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MANEJO DE LA NUTRICIÓN INTEGRAL PARA LA ESTIMULACIÓN DE PRECURSORES DE METABOLITOS SECUNDARIOS EN CAÑAMO MEDICINAL

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Presenta:

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Bajo la supervisión de:

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MANEJO DE LA NUTRICIÓN INTEGRAL PARA LA ESTIMULACIÓN DE PRECURSORES DE METABOLITOS SECUNDARIOS EN CAÑAMO MEDICINAL

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CONTENIDO

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El desarrollo de esta tesis doctoral ha sido un camino de profundo crecimiento, no solo profesional y científico, sino también personal. Esta investigación, como toda buena cosecha, es el resultado de la paciencia, el cuidado y el esfuerzo conjunto de muchas personas a las que hoy deseo expresar mi más profunda gratitud.

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Formación y trayectoria profesional

Realicé mis estudios de Licenciatura como Ingeniero Agrónomo Especialista en Suelos en la Universidad Autónoma Chapingo, donde me titulé con mención por mi investigación en “Diagnóstico nutrimental del duraznero cultivado en cepa-zanja en Altzayanca, Tlaxcala”. Posteriormente, obtuve el grado de Maestro en Ciencias en Agroforestería para el Desarrollo Sostenible por la Universidad Autónoma Chapingo, especializándome en la dinámica de nutrientes, fisiología vegetal y la bioestimulación integral.

Mi trayectoria profesional se ha centrado en el sector agroindustrial, con un enfoque particular en el cumplimiento de las normativas para la producción agrícola, destacando el dominio de la NOM-021-RECNAT-2000 para el análisis de suelos, la calidad e inocuidad para la certificación de los sistemas productivos y la NOM-051 para el etiquetado de alimentos. En el ámbito regional, he colaborado en proyectos productivos agrícolas, diseñando planes de fertirrigación y optimizando la producción agrícola de las comunidades locales.

Como investigador, mi interés primordial radica en el metabolismo secundario de las plantas, específicamente en la biosíntesis de cannabinoides, terpenos y antioxidantes en especies medicinales como el *Cannabis* spp. He explorado profundamente la intervención fisiológica mediante el uso de bioestimulantes innovadores como los brasinoesteroides, silicio y moléculas farmacológicas para la manipulación de perfiles fitoquímicos y la resiliencia ante el estrés biótico y abiótico.

MANEJO DE LA NUTRICIÓN INTEGRAL PARA LA ESTIMULACIÓN DE PRECURSORES DE METABOLITOS SECUNDARIOS EN CAÑAMO MEDICINAL†

RESUMEN GENERAL

El cáñamo es una especie de gran interés por su capacidad para sintetizar metabolitos secundarios con aplicaciones terapéuticas, medicinales y nutracéuticas. Sin embargo, se ha documentado que la producción y calidad fitoquímica dependen de factores fisiológicos y del manejo agronómico, incluida la nutrición especializada. En esta investigación se evaluó el efecto del manejo integral de la nutrición sobre la estimulación de precursores y metabolitos en *Cannabis* spp., integrando nutrición mineral, bioestimulantes, microorganismos benéficos y fitorreguladores. Los resultados mostraron que la suplementación nutricional mejoró el balance mineral y la acumulación de fenoles y flavonoides; la simbiosis microbiana incrementó la biomasa y la capacidad antioxidante; y la dosis optima de brasinólidos (1.0 mg L^{-1}) elevó las concentraciones de cannabinoides (CBD, THC y CBG) y diversificó el perfil terpénico sin afectar el rendimiento. En su conjunto, el manejo nutricional integrativo moduló positivamente diferentes rutas metabólicas como la del shikimato, la MAV y la MEP, estimulando la producción de compuestos bioactivos para la salud humana. Se concluye que la nutrición integral representa una estrategia sustentable y eficiente para mejorar la calidad fitoquímica del cáñamo, fortaleciendo su potencial como cultivo de alto valor para la industria farmacéutica.

Palabras clave: Microorganismos benéficos, nutrición integrativa, brasinólidos, metabolitos secundarios, cannabinoides.

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INTEGRATED NUTRITIONAL MANAGEMENT TO STIMULATE SECONDARY METABOLITE PRECURSORS IN MEDICAL HEMP†

GENERAL ABSTRACT

Hemp is a species of great interest due to its ability to synthesize secondary metabolites with therapeutic, medicinal, and nutraceutical applications. However, it has been documented that the production and phytochemical quality of the plant depend on physiological factors and agronomic management, including specialized nutrition. In this study, the effect of integrated nutritional management on the stimulation of precursors and metabolites in *Cannabis* spp. was evaluated, integrating mineral nutrition, biostimulants, beneficial microorganisms, and phytohormones. The results showed that nutritional supplementation improved mineral balance and the accumulation of phenols and flavonoids; microbial symbiosis increased biomass and antioxidant capacity; and the optimal dose of brassinolide (1.0 mg L^{-1}) enhanced cannabinoid concentrations (CBD, THC, and CBG) and diversified the terpene profile without affecting yield. Overall, the integrative nutritional management positively modulated different metabolic pathways such as the shikimate, MVA, and MEP pathways, stimulating the production of bioactive compounds beneficial to human health. It is concluded that comprehensive nutrition represents a sustainable and efficient strategy to improve the phytochemical quality of hemp, strengthening its potential as a high-value crop for the pharmaceutical industry.

Keywords: Beneficial microorganisms, integrative nutrition, brassinolide, secondary metabolites, cannabinoids.

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1. INTRODUCCIÓN

La planta de cáñamo (*Cannabis* spp.) ha sido domesticada durante aproximadamente 5,000 años hasta la fecha en Asia central (Booth y Bohlmann, 2019). De acuerdo con Andre et al. (2016), el cultivado del cáñamo, en la actualidad, está adquiriendo gran importancia por su versatilidad, ya que provee fibras textiles, proteína cruda, compuestos fitoquímicos y servicios ambientales (captura de carbono). Para la industria, existen variedades bajas en concentraciones de compuestos psicoactivos, utilizadas en la producción de fibras textiles y aceites esenciales. En particular, el sector farmacéutico muestra interés por sus metabolitos con propiedades bioactivas para la salud humana (Andre et al., 2016). Las leyes sobre el cultivo de *Cannabis* spp. están cambiando rápidamente a nivel mundial y México no es la excepción; con la legalización judicial para el uso médico y recreativo, está emergiendo una nueva industria que demandará materia prima sustentable y de alta calidad nutracéutica (Beitlich, 2019).

De manera general, Carranza (2012) afirmó que la composición química de *Cannabis* spp. es muy compleja, debido a que contiene más de 400 productos fitoquímicos (terpenos, hidrocarburos, azúcares, flavonoides, esteroides, compuestos nitrogenados y aminoácidos). Aunado a lo anterior, posee un total de 66 cannabinoides, sobresaliendo el 9-tetrahidrocannabinol (THC), un compuesto psicoestimulante, y el cannabidiol (CBD), una sustancia terapéutica. Andre et al. (2016) señalaron que los quimiotipos de *Cannabis* sintetizan la gran mayoría de sus precursores y metabolitos finales mediante diferentes rutas metabólicas: la vía del ácido shikímico, ácido malónico, mevalonato, policétida (OLA) y plastidal (MEP) (Raharjo et al., 2004; Baud et al., 2007).

El manejo de la nutrición y la bioestimulación con microorganismos benéficos puede impactar directamente en el mecanismo de las rutas bioquímicas mencionadas previamente (Bernstein et al., 2019; Yu et al., 2022). Por un lado, investigaciones recientes demostraron que las cantidades de nutrientes suministrados a la planta modificaron la concentración interna de metabolitos. Por ejemplo, Saloner y Bernstein (2021) evidenciaron que al incrementar las dosis de nitrógeno en la

solución nutritiva la producción de cannabinoides decrecía entre un 60 y 70 %; Saloner y Bernstein (2022a) también observaron el mismo efecto con altas concentraciones de potasio en solución. Por otro lado, se ha documentado resultados satisfactorios con el empleo de microorganismos. La inoculación de *Bacillus* sp., *Mucilaginibacter* sp. y *Pseudomonas* sp. en el cultivar CBD Kush indujo un buen desarrollo fisiológico en etapa de vivero (Lyu et al., 2022). Otro trabajo comprobó que la inoculación con 2×10^{12} UFC kg^{-1} de Trichoderma*. harzianum* aplicado vía fertirriego en cultivares Fedora 17 y Felina, incrementó significativamente la concentración de CBD en las inflorescencias (Kakabouki et al., 2021).

Asimismo, las moléculas señalizadoras (bioestimulantes y reguladores del crecimiento) han demostrado un efecto positivo al impulsar la síntesis de compuestos fitoquímicos. Dentro de los bioestimulantes, el trabajo realizado en *Echinacea purpurea* reveló que la adición de aminoácidos aromáticos al medio de cultivo hidropónico incrementó la concentración de metabolitos derivados del ácido cafeico en tejidos foliares (Tabar et al., 2019). En fresa, la aplicación de silicato de potasio en la solución nutritiva elevó significativamente el contenido de compuestos fenólicos totales y la capacidad antioxidante en el producto cosechado (El-Sayed et al., 2023). Para el caso de las fitohormonas, Zhu-mei et al. (2013) encontraron que la aplicación foliar de brasinoesteroides en uva 'Cabernet Sauvignon' generó una mayor síntesis de metabolitos secundarios como antocianinas, compuestos fenólicos, flavonoides y taninos. Un estudio en pepino mostró que la irrigación con ácido salicílico a bajas concentraciones promovía una mayor actividad enzimática de compuestos antioxidantes (Nie et al., 2021). La nutrición especializada se refiere a la aplicación de macronutrientes, micronutrientes y compuestos bioactivos (bioestimulantes, elicitores y aminoácidos), diseñada para satisfacer demandas fisiológicas específicas de la planta bajo condiciones particulares, como situaciones de estrés, etapas fenológicas, potenciación de cualidades organolépticas o incremento del valor nutricional (Selim, 2020). No obstante, a pesar de ser amplio el campo de estudio del *Cannabis*, existe escasa información sobre el empleo integrado de soluciones nutritivas, microorganismos, bioestimulantes y

fitorreguladores específicos para incrementar la producción de compuestos fitoquímicos y garantizar la calidad nutracéutica de sus tejidos aprovechables.

Los desafíos actuales se centran en resolver el déficit de materia prima de calidad para su procesamiento por las industrias nacionales, que abastecen a los mercados con productos beneficiosos para la salud. Este trabajo puede servir de base para el aprovechamiento de otros cultivares y especies vegetales con la finalidad de manipular la producción de metabolitos secundarios a través de técnicas nutricionales integrativas, lo que repercutirá favorablemente en la rentabilidad de pequeños y medianos productores, comunidades rurales y organizaciones de la salud de nuestro país. Es por ello que, el objetivo de la presente investigación fue evaluar distintas soluciones nutritivas, dosis de microorganismos benéficos y niveles de bioestimulantes en sistemas sin suelo, como parte del manejo de la nutrición especializada para intensificar la producción de metabolitos secundarios en cáñamo.

OBJETIVOS

Objetivo General

Evaluar distintas soluciones nutritivas, dosis de microorganismos benéficos y niveles de bioestimulantes en sistemas de cultivo sin suelo, como parte del manejo de la nutrición integral durante la producción de metabolitos secundarios en cáñamo (*Cannabis* spp.).

Objetivos Específicos

- Medir la efectividad de diferentes soluciones nutritivas complementadas con bioestimulantes a través de indicadores nutrimentales, metabólicos, morfológicos y de rendimiento para establecer la de mejor desempeño en *Cannabis indica*.

- Determinar el efecto de diferentes dosis de *Trichoderma* spp. y *Pseudomonas fluorescens* mediante inoculación asociativa al sistema hidropónico para definir la de mejor capacidad fisiológica y nutraceútica durante el periodo vegetativo de *Cannabis sativa* L.

- Evaluar el impacto de diferentes niveles de brasinoesteroides mediante aplicación foliar durante la etapa de floración en *Cannabis sativa* L. bajo un esquema nutricional integrativo para delimitar la concentración potenciadora de mayor rendimiento de compuestos fitoquímicos.

HIPÓTESIS

- La adición de soluciones nutritivas complementadas con diferentes bioestimulantes repercutirán en un desarrollo óptimo de la planta y mayor producción de metabolitos secundarios medicinales en *C. indica*.

- La coinoculación de microorganismos benéficos al sistema hidropónico sin sustrato promoverá un medio de crecimiento óptimo para aumentar la producción de metabolitos antioxidantes en tejidos foliares de *C. sativa* L.

- La aplicación de brasinoesteroides vía foliar actuará positivamente sobre el rendimiento de inflorescencias y metabolitos secundarios en el cultivo de *C. sativa* L.

2. MARCO TEÓRICO

2.1. Síntesis bioquímica de metabolitos secundarios

El cáñamo (*Cannabis* spp.) se ha aprovechado desde el neolítico (5000 a. C.) hasta la actualidad en países asiáticos con fines industriales y medicinales (Booth y Bohlmann, 2019). Esto se debe a que es una especie multipropósito de la cual se extraen aceites esenciales, fibras textiles, proteínas vegetales y compuestos farmacéuticos (Andre et al., 2016). La composición fitoquímica de esta planta es compleja, se han identificado más de 500 compuestos derivados del metabolismo secundario, principalmente terpenos, flavonoides, fenoles, alcaloides, cannabinoides y taninos (Ángeles et al., 2014).

2.1.1. Fenoles

Los compuestos fenólicos, polifenoles o fenilpropanoides son metabolitos secundarios caracterizados por la presencia de, al menos, un grupo fenol (anillo aromático unido a un radical hidroxilo) (Flores-Sánchez, 2008). Estos compuestos abarcan desde moléculas sencillas (ácidos fenólicos) hasta polímeros complejos (taninos y lignina). Los ácidos fenólicos son precursores de otros fitoquímicos importantes como taninos, cumarinas, benzoquinonas y naftoquinonas. Entre estos ácidos, se encuentran comúnmente el ácido cafeico, ferúlico, p-hidroxibenzoico, protocatecuico, vainíllico, salicílico y gálico. Este último, junto con sus derivados ésteres, destacan por su papel medicinal en la salud, debido a sus efectos antioxidantes, antimicrobianos, antiinflamatorios, anticancerígenos, cardioprotectores, gastroprotectores y neuroprotectores (Kahkeshani et al., 2019). Según García y Carril (2011), se han descrito dos rutas para la biosíntesis de dichas moléculas en plantas superiores: la del ácido shikímico y la del ácido malónico.

La ruta del shikimato es responsable de la síntesis de la mayoría de los compuestos fenólicos. Esta comienza con la acumulación de ácido fosfoenolpirúvico (PEP) proveniente de la glucólisis y eritrosa-4-fosfato (E4P) del ciclo de las pentosas fosfato. Mediante enzimas como la fenilalanina amonio liasa (PAL) y policétido sintasas (PKS), éstos son transformados en aminoácidos aromáticos para obtener

a los ácidos cinámico y cumárico como precursores base (Ziemert y Jensen, 2012). Finalmente, las enzimas cinamato-4-hidroxilasa (C4H) y 4-cumarato-CoA ligasa (4CL) catalizan una serie de hidroxilaciones y metilaciones de los ácidos mencionados para originar ligninas, alcaloides, flavonas, isoflavonas y flavonoides (Fraser y Chapple, 2011; Yadav et al., 2020).

Aunque la ruta del ácido malónico es más destacada en hongos y bacterias, también opera en plantas, a menudo interconectada con la ruta del ácido shikímico (Austin y Noel, 2003). En este proceso metabólico, el precursor inicial es el acetil-CoA, que se carboxila para formar malonil-CoA mediante las acetil-CoA carboxilasas (ACC). El malonil-CoA actúa como donante de unidades de carbono para las reacciones de condensación catalizadas por enzimas PKS, lo que permite originar productos fenólicos. Por ejemplo, tres moléculas de malonil-CoA se condensan con una molécula de p-cumaroil-CoA (derivado del ácido shikímico) para formar naringenina chalcona, el esqueleto principal de todos los flavonoides (Dibyendu, 2015; Yu y Jez, 2008).

2.1.2. Flavonoides

Los flavonoides son una subclase de compuestos fenólicos que comparten un esqueleto base (difenilpropano) de 15 carbonos, conformado por dos anillos de fenilo (A y B) y uno heterocíclico (C) (Karak, 2019; Panche et al., 2016). Entre las funciones generales de los flavonoides, se destaca la generación de una amplia gama de colores en los tejidos de las plantas, así como el desempeño de funciones de protección contra la radiación UV, la defensa contra patógenos y la señalización (Panche et al., 2016). Actualmente, se han descrito alrededor de 8,000 tipos de flavonoides, incluidos agliconas, glucósidos y polímeros (Terahara, 2015).

De acuerdo con Panche et al. (2016), los compuestos flavónicos se pueden clasificar en flavonas, flavonoles, isoflavonas, flavanonas, flavanonoles, flavanoles o catequinas, antocianinas y chalconas. Los flavonoles se caracterizan por presentar un grupo cetona en su estructura principal, resaltan por ser los más abundantes en productos vegetales como raíz, follaje, flor y fruto (Terahara, 2015). Los compuestos con mayor número de trabajos de investigación son el kaempferol,

la quercetina, la miricetina y la fisetina, ya que se les atribuyen propiedades nutracéuticas y medicinales para la salud humana (Karak, 2019). La quercetina es un metabolito vegetal de gran importancia en el metabolismo humano como parte de una dieta equilibrada, ya que ha demostrado ser un potente antioxidante, antiviral, antiinflamatorio crónico, antienvejecimiento celular y anticancerígeno. Por lo que, se recomienda un consumo promedio de 50 - 1000 mg día^{-1} (Terahara, 2015; Karak, 2019).

La biosíntesis de este subgrupo de metabolitos evidencia una sinergia entre las rutas del metabolismo secundario. El anillo A y parte del anillo C se derivan de la condensación de tres moléculas de malonil-CoA (ruta del ácido malónico), mientras que el anillo B y la otra parte del anillo C se originan a partir del p-cumaroil-CoA (ruta del ácido shikímico) (Liu et al., 2021). Una vez formado el esqueleto base (naringenina chalcona) a través de la enzima chalcona sintasa (CHS), la chalcona isomerasa (CHI) cataliza la ciclación de la chalcona para formar naringenina. A partir de esta, la ruta se ramifica para producir las diferentes clases de flavonoides previamente descritos, mediante la activación de una serie de enzimas como la flavanona 3-hidroxilasa (F3H), la flavonol sintasa (FLS) y la flavona sintasa (FNS). (Nabavi et al., 2020; Li et al., 2021). La expresión de los genes implicados en el proceso anterior está controlada por el complejo de transcripción MBW (factores MYB, bHLH y WD40), el cual responde a señales de desarrollo celular y a estímulos ambientales (Xu et al., 2015). En *C. sativa* L., se han identificado varias de estas enzimas, y su expresión varía según el quimiotipo y las condiciones del cultivo (Bautista et al., 2021).

2.1.3. Cannabinoides

Los cannabinoides son un grupo de metabolitos secundarios constituidos por 21 o 22 carbonos (formas carboxiladas) y que son predominantes dentro de la planta (Andre et al., 2016). Se han descrito alrededor de 70 tipos de estos compuestos fitoquímicos y se ha caracterizado que son los metabolitos más abundantes en toda la planta (Ángeles et al., 2014). El tetrahidrocannabinol (THC) o dronabinol es importante debido a su actividad psicoactiva, aunque se le atribuyen

propiedades medicinales contra náuseas, vómitos y pérdida de apetito ocasionados por quimioterapias agresivas y el síndrome de inmunodeficiencia adquirida (SIDA), entre otros (Vilchis-Valentín et al., 2021). Por otro lado, el cannabidiol (CBD) presenta efectos positivos contra la epilepsia y problemas cardiovasculares; además, de contrarrestar síndromes psiquiátricos (depresión y ansiedad), desórdenes del sueño, inmunosupresivo, antiemético, antiinflamatorio y neuroprotector (Beitlich, 2019). Los fitocannabinoides se concentran principalmente en la cavidad secretora de los tricomas glandulares presentes en flores femeninas; sin embargo, también pueden sintetizarse en otras partes de la planta (Andre et al., 2016), y son metabolizados en su forma ácida. No obstante, para ser reconocidos por los receptores neuronales se requiere descarboxilarlos mediante calentamiento (>80 °C) o incineración (Ángeles et al., 2014).

En consecuencia, es vital comprender su proceso de formación. Por ello, Andre et al. (2016) describieron el origen de los compuestos cannábicos a partir de dos vías principales: la ruta policétida (ácido olivetólico) y la plastidal (MEP) para obtener pirofosfato de geranilo (GPP) (Booth y Bohlmann, 2019). La biosíntesis de cannabinoides comienza con la producción del primer intermediario, conocido como ácido olivetólico (OLA). La formación de OLA se logra mediante la ciclización de un compuesto policétido, el cual, proviene de la condensación de una molécula de n-hexanoil-CoA con tres moléculas de malonil-CoA (Figura 3), catalizada por la enzima policétida sintasa (PKS). Después, se lleva a cabo la prenilación del OLA por GPP o por pirofosfato de farnesilo (FPP), obteniendo ácido cannabigerólico (CBGA). Este producto es el precursor base de las formas ácidas de los principales cannabinoides, como el ácido 9-tetrahidrocannabinólico (THCA) y el ácido cannabinólico (CBDA) (Lyu et al., 2023). Por último, la actividad enzimática será responsable de la diversidad de cannabinoides, por ejemplo, la THCA sintasa (THCAS) convierte el CBGA en THC, la CBDA sintasa (CBDAS) forma CBD y la CBCA sintasa (CBCAS) produce CBC (Raharjo et al., 2004).

2.1.4. Terpenos

Las interacciones mediante señales bioquímicas desempeñan un papel fundamental en el crecimiento y desarrollo de las plantas, la respuesta ambiental y los procesos fisiológicos (Yang et al., 2020). Boncan et al. (2020) afirmaron que las interacciones bidireccionales y dinámicas de las plantas con el medio ambiente propician la síntesis de compuestos orgánicos volátiles (COV), incluyendo a los terpenos. Estos actúan como medio de comunicación en forma de señales atractivas o disuasorias entre plantas vecinas y organismos benéficos o patogénicos. Los terpenos y terpenoides son la clase más diversa de metabolitos secundarios en plantas, con más de 80,000 compuestos identificados hasta la fecha (Yang et al., 2020). Entre sus principales funciones, se les atribuye ser los causantes de los aromas característicos de cada especie vegetal, lo que contribuye a la defensa contra herbívoros y patógenos, a la atracción de polinizadores y a la formación de precursores de hormonas vegetales (Vranová et al., 2013).

Investigaciones previas han descrito dos vías biosintéticas importantes para la formación de los compuestos mencionados. La primera ruta, la del mevalonato (MVA), inicia en el citosol celular con la condensación de tres moléculas de acetil-CoA, mediante una serie de reacciones catalizadas por la enzima HMG-CoA reductasa, para producir ácido mevalónico. Posteriormente, este ácido se convierte en isopentenil pirofosfato (IPP) y su isómero dimetilalil pirofosfato (DMAPP), que son los esqueletos básicos (C5) de todos los terpenos. Finalmente, se obtienen los sesquiterpenos (C15), triterpenos (C30) y esteroles (Dubey et al., 2003; Vranová et al., 2013; Yang et al. 2020). La segunda ruta, la del metileritritol fosfato (MEP), se lleva a cabo en los plástidos empleando piruvato y gliceraldehído-3-fosfato (G3P) como sustratos iniciales. Las enzimas 1-deoxi-D-xilulosa-5-fosfato sintasa (DXS) y 1-deoxi-D-xilulosa-5-fosfato reductoisomerasa (DXR) catalizan una serie de reacciones para producir IPP y DMAPP. Luego, el IPP y el DMAPP son condensados por la acción de preniltransferasas como la geranil pirofosfato sintasa (GPPS) y la farnesil pirofosfato sintasa (FPPS). Por último, las terpeno sintasas (TPS) catalizan la ciclación y el reordenamiento de grupos funcionales para generar monoterpenos (C10), diterpenos (C20) y carotenoides (Chen et al., 2011; Zager et

al., 2019; Sharma et al., 2025). En *Cannabis* spp. esta vía converge con la producción del precursor principal de los cannabinoides (GPP). Además, se ha identificado un gran número de genes TPS, lo que explica la gran variedad de perfiles de terpenos entre diferentes cultivares (Booth et al., 2019).

2.2. Nutrición mineral

La nutrición mineral es un factor importante que impacta el desarrollo y las funciones metabólicas de las plantas. Cada vez hay más evidencia de que el aporte de macro y micronutrientes influye no solo en el metabolismo primario, sino también en el secundario. Por un lado, los macronutrimentos (N, P, K, Ca, Mg, S) son los elementos que más demandan las plantas durante toda su vida útil. Su participación no se limita a ser solo componentes estructurales, sino que actúan como cofactores enzimáticos, osmolitos y moléculas de señalización que orquestan ambos metabolismos (Kirkby, 2012). Por otro lado, los micronutrientes (Fe, Mn, Zn, Cu, B, Mo, Cl, Ni) desempeñan roles catalíticos y estructurales, actuando principalmente como cofactores de enzimas. Participan en procesos vitales, desde la fotosíntesis y la respiración hasta la defensa contra el estrés (Broadley et al., 2012). Por lo tanto, el manejo apropiado de fertilizantes (orgánicos e inorgánicos), suplementos y bioestimulantes es vital para plantas medicinales, incluyendo el cáñamo (Bernstein et al., 2019).

2.2.1. Nitrógeno

El nitrógeno es uno de los macronutrientes más importantes, ya que participa en la formación de ácidos nucleicos, aminoácidos, proteínas, AIA, citocininas y clorofila. Además, promueve el crecimiento vigoroso, una buena coloración en las hojas y eficientiza la fotosíntesis (Kochhar et al., 2020). Dentro del metabolismo primario, el N es crucial para la fotosíntesis, ya que una gran proporción del nitrógeno foliar se invierte en la enzima RuBisCO y otra parte en la síntesis de porfirinas para la formación de clorofila (Li et al., 2016). En el metabolismo secundario, el N es un constituyente de numerosos compuestos de defensa, como alcaloides y glucosinolatos, y de moléculas de señalización como las poliaminas (Kirkby, 2012).

En estudios realizados en *C. sativa* sobre el impacto del nitrógeno en el desarrollo de la planta, Saloner y Bernstein (2021) demostraron que, al incrementar las dosis de nitrógeno de 30 a 320 ppm en la solución nutritiva, la concentración de THC y CBD decrecía entre un 63 y 69 % con respecto al testigo. La concentración más alta de cannabinoides se obtuvo por debajo de 30 ppm de N, mientras que el mayor rendimiento de inflorescencias se alcanzó bajo 160 ppm de N.

La forma de suministrar el nitrógeno como nitrato (NO_3^-) o como amonio (NH_4^+) juega un papel importante en la respuesta metabólica de la planta. Saloner y Bernstein (2022b) encontraron que una relación NH_4^+/NO_3^- de 0 % NH_4^+ y 100 % NO_3^- bajo una concentración base de 200 $mg \cdot L^{-1}$ N evidenció la mayor producción de cannabinoides, rendimiento de inflorescencias y desarrollo de la planta en comparación con sustituir NO_3^- por NH_4^+ por arriba del 10 %, lo que además de disminuir rendimiento impulsó la presencia de inflorescencias de menor tamaño; cabe destacar que sustituciones por encima del 50 % de NO_3^- provocó fitotoxicidad en la planta e inclusive la muerte. Gorelick y Bernstein (2017) mencionaron que la nutrición es un factor importante en la síntesis de metabolitos secundarios, Por ende, para la producción de cannabinoides se ha sugerido que existe una correlación entre el incremento de los contenidos minerales de N, Ca^{2+}, Mg^{2+} y Fe^{3+} con el aumento de la concentración de éstos dentro de la planta.

2.2.2. Fósforo

El fosforo es un nutriente fundamental para el metabolismo primario ya que participa en la formación de ácidos nucleicos y nucleoproteínas, además de los fosfolípidos que conforman las membranas celulares y síntesis de moléculas de reserva de energía como ATP, ADP, UDP, UTP, CDP, CTP, GTP y GDP (Kochhar et al., 2020).

De acuerdo con Shiponi y Bernstein (2021), la planta de *C. sativa* L. modifica la producción de cannabinoides linealmente conforme se adicionan dosis de fósforo a ésta. Ellos determinaron que para un desarrollo óptimo de la planta se requiere una concentración de 30 a 90 $mg \cdot L^{-1}$ de P, en conjunto del máximo rendimiento de biomasa de inflorescencias. Las deficiencias de fósforo, caracterizadas por concentraciones inferiores al 0.09 % de P en el tejido foliar, se manifiestan con

síntomas visuales de retraso en el crecimiento, acompañados de un patrón irregular de manchas color verde oliva en foliolos jóvenes y maduros (Cockson et al., 2019). En otro estudio en *C. sativa* L., la adición de P aumentó los niveles de P en las hojas de las inflorescencias y flores, junto con un incremento del 1.3 al 2.9 % de Ca en éstas, además de potencializar la absorción de zinc en todas las partes de la planta (Bernstein et al., 2019). Cabe resaltar que la dosificación de P por encima de 5 mg L^{-1} redujo en un 25 % la producción de THCA y CDBA en las inflorescencias (Shiponi y Bernstein, 2021).

En el metabolismo secundario, la fosforilación y desfosforilación de proteínas son mecanismos clave en las reacciones en cascada para activar respuestas a estímulos bióticos y abióticos, modulando la síntesis de compuestos como los fenoles y flavonoides (Tuteja y Singh, 2012).

2.2.3. Potasio

El potasio es un nutriente que participa principalmente como regulador del potencial osmótico de las células guardas y en los balances iónicos, además de otras funciones como cofactor en más de 50 enzimas catalizadoras durante la fotosíntesis y respiración, y su participación directa en procesos como la síntesis de proteínas y de almidón (Kulcheski et al., 2015), la apertura y cierre de estomas, incremento de la calidad de flores y frutos, aumento de la resistencia a plagas y enfermedades, y mayor tolerancia a temperaturas extremas (Kochhar et al., 2020). A diferencia de los nutrientes mencionados, este no es un constituyente directo de metabolitos secundarios, pero su rol está en la asignación de azúcares desde las hojas hacia los órganos demanda, lo que impacta indirectamente en la disponibilidad de fotosintatos para la síntesis de estos compuestos (Tuteja y Gill, 2012).

En estudios previos realizados sobre *C. sativa* L. por Saloner y Bernstein (2022a), se determinó que concentraciones menores a 15 $mg \cdot L^{-1}$ en solución nutritiva promovieron clorosis amarilla en el margen de foliolos maduros y bajos rendimientos de inflorescencias. Complementando lo anterior, Cockson et al. (2019) diagnosticaron que la deficiencia visual iba acompañada con valores inferiores al 1 % de K en tejido foliar en el cultivar T1. En contraste, altas concentraciones por

encima de 175 $mg \cdot L^{-1}$ mostraron un desarrollo óptimo de la planta y altos rendimientos de inflorescencias, sin embargo, la cantidad de cannabinoides y terpenos disminuyeron considerablemente conforme aumentaban la concentración del ion en solución; los autores concluyeron que la concentración óptima para la producción de metabolitos medicinales en combinación del desarrollo de la planta oscila los 60 $mg \cdot L^{-1}$ de K^+. Por otro lado, Bevan et al. (2021) corroboraron el impacto del K^+ en concentraciones que van de 60 a 340 $mg \cdot L^{-1}$ para el cultivar Gelato y no encontraron diferencias significativas en altura, número de nudos, diámetro de tallo y rendimiento de inflorescencias por planta.

2.2.4. Calcio

La utilidad del calcio tiene un doble rol en el metabolismo primario (estructural) y secundario (señalización). Dentro del estructural, este ion es componente de la lámina media de las paredes celulares como pectato de calcio, lo que confiere estabilidad y rigidez a estas mismas. Como mensajero secundario, este catión en forma de calmodulina y en concentraciones graduales dentro del citosol genera señales intracelulares que desencadenan una amplia gama de respuestas fisiológicas, que abarca desde la respuesta hormonal hasta la defensa contra patógenos y la adaptación al estrés abiótico (Tuteja y Gill, 2012). Esta señalización modula la expresión de genes y la actividad de enzimas involucradas tanto en el metabolismo primario como en el secundario.

Para este nutriente, existen pocos estudios que profundicen en su función dentro de la planta de *Cannabis* spp. Cockson et al. (2019) delimitaron la deficiencia visual de este elemento en el cultivar T1 y correlacionaron que una deficiencia avanzada de calcio manifiesta una clorosis amarilla en la base de los foliolos, manteniendo la punta con su color normal. Concentraciones foliares de Ca por debajo del 0.40 % generan un crecimiento irregular y asimétrico durante la expansión del foliolo. Síntomas severos promueven la necrosis apical de los puntos de crecimiento, generando brotación lateral.

2.2.5. Magnesio

El magnesio es un elemento que participa en la fotosíntesis, ya que es el principal constituyente mineral de la molécula de clorofila. Otras funciones incluyen estabilizar la estructura de los ribosomas y actuar como activador de la RuBP carboxilasa y PEP carboxilasa, entre otras (Kochhar et al., 2020). Además, actúa como un cofactor esencial de enzimas involucradas en la transferencia de grupos fosfato (quinasas y ATPasas). Su participación en la catálisis enzimática lo vincula directamente con casi todas las rutas del metabolismo primario y muy escasa participación en el secundario.

Morad y Bernstein (2023) caracterizaron en *C. sativa* 'Anafurna' que a concentraciones de 35 a 70 mg L^{-1} en solución promueven un desarrollo óptimo durante el período vegetativo. Por otro lado, si se suministran concentraciones menores a 20 mg·L^{-1}, se manifestarán signos de deficiencia con alteraciones en la fisiología de la planta, como disminución de la altura, reducción de la tasa fotosintética/respiración y conductividad estomática. Asimismo, se potencializará la absorción y acumulación de N, P, K, Ca, Mn y Zn. La clorosis fue diagnosticada por Cockson et al. (2019), donde los síntomas iniciales se presentaron en hojas maduras basales con un ligero color amarillo en la región intervenal, las cuales se asocian a concentraciones foliares de este elemento por abajo del 0.12 %. Excesos de Mg (> 140 mg·L^{-1}) redujeron la función fisiológica de manera no significativa, ya que no afectaron el desarrollo morfológico ni la acumulación de biomasa (Morad y Bernstein, 2023).

2.2.6. Azufre

La funcionalidad del azufre ha sido descrita por su participación en la síntesis de aminoácidos azufrados y proteínas como: cisteína, cistina y metionina, además, de ser constituyente de vitaminas (biotina y tiamina), CoA, ácido lipoíco, tiamina pirofosfato y glutatión, aunado de ser importante en las reacciones de transporte de electrones por ferredoxina durante la fotosíntesis y fijación de nitrógeno (Kochhar et al., 2020). de Kok y Hawkesford (2007) aseguran que la metionina es la de mayor actividad dentro del sistema metabolómico como precursora de la S-

adenosilmetionina (SAM), un donador universal de grupos metilo hacia la ruta de síntesis de lignina, pectinas y algunos metabolitos secundarios. El S también forma parte de compuestos de defensa clave como los glucosinolatos en las brasicáceas y los compuestos sulfurosos volátiles en el género *Allium* (Kirkby, 2012). Además, el glutatión actúa como uno de los antioxidantes no enzimáticos más importantes de la célula, participando en la detoxificación de especies reactivas de oxígeno (ROS) generadas por el estrés (Kok y Hawkesford, 2007).

Debido a la escasa información sobre la influencia de este elemento en el metabolismo secundario de *Cannabis* spp., no quedan claros los niveles óptimos para un buen desarrollo y síntesis de metabolitos dentro de la planta. Sin embargo, Cockson et al. (2019) determinaron los síntomas de deficiencia en el cultivar T1 donde los síntomas típicos se manifiestan como clorosis amarilla en el follaje, siguiendo un patrón desde la base de los foliolos hacia el centro de la planta. La deficiencia en esta variedad se presenta con valores de S por debajo del 0.11% en tejido foliar.

2.2.7. Hierro

El hierro es fundamental para las reacciones de óxido-reducción, debido a que es un componente clave de los citocromos y las proteínas ferrosulfuradas (ferredoxinas) de la cadena de transporte de electrones en la fotosíntesis y la respiración (White, 2012). Además, conforma enzimas oxidasas, catalasas y peroxidasas, enzimas cruciales para la detoxificación de ROS (Clemens, 2006) y es indispensable para la síntesis de clorofila y proteínas de cloroplastos en hojas (Kochhar et al., 2020).

Los síntomas de deficiencias de este elemento, se manifiesta con una clorosis intervenal en las hojas jóvenes superiores, se desarrolla una coloración amarillenta de los folíolos alrededor de la punta de crecimiento y las hojas recién expandidas, mientras que el follaje inferior y medio se muestra normal y saludable. Los síntomas de deficiencias se asocian a concentraciones foliares por debajo de 100 $mg \cdot kg^{-1}$ en plantas del cultivar T1 (Cockson et al., 2019).

2.2.8. Manganeso

El manganeso es indispensable para la formación del complejo que oxida el agua dentro del fotosistema II (PSII), siendo responsable de la fotólisis del agua (reacción de Hill) y la liberación de oxígeno a la atmósfera (Millaleo et al., 2010). Además, es cofactor de la superóxido dismutasa (Mn-SOD), una enzima antioxidante en la mitocondria y participa en la biosíntesis de lignina y otros metabolitos secundarios, contribuyendo a la defensa estructural de la planta (Clemens, 2006). Este microelemento también es requerido como cofactor para otras enzimas como: descarboxilasas, deshidrogenasas, oxidasas, peroxidasas, quinasas, arginasas e hidroxilamino reductasa (Kochhar et al., 2020).

Cockson et al. (2019) mencionaron que las deficiencias de Mn son similares a las de Fe, presentando clorosis intervenal en las mismas partes de la planta, pero con pequeñas diferencias en los patrones. Principalmente, comienza con la nervadura central de los folíolos y se extiende hacia el margen de la hoja a medida que avanzan los síntomas. El trastorno fisiológico aparece cuando las concentraciones en el follaje están por debajo de 10 $mg \cdot kg^{-1}$. Por otro lado, encontraron que concentraciones foliares por encima de 45 $mg \cdot L^{-1}$ de Mn en el tejido foliar generan toxicidad en la planta y los síntomas consisten en un amarillamiento marginal en las hojas inferiores y necrosamiento en casos más severos.

2.2.9. Zinc

El zinc es un micronutriente con funciones tanto catalíticas como estructurales en la síntesis de enzimas y fitohormonas. Es cofactor de más de 300 enzimas, incluyendo la anhidrasa carbónica (transportadora de CO_2), alcohol deshidrogenasa y superóxido dismutasa de cobre-zinc (Cu/Zn-SOD) (Sinclair y Krämer, 2012). Estructuralmente, el Zn es fundamental para la integridad de las membranas celulares y para la estabilización de proteínas a través de los "dedos de zinc", que son dominios que se unen al ADN y regulan la transcripción de genes. También participa en la síntesis del triptófano, un aminoácido precursor del ácido indol-3-acético (AIA), una hormona primordial del crecimiento vegetal (Gindri et al., 2020).

Los síntomas de deficiencia en *C. sativa* se manifiestan como un amarillamiento en los brotes jovenes y en las hojas en expansión. El amarillamiento se concentra principalmente en el margen de la hoja y a lo largo de las partes dentadas de los folíolos. En casos severos, se desarrollan regiones necróticas de color canela de forma irregular a lo largo del margen de la hoja. Por lo general, estos problemas se notan en concentraciones por debajo de 10 $mg \cdot kg^{-1}$ en el follaje (Cockson et al., 2019).

2.2.10. Cobre

El cobre actúa como cofactor en enzimas oxidativas, como citocromo oxidasa, ácido ascórbico oxidasa y polifenol oxidasa. Además, es parte de las plastocianinas y plastoquinonas en los centros de reacción para el transporte de electrones. Al igual que el zinc, el cobre participa en la estructura de la Cu/Zn-SOD (Kochhar et al., 2020), enzima involucrada en la biosíntesis de lignina y la formación de enlaces cruzados con otros componentes de la pared celular (Broadley, 2012).

Durante una deficiencia de este elemento, la planta de *C. sativa* presenta un retraso en el crecimiento junto con una deformación de las hojas nuevas y en expansión, principalmente en la base de los folíolos. En casos severos, la hoja entera muestra una fina clorosis intervenal y el margen se enrosca de afuera hacia adentro y hacia abajo. Las plantas con deficiencia presentan concentraciones foliares de cobre por debajo de 2 $mg \cdot kg^{-1}$ (Cockson et al., 2019).

2.2.11. Boro

El boro es indispensable para la estructura de paredes y membranas celulares, Brown et al. (2002) mencionaron que este elemento forma complejos diéster con los residuos de apiosa del ramnogalacturonano II (polisacárido péctico), creando enlaces cruzados que son esenciales para la correcta expansión de la pared celular. Además, de participar en la división y elongación celular de las raíces primarias y secundarias. Es indispensable para la síntesis de ácidos nucleicos, estimulación de la germinación y elongación del tubo polínico, también es considerado como un transportador de azúcares dentro de la planta (Kochhar et al., 2020).

Los síntomas de deficiencia de B se desarrollan, en promedio, seis semanas después de que la planta de *Cannabis* spp. se expone al déficit del elemento en el medio de crecimiento. Éstos comienzan con un retraso en el crecimiento, principalmente en los brotes apicales y en los nuevos, los cuales presentan tamaños más pequeños y estrechos en la base del folíolo en comparación con la punta. Los problemas avanzados se evidencian en las hojas jóvenes con una deformación severa y los márgenes de los folíolos se tornan necróticos, enroscándose hacia abajo. Los síntomas severos muestran necrosis de meristemos apicales (Cockson et al., 2019). Las concentraciones foliares de B relacionadas con las deficiencias visuales están por debajo de 2.5 mg kg^{-1} del elemento en materia seca.

2.2.12. Molibdeno

El molibdeno es un componente del cofactor de molibdeno (MoCo). Kaiser et al. (2005) mencionaron que su esencialidad radica en la actividad de un pequeño, pero vital, número de enzimas. Dentro de este grupo se localizan la nitrato reductasa, que cataliza el primer paso durante la asimilación del nitrato, y la nitrogenasa, que fija el nitrógeno atmosférico en bacterias simbiontes. Por tanto, el Mo es crucial para el metabolismo integrado del nitrógeno. Del mismo modo, también participa en la síntesis del ácido abscísico (ABA).

2.2.13. Níquel

El níquel es un micronutriente clasificado recientemente como esencial, ya que es el componente metálico de la enzima ureasa, la cual hidroliza la urea para liberar amonio y permitir su asimilación por la planta (Hänsch y Mendel, 2009), la ausencia de Ni provocaría la acumulación de urea a niveles tóxicos (Clemens, 2006). En leguminosas, el Ni es también un componente de enzimas hidrogenasas en bacterias fijadoras de N_2, lo que mejora la eficiencia de este proceso (White, 2012).

2.3. Diagnóstico nutrimental

El análisis de tejido foliar se emplea para medir los nutrientes absorbidos por las plantas y evaluar la disponibilidad de nutrientes minerales en el suelo (Etchevers, 1999). Es posible medir una fracción o la concentración total de un elemento dentro de la planta mediante técnicas analíticas. Por lo tanto, el análisis foliar se utiliza para

diagnosticar deficiencias nutrimentales y como apoyo en la elaboración de dosis de fertilización. Para ello, se requiere el empleo de métodos de diagnóstico nutrimental como: Sistema Integrado de Diagnóstico y Recomendación (DRIS), Desviación Óptima Porcentual (DOP), Índices de Balance Kenworthy (IBK), Diagnóstico de Composición Nutrimental (CDN), Rangos de Suficiencia (RS), Niveles Críticos (NC) y Propuesta de Interpretación de Resultados de Análisis (PIRA) (Oltra, 2016). El Cuadro 1 presenta la concentración nutrimental en tejido de plantas con altos contenidos de CBD.

2.3.1. Índices de Balance Kenworthy (IBK)

El diagnóstico por normas Kenworthy es un método que compara la concentración de nutrientes en una muestra con respecto a un valor estándar y su coeficiente de variación (CV) asociado a un estado particular de la planta (Kenworthy, 1961). Kenworthy (1967) menciona que un valor estándar puede considerarse como la media de la concentración del nutriente en plantas con desarrollo óptimo, la cual debe ajustarse conforme a la variación normal de cada nutriente dentro de esta para evitar una interpretación errónea o sesgada. Es por ello que se plantearon las siguientes ecuaciones para calcular los índices nutricionales para cada nutriente:

- Si $\bar{\chi}_{muestra} < \bar{\chi}_{estándar}$

$$P = \left(\frac{\chi}{S}\right) \times 100$$

$$I = (100 - P) \times \left(\frac{CV}{100}\right)$$

$$IBK = P + I$$

- Si $\bar{\chi}_{muestra} > \bar{\chi}_{estándar}$

$$P = \left(\frac{\chi}{S}\right) \times 100$$

$$I = (P - 100) \times \left(\frac{CV}{100}\right)$$

$$IBK = P - I$$

Donde:

x: Concentración de la muestra

s: Valor estándar del elemento

P: Porcentaje del nutriente evaluado

I: Influencia de la variación

CV: Coeficiente de variación IBK: Índice de balance Kenworthy

La interpretación de cada índice es la siguiente: la deficiencia severa del nutriente se asocia cuando el IBK es menor a 50. La deficiencia marginal se presenta si el IBK está entre 50 y 83. El nivel óptimo se logra con el rango de 83 a 117. Un exceso marginal se manifiesta con índices entre 117 y 150. La toxicidad del elemento se alcanza cuando se superan las 150 unidades del indicador Kenworthy.

Cuadro 1. Valores de referencia de concentración nutrimental en hojas de cáñamo (*Cannabis indica*) para cultivares con altos contenidos de CBD.

Estándares de referencia	N	P	K	Ca	Mg	S	Fe	Mn	Zn	Cu	B
	(%)						($mg \cdot kg^{-1}$)				
(Kalinowski et al., 2020)	4.30	0.36	2.57	2.91	0.48	0.31	114.20	140.00	41.10	4.60	43.80
(Landis et al., 2019)	3.75	0.35	2.42	1.15	0.32	0.24	82.20	37.10	31.00	3.50	35.90
(Bryson et al., 2014)	4.03	0.37	2.09	2.95	0.61	0.22	125.00	67.00	38.00	6.05	80.50

2.4. Bioestimuladores

Yakhin et al. (2017) definieron a los bioestimuladores como sustancias o complejos de origen biológico o mineral que mejoran la productividad de las plantas debido a las propiedades químicas de sus constituyentes, y no como consecuencia de efectos de nutrientes esenciales, reguladores del crecimiento o compuestos protectores. A continuación, se mencionan algunos que pueden impactar en la producción de metabolitos secundarios.

2.4.1. Aminoácidos aromáticos

Según García y Carril (2011), los aminoácidos aromáticos (fenilalanina, tirosina y triptófano) pueden dirigirse tanto al metabolismo primario como al secundario. La biosíntesis de estos monómeros peptídicos es exclusiva de plantas, microorganismos y hongos, y son derivados de la ruta del ácido shikímico en una secuencia de siete pasos enzimáticos dentro de los plastidios (cloroplastos, mitocondrias, etc.), los cuales convierten a los productos del metabolismo primario (PEP y E4P) en corismato (Maeda y Dudareva, 2012). Este último es el intermediario final para ramificar la ruta shikímica, por un lado, hacia la producción del triptófano y, por el otro, la de fenilalanina y tirosina

(Tzin y Galili, 2010). Las enzimas clave para este proceso son la 3-deoxi-D-arabino-heptulosonato-7-fosfato sintasa (DAHPS) y la corismato sintasa, ya que actúan como puntos de control homeostático de metabolitos secundarios (Barros y Dixon, 2020).

La fenilalanina (Phe) es el precursor principal de los fenilpropanoides. La enzima PAL cataliza la desaminación de la fenilalanina para producir ácido cinámico como primer paso de esta ruta (Vogt, 2010). A partir de aquí, se sintetizan ligninas, flavonoides, isoflavonoides, taninos y compuestos volátiles ya descritos previamente (Maeda y Dudareva, 2012). La tirosina (Tyr) en algunas plantas puede ser convertida en p-ácido cumárico por la tirosina amonio liasa (TAL). No obstante, su importancia radica en ser precursora de alcaloides (morfina y codeína) en amapola (*Papaver somniferum*), la capsaicina en chiles (*Capsicum* spp.), betalaínas en remolacha (*Beta vulgaris*), tocoferoles (vitamina E) y plastoquinonas en membranas (Gershenzon y Ullah, 2020). El triptófano (Trp) es el aminoácido base para la síntesis de AIA, el cual regula casi todos los procesos implicados en el crecimiento de la planta (Zhao, 2018). Además, es el precursor de una amplia gama de metabolitos secundarios, incluyendo los alcaloides quimioterapéuticos (vincristina y vinblastina) en *Catharanthus roseus*, fitoalexinas y glucosinolatos en brasicáceas (Wang et al., 2025).

La aplicación foliar permite una absorción y translocación directa a los tejidos metabólicamente activos, como hojas y frutos, evitando la posible degradación por microorganismos, fotolisis u otro agente. Cerdán et al. (2014) aplicaron diferentes concentraciones foliares de fenilalanina y concluyeron que la de 750 $mg \cdot L^{-1}$ optimizó la producción de estilbenos totales (fitoalexinas) en el cultivo de *Vitis vinifera*, lo que resultó en una mayor concentración de trans-piceido en el vino. Otro estudio en el cultivar Merlot demostró que la coaplicación de 750 $mg \cdot L^{-1}$ fenilalanina y tirosina en el inicio de la maduración del fruto mejoró la acumulación de antocianinas y flavonoles en la piel de la uva, compuestos clave para mejorar las propiedades organolépticas del vino tinto (Portu et al., 2016). En albahaca (*Ocimum basilicum*), la aplicación individual de fenilalanina (100 y 200 $mg \cdot L^{-1}$) incrementó significativamente el contenido de fenoles totales y la capacidad antioxidante de los extractos (Autaijamsripon et al., 2023).

Otra forma de suministrarlos es a través del fertirriego, esta labor promoverá una absorción sostenida y constante mediante las raíces, aunque puede estar sujeta a la competencia microbiana y a la adsorción con las partículas del medio de crecimiento. El trabajo realizado en equinácea (*Echinacea purpurea*) reveló que la adición entre 50 y 100 $mg \cdot L^{-1}$ de triptófano al medio de cultivo hidropónico incrementó la concentración de metabolitos derivados del ácido cafeico como el ácido cicórico, potenciando su calidad medicinal (Tabar et al., 2019).

2.4.2. Silicio

El silicio es un componente vegetal importante, ya que comprende del 0.1 al 10 % de la materia seca de las plantas. Aunque ha sido subestimado en la fisiología vegetal por clasificarlo como elemento útil o benéfico, es casi esencial, puesto que contribuye significativamente a la salud de las plantas debido a la alteración del tejido vegetal y a su interacción con otros mecanismos de absorción nutrimental (Epstein, 1994). El Si es absorbido por las raíces preferiblemente en su forma neutra como ácido monosilícico (H_4SO_4). Su absorción y transporte hacia los órganos de demanda es un proceso mayoritariamente pasivo, el cual se lleva a cabo por flujo de masas durante la evapotranspiración (Guerriero et al., 2016). Una vez depositado en los tejidos aéreos, el agua se evapora y éste se polimeriza como sílice amorfo ($SiO_2 \cdot nH_2O$) en espacios inter e intracelulares, especialmente en la epidermis de hojas y tallos, y en estructuras externas como los tricomas.

Los beneficios del Si tradicionalmente se le atribuye a la formaciín de una barrera física, que dificulta la penetración de hifas de hongos y el aparato bucal de insectos. Sin embargo, la investigación de la última década ha revelado que este también actúa como un agente de señalización celular (Luyckx et al., 2017). Se ha demostrado que el Si prepara (“efecto priming”) el sistema de defensa de la planta, permitiendo una respuesta más rápida y robusta frente a un ataque posterior. Este priming no se limita solo a la defensa física, sino que involucra la activación de rutas bioquímicas secundarias (Debona et al., 2017). La conexión entre el Si y el metabolismo secundario es intrínseca a su función como inductor de defensas ya que, al ser percibido por la planta, el Si puede desencadenar señales que activan genes clave en la producción de compuestos

fitoquímicos. Luyckx et al. (2017) aseguran que el suministro de Si mejora la biosíntesis de ácido jazmónico (AJ) y etileno (ET). Demostraron que, en plantas de tomate tratadas con Si y expuestas a *Ralstonia solanacearum* se activaron las vías de señalización de AJ y ET para tolerar la infección.

La aplicación mediante fertirrigación garantiza una disponibilidad continua para la absorción radical. En fresa (*Fragaria* × *ananassa*), la aplicación de silicato de potasio en la solución nutritiva a una concentración de 1.7 mM (≈100 ppm de SiO_2) no solo aumentó la firmeza del fruto, sino que también elevó significativamente el contenido de compuestos fenólicos totales y la capacidad antioxidante (El-Sayed et al., 2023). En tomate (*Solanum lycopersicum*), 1.5 mM de Si en la solución nutritiva mejoraron la resistencia contra *Fusarium oxysporum* al potenciar la acumulación de compuestos fenólicos y la actividad de enzimas de defensa como la peroxidasa y la polifenoloxidasa (López-Pérez et al., 2024). Danny y Muir (2002) demostraron que dosis de 2.7 a 14.6 mg L^{-1} de Si aplicadas al sustrato como H_4SiO_4 en plantas de guisante activan metabolitos de bajo peso molecular e incrementan la actividad de proteínas quitinasas y gluconasas relacionadas con mecanismos de defensa.

Aplicaciones foliares también tienen un impacto positivo en el metabolismo secundario. Concentraciones evaluadas de 0.1 y 0.2 % de SiO_2 en plantas de tomate de cáscara presentaron mayor resistencia a *F. oxysporum*, además del incremento de las concentraciones de boro y calcio en el tejido foliar (Camacho et al., 2006). Un estudio en albahaca (*Ocimum basilicum*) encontró que concentraciones de 2 y 4 mM de silicato de sodio incrementaron el contenido de aceite esencial y la concentración de terpenos como linalool y metil chavicol (Farouk y Omar, 2020).

2.5. Reguladores del crecimiento

2.5.1. Ácido salicílico

El ácido salicílico (AS) es un compuesto fenólico (ácido β-hidroxifenólico) catalogado como fitorregulador del crecimiento y reconocido por su acción en la defensa de la planta contra patógenos (Ding y Ding, 2020). Su estudio ha revelado una profunda implicación en la regulación de una amplia gama de procesos fisiológicos vegetales y bacterianos, que incluyen la germinación de semillas, la formación de poblaciones microbianas en las

raíces, el crecimiento y desarrollo, la senescencia celular y, de manera crucial, la modulación del metabolismo secundario (Rivas-San Vicente y Plasencia, 2011). El AS actúa como un señalizador endógeno clave que, al acoplarse en los receptores de la membrana celular, desencadena una reacción en cascada que culmina en la activación de respuestas de defensa y aclimatación al estrés, proceso conocido como Resistencia Sistémica Adquirida (RSA).

De acuerdo con Ding y Ding (2020), la biosíntesis de AS se deriva de dos vías principales que parten del corismato (metabolito del ciclo shikímico): la del isocorismato (IC) y la mediada por la PAL. Por un lado, la primera ruta isómera el corismato mediante las enzimas isocorismato sintasas (SID2 o ICS1 e ICS2) dentro del plasto para ser transportado hacia el citosol por la proteína EDS5. Posteriormente, el IC, al acoplarse a PBS3, se transforma en isocorismoil-9-glutamato (IC-9-Glu) por la isocorismoil-glutamato sintasa (IGS), empleando ATP y L-glutamina como sustrato. Finalmente, el IC-9-Glu puede descomponerse espontáneamente en AS y 2-hidroxi-acriloil-N-glutamato (2HNG) o N-piruvoil-L-glutamato (NPG). Especies como *Arabidopsis* spp. eficientizaron este proceso con otro transportador intermedio, la EPS1, y la enzima piruvoil-glutamato liasa (IPGL) para convertir el IC-9-Glu en AS. En organismos procariontes (*Pseudomonas* spp.), el IC se puede convertir directamente en AS y piruvato por la isocorismato liasa (IPL) (Bakker et al., 2014).

Por otro lado, para la segunda ruta metabólica, el corismato se convierte en aminoácidos aromáticos a través de diferentes vías dentro del plastidio y es transportado hacia el citosol. La fenilalanina (Phe), producida a partir de las vías plastidiales y citosólicas, se transforma en ácido transcinámico (t-CA) por la PAL y, finalmente, éste produce AS mediante el ácido ortocumárico. De forma alternativa, el t-CA puede generar benzaldehído y luego ácido benzoico (BA) que, a su vez, es hidroxilado a AS por una hidroxilasa de ácido benzoico (BA2H).

El mecanismo de señalización del AS es complejo y está controlado por el estado de la proteína NPR1. A bajos niveles celulares de AS, la NPR1 se encuentra en el citoplasma como un oligómero inactivo. Ante una acumulación natural o inducida de este fitorregulador, el medio químico del citosol sufre cambios de óxido-reducción, lo que

provoca la monomerización de NPR1 para su translocación al núcleo. Una vez en el núcleo, la NPR1 interactúa con factores de transcripción de la familia TGA, lo cual activa la expresión de genes relacionados con la patogénesis (PR). Estos incluyen los de enzimas clave para la síntesis de metabolitos secundarios (An y Mou, 2011; Herrera-Vásquez et al., 2015). La activación de la RSA implica una reprogramación metabólica que desvía recursos del metabolismo primario hacia la síntesis de metabolitos de defensa, como fenoles, terpenoides y alcaloides (An y Mou, 2011). La capacidad del AS para actuar como elicitor lo ha posicionado como una herramienta biotecnológica de bajo costo y alta eficacia en la agricultura moderna. Su aplicación exógena busca imitar una señal de ataque patogénico para acondicionar la planta, mejorando no solo su resistencia, sino también la concentración de compuestos de interés nutracéutico, farmacéutico o industrial.

La eficacia de la aplicación exógena de AS depende de la concentración, el método de aplicación, la etapa fenológica y la especie vegetal. Concentraciones muy altas pueden ser fitotóxicas, causando estrés oxidativo y necrosis. Un estudio en pepino (*Cucumis sativus*) mostró que la irrigación con AS a bajas concentraciones (75 µM) mejoraba la tolerancia al estrés salino y promovía un nivel más alto de la actividad enzimática (SOD, CAT) para la síntesis de compuestos antioxidantes (Nie et al., 2021). Wang et al. (2015) aplicaron dosis foliares de AS (1 y 2 mM) antes del envero del fruto en *Vitis vinifera* y obtuvieron una mejor concentración de resveratrol (estilbeno) y de antocianinas en el hollejo de las bayas, compuestos de alto valor para la calidad del vino.

2.5.2. Brasinoesteroides

Los brasinosteroides (BR) son un grupo de hormonas esteroideas polihidroxiladas que ejercen un efecto fisiológico en el desarrollo de las plantas a bajas concentraciones (Rodríguez et al., 2022). Actualmente, se han descrito alrededor de cuarenta tipos de compuestos con aplicación en la actividad agrícola. Dentro del grupo de los BR naturales, destaca la brasinólida (BL) como la hormona primitiva descubierta en el polen de colza o canola (*Brassica napus*), así como sus isómeros funcionales 28-homobrasinólida (HBR) y 24-epibrasinólida (EBR), derivadas del estigmasterol y ergosterol, respectivamente (Hernández y García-Martínez, 2016). Entre los análogos sintéticos se encuentran el

Biobras-16 (BB-16), Biobras-6 (BB-6), MH5, DI-43, CR-44 e IRA-67 (Núñez y Mazorra, 2001). La proteína BRI1 es el receptor especializado para los BR, la cual permite su acoplamiento, reconocimiento y desencadenamiento de la reacción en cascada pertinente. Esta reacción puede ser unitaria o sinérgica con otros reguladores del crecimiento, como auxinas, citocininas y giberelinas, e incluso puede sustituir el efecto de estos últimos (Hernández y García-Martínez, 2016). Rodríguez et al. (2022) mencionaron que las principales funciones fisiológicas de los BR es el alargamiento y división celular, fotomorfogénesis, diferenciación de tejidos, estimulación de la actividad antioxidante, tolerancia al estrés abiótico y biótico, e incremento de la producción. A nivel molecular, estos modifican la expresión génica y la síntesis de ácidos nucleicos y proteínas (Hernández y García-Martínez, 2016).

En consecuencia, se ha estudiado el impacto de los BR en las rutas metabólicas secundarias, tal como lo realizaron Zhu-mei et al. (2013). Ellos delimitaron el efecto de la aplicación foliar de EBR (0.1 - 0.8 $mg \cdot L^{-1}$) en *Vitis vinifera* 'Cabernet Sauvignon', denotando que la concentración de 0.4 $mg \cdot L^{-1}$ resultó en una mayor síntesis de metabolitos secundarios como antocianinas (2.17 ± 0.19 $mg \cdot g^{-1}$), compuestos fenólicos, flavonoides y taninos. Adicionalmente, se observó un aumento de la actividad de las enzimas PAL (240 U g^{-1} FW) y UFGT (8 $mmol \cdot g^{-1}$ FW h^{-1}). En otros cultivos, Mohammadi et al. (2020) encontraron que la aplicación de 100 $mg \cdot L^{-1}$ de EBR en conjunto de estrés hídrico (60 %) repercutieron positivamente en el incremento de aceites esenciales y constituyentes volátiles de hidratos de cis-sabinina en plantas de salvia (*Salvia officinalis*). Çoban y Baydar (2016) evaluaron diferentes niveles de EBR (0, 0.5, 1.5 y 2.5 $mg \cdot L^{-1}$) en plantas de menta (*Mentha piperita* L.) bajo condiciones de salinidad (0, 100 y 150 mM NaCl) y observaron efectos positivos como una reducción en la permeabilidad de membranas, peroxidación lipídica y porcentaje de mortalidad, además de un incremento en la síntesis de aceites esenciales y la actividad de enzimas antioxidantes. Delimitaron la dosis de 0.5 $mg \cdot L^{-1}$ de EBR como la de mejor desempeño para la producción de aceites esenciales en condiciones óptimas y de estrés salino.

2.6. Microorganismos potencializadores de metabolitos secundarios

La intervención de los microorganismos benéficos en la fisiología vegetal para potenciar la producción de compuestos fitoquímicos es un campo de estudio en plena expansión. Lejos de ser organismos pasivos y aislados, las plantas coexisten con una vasta diversidad de microorganismos mediante una serie de reacciones, tanto con endófitos como con los de vida libre en la rizosfera y filosfera (Raaijmakers et al., 2007).

Investigaciones en varias cepas de *Trichoderma* spp. han revelado que las hifas que colonizan las raíces se enrollan alrededor de estas y, finalmente, penetran la corteza de la raíz. En respuesta, las células vegetales circundantes producen compuestos fenólicos y son depositados en la periferia para formar una pared celular más robusta. Esta reacción de la planta impide el crecimiento de *Trichoderma* hacia dentro de la raíz (Shoresh et al., 2010). Nishad et al. (2015) afirmaron que dichos mecanismos implicados durante las interacciones de los microorganismos huéspedes sobre la planta hospedante se ajustan al modelo coevolutivo en zigzag, como una estrategia de defensa ante patógenos y microorganismos benéficos. La ruta de señalización principal emplea el reconocimiento de patrones moleculares asociados a microbios (MAMP), patógenos (PAMP) o daños (DAMP), como los de la flagelina bacteriana y la quitina fúngica, mediante receptores ubicados en la superficie de las células vegetales. Esto desencadena un conjunto de respuestas inmunitarias, tales como inmunidad por MAMP (MTI), PAMP (PTI) y DAMP. La activación de estas respuestas genera una compleja cascada de señalización a partir del ion calcio como mensajero secundario y la fosforilación de varios genes involucrados en la síntesis del AS, AJ y ET, así como la producción de compuestos antimicrobianos (Yu et al., 2022).

En consecuencia, los microorganismos benéficos pueden mejorar la absorción de nutrientes, producción de fitohormonas, inducción de resistencia sistémica y estimulación de compuestos que actúan como precursores o elicitores de las rutas biosintéticas de metabolitos (Gouda et al., 2018). Aunque, la eficacia de la inoculación con estos microorganismos depende en gran medida de la especie microbiana y su manejo, la planta huésped, la competencia rizosférica, las condiciones ambientales y, de manera crítica, la cantidad de inoculación (Compant et al., 2005). La determinación de la

concentración óptima de inóculo es fundamental para maximizar los efectos deseados sin incurrir en costos innecesarios o incluso en efectos contraproducentes.

2.7. Métodos analíticos de determinación de metabolitos secundarios

2.7.1. Métodos de cuantificación de cannabinoides por cromatografía

En los últimos años, a nivel internacional se han desarrollado y validado varios métodos analíticos mediante cromatografía para cuantificar los principales cannabinoides (THC y CBD) en inflorescencias de *Cannabis* spp. Las técnicas analíticas más empleadas son cromatografía líquida de alta resolución (CLAR) y cromatografía de gases (CG).

En general, los métodos de CLAR emplean columnas de sílice de fase inversa (C18 o C8), utilizando una fase móvil compuesta por una mezcla de agua y un solvente orgánico (acetonitrilo o metanol), a menudo con aditivos como ácido fórmico o formiato de amonio para mejorar la forma de los picos cromatográficos; y la cuantificación de los analitos se realiza mediante detectores de arreglo de diodos (DAD) y ultravioleta-visible (UV-Vis) (Patel et al., 2017). La preparación de la muestra típicamente involucra una extracción sólido-líquido utilizando solventes orgánicos como metanol, etanol o acetonitrilo, seguida de una etapa de limpieza para eliminar interferencias de la matriz, como pigmentos, partículas y otras moléculas orgánicas, especialmente en muestras complejas (Wang et al., 2016). Existen diferentes metodologías dentro del CLAR para la extracción de cannabinoides, como la que describen Mandrioli et al. (2019), donde emplearon 25 mg de muestra y la extracción se realizó con 10 mL de metanol:cloroformo (9:1), se filtró y concentró el extracto, y finalmente, se inyectó 5 µL en un HPLC-UV (220 nm); este método obtuvo recuperaciones por encima del 85 % y un LOD desde 0.3 a 1.3 $\mu g \cdot mL^{-1}$. Otros estudios usan extracción con metanol:agua y columnas de partículas superficialmente porosas (core shell) para lograr tiempos cortos (<10 min), con recuperaciones mayores al 90 % y una incertidumbre menor del 6 % (Patel et al., 2017).

En CG, los métodos convencionales se desarrollaron para detectores de ionización de llama (CG-FID) y espectrometría de masas (CG-EM) con derivatización TMS. La importancia de la derivatización antes del análisis radica en que convierte los analitos de interés en derivados más volátiles y térmicamente estables, mejorando la forma de los picos y la sensibilidad (Wang et al., 2016). Gul et al. (2023) validaron un método CG-FID

rápido para 20 cannabinoides (ácidos y neutros), logrando un LOD de 0.10 $\mu g \cdot mL^{-1}$ y un LOQ entre 0.25 y 0.50 $\mu g \cdot mL^{-1}$ (Figura 19). Cardenia et al. (2018) reportan un método rápido para GC/MS (<7 min) tras sililación, con LOD menor a 11 $ng \cdot mL^{-1}$.

La CLAR permite medir los cannabinoides tanto en su forma ácida como neutra sin necesidad de un paso de derivatización, mientras que la CG requiere derivatización para evitar la descarboxilación de los cannabinoides ácidos, aunque esta última ofrece una alta sensibilidad, mayor resolución de pico y un rango dinámico muy amplio (Pourseyed et al., 2020). Por ejemplo, para cuantificar los hidrógenos de los grupos funcionales polares (alcoholes, aminas, ácidos carboxílicos y amidas) presentes en las moléculas de THCA y CBDA se emplea N,O-bis(trimetilsilil)trifluoroacetamida (BSTFA) como agente reactante y derivatizar en trimetilsililo (TMS) (Gul et al., 2023). En contraste, métodos de HPLC permiten la cuantificación directa de los principales cannabinoides sin ningún factor de ajuste (Mandrioli et al., 2019). La validación rigurosa del método, incluyendo parámetros clave como los descritos en los Cuadros 2 y 3 (recuperación, linealidad, LOD, LOQ), y el uso de materiales de referencia certificados son indispensables para garantizar la exactitud y comparabilidad de los resultados entre laboratorios, un pilar fundamental para la consolidación de una industria del *Cannabis* segura y transparente.

Cuadro 2. Métodos de extracción y condiciones cromatográficas para la cuantificación de cannabinoides mediante CLAR-UV/DAD.

Matriz	Extractante	Método de limpieza	Fase móvil	Columna	λ (nm)	Recuperación (%)	LOD (µg mL^{-1})	LOQ (µg mL^{-1})	Referencia
100 mg (Flor)	Metanol	Filtración por PTFE (0.45 µm)	A: H_2O + 0.1 % ácido fórmico (AF) B: Acetonitrilo (ACN) + 0.1 % AF	Kinetex C18 (150 x 4.6 mm, 2.6 µm)	228	95 - 105	0.05	0.15	(de Backer et al., 2009)
200 mg (Aceite)	Etanol	Dilución y filtración por PTFE (0.22 µm)	A: Formiato de amonio 5mM B: Metanol	Restek Raptor ARC-18 (150 x 4.6 mm, 2.7 µm)	230	92 - 108	0.30	1.00	(Ciolino et al., 2018)
10 mg (Extracto)	Isopropanol	Dilución y centrifugación	A: H_2O + 0.1 % AF B: ACN	Waters Cortecs C18 (100 x 2.1 mm, 1.6 µm)	228	97 - 104	0.02	0.06	(Gülck y Møller, 2020)
25 mg (Flor)	Mezcla	Filtración, secado y reconcentración con acetonitrilo	A: H_2O + 0.085 % H_3PO_4 B: ACN + 0.085 % H_3PO_4	Nex-Leaf CBX Potency (150 × 4.6 mm, 2.7 µm)	220	84 - 100	0.28	0.84	(Mandrioli et al., 2019)
200 mg (Flor)	Mezcla	Centrifugación, evaporación y reconcentración	A: buffer acetato amónico 25 mM pH 4.75 B: metanol	Poroshell SB-C18 (100 × 4.6 mm, 2.7 µm)	214 y 228	>90%	0.06	0.25	(Patel et al., 2017)

LOD: Límite Mínimo Detectable. LOQ: Límite Mínimo Cuantificable. PTFE: Politetrafluoroetileno.

Cuadro 3. Métodos de extracción y condiciones cromatográficas para la cuantificación de cannabinoides mediante CG-FID/EM.

Matriz	Extractante	Método de limpieza	Agente derivatizante	Columna	Detector	Recuperación (%)	LOD (ng / inyección)	LOQ (ng / inyección)	Referencia
50 mg (flor)	Hexano - isopropanol (9:1)	Filtración	BSTFA + 1 % TMCS	Agilent DB-5MS (30 m x 0.25 mm, 0.25 µm)	MS	92 - 103	0.05	0.15	(Raharjo y Verpoorte, 2004)
1 mL (plasma)	Hexano - Acetato de Etilo (9:1)	Extracción líquido-líquido y SPE C18	MSTFA	HP-5ms (30 m x 0.25 mm, 0.25 µm)	MS	85 - 95	0.10	0.50	(Lo Faro et al., 2022)
100 mg (extracto)	Etanol	Dilución y filtración	BSTFA	Phenomenex ZB-5 (30 m x 0.25 mm, 0.25 µm)	FID	94 - 106	1.00	3.00	(Fischedick et al., 2010)
100 mg (flor)	Metanol	Sonicación, centrifugación, reconcentración	BSTFA	Agilent DB-1MS (15 m × 0.25 mm, 0.25 µm)	FID	90 - 107	0.10	0.25	(Gul et al., 2023)
200 mg (flor)	Metanol	Extracción en agitación, centrifugación	-	Rxi-35sil MS (15 m × 0.25 mm, 0.25 µm)	FID	ND	ND	ND	(Arsenault et al., 2024)

LOD: Límite Mínimo Detectable. LOQ: Límite Mínimo Cuantificable. BSTFA: N,O-bis(trimetilsilil)trifluoroacetamida. TMCS: Trimetilclorosilano. MSTFA: N-metil-N-(trimetilsilil)trifluoroacetamida. ND: No detectado.

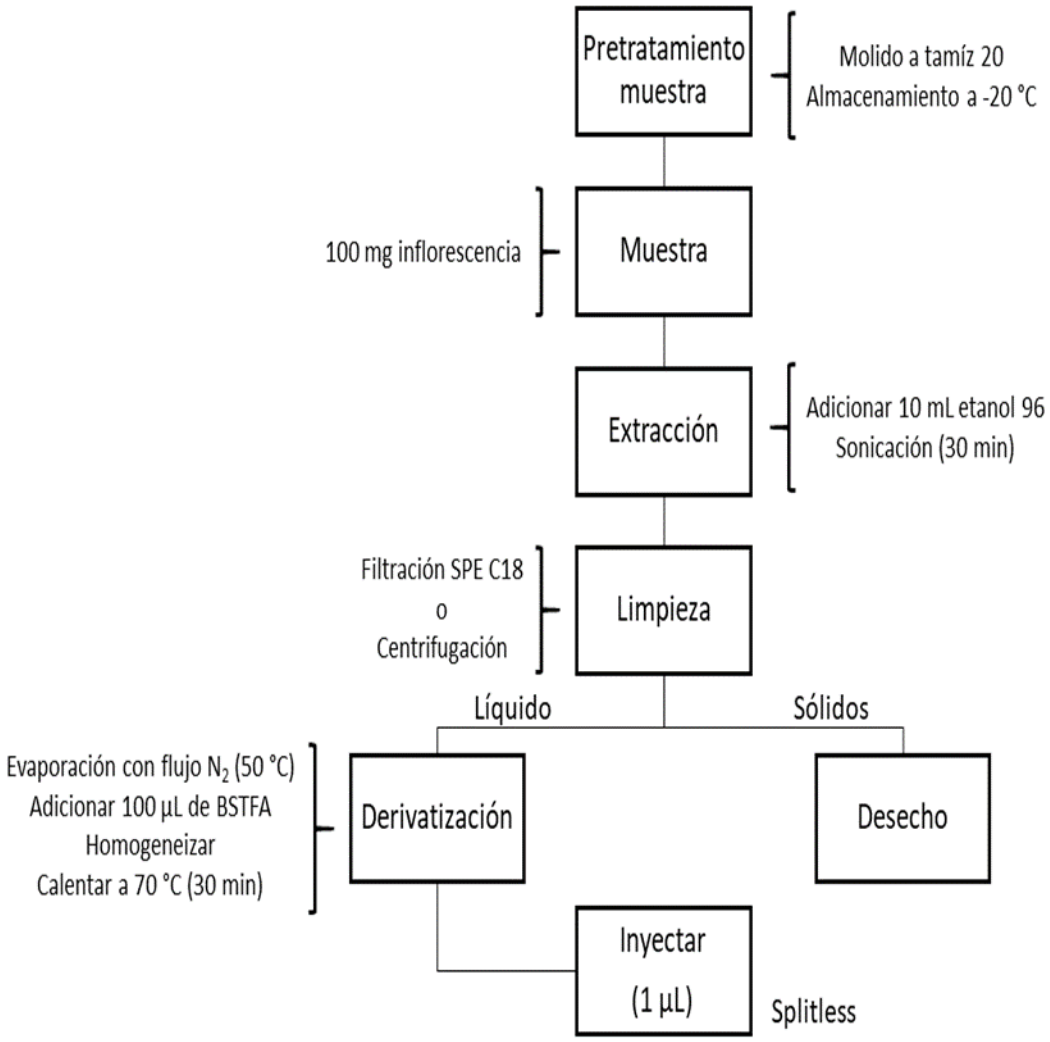

Figura 1. Diagrama de flujo para la extracción de cannabinoides en inflorescencias de cáñamo en GC/MS (Gul et al., 2023).

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3. Production of nutraceutical compounds from *Cannabis indica* by different integrative treatments in nutritive solution[1]

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ABSTRACT

Cannabis indica is a multipurpose species rich in phytochemical compounds with nutraceutical and medicinal properties. Mineral nutrition has been shown to be an important factor for the synthesis of these molecules. However, the influence of ion balances with signaling and stimulating molecules applied through the nutrient solution on the synthesis of metabolites has not been studied. Therefore, the purpose of this work was to delimit the impact of ion balances complemented with acetylsalicylic acid (ASA) and phenylalanine (Phe) on the development of the plant and on the production of nutraceutical compounds. Nine treatments were evaluated: 12 $mmol_c\ L^{-1}$; 15 $mmol_c\ L^{-1}$; 15 $mmol_c\ L^{-1}$ + 20 mg L^{-1} ASA; 15 $mmol_c\ L^{-1}$ + 500 mg L^{-1} Phe; 15 $mmol_c\ L^{-1}$ + 20 mg L^{-1} ASA + 500 mg L^{-1} Phe; 19 and 20 $mmol_c\ L^{-1}$; 19 and 20 $mmol_c\ L^{-1}$ + 20 mg L^{-1} ASA; 19 and 20 $mmol_c\ L^{-1}$ + 500 mg L^{-1} Phe; 19 and 20 $mmol_c\ L^{-1}$ + 20 mg L^{-1} ASA + 500 mg L^{-1} Phe in a randomized block design under controlled conditions. The commercial solution (12 $mmol_c\ L^{-1}$) performed better in terms of quercetin production (1878 mg 100 g^{-1}) in inflorescences, accompanied by an antagonism for Ca. The Hoagland solution (19 $mmol_c\ L^{-1}$) obtained better vegetative development with higher heights (9.9 - 10.8 cm) and SPAD indices (50.1 - 52.8). The combination of Hoagland and Steiner generated high levels of foliar potassium (7.74 to 8.86 %) due to cationic antagonisms, but produced lower concentration of quercetin. Supplementation of the nutrient solution promoted higher leaf biomass in treatments 3 and 8 with 8.3 g of dry matter, and improved calcium absorption in treatments 3 and 5.

Keywords: Hemp, phenylalanine, ionic balances, acetylsalicylic acid, flavonoids.

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INTRODUCTION

Cannabis (*Cannabis indica*) has been utilized by Central Asian countries from the Neolithic period to the present (Booth and Bohlmann, 2019). Its importance lies in being a multipurpose species for various sectors, including industry, environment, livestock, and pharmaceuticals. The latter is particularly relevant due to its potential for stimulating phytochemical compounds with nutraceutical and medicinal properties beneficial to human health (Andre et al., 2016). These molecules result from secondary metabolism through the following biochemical pathways: shikimic acid, polyketide, and plastidial (Gordo, 2018). Key enzymes involved in these processes include phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), chalcone synthase (CHS), and polyketide synthase (PKS) (Bautista et al., 2021).

On one hand, previous research has correlated the impact of mineral nutrition with the activation of the aforementioned pathways. Nitrogen is essential for the

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synthesis of amino acids and proteins, directly influencing photosynthesis and growth (Song et al., 2023). Saloner and Bernstein (2021) demonstrated that increasing nitrogen doses from 30 to 320 mg L^{-1} in the nutrient solution resulted in a 60–70% decrease in cannabinoid concentration compared to the control. A similar effect was observed when the NH_4^+/NO_3^- ratio exceeded 30%, as the plant is highly sensitive to ammonium toxicity (Saloner and Bernstein, 2022b). The optimal average value is 160 mg L^{-1} N with an NH_4^+/NO_3^- ratio of 0 to 30%.

Phosphorus is an essential element for the formation of nucleic acids, nucleoproteins, phospholipids, and energy storage molecules, as well as for stimulating metabolomic pathways (Kochhar and Gujral, 2020). Shiponi and Bernstein (2021) stated that phosphorus application linearly modifies cannabinoid production and determined that a concentration of 30 to 90 mg L^{-1} of P in solution is required for optimal plant development without affecting cannabinoid synthesis. Potassium is an essential macronutrient that plays a crucial role in various physiological processes, acting as an activator of catalytic enzymes during photosynthesis and respiration (Mengel and Kirkby, 2000). Saloner and Bernstein (2022a) outlined the effects of potassium on the production of medicinal compounds, noting that concentrations below 15 mg L^{-1} indicate visual deficiency symptoms, while concentrations above 175 mg L^{-1} resulted in high inflorescence yields but significantly decreased the production of cannabinoids and terpenes. Magnesium is an essential element whose primary function is to be a structural component of chlorophyll, as well as to stabilize ribosome structures and activate enzymes such as RuBP carboxylase and PEP carboxylase (Kochhar and Gujral, 2020). Optimal development requires a magnesium supply of 35–70 mg L^{-1} in solution; lower concentrations lead to visual deficiency symptoms, physiological disorders, and a 28% reduction in the total biomass of *Cannabis sativa* (Morad and Bernstein, 2023).

On the other hand, Phe has been studied for its high potential in agriculture as a stimulant that enhances plant tolerance and resistance. Phe is an essential aromatic amino acid with neutral and nonpolar properties (Das et al., 2018). It serves as a precursor to the enzyme phenylalanine ammonia-lyase (PAL) in the phenylpropanoid pathway and the shikimic acid pathway (Aghdam et al., 2019). Exogenous applications of Phe have been shown to increase the concentration of metabolites such as phenols, flavonoids, anthocyanins, and lignin in harvested crops (Das et al., 2018).

Another molecule with hormonal effects capable of influencing the plant metabolomic system is salicylic acid (SA). Within this group, there is an analog known as ASA, which performs the same function as salicylic acid despite being of synthetic origin and easily accessible in Mexico. SA is a hormone associated with plant defense and, therefore, promotes immunity against pathogens (Ding and Ding, 2020). According to Tucuch-Haas et al. (2021), exogenous application of ASA to foliage has been documented to regulate stomatal opening and closing, promote systemic acquired resistance (SAR), induce cell apoptosis, and stimulate metabolite production through the expression of regulatory genes in the aforementioned metabolic pathways.

However, little research has been conducted on the impact of ionic balance and the described molecules on the secondary metabolism of *C. indica*. For this reason, the present study aimed to determine the impact of ionic balance, supplemented with Phe and ASA in the nutrient solution, on the development of *C. indica* (hemp) and the production of phenols and total flavonoids.

MATERIALS AND METHODS

Plant material

The hemp seeds (*C. indica*) used in this research were of the regular (dioecious) type, intended for medicinal purposes. They were donated by producers from the community of La Cercada, Dolores Hidalgo, Guanajuato, located at 21º 11' 10.87" N latitude and 100º 53' 40.79" W longitude.

Seedling management

The seeds were soaked the night before sowing. The most vigorous seeds were selected and sown in polystyrene trays with 72 cavities. The substrate used was a 50:50 mixture of peat and perlite. Watering was done manually according to the substrate's needs. The seedlings were placed under artificial lighting at an intensity of 150 µmol s^{-1} m^{-2} using 100 W Citizen COB LED lamps emitting white light, positioned 30 cm above the plants. The photoperiod consisted of 16 hours of light and 8 hours of darkness throughout this stage. The seedling phase lasted for 30 days.

Treatment design

Nine treatments were evaluated (Table 1). Some treatments were supplemented with 20 mg L^{-1} acetylsalicylic acid (ASA) (treatments 3 and 7), 500 mg L^{-1} phenylalanine (Phe) (treatments 4 and 8), and a combination of 20 mg L^{-1} ASA + 500 mg L^{-1} Phe (treatments 5 and 9), which were provided in the nutrient solution during the flowering stage.

As a source of micronutrients, all treatments included Fe^{2+} (2 mg L^{-1}), Mn^{2+} (1 mg L^{-1}), Zn^{2+} (0.1 mg L^{-1}), Cu^{2+}

Table 1. Design of treatments applied via nutrient solution under different total ionic balances and biostimulants in hemp (*Cannabis indica*).

Treatment†	NO_3^-	$H_2PO_4^-$	SO_4^{2-}	NH_4^+	K^+	Ca^{2+}	Mg^{2+}	RIT††	Stage ¶	Biostimulant ¶¶
				($mmol_c$ L^{-1})						
1	7.5	1.0	3.5	1.5	3.0	5.0	2.5	12.0	V	A
	9.0	1.5	1.5	-	5.0	4.0	3.0	12.0	F	A
2	10.0	1.0	4.0	0.5	4.5	7.0	3.0	15.0	V, F	A, A
3	10.0	1.0	4.0	0.5	4.5	7.0	3.0	15.0	V, F	A, P
4	10.0	1.0	4.0	0.5	4.5	7.0	3.0	15.0	V, F	A, P
5	10.0	1.0	4.0	0.5	4.5	7.0	3.0	15.0	V, F	A, P
6	14.0	1.0	4.0	1.0	6.0	8.0	4.0	19.0	V	A
	12.0	1.0	7.0	-	7.0	9.0	4.0	20.0	F	A
7	14.0	1.0	4.0	1.0	6.0	8.0	4.0	19.0	V	A
	12.0	1.0	7.0	-	7.0	9.0	4.0	20.0	F	P
8	14.0	1.0	4.0	1.0	6.0	8.0	4.0	19.0	V	A
	12.0	1.0	7.0	-	7.0	9.0	4.0	20.0	F	P
9	14.0	1.0	4.0	1.0	6.0	8.0	4.0	19.0	V	A
	12.0	1.0	7.0	-	7.0	9.0	4.0	20.0	F	P

†1: Trade balance; 2, 3, 4, and 5: Modified equilibrium (Saloner and Bernstein, 2022b); 6-V, 7-V, 8-V, and 9-V: Equilibrium (Hoagland and Arnon, 1938); 6-F, 7-F, 8-F, and 9-F: Equilibrium (Steiner, 1980). ††Total ionic balance of anions and cations in the nutrient solution. ¶V: Vegetative phase, F: Flowering phase. ¶¶A: Without biostimulant, P: With biostimulant.

(0.1 mg L^{-1}), B^{2-} (0.5 mg L^{-1}), and EDTA-chelated Mo^{2-} (0.05 mg L^{-1}). A completely randomized block design (CRBD) was used, with three replications and a planting density of 9 plants/m². The nutrient solution was applied immediately after transplanting. Each experimental unit consisted of 4 L pots filled with the same substrate mixture used for sowing, following a "Sea of Green" (SOG) cultivation system.

During the vegetative phase (weeks 1-4), the photoperiod was set to 16 hours of light and 8 hours of darkness. During the flowering phase (weeks 5-12), the photoperiod was adjusted to 12 hours of light and 12 hours of darkness. Harvesting took place between 120 and 130 days after transplanting (DAT).

Sample pretreatment

All fresh and senescent foliar tissues were washed with distilled water to remove impurities. The foliar samples and inflorescences were dried in an oven at 50 ± 5 ºC until reaching a constant weight. The dried samples were ground using a 20-mesh sieve and homogenized for subsequent measurements, including total flavonoids, total phenols, and nutrient content.

Evaluation of physiological variables

Plant height

Height was measured using a tape measure from the base to the apex of the main stem at 30 days after transplanting (DAT) in each experimental unit before apical pruning.

SPAD indices

SPAD indices were determined using a SPAD 502 Minolta LTD in physiologically mature leaves at 30 and 60 DAT. Measurements were taken with five repetitions per experimental unit, and the obtained values were averaged.

Chlorophyll a and b

Chlorophyll determination was conducted following the methodology of Witham et al. (1971). Absorbance was measured at 645 nm and 663 nm for chlorophyll b and a, respectively, using a UV-visible spectrophotometer (10UV, Thermo Fisher Scientific, Waltham, Massachusetts, USA). The obtained values were applied to the following equations:

- Chlorophyll a (µg mL^{-1}) = 12.21 × Abs 663 - 2.81 × Abs 645
- Chlorophyll b (µg mL^{-1}) = 20.13 × Abs 645 - 5.03 × Abs 663.

Biomass

Foliar tissue and inflorescences were harvested at 130 DAT. The samples were stored in paper bags at room temperature for two weeks. The dry plant parts were weighed to a constant weight using a precision balance.

Evaluation of nutraceutical compounds

Total flavonoids

The total flavonoid content in inflorescences was determined using the UV-VIS spectrophotometric method proposed by Bakar et al. (2009). A calibration curve was prepared using ten points (0 to 450 µg mL^{-1}) of quercetin (95%, Sigma-Aldrich).

Total phenols

The total phenol content in leaves was determined using the Folin-Ciocalteu spectrophotometric method, following the procedure described by Koch et al. (2015). From a stock solution of 1128 µg mL^{-1}, a series of dilutions were prepared: 112, 225, 338, 451, 563, 676, 789, 902, and 1015 µg mL^{-1} of gallic acid (98%, Sigma-Aldrich).

Nutrient content evaluation

The macronutrient content (N, P, K, Ca, and Mg) was determined using a wet digestion method with an H_2SO_4:$HClO_4$ (4:1, v/v) mixture. Digestion was carried out with 0.25 g of foliar sample, adding 4 mL of the acid mixture and 2 mL of H_2O_2. The digestate was diluted to 50 mL with distilled water and stored at 4°C until analysis. Nitrogen (N) was quantified using the micro Kjeldahl method (Fleck and Munro, 1965). Phosphorus (P) was determined by forming a vanadomolybdate complex and measuring absorbance using UV-VIS spectrophotometry at 420 nm. Potassium (K) was analyzed via flame photometry using a 410 flame photometer (Sherwood, Cambridge, UK), while calcium (Ca) and magnesium (Mg) were measured using atomic absorption spectrophotometry (GBC-SavantAA, Victoria, Keysborough, Australia).

Statistical analysis

The results for plant height, SPAD readings, chlorophyll a and b, foliar biomass and inflorescences, total flavonoids, total phenols, and nutrient concentration in foliar tissue were statistically analyzed using SAS Student (Statistical Analysis System). The analysis included orthogonal contrasts, multiple mean comparisons, and mixed models (Montesinos et al., 2022).

RESULTS

Evaluation of physiological variables

The results (Table 2) show that the evaluated ionic relationships, both alone and in combination with ASA and Phe, did not induce significant effects on SPAD indices during flowering, chlorophyll a and b levels, or inflorescence biomass.

Table 2. Average values of height, indices, photosynthetic pigments, and hemp (*Cannabis indica*) biomass under different nutrient solutions.

Treatment	Height (cm)	SPAD		Clorophyll		Biomass	
				a	b	foliage	flowers
		30 dat	60 dat	(mg g^{-1})		(g)	
1	9.35 ab	51.23 a	63.00 a	1.93 a	0.52 a	5.22 b	1.70 a
2	6.70 bc	46.53 ab	65.40 a	1.37 a	0.39 a	6.88 ab	3.41 a
3	8.30 abc	48.90 a	65.97 a	1.52 a	0.41 a	8.30 a	2.30 a
4	9.10 ab	46.70 ab	66.50 a	1.60 a	0.40 a	6.86 ab	3.49 a
5	6.10 c	47.83 ab	66.80 a	1.47 a	0.37 a	6.78 ab	2.35 a
6	9.90 a	52.80 a	66.57 a	1.21 a	0.31 a	6.73 ab	2.11 a
7	10.80 a	55.17 a	67.70 a	1.70 a	0.43 a	6.69 ab	1.78 a
8	10.35 a	50.13 a	66.37 a	1.56 a	0.39 a	8.29 a	1.21 a
9	9.20 ab	38.20 b	67.10 a	1.11 a	0.26 a	5.52 ab	2.28 a
p-value	0.0400	0.0423	0.8407	0.3199	0.1452	0.0400	0.6314
DMS	2.70	10.65	6.27	1.09	0.27	2.93	2.84

Different letters within rows indicate statistically significant differences (LSD, $P \leq 0.05$).

During the vegetative stage, Treatment 7, corresponding to 19 mmolc L^{-1}, resulted in the greatest height and SPAD index, measuring 10.8 cm and 55.17, respectively, compared to solutions with total ionic balances of 12 and 15 mmolc L^{-1}. Treatments 3 and 8 (Bernstein + 20 mg L^{-1} ASA and Hoagland + Steiner + 500 mg L^{-1} Phe) produced the highest foliar biomass, both yielding 8.30 g of dry matter. In contrast, Treatment 1 promoted the lowest foliar biomass accumulation, with 5.22 g.

Table 3. Average concentration of secondary metabolites and macronutrients in the foliar tissue of hemp (*Cannabis indica*) under different ionic balances and biostimulation in a nutrient solution.

Treatment	Total Flavonoids†	Total Phenols††	N	P	K	Ca	Mg
	(mg 100 g^{-1})				(%)		
1	1878.00 a	438.53 a	2.61 a	0.26 a	5.03 d	0.24 ab	0.10 a
2	1340.22 bc	394.41 a	2.89 a	0.22 a	7.15 bc	0.23 abc	0.11 a
3	1339.48 bc	360.29 a	2.60 a	0.22 a	7.62 b	0.25 a	0.11 a
4	1483.19 ab	436.76 a	2.59 a	0.20 a	7.26 bc	0.22 bcd	0.10 a
5	1687.63 ab	382.65 a	2.71 a	0.23 a	6.22 c	0.25 a	0.09 a
6	1374.30 bc	316.76 a	2.82 a	0.22 a	8.19 ab	0.23 abcd	0.10 a
7	1229.75 bc	393.82 a	3.12 a	0.22 a	7.74 ab	0.21 cd	0.09 a
8	999.48 c	326.76 a	2.79 a	0.21 a	8.86 a	0.21 d	0.10 a
9	1308.05 bc	397.94 a	3.07 a	0.23 a	7.81 ab	0.24 ab	0.09 a
p-value	0.0499	0.4034	0.2674	0.7457	0.0020	0.0118	0.4409
DMS	457.68	114.05	0.4706	0.1223	1.101	0.0218	0.0177

Different letters within rows indicate statistically significant differences (LSD, $P \leq 0.05$).
† Total flavonoids quantified as total quercetin in inflorescences ($n = 3$).
†† Total phenols quantified as total gallic acid in mature leaves ($n = 3$).

Evaluation of nutraceutical compounds

According to Table 3, the evaluated treatments did not have a significant effect on the total phenol content in leaves. Treatment 1 (12 mmolc L^{-1}) exhibited the highest yield of nutraceutical metabolites, expressed as total flavonoids (1,878 mg per 100 g of quercetin) in flowers, and showed the highest trend in total phenol content (439 mg per 100 g of gallic acid) in leaves.

Evaluation of nutrient content

The average macronutrient concentrations (Table 3) showed no significant differences among treatments concerning RIT or the application of ASA and Phe for N, P, and Mg in foliar tissue. The highest potassium absorption was observed in Treatment 8 (20 mmolc L^{-1} + 500 ppm Phe), with an average concentration of 8.86%, while the lowest was recorded in Treatment 1 (12 mmolc L^{-1}), with 5.03% in foliar tissue. Treatments 3 and 5 resulted in the highest calcium absorption in foliar tissue, at 0.25%, whereas Treatments 7 and 8 exhibited the lowest values at 0.21%.

DISCUSSION

Interactions between nutrient solutions and physiological parameters

The behavior of plant height and SPAD variables can be attributed to the proportions of NO_3^- and NH_4^+ present in the nutrient solutions. Saloner and Bernstein (2022b) obtained similar effects by modifying the NH_4^+/NO_3^- ratio, determining that when this ratio exceeded 10%, total plant height and inflorescence length decreased due to NH_4^+ phytotoxicity in *C. sativa*. In Treatment 7, the NH_4^+/NO_3^- ratio was 7%, compared to 5% in Treatment 5 and 20% in Treatment 1. This effect can be explained by the role of nitrogen and its ionic form in determining plant size, as nitrogen is an essential component of proteins, porphyrins, and the enzyme Rubisco (Bevan et al., 2021). Although plants can absorb the reduced form of nitrogen to save energy during cellular denitrification and more efficiently form amino groups, they are also susceptible to ammonia toxicity within vacuoles (Yep and Zheng, 2020). In Solutions 3 and 8, the presence of ASA and Phe enhanced foliar biomass accumulation. ASA is processed and converted into salicylic acid within the cell (Peng et al., 2017), and the evaluated dose (20 mg L^{-1}) improved CO_2 fixation during photosynthesis, resulting in a greater amount of structural carbon in the foliage of *C. indica*, as reported by Tucuch-Haas et al. (2020) for maize treated with 0.2 mg L^{-1} ASA. The positive effect of Phe application (500 mg L^{-1}) via the root system in *C. indica* can be attributed to the plant's ability to use it as a temporary nitrogen source. This behavior aligns with a study on *Populus* × *canescens*, in which partial or total substitution of inorganic nitrogen with Phe stimulated greater aerial and root biomass production (Jiao et al., 2018). However, it has been studied that other amino acids have the same property as Phe as a provisional source (Näsholm et al. 2009).

Interactions between nutrient solutions and nutraceutical compounds

During this research, the behavior of cannabinoids was not addressed because, at the time of laboratory determinations, the reference standards had import

restrictions in Mexico. When analyzing the trend of flavonoid data across treatments, it was observed that increasing the total ion concentration in the solution significantly reduced the quercetin concentration in *C. indica* flowers. This response may be influenced by the nutritional dynamics present in the plant's nutrient medium. Saloner and Bernstein (2020) proposed optimal values using response surface methodology for a specialized nutrient solution for *C. sativa*, with concentrations ranging from 11 mmolc L^{-1} of NO_3, 1 to 3 mmolc L^{-1} of H_2PO_4 (Shiponi and Bernstein, 2021), 4.5 mmolc L^{-1} of K (Saloner and Bernstein, 2022a), and 3 to 6 mmolc L^{-1} of Mg (Morad and Bernstein, 2023). These values successfully optimized primary growth while maintaining the synthesis of terpenes and therapeutic cannabinoids. However, in *C. indica*, the production of inflorescences with the highest nutraceutical quality was not observed. This contrasts with Treatment 1, based on the principle of electroneutrality, which resulted in a higher flavonoid concentration in the flowers compared to Treatment 2, which was designed using response surface methodology and yielded lower flavonoid concentrations in this hemp variety.

The group of treatments (2, 3, 4, and 5), according to orthogonal contrast analysis (data not shown), exhibited a synergy with ASA and Phe, possibly due to the overexpression of enzymatic genes activating the plastidial (MEP) and shikimic acid pathways. This led to abnormal enzymatic activity, as noted by Pott et al. (2019), who stated that the internal accumulation of Phe and ASA stimulates precursors of lignin, terpenes, and flavonoids, as well as precursors for phenylpropanoid synthesis. This phenomenon is related to the disruption of the natural balance between primary and secondary metabolism, shifting the demand and transport of sugars toward secondary metabolism to enhance the plant's defense mechanisms (Sun et al., 2019). Naturally, 30% of the fixed carbon is allocated to phenylpropanoid production (Pott et al., 2019).

The Hoagland (1938) and Steiner (1980) solutions were developed to enhance productivity and yield in high-value commercial vegetables. However, the results indicated that *C. indica* does not conform to the principles established by these solutions, as the modified treatments (6, 7, 8, and 9) did not promote the production of nutraceutical metabolites. On the contrary, the addition of ASA and Phe inhibited flavonoid synthesis, possibly because their potassium levels were higher than those in the other solution groups. Saloner and Bernstein (2022a) reported that elevated potassium levels could hinder the biosynthesis of certain secondary metabolites by altering enzymatic activity, potentially leading to excessive vegetative growth at the expense of defensive compound production. The data from this study align with that statement; however, there is no evidence to support the presence of antagonism between ASA and Phe in flavonoid production in *C. indica*.

Interactions between nutrient solutions and nutritional content

The explanation for these results may lie in the selectivity exerted by root membranes. Despite working with widely differing concentrations of nitrogen and magnesium in solution, there was no reciprocal absorption of these nutrients. Rengel et al. (2022) state that the primary site of selectivity in the absorption of cations and anions is the plasma membrane of the cells, where the lipid bilayer prevents the indiscriminate flow of ions and polar molecules from the apoplast to the cytoplasm (influx) and from the cytoplasm to the apoplast (efflux).

The results exceeded those reported by Cockson et al. (2019) for the 'T1' variety of *C. sativa* using Hoagland and Arnon (1938) as a nutrient medium throughout the production cycle. In their study, the average foliar potassium value was 2.85%, and as the concentration decreased to 0.41%, biomass was reduced by 27% compared to the control. Yep and Zheng (2020) tested lower potassium doses (1.9 and 2.9 mmolc L^{-1}) than those used in the present study on *C. sativa* cv. Mandarin and found an increase in the number of marketable inflorescences by 16% and 22%, associated with foliar potassium concentrations of 1.95% and 2.69%, respectively.

Furthermore, orthogonal contrast analysis (data not shown) indicated that potassium absorption was more significantly influenced by the RIT than by the effects of ASA and Phe. Therefore, foliar potassium dynamics are directly proportional to the ion's available concentrations in the nutrient solution. When comparing flavonoid content with foliar potassium levels, it was observed that as potassium concentration in the foliage increased, total flavonoid production in the inflorescences decreased. This confirms the premise of Saloner and Bernstein (2022a) that high potassium concentrations inhibit the production of certain secondary metabolites, leading to greater vegetative growth.

The treatment group analysis (data not shown) indicated that calcium absorption is more closely linked to signaling molecules than to the RIT. The impact of ASA and Phe on calcium assimilation lies in their ability to directly influence calcium metabolism by regulating specific ion transporters in cell membranes and enhancing cell permeability (Khan et al., 2015). This facilitates greater calcium ion uptake by the roots. The absorbed calcium followed an inverse relationship with the RIT, similar to the behavior of quercetin in inflorescences. However, when comparing the trends of potassium and calcium, an antagonistic effect can be inferred.

In agreement with these results, Yep and Zheng (2020)

found that an increase in potassium ions in solution reduced the concentration of divalent cations (calcium and magnesium) in the leaf tissue of *C. sativa*. This antagonism arises from competition among ions with similar properties (group, valency, and ionic radius) for transport sites (carrier proteins), as transporters are rarely specific and depend on ion concentrations in solution (Rengel et al., 2022). This highlights that potassium may have inhibited or blocked enzymatic activities in the secondary metabolic system in solutions combined with Hoagland and Steiner. In contrast, calcium exerted a synergistic effect on the metabolomic system of
C. indica with Bernstein and commercial solutions.

This research supports the claims of Yep and Zheng (2020) and Bevan et al. (2021) regarding the taboos surrounding the empirical management of this crop's nutrition by commercial companies and technical advisors. It is common practice to apply excessive doses of phosphorus and potassium starting from the pre-flowering stage. However, evidence shows that this approach is counterproductive, as it promotes the allocation of sugars to primary pathways rather than secondary ones, resulting in raw materials of very low nutraceutical and medicinal quality. Consequently, Treatment 1 (12 mmolc L^{-1}) is recommended as the best-performing option for both stages of medicinal compound production, not only for its productivity but also for its contribution to sustainable cultivation.

CONCLUSIONS

The results showed that during the vegetative stage, Treatments 6, 7, and 8, based on Hoagland's solution, achieved better development, with plant heights of 9.9, 10.8, and 10.3 cm and SPAD indices of 52.8, 55.2, and 50.1, respectively. During the reproductive stage, the group of treatments (6, 7, 8, and 9) using the Steiner solution base generated high foliar potassium levels (7.74-8.86%) but resulted in lower metabolite production compared to other groups. Treatment 1 exhibited the highest quercetin concentration in inflorescences (1878 mg 100 g^{-1}) and the lowest potassium level (5%) in foliar tissue. The addition of ASA and Phe in the solution promoted greater foliar biomass in Treatments 3 and 8, with 8.3 g of dry matter, and improved calcium absorption in Treatments 3 and 5.

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4. Complemento. PRODUCTION OF NUTRACEUTICAL COMPOUNDS FROM *Cannabis indica* BY DIFFERENT INTEGRATIVE TREATMENTS IN NUTRITIVE SOLUTION[2]

Keywords: Hemp, phenylalanine, kenwhorthy, acetylsalicylic acid, flavonoids.

MATERIALES Y MÉTODOS

Determinación de normas Kenworthy

De los resultados de tejido foliar, se seleccionó el 30 % de los valores relacionados con los mejores rendimientos de flavonoides y fenoles totales. Se promediaron las concentraciones y se obtuvo su desviación estándar (DE) y coeficiente de variación (CV) para cada nutriente conforme a la metodología de Kenworthy (1961). Con las normas anteriores, se realizó el diagnóstico nutrimental de cada tratamiento.

Análisis estadístico

Los resultados de concentración nutrimental en tejido foliar se procesaron estadísticamente mediante SAS Student (Statistic Analysis System) con regresión polinomial, contrastes ortogonales, comparación múltiple de medias y modelos mixtos (Montesinos et al., 2022).

RESULTADOS Y DISCUSIÓN

Diagnóstico nutrimental Kenwhorty

Se delimitaron las normas nutrimentales (Cuadro 5) para cada macronutriente (N, P, K, Ca y Mg) para una producción promedio de 1762 ± 313 y 465 ± 49 mg 100 g^{-1} de flavonoides y fenoles totales en inflorescencias y tejido foliar, respectivamente. De acuerdo con el orden de requerimiento nutrimental (Cuadro 4), todos los tratamientos presentaron hambre oculta de fósforo debido a que se suministró 1 $mmol_c$ L^{-1} en estos, lo cual los resultados sugieren adicionar de 1 a 2 $mmol_c$ de P en solución, manteniendo el rango propuesto por Shiponi y Bernstein (2021) para fósforo. En consecuencia, esta puede ser una limitante nutrimental significante para alcanzar el máximo rendimiento de metabolitos medicinales (Kenworthy, 1961), ya que el fósforo tiene una relación lineal con la síntesis de cannabinoides y terpenos, tal como lo aseguran Shiponi y Bernstein (2021).

La metodología de diagnóstico nutrimental respaldó que los tratamientos 2, 3, 4, 6, 7, 8 y 9 comparten un excedente de potasio en tejidos foliares, lo que correlaciona muy bien el efecto de antagonismo iónico descrito en el apartado anterior sobre el calcio y magnesio absorbido, además del impacto negativo en la síntesis de compuestos metabolómicos en los tejidos estudiados.

Los cationes K y Mg de los tratamientos 1 y 5 manifestaron antagonismo por el exceso de calcio. Sin embargo, al relacionarlos, ambos tratamientos resultaron ser los más efectivos para la producción de flavonoides totales, acompañados de concentraciones muy bajas de K foliar.

[2]Sometido a: No sometido
Estado: No publicado

Cuadro 4. Concentración promedio de macronutrientes en tejido foliar de cáñamo (*C. indica*) bajo diferentes balances iónicos y bioestimulación en solución nutritiva.

Tratamiento	N	P	K	Ca	Mg	IDN[†]	ORN[††]
			(%)				
1	2.61 a	0.26 a	5.03 d	0.24 ab	0.10 a	463.00	P<K<Mg<N<Ca
2	2.89 a	0.22 a	7.15 bc	0.23 abc	0.11 a	498.00	P<Ca<Mg<K<N
3	2.60 a	0.22 a	7.62 b	0.25 a	0.11 a	500.00	P<N<Ca<Mg<K
4	2.59 a	0.20 a	7.26 bc	0.22 bcd	0.10 a	471.00	P<Ca<Mg<N<K
5	2.71 a	0.23 a	6.22 c	0.25 a	0.09 a	471.00	P<Mg<K<N<Ca
6	2.82 a	0.22 a	8.19 ab	0.23 abcd	0.10 a	499.00	P<Mg<Ca<N<K
7	3.12 a	0.22 a	7.74 ab	0.21 cd	0.09 a	488.00	P<Mg<Ca<K<N
8	2.79 a	0.21 a	8.86 a	0.21 d	0.10 a	496.00	P<Ca<Mg<N<K
9	3.07 a	0.23 a	7.81 ab	0.24 ab	0.09 a	501.00	P<Mg<Ca<K<N
Prob.	0.2674	0.7457	0.0020	0.0118	0.4409		
DMS	0.4706	0.1223	1.101	0.0218	0.0177		

Letras diferentes por columnas son estadísticamente significantes (LSD, P = 0.05). [†]Índice de desbalance nutricional. [††]Orden de requerimiento nutrimental comenzando desde el más deficiente.

Esto resalta que el K inhibió o bloqueó las actividades enzimáticas del sistema secundario (Sun et al., 2021). En contraste, el Ca ejerció un efecto sinérgico para el sistema metabolómico. Esta investigación respalda lo que Yep y Zheng (2020) y Bevan et al. (2021) afirmaron sobre los tabús generados durante el manejo empírico de la nutrición de este cultivo por parte de empresas comerciales y asesores técnicos. Ellos recomiendan aplicar dosis excesivas de P y K durante la etapa de prefloración, además de adicionar al riego suplementos para intensificar la síntesis de precursores. Sin embargo, se evidenció que esto es contraproducente, ya que promueve la asignación de azúcares hacia las rutas de crecimiento primario y no a las de defensa, obteniendo materias primas de muy baja calidad nutraceútica y medicinal. En consecuencia, se recomienda al tratamiento 1 (12 $mmol_c$ L^{-1}) como el de mejor desempeño para ambas etapas en la producción de compuestos medicinales, no solo por la productividad, sino también por la parte sustentable del cultivo, ya que puede reducir el impacto ambiental y económico generado por el exceso de fertilizantes empleados, los cuales terminan contaminando los recursos suelo y agua.

Cuadro 5. Estándares (media ± DE) de diagnóstico nutrimental Kenwhorty para la producción de metabolitos secundarios en función de macronutrientes en cáñamo (*C. indica*).

Nutrimento	Estándar	CV	Regresión estimadora[†]	R^2
	(%)			
Nitrógeno	2.51 ± 0.09	3.46	Y_f= -3147.90x^2+16263x-19069	0.52
			Y_p= -816.59x^2+4207.12x-4943.81	0.82
Fósforo	0.42 ± 0.10	24.00	Y_f= -20377x^2+1942x-3125.15	0.10
			Y_p= -805.87x^2+749.01x+173.70	0.10
Potasio	6.31 ± 1.44	22.76	Y_f= -10.10x^2+2071.15	0.28
			Y_p= -22.81x^2+264.39x-275039	0.58
Calcio	0.23 ± 0.02	8.07	Y_f= -107647x^2+64112x-7354.77	0.60
			Y_p= -776538x^2+37596x-4143.85	0.47
Magnesio	0.10 ± 0.01	10.69	Y_f= -1390704x^2+289669x-13117	0.66
			Y_p= -15826039x^3+4785872x^2-474482x+15872	0.66
Sodio	0.11±0.06	53.92	Y_f= -44728x^2+16889x+427.10	0.40
			Y_p= -8312.22x^2+2934.79x+209.37	0.38

[†]Y_f: estimador de flavonoides totales en inflorescencia. Y_p: estimador de fenoles totales en hoja.

Se sugiere que en futuros estudios se aborden otros bioestimulantes empleando un espaciado más amplio entre dosis para manipular la ingeniería del sistema metabolómico y considerar un incremento de 1 o 2 $mmol_c$ de P y Ca para solventar el hambre oculta.

CONCLUSIONES

El diagnostico Kenwhorty confirmó que todos los tratamientos presentaron antagonismo iónico debido a altas concentraciones de potasio en la solución nutritiva, a excepción del tratamiento 1 (12 $mmol_c$ L^{-1} de K) que mostró antagonismo por parte del calcio. Además, todos manifestaron una deficiencia oculta de fósforo.

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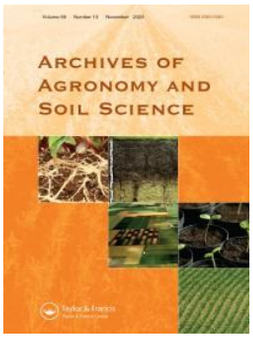

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Boosting antioxidant compounds in *Cannabis sativa* L. By co-inoculation to the hydroponic system in vegetative stage[3]

Víctor M. Marín-Campos, Joel Pineda-Pineda, Langen Corlay-Chee, Mateo Vargas-Hernández, Gustavo Almaguer-Vargas, Rosa M. López-Romero, Vicente Espinosa-Hernández & Cecilia García-Osorio

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5. Boosting antioxidant compounds in *Cannabis sativa* L. By co-inoculation to the hydroponic system in vegetative stage

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ABSTRACT

Co-inoculation with beneficial microbiology has become important in different hydroponic crops. *Cannabis sativa* L. is a plant rich in products of secondary metabolism. Phenolic compounds and flavonoids act as anti-inflammatory, anti-aging, and anticancer agents due to their antioxidant properties. However, the interactions between microbiota and their effect on the synthesis of antioxidant compounds during the vegetative stage have not been studied in depth. Therefore, this research focus to evaluate the impact of *Pseudomonas fluorescens* and *Trichoderma* spp. on the dynamics of the nutrient environment, physiological development and the production of antioxidant compounds. Five treatments were evaluated: without inoculation; 3.33×10^4; 6.67×10^4; 1.00×10^5; 1.33×10^5 CFU L^{-1} of *P. fluorescens* and *Trichoderma* spp. in a divided plot design under controlled conditions. The results showed that 6.67×10^4 CFU L^{-1} stimulated the best development in terms of height (51.24 cm), stem diameter (9.83 mm) and aboveground biomass, as well as increased production of antioxidant compounds, specifically quercetin (353.64 mg) and gallic acid (51.25 mg) in the plant. Finally, high densities of microorganisms in the rhizosphere inhibited vegetative growth. Co-inoculation in the hydroponic system is a biotechnological alternative for the production of antioxidant metabolites.

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KEYWORDS
Rhizosphere; co-inoculation; nutraceutical compounds; secondary metabolites

Introduction

Conventional cannabis cultivation systems include soil and alternative substrates (e.g. peat moss, rock wool, plant fibers). However, in recent years, hydroponic systems (HS) have gained popularity due to their productive potential in controlled growth chambers with artificial light (Alneyadi et al. 2024). Pertierra Lazo and Quispe (2020) state that deep water culture hydroponic systems exhibit high productivity in smaller areas, allow for year-round harvesting, and result in considerable water savings. Nevertheless, they are more expensive than open-field cultivation due to high electricity consumption and the required infrastructure investment, costs that only high-value crops could recover (Alkaabi et al. 2025).

The cultivation of *C. sativa* L. is considered of high commercial value due to its ability to produce a wide range of phytochemical compounds with nutraceutical and medicinal properties of interest to the pharmaceutical industry (Balthazar et al. 2022). These molecules include cannabinoids, phenols, terpenes, flavonoids, and anthocyanins, produced through the activation of secondary metabolism as a plant defense mechanism against biotic and/or abiotic stress (Ahmed and Hijri 2021). Phenolic compounds and flavonoids have received growing interest for their beneficial health effects as anti-inflammatory, anti-aging, and anticancer agents, owing to their ability to neutralize free radicals in the human body (Kim et al. 2002; Aldhanhani et al. 2022). Consequently, technologies for their hydroponic utilization are being developed. As stated by Malík et al. (2023), the recirculation and reuse of nutrient solutions in HS reduce the environmental and economic impact associated with the excessive disposal of solutions into drains and surface water flows, in addition to increasing the concentration of tetrahydrocannabinolic acid (THCA) with lower water and nutrient consumption.

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These developments include the use and management of beneficial microorganisms in the nutrient solution. Innoculation with Plant Growth Promoting Rhizobacteria (PGPR) has gained interest in hydroponic production for its ability to enhance plant development and health (Asif et al. 2023). Currently, mixtures of PGPR and fungi have been introduced for their capacity to promote physiological development through the production of phytohormones, nutrient solubilization, and protection against pathogens (Pineda-Acosta et al. 2022). Previous studies have demonstrated these effects with microorganisms from the Proteobacteria (*Pseudomonas* spp. and *Burkholderia* spp.), Firmicutes (*Bacillus* spp.), and Deuteromycota (*Trichoderma* spp., *Gliocladium* spp. and non-pathogenic *Fusarium oxysporum*) groups (Raaijmakers et al. 2009).

In different hydroponic crops, such as lettuce, celery, and pak choi, an improvement in fresh weight gain, height, biomass, and leaf size was found with the inoculation of different microbial groups (alone or combined) (Pineda-Acosta et al. 2022; Wang et al. 2023; Oliveira et al. 2025a; Oliveira et al., 2025b). In semi-hydroponic tomato cultivation, co-inoculation with *Bacillus subtilis* and *Rhizoglomus intraradices* promoted better growth and fruiting indicators (Zulueta-Rodríguez et al. 2020). Another work in soybean carried out by Jabborova et al. (2018), showed that the joint inoculation of *Bradyrhizobium japonicum* and *Pseudomonas putida* positively modified the architecture of the root to take advantage of phosphorus in solution. In addition to the above, Gato et al. (2023) showed that combined inoculations of *Azospirillum brasilense* and *Trichoderma harzianum* stimulated better mineral biofortification of K, S, Ca, Mg, Zn, Fe, and Mn in arugula plants. Nevertheless, one of the gaps in hydroponic research with *C. sativa* L. is the lack of information on co-inoculation with beneficial microorganisms and the interactions between said microbiota within the plant's growth medium, as well as its impact on the production of antioxidant compounds during the vegetative stage. According to Yu et al. (2022), following colonization by beneficial microbes, the plant 'primes' its defense mechanisms through induced systemic resistance (ISR), which is associated with the intensification of secondary metabolism, consequently leading to a higher production of nutraceutical metabolites. This would add value to pruning waste, a constant activity throughout the plant's life cycle.

Therefore, the present research aimed to evaluate the impact of different inoculation levels of *P. fluorescens* and *Trichoderma* spp. on the dynamics of the nutrient medium, rhizosphere colonization, physiological development, and the production of antioxidant compounds in *C. sativa* as a biostimulation method within the hydroponic system during the vegetative stage.

Materials and methods

Plant material

The *C. sativa* seeds used were of the Belmont cultivar, of regular type (dioecious), obtained through regional participatory breeding among farmers of the peasant union in Tetecala, Morelos, México.

Microbiology source

The commercial product 'Micorrizas' from the company Hydro Farms®, México was used as the source of inoculant material, with a composition of 1×10^8 CFU of bacteria (*P. fluorescens*) and beneficial fungi (*T. harzianum*, *T. viride*, and *T. reesei*).

Seedling management

Seeds were placed inside a hermetic container on a layer of inert cotton and moistened with distilled water. The container was stored for 72 h in total darkness at room temperature (20– 24°C). Afterwards, germinated seeds were selected and sown in rock wool cubes (2.5 × 2.5 × 2.5 cm). They were manually irrigated with tap water to maintain the substrate at container capacity moisture. The seedlings were placed in a reflective tent-type protected environment system of 1.2 × 1.2 × 2 m, monitoring a vapor pressure deficit (DPV) of 1.20 within it. The seedlings were placed under artificial illumination at 300 µmol s^{-1} m^{-2} with 100 W Citizen COB-type LED lamps (yellow and white light), at a distance of 30 cm. The photoperiod employed consisted of 16 h of light and 8 h of darkness throughout the seedling stage. The nursery period lasted for 14 days after sowing (DAS).

Treatment design

The following treatments were evaluated: T1: without inoculation; T2: 3.33×10^4; T3: 6.67×10^4; T4: 1.00×10^5; and T5: 1.33×10^5 CFU L^{-1} of bacteria and fungi, respectively. The inoculation was performed by incorporating the microorganisms into the nutrient solution immediately after the nursery stage, maintaining them until the first solution change. The experimental design was a split-plot with three replications under the same conditions "indoor" as in the nursery stage. For each experimental unit, 3 L capacity plastic containers were used, where the plants were placed on a floating polyurethane base with 2-inch diameter plastic baskets, filled with nutrient solution. Oxygenation was supplied by air pumps with a flow rate of 210 L h^{-1} from two lines. The light condition for the vegetative period was the same as for the nursery stage. The nutrient solution was prepared according to Marín-Campos et al. (2025) with the following final proportions: 7.5 NO_3^-, 1.0 $H_2PO_4^-$, 3.5 SO_4^{2-}, 1.5 NH_4^+, 3.0 K^+, 5.0 Ca^{2+}, and 2.5 Mg^{2+} millimoles of charge per liter ($mmol_c$ L^{-1}), and a microelement dosage of: 2.0 Fe^{2+}, 0.8 Mn^{2+}, 0.1 Zn^{2+}, 0.1 Cu^{2+}, 0.2 B^{2-}, and 0.03 Mo^{2-} mg L^{-1}. Nutrition started at 14 DAS, and the solution was changed every 7 days.

Evaluation of physiological variables

Height, diameter and root length

These variables were evaluated weekly from 21 to 49 DAS. Plant height was measured with a flexible tape measure from the base to the apex of the main apical stem. Stem diameter was measured with a vernier caliper at the midpoint between the cotyledons and the first true leaves. Root length was measured from the base to the tip of the longest root.

Biomass

The biomass of root, stem, and leaf tissue was evaluated at 49 DAS. The samples were stored in paper bags at room temperature and then taken to the oven as described in the sample pretreatment section. The dry parts were weighed with a precision balance (A&D GX-2000).

Evaluation of buffering capacity

The nutrient solution was adjusted to a concentration of 1.50 mM with citric acid (CA) as an organic carbon source to evaluate the behavior of the treatments on pH, electrical conductivity (EC), and redox potential (ORP) variables (Samaniego-Gaxiola et al. 2019). Measurements were taken with a multiple potentiometer (pH/EC/ORP) within the first 19 h after changing the nutrient solution. The measurement of buffering capacity started at 21 DAS and continued with each solution change until 49 DAS.

Evaluation of nutrient dynamics

The measurement of NH_4^+, K^+, Ca^{2+} and Mg^{2+} in the solution was performed using a portable Imacimus® 10 Multi ION selective electrode meter (NT Sensors, Jaume, El Catilar, Spain). Prior to sampling, each treatment was brought to its original volume with distilled water. The sampling of the solution and the determination of the residual nutrient concentration were carried out at the time of changing the in-use solution, recalibrating every 10 samples with the reference standard solutions.

Sample pretreatment

Fresh root samples were stored in plastic bags immediately after harvest and placed in refrigeration for subsequent use in microbiological determinations. All fresh leaf tissues were washed with distilled water to remove impurities. The leaf samples were dried in an oven at 65°C until a constant weight was reached, ground through a 20-mesh sieve, and homogenized to be used in the following measurements: total flavonoids and phenols.

Evaluation of rhizosphere microbiota

Extraction of rhizosphere microorganisms

The extraction and quantification of microorganisms were performed according to Döbereiner et al. (1995). A 5 g sample of fresh root per treatment was taken and placed in glass bottles with 45 mL of phosphate buffer solution (1.10 g K_2HPO_4 +0.32 g KH_2PO_4 L^{-1} of water, pH: 7.2 ± 0.1). The bottles were previously sterilized along with the extractor solution and three glass beads at 300°C in an autoclave for 15 min. The samples were placed on a reciprocal action shaker for 5 min. From each stock solution, four successive dilutions (1:10, v/v) were prepared in a sterile medium, using an extraction hood disinfected with 96% ethanol.

Quantification of total microbial colonies

The development of fungal colonies present in the dilutions was carried out on potato dextrose agar (PDA). The culture medium was prepared with commercial PDA according to the manufacturer's indicated doses per liter of distilled water and sterilized for 15 min. Subsequently, ampicillin was added to the nutrient medium at a dose of 75 mg L^{-1}. Plating was done in triplicate using a 1 mL aliquot from the 10^{-3} to 10^{-5} dilutions in disposable Petri dishes with 20 mL of medium. It was manually homogenized by shaking three times clockwise and three times counter-clockwise. All cultures were incubated at 30°C for 48 h. For *Pseudomonas* spp., the methodology by Lyu et al. (2022) was used. King B was used as the nutrient medium with the following composition: 20 g bovine peptone, 1.5 g K_2HPO_4, 1.5 g $MgSO_4$ and 15 g agar per liter of water. Sterilization, plating, homogenization, and incubation were performed in the same way as for fungi, with the exception of the antibiotic. At the end of the incubation period, the colony-forming units (CFU) of fungi were counted directly, and for *P. fluorescens*, colonies that showed green fluorescence at 395 nm with the aid of an ultraviolet lamp were considered.

Evaluation of nutraceutical compounds

Total flavonoids

The UV-VIS spectrophotometric method proposed by Bakar et al. (2009) was used for the determination of total flavonoids. Ten points (0 to 800 µg mL^{-1}) of quercetin (95%, Sigma-Aldrich) were prepared for the calibration curve. 1:2 dilutions were prepared for samples that did not fall within the curve.

Total phenols

The total phenol content was determined by the Folin-Ciocalteu spectrophotometric method (Koch et al. 2015). From a stock solution (627 µg mL^{-1}) of gallic acid (98%, Sigma-Aldrich), concentrations of 63, 126, 188, 314, 376, 439, 502, and 564 µg mL^{-1} were prepared to create the calibration curve.

Antioxidant capacity

With the phenol extracts, the antioxidant capacity was quantified using 0.1 mM DPPH in UV-VIS spectrophotometry (Kim et al. 2002). Absorbance was measured at 517 nm for each sample with 15-min intervals between measurements using a 10UV UV-visible spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A.).

Statistical analysis

For the physiological and nutritional variables, a repeated measures analysis was applied using mixed models with a first-order autoregressive (1AR) covariance structure and its respective multiple comparison of adjusted means, in SAS® student. Mixed models with subsampling were used for the microbiological and metabolic variables. With the pH, EC, and ORP variables, descriptive graphs were created using the OpenOffice package.

Results

Evaluation of physiological variables

The results in Table 1 show that inoculation with a dose of 6.67×10^4 CFU L^{-1} yielded better growth in plant height (51.24 cm), basal stem diameter (9.83 mm), stem biomass (2.30 g), and leaf biomass (8.30 g); there is a trend of greater root biomass (2.31 g), although there were no significant differences between treatments for the latter. The low dose (3.33×10^4 CFU L^{-1}) promoted greater primary root length (57.10 cm) compared to the other treatments. The highest inoculant dose (1.33×10^5 CFU L^{-1}) generated values similar to the control in stem diameter (4.10 ± 0.2 mm) and a trend of root biomass (0.72 ± 0.02 g). However, for variables such as plant height and root length, it remained below the control, with differences of 10.58 and 7.90 cm, respectively.

Qualitatively (Figure 1), treatment 3 showed better visual development, as it had a more vigorous appearance with more budding, no chlorosis, and large leaflets. The control treatment showed symptoms of interveinal chlorosis in mature and young leaves, similar to those of iron or manganese deficiency, with small leaflets and little secondary budding. Treatment 5 presented symptoms similar to the control, but with burns and necrosis on the leaves instead of chlorosis, resembling symptoms of microelement toxicity.

Evaluation of buffering capacity

According to Figure 2(A), the control showed a more rapid neutralization of citric acid, maintaining a pH above 8 compared to the other treatments. Treatments 2 and 3 exhibited very similar behavior in their buffering curves, reaching stability around pH 8. The curve for treatment 4 showed an intermediate trend between treatments 3 and 5 during the first 12 h; however, the final pH of the solution stabilized very close to treatment 2. The 1.33×10^5 CFU L^{-1} dose maintained the average pH one unit below the control, buffering the nutrient solution at a low alkalinity pH during the plant's weekly development.

Regarding the behavior of EC (Figure 2(B)), the control showed a decrease to 1220 µS cm^{-1} during the first 4 h after changing the nutrient solution. Subsequently, in the next 4 h, it increased by 60 units and finally stabilized at 11 h, oscillating around 1240 µS cm^{-1}. On the other hand, the remaining treatments (2, 3, 4, and 5) showed an increase in EC in the first hour after the solution change. After that hour, it began to decrease, reaching its lowest point in the subsequent three hours. Finally, each treatment's EC increased with certain differences among them until reaching stability, well above the initial EC of the solution. It is important to note that the highest inoculant dose produced the highest EC, followed by treatments 4, 3, and 2.

For ORP (Figure 2(C)), all treatments had a similar behavior until 8 h after the solution change, indicating average values between 150 and 160 mV. However, at 11 h, a deviation of treatments 2, 3, 4, and 5 from the control was observed. At 19 h, the treatments with microorganisms stabilized the nutrient medium between 80 and 145 mV, while the control remained constant at approximately 175 mV.

Evaluation of nutrient dynamics

The results of the cumulative uptake curve for ammonium (Figure 3(A)) and magnesium (Figure 3(D)) showed a non-significant linear trend per week, indicating an average weekly consumption of 1.0 ± 0.1 and

Table 1. Average values of plant height, stem diameter, root length, and biomass during the vegetative stage of *C. sativa* under different combined doses of *P. fluorescens* and *trichoderma* spp. In the hydroponic medium.

Treatment	Height (cm)	Stem diameter (mm)	Root length (cm)	Biomass		
				Root	Stem (g)	Leaves
Control	30.46 bc*	4.10 c	27.12 bc	0.72 a	0.35 b	1.35 b
3.33×10^4	44.58 a	8.35 ab	57.10 a	1.53 a	0.99 b	4.54 b
6.67×10^4	51.24 a	9.83 a	54.96 a	2.31 a	2.30 a	8.30 a
1.00×10^5	32.60 b	5.82b c	34.00 b	1.39 a	0.52 b	3.40 b
1.33×10^5	19.88 c	4.27 c	19.22 c	0.74 a	0.55 b	2.52 b
HSD**	10.9340	2.7531	11.9660	1.1542	0.8865	3.5432
Prob	0.0009	0.0083	< 0.0001	0.1711	0.0093	0.0414

*Means with the same letter per column are not statistically different (Tukey, $p = 0.05$). ** Tukey's honest significant difference.

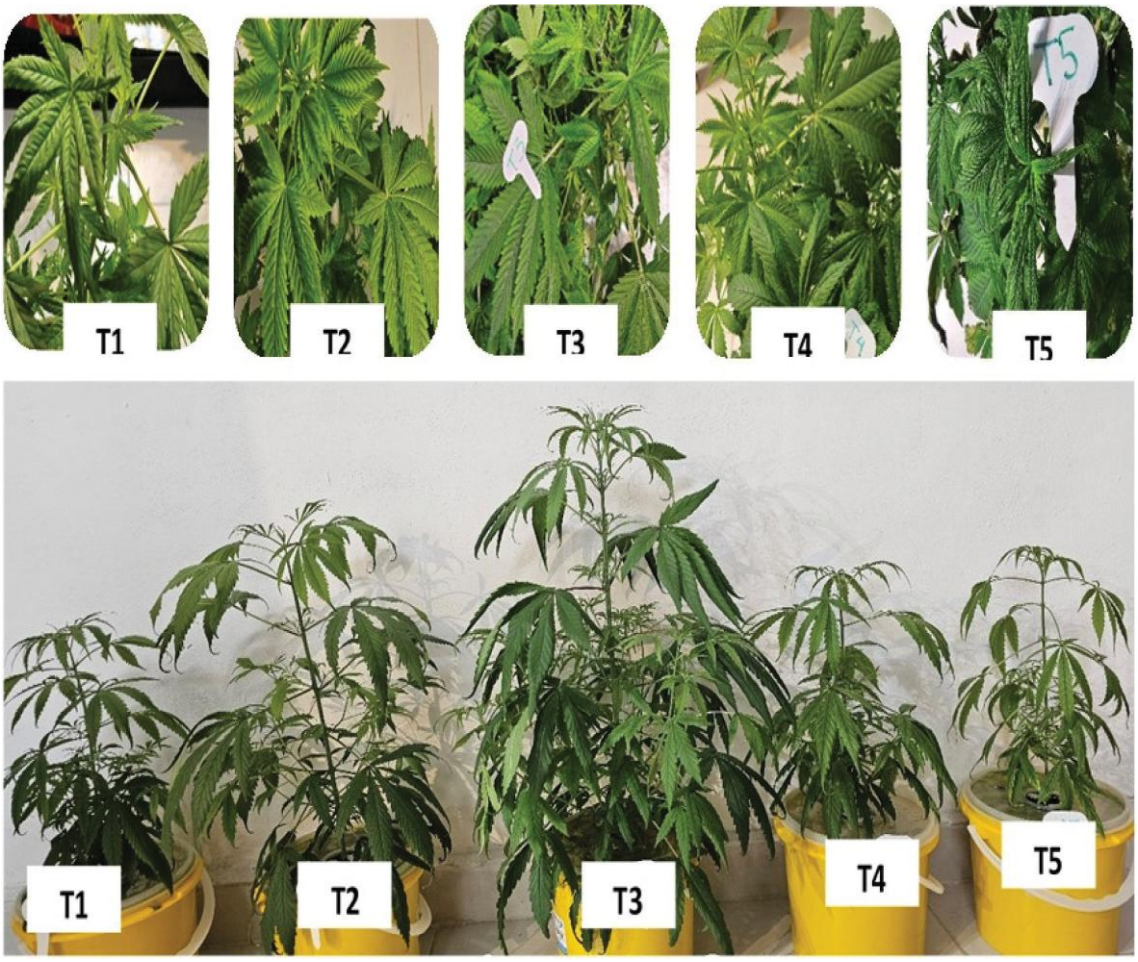

Figure 1. Visual development of *C. sativa* plants during the vegetative stage under different combined doses of *P. fluorescens* and *Trichoderma* spp. In the hydroponic system. T1: without inoculation. T2: 3.33×10^4. T3: 6.67×10^4. T4: 1.00×10^5. T5: 1.33×10^5 CFU L^{-1}.

3.2 ± 0.1 $mmol_c$ L^{-1} of NH_4^+ and Mg^{2+}, respectively, throughout the vegetative stage. For potassium uptake (Figure 3(B)), it was observed that treatment 2 extracted a significantly higher amount of this element compared to the other treatments, with an average consumption of 0.45 $mmol_c$ L^{-1} throughout the vegetative stage. The average consumption for the remaining treatments ranged between 0.3 and 0.4 $mmol_c$ L^{-1} of potassium. In addition, treatments 4 and 5 showed a similar consumption pattern of 0.3 $mmol_c$ L^{-1}, with a change in the last week of the vegetative period where treatment 5 consumed amounts similar to the control. The weekly uptake of calcium (Figure 3(C)) in the treatments ranged from 2.5 to 3.2 $mmol_c$ L^{-1}, with the control showing the highest absorption ($p \leq 0.05$) of this cation from the solution. The concentrations extracted by the inoculated treatments decreased as the microorganism doses increased, with treatment 5 having the lowest calcium uptake, averaging 2.5 $mmol_c$ L^{-1}.

Evaluation of rhizosphere microbiota

Comparing microorganisms per gram of fresh root, the results in Table 2 indicated that treatment 5 promoted the highest colonization of *P. fluorescens* and other fungi, with 1.35×10^7 and 8.53×10^6 CFU, respectively. On the other hand, the highest concentration of *Trichoderma* spp. was established in the control, with 8.98×10^5 CFU, accompanied by the lowest level of *P. fluorescens* (2.28×10^6 CFU). Treatment 3 had the minimum value of *Trichoderma* spp. (9.30×10^3 CFU) and showed the same behavior for other fungi (3.64×10^5 CFU), in addition to an intermediate value of *P. fluorescens* (2.42×10^8 CFU).

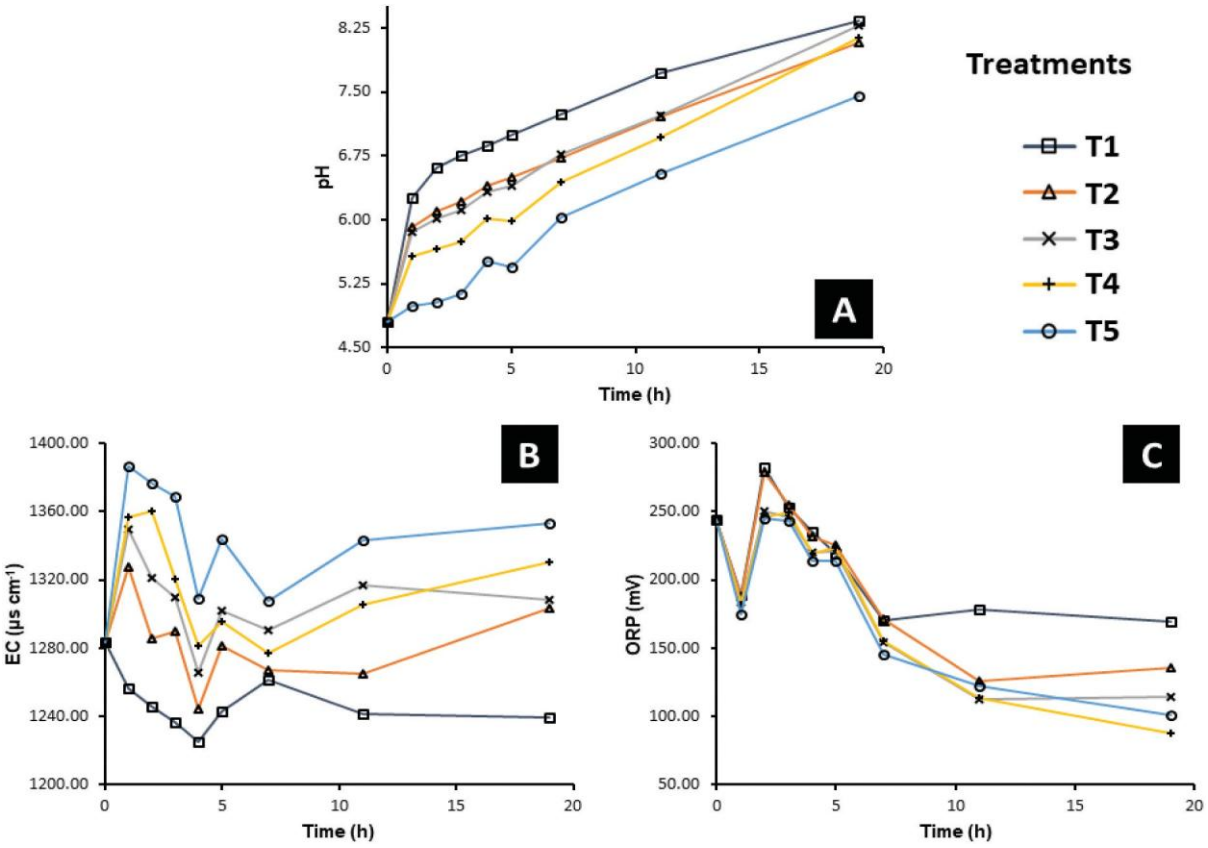

Figure 2. Average behavior of chemical variables (pH, EC, and ORP) for each treatment in the first hours after adjusting the nutrient solution in C. sativa during the vegetative period. T1: without inoculation. T2: 3.33×10^4. T3: 6.67×10^4. T4: 1.00×10^5. T5: 1.33×10^5 CFU of P. fluorescens and Trichoderma spp. L^{-1}.

Considering colonization on the entire root of *C. sativa*, treatment 4 showed a better establishment of *P. fluorescens* (1.10×10^8 CFU), while the control had the poorest performance (9.04×10^6 CFU). Conversely, plants without inoculation in the hydroponic medium maintained a significant colonization of their rhizosphere with high populations (3.75×10^6 CFU) of *Trichoderma* spp. The low co-inoculant dose generated a better establishment of other fungi (4.33×10^7 CFU) on the root surface.

Evaluation of antioxidant metabolites

The average content of total phenols (Table 3) present in the leaf biomass of *C. sativa* showed no significant differences between treatments. The highest content of leaf quercetin (353.64 mg per plant) was obtained with the 6.67×10^4 CFU inoculant dose, compared to the control, which had the lowest production of total flavonoids (51.42 mg per plant). However, the latter exhibited the best neutralization of free radicals (70–83% DPPH) by antioxidant compounds present in the leaf tissue, whereas treatment 3 concentrated fewer antioxidant metabolites (43–62% DPPH) per biomass produced.

Discussion

Effect of microorganisms on the physiological development of the plant

The results obtained in this investigation indicated that treatment 3 showed better physiological and visual development during the vegetative stage, associated with a distribution of 9.51×10^7, 2.12×10^5 and 8.85×10^6 CFU of *P. fluorescens*, *Trichoderma* spp., and other fungi, respectively, across the entire surface of the floating root. This co-inoculation dose showed positive stimulation, possibly due to the release of microbial compounds towards the root of C. sativa. The beneficial effect of the studied

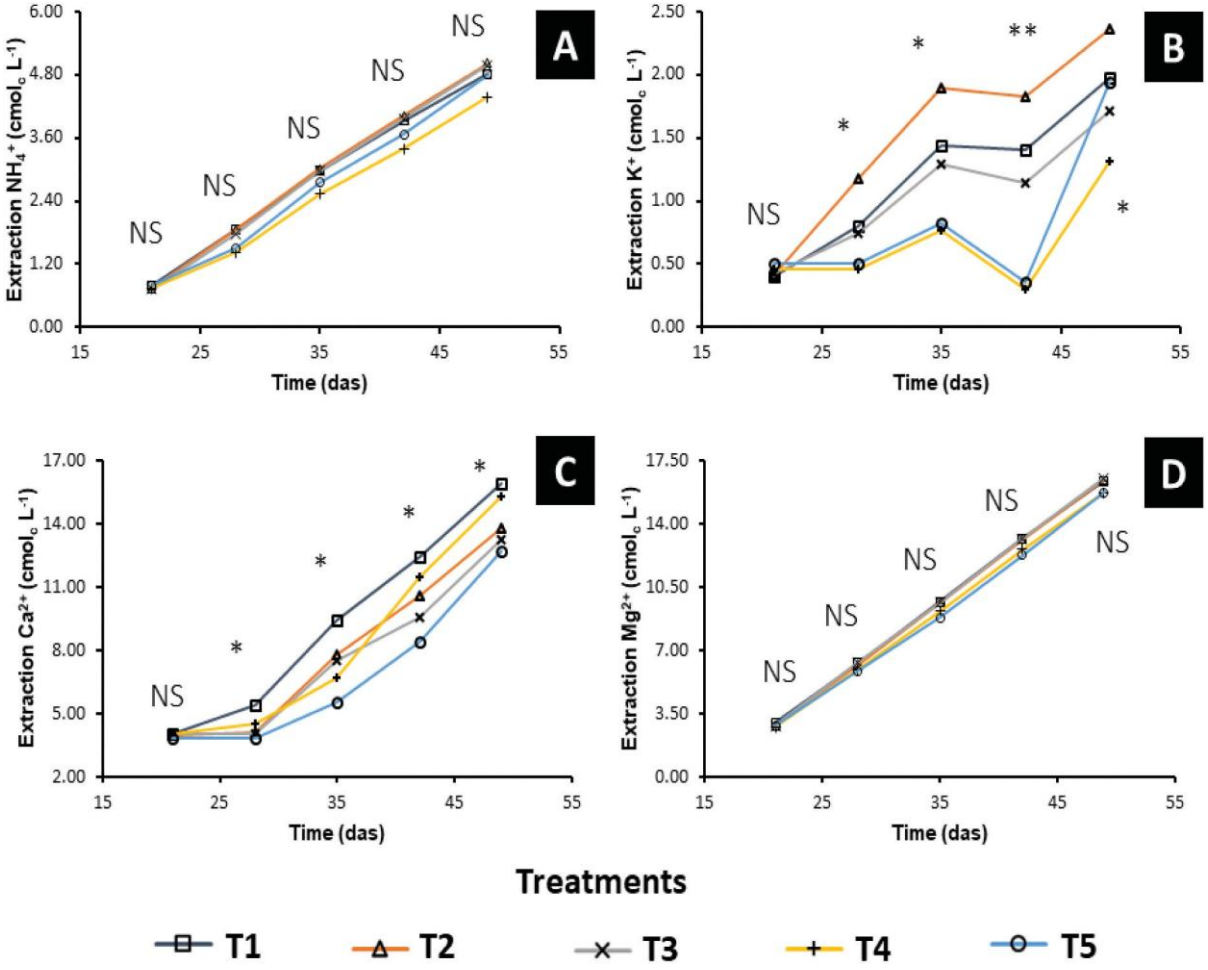

Figure 3. Average cumulative uptake of cations (NH_4^+, K^+, Ca^{2+}, and Mg^{2+}) in the nutrient solution for each treatment during the vegetative period of *C. sativa*. T1: without inoculation. T2: 3.33×10^4. T3: 6.67×10^4. T4: 1.00×10^5. T5: 1.33×10^5 CFU of P. fluorescens and Trichoderma spp. L^{-1}.

Table 2. Average CFU values of PGPR bacteria and fungi established in the root of *C. sativa* at the end of the vegetative stage in a hydroponic system.

Treatment	*P. fluorescens*	*Trichoderma* sp. CFU g^{-1} fresh root	Other fungi	*P. fluorescens*	*Trichoderma* sp. CFU/plant	Other fungi
Control	2.28×10^6 b*	8.98×10^5 a	2.15×10^6 b	9.04×10^6 b	3.75×10^6 a	1.01×10^7 bc
3.33×10^4	2.30×10^6 b	1.02×10^4 b	1.98×10^6 b	5.80×10^7 ab	2.36×10^5 b	4.33×10^7 a
6.67×10^4	2.42×10^6 b	9.30×10^3 b	3.64×10^5 b	9.51×10^7 a	2.12×10^5 b	8.85×10^6 bc
1.00×10^5	2.54×10^6 b	5.60×10^4 b	5.82×10^5 b	1.10×10^8 a	1.84×10^6 ab	4.28×10^6 c
1.33×10^5	1.35×10^7 a	6.11×10^5 a	8.53×10^6 a	8.94×10^7 a	2.95×10^6 ab	3.13×10^7 ab
HSD**	7.88×10^5	8.18×10^4	7.83×10^5	1.55×10^7	5.66×10^5	4.48×10^6
Prob	< 0.0001	< 0.0001	< 0.0001	0.0049	0.0014	< 0.0001

*Means with the same letter per column are not statistically different (Tukey, $p = 0.05$). **Tukey's honest significant difference.

microorganisms lies in their ability to synthesize growth regulators (auxins, cytokinins, and gibberellins), which promote cell differentiation, division, and elongation in the plant (Sandheep et al. 2013; Contreras-Cornejo et al. 2016; Balthazar et al. 2022). Additionally, they release siderophores (pyoverdine) and hydrolytic enzymes into the medium to increase the availability of micronutrients (Weller 2007) and improve nitrogen absorption (Halifu et al. 2019).

Similar results have been documented in other crops under inoculation in hydroponic systems. Pineda-Acosta et al. (2022) evaluated the impact of *Azospirillum brasilense*, *T. harzianum*, and *Bacillus subtilis* (alone and in combination) on *Lactuca sativa* L. 'Parris Island' and noted a significant increase in height, biomass

Table 3. Average yield of flavonoids, total phenols, and antioxidant capacity (%) present in dry matter of leaf tissue in *C. sativa* under different combined doses of *P. fluorescens* and *trichoderma* spp. In the nutrient solution.

Treatment	Total flavonoids† (mg plant^{-1})	Total phenols†† (mg plant^{-1})	Antioxidant capacity ††† (min) 15	30	45	60	75
Control	51.42 b*	12.41 a	70.15 a	75.90 a	79.26 a	83.00 a	82.88 a
3.33×10^4	163.53 ab	33.54 a	45.94 c	49.97 c	55.40 c	58.50 bc	62.96 b
6.67×10^4	353.64 a	51.25 a	42.59 c	49.33 c	53.49 c	55.13 c	61.61 b
1.00×10^5	141.35 ab	27.32 a	54.18 b	59.37 b	62.83 b	65.61 b	67.76 b
1.33×10^5	84.01 b	24.11 a	52.88 b	57.55 b	61.30 b	63.74 b	65.99 b
HSD**	235.99	47.462	5.2874	5.1235	4.2674	7.7667	6.5677
Prob	0.0416	0.2074	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001

*Means with the same letter per column are not statistically different (Tukey, $p = 0.05$). **Tukey's honest significant difference. †Total flavonoids expressed as mg of quercetin present in leaf tissue. ††Total phenolic compounds expressed as mg of gallic acid present in leaf tissue. †††Antioxidant capacity expressed as a percentage of DPPH neutralized by antioxidant compounds present in 0.50 g of leaf tissue.

leaf area, and root volume when using *A. brasilense* compared to the control. However, the interaction of the three strains as a microbial consortium inhibited favorable development, resulting in parameters equal to or even lower than the control due to the absence of oxygen. On the other hand, Oliveira et al. (2025a) worked on the same lettuce species under a nutrient film technique, incorporating co-inoculation of *B. subtilis*, *P. fluorescens*, and *A. brasilense* into the system and found that the combination of PGPR bacteria increased shoot length, fresh weight, number of leaves, and accumulated biomass by 68%, 92%, 30%, and 160%, respectively, compared to the uninoculated treatment. Similarly, positive developments were observed in other plants such as celery and pak choi (Oliveira et al., 2025b; Wang et al. 2023).

In production systems with alternative substrates, the inoculation of low (2×10^{12} CFU kg^{-1}) and high (4×10^{12} CFU kg^{-1}) doses of *T. harzianum* via fertigation improved plant height and biomass in *C. sativa* cv. Fedora 17 and Felina, compared to its absence (Kakabouki et al. 2021). In the 'CBD Kush' cultivar, inoculation with 1×10^8 CFU kg^{-1} of *Bacillus* sp., *Mucilaginibacter* sp., and *Pseudomonas* sp. promoted greater height, aerial biomass, and root exploration length and volume during the cutting stage, with *Pseudomonas* sp. being highlighted as the best promoter of vegetative and reproductive growth in *Cannabis* (Lyu et al. 2022). The same physiological trend has also been demonstrated in *Vanilla planifolia* (Sandheep et al. 2013) and tomato (Anbazhagan et al. 2020) plants with the joint inoculation of *P. fluorescens* and *T. harzianum*.

The data obtained showed that high amounts of inoculant (4 and 5) in the hydroponic system inhibit the optimal development of the plants, although the present research does not delve into what type of microbial exudates were involved during the excessive microbial activity to manifest this negative effect. However, several authors have explained that the main effect of microbiological overactivity is anoxia and competition for available nutrients in the plant's growth medium (Plocek et al. 2024). Subsequently, toxicities from excreted metabolites such as hydrogen cyanide predominate, as several *Pseudomonas* strains (*putida*, *fluorescens*, *aeruginosa*, etc.) have the ability to produce it as a defensive antibiotic to inhibit other bacterial and fungal species that compete with them for carbon and iron sources, among other molecules that ensure their survival (phenazines, 2,4-diacetylphloroglucinol, pyrrolnitrin, harzianic acid, and pyoluteorin) (Compant et al. 2005; Weller 2007; Raaijmakers et al. 2009; Shoresh et al. 2010; Balthazar et al. 2022; Yu et al. 2022).

Effect of microorganisms on the buffering capacity of the nutrient medium

In previous studies, Harman et al. (2004) found that inoculation with *Trichoderma* sp. acidified the pH of the nutrient medium by 0.3 to 0.7 units, as was perceived in Figure 2A, where increasing doses of the inoculum tend to maintain an acidic pH for a longer time compared to the control. On the other hand, *P. fluorescens* contributed to lowering the medium's pH. This is because these same bacteria can acidify or alkalinize, depending on the strain and the substrate used (Compant et al. 2005). The behavior observed for *C. sativa* is attributed to a synergy between microorganisms for this parameter, where the treatment with the highest dose shifted the pH by up to 1 unit with respect to the control. This buffering effect lies in the release of organic acids (citric, malic, acetic, and oxalic acid) during aerobic microbial metabolism into the root's growth medium (Samaniego-Gaxiola et al. 2018; Halifu et al. 2019).

Regarding EC, both microorganisms manage to increase it through the release of organic salts, lytic enzymes, siderophores, metabolites, and growth regulators resulting from microbial activity that breaks down easily assimilable organic molecules like CA, and sugars, lipopolysaccharides (LPS), and signaling metabolites available in the root periphery (Weller 2007; Kakabouki et al. 2021). Yu et al. (2022) mention that, among the exudates, microbial metabolites such as flagellin, LPS, exopolysaccharides, and chitin oligosaccharides have been characterized, which are compounds with low electro-conductivity and the ability to couple to root receptors to initiate ISR pathways. According to Mastouri et al. (2012), *Trichoderma* sp. usually raises the EC by 15% to 30% relative to the control, while *P. fluorescens* tends to increase it by 10% to 25% (Raaijmakers et al. 2009). The average values obtained (Figure 2(B)) support this premise, given that treatments 2, 3, 4, and 5 increased the EC in the nutrient solution by 4, 5, 8, and 10% in relation to the control, respectively, which translates into greater availability of nutrient solutes for the plant (Halifu et al. 2019).

The microbial metabolic activity impacted the ORP in the nutrient medium, as the results obtained for *C. sativa* grown under a hydroponic system are consistent with those of Samaniego-Gaxiola et al. (2019) for the cultivation of pecan, where the inoculation of *T. harzianum* in combination with 2 mg of glucose g^{-1} of soil caused the ORP to decrease from 145 to 10 mV on the second day after applying reductive disinfection treatments. This trend was observed among the treatments (Figure 2(C)) on the first day after the solution change, where the evaluated doses promoted average values between 145 and 80 mV. Shoresh et al. (2010) mention that *Trichoderma* sp. tends to reduce the ORP by an average value of 20–80 mV, while *P. fluorescens* can modify it by ± 15 to 30 mV. Both microbiological groups consume oxygen during their metabolism and respiratory processes, decreasing the concentration of oxygen and reactive oxygen species (ROS) in solution. Therefore, the oxidizing potential of the medium is reduced, and the transfer of electrons to other elements and present complexes increases (Halifu et al. 2019), creating a more favorable environment due to reducing conditions for the absorption of certain nutrients and compatible microbial activity (Vinale et al. 2008). The synergistic reduction of ORP through combined inoculation correlates with better morphological parameters such as height, stem diameter, and fixed biomass, and it helped to resolve the ferric chlorosis present in the untreated plants, a characteristic problem of aerated conditions and a neutral or alkaline pH where most of the iron is in an insoluble form (Fe^{3+}) (Sinha et al. 2018).

Effect of microorganisms on nutrient uptake

The control treatment significantly led in calcium uptake, while the lowest inoculation dose did so for potassium. Nishad et al. (2020) and Yu et al. (2022) affirm that the stimulation of elicitation through false pathogenesis by beneficial microorganisms promotes a rapid accumulation of ROS and an influx of peripheral Ca^{2+} into the cytosol to couple with phospholipase C and release calmodulin, blocking the influx of K^+, Cl^-, and NO_3^- across the root membranes. Calmodulin acts as a sensory molecule for the phosphorylation of protein kinase, which activates the expression of genes for enzymes that catalyze secondary metabolites at the cost of intensifying the efflux of these ions into the nutrient solution. Therefore, the control, being in more oxidized conditions and with active ROS, needed to absorb more calcium from the medium to synthesize a greater quantity of antioxidants per unit of dry matter to neutralize them. It is noteworthy that among the other inoculated treatments, there was a blockage of Ca^{2+} influx. Conversely, treatment 2 consumed more potassium and showed an antagonism towards calcium, but it had the lowest values of these compounds, which reaffirms the findings by Marín-Campos et al. (2025), where an antagonism on potassium caused by the calcium ion in *C. indica* potentiates the production of nutraceutical compounds.

This work on *C. sativa* correlates with trials on hydroponic 'Black Summer' pak choi by Plocek et al. (2024), where they observed low absorption efficiencies of potassium and calcium in treatments with *Bacillus amyloliquefaciens* combined with *Trichoderma* sp., compared to the control treatment, and no significant effect on ammoniacal nitrogen and magnesium. Moreira et al. (2022) document similar results in which the inoculation of *T. harzianum* in lettuce under the same cultivation system did not generate significant effects on nutrient absorption. In accordance with the previous studies, *C. sativa* 'Belmont' would be categorized within these cases, as most authors claim improvements in nutrient absorption with the inoculation of beneficial microorganisms in hydroponic systems (Pineda-Acosta et al. 2022; Oliveira et al. 2025a).

Effect of microorganisms on the production of antioxidant metabolites

Treatment 3 also demonstrated a higher production of secondary metabolites in leaf tissue per plant. The data align with the assertion by Balthazar et al. (2022) in other medicinal species, where inoculation with *P. fluorescens* manages to stimulate the synthesis of phytochemical compounds (terpenes, phenols, flavonoids, alkaloids, and anthocyanins) due to the detection of beneficial microorganisms as a potential threat, triggering ISR chemical reactions in the plant. Within these reactions, the plant allocates more reserves towards the production of salicylic acid, jasmonates, and ethylene, beginning with the accumulation of phenylpropanoid precursors in the vacuoles (Pott et al. 2019). *Trichoderma* spp. tends to activate the jasmonic acid and ethylene pathways, while *P. fluorescens* induces responses mediated by salicylic acid and other hormones (Yu et al. 2022). Previous studies support this premise, as substrate inoculations with *T. harzianum* significantly increased the CBD content in medicinal hemp (Kakabouki et al. 2021), because *Trichoderma* spp. tends to colonize between the epidermis and the root cortex, which induces the surrounding plant cells to deposit more cell wall material and increase the synthesis of phenolic compounds destined for lignin, preventing it from growing inwards (Compant et al. 2005), including the overproduction of ethylene precursors (Shoresh et al. 2010). It is suggested that future research should delve deeper into the cascade reactions associated with the plant's systemic defense caused by the inoculation of both species, given that there are few experimental works that support the theoretical explanation of the metabolic pathways activated by beneficial microbiology in *C. sativa*.

The reason why the antioxidant capacity was higher in the control compared to the other treatments lies in the distribution of phytochemical compounds throughout the total biomass produced. In crops such as basil (*Ocimum basilicum*), sage (*Salvia officinalis*), and sweet orange (*Citrus sinensis*), it has been observed that more productive plants have lower concentrations of total phenols and flavonoids per gram of dry matter (Nguyen and Niemeyer 2008; Asai et al. 2016; Vosoughi et al. 2018). This is attributed to a dilution effect, as primary metabolism is prioritized over secondary metabolism during carbohydrate allocation.

Consequently, the greater the total biomass produced, the lower the concentration of antioxidant metabolites in tissues, and vice versa. By relating different research works with the present one, it is inferred that the optimal quantity of microorganisms to promote a beneficial stimulation for the plant is species-specific and depends on the desired objective, such as higher productivity, disease resistance, post-harvest quality, and nutraceutical quality.

Conclusions

The results showed that co-inoculation in the hydroponic system with 6.67×10^4 CFU L^{-1} of *P. fluorescens* and *Trichoderma* spp. stimulated the best physiological development in *C. sativa* 'Belmont' in terms of plant height (51.2 cm), stem diameter (9.8 mm), and aerial biomass (2.3 g of stem and 8.3 g of leaf) during the vegetative stage; as well as a higher production per plant of antioxidant compounds, specifically quercetin (353.6 mg) and gallic acid (51.3 mg). This was accompanied by favorable reducing conditions (pH 8.3, EC 1308.3 µS cm^{-1}, and ORP 114.1 mV) in the growth medium, and an average weekly uptake of 1.0, 0.3, 2.7, and 3.3 $mmol_c$ L^{-1} of ammonium, potassium, calcium, and magnesium, respectively. Furthermore, these effects were associated with a colonization of 9.51×10^7, 2.12×10^5, and 8.85×10^6 CFU of *P. fluorescens*, *Trichoderma* spp., and other fungi throughout the root, respectively. The uninoculated treatment showed a higher antioxidant capacity (70 to 83% of DPPH) per sample of dry matter. High densities of microorganism populations (1.35×10^7 *P. fluorescens*, 6.11×10^5 *Trichoderma* spp., and 8.53×10^6 other fungi) established per unit of fresh root inhibit the vegetative growth of the plant. Co-inoculation in the hydroponic system is a biotechnological alternative for the production of antioxidant metabolites. However, it will favor or harm the development of the plant according to the microbial population density established in the rhizosphere.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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POTENTIATION OF PHYTOCHEMICAL QUALITY IN INFLORESCENCES OF *Cannabis sativa* L. BY EXOGENOUS APPLICATION OF BRASSINOLIDE[4]

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6. POTENTIATION OF PHYTOCHEMICAL QUALITY IN INFLORESCENCES OF *Cannabis sativa* L. BY EXOGENOUS APPLICATION OF BRASSINOLIDE

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ABSTRACT

Cannabis sativa L. is a high-value crop due to its ability to synthesize secondary metabolites with nutraceutical, pharmacological and therapeutic applications. The manipulation of the biochemical pathways of these compounds by phytoregulators is an alternative to improve the phytochemical quality of the inflorescences. However, the impact of brassinosteroids on the intensification of phytochemical compounds has not been addressed. Therefore, in this study the effect of foliar application of different levels of brassinolide (0 - 1.00 mg L^{-1}) during the flowering stage in the cultivar 'Belmont' was evaluated, under an integrative nutritional scheme with biostimulants, growth regulators and beneficial microorganisms. The experimental design was randomized complete blocks with three replicates per treatment. The results indicated that leaf biomass and flower yield did not present significant differences between treatments ($P \geq 0.05$). However, the dose of 1.00 mg L^{-1} promoted higher concentrations of cannabidiol (29.4 mg), tetrahydrocannabinol (1451.9 mg), and cannabigerol per plant, in addition to a greater diversity of sesquiterpenes such as caryophyllene and α-farnesene. In contrast, low doses tended to inhibit the accumulation of secondary metabolites. Multivariate analysis revealed interactions between phenols, flavonoids, and cannabinoids, with a metabolomic break-even between 190 - 205 mg gallic acid. It is concluded that the application of brassinolide at high doses enhance the MEP pathway, favoring the accumulation of cannabinoids and terpenes without compromising productive performance. These findings highlight the potential of brassinosteroids to improve phytochemical quality in integrative management schemes.

Keywords: brassinosteroids, secondary metabolites, biochemical pathways, integrative nutrition, *Cannabis sativa* L.

INTRODUCTION

Cannabis is considered a crop of high commercial value due to its capacity to produce a wide range of phytochemical compounds with psychoactive, nutraceutical, and medicinal properties for humans (Bautista et al., 2021). Ahmed & Hijri (2021) mention that, within its composition, different groups of metabolites can be found, such as cannabinoids, phenols, terpenes, flavonoids, and alkaloids, which are produced through the activation of secondary metabolism as a plant defense mechanism. Currently, these compounds have aroused great interest due to their potential to combat nervous system disorders, such as psychiatric (anxiety and depression), neurodegenerative (dementia, Alzheimer's, and Parkinson's), and neurological (epilepsy) conditions, among others (Martínez et al., 2007; Enciu et al., 2011; Quinapanta et al., 2023), as well as for their anti-inflammatory, anti-aging, and anticancer properties in the human body (Kim et al., 2002).

Andre et al. (2016) point out that the different chemotypes of *Cannabis* synthesize the vast majority of their precursors and final phytochemicals through metabolic pathways such as the shikimic acid, malonic acid, mevalonate, polyketide (OAA), and plastidial (MEP) pathways (Raharjo et al., 2004; Baud et al., 2007; Karak, 2019; Yang et al., 2020). Consequently, *Cannabis* spp. growers have sought alternatives to intensify or manipulate these pathways in order to improve phytochemical production through different cultivation techniques (Trono, 2025), including the use of biostimulants and plant growth regulators (PGRs).

Within the first group, Malík et al. (2022) evaluated a mixture of 17 amino acids on *Cannabis sativa* L. 'McLove' plants and found that cannabinoid content in the inflorescences decreased by 44%; however, d-limonene increased between 81% and 123% compared to the control. In another study on the cv. NB100, the application of humic substances as a supplement to the nutrient solution improved the spatial distribution of cannabinoids towards the upper part of the plant, rather than increasing concentrations in the treated flowers (Bernstein et al., 2019). Beleggia et al. (2023) tested elicitation with chitosan at different molecular weights (low, medium, and high) and two foliar doses (50 and 250 mg L^{-1}) in 'Codimono' hemp. The low dose positively stimulated the content of total phenols, β-tocopherol, and antioxidant capacity, but there were no major changes in cannabinoid production.

Regarding phytoregulators, Burgel et al. (2020) applied, individually and combined, naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP) via foliar spray on the Kanada, 0.2x, and FED cultivars. These treatments promoted better plant architecture but did not favorably modify the production of inflorescences or cannabinoids; in fact, flower yield decreased considerably in the 0.2x and FED cultivars. The exogenous application of ethephon (60, 121, 1041, and 1394 mg L^{-1}) as an ethylene precursor significantly modified the cannabinoid content in the Felina 32, Kompolti, and Santhica 27 cultivars; notably, the low dose induced feminization of monoecious phenotypes (Ruzgas et al., 2024). The effect of joint stimulation, using the two aforementioned groups, was described by Marín-Campos et al. (2025), who found a significant synergism between phenylalanine and acetylsalicylic acid on quercetin synthesis in *Cannabis indica* inflorescences in phenotypes under peasant breeding. However, the impact of brassinosteroids as a complementary tool to potentiate the production of these phytochemical compounds in other cultivars under an integrative nutrition scheme has not been deeply explored.

Brassinosteroids (BRs) are a group of polyhydroxylated steroidal hormones that exert a physiological effect on plant development at low concentrations (Rodríguez et al., 2022). Within the BR group, brassinolide (BL) stands out as the primitive natural phytohormone discovered in rapeseed or canola (*Brassica napus*) pollen, along with its functional isomers 28-homobrassinolide (HBR) and 24-epibrassinolide (EBR), derived from stigmasterol and ergosterol, respectively (Hernández & García-Martínez, 2016). Previous studies on other crops have measured the impact of BRs on secondary metabolic pathways, as demonstrated by Xu et al. (2015), who defined the effect of foliar EBR application (0.1 - 0.8 mg L^{-1}) on *Vitis vinifera* 'Cabernet Sauvignon', noting that the 0.4 mg L^{-1} concentration resulted in a higher synthesis of secondary metabolites such as anthocyanins, phenolic compounds, flavonoids, and tannins. Çoban & Baydar (2016) also evaluated different levels of EBR (0, 0.5, 1.5, and 2.5 mg L^{-1}) on *Mentha piperita* L. plants under salinity conditions and observed positive effects such as a reduction in membrane permeability, lipid peroxidation, and mortality rate, alongside an increase in essential oil concentration and antioxidant enzyme activity.

Manipulating the metabolomic system could directly influence the obtainment of raw materials of superior quality, both nutraceutical and medicinal. This would benefit producers with higher

incomes from the commercialization of their harvested products, and final consumers through better utilization of health-beneficial metabolites. Likewise, it could lay the foundation for future research focused on intensifying secondary metabolism over primary metabolism through an integrative and holistic strategy. Therefore, the objective of this work was to evaluate the impact of different levels of brassinolide, applied via foliar spray during the flowering stage in *C. sativa* 'Belmont' under an integrative nutritional scheme, to determine the physiological dose that potentiates the yield of phytochemical compounds.

MATERIALS AND METHODS

Analytical equipment

For spectrophotometric methods, a 10UV UV-visible spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) was used. For gas chromatography analysis, an Agilent 7890A chromatograph (Agilent Technologies, Santa Clara, California, USA) coupled to an Agilent 5975C mass spectrometer was utilized. Analyte separation was performed using a DB-35MS Ultra Inert column (30 m × 0.25 mm, 0.25 µm). The chromatographic conditions were: initial temperature of 50 ºC for 2 min, followed by an increase of 10 ºC/min up to 200 ºC for 1 min, and finally 20 ºC/min up to 300 ºC for 5 min. Helium was used as the carrier gas at a flow rate of 1 mL/min (Pellegrini et al., 2005).

Plant material

C. sativa L. plants obtained through vegetative propagation from female mother plants of the Belmont cultivar, donated by producers from the peasant union of Tetecala, Morelos, Mexico, were used.

Management of plant material

Three-week-old rooted cuttings were transplanted into 4 L plastic pots with microorganism-enriched substrate. The day prior to transplanting, the coconut coir substrate (70:30, fiber:dust) was soaked with tap water to remove residual salinity. The following day, the supernatant was removed, and it was washed three times with tap water. Subsequently, 1 L of the granulated product Hydro Farms® (1×10^8 CFU of *Pseudomonas fluorescens*, *Trichoderma harzianum*, *T. viride*, and *T. reesei*) was added per 100 L of final substrate as a source of beneficial microorganisms and homogenized. Irrigation was performed manually with a 50% nutrient solution appropriate for the vegetative stage to maintain substrate moisture and feed the cuttings. Plants were placed under artificial lighting at 250 µmol s^{-1} m^{-2} with 300 W Full Spectrum high-efficiency LED lamps at a distance of 30 cm. The photoperiod used was 16 hours of light and 8 hours of darkness throughout this stage. The conditioning period for the cuttings lasted 2 weeks after transplanting (WAT).

Treatment design

The following foliar treatments were evaluated: an untreated control and five levels of BL (90%, XenanXinyu Chemical Technology Ltd): 0.2, 0.4, 0.6, 0.8, and 1.0 mg L^{-1}. Treatment application was carried out twice: the first after defoliation and the second 14 days later. Inex-A Cosmocel® was used as a surfactant at 1 mL per liter of water for the foliar treatments. The experimental design was randomized blocks with three replicates under controlled conditions in a 1.2 × 2.4 × 2 m reflective tent system, maintaining an internal vapor pressure deficit between 1.0 and 1.2. Inflorescence production was standardized to four main branches through formative pruning and the removal of new shoots. Severe defoliation was performed at the transition from the vegetative to the reproductive cycle (6 WAT), preserving the first three apical shoots and removing all subsequent ones. Lighting conditions for the vegetative stage were the same as those for conditioning. For the reproductive stage, it was adjusted to 350 µmol s^{-1} m^{-2} with a 12/12 cycle at a distance of 50 cm. As a nutrition source, High Nutrients® kits were employed: Base + Protecta + Veg A + Veg B + God Root for the vegetative phase; and Base + Protecta + King Bloom A + King Bloom B + God Root + Bloomer for the flowering phase, following the nutritional schedule proposed by the commercial company. The nutrient solution was supplemented during the flowering stage according to Marín-Campos et al. (2025), adding 100 and 500 mg L^{-1} of acetylsalicylic acid (ASA) and phenylalanine (Phe), respectively. Nutrition was provided by drip irrigation, according to the plant needs, and was suspended at 14 WAT, replacing it with tap water to flush excess nitrates from the inflorescences. Thrips and mite control was performed using Confidor Bayer® (imidacloprid) at a dose of 1.5 mL per liter of water, depending on the frequency of appearance.

Evaluation of physiological variables

Leaf biomass Leaf biomass after defoliation was measured at the end of the harvest. Samples were stored in paper bags at room temperature and subsequently oven-dried at 60 ºC until a constant weight was reached. Dry tissues were weighed using a precision balance (A&D GX-2000). Inflorescence yield Harvesting was carried out according to the commercial maturity of the trichomes (30% amber color) between 16 and 17 WAT, removing leaves that sprouted between the inflorescences. For drying, the inflorescences were hung inside a tent at room

temperature in total darkness for three weeks. Afterwards, they were weighed on a balance and stored in airtight glass jars.

Sample pretreatment

Dried inflorescence samples were ground with a mortar for the measurements of total flavonoids and phenols. For total cannabinoid and terpene determinations, the samples were crushed with a hand grinder. Each sample was manually homogenized and stored in plastic tubes under refrigeration until further use.

Evaluation of the phytochemical profile

Total phenols

Total phenol content was determined by the Folin-Ciocalteu spectrophotometric method (Koch et al., 2015). Extraction was performed from 0.5 g of sample, to which 10 mL of methanol:water (80:20, v/v) was added in amber vials. Subsequently, the samples were magnetically stirred for 60 minutes. Phase separation was carried out in a 5804R centrifuge (Eppendorf, Barkhausenweg, Hamburg, Germany) at 5000 rpm for 10 minutes. The supernatant was filtered and brought to a volume of 10 mL with additional extraction solvent. The colored complex for samples and standards was developed following the authors' procedure. From a stock solution of gallic acid (98%, Sigma-Aldrich), eight calibration points were prepared ranging from 0 to 627 µg mL^{-1}.

Total flavonoids

The method proposed by Aparna & Hema (2022), modified for the determination of total flavonoids, was used. The extraction phase was carried out in amber vials by adding 10 mL of ethanol (96%) to 0.5 g of sample. Subsequently, they were placed on a magnetic stirrer for 120 min at room temperature and left refrigerated at 4°C overnight. The next day, the samples were shaken again, the supernatant was collected in tubes, and centrifuged at 5000 rpm for 5 min. The samples were filtered and brought up to 10 mL again with the same solvent. An aliquot (300 µL) was taken from each extract, using ethanol as a blank. Color development was performed according to the consulted methodology. Ten points of quercetin (95%, Sigma-Aldrich) ranging from 0 to 1000 µg mL^{-1} were prepared for the calibration curve. Samples that exceeded the curve were diluted 1:2.

Cannabinoids and terpenes

For the determination of total cannabinoids, a modified version of the method proposed by Fischedick et al. (2010) was used; based on previous trials, the sample:solvent ratio, time, and sonication power were modified. Ten mL of absolute ethanol was added to 100 mg of sample in plastic tubes. Each sample was sonicated at 50% power for 5 min. The extracts were filtered with slow-filtration paper and brought to the initial volume with the same solvent. Finally, they were passed through an acrodisc syringe filter (0.2 µm) for final cleaning of trace impurities and placed in amber vials. An aliquot (1 µL) of the clean extract was taken and injected at a split ratio (15:1) in duplicate. All obtained extracts and curve points were run in SCAN mode. For the cannabidiol (CBD, 99%) calibration curve, 6 points were prepared (Figure 4A), and for tetrahydrocannabinol (THC, >99%), ranges from 220 to 1000 µg g^{-1} were prepared (Figure 4B). Cannabigerol (CBG) and traces of terpenes were found in the extracts. Identification was performed using the National Institute of Standards and Technology (NIST) library.

Statistical analysis

Morphological and metabolic variables (phenols, flavonoids, and cannabinoids) were processed in SAS® Student using mixed models, linear and non-linear regressions, multiple mean comparisons, and orthogonal polynomials. For terpene analysis, the set of areas obtained for each compound was considered and processed using generalized linear mixed models (GLMz) with a logarithmic link function under a gamma distribution, and principal component analysis (PCA) in R, including all internal replicates.

RESULTS

Evaluation of physiological variables

Leaf biomass (Figure 1A) and inflorescence yield (Figure 1B) results per plant did not show significant differences ($P \geq 0.05$) among the evaluated treatments, although a trend was observed. The 0.4 mg L^{-1} dose of BL evidenced the highest leaf biomass trend (5.70 g per plant). For the yield of commercial flowers, the 0.6 and 1.0 mg L^{-1} concentrations produced average values of 15.09 and 14.77 g per plant, respectively.

Evaluation of the phytochemical profile

Total phenols

Significant effects ($P < 0.05$) of the evaluated BL doses on total phenol concentrations in *C. sativa* 'Belmont' flowers were observed, represented as gallic acid content (Figure 1C). The highest production was obtained in the control treatment and the highest dose (1.0 mg L^{-1}), with an average value of 363.46 and 339.73 mg per plant, respectively. Gallic acid concentration increased as did the applied doses of BRs, except at the 0.8 mg L^{-1} point.

Total flavonoids

Similar to phenols, flavonoid contents (Figure 1D) were not significant ($P \geq 0.05$) under the applied treatments. The best trend was achieved with the application of 1.0 mg L^{-1} of BL, which generated 1391.52 mg of quercetin per plant. The other BL doses showed a constant increase, except for the 0.8 mg L^{-1} treatment, and remained below the control.

Cannabinoids

As an example, Figure 2 shows the profile of all metabolites detected by GC/MS in the inflorescence extracts. Seventeen volatile compounds originating from secondary metabolism were identified, including phytocannabinoids up to the end of the chromatographic run. However, the most important terpenes were considered during the statistical analyses. According to the group in Figure 3 (A, B, and C), the application of 1.0 mg L^{-1} during the flowering stage showed the best average production of CBD (29.40 mg), THC (1451.91 mg), and CBG (454,892,243 units) per plant in the *C. sativa* 'Belmont' inflorescences compared to the control. The orthogonal models associated with the evaluated treatments significantly

evidenced ($P \leq 0.05$) that CBD and THC fit a quadratic model, while CBG fits a linear regression. Furthermore, as the doses of foliar BL increased, cannabinoid concentrations responded proportionally. The application of low doses of BL (0.2 and 0.4 mg L^{-1}) promotes a concentration similar to or lower than that of the control treatment.

Terpenes

The effects of the evaluated treatments on the terpenes produced by the *C. sativa* 'Belmont' plant are shown in Table 1. A qualitative analysis was performed, and the results evidenced that, for aromatic compounds such as α-pinene, β-pinene, and d-limonene, the control treatment had a better response. The 1.00 mg L^{-1} dose of BL promoted a better synthesis of terpenes such as caryophyllene, α-caryophyllene, α-farnesene, and β-maaliene, maintaining a ratio slightly lower than the control. No significant differences between treatments were observed for guaiol, γ-selinene, elemol, and α-eudesmol; however, the control showed an upward trend for γ-selinene and elemol. The treatments with 0.6 and 0.8 mg L^{-1} of BL had the least impact on the production of the significant terpenes.

The PCA (Figure 5) allowed for the discrimination of variability in the terpene profile of the *C. sativa* 'Belmont' inflorescence samples. Components 1 (40%) and 2 (27%) jointly explained 67% of the total variability. On one hand, component 1 was mainly associated with the variation of compounds such as β-pinene, α-pinene, α-eudesmol, γ-selinene, α-farnesene, and guaiol. In this quadrant, the groups of monoterpenes (α- and β-pinene), sesquiterpenes (α-farnesene, γ-selinene), and oxygenated sesquiterpenes (α-eudesmol and guaiol) prevailed. On the other hand, Component 2 was strongly influenced by the content of β-maaliene, caryophyllene, and α-caryophyllene, compounds from the cyclic sesquiterpene group. Likewise, d-limonene and elemol also showed high correlations with this component, indicating their contribution to the discrimination of the phytochemical profiles.

Interactions between different phytochemical groups

Figures 6A and 6B exemplify the intrinsic relationships between the production of quercetin (flavonoids), gallic acid (phenols), CBD, and THC (cannabinoids). It can be observed that, as the synthesis of phenolic compounds increases in the aerial part of the plant, the other two phytochemical groups grow in parallel. However, a high production (> 250 mg) of gallic acid

decreases that of the other compounds. By evaluating the second derivative of the equations for each polynomial regression, it was determined that these compounds conform to a maximum response (negative value). Consequently, by applying the first derivative to each equation, it could be established that the metabolomic break-even is achieved between 190 and 205 mg of gallic acid, with response intervals of 1236.74 to 1246.73 mg of quercetin, 22.15 to 22.18 mg of CBD, and 1025.06 to 1036.72 mg of THC.

DISCUSSION

Effect of brassinolide on plant development

This study evidenced that an integrative nutritional management aimed at boosting secondary metabolic pathways through nutritional relationships, microorganisms, elicitors, and PGRs in solution, may be blocking primary metabolism. This occurs even when applying physiological doses of BRs, which have a history of promoting plant growth under unfavorable conditions (Rodríguez et al., 2022). Previous studies highlight the impact of BRs on crop productivity. In mung bean (*Vigna radiata* L.), the second foliar application of 0.25 mg L^{-1} of BL produced a greater gain in aerial biomass and grain yield, with 377.35 g per plant and 724.50 kg ha^{-1}, respectively, compared to higher doses (Sengupta et al., 2011). In strawberry (*Fragaria* × *ananassa* Duch.) cv. Winter, the triple application of 0.2 mg L^{-1} during the flowering and fruiting stages also significantly promoted a higher number of flowers (38), fruits (33), and marketable achene yield (402 g per plant) (Khatoon et al., 2023). Nasser & Sarhan (2023) evaluated four doses of BL (0, 0.5, 1.0, and 1.5 mg L^{-1}) on various sesame cultivars (*Sesamum indicum* L.) and found that the highest concentration produced the highest number of capsules (274), seeds per capsule (96), and grain yield (2.22 Mg ha^{-1}) in 'Hadd' and 'Sumeria'.

It should be noted that the consulted works do not focus on secondary metabolism, but rather on the primary mechanism. Although BRs have been shown to beneficially affect plant physiological characteristics by modifying carbon metabolism and nutrient transport efficiency (Nasser & Sarhan, 2023), the data did not reveal a significant effect on leaf biomass gain or flower yield per plant for the *C. sativa* Belmont cultivar. However, a marked trend was observed across the varying BL application levels. Specifically, the slight increases observed at 0.2 to 0.6 mg L^{-1} corresponds to biphasic effect dose-dependent (hormetic effect) of BRs on primary metabolism. At low to intermediate concentrations, BRs promote cell elongation and division through canonical signaling cascades that prevent the degradation of key transcription factors like BZR1 and BES1, temporarily stimulating growth (Basit et al., 2021). As the dose increases toward 0.8 and 1.0 mg L^{-1}, this growth promotion reaches a threshold and becomes mildly inhibitory, diverting metabolic flux away from biomass accumulation. The possible reason for this behavior lies in the allocation of carbon sources destined for primary pathways, which are diverted towards secondary ones due to the integrative management, as mentioned by Pott et

al. (2019). This resource diversion creates a metabolic "trade-off," known as the dilemma of growing or defending (Züst and Agrawal, 2017). This internal competition is not limited to carbon skeletons but also includes energy. The production of complex metabolites is a process that demands a large amount of ATP and reducing energetic molecules, which could otherwise be used for biomass synthesis, such as structural proteins and lipids (Huot et al., 2014), thereby stalling growth in favor of defense. Nonetheless, another important property of this group of phytoregulators is driving defense pathways against biotic and abiotic stress.

Effect of brassinolide on the production of nutraceutical metabolites

In the case of total phenols, the foliar addition of increasing doses of BL did not promote a favorable effect on gallic acid. Similar to the morphological variables, flavonoids showed a trend in which the 1.0 mg L^{-1} dose of BL stood out. These phytochemical compounds originate from shared biosynthetic routes, given that the vast majority of phenolic compounds and a part of the structural skeleton of flavonoids are obtained from the shikimic acid pathway (Yadav et al., 2020; Liu et al., 2021). ~~Phenylalanine ammonia-lyase (PAL) is the main enzyme in this mechanism for the synthesis of phenylpropanoids (Aghdam et al., 2019).~~

The combined application of inducers such as silicon (Si), salicylic acid (SA), and supplementation with Phe represents a comprehensive strategy to enhance this metabolic pathway. Although Si is not an essential element, it acts as a defensive pre-conditioning agent or "priming". Upon absorption, it deposits in the cell walls as amorphous silica, creating a physical barrier. At the same time, it modulates the expression of defense genes, increasing the transcription and activity of PAL and chalcone synthase (CHS) (Debona et al., 2017). For its part, SA applied as ASA triggers a signaling cascade that culminates in the activation of transcription factors of the plant's systemic acquired resistance (SAR), such as those of the WRKY family. These factors bind to pathogen-response genes, including those encoding PAL and other intermediary enzymes (Peng et al., 2021). Finally, by providing a surplus of Phe as an initial substrate, metabolic flux is driven toward the production of trans-cinnamic acid and its derivatives, activating the available enzymatic machinery (Tohge et al., 2013).

However, our results suggest that this synergistic stimulation leads to a hypothetical enzymatic saturation point in the addressed pathway, a bottleneck that is not overcome with the addition of growth promoters like BRs, since metabolite production is not infinite. Castañeda et al. (2019)

state that PAL operates under the principles of Michaelis-Menten kinetics. The reaction rate increases as substrate concentration rises until reaching a maximum velocity, at which point the active sites of all enzyme molecules are occupied. In this state of saturation, a further increase in substrate does not translate into an over-increase in maximum velocity. This means that once PAL becomes saturated, flux into the entire phenylpropanoid pathway stagnates, regardless of the surplus of signaling molecules and available Phe. The same occurs with downstream enzymes like CHS and chalcone isomerase (CHI); even if there is a high flux of p-coumaroyl-CoA, if CHS and CHI are saturated, flavonoid synthesis will not significantly increase (Lewis et al., 2024). For this reason, the control treatment generated more phenolic compounds, while BR doses attempted to activate the adjacent flavonoid pathway, diverting surplus resources such as pyruvate, erythrose-4-phosphate (E4P), glyceraldehyde-3-phosphate (G3P), and acetyl-CoA from the shikimic acid pathway towards the malonic acid pathway and even to those of other phytochemical groups.

Furthermore, the mechanisms driving the varied application levels highlight a clear dose-dependency. The observed variation across the 0.2 to 0.8 mg L^{-1} range, where phenols and flavonoids values were largely inferior to the control, indicates that low and intermediate doses of BRs prioritize primary growth pathways and fail to generate the stress signaling required to overcome the established enzymatic bottleneck in the phenylpropanoid pathway. In contrast, the recovery and upward trend seen at the 1.0 mg L^{-1} concentration suggests that a higher physiological threshold must be crossed to induce an abiotic-like stress response. This high-dose stress acts as a secondary messenger mechanism capable of redirecting carbon flux toward defense metabolites (Basit et al., 2021), explaining why only the highest level of BL managed to positively influence these compounds.

Effect of brassinolide on the production of cannabinoids and terpenes

Increasing doses of BL impacted cannabinoids (CBD, THC, and CBG) with an accumulative effect in the inflorescences of the studied cultivar. Likewise, they generated an overexpression of other terpenes (caryophyllene, α-caryophyllene, α-farnesene, and β-maaliene) foreign to those that predominated in the base treatment without BRs (pinene isomers and d-limonene).

The mechanisms of BRs are strictly biphasic and highly dependent on the applied dose, which explains the distinct outcomes across the 0 to 1.0 mg L^{-1} range. At low concentrations (0.2 to

0.6 mg L^{-1}), BRs focus on maintaining metabolic homeostasis by promoting cell elongation and division. This activates the canonical signaling of the BZR1/BES1 transcription factor group (Al-Mamun et al., 2024; Basit et al., 2021), keeping oxidative stress minimal and thus resulting in cannabinoid and terpene accumulation that is similar to or lower than the control. However, increasing the exogenous application to 0.8 and 1.0 mg L^{-1} deliberately disrupts this homeostasis. High doses cross a toxicity threshold that induces a controlled abiotic stress response within the plant tissues (Gruszka et al., 2021). This stress manifests through the targeted accumulation of reactive oxygen species (ROS) and the deployment of mitogen-activated protein kinases (MAPK) signaling cascades (Xiong et al., 2022). These ROS act as secondary messengers that aggressively upregulate the expression of the DXPS gene in the MEP pathway, successfully shifting the metabolic flux toward the overproduction of GPP, ultimately explaining the maximized synthesis of cannabinoids and diverse sesquiterpenes strictly at the highest evaluated doses.

Phytocannabinoids and terpenes share geranyl pyrophosphate (GPP) as their base precursor. GPP is synthesized from glycolysis intermediates (pyruvate and G3P) through the methylerythritol phosphate (MEP) pathway, located in the plastids, where the enzyme 1-deoxy-D-xylulose-5-phosphate synthase (DXPS) regulates the flux of this pathway (Lichtenthaler, 1999). Stress signals, including MAPK cascades induced by ROS from treatments, are potent transcriptional activators of the DXPS gene (Singh et al., 2024). In this way, the high dose (1.0 mg L^{-1}) of foliar BL added an extra layer of activation on top of the base induction derived from Si, ASA, Phe, and microorganisms, theoretically increasing DXPS enzymatic activity and, consequently, carbon flux toward GPP production. Therefore, it is proposed that high doses of BRs do not act as an antagonistic growth signal to SA because the "trade-off" does not manifest, but rather as a second stress elicitor. Consequently, there was no competition with the pre-activated defense pathway, but rather a metabolic synergy.

Complementing the above, the distribution of terpenes in the PCA biplot reflected a separation between samples with low contents of these, located near the origin of the axes, and those with specific profiles dominated by certain compounds, projected in the distal quadrants. Samples 33 and 34 were associated with high levels of caryophyllene and β-maaliene, while sample 6 was distinguished by a high β-pinene content. In contrast, most samples clustered around the

centroid, suggesting a more balanced terpenic profile without marked predominance of a particular compound. The results evidenced that terpenic variability in *C. sativa* 'Belmont' is mainly explained by differences in the relative accumulation of sesquiterpenes (caryophyllene, α-caryophyllene, and β-maaliene) versus monoterpenes (β-pinene and α-pinene).

The rationale for enhancing and symmetrically diversifying the terpene profile lies in the fact that they exhibit therapeutic properties, often complementary to those of cannabinoids. This synergistic interaction (entourage effect) manages to amplify and produce superior therapeutic effects compared to the isolated and individual form of each compound (Finlay et al., 2020). On one hand, Gertsch et al. (2008) state that caryophyllene and its isomers act as selective agonists for the cannabinoid receptor type 2 (CB2). CB2 is predominantly expressed in immune system cells, and its activation is associated with anti-inflammatory and analgesic responses without inducing psychoactivity via the CB1 receptor. Caryophyllene directly contributes to the anti-inflammatory effect of the CBD phytocomplex through the endocannabinoid system. Turkez et al. (2014) characterized the neuroprotective potential of different farnesene isomers (acyclic sesquiterpene), such as α and β farnesene, against oxidative stress causing neuronal loss and impairment in various neurodegenerative disorders.

On the other hand, monoterpenes tend to modulate neurotransmitter system activity, as d-limonene has demonstrated anxiolytic and antidepressant effects, possibly due to serotonergic system (5-HT) modulation, which complements CBD's effects on the 5-HT1A receptor (López et al., 2017). α-Pinene acts as an acetylcholinesterase inhibitor, which may counteract short-term memory deficit (Russo, 2011). Furthermore, both terpenes and certain flavonoids can interact with cytochrome P450 (CYP) enzymes in the liver, which are responsible for the metabolism of most drugs, including cannabinoids (Yamaori et al., 2012). Therefore, the use of BRs such as BL in integrated productive management promises a holistic utilization of Cannabis therapeutic compounds.

Internal interactions between different phytochemical groups

The behavioral models between phenolic compounds, flavonoids, and cannabinoids showed that internal interactions exist among them. These compounds share key biosynthetic precursors derived from the shikimic acid pathway and the malonyl-CoA pathway. On one hand, shikimic acid produces aromatic amino acids as a substrate for the phenylpropanoid family,

including phenols and flavonoids (Maeda & Dudareva, 2012). On the other hand, malonyl-CoA is crucial for fatty acid chain elongation and is also an essential substrate for the synthesis of polyketides, which are precursors to certain phenolic compounds and the polyketide portion of cannabinoids (Milke et al., 2019). This dependence on common substrates generates direct competition between the different biosynthetic pathways, as the plant must efficiently allocate available carbon and energy among them to modulate the expression of genes encoding primordial enzymes (Wang et al., 2024). Besides competition, interactions between these pathways can arise, such as synergisms or antagonisms.

The obtained quadratic models suggest that an increase in the production of one class of compounds can coexist with or even slightly favor the production of others (synergism). However, upon exceeding a break-even point or optimal threshold, an additional increase in the synthesis of one group causes a significant decrease in the production of the others (antagonism), as affirmed by Atkinson (1965) at the enzymatic level. This antagonistic phenomenon is due to the overload of a specific metabolic pathway that sequesters the majority of shared precursors and cellular energy (ATP, NADPH). The massive induction of expression of the phenol pathway enzymes monopolizes substrates, such as malonyl-CoA and phenylalanine derived precursors, generating a deficit that severely limits or inhibits the plant's capacity to synthesize flavonoids and cannabinoids. This excess not only depletes resources but can also generate metabolic stress, affecting overall cell health and the functionality of other biosynthetic pathways (Small, 2016). Therefore, this regulation guarantees that resources are not wasted and maintains metabolic homeostasis when a compound has accumulated in sufficient levels, as mentioned by Hartman et al. (2023) regarding primary metabolism. The ideal is to find the optimal point where the plant achieves a balanced and functionally diverse production of compounds, as observed with the participation of BL in the proposed integrative management.

From a biotechnological perspective, understanding this equilibrium point is fundamental. Genetic manipulation or the application of elicitors to maximize the production of a single compound, without considering these interactions, can lead to unintended consequences, such as a decrease in other beneficial compounds or the induction of metabolic stress that, paradoxically, could reduce overall yield (Li et al., 2024). Although this study did not address

the stimulated transcriptions to observe the involved enzymatic activities, nor the traceability of reallocated resources and precursors among different secondary metabolism biosynthetic pathways, it is recommended that future research delve into the exposed mechanisms to understand how integrated management can generate a diverse and balanced phytochemical environment within the metabolomic system.

CONCLUSIONS

The foliar addition of brassinolide, as a source of brassinosteroids, during the flowering stage in *C. sativa* 'Belmont' under an integrative nutritional system did not significantly impact primary metabolism, such as leaf biomass gain or commercial inflorescence yield, nor flavonoid production. The effects of BRs are highly dependent on the applied dose. At low doses of brassinolide, downward trends and even the inhibition of phytochemical compound production were observed. In contrast, high doses (1.0 mg L^{-1}) had a positive result on metabolites derived from the MEP pathway (cannabinoids and terpenes) and total phenols, accompanied by a balanced diversity of therapeutic volatile compounds and good trends in the other metabolites. This potentiated a better phytochemical quality of the harvested product for its subsequent pharmaceutical or medicinal utilization.

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DISCLOSURE STATEMENT

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DATA AVAILABILITY STATEMENT

Not applicable.

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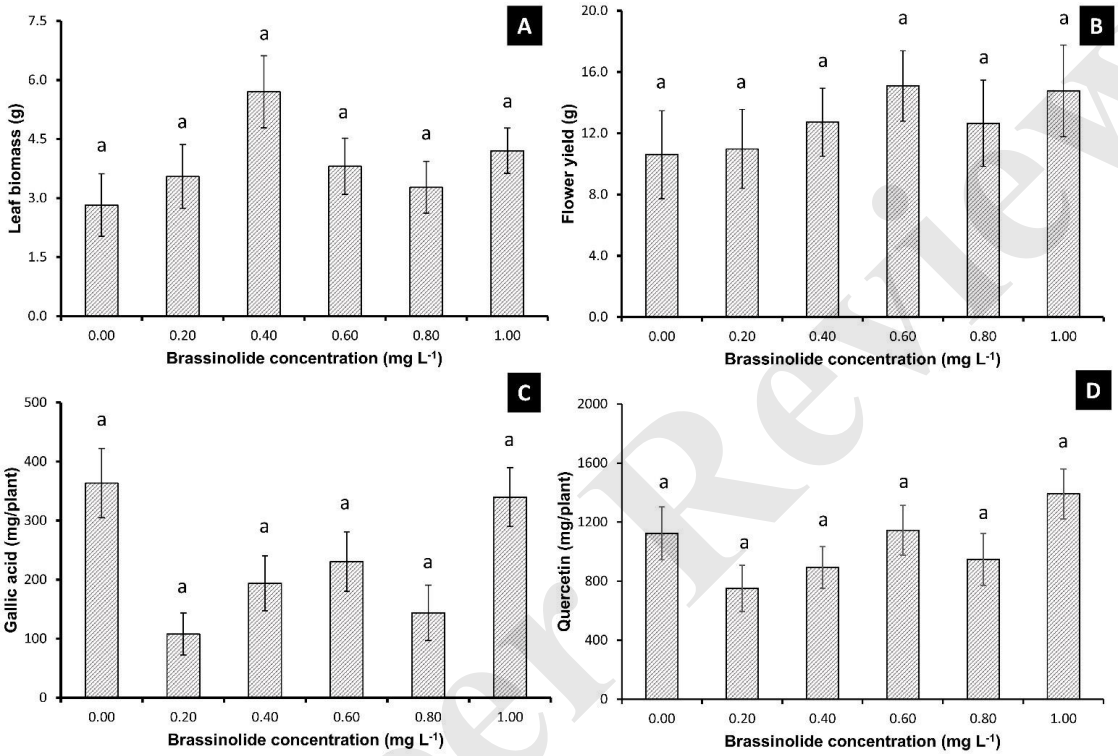
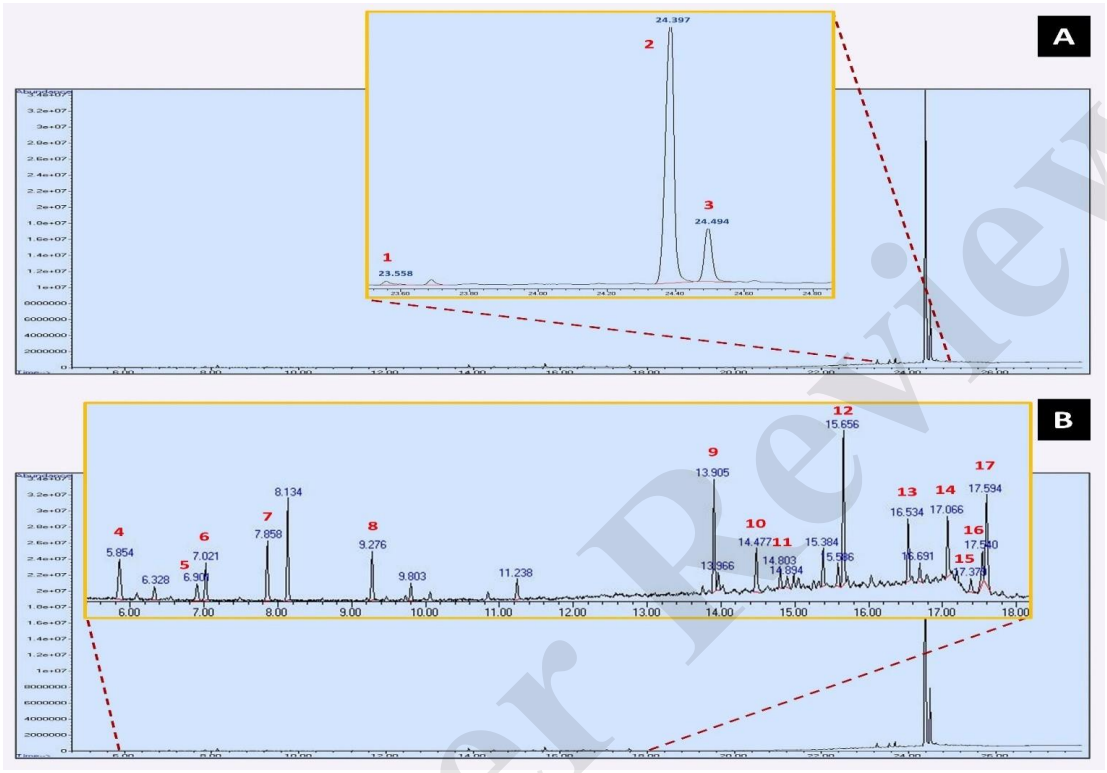
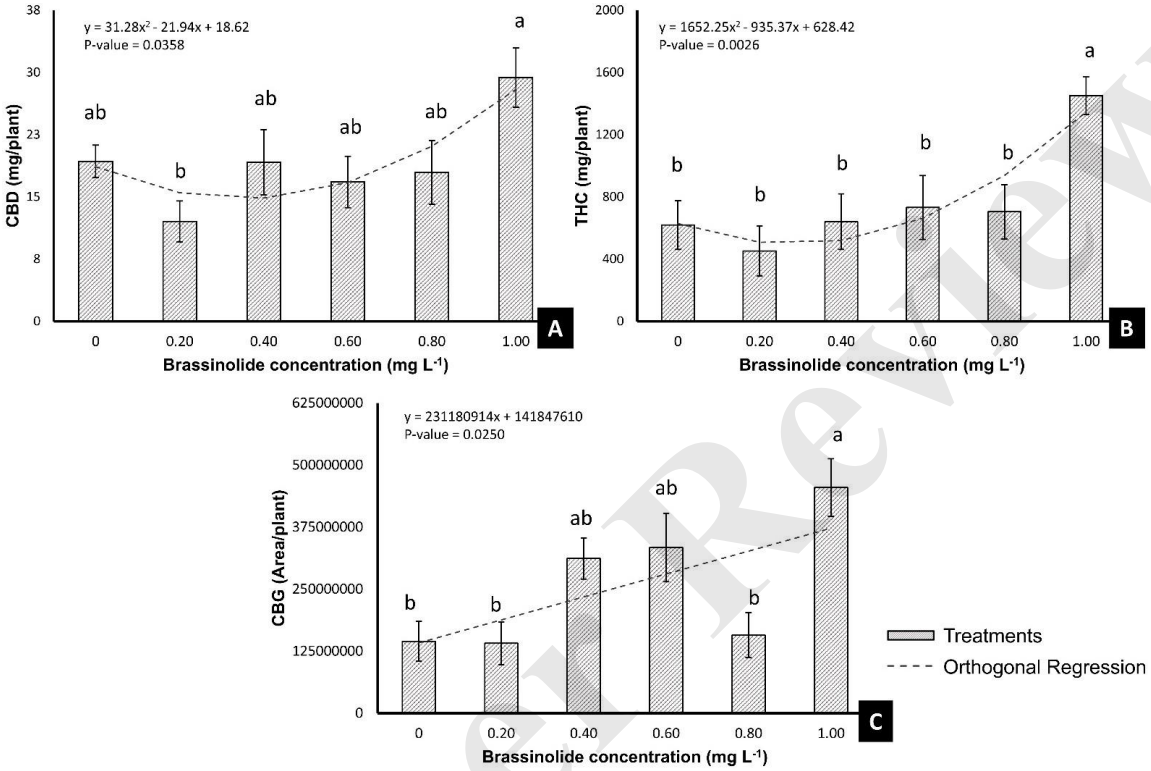
Table 1. Average areas of the major terpenes present in 100 mg of *C. sativa* 'Belmont' inflorescences under six levels of foliar brassinolide.

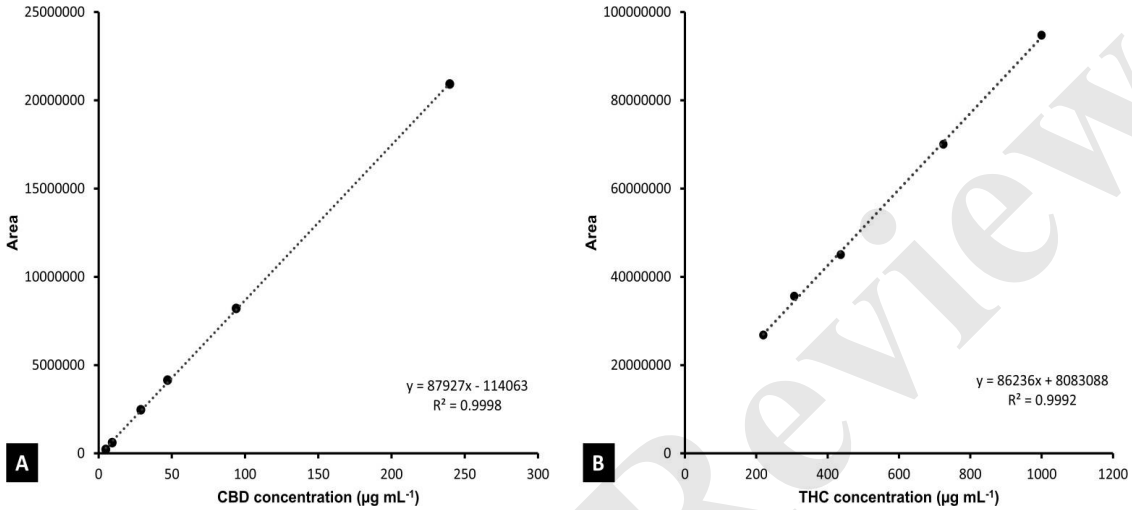
Treatment	Alpha pinene	Beta pinene	d-limonene	Caryophyllene	Alpha Caryophyllene	Alpha farnesene	Beta maliene	Guaiol	Gamma selinene	Elemol	Alpha eudesmol
Control**	2622660 a*	5180317 a	1410208 a	2642612 ab	961990 ab	149785 b	739121 c	904923 a	1576799 a	380661 a	765685 a
0.20	935758 b	1592192 bc	658629 bc	1387650 bc	519424 bc	159745 ab	1104930 bc	1073596 a	1070911 a	221807 a	1022504 a
0.40	1624369 ab	1437127 bc	723983 abc	1348715 bc	446097 bc	116822 b	2385010 ab	831804 a	623254 a	278337 a	1141575 a
0.60	376548 c	588516 c	446075 c	1282655 bc	425650 c	148093 b	1056009 bc	984494 a	1054550 a	192453 a	994096 a
0.80	2635155 a	1636579 bc	375135 c	952428 c	311992 c	134429 b	3088291 a	716864 a	801628 a	153430 a	1211864 a
1.00	847312 bc	2935252 ab	1199355 ab	4615280 a	1226119 a	229131 a	4305948 a	483053 a	677893 a	209256 a	768602 a
Prob	0.0010	0.0081	0.0059	0.0064	0.0086	0.0203	0.0087	0.5514	0.3164	0.3135	0.3770

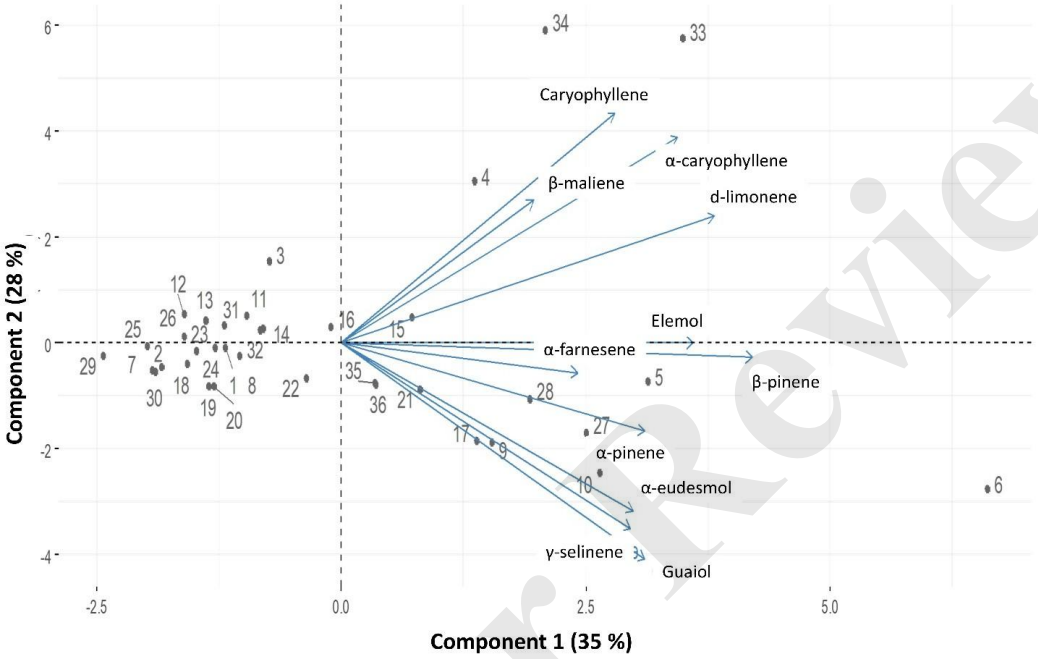
*Means with the same letter per column are not statistically different (Tukey, P = 0.05). **Evaluated doses expressed as mg L^{-1} of foliar brassinolide.











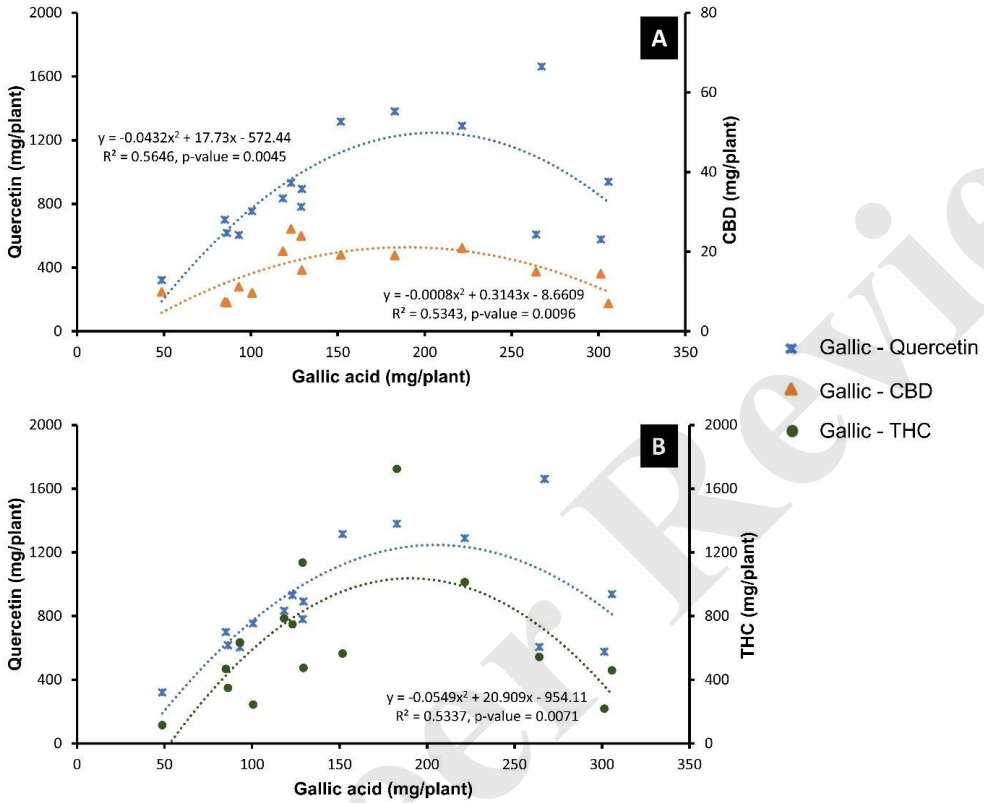


Figure legends

Figure 1. Effect of varying concentrations (mg L^{-1}) of foliar brassinolide on physiological parameters (leaf biomass and commercial inflorescence yield) and the production of nutraceutical compounds (gallic acid and quercetin) in *C. sativa* L. 'Belmont' inflorescences. Means followed by the same letter are not significantly different between treatments (LSD, $P \geq 0.05$, $n = 3$).

Figure 2. Chromatographic profile of the major cannabinoids (A) and terpenes (B) identified in extracts from the highest brassinolide concentration (1.0 mg L^{-1}) in *C. sativa* L. 'Belmont'. 1: CBD; 2: THC; 3: CBG; 4: α-pinene; 5: β-pinene; 6: d-limonene; 7: trans-β-ocimene; 8: linalool; 9: caryophyllene; 10: α-caryophyllene; 11: α-farnesene; 12: β-maaliene; 13: guaiol; 14: γ-selinene; 15: eudesm-7(11)-en-4-ol; 16: elemol; 17: α-eudesmol.

Figure 3. Effect of exogenous brassinolide application at varying concentrations (mg L^{-1}) on cannabinoid yield (CBD, THC, and CBG) in *C. sativa* L. 'Belmont' inflorescences. Means followed by the same letter are not significantly different between treatments (LSD, $P \geq 0.05$, $n = 3$).

Figure 4. Calibration curves of the two major cannabinoids (CBD and THC) for quantification in *C. sativa* L. 'Belmont' inflorescences.

Figure 5. Principal Component Analysis (PCA) of terpenes in *C. sativa* L. 'Belmont' inflorescence extracts.

Figure 6. Behavioral models among compounds from different metabolic groups identified in *C. sativa L.* 'Belmont' inflorescences.

7. CONCLUSIONES GENERALES

Se concluye que el manejo integral de la nutrición en *Cannabis* spp. demostró ser una estrategia eficaz para modular la síntesis de metabolitos secundarios de interés medicinal y nutracéutico. La combinación de soluciones nutritivas con aminoácidos aromáticos y silicio favoreció el equilibrio nutrimental, mejoró la actividad fotosintética y promovió la acumulación de fenoles y flavonoides, confirmando que la nutrición mineral optimizada potenció las rutas del shikimato y del ácido malónico. La inoculación conjunta de *Trichoderma* spp. y *P. fluorescens* en sistemas hidropónicos incrementó significativamente la biomasa foliar y la acumulación de compuestos fenólicos durante la fase vegetativa, demostrando que la interacción simbiótica activó mecanismos fisiológicos y metabolómicos asociados con la resistencia sistémica y la producción de metabolitos antioxidantes. Finalmente, la aplicación foliar de brasinólida (1.0 mg L^{-1}) en floración promovió una mayor acumulación de cannabinoides (CBD, THC y CBG) y diversificó el perfil de terpenos sin afectar negativamente el rendimiento productivo, evidenciando que los brasinoesteroides estimulan la ruta MEP para complementar la biosíntesis de metabolitos especializados. Este modelo de manejo puede aplicarse en sistemas de producción tecnificados o a pequeña escala, lo que contribuye al desarrollo de una agricultura de alto valor orientada a la obtención de materia prima con potencial medicinal y abriendo nuevas perspectivas para la industria farmacéutica basada en el *Cannabis*.