm in a microplate reader every 10 seconds for 10 min to obtain kinetics of PAL enzyme activity. This was done at a constant temperature of 37 °C. One unit of PAL activity is defined as the amount of enzyme that generates an increase in absorbance of 0.01 at 290 nm h^{-1}. The enzyme activity was expressed as units of enzyme activity per mg of protein ($U*mg^{-1}$).

2.8. Peroxidase (POD) activity

The method to evaluate POD activity followed the procedure based on what was reported by Sellamuthu et al. [21], with some modifications: extraction buffer was prepared (100 mM Na-phosphate buffer, pH 7.0) and reaction buffer or substrate (100 mM Na-phosphate buffer, 20 mM guaiacol, pH 7.0). In a 1:20 ratio, the extraction buffer was applied to the sample and homogenized. Subsequently, they were centrifuged at 11,000 rpm, 4 °C, 30 min. The supernatant (enzymatic extract) was taken. 144 µL of reaction buffer was applied to 36 µL of enzyme extract. They were incubated for 5 min at 30 °C. 72 µL of H_2O_2 (100 mM) was applied to the mixture and the absorbance at 460 nm for 2 min was measured. The specific activity of the enzyme was calculated as ΔAbs 460 min^{-1}mg $protein^{-1}$ and expressed as units of enzyme activity per mg protein ($U*mg^{-1}$).

2.9. Polyphenol oxidase (PPO) activity

The extraction buffer (100 mM Na-phosphate buffer, pH 7.0) and the reaction buffer (0.1 M Na-phosphate buffer, 0.1 M catechol, pH 7.4) were prepared. In a 1:20 ratio, the extraction buffer was applied to the sample. Subsequently, it was centrifuged at 11,000 rpm, 4 °C, 20 min. The supernatant (enzyme extract) was taken. 3 mL of reaction buffer or substrate were applied to 100 µL of enzyme extract. The oxidation rate of catechol was monitored at 410 nm, at 25 °C for 1 min. PPO activity was calculated as the change in optical density unit (410 nm) g^{-1} FW min^{-1} [22] and expressed as units of enzymatic activity per mg of protein ($U*mg^{-1}$ of protein)

2.10. Protein quantification of enzyme extracts

Protein quantification in enzyme extracts was determined by Bradford [23] method with appropriate modifications at a protein concentration between 100 and 1 µg/mL [24].

2.11. Statistical analysis

The reported results correspond to the mean of nine determinations ± standard deviation. A one-way analysis of variance (ANOVA) was performed with a significance level of 0.05, to which the assumptions were verified [Levene's test confirms the assumption of homogeneity between treatments ($p>0.01$) and the Shapiro-Wilk test confirms that the data show a normal distribution ($p>0.05$)]. Additionally, a comparison of means was performed using the Tukey test.

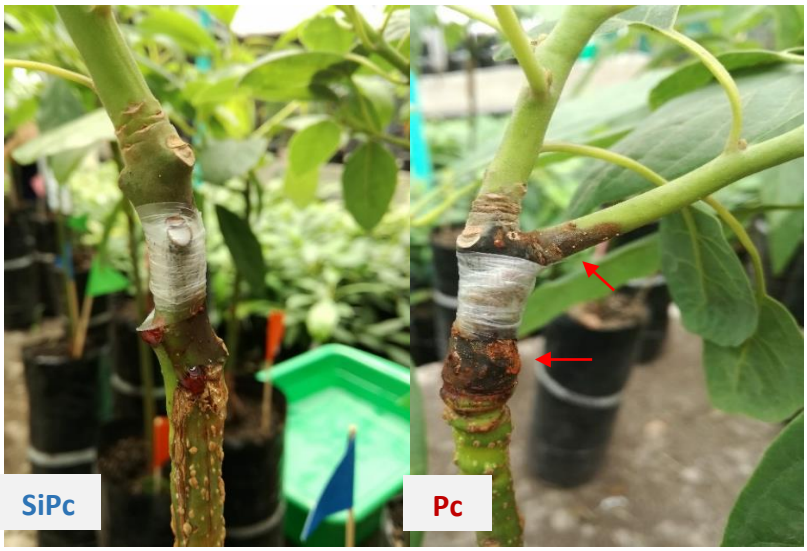
3. Results

3.1. Silicon pre-treated plants had fewer symptoms after being inoculated with *P. cinnamomi*.

The irrigation with SiO_2 was directly on the moistened soil of each Hass avocado plant, for 10 consecutive days with a 24 h difference between each application, until inoculation with the pathogen on day 11. The application of silicon was carried out previously to allow the assimilation of the nutrient via the root, corresponding to what was observed by different authors during similar tests of defense induction with silicon in commercial species [25-28]. Visually, the plants to which silicon was supplied did not show any difference from the others at the end of 10 days.

The symptomatology of the inoculated plants was notable for the darkening of the wound area, the tissue around it becoming necrotic, followed by a radial growth of the necrosis along the stem, with a mainly apical orientation (Figure 1a); Subsequently, necrosis invaded the space of the branches and a generalized wilting of the plant with the loss of tonicity was manifested, despite having an adequate water supply (Figure 1b). Those leaves and plants that showed accelerated wilting over the others, with signs of external conditions resulting from mechanical damage or possible herbivory, were not considered in the sampling carried out for the biochemical analyses.

(a)



(b)

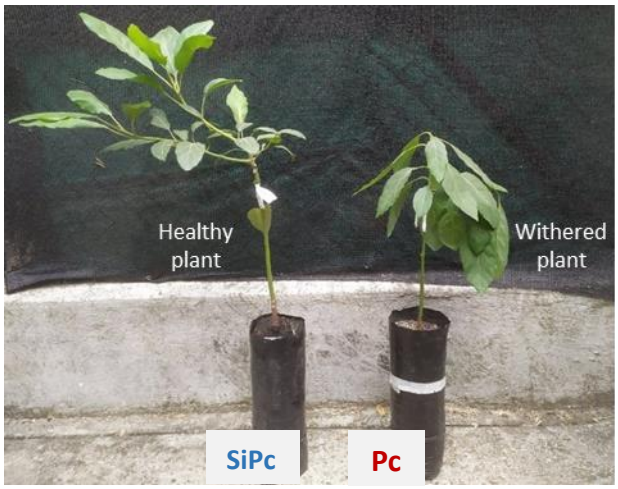
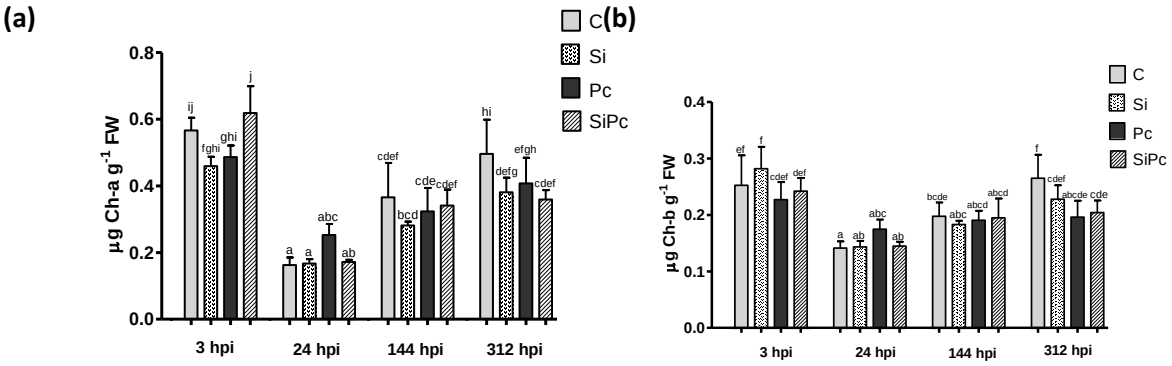


Figure 1. Comparison of the state of the plants in the SiPc and Pc treatments, 312 hpi. (a) The appearance of the lesion in the inoculation zone of *P. cinnamomi*. (b) Fitness of plants inoculated with *P. cinnamomi* with and without silicon treatment.

Chlorophyll content showed a significant decrease in the Pc treatment, from SiPc after 3 hpi; however, there were no significant changes between treatments in the content of chlorophylls a, b, and carotenoids (Figure 2), in the other times evaluated. These results suggest that under the evaluated conditions there was no affectation of the photosystems in the Hass avocado plants, even in those plants that presented a greater decay, as in the case of those subjected to the Pc 312 hpi treatment (Figure 1b).



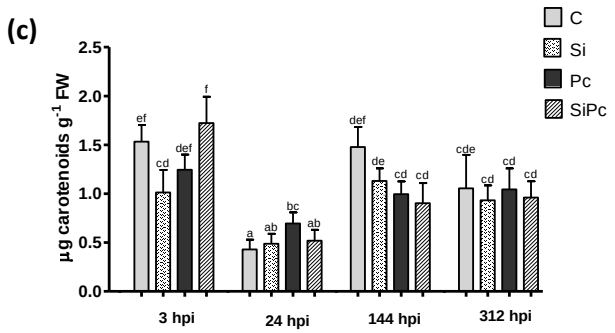


Figure 2. The total content of pigments in each treatment over time. a) Chlorophyll a content. b) Chlorophyll content b. c) Content of carotenoids. SiPc, Pc, Si and C represent potassium silicate application one day before *P. cinnamomi* inoculation, *P. cinnamomi* inoculation, potassium silicate application without *P. cinnamomi* inoculation and control (distilled water), respectively. Data are presented as the mean ± standard deviation of nine replicates. Means with the same letter are not significantly different (ANOVA followed by Tukey's test with $p<0.05$).

3.2. The presence of *P. cinnamomi* induces a higher content of phenolic compounds in the first hours of inoculation

For the quantification of total phenols, the leaf samples were kept frozen at -80 °C from the moment of collection until obtaining the extracts, to avoid the action of enzymes such as polyphenol oxidases, which could degrade these components of interest [29]. The quantification method used sought to know the total phenolic compounds, however, the type of phenols found in the extracts or the proportion of these, against other secondary metabolites was not discriminated. Meanwhile, it was wanted to know the radical stabilization capacity of these phenolic compounds, so the $DPPH^{\bullet}$ anion stabilization test was carried out, a chromophore agent that decreases its absorbance at 517 nm when it is reduced by an antioxidant either by electron transfer or by giving it protons (H^{+}) [30].

Precisely, Figure 3 shows that the Pc treatment showed the highest content of phenolic compounds at 3 hpi, differing significantly from the others. This seems to coincide with what is shown in the stabilization capacity of the radical $DPPH^{\bullet}$ (Figure 4). These data suggest that during this period, there is a marked metabolic activity that could be involved in the defensive response of Hass avocado plants. It has been shown that the increase in phenolic compounds is a response mechanism in the presence of mycelium [31].

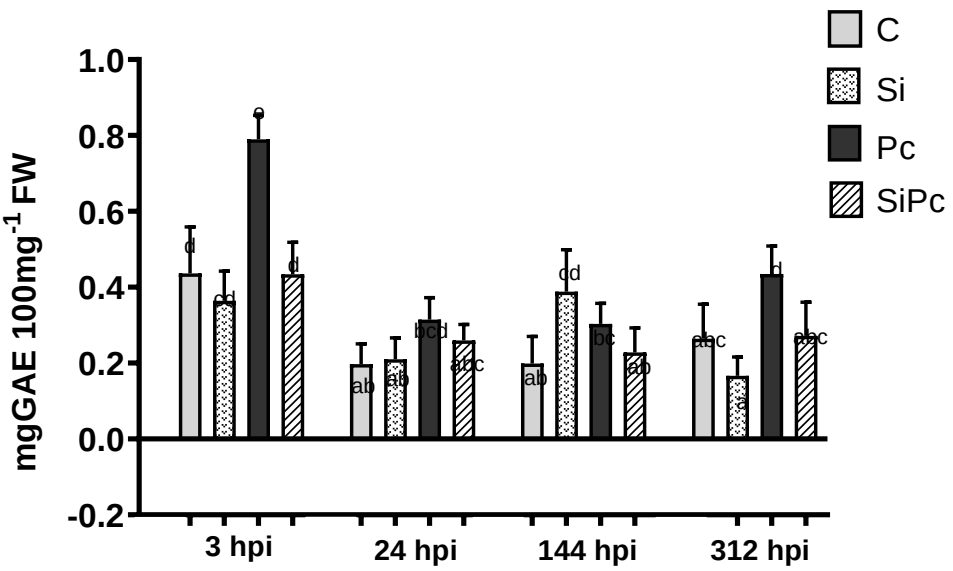


Figure 3. Total phenol content per treatment over time. GAE: gallic acid equivalents. FW: fresh weight. SiPc, Pc, Si and C represent potassium silicate application one day before *P. cinnamomi* inoculation, *P. cinnamomi* inoculation, potassium silicate application without *P. cinnamomi* inoculation and control (distilled water), respectively. Data are presented as the mean ± standard deviation of nine replicates. Means with the same letter are not significantly different (ANOVA followed by Tukey's test with $p<0.05$).

The stabilization capacity of DPPH$^{\bullet}$ shows a marked decrease up to 24 hpi in all treatments. Just as the Pc treatment showed the highest content of phenols at 3 hpi, it also showed the most abrupt decrease in the concentration of phenols and antiradical activity, this in the period between 3 h and 24 h; Taking into account that Hass avocado is susceptible to the disease caused by *P. cinnamomi*, it can be thought that this decrease in the concentration of phenols in plants without the presence of silicon as an inducer implies a decrease in the ability of the host to regulate ROS and RNS via these metabolites. From 24 hpi, the variations in the phenol content and antiradical activity of the Pc treatment are less marked; It should be noted that in the stem of each inoculated plant the growth of the necrotic lesion of the tissue surrounding the inoculation wound was maintained (Figure 1).

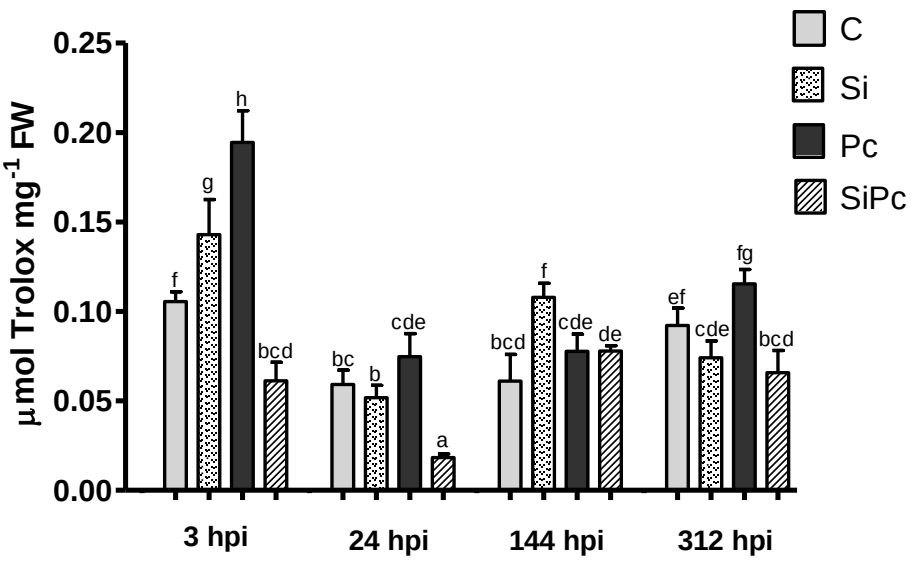


Figure 4. Stabilization capacity of the DPPH● radical over time. FW: fresh weight. SiPc, Pc, Si and C represent potassium silicate application one day before *P. cinnamomi* inoculation, *P. cinnamomi* inoculation, potassium silicate application without *P. cinnamomi* inoculation and control (distilled water), respectively. Data are presented as the mean ± standard deviation of nine replicates. Means with the same letter are not significantly different (ANOVA followed by Tukey's test with $p<0.05$).

3.3. PAL, POD, and PPO enzyme activities, early defense response

From the vegetal material of Hass avocado plants, the enzymatic extracts corresponding to the PAL, POD, and PPO enzymes were obtained. Activities were calculated based on enzyme kinetics and expressed as U*mg-1 of protein. Figures 5, 6, and 7 show the behavior over time of the PAL, POD, and PPO enzymatic activities, respectively.

Regarding the enzymatic activity of PAL, the application of silicon in Hass avocado plants does not seem to induce an increase in the presence of *P. cinnamomi* (Figure 5), this is contrary to what has been reported in different crops of commercial interest against P. their respective pathogen, after the application of silicon as a defense inducer [28,32,33]. This situation seems to coincide with what was evidenced in this study in the content of phenols (Figure 3), where instead of increasing the content of these metabolites, it decreased in the SiPc treatment.

Concerning the other treatments, at 3 hpi, treatment C presented the lowest PAL activity of the entire experiment, with statistically significant differences compared to the other treatments. At the same time, the SiPc treatment was the closest to the activity of the plants without infection and did not present significant changes in the activity of this enzyme over time. Treatment C presented a similar behavior, added to a marked increase in the activity of the PAL enzyme up to 24 hpi.

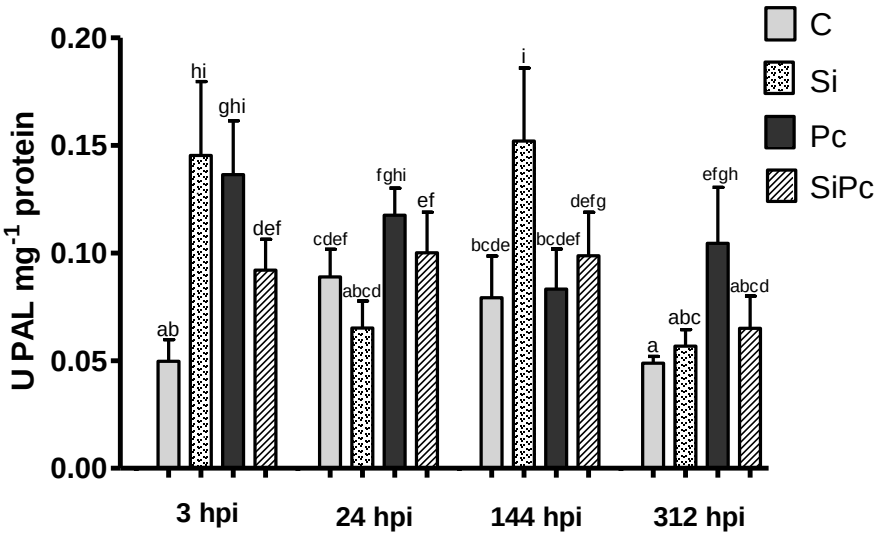


Figure 5. PAL activity per treatment over time. SiPc, Pc, Si and C represent potassium silicate application one day before *P. cinnamomi* inoculation, *P. cinnamomi* inoculation, potassium silicate application without *P. cinnamomi* inoculation and control (distilled water), respectively. Data are presented as the mean ± standard deviation of nine replicates. Means with the same letter are not significantly different (ANOVA followed by Tukey's test with $p<0.05$). U: enzyme activity units.

In Figure 6, it is possible to show the behavior in the different treatments over time of the POD enzymatic activity. As mentioned, at 3 hpi of the SiPc treatment, the highest activity of this enzyme was present, for an abrupt decrease in activity in said treatment up to 24 hpi; It is from this last time that the behavior in the different treatments is similar, with an increase in enzymatic activity until 312 hpi, unlike treatment C, which between 144 and 312 hpi does not present significant changes.

At 24 hpi, it cannot be affirmed that there are significant differences between the 4 treatments studied. Treatment C did not show significant changes over time; this was the only treatment that presented such behavior.

The behavior of the PPO enzyme evidenced in Figure 7 is like that described for POD, its activity being inversely proportional to the content of phenolic compounds of the SiPc and Pc treatments at 3 hpi. This situation is natural, considering that PPO is the main enzyme in the oxidation of phenols [34]; PPO activity has been positively correlated with resistance to diseases induced by silicon, thanks to its participation in the synthesis of lignin and the increase in the antimicrobial capacity of host plants [35].

The PPO activity also makes it possible to show again that the greatest differentiation between the treatments occurs only 3 hpi after the inoculation of the pathogen. In this time each treatment presented significant differences from the others; This is perhaps since the main metabolic changes generated in the plant defense response occur in the first hours, emphasizing that for future research it is necessary to evaluate this response at closer intervals between 0 and 24 hpi.

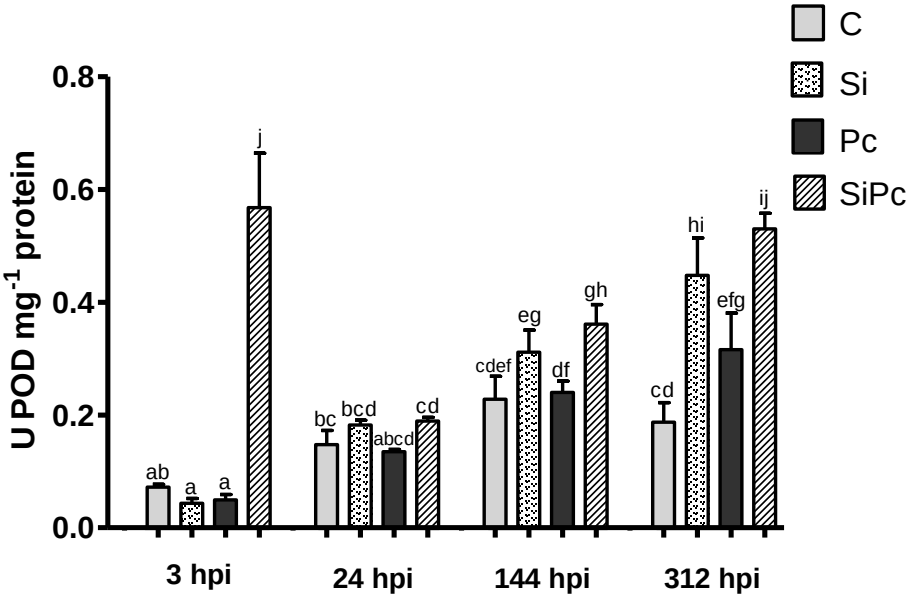


Figure 6. POD activity per treatment over time. SiPc, Pc, Si and C represent potassium silicate application one day before *P. cinnamomi* inoculation, *P. cinnamomi* inoculation, potassium silicate application without *P. cinnamomi* inoculation and control (distilled water), respectively. Data are presented as the mean ± standard deviation of nine replicates. Means with the same letter are not significantly different (ANOVA followed by Tukey's test with $p<0.05$). U: units of enzyme activity.

After 24 hpi, the treatments with the presence of the pathogen behaved similarly, with an increase in PPO activity up to 144 hpi, followed by a decrease in it up to 312 h. On the other hand,

the plants treated with silicon, without the presence of the pathogen, did not show abrupt changes in their enzymatic activity.

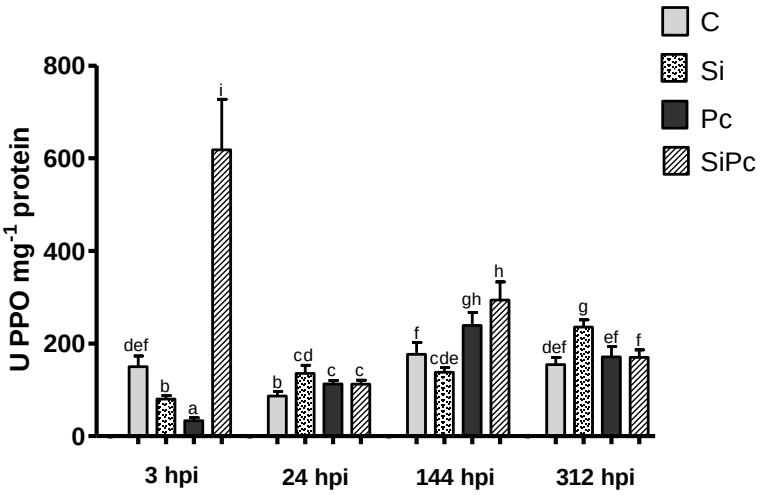


Figure 7. PPO activity per treatment over time. SiPc, Pc, Si and C represent potassium silicate application one day before *P. cinnamomi* inoculation, *P. cinnamomi* inoculation, potassium silicate application without *P. cinnamomi* inoculation and control (distilled water), respectively. Data are presented as the mean ± standard deviation of nine replicates. Means with the same letter are not significantly different (ANOVA followed by Tukey's test with $p<0.05$). U: units of enzyme activity.

4. Discussion

4.1.Pigments content suggests similar oxidative stress levels between treatments

Chlorophyll a and b levels tend to change under conditions of high oxidative stress in plants, as their synthesis and accumulation are inhibited [36]. The low variation in pigments content, between treatments over time, suggests that the oxidative conditions were not contrasting beyond 3 hpi, in the presence of the pathogen and the inducer in the Hass avocado plants.

From the above, only at the time of 3 hpi was there a significant difference in the content of chlorophyll a between SiPc and Pc. In this sense, it has been reported that silicylated plants tend to increase the activity of enzymes related to the protection of photosystems, such as superoxide dismutase (SOD), ascorbate peroxidase (APX), and peroxidases (POD), increasing in consequently the content of chlorophylls [37]. This coincides with what was evidenced in the SiPc treatment at 3 hpi, where the POD activity was significantly higher than that of the other treatments (Figure 6).

The inoculation with *P. cinnamomi* in the Pc treatment showed more noticeable symptoms (necrosis and loss of turgor) compared to that evidenced in the SiPc treatment at 312 hpi (Figure

1, b). The symptoms shown have been associated with the disease generated by the oomycete under study [38,39]. Despite this effect on plant fitness, photosynthetic pigments and carotenoid content did not show significant differences between treatments inoculated, and with or without silicon at 312 hpi. Therefore, it could be thought that the loss of turgor observed is a consequence of parameters not evaluated, unrelated to oxidative stress.

Despite the above, changes in chlorophyll content are a suggested parameter for the detection and monitoring of diseases caused by pathogens [40]. Similarly, changes in pigment content have been associated with abiotic stress in plants (mainly salinity stress) [37,41].

4.2.Phenolic compounds would be precursors of physical barriers and bioactive metabolites during the defense response of Hass avocado

The non-enzymatic mechanisms for regulating the oxidative processes that occur in the plant defense response involve phenols [42]. However, the role of phenolic compounds in the plant defense response is not always related to the regulation of ROS and RNS [43,44]. These compounds would fulfill different roles: structural and cell wall strengthening compounds [45,46], phytoalexins, phytoanticipins, antimicrobial compounds, pathogenicity modulators, defense gene activators [47], and also as radical stabilizing agents [42].

The regulation of ROS and RNS is essential in the plant defense response, since these substances can become toxic to the plant, leading to cell death [48], however, this can be desired under certain conditions to prevent the advancement of biotrophic pathogens during the hypersensitive response (HR) [49]. In addition, ROS and RNS can trigger intracellular and intercellular signaling behind the systemic host response [50], or act as antimicrobial compounds [51].

The results of the Pc treatment coincide with the report by [52]Andrade-Hoyos et al., (2015), where the content of phenols in avocado rootstocks increases in the presence of *P. cinnamomi*. In this sense, it has been found that in roots of avocado trees infected with the same pathogen, and subjected to previous treatment of silicon dioxide, the concentration of these metabolites also increased, suggesting that upon contact with the pathogen they function as physical barriers, conferring a certain resistance to the penetration of *P. cinnamomi* to the cell wall [13].

One of the characteristics of the defense responses based on phenols is the rapid and early accumulation in the infection zones, which isolate the pathogen right at the original site of entry to the host [52]. This would explain the increase in these compounds at 3 hpi in the presence of *P. cinnamomi*. Despite this, in the present study, no increase in phenols was generated in the plants inoculated with the pathogen, previously treated with silicon. That the SiPc treatment had a behavior-oriented mainly to the decrease in the concentration of phenols and the radical stabilization capacity, suggests that the defense response against the pathogen in silicylated Hass avocado plants is not directly dependent on the biological activity derived from phenolic compounds.

The SiPc and C treatments show similar behaviors during the first hours of the experiment concerning total phenols, from which it can be inferred that despite the presence of the pathogen *P. cinnamomi*, SiPc behaves in the same way as a not inoculated plant, thus avoiding the metabolic wear that entails the de novo synthesis of these metabolites [53].

The results shown in figures 3 and 4 indicate that there is a directly proportional relationship between the content of phenolic compounds and the antiradical capacity of the treatments. This correlation is not manifested in all times and treatments, because although there are significant differences in the content of phenolic compounds between treatments, there will not necessarily be differences in the reduction capacity of DPPH●.

In avocado cultivation, potassium silicate has been used to control fruit diseases, in addition to root rot, varying the mode of application between foliar, root, and stem injection; among these, the foliar application has not shown an apparent effect on the defense response [38,54,55]. The root application of silicon is more effective for the induction of defense in avocado; likewise, an increase in soluble phenolic compounds has been reported due to the root application of potassium silicate, being mainly glycosylated phenols, further suggesting that the undetected phenols are due to this fact as they are not water-soluble and are bound to the cell wall [13,56].

It could be assumed that the decrease in the content of phenolic compounds in the SiPc treatment was due to the polymerization of such compounds in structures for strengthening the cell

wall, or due to the oxidation of these metabolites. The production and transformation of soluble phenols are regulated by defense enzymes such as PAL, POD, and PPO [32]. Both, the polymerization, and the oxidation of phenols would explain the decrease in antiradical activity.

4.3.The activity of the enzymes PAL, POD, and PPO respond to the presence of the pathogen before that of Silicon, explaining the changes in the content of phenols

It has been shown that silicon can stimulate the activity of enzymes related to disease resistance during plant-pathogen interaction [57,58]. Among these enzymes are phenylalanine ammonium lyase (PAL), peroxidases (PODs), and polyphenol oxidases (PPOs), which influence the content of phenolic compounds [59].

This is how PAL catalyzes the deamination of the amino acid L-phenylalanine, generating trans-cinnamic acid as a product, which is the precursor of multiple types of phenols in the phenylpropanoid pathway, with lignin as the final product [60]. On the other hand, cell wall stiffness is, in most cases, the result of POD-mediated H_2O_2-dependent crosslinking and its participation in the final steps of lignin biosynthesis from phenolic compounds [59,61]. While PPOs are enzymes that catalyze the oxidation of monophenols and o-diphenols to o-quinones; the latter being highly reactive, generating secondary reaction products that include potentially cytotoxic ROS and o-quinone protein complexes, which generate the browning commonly observed in a wound on fruits [62].

The increase in PAL activity is a typical behavior of this enzyme against stress generated by cuts, such as that performed for the inoculation of *P. cinnamomi*, in a defensive response to strengthen the cell wall around a wound (SiPc treatments and PC) [63,64]. The PAL enzyme increased its activity in the Pc treatment but not in SiPc at 3 hpi; it is known that the expression of genes that code for phenylalanine ammonia-lyase (PALa and PALb), can be regulated with silicon treatments [65]. This decrease in PAL activity in SiPc could be due to a preexisting systemic strengthening in the tissue of Hass avocado plants due to the accumulation of silicon [55,56,59,66]. Regarding this, [67]Viviancos et al., (2015) proposed a hypothesis that silicon deposition in the plant apoplast can interfere with pathogen effectors, preventing the pathogen from inhibiting the plant defense response [68].

The Si treatment showed one of the most erratic behaviors through the different times evaluated for the PAL activity; comportment was associated with the changes seen in the content of phenols. The main differences between treatments in the activity of the PAL enzyme are evident before 24 hpi. It is recommended for future research to evaluate the behavior of PAL activity in Hass avocado at multiple points throughout the period between 0 and 24 hpi.

It has also been shown that the activity of the PAL enzyme tends to increase through elicitation with salicylic acid (SA) [69], while it is through PAL that the biosynthetic pathway of the same phytohormone occurs [70]. This allows us to infer that the SA signaling pathway did not have significant activity in Hass avocado plants inoculated with *P. cinnamomi* and treated with silicon (SiPc). At the same time, SA can also negatively interfere with POD-mediated metabolic pathways; SA-induced systemic acquired resistance (SAR) is normally mediated by elevated ROS levels, achieved through inhibition of enzymes such as catalase and POD [61]. Silicon may then have enhanced signaling by jasmonic acid (JA) in the evaluated treatments.

Regarding the regulation of POD expression, it is known that there is a joint action between SA and JA, however, a large number of POD isoforms can be induced by SA [71,72], but not in a generalized way, since some PODs do not respond to this induction [61,73]. For its part, JA and its derivative, methyl jasmonate (MeJA), have been proposed as key compounds in the positive regulation of POD enzyme expression during its participation in plant defense responses, serving as a factor of transcription of Prx genes (genes encoding enzymes with peroxidase activity) [74-76]. This information reinforces the approach that the main signaling pathway in the interaction between silicylated avocado plants and *P. cinnamomi* is given by the JA.

The POD would allow a strengthening of the cell wall of the Hass avocado plants, as it is an oxide-reducing enzyme with participation in the suberization of cellulose and the oxidation of phenols for the lignification of the cells in the defense response [77]. Both, lignification and suberization, involve the formation of a three-dimensional polyphenolic matrix within the carbohydrate matrix of the primary cell wall [78], making these compounds neither soluble nor available for quantification with the Folin-Ciocalteu method used in the present study. This phenolic component is distinguished by the presence mainly of p-hydroxycinnamic, p-coumaric,

caffeic, and ferulic acids, which constitute the cell wall-bound polyphenolic domain (PPD) [79]. A more detailed study, of the composition of phenols present in the evaluated samples, would allow the presence of the mentioned phenolic acids to be evidenced.

Similarly, the decrease in the content of soluble phenols between 3 and 24 hpi of the SiPc treatment (Figure 3), implies that these metabolites were transformed, and the biological activities of the enzymes POD and PPO, induced by silicon, could support it [59]. These changes did not occur in the Si or Pc treatment, where the content of total (soluble) phenols was higher than that of the other treatments. There it could be inferred that the phenolic compounds were not modified into structures for the strengthening of the cell wall, allowing infection with the pathogen (Pc treatment).

For the SiPc treatment, which presented the highest activity of the PPO enzyme at 3 hpi, it could be inferred that it also presented the highest concentration of the product of the enzyme activity, that is, the quinones, which are known to reach be more toxic to the pathogen than the same phenols [35]. It makes sense then that the Pc treatment was the one that presented the lowest activity of the PPO enzyme at the same time, thus avoiding the generation of antimicrobial compounds that could minimize the advance of *P. cinnamomi* in plants without an inducer.

Regarding the signaling involved in the expression of PPO, it seems that the participation of JA also plays a fundamental role when it corresponds to the defense response against a pathogen since this phytohormone is capable of positively regulating the expression of PPO [68,80].

Although multiple studies suggest the induction of phenol production by silicon as a defense response [13,14,81,82], the analysis of the plant-pathogen interaction between Hass avocado and *P. cinnamomi*, allows us to state that these metabolites are not the main line of defense, at least directly, in Hass avocado plants elicited with silicon; PPO activity is proof of the above, however, it is necessary to evaluate the presence of antimicrobial compounds that could derive from the activity of the said enzyme, such as phytoalexins and/or quinones [29], to expand knowledge about the metabolism of phenolic compounds in the studied plant-pathogen interaction.

Finally, the high enzymatic activity of PPO in the SiPc treatment suggests that substrate availability must be present; however, the decrease in the content of phenols and the low activity of the PAL enzyme seem to show that the de novo synthesis of these metabolites was low in the defense response of Hass avocado under the conditions studied. Supporting the above, [83]Rodrigues & Datnoff (2015) report that the beneficial effects of silicon become evident when plants are subjected to stress (biotic or abiotic), more than in those that grow under optimal conditions. All the above would strengthen the idea that the enzymatic activities of POD and PPO are the main responses related to the induction of defense with silicon in Hass avocado, as the maximum activity of such enzymes occurs in the SiPc treatment.

5. Conclusion

The action of the phenolic compounds was not direct during the defense response of Hass avocado elicited with silicon. That response depended mainly on the available enzymatic mechanisms, on which the phenolic compounds would possibly fulfill their role as a substrate for the generation of structural components required in the strengthening of the wall. A more detailed study on the composition of the phenols present in the evaluated samples would allow demonstrating the presence of these metabolites in more complex structures with a possible role in strengthening the cell wall. The possible deposition of silicon in plant tissue and the strengthening of the cell wall through the synthesis of compounds with a structural role is proposed as the main defense tool during the interaction of Hass avocado with the pathogen of interest.

Additionally, the action of the enzymes POD and PPO could have regulated the ROS and RNS, in addition to the generation of antimicrobial compounds as a product of the enzymatic activity induced by silicon. The main signaling pathway, suggested by the data obtained in plants induced with silicon and inoculated with the pathogen, corresponds to that of jasmonic acid, due in part to the low activity of the PAL enzyme, which is normally induced by salicylic acid; Additionally, jasmonic acid plays a fundamental role in the expression of POD and PPO enzymes. This research revealed fundamental aspects in the process of plant-pathogen interaction between Hass avocado plants in the greenhouse stage and the phytopathogen *Phytophthora cinnamomi*.

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DISCUSIÓN GENERAL

Las interacciones compatibles entre plantas y patógenos suponen un reto para la agricultura. Son grandes las pérdidas que genera y muy altos los costos del manejo de enfermedades, sumado a los problemas poscosecha, por la trazabilidad de los productos de síntesis química que deben emplearse. Sin embargo, la inducción de resistencia para el control de patógenos parece ser una alternativa viable y que cobra cada día mayor relevancia, por la tendencia hacia una agricultura sostenible. La inducción de resistencia puede definirse como el incremento de las defensas naturales de la planta contra varios patógenos y se basa en la expresión de la información genética propia de la planta, sin ocasionar alteraciones en el genoma. Esto puede ser activado por cepas de patógeno atenuadas, elicitores (glucanos, proteínas, lípidos, entre otros) o incluso compuestos químicos como el ácido salicílico, dióxido de silicio, compuestos fenólicos, ácido jasmónico o metil jasmonato, ácido β-aminobutírico, etc.

En esta tesis, se estudió el uso del elicitor de tipo β-glucano, curlano, para controlar *P. cinnamomi*. Este elicitor estimula inmunidad desencadenada por efectores (ETI) gracias a la imitación del oligosacárido de tipo β-1,3-glucano, que compone la pared celular del *Phytophthora*.

De igual manera, el uso de silicato de potasio como inductor de resistencia en plantas de aguacate Hass frente a *P. cinnamomi*, fue evaluado. De este elicitor se sabe que puede generar un reforzamiento estructural, la activación de diferentes rutas metabólicas o actuar a nivel molecular regulando diferentes genes asociados a resistencia. Por tanto, este trabajo contribuye al entendimiento de la eficacia de la inducción de resistencia con los elicitores curlano y silicio y la respuesta metabólica que tienen las plantas de aguacate Hass cuando se ven enfrentadas a la enfermedad ocasionada por el oomycete hemibiótrofo *P. cinnamomi*.

La aplicación de los elicitores en plantas de aguacate Hass, disminuyó los síntomas de la enfermedad causada por *P. cinnamomi*. Las respuestas entre las plantas inoculadas con el patógeno con (Curd+Phy) y sin el inductor (Phy), fueron en su mayoría contrastantes (Figura 1), pues el curlano moduló la expresión de diferentes enzimas como SOD, PAL, POD, PPO, contenido fenólico total, concentración de compuestos fenólicos, clorofila y fitohormonas, siendo su expresión, en casi todos los tiempos, opuesta en estos dos tratamientos. Dicha respuesta bioquímica se corresponde con el seguimiento visual, las plantas con el patógeno murieron o se

marchitaron considerablemente, mientras que las que contaban con la aplicación previa del inductor, no tenían signos de marchitamiento o si los tenían eran mínimos, todo lo cual lleva a pensar que si hubo acción protectora del curlano.

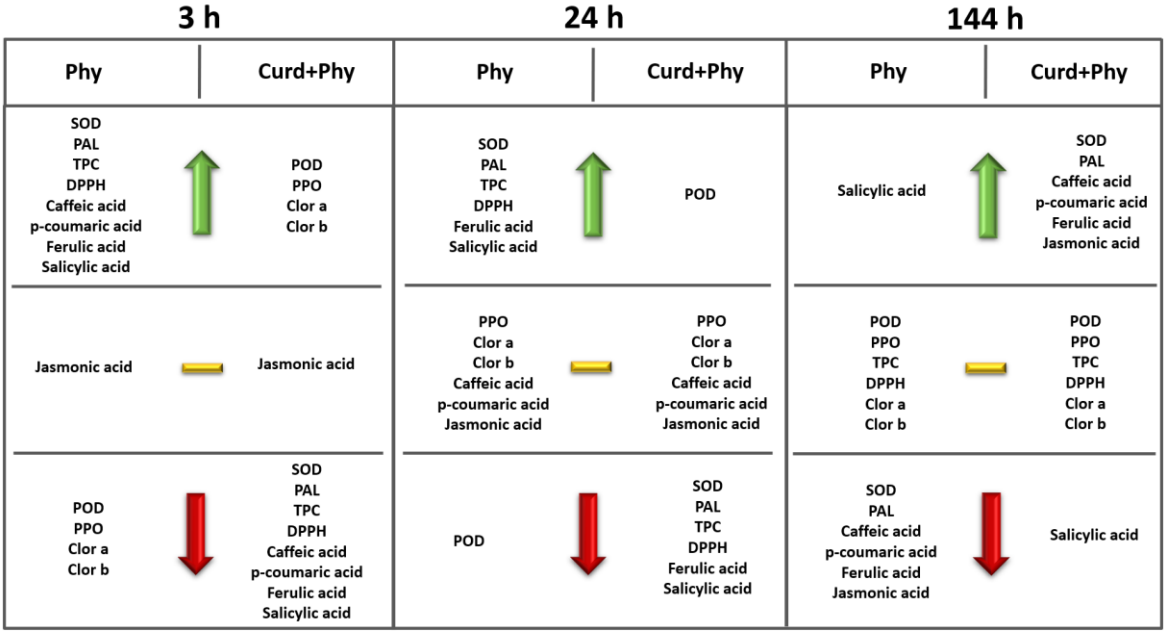

Figura 1. Resumen del efecto del tratamiento del curlano sobre las respuestas evaluadas en plantas de aguacate Hass inoculadas con *P. cinnamomi*. Phy y Curd+ Phy representan la inoculación de *P. cinnamomi* y la aplicación de curlano un día antes de la inoculación de *P. cinnamomi*, respectivamente. Las flechas de color verde indican aumento de un compuesto en un tratamiento respecto del otro, las de color rojo lo contrario, es decir, disminución de un compuesto en un tratamiento con referencia al otro y la línea amarilla muestra que no hay diferencias en la expresión en ese compuesto entre los tratamientos.

Por su parte, la aplicación de silicio al suelo tuvo un efecto positivo cuando las plantas se enfrentaron al patógeno (SiPc). Los resultados sobre el estado general de las plantas fueron similares al uso de curlano. Se observó la modulación de enzimas como PPO, POD, PAL y contenido fenólico total.

Respecto a las actividades enzimáticas, la inducción de la SOD en las células vegetales en respuesta a diferentes ambientes estresantes refleja su importante papel en el mecanismo de defensa de las plantas [1]. En el presente estudio, la enzima SOD se incrementó en las plantas inoculadas con el patógeno (Phy) en etapas tempranas, pero disminuyó en los tiempos posteriores. Mientras que las plantas inoculadas con aplicación previa de curlano (Curd+Phy) tiene un

incremento en los tiempos finales, tal vez asociado a una mayor resistencia al patógeno. Esto ha sido reportado por muchos autores, quienes han asociado el aumento de la producción de la enzima SOD con la resistencia [2-4].

Por su parte, los tratamientos con los inductores previo a la inoculación de *P. cinnamomi* incrementa las enzimas PPO y POD con un comportamiento muy similar, la mayor actividad de la enzima se observó a los 3 hai. Estas enzimas PPO y POD están implicadas en la producción de quinonas, con acción antimicrobiana, eliminación de H_2O_2 para regular diferentes vías de señalización y lignificación, con el fin de impedir la entrada en la célula de patógenos, como *P. cinnamomi*, que lo hace a través de presión mecánica. [5-7]. También se ha demostrado que el uso de elicitores aumenta tanto la actividad de la enzima POD [8,9] como de la PPO [10-12].

Ahora bien, los compuestos fenólicos forman parte de la defensa de la planta frente a los patógenos, ya sea porque se produce una oxidación de los mismos cuando hay una producción de especies reactivas de oxígeno o porque tienen una función antimicrobiana. Además, son precursores de polímeros estructurales o sirven como moléculas de señalización. Así, cuando hay una disminución de fenoles en la interacción planta-patógeno, se atribuye a su oxidación [13]. El presente estudio indicó que los tratamientos con los inductores, no afectaron la producción de fenoles ni a la actividad antioxidante, al contrario que el tratamiento que sólo tenía la inoculación del patógeno (Phy/Pc), pudiendo relacionarse esto, con la transformación enzimática de los fenoles, por parte de la peroxidasa [14] o la polifenoloxidasa (PPO), lo que significa que su concentración puede estar relacionada con otros mecanismos de defensa.

Respecto a la actividad de la enzima PAL, se presentó un comportamiento similar a la SOD para el caso del inductor curlano. Este es un resultado que se corresponde con lo reportado en la literatura donde se establece la relación entre una alta producción de esta enzima tanto con genotipos resistentes [2] como con plantas que tenían aplicación previa de un inductor [8,9,15-17]. No obstante, para el caso del silicio, la actividad de la enzima PAL para el tratamiento SiPc, no incrementó, como sí sucedió en plantas inoculadas con el patógeno (Pc). Lo anterior puede indicar que, si no hay un aumento de PAL, es probable que tampoco lo haya de SA, ya que la vía de biosíntesis de este compuesto tiene la participación de esta enzima. De hecho, autores como

Vivancos, *et al.* [18] han reportado incremento de genes que codifican para enzimas implicadas en la vía del SA, pero disminución significativa en el contenido del mismo para el genotipo resistente, sugiriendo que el Si aplicado, actuó a través de mecanismos diferentes, similar a lo que muestran los resultados de este estudio con plantas de aguacate Hass. En relación a ello, es probable inferir que el silicio pudo haber potenciado la ruta del JA, que está relacionada con la regulación positiva en la expresión de la enzima POD [19,20] , lo cual puede reforzar este planteamiento.

Entre tanto, para clorofila y carotenoides, los resultados encontrados en esta investigación se corresponden con lo reportado en la literatura. Cuando se ha aplicado un elicitor foliar de tipo oligosacárido en diferentes estreses, los contenidos de clorofila y carotenoides han aumentado o se han mantenido estables [21-25].

Sumado a los resultados de actividad enzimática, tenemos la comparación de los perfiles metabólicos, cuando el elicitor curlano fue empleado como inductor de resistencia. Con lo obtenido, es posible identificar algunas similitudes en los perfiles metabólicos de las plantas inoculadas con el patógeno con (Curd+Phy) y sin (Phy) aplicación previa del inductor: No obstante, también se observan diferencias en muchos de los metabolitos generados.

Lo anterior, es una clara señal de que, en ambos tratamientos, las plantas se enfrentaron al patógeno, ya que la mayoría de los compuestos producidos que aumentaron su concentración, tienen actividades biológicas que las protegen contra el estrés que representa el ataque de un microorganismo. Estos son: ácido caféico, ferúlico, *p*-cumárico, malónico, catequina, ácido benzoico, hidroxibenzóico, timol, estigmasterol, sitosterol, vitamina E, ácido protocatequico, floroglucionol [26,27], entre otros. De la misma forma, compuestos del metabolismo primario como ácido málico, oxálico, fumárico y succínico, aumentaron su concentración, en respuesta al gasto energético que representa un estrés biótico como lo es el ingreso de un patógeno [28]. Igualmente, existe una sobreproducción de ácido cítrico que se presume que juega un papel importante en la expresión de genes y en la señalización de metabolitos en varios organismos [29].

Sin embargo, es interesante observar cómo la aplicación de curlano, previa a la inoculación del patógeno (Curd+Phy), provoca la generación de metabolitos diferentes o los mismos, pero en

momentos opuestos a las plantas inoculadas sólo con el patógeno. Siendo esto lo que posiblemente provoque una respuesta diferencial a favor de las plantas a las que se les aplicó el inductor, para que sean capaces de sobrevivir a la enfermedad generada por la entrada de *P. cinnamomi*.

Todos estos resultados de metabolómica, coinciden con estudios previos de transcriptómica de la especie, en los que se han encontrado genes asociados a la defensa frente a oomycetos [30]. Estos permiten concluir que las plantas de aguacate Hass, susceptibles al patógeno *P. cinnamomi*, tienen la capacidad de generar todos los metabolitos necesarios para defenderse de él. Sin embargo, no lo hacen, debido a una inadecuada biosíntesis de estos, en tiempo o concentraciones incorrectas, similar a lo observado en investigaciones con otras especies de plantas, como el banano, donde se hicieron estudios al respecto [31,32].

Con base en los resultados de los compuestos fenólicos cuantificados y al contenido fenólico total, se podría establecer que la rápida producción de compuestos fenólicos en las plantas inoculadas con el patógeno (Phy) se debe a una respuesta eficaz que busca contrarrestar su efecto. Algo que se ha reportado en otros estudios en los que se asocia un incremento temprano de estos compuestos con la resistencia [10]. Sin embargo, también se ha reportado un aumento de los compuestos fenólicos en variedades susceptibles o en un estado más avanzado de la enfermedad [14,33].

En el mismo sentido, no hay que olvidar que el patógeno tiene la posibilidad de percibir continuamente la respuesta de la planta y secretar proteínas efectoras que actúan sobre el huésped, desarmando sus defensas y promoviendo la colonización [[34]. Por lo tanto, también podría ser posible que las plantas con aplicación previa del inductor, sinteticen compuestos fenólicos en momentos más avanzados buscando regular mejor la respuesta de defensa.

De igual forma, queda patente que una de las vías de señalización que estuvo involucrada en la respuesta de defensa cuando se aplicó el inductor curlano, es la del ácido jasmónico, que es opuesta a la ruta del ácido salicílico. Teniendo esto soporte en la literatura donde es claro que, las vías deben ser opuestas y estas fitohormonas deben sintetizarse en tiempos y concentraciones adecuadas, dependiendo el patógeno que se quiera controlar. Para el caso de una planta de aguacate resistente a *P. cinnamomi*, que es un patógeno hemibiótrofo, se ha determinado que el SA debe

aumentar en las primeras horas, e ir disminuyendo paulatinamente para dar paso en las 24 h, a un aumento de JA, tal como se observó en esta investigación. Cuando el elicitor se aplicó a las plantas de aguacate un día antes de la inoculación del patógeno se modulo la expresión de estas fitohormonas

Lo anterior podría explicar lo sucedido en el tratamiento Phy, donde hay un aumento de SA tan fuerte, que puede estar generando un escenario que favorece al patógeno en su etapa necrótrofa. Se ha observado que patógenos de este tipo, potencian la vía de señalización de SA para antagonizar con el ácido jasmónico y promover el desarrollo de la enfermedad en otras plantas como el tomate [35].

Además de lo anterior, existen reportes en la literatura que mencionan cuál es el comportamiento adecuado de fitohormonas para que la planta responda correctamente a *P. cinnamomi*. En estos, se menciona el papel del SA en la defensa contra patógenos biotróficos, mientras que el JA activa la defensa contra los patógenos necrótrofos [36]. Del mismo modo, estudios de genes y de transcriptómica en plantas de aguacate han encontrado que la resistencia se ha asociado a un inicio temprano de vías de señalización mediadas por SA y ET [37] pasando a un aumento de JA, después de las 24 h [30]. Así queda claro que la interacción hormonal ajusta la respuesta de defensa de las plantas contra atacantes específicos, por lo que se sabe que la interacción entre SA y JA es a menudo antagónica [38].

En general, los resultados de este estudio proporcionan nuevo conocimiento sobre la eficiencia de curlano y silicio como inductores de la respuesta de defensa en plantas de aguacate Hass frente a *P. cinnamomi*. Además, permitió plantear las posibles rutas bioquímicas a través de las cuales, las plantas de aguacate Hass inducidas, se defiendan de este patógeno, siendo la principal la del JA. Así como también, establecer que para la inducción con curlano, los perfiles metabólicos muestran la producción de diversos compuestos relacionados con la defensa, los cuales, al parecer, deben sintetizarse en determinada concentración o en un tiempo específico después de que el patógeno ingresa a la planta. Mientras que para ambos inductores, hay una rápida respuesta de enzimas relacionadas con la síntesis de compuestos con función estructural y antimicrobiana.

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CONCLUSIONES Y PERSPECTIVAS

Las principales conclusiones obtenidas de los estudios presentados en esta tesis doctoral se presentan a continuación:

1. La aplicación del elicitor curlano en plantas de aguacate Hass redujo los síntomas causados por *P. cinnamomi*, evidenciado en una menor longitud de la lesión y apariencia saludable. La respuesta entre plantas inoculadas con el patógeno con (Curd+Phy) y sin aplicación del inductor (Phy) fueron en su mayoría contrastantes. El curlano moduló la expresión de diferentes enzimas como SOD, PAL, POD y PPO. Las primeras dos aumentaron en el tiempo, mientras las últimas tuvieron una respuesta muy rápida y fuerte lo cual demuestra el efecto sobre las defensas que tuvo el curlano sobre las plantas.

2. Las plantas de aguacate Hass inoculadas con P. cinnamomi, con aplicación previa del inductor (Curd+Phy) o sin aplicación del mismo (Phy) presentan perfiles metabólicos similares en cuanto a la composición de metabolitos, es decir, generaron muchos compuestos relacionados a la defensa como ácido sinápico, protocatecúico, vitamina E, timol, entre muchos otros. Sin embargo, el tiempo de producción y la cantidad no es igual, de hecho, son contrastantes entre los tiempos de medición, sugiriendo que una adecuada defensa implica la activación de determinadas rutas metabólicas en tiempos específico, como previamente se ha evidenciado e estudios con inductores.

3. El comportamiento de las fitohormonas en el las plantas de aguacate Hass inoculadas con el patógeno (Phy) muestra un aumento sostenido en el tiempo de SA y una concentración estable de JA. Por su parte, las plantas inoculadas con *P. cinnamomi* que tenían previa aplicación del inductor (Curd+Phy), la concentración de SA se mantuvo estable mientras que la de JA aumentó a las 144 hai. El primer escenario (Phy) favorece al patógeno, entre tanto, el segundo (Curd+Phy) muestra una regulación más adecuada. Afirmación basada en lo reportado por la literatura, que indica que el JA favorece la defensa ante patógenos necrotróficos y se produce en mayor concentración después de las 24 hai en genotipos de aguacate resistentes a *P. cinnamomi*.

4. El silicio como inductor de resistencia mostró ser efectivo en la protección de plantas de aguacate Hass inoculadas con *P. cinnamomi*. Se propone que su efecto pudo estar sobre el fortalecimiento del tejido vegetal por deposición de silicio. Igualmente, las enzimas PPO y POD podrían haber regulado las ROS para modular diferentes vías enzimáticas, así como participar en la generación de compuestos antimicrobianos. Adicional, la principal vía de señalización podría ser la del JA debido a la baja actividad de la enzima PAL que normalmente es inducida por el SA.

En general, los resultados muestran efectos positivos de los inductores (curlano y silicio) sobre la regulación de la respuesta de defensa de plantas de aguacate Hass inoculadas con *P. cinnamomi*. Sin embargo, se requieren investigaciones adicionales que a futuro permitan establecer dosis adecuadas, frecuencia o intervalos de aplicación para mantener el efecto deseado. Para el caso del curlano, sería importante determinar si en campo se comporta de manera similar o se ve afectado por las condiciones climáticas y cuál es su impacto en la productividad del cultivo.

Además, sería pertinente estudiar más a fondo el efecto del silicio en la respuesta bioquímica, para ello se podría cuantificar compuestos fenólicos o estudiar los perfiles metabólicos de las plantas a través de técnicas analíticas como cromatografía de alta resolución, para obtener información que permita reforzar lo obtenido. De igual manera, sería adecuado conocer qué otros inductores son efectivos o si generan una mejor respuesta a la encontrada en esta investigación.

APÉNDICE

Apéndice 1. Abreviaturas empleadas en la tesis Doctoral

IPM	Manejo integrado de la enfermedad
P. cinnamomi	*Phytophthora cinnamomi* Rands
MAMPs	Patrones moleculares asociados a microorganismos
DAMPs	Patrones moleculares asociados a patógenos
HAMPs	Patrones moleculares asociados a herbivoría
PRRs	Patrones de receptores de reconocimiento
MTI	Inmunidad desencadenada por microorganismos
PTI	Inmunidad desencadenada por patógenos
RLKs	Cinasas tipo receptor
RLPs	Proteínas tipo receptor
ETI	Inmunidad desencadenada por efectores
ROS	Especies reactivas de oxígeno
NO	Óxido nítrico
HR	Respuesta hipersensible
SA	Ácido salicílico
JA	Ácido jasmónico
ET	Etileno
H_2O_2	Peróxido de hidrógeno
APX	Ascorbato peroxidasa
MDHAR	Monodehidroascorbato reductasa
DHAR	Dehidroascorbato reductasa
GR	Glutatión reductasa
GSH	Glutatión reducido
SOD	Superóxido dismutasa
GSSG	Glutatión oxidado
SAR	Resistencia sistémica adquirida
PAL	Fenilalanina amonio liasa

POD	Peroxidasa
PPO	Polifenol oxidasa
CDPKs	Proteína cinasa dependiente de calcio
MAPKs	Proteína cinasa activada por mitógeno
TFs	Factores de transcripción
PR	Proteínas relacionadas a patogénesis
ISR	Resistencia sistémica inducida
PRIs	Inductores de resistencia en plantas
OGAs	Oligosacáridos
DP	Grado de polimerización
CERK 1	Receptor de quitina
PME	Enzima pectinametilesterasa
PGN	Péptido glucanos
NB-LRR	Dominio de repetición rica en leucina, enlazada a nucleótido
LecRK	Cinasa receptor de lectina
CC-NB-LRR	Proteínas de hélice superenrollada de unión a nucleótidos, con repetición rica en leucina
PDA	Agar papa dextrosa
DMSO	Dimetil sulfóxido
NaOH	Hidróxido de sodio
Curd+Phy	Plantas tratadas con curlano 1 día antes de la inoculación de *P. cinnamomi*
Curd	Plantas tratadas con curlano sin la inoculación del patógeno
Phy/Pc	Plantas inoculadas con Phytophthora cinnamomi
hai	Horas después de la inoculación
EDTA	Ácido etilendiaminotetracético
DTNB	Ácido ditionitrobenzoico (Reactivo de Ellman)
NADPH	Nicotinamida adenina dinucleótido fosfato oxidado
HCl	Ácido clorhídrico

TPC	Contenido fenólico total
FW	Peso fresco
DPPH	2,2-Difenil-1-Picrilhidrazilo
Chl a	Clorofila a
Chl b	Clorofila b
PSI	Fotosistema I
PSII	Fotosistema II
GC-MS	Cromatografía de gases acoplada a masas
UPLC-MS	Cromatografía líquida acoplada a masas de ultra alta resolución
BHT	2,6-di-ter-butil-4metilfenol
MEOX	Clorhidrato de metoxiamina
MTSF	Metil-N-(trimetilsilil)trifluoroacetamida
MeOH	Metanol
EI	Impacto electrónico
ESI	Ionización electroespray
PCA	Análisis de componentes principales
Si	Silicio/Plantas con aplicación de silicio
SiPc	Plantas con aplicación de silicio e inoculadas con *P. cinnamomi*
C	Planas sin aplicación de silicio y no inoculadas
hpi	Horas después de la inoculación de *P. cinnamomi*
SiO_2	Dióxido de silicio
RNS	Especies reactivas de nitrógeno
MeJA	Metil jasmonato
Prx genes	Genes que codifican para enzimas con actividad peroxidasa
PPD	Dominio polifenólico unido a la pared celular

APÉNDICE II.

LISTA DE PUBLICACIONES

Guarnizo, N., Oliveros, D., Murillo-Arango, W., & Bermúdez-Cardona, M. B. **Oligosaccharides: Defense inducers, their recognition in plants, commercial uses and perspectives.** *Molecules* (2020),25, 5972. Doi:10.3390/molecules25245972

Guarnizo, N.; Álvarez, A.; Oliveros, D.; Barbosa, O.; Eras, J.; Bermúdez-Cardona, M.B.; Murillo-Arango, W. **Elicitor Activity of Curdlan and Its Potential Application in Protection of Hass Avocado Plants against *Phytophthora cinnamomi* Rands**. *Horticulturae* (2022), 8, 646. Doi:10.3390/horticulturae8070646.

MANUSCRITOS SOMETIDOS

Metabolomic analysis of Hass avocado plants inoculated with *Phytophthora cinnamomi* responses to curdlan elicitor. Guarnizo, N., Álvarez, A., Oliveros, D., Peñaloza-Atuesta, G.C., Murillo-Arango, W., & Eras, J. Sometido en *International Journal of Agricultural Sustainability*.

Resistance induction with silicon in Hass avocado plants inoculated with *Phytophthora cinnamomi* Rands. Álvarez, A., Oliveros, D., Ávila, Y. C., Sabogal-Palma, A.C., Murillo-Arango, W., Eras, J., Bermúdez-Cardona, M. B., & Guarnizo, N. Sometido en *Plant Signaling & Behavior*.

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Análisis metabolómico de plantas de aguacate Hass inoculadas con Phytophthora cinnamomi. Guarnizo, N.; Álvarez, A.; Oliveros, D.; Bermúdez-Cardona, M.B.; Eras, J.; Murillo-Arango, W. 34° Congreso Latinoamericano de Química CLAQ 2020, el XVIII COLACRO, el X COCOCRO, el II SPAE y el IV C2B2, 2021. Cartagena (Colombia). **Ponencia oral.**

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Efecto de la inducción de resistencia sobre la respuesta de defensa en plantas de aguacate Hass inoculadas con *Phytophthora cinnamomi*. Guarnizo, N.; Álvarez, A.; Oliveros, D.; Bermúdez-Cardona, M.B.; Murillo-Arango, W. I Congreso internacional de ciencia y tecnología en el trópico: aguacate Hass y cacao, 2019. Ibagué (Colombia). **Poster**.