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**Influencia de diferentes manejos agronómicos en la actividad microbiológica del suelo y capacidad antioxidante del fruto de calafate (*Berberis microphylla* G. Forst)**

**Influence of different agronomic management on soil microbiological activity and antioxidant capacity of calafate (*Berberis microphylla* G. Forst) fruit**

Tesis para optar al grado de Doctor en Ciencias de la Agronomía

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## RESUMEN

El calafate (*Berberis microphylla* G. Forts) tiene frutos con polifenoles beneficiosos para la salud humana; sin embargo, su producción se limita a poblaciones silvestre de baja productividad. El manejo agronómico es una alternativa para mejorar la productividad y/o características bioactivas de fruta en huertos, ya que, mediante distintas prácticas como el riego, fertilización, control de malezas o densidad de plantación, modifica las condiciones fisicoquímicas y microbiológicas del suelo necesarias para el ciclo de nutrientes involucrados en el desarrollo vegetativo, funcionamiento fisiológico y productividad de las plantas. Este estudio evaluó el efecto sobre el suelo, planta y frutos de cuatro manejos agronómicos sobre un huerto de calafate del centro-sur de Chile: dosis de riego (0, 50, 100 y 150% de la evapotranspiración de referencia [ET<sub>0</sub>]), tipos de mulch (sin mulch, geotextil, paja de avena, cáscara de avellana), dosis de fertilización orgánica (0, 5, 10 y 15 t ha<sup>-1</sup> de compost) y densidades de plantación (6667, 3333, 2222 y 1667 plantas ha<sup>-1</sup>). Los resultados demostraron que, el riego, mulch, fertilización y densidad de plantación favoreció la actividad biológica del suelo, desarrollo de la planta y productividad de frutos de calafate. Regar con 50% de la ET<sub>0</sub>, optimiza el agua, y mejoró el suelo con más biomasa microbiana (33%), respiración basal (18%) y actividad enzimática (46% deshidrogenasa y 12% fosfatasa) que sin riego. Además, favoreció a la planta con mayor conductancia estomática (34%) e índice de clorofila (45%) que sin riego, manteniendo frutos con similares antocianinas (286,4 mg 100 g<sup>-1</sup>) y capacidad antioxidante ORAC (1011 μmol TE 100 g<sup>-1</sup>) que riego de 100% de la ET<sub>0</sub>. El mulch favoreció la actividad biológica del suelo y calidad de los frutos de calafate. La cascara de avellana mejoró el suelo con más actividad microbiana (40%), respiración basal (31%) y actividad de la ureasa (20%) que sin mulch. Aunque las funciones fisiológicas de la planta y rendimiento no mejoraron con la cascara de avellana, la calidad de fruta mejoró, con incrementos de 24% en antocianinas y 30% en capacidad antioxidante ORAC que paja de avena. La fertilización orgánica benefició la biomasa microbiana del suelo, desarrollo de la planta, rendimiento y calidad de

frutos. La dosis de 10 t ha<sup>-1</sup> mejoró la microbiología del suelo más biomasa microbiana (33%) y respiración basal (40%) que sin fertilización. También, favoreció a la planta con aumento del índice de área foliar (33%) e índice de clorofila (30%), y condujo a una producción de fruta 37% más alta con 2.7 veces más antocianinas y 12% más capacidad antioxidante ORAC que sin fertilización. Las densidades de plantación mejoraron la respuesta microbiológica del suelo, el desarrollo de la planta y calidad de los frutos. La densidad tradicional (3333 plantas ha<sup>-1</sup>) produjo un suelo con mayor respiración basal (13%) y actividad de ureasa (29%) que alta densidad (6667 plantas ha<sup>-1</sup>). Además, la planta se vio favorecida aumentando 42% el índice de área foliar y 17% la conductancia estomática que una alta densidad, mientras que hubo un aumento en la calidad de fruta en comparación a una densidad alta, con 39% más contenido de polifenoles totales y 58% más capacidad antioxidante DPPH. Estos resultados son consistentes en demostrar que es posible aumentar la productividad y calidad del calafate, así como la actividad microbiológica y enzimática del suelo, mediante manejos agronómicos de riego, mulch, fertilización orgánica y densidad de plantación. Esta información es relevante para el establecimiento de nuevos huertos de calafate en Chile.

## **ABSTRACT**

Calafate (*Berberis microphylla* G. Forts.) has fruits with polyphenols that are beneficial to human health; however, its production is limited to wild populations with low productivity. Agronomic management is an alternative to improve the productivity and/or bioactive characteristics of fruits in orchards because, through different practices such as irrigation, fertilization, weed control, or planting density, it modifies the physicochemical and microbiological conditions of the soil necessary for the cycling of nutrients involved in vegetative development, physiological functioning, and plant productivity. This study evaluated the effect on soil, plant and fruit of four agronomic managements on a calafate orchard in south-central Chile: irrigation doses (0, 50, 100 and 150% of reference evapotranspiration [ET<sub>0</sub>]), mulch types (no mulch,

geotextile, oat straw, hazelnut shell), organic fertilization doses (0, 5, 10 and 15 t ha<sup>-1</sup> of compost) and planting densities (6667, 3333, 2222 and 1667 plants ha<sup>-1</sup>). The results showed that irrigation, mulching, fertilization, and planting density favored soil biological activity, plant development, and calafate fruit productivity. Irrigation at 50% of ET<sub>0</sub> optimized water and improved soil microbial biomass (33%), basal respiration (18%), and enzyme activity (46% dehydrogenase and 12% phosphatase) than without irrigation. In addition, it favored the plant with higher stomatal conductance (34%) and chlorophyll index (45%) than without irrigation, maintaining fruits with similar anthocyanins (286.4 mg 100 g<sup>-1</sup>) and ORAC antioxidant capacity (1011 μmol TE 100 g<sup>-1</sup>) than irrigation of 100% of ET<sub>0</sub>. Mulching favored soil biological activity and the quality of calafate fruits. Hazelnut husks improved soil microbial activity (40%), basal respiration (31%), and urease activity (20%) compared to those without mulch. Although plant physiological functions and yield did not improve with hazelnut hulls, fruit quality improved, with 24% higher anthocyanin content and 30% higher ORAC antioxidant capacity than oat straw. Organic fertilization improves soil microbial biomass, plant development, yield, and fruit quality. A dose of 10 t ha<sup>-1</sup> improved soil microbiology by increasing microbial biomass (33%) and basal respiration (40%) than without fertilization. It also favored plants with an increased leaf area index (33%) and chlorophyll index (30%), and led to a 37% higher fruit yield with 2.7 times more anthocyanins and 12% more ORAC antioxidant capacity than those without fertilization. Planting densities improved the soil microbiological response, plant development, and fruit quality. Traditional density (3333 plants ha<sup>-1</sup>) produced soil with higher basal respiration (13%) and urease activity (29%) than high density (6667 plants ha<sup>-1</sup>). In addition, the plant had a 42% higher leaf area index and 17% higher stomatal conductance than high-density plants, while there was an increase in fruit quality compared to high-density plants, with 39% higher total polyphenol content and 58% higher DPPH antioxidant capacity. These results are consistent in demonstrating that it is possible to increase the productivity and quality of calafate, as well as the microbiological and enzymatic activity of the soil, through agronomic management of irrigation, mulching, organic fertilization, and planting

density. This information is relevant to the establishment of new calafate orchards in Chile.

## I. INTRODUCCION GENERAL

El género *Berberis* contiene un total 650 especies, de las cuales 18 especies se encuentran en Sudamérica (Silva et al., 2020). A este género pertenece la especie *Berberis microphylla* G. Forts, que, según la última clasificación botánica, se compone de otras tres especies, *B. Berberis buxifolia*, *Berberis microphylla* y *Berberis heterophylla*, compartiendo características morfológicas como, hojas enteras, flores solitarias, estambres con apéndices laterales, espinas en ángulos muy abiertos palmadas y, frutos negros azulados pruinosos con dos a nueve semillas (Landrum 1999; Radice y Arena 2021). *Berberis microphylla* G. Forts, comúnmente es llamado calafate y crece en la Patagonia de Chile y Argentina asociado a especies acompañante como, chaura (*Gaultheria mucronata*), notro (*Embothrium coccineum*), ñire (*Nothofagus antarctica*) o coirón (*Festuca pallescens*). En Chile, se distribuye geográficamente desde la Región de Punta Arenas hasta la Región Metropolitana (34° 59'0" Latitud Sur a 53° 28'33" Latitud Sur), pero, la mayor abundancia de poblaciones está en bosques nativos de la Región de Aysén con 82.000 ha (Salinas et al., 2019). El calafate, desde tiempos ancestrales contribuyó con servicios ecosistémicos para poblaciones autóctonas de Chile como los Mapuches, Aónikenks y Yámanes (Fredes et al., 2020). Las hojas, corteza y la madera eran efectivas contrarrestando afecciones hepáticas y usadas como refrescantes y laxantes, los frutos frescos, secos, o procesados generaban aportes nutricionales a través de la dieta y se utilizaban para colorear lanas de púrpura, mientras que las raíces, para teñir de amarillo (Fredes et al., 2020; Schmeda-Hirschmann et al., 2019). A nivel global, el cambio de paradigma por un estilo de vida más saludable, aumentando el consumo de frutos con propiedades funcionales, así como, el remplazo de colorantes sintéticos por colorantes naturales de origen vegetal, generó un interés significativo por el fruto de calafate que contiene alta calidad representada por atributos nutrifuncionales.

En las últimas tres décadas ha sido ampliamente estudiado el fruto de calafate. La baya de calafate, al igual que otras bayas, aporta vitaminas, minerales,

fibra y presenta un sabor astringente (Mariangel et al., 2013). En términos nutricionales, calafate puede contener 4.8 g 100 g<sup>-1</sup> de ácidos grasos, 8.37 g 100 g<sup>-1</sup> de fibra, 13 g 100 g<sup>-1</sup> de proteína y 70 g 100 g<sup>-1</sup> de humedad (Araya, 2010). En términos funcionales y de calidad, el fruto de calafate tiene mayor reconocimiento porque presenta capacidad antioxidante siete veces superior que la fresa y cuatro veces que el arándano (Rodoni et al., 2014). Asimismo, se encontraron para las frutas de calafate los valores más altos de ORAC (Oxygen Radical Absorbance Capacity) entre 120 especies de frutas chilenas analizadas (Speisky et al., 2012). Esta capacidad antioxidante, se atribuye a compuestos fenólicos como los flavonoides, que se encuentran en cantidades significativas en bayas cultivadas y silvestre (Seeram et al., 2006). Específicamente, la capacidad antioxidante del calafate se ha correlacionado con un mayor contenido de flavonoides, como las antocianinas, de las cuales se han identificado dieciocho, siendo los conjugados de 3-glucósido las más abundantes (Ruiz et al., 2013; Ruiz et al., 2014). Estos compuestos han permitido que el calafate sea capaz de contrarrestar el estrés oxidativo en sangre humana generado por metabolitos oxidados originados por el antibiótico clorofenicol (Albrecht et al., 2010). Además, investigaciones recientes *in vivo* han demostrado que suplementar la dietética con bayas de calafate, estimuló significativamente la tolerancia a la glucosa en sangre contrarrestando la diabetes, y previno la obesidad (Ramírez et al., 2021; Soto-Covasich et al., 2020). Estos avances, causaron interés en industrias de alimentos, farmacéutica y colorantes naturales. Sin embargo, la superficie limitada de poblaciones silvestres de calafate no es suficiente para proveer los volúmenes de fruta que se requieren para su procesamiento nacional e internacional. Por otro lado, se ha reportado baja productividad y calidad de fruta desde plantas silvestres de calafate, por crecer en condiciones climáticas adversas, suelos de baja fertilidad, y la sobreexplotación humana (Arena et al., 2003; Romero-Román et al., 2021). El manejo agronómico, es usado para mejorar la productividad y calidad de frutos en huertos comerciales, incluso, en Chile se ha implementado en especies silvestres de alto interés nutraceútico, como ha sido el caso de Maqui (*Aristotelia chilensis* Mol.) (Doll et al.,

2017; González et al., 2015). No obstante, además de los efectos del manejo agronómico en la productividad, se requiere una evaluación exhaustiva de los efectos en el suelo ya que mantiene una amplia variedad de formas de vida que contribuyen a la fertilidad y a los ciclos de nutrientes que luego utilizan las plantas (Bardgett y Van Der Putten, 2014).

El suelo contiene variadas formas de vida, decenas de millones de especies de bacterias, arqueas, hongos, virus y microeucariotas viven en él y producen enzimas que intervienen en procesos como el ciclo de nutrientes (Henríquez et al., 2014; Wu et al., 2024). Además, las comunidades de microorganismos del suelo son relevantes por representar una fracción significativa de la biomasa de la tierra (Banerjee y Van Der Heijden, 2023). La microbiología del suelo se ha convertido en un indicador importante de la calidad general del suelo en huertos frutales, ya que la biodiversidad y la actividad de las comunidades microbianas del suelo son responsables de múltiples procesos involucrados en los ciclos biogeoquímicos (Wang et al., 2019). Por tanto, un suelo saludable será aquel que presente adecuada fertilidad, capacidad para sustentar el crecimiento vegetal y resiliencia frente a perturbaciones ambientales. Sin embargo, la actividad de los organismos del suelo y su funcionalidad, está determinada en gran medida por factores ambientales como temperatura, humedad o sustrato (Długosz, et al., 2023), por lo que, modificar las características físico-químicas del suelo mediante manejo agronómico, afectaría a las comunidades de microorganismos que viven en él. La respuesta microbiológica del suelo se puede estimar a través de una serie de indicadores que describen la actividad, biomasa, diversidad y funciones de las comunidades microbianas presentes en el suelo (Anderson, 2003; Nielsen et al., 2022). La respuesta fisiológica de los microorganismos del suelo que determina su actividad y funcionamiento en respuesta a su entorno, se puede medir con métodos comunes como la respirometría del suelo que está relacionada con la actividad general (Celis et al., 2009). También, la actividad enzimática es un indicador biológico del suelo ya que las enzimas están involucradas en muchos procesos en ciclos de nutrientes del suelo y permite estimar

la cantidad y rapidez con la que las enzimas producidas por los microorganismos descomponen la materia orgánica (Henríquez et al., 2014).

Algunos manejos agronómicos que afectan las condiciones del suelo, pero son necesarios para mejorar la productividad de huertos frutales son: el riego, control de malezas, fertilización y densidad de plantación. Un sistema de riego bien gestionado permite optimizar la disponibilidad de agua y las respuestas microbiológicas vinculadas a la salud del suelo. Por efecto del riego, se ha incrementado hasta 131% la biomasa microbiana, 148% la enzima ureasa, y 102% la enzima fosfatasa del suelo en un huerto de Almendros (*Amygdalus communis* L.) (Romero-Trigueros et al., 2021); mientras que, la deficiencia de riego disminuyó 33% la biomasa microbiana del suelo y 26% el rendimiento de fruta en un huerto de pomelos (*Citrus paradisi* Macf) (Bastida et al., 2019). Por otro lado, el control de malezas con mulch, reduce la necesidad de herbicidas químicos, evitando la contaminación y favorece la economía circular al reutilizar residuos orgánicos provenientes de huertos frutales. Los mulch, aumentan las respuestas microbiológicas vinculadas a la salud del suelo, por ejemplo, fue efectivo para aumentar más de 50% el C y N del suelo, e incrementar significativamente la riqueza y diversidad de las comunidades bacterianas y fúngicas del suelo en huertos de manzanos (*Malus baccata* L. y *Malus pumilla* Mill.) (Chen et al., 2014; Xie et al., 2022). Además, los mulch mejoraron la fertilidad del suelo aumentando la calidad de fruta con 33% más diámetro de baya y 15% más rendimiento en vid (*Vitis vinifera* L.) (Zhang et al., 2014). La fertilización orgánica, a la vez que reduce desechos orgánicos transformándolos en fertilizantes, reduce el uso de fertilizantes sintéticos de fáciles pérdidas que pueden contaminar el ambiente. La fertilización, fue efectiva para aumentar indicadores de la salud del suelo como, abundancia bacteriana (11.6%), de hongos (113.6%), la enzima  $\beta$ -glucosidasa (20.7%), la enzima  $\beta$ -xilosidasa (32.5%) y la enzima N-acetilglucosaminidasa (32.8%) que suelos sin fertilizar (Zhao et al., 2016). Además, la fertilización orgánica, produjo frutas con altos niveles de fenoles, flavonoles y contenido de antocianinas en cultivares Allstar y Honeoye de fresas (*Fragaria x ananassa* Duch.) (Wang y Lin, 2003). La densidad de plantas puede contribuir a una

gestión eficiente del espacio o, favorecer el desarrollo vegetativo, productivo y calidad de la fruta en huertos a través de la actividad microbiológica y disponibilidad de nutrientes en el suelo. Una selección adecuada incrementó 29% en el carbono orgánico del suelo, mientras que en fruto mejoró 1.2% el contenido de aceite en huertos de olivos (*Olea europaea* L.) (Gómez et al., 2022; Lavee et al., 2012). A pesar de estos antecedentes, es escaso el conocimiento sobre los efectos del manejo agronómico en plantas de calafate, y más aún, sobre las propiedades microbiológicas del suelo.

Prácticas agronómicas como riego y fertilización, fueron probadas en plantas silvestre de calafate en Chile y Argentina, para evaluar su efecto en la calidad del fruto principalmente. En la Región de Coyhaique, Chile, Arribillaga (2000) demostró que un riego (100% de la evapotranspiración) y fertilización (50 gr de  $P_2O_5$  planta<sup>-1</sup> y 50 gr de  $K_2O$  planta<sup>-1</sup>) aumentó 37% el contenido de antocianinas del fruto que plantas sin riego ni fertilización. Posteriormente, Arena et al. (2017) con plantas de Tierra del Fuego, Argentina, demostraron que una fertilización con 6.72 g planta<sup>-1</sup> de  $NH_4NO_3$ ,  $Ca(H_2PO_4)$  y  $K_2SO_4$  en proporción 1:0.6:1.2, aumento 20% los polifenoles totales y 8% la capacidad antioxidante DPPH. En bases a estos resultados, y a la capacidad del calafate para resistir condiciones climáticas adversas (Mariangel et al., 2013), en 2017 se estableció por primera vez un huerto de calafate en la Región de Ñuble, centro-sur de Chile. Luego, en dicho huerto comercial de calafate, Pinto-Morales et al. (2022), demostraron que la fertilización orgánica en dosis crecientes, además de mejorar significativamente la respuesta fisiológica de la planta, aumentó hasta 53% el rendimiento planta<sup>-1</sup> y 24% la capacidad antioxidante DPPH del fruto, respecto a plantas sin fertilizar. No obstante, queda por evaluar el efecto de estas y otras prácticas de manejo agronómico de común uso sobre las propiedades microbiológicas del suelo, la fisiología de planta y calidad del fruto de calafate para entender cómo establecer futuros huertos de una forma sostenible. La sostenibilidad de los huertos incluye proveer un suelo funcional y la eficiencia en el uso de recursos como el agua, subproductos orgánicos, fertilizantes orgánicos y número de plantas, al momento de regar, aplicar mulch, fertilizar con compost o estimar la densidad de

plantación. A pesar de los estudios realizados sobre el manejo agronómico en especies frutales nativas de interés nutracéutico, persisten importantes brechas de conocimiento. En calafate, los estudios se han centrado principalmente en la fertilización y el riego como métodos para mejorar la calidad del fruto, pero se ha descuidado el impacto que estos manejos agronómicos pueden tener en las propiedades microbiológicas del suelo, un aspecto clave para la sostenibilidad de los huertos. Además, es limitada la información sobre otros manejos agronómicos, como el uso de mulch y la densidad de plantación, en relación con la microbiología del suelo y la productividad del calafate. Estas lagunas de conocimiento dificultan el establecimiento de huertos sostenibles que optimicen tanto la productividad como la calidad de los frutos de calafate. Además, no se ha evaluado de manera integral la interacción entre las prácticas de manejo agronómico, la microbiología del suelo y la calidad del fruto de calafate. Por lo tanto, el objetivo de esta investigación fue evaluar, en un huerto de calafate, los manejos agronómicos de uso frecuente en fruticultura (riego, fertilización orgánica, mulch y densidad de plantación), que mejoren la salud del suelo (biomasa microbiana, respiración y actividad enzimática de los ciclos del C, N y P), mejoren el desarrollo vegetativo y fisiológico de la planta (índice de área foliar, índice de clorofila, conductancia estomática, rendimiento cuántico máximo del fotosistema II), y conduzcan a una adecuada productividad y calidad de los frutos (rendimiento por planta, contenido de polifenoles y actividad antioxidante).

## **HIPÓTESIS**

1. Una reposición hídrica sobre 0% y bajo 150% de la evapotranspiración de referencia ( $ET_0$ ), es adecuado para aumentar significativamente la actividad microbiana y enzimática del suelo, sin generar estrés hídrico por exceso o déficit en la planta de calafate y, con aumento significativo en la productividad y calidad de fruta.

2. Los mulch orgánicos de paja de avena y cascara de avellano son mejores para promover un aumento significativo en la actividad microbiana y enzimática del suelo, desarrollo vegetativo, el rendimiento y calidad de fruta del calafate en comparación a suelos sin cobertura y mulch de geotextil.
3. Dosis de compost sobre 0 ton ha<sup>-1</sup> genera un aumento significativo en la actividad microbiana y enzimática del suelo, mejorar la respuesta fisiológica, desarrollo vegetativo y calidad de fruta de calafate. Las dosis más altas de compost (10 y 15 ton ha<sup>-1</sup>) mejora significativamente la salud del suelo y el rendimiento del cultivo de calafate.
4. Disminuir la densidad de plantación mejora significativamente la actividad microbiana del suelo y, a la vez, aumenta el desarrollo vegetativo, productividad y calidad de fruta en calafate. Densidades de plantación menores a 6667 plantas ha<sup>-1</sup>, mejora significativamente la salud del suelo y el rendimiento del cultivo de calafate.

## **OBJETIVOS ESPECIFICOS**

1. Determinar el efecto de dosis de reposición hídrica (0, 50, 100 y 150% de la Evapotranspiración de referencia [ET<sub>0</sub>]) sobre indicadores de actividad microbiológica del suelo, desarrollo vegetativo de planta y rendimiento del fruto en un huerto de calafate.
2. Estimar el efecto de diferentes tipos de cobertura (sin cobertura, geotextil, paja de avena y cáscara de avellano) en parámetros microbiológicos de la salud del suelo, desarrollo vegetativo de planta y atributos de calidad del fruto en un huerto de calafate.
3. Evaluar la influencia de dosis de compost (0, 5, 10 y 15 ton ha<sup>-1</sup>) sobre parámetros microbiológicos de la salud del suelo, fisiológicos y foliares de planta, físico-químicos del fruto en un huerto de calafate.
4. Determinar la influencia de diferentes densidades de plantación (6667, 3333, 2222 y 1667 plantas ha<sup>-1</sup>) sobre indicadores de actividad microbiológica del suelo, fisiológicas de la planta, y composición funcional de fruta en un huerto de calafate.

## II. CAPÍTULO I: PLANT PERFORMANCE AND SOIL MICROBIAL RESPONSES TO IRRIGATION MANAGEMENT: A NOVEL STUDY IN A CALAFATE ORCHARD

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### ABSTRACT

Calafate fruits have a high content of phenolic compounds and an antioxidant activity up to four times higher than that of blueberries. The establishment of a calafate orchard and irrigation responses on fruit and soil characteristics have been scarcely studied. Therefore, the objective of this study was to evaluate the effect of water replenishment rate: 0%, 50%, 100% and 150% of reference evapotranspiration (ET<sub>0</sub>), on soil microbiological activity, plant physiological response, fruit yield and chemical composition in a calafate orchard. The results showed that irrigation at 50% ET<sub>0</sub> presented significant increases in soil urease, dehydrogenase and acid phosphatase

activity. Likewise, irrigation at 50% ET<sub>0</sub> significantly increased stomatal conductance and plant chlorophyll index, which led to a significant increase in fruit yield being 60% higher compared to the other treatments. Despite the higher fruit yield, 50% ET<sub>0</sub> irrigation had a similar level of total anthocyanins and ORAC antioxidant capacity as the 100% ET<sub>0</sub> treatment. In contrast, 0% and 150% ET<sub>0</sub> treatments showed a higher degree of stress and got higher values for total anthocyanins and fruit antioxidant capacity. Irrigation rates 50% ET<sub>0</sub> increases fruit yield while maintaining fruit quality and optimizing water resources in commercial orchards of calafate.

**Keywords:** berberis; soil enzyme activity; polyphenols; anthocyanins; antioxidant capacity

## 1. INTRODUCTION

*Berberis microphylla* G. Forst., commonly called calafate, is a very thorny shrub-like species, with arching branches and blackish-blue berries, which grows under different agroclimatic conditions in Chile and Argentina [1].

Currently, the calafate has become known for the properties of its blackish blue fruits, as they have a high content of phenolic compounds and antioxidant activity [2], being up to ten times higher than oranges, apples and pears, and up to four times more than blueberries [3]. Despite its functional and nutritional properties, it is only grown in the wild, without cultural management to improve its phytosanitary status, and longevity of branches or shoots [4], which leads to a low fruit production per plant, resulting in total production of calafate fruit in Chile that did not exceed 1 t in 2019 [5]. In this context, to stimulate its productive development, in 2017, the first commercial orchard was established in the central-southern zone of Chile, for domestication and development of agronomic management [6]. Pinto-Morales et al. [6] pointed out that agronomic management is being addressed, but the implementation of commercial orchards brings new questions, such as how the interaction of these agronomic managements is altering soil biological properties.

The calafate is a highly adaptable species, being able to develop and bear fruit in regions with a Mediterranean climate [7]. However, it has been shown that environmental factors such as ultraviolet (UV) radiation and temperature can alter phenol and anthocyanin content of the fruit [8], a relevant factor due to the high impact of environmental changes resulting from climate change reported in recent times [9] and the current food trend, which demands healthier foods [10]. Therefore, the evaluation of water replenishment for the establishment of this species becomes relevant, since this management practice attenuates soil water losses [11] and defines nutritional status of the plant, which affects fruits production and chemical characteristics [12]. In this sense, water replacement in fruit crops is used as a strategy to enhance the accumulation of secondary metabolites in the fruit [13] and to reduce the consumption and losses of water, which is increasingly scarce [14]. Accordingly, it has been shown that the use of controlled deficit irrigation (CDI) benefited 'Barnea' and 'Askal' olive oil quality, reaching mean polyphenol contents of up to 372 and 487 mg kg<sup>-1</sup>, respectively, which represented increases of up to 150% and 385%. However, it was also shown that this type of controlled water stress produced lower fruit yields, from 305 to 265 kg/tree<sup>-1</sup> in 'Barnea' and from 205 to 190 kg/tree<sup>-1</sup> in 'Askal', respectively [14]. Consequently, knowing the water requirements of native plants of high nutritional and pharmacological value [2] would allow for setting up the best establishment conditions to increase the quality of fruits and efficiency of water resources.

It should be noted that variation in water availability alters soil physical [15], chemical [16] and microbiological properties [17]. The latter is relevant because, according to several authors, some soil bacterial communities can alter the functional characteristics of plants [18] and allow greater resistance to pathogen invasion [19]. Likewise, soil fungi such as arbuscular mycorrhizal fungi (AMF) form symbiotic associations with roots and improve the hormonal balance of the plant to resist water deficits [20,21]. In accordance with the above, previous studies have shown that soil water availability through different doses can determine the size, distribution, and activity of soil microorganisms [22], with bacterial communities being more sensitive

to changes in soil moisture and osmotic stress than fungi [23], which is consistent with what has been reported in an almond orchard under different water management strategies [24].

To date, there are no studies that evaluate the interaction of soil microorganisms with the degree of adaptability and chemical characteristics of the fruit, nor how these would be influenced by different levels of water replenishment. In fact, as far as fruit-bearing species are concerned, studies showing the influence of irrigation dose on soil microbiological properties and plant productive parameters are still incipient and their results are dissimilar. For example, in a grapefruit orchard irrigated with different water quantities, restricted water replenishment decreased soil microbial biomass by up to 33%, leading to a decrease in fruit production from 232 to 184 kg/fruit tree<sup>-1</sup> [24]. In contrast, in an almond orchard, restricted water replenishment significantly increased soil microbial biomass by up to 131%, as well as the activity of enzymes such as urease by 148% and phosphatase by 102%, but reduced fruit production by 86% [25].

This is the first study that proposes to evaluate the water replenishment rates of calafate under a commercial system and its effect on soil microbiological and enzyme activity, plant physiology, fruit production, and fruit secondary metabolite accumulation.

## **2. MATERIALS AND METHODS**

### **2.1. Orchard Establishment and Edaphoclimatic Characteristics of the Study Site**

The calafate (*Berberis microphylla* G. Forst) orchard was established in 2017 with two-year-old plants. The orchard was located on the road to Tanilvoro, kilometer 12, from Chillán (36°31' S; 71°54' W), Ñuble Region, Chile, with a temperate Mediterranean climate with mean annual temperatures of 14.5 °C and maximum temperatures of 31.1 °C in the hottest months, and accumulated annual precipitation of 521.1 mm concentrated between May and October [26]. The Andisol (Melanoxerand) [27] was characterized by chemical analysis at the soil laboratory of

the Instituto de Investigaciones Agropecuarias (INIA Quilamapu), located in Chillan (Table 1).

Table 1. Soil chemical analysis of the study site in the Ñuble Region of Chile.

<b>Analysis</b>	<b>Unit</b>	<b>Result</b>
Organic matter	%	9.7
pH (water)		6.4
N availability	mg kg <sup>-1</sup>	19.0
Olsen P	mg kg <sup>-1</sup>	15.3
K availability	mg kg <sup>-1</sup>	496.0
S availability	mg kg <sup>-1</sup>	24.0
Exchangeable Ca	cmol <sub>c</sub> kg <sup>-1</sup>	8.7
Exchangeable Mg	cmol <sub>c</sub> kg <sup>-1</sup>	1.6
Exchangeable K	cmol <sub>c</sub> kg <sup>-1</sup>	1.3
Exchangeable Na	cmol <sub>c</sub> kg <sup>-1</sup>	0.01
Sum of bases	cmol <sub>c</sub> kg <sup>-1</sup>	11.6
Interchangeable Al	cmol <sub>c</sub> kg <sup>-1</sup>	0.02
CEC*	cmol <sub>c</sub> kg <sup>-1</sup>	11.6
Al saturation	%	0.1
B	mg kg <sup>-1</sup>	0.4
Cu	mg kg <sup>-1</sup>	1.6
Zn	mg kg <sup>-1</sup>	0.9
Fe	mg kg <sup>-1</sup>	44.0
Mn	mg kg <sup>-1</sup>	3.0

\*CEC: Cation exchange capacity of the soil; samples were obtained in August 2020, at a depth of 0-40 cm.

The total number of plants in the orchard was 352 in an area of 1056 m<sup>2</sup>, distributed in 16 plants per row with a total of 22 rows and a planting density of 1 m above row and 3 m between rows (Figure 1a,b). Only at the time of orchard establishment, fertilization was applied with 150 g urea (45% N), 200 g triple superphosphate (46% P<sub>2</sub>O<sub>5</sub>), and 200 g potassium sulfate (50% K<sub>2</sub>O) per planting hole [28]. Phytosanitary

management consisted of six alternating applications per year of tebuconazole (Orius 43 SC, Adama Brasil, Londrina B, Brasil) at a concentration of 25.8 g hL<sup>-1</sup> and cuprous oxide (Cuprodul WG, Quimetal Industrial S.A., Santiago, Chile) at a concentration of 180 g hL<sup>-1</sup>, during the first year of orchard establishment.

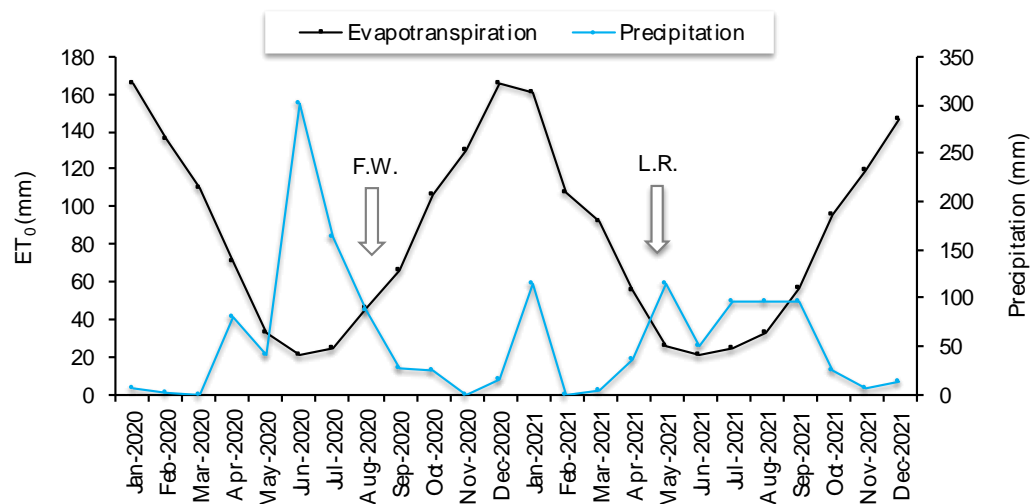


**(a)** **(b)**  
**Figure 1.** (a) Flowering calafate orchard, September 2021. (b) calafate orchard in fruiting, December 2021.

## 2.2. Experimental Setup of the Test

Four irrigation treatments were established consisting of 0%, 50%, 100%, and 150% water replenishment of reference evapotranspiration (ET<sub>0</sub>). The irrigation treatments were applied from the establishment of the orchard (year 2017) in the same period indicated for the evaluation. To determine the actual daily irrigation, ET<sub>0</sub> was calculated monthly using the Pen-man-Monteith method, taking as a reference what was suggested by Romero et al. [28], with data obtained from the INIA Quilamapu agroclimatic station located near the study site [29]. Irrigation treatments were applied using one irrigation lateral for the 50% ET<sub>0</sub> treatment, two irrigation laterals for the 100% ET<sub>0</sub> treatment, and three irrigation laterals for the 150% ET<sub>0</sub> treatment, with self-compensating pressure drippers (UniRam, Netafim, Hatzerim, Israel) spaced at 50 cm with a flow rate of 2.0 Lh<sup>-1</sup>. The control treatment did not consider irrigation

laterals. The irrigation period for the evaluation started in September 2020, until March 2021 (Figure 2), with a total evapotranspiration of 828.5 mm. The actual daily irrigation was applied every two days, where the daily irrigation time ( $\text{h day}^{-1}$ ) was calculated based on the monthly evapotranspiration (mm) divided by the number of irrigation days, being the actual monthly evapotranspiration and daily irrigation time for September were 33 mm and 2.2 h; October 53 mm and 3.5 h; November 65 mm and 4.3 h; December 166 mm and 5.5 h; January 160 mm and 5.3 h; February 107 mm and 3.6 h; and March 92 mm and 3 h, respectively.



**Figure 2.** Irrigation period for calafate plants: FI: First irrigation; LR: last watering, based on reference evapotranspiration (ET<sub>0</sub>) and precipitation, according to information from the agroclimatic station of the Instituto de Investigaciones Agropecuarias (INIA Quilamapu), Chile.

Soil volumetric water content (%) was monitored every 15 days (Table 2) with a Diviner 2000 portable soil moisture probe (Sentek, Stepney, Australia) that provides the soil moisture value in percent, from November 2021 to February 2022. The statistical design was randomized complete blocks. Soil microbiological, plant physiological and fruit chemical analyses were determined only once at the end of the

harvest period, during the month of January. Samples were taken from each experimental unit, where the experimental unit consisted of the average of two subsamples, each subsample being a calafate plant, and four replicates per treatment (n = 16) were carried out in all blocks.

**Table 2.** Soil moisture monitoring with a frequency of 15 days.

Treatments (ET <sub>0</sub> %)	soil moisture (%)							
	15-11- 2021	30-11- 2021	15-12- 2021	30-12- 2021	15-01- 2022	30-01- 2022	15-02- 2022	30-02- 2022
0	6.54	5.53	3.12	2.30	6.18	5.79	2.00	1.93
50	9.68	11.43	7.70	5.43	21.73	28.66	24.43	8.80
100	10.71	13.25	9.25	7.23	24.85	31.77	30.63	9.60
150	15.57	16.55	11.40	8.80	30.02	35.10	34.90	10.65

Treatments: 0%, 50%, 100% and 150% ET<sub>0</sub>. Measurements on 15 November 2021, 30 November 2021, 15 December 2021, 30 December 2021, 30 December 2021, and 30 February 2022 were measured 48 h after irrigation. Measurements on 15 January 2022, 30 January 2022, and 15 February 2022 were measured 24 h after irrigation.

### 2.3. Microbiological Analysis of Soil and Roots

Soil microbiological activity was determined by fluorescein diacetate (FDA) hydrolysis using 1.0 g wet soil sample in triplicate including a blank. In glass tubes, 9.9 mL sodium phosphate buffer (60 mM; pH 7.8) and 0.1 mL FDA were added, while only 10 mL buffer were added to the blanks, then vortexed and incubated at 20 °C for 1 h in a thermostatic bath. After incubation, the reaction was stopped in an ice water bath and 10 mL acetone was added to each tube, stirring to homogenize, and then filtered using Whatman No. 40 filter paper. Once each sample was obtained, the absorbance was measured using a spectrophotometer (Rayleigh-Model UV1601 UV/VIS, Beijing, China) at 490 nm. The results were expressed as µg FDA g<sup>-1</sup> [30].

Soil respiration was determined in duplicate using 25 g soil, which was placed in a flask together with a centrifuge tube with a volume of 7.5 mL 0.5 M NaOH. Both were placed in a hermetically sealed system and incubated at 22 °C for 7 days. After the incubation time, 1 mL 0.5 M NaOH was taken from the centrifuge tube and mixed

with 2 mL 1 M BaCl<sub>2</sub>; 2 to 3 drops of phenolphthalein were previously added as an indicator. The solution was titrated with 0.1 M HCl and data were expressed as  $\mu\text{g CO}_2 \text{ g}^{-1} \text{ h}^{-1}$  [30].

Urease activity was determined in 0.1 M phosphate buffer at pH 7 using 1 M urea as substrate. Aliquots of 2 mL buffer and 0.5 mL substrate were added to 0.5 g sample and incubated at 30 °C for 90 min. Urease activity was expressed as NH<sub>4</sub><sup>+</sup> released in the hydrolysis reaction [31], according to the ammonium sulfate standard curve. The standard curve was prepared with a dilution of 4 mL of ammonium sulfate in 200 mL of distilled water. Amounts of 0, 2, 4, 6, 6, 8, 10 and 12 mL of the dilution were added in 25 mL volumetric flasks to which 5 mL of EDTA, 1 mL of phenol nitroprusside, and 4 mL hypochlorite buffer were added. The absorbance was read against reagent blank at 636 nm. Once the data was recorded, it was fitted to a straight line of  $\mu\text{g NH}_4^+$  versus absorbance and  $\mu\text{mol NH}_4^+ \text{ g}^{-1} \text{ h}^{-1} = (\text{NH}_4^+ \mu\text{g/mL})/\text{dry soil weight (g)} \cdot \text{Incubation time (h)}$ . Acid phosphatase activity was determined with P-nitrophenyl disodium phosphate (PNPP 0.115 M) substrate, for which, 2 mL 0.5 M sodium acetate buffer at pH 6 with acetic acid [32] and 0.5 mL substrate was added to 0.5 g soil sieved at <2 mm and then incubated at 37 °C for 90 min. The reaction was stopped by cooling to 0 °C for 10 min in a cuvette of water with crushed ice. Then, 0.5 mL 0.5 M CaCl<sub>2</sub> and 2 mL 0.5 M NaOH were added and the mixture was centrifuged at 1382 g. The P-nitrophenol (PNP) formed was determined spectrophotometrically at 398 nm [33]. Los  $\mu\text{mol PNF g}^{-1} \text{ soil h}^{-1} = ((80.093 \cdot \text{absorbance} - 0.4026)/\text{peso} \cdot \text{weight} \cdot \text{incubation time} \cdot 139)$ .

Dehydrogenase activity was determined with 1 g soil at 60% field capacity exposed to 0.2 mL 0.4% INT (2-p-iodophenyl-3-p-nitrophenyl-5-phenyltetrazolium chloride) and carried in darkness for 20 h at 22 °C. The idonitrotetrazoliumformazan (INTF) formed was extracted with 10 mL methanol by shaking for 1 min with a vortex and filtering through Whatman No. 5 filter paper. Subsequently, INTF was measured spectrophotometrically at 490 nm and the results were expressed in terms of micrograms of INTF per gram of soil concerning an INTF standard curve [34],  $\text{mg INTF g}^{-1} = ((39.997 \cdot \text{absorbance} - 0.3834) \cdot \text{final extraction volume}/\text{weight})$ .

The colonization of arbuscular mycorrhizal fungi (AMF) in roots was determined by cutting 1 cm pieces of fine roots which were washed and clarified with KOH (2.5% w/v) at 120 °C for 15 min. Subsequently, roots were covered with HCl (1% w/w) for 1 day and then washed with abundant water to cover them with trypan blue (0.05% w/v) for 1 day [35]. To determine the percentage of mycorrhizal colonization in the roots, these were randomly distributed on a grid plate to subsequently visualize structures such as mycelium, spores, hyphae, arbuscules, and vesicles in the root tissues through a microscope at 40x (Siedentopf, United Scope, Irvine, CA, USA) and by counting all root intersections with horizontal lines according to the presence or absence of AMF structures [36,37].

#### 2.4. Plant Physiological Analysis

Maximum chlorophyll fluorescence ( $F_m$ ) and minimum chlorophyll fluorescence ( $F_o$ ) were measured in leaves on a clear day after harvest at four times of the day, at 09:00, 12:00, 15:00, and 18:00 h using a portable fluorimeter model OS-5p (Opti-Sciences, Hudson, NH, USA). For determination of  $F_o$  and  $F_m$ , leaves were dark adapted using leaf clips including a movable shutter plate for 30 min [38]. Maximum photosystem II photochemical efficiency ( $F_v/F_m$ ) was quantified using the ratio  $F_v/F_m = (F_m - F_o)/F_m$  [39].

Stomatal conductance ( $g_s$ ,  $\text{mmol m}^{-2} \text{s}^{-1}$ ) was measured on leaves on a clear day after harvest at four times of the day, at 09:00, 12:00, 15:00, and 18:00 h using a portable porometer model SC-1 (Decagon Devices, Washington, DC, USA). For data representativeness, the leaf selection criteria were leaves exposed to the sun, in the second third of a branch of the season as with the data obtained from chlorophyll fluorescence [38].

The SPAD (Soil Plant Analysis Development) index was measured on leaves at midday with the criteria used for chlorophyll fluorescence and  $g_s$  measurements, after harvest. The data were obtained through a portable chlorophyll meter (MC-100, Apogee Instruments, Logan, UT, USA) equipment that determines a value proportional to the amount of chlorophyll present in the leaf [40].

Leaf area index (LAI;  $\text{m}^2 \text{m}^{-2}$ ) was measured on a clear day at midday, after harvest, when plant growth had already stopped, for which an AccuPAR LP-80 ceptometer (Decagon Devices Inc., Washington, DC, USA) was used that delivers the average of 80 quantum sensors that determine direct, diffuse, residual and reflected photosynthetically active radiation from the soil [41].

## 2.5. Fruit Yield and Chemical Compounds

Fruit productivity ( $\text{g plant}^{-1}$ ) was measured immediately after hand harvesting at 130 d after full flowering [6]. The period of full flowering took place at the beginning of September and the manual harvest was carried out at the end of December.

The total polyphenol content was determined using the Folin–Ciocalteu method following the indications by Romero-Román et al. [8], where first, an extract was obtained from the harvested sample, using 0.25 g sample, 2.5 mL  $\text{H}_2\text{O}:\text{MeOH}:\text{formic acid}$  solution (24:25:1) and taken to ultrasound CPX 5800 Branson (Branson Ultrasonics Corp., Danbury, CT, USA) for 1 h. Then, it was left for the rest of 24 h and taken for an ultrasound for 1 h, then; the sample was centrifuged for 15 min at 1209 g and filtered. Subsequently, a standard curve was prepared with gallic acid and then, the samples prepared with Folin–Ciocalteu reagent, distilled  $\text{H}_2\text{O}$  and  $\text{Na}_2\text{CO}_3$  20% were mixed and incubated for 2 h in the dark, to be measured in the spectrophotometer at 760 nm [42]. The standard curve equation used was  $0.0013x + 0.0573$  and  $R^2 = 0.99$ . The results are expressed as mg gallic acid  $100 \text{g}^{-1}$  FW [43].

The DPPH antioxidant capacity was performed after Romero-Román et al. [44] by diluting the extract and incorporating DPPH solution, which was shaken and kept in the dark for 1 h, at room temperature, and then spectrophotometer readings were taken at 515 nm. Subsequently, a standard curve of Trolox was carried out [45]. The standard curve equation used was  $0.0008x + 0.0272$  and  $R^2 = 0.99$ . The results were expressed in  $\mu\text{mol Trolox equivalent (TE)} 100 \text{g}^{-1}$  FW [6].

ORAC antioxidant capacity was performed by Romero-Román et al. [8]. Diluting 100  $\mu\text{L}$  sample in phosphate buffer pH 7.4, fluorescein intensity was

measured every 1 min for 1 h with excitation and emission wavelengths of 485 and 520 nm at 37 °C. The results were expressed as  $\mu\text{mol TE } 100 \text{ g}^{-1} \text{ FW}$  [45].

Anthocyanins were identified by high-performance liquid chromatography with a diode array detector (HPLC-DAD) on a Hitachi primaide apparatus equipped with a photodiode array detector (Model 1430, Hitachi, Tokyo, Japan). The equipment consisted of a binary pump with a degasser (Model 1110, Hitachi, Tokyo, Japan) and an autosampler (Model 1430, Hitachi, Tokyo, Japan). The HPLC system was controlled by ChemStation software (version 08.03, Agilent Technologies, Palo Alto, CA, USA) [46]. Anthocyanin quantification was performed with the sample extracts used for the determination of the mentioned analyses, which were filtered through a 0.22  $\mu\text{m}$  PVDF membrane (Millex V13, Millipore, Bedford, MA, USA). The results were expressed as  $\text{mg } 100 \text{ g}^{-1} \text{ FW}$  [45].

## 2.6. Statistical Analysis

Data were subjected to analysis of variance (ANOVA) with a significance level of  $p < 0.05$ . Comparison of means was performed using Fisher's least significant difference (LSD) test with a significance level of 0.05, and in addition, principal component analysis (PCA) was performed using mean-centered data based on eigenvalues to determine the correlation and discrimination between soil, plant, and fruit variables under different water replenishment conditions using R software [47] with FactoMineR and ggplot2 packages [48].

## 3. RESULTS

### 3.1. Biological Properties of Soil and Roots

Soil moisture monitoring showed that the treatment of 150% of ET<sub>0</sub> > 100% of ET<sub>0</sub> > 50% of ET<sub>0</sub> > 0% of ET<sub>0</sub>, both in measurements taken 24 h after irrigation and 48 h after irrigation (Table 2).

Soil microbiological and enzyme activity showed significant differences among the different treatments (Table 3). The highest fluorescein diacetate (FDA) activity

was reached with the treatment containing 100% ET<sub>0</sub>, which presented 41.8  $\mu\text{g FDA g}^{-1}$ , being significantly superior to the treatments containing 50% and 150% ET<sub>0</sub>, which presented average values of 34.1 and 30.3  $\mu\text{g FDA g}^{-1}$ , respectively, both significantly higher than the treatment without irrigation, which reached a value of 22.8  $\mu\text{g FDA g}^{-1}$  dry soil. It should be noted that irrigation treatments significantly influenced soil microbial respiration. The treatments with the highest irrigation dose, 100% and 150% ET<sub>0</sub>, presented higher soil basal respiration with values of 2.2 and 2.1  $\mu\text{g CO}_2 \text{ g}^{-1} \text{ h}^{-1}$ , without significant differences between them, but significantly higher than 50% ET<sub>0</sub> treatment with 1.7  $\mu\text{g CO}_2 \text{ g}^{-1} \text{ h}^{-1}$ . It is important to note that in soil basal respiration, all irrigation treatments showed significant increase in comparison to the non-irrigated treatment.

**Table 3.** Soil microbiological properties and enzyme activity in response to water replenishment.

Treatments (ET <sub>0</sub> %)	FDA activity ( $\mu\text{g FDA g}^{-1}$ )	Soil basal respiration ( $\mu\text{g CO}_2 \text{ g}^{-1} \text{ h}^{-1}$ )	Urease activity ( $\mu\text{mol NH}_4^+ \text{ g}^{-1} \text{ h}^{-1}$ )	Dehydrogenase activity ( $\mu\text{g INTF g}^{-1}$ )	Acid phosphatase activity ( $\mu\text{mol PNP} \text{ g}^{-1} \text{ h}^{-1}$ )	AMF colonization in roots (%)
0	22.8 ± 1.70 c	1.4 ± 0.10 c	2.5 ± 0.09 b	59.1 ± 0.72 c	12.8 ± 0.48 b	62.5 ± 4.79 a
50	34.1 ± 1.27 b	1.7 ± 0.08 b	2.7 ± 0.06 ab	109.8 ± 3.02 a	14.6 ± 0.32 a	82.5 ± 4.79 a
100	41.8 ± 1.49 a	2.2 ± 0.16 a	2.5 ± 0.04 b	83.9 ± 4.74 b	14.0 ± 0.64 a	65.0 ± 8.66 a
150	30.3 ± 1.19 b	2.1 ± 0.08 a	2.8 ± 0.07 a	76.0 ± 5.34 b	7.7 ± 0.86 c	72.5 ± 4.79 a
Anova <i>p</i> values	0.0001	0.0007	0.047	0.0001	0.0001	0.1413

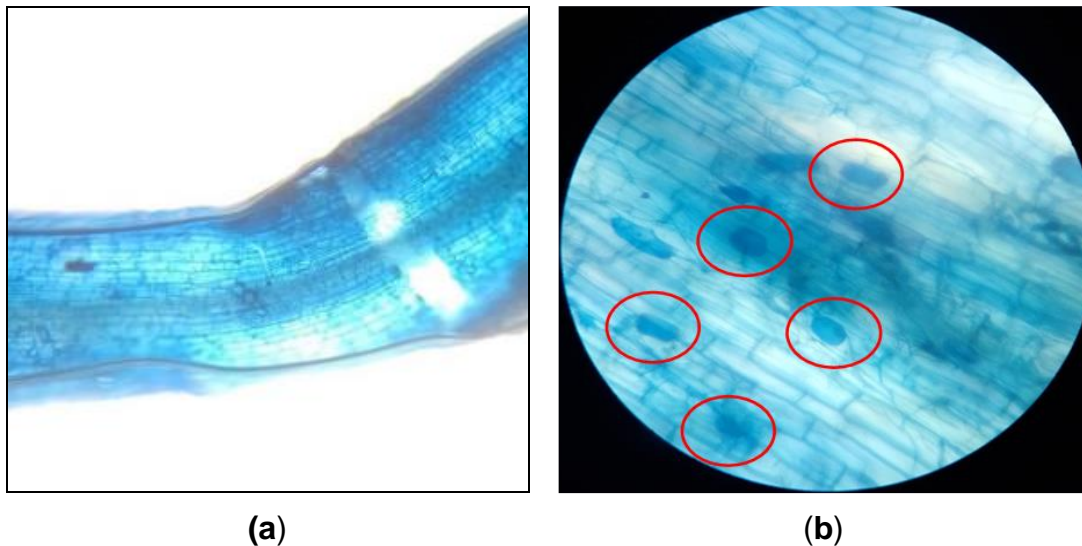
Treatments: 0%, 50%, 100%, and 150% ET<sub>0</sub>. Different lowercase letters indicate significant differences between treatments according to Fischer's LSD test ( $p < 0.05$ ). Mean ± standard error ( $n = 4$ ). AMF: Arbuscular mycorrhizal fungi. Data on arbuscular mycorrhizal fungi (AMF) colonization on roots was analyzed by Kruskal–Wallis nonparametric analysis.

On the other hand, urease enzymatic activity increased significantly with the replacement treatment of 150% ET0, presenting an increase of 12% with respect to 0% ET0 treatment, which presented a value of  $2.5 \mu\text{mol NH}_4^+ \text{g}^{-1} \text{h}^{-1}$ .

As for dehydrogenase activity, the highest value reached was obtained with 50% replacement of ET0, being 86% higher than the treatment without irrigation. Meanwhile, 100% and 150% ET0 replenishment treatments with values of 83.9 and  $76.0 \mu\text{g INTF g}^{-1}$  presented increases of 42% and 29%, respectively, with respect to the treatment without irrigation.

Regarding acid phosphatase activity, irrigation of 50% and 100% ET0, had increases of 14% and 9%, respectively, with respect to 0% ET0. It should be noted that in acid phosphatase activity, the maximum irrigation rate applied to the crop (150% ET0) was significantly lower than the rest of the treatments including the treatment without irrigation, registering a value of  $7.7 \mu\text{mol PNP g}^{-1} \text{h}^{-1}$ .

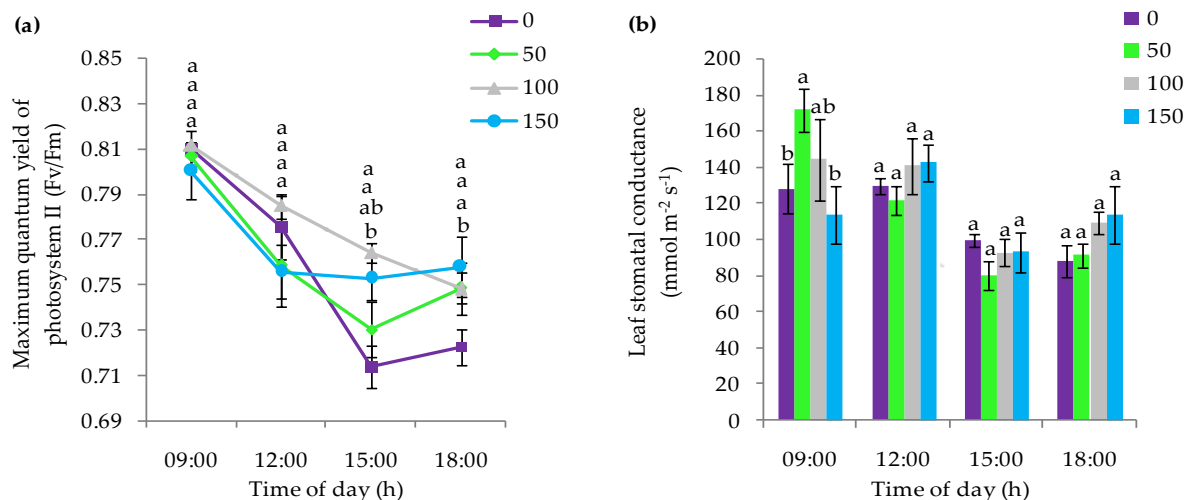
On the other hand, AMF colonization in roots (Figure 3a,b) did not show significant differences between treatments ( $p > 0.05$ ).



**Figure 3. (a)** Stained calafate fine root section, viewed microscopically at 10X; hydric replenishment rate of 50% ET0. **(b)** AMF vesicles in stained calafate fine roots (structures circled in red), viewed microscopically at 40X; hydric replenishment rate of 50% ET0. AMF: Arbuscular mycorrhizal fungi.

### 3.2. Plant Physiological Parameters

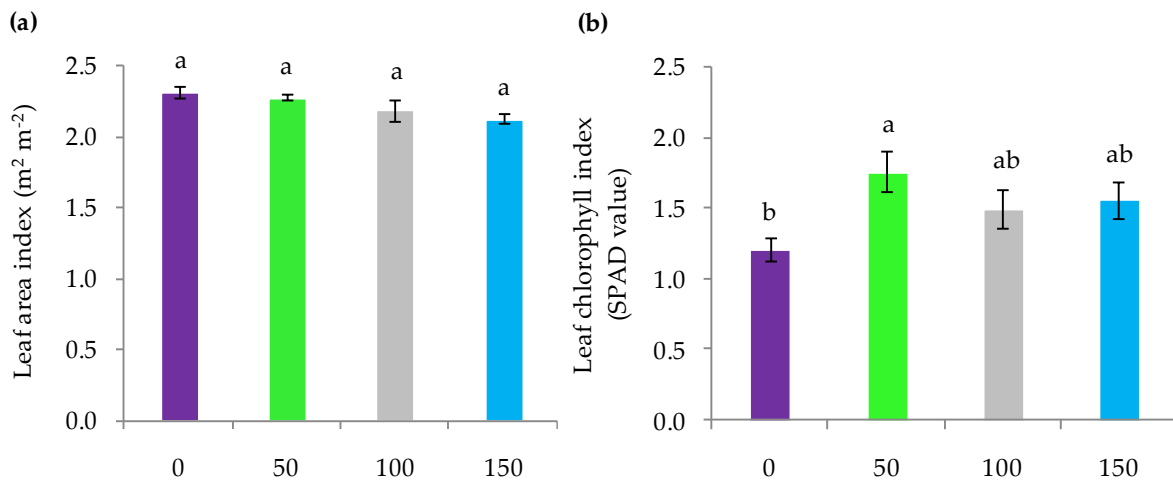
The maximum quantum yield of photosystem II (Fv/Fm; Figure 4a), measured at 09:00 and 12:00 h, did not show significant differences among treatments, reaching values close to 0.80 and 0.77, respectively. At 15:00 h, the 100%, 150% and 50% ET0 replenishment rates had a 7%, 6% and 3% lower Fv/Fm decrease than the 0% ET0 treatment, which only reached 0.71 (Figure 4a). At 18:00 h, all treatments irrigated at 50%, 100% and 150% ET0, reached a mean Fv/Fm value of 0.76, significantly higher than 0% ET0 treatment, which only reached Fv/Fm 0.72 (Figure 4a). Stomatal conductance (Figure 4b) presented significant statistical differences among treatments at 09:00 h, with 50% and 100% ET0 irrigation presenting the greatest increases, 34% and 13%, respectively, with respect to the treatment without irrigation, which had a value of 128 mmol m<sup>-2</sup> s<sup>-1</sup>. On the other hand, the irrigation of 150% ET0 presented the lowest value of stomatal conductance measured at 09:00 h with 114 mmol m<sup>-2</sup> s<sup>-1</sup> (p < 0.05). In the subsequent measurements, 12:00, 15:00 and 18:00 h, nonsignificant differences were found between treatments, with mean values of stomatal conductance close to 133, 91 and 101 mmol m<sup>-2</sup> s<sup>-1</sup>, respectively for each of the aforementioned times (Figure 4b).



**Figure 4.** (a) Variation of the maximum quantum yield of photosystem II (Fv/Fm) in calafate plants; (b) Values recorded for stomatal conductance (mmol m<sup>-2</sup> s<sup>-1</sup>), in calafate plants subjected to different doses of water replenishment, evaluated at

different times of the day: 09:00, 12:00, 15:00 and 18:00 h. Treatments: 0%, 50%, 100% and 150% ET<sub>0</sub>. Different lowercase letters indicate significant differences between treatments according to Fischer's LSD test ( $p < 0.05$ ). Mean  $\pm$  standard error ( $n = 4$ ). Bars correspond to experimental error for each treatment.

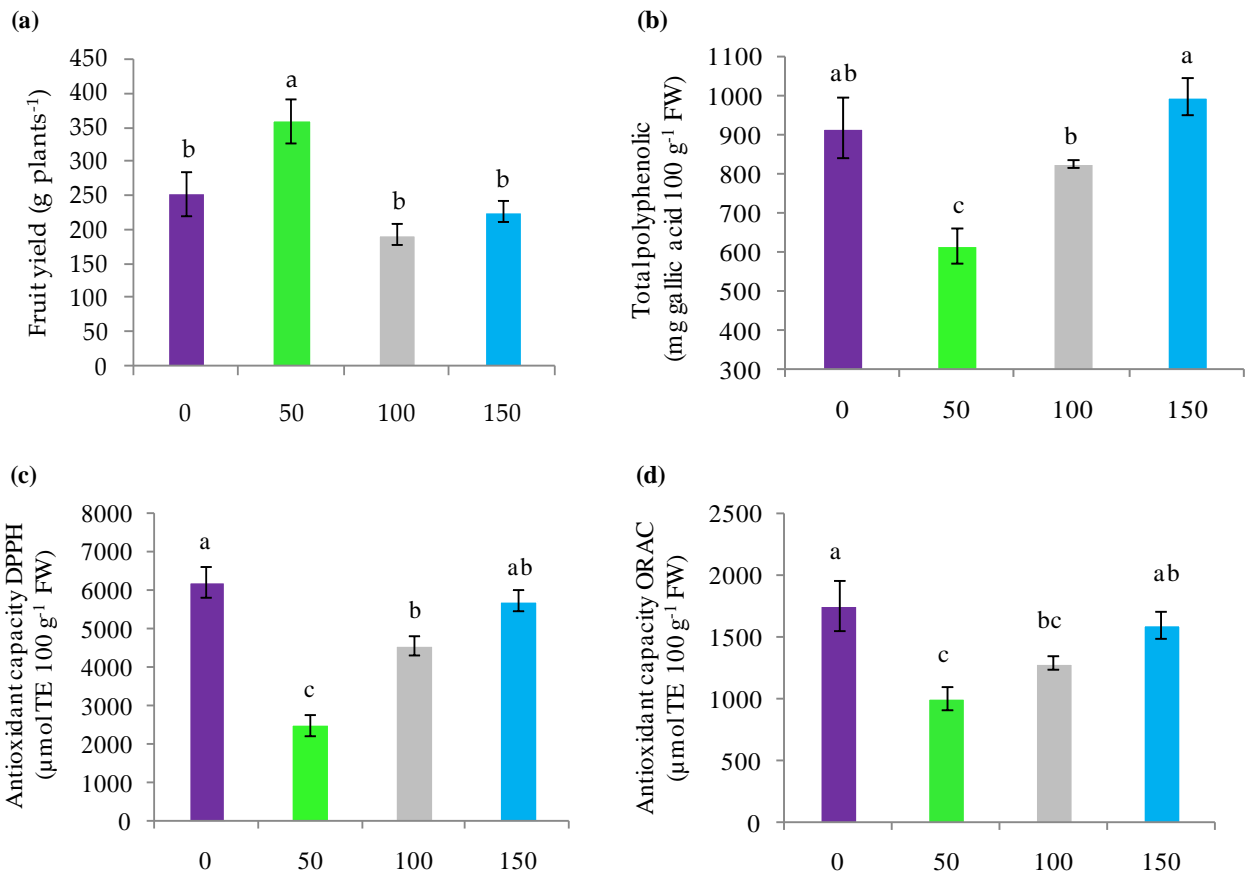
The Leaf area index (LAI) was not influenced by the water replenishment treatments ( $p > 0.05$ ), all of them reaching a mean value of LAI close to 2.2  $\text{m}^2 \text{m}^{-2}$  (Figure 5a). Leaf chlorophyll index was influenced by irrigation dose ( $p < 0.05$ ), where 50% ET<sub>0</sub> replenishment significantly increased SPAD (Soil Plant Analysis Development) value by 45% with respect to the 0% ET<sub>0</sub> treatment. On the other hand, the treatments with higher irrigation, replenishment of 100% and 150% ET<sub>0</sub>, without statistical differences, increased their SPAD value by 33% with respect to the treatment without irrigation (Figure 5b).



**Figure 5. (a)** Leaf area index values recorded in calafate plants; **(b)** leaf chlorophyll index recorded in calafate leaves subjected to different doses of water replenishment. Treatments: 0%, 50%, 100% and 150% ET<sub>0</sub>. Different lowercase letters indicate significant differences between treatments according to Fischer's LSD test ( $p < 0.05$ ). Mean  $\pm$  standard error ( $n = 4$ ). Bars correspond to experimental error for each treatment.

### 3.3. Fruit Yield and Chemical Parameters

Fruit yield per plant was significantly higher with 50% ET0 irrigation ( $p < 0.05$ ) compared to the rest of the treatments, reaching a mean value of  $359 \text{ g plant}^{-1}$ , while the treatments of 0%, 150% and 100% ET0 replenishment registered mean values of 253, 226 and  $193 \text{ g plant}^{-1}$ , respectively, with nonsignificant differences among them (Figure 6a). It should be noted that irrigation treatments had a significant influence on the total polyphenol content of the fruit ( $p < 0.05$ ). The treatments without irrigation and with an irrigation of 150% ET0, reached the highest values recorded, being these 920 and  $1001 \text{ mg gallic acid } 100 \text{ g}^{-1} \text{ FW}$ , respectively. On the other hand, the irrigation treatment of 100% ET0 with  $830 \text{ mg gallic acid } 100 \text{ g}^{-1} \text{ FW}$  was superior to the treatment with 50% ET0 ( $p < 0.05$ ), which presented a value of  $620 \text{ mg gallic acid } 100 \text{ g}^{-1} \text{ FW}$  (Figure 6b).



**Figure 6. (a)** Average yield of fresh fruits of calafate ( $\text{g plant}^{-1}$ ); **(b)** total polyphenols; **(c)** DPPH antioxidant capacity; and **(d)** ORAC antioxidant capacity in fruits of calafate plants subjected to different doses of water replenishment. Treatments: 0%, 50%, 100% and 150% ET0. Different lowercase letters indicate significant differences between treatments according to Fischer's LSD test ( $p < 0.05$ ). Mean  $\pm$  standard error ( $n = 4$ ). Bars correspond to experimental error for each treatment.

Likewise, treatments with irrigation significantly influenced the antioxidant capacity 2,2-diphenyl-picryl-hidrazyl (DPPH) (Figure 6c) and oxygen radical absorbance capacity (ORAC) (Figure 6d). The treatments without irrigation and with 150% ET0 replenishment presented the highest DPPH values corresponding to 6236 and 5764  $\mu\text{mol TE } 100 \text{ g}^{-1} \text{ FW}$ , which were followed by the 100% ET0 hydric replenishment treatment, which reached an average value of 4592  $\mu\text{mol TE } 100 \text{ g}^{-1} \text{ FW}$ , being significantly higher than 50% ET0, which presented 2528  $\mu\text{mol TE } 100 \text{ g}^{-1} \text{ FW}$ . Likewise, the ORAC antioxidant capacity was higher with the 0% and 150% ET0 replenishment treatments, with values of 1757 and 1609  $\mu\text{mol TE } 100 \text{ g}^{-1} \text{ FW}$ , respectively (Figure 6d). On the other hand, 50% and 100% ET0 replenishment did not present significant differences between them ( $p > 0.05$ ), with ORAC antioxidant capacity values of 1011 and 1297  $\mu\text{mol TE } 100 \text{ g}^{-1} \text{ FW}$ , respectively. As for the total anthocyanin content, it presented the same trend as the ORAC antioxidant capacity, with the 0% and 150% ET0 replenishment treatments presenting the highest mean content with 610.75 and 484.68  $\text{mg } 100 \text{ g}^{-1} \text{ FW}$ , respectively, followed by 50% and 100% ET0 with 286.37 and 468.26  $\text{mg } 100 \text{ g}^{-1} \text{ FW}$ , respectively, which did not present significant differences between them. The total anthocyanin content was represented by 82% to 85% by three main anthocyanins: delphinidin, petunidin and malvidin 3-glucoside (Table 4).

Table 4. Anthocyanins (mg 100 g<sup>-1</sup>) of fresh calafate fruit by HPLC according to water replenishment treatments.

Anthocyanins	Treatments (ET <sub>0</sub> %)				Anova p-values
	0	50	100	150	
Petunidin 3,5-dihexoside	13.7 ± 2.4 a	6.6 ± 0.8 b	12.1 ± 0.8 ab	16.5 ± 1.7 a	0.0018
Malvidin 3,5-dihexoside	13.6 ± 1.9 a	5.4 ± 0.9 b	6.8 ± 0.7 b	12.0 ± 1.6 a	0.0013
Delphinidin 3-glucoside	208.7 ± 27.2 a	112.6 ± 21.3 b	201.2 ± 15.1 ab	196.6 ± 30.5 ab	0.0277
Delphinidin 3-rutinoside	6.1 ± 1.0 a	3.9 ± 0.8 a	3.8 ± 1.0 a	4.2 ± 1.0 a	0.1741
Cyanidin 3-glucoside	37.9 ± 6.7 a	12.0 ± 2.1 b	23.8 ± 3.3 ab	36.1 ± 6.8 a	0.004
Petunidin 3-glucoside	154.3 ± 16.9 a	77.3 ± 14.7 b	126.3 ± 8.7 ab	125.9 ± 13.3 ab	0.0048
Petunidin 3-rutinoside	8.5 ± 1.6 a	4.5 ± 1.0 b	5.1 ± 0.7 b	5.2 ± 0.9 b	0.0297
Peonidin 3-glucoside	29.5 ± 4.15 a	6.1 ± 0.96 b	12.1 ± 1.62 b	11.8 ± 2.00 b	0.0001
Malvidin 3-glucoside	138.4 ± 10.1 a	54.3 ± 6.7 b	66.5 ± 8.6 b	79.7 ± 8.5 b	0.0001
Total anthocyanins	610.8 ± 61.1 a	286.4 ± 46.3 b	468.3 ± 24.4 ab	484.7 ± 55.3 a	0.0016

Treatments: 0%, 50%, 100% and 150% ET<sub>0</sub>. Different lowercase letters indicate significant differences between treatments according to Fischer's LSD test ( $p < 0.05$ ). Mean ± standard error (n = 4).

### 3.4. Correlations

The correlation matrix (Figure 7) indicates that soil FDA was negatively correlated with fruit DPPH antioxidant activity; the higher the FDA, the lower the DPPH antioxidant activity. Likewise, dehydrogenase activity was negatively related to total polyphenol content, total anthocyanins, and fruit DPPH and ORAC antioxidant capacity. On the other hand, acid phosphatase activity was negatively correlated with fruit total polyphenol content. Among the soil parameters measured, FDA was positively correlated with soil respiration and urease activity was negatively correlated with acid phosphatase activity. Dehydrogenase activity was positively correlated with soil respiration and acid phosphatase activity was positively correlated with AMF colonization. On the other hand, the leaf area index (LAI) of the plant was positively correlated with total polyphenol content, total anthocyanins and antioxidant activity

ORAC of fruits. The correlation between fruit parameters was positive for antioxidant capacity DPPH and ORAC, with total polyphenol content. In addition, fruit total anthocyanin content was positively correlated with total polyphenol content and antioxidant activity DPPH and ORAC. In contrast, fruit yield was negatively correlated with fruit total polyphenol and total anthocyanin content (Figure 7).

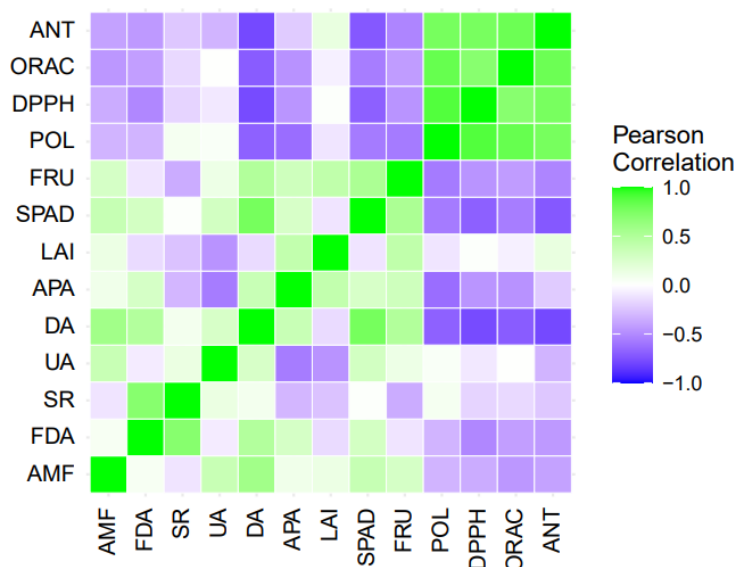


Figure 7. Correlation matrix for soil variables, plant physiological parameters, yield parameters and fruit bioactive compounds. AMF: Arbuscular mycorrhizal fungi; FDA: soil microbiological activity; SR: soil microbial respiration; UA: urease activity; DA: dehydrogenase activity; APA: acid phosphatase activity; LAI: leaf area index; SPAD: chlorophyll index; FRU: fruit yield; POL: total polyphenols; DPPH: DPPH antioxidant capacity; ORAC: ORAC antioxidant capacity; and ANT: total anthocyanins.

Principal component analysis (PCA) (Figure 8a) was performed for 13 parameters: arbuscular mycorrhizal fungi, soil microbiological activity, soil microbial respiration, urease activity, dehydrogenase activity, acid phosphatase activity, LAI, chlorophyll index, fruit yield, total polyphenols, DPPH antioxidant capacity, ORAC antioxidant capacity and total anthocyanins. The principal components PC1 and PC2 retained 44.5% and 18.7%, respectively. This represents all parameters as vectors in the

biplot, while the vector length shows how well-represented the variables are in this plot. The treatments in the PCA (Figure 8b) are represented by the numbers 1–4 for the 0% ET<sub>0</sub>; 5–8 for the treatment with 50% ET<sub>0</sub>; 9–12 for the treatment with 100% ET<sub>0</sub>; and 13–16 for the treatment with 150% ET<sub>0</sub>. These results confirm what was previously indicated in the correlation matrix.

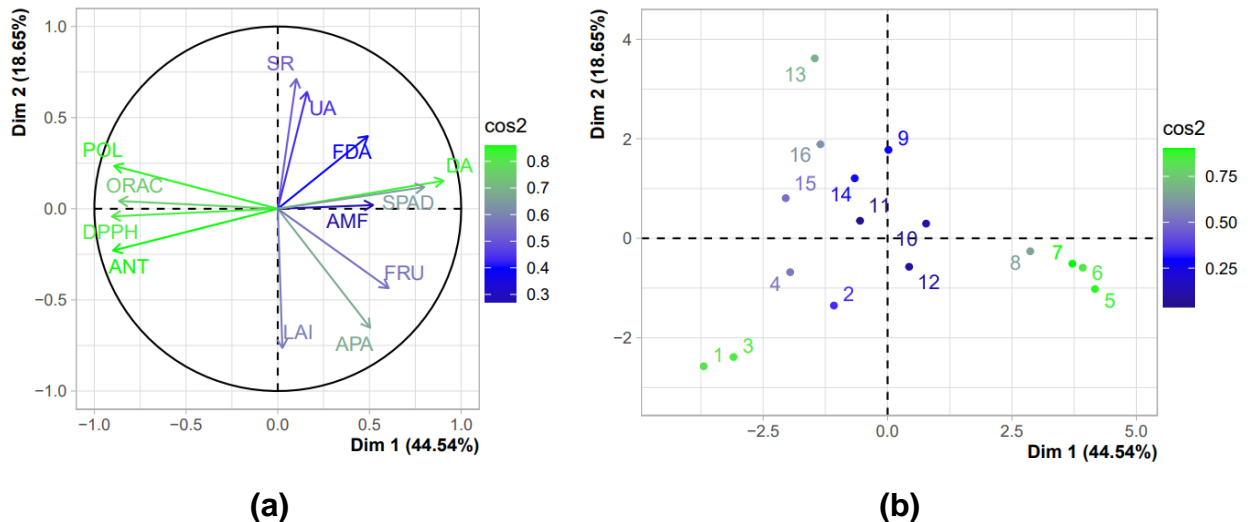


Figure 8. Principal component analysis (PCA) of arbuscular mycorrhizal fungi (AMF); soil microbiological activity (FDA); soil microbial respiration (SR); urease activity (UA); dehydrogenase activity (DA); acid phosphatase activity (APA); leaf area index (LAI); chlorophyll index (SPAD); fruit yield (FRU); total polyphenols (POL); DPPH antioxidant capacity (DPPH); ORAC antioxidant capacity (ORAC); and total anthocyanins (ANT). (a) PCA of variables; (b) PCA of individuals.

## 4. DISCUSSION

### 4.1. Microbiological Parameters of Soil and Roots

Soil microbial communities and their activity define soil quality, as they carry out fundamental processes related to nutrient cycling and biodegradation of soil organic compounds [49]. In the present investigation, soil microbial activity, measured as FDA and soil microbial respiration, was influenced by irrigation dose, with significant increases ( $p < 0.05$ ) in treatments irrigated with 50%, 100% and 150% ET<sub>0</sub>. In

agreement with the present results, previous studies have shown that water deficit through frequent or intermittent drought reduces soil microbial activity and modifies community composition, as microbes focus on synthesizing osmolytes and intracellular maintenance strategies [50]. On the other hand, irrigation increases the availability of organic matter due to release by physical disruption of soil aggregates and/or cell lysis, thus increasing microbial growth and activity [51]. Therefore, we can infer that the irrigation doses that had the greatest effect on soil microbial activity (50% and 100% ET<sub>0</sub>) increased soil water potential, which produced a decrease in the compensatory intracellular solute concentrations of soil microorganisms, accelerating biochemical functions and preventing cytoplasmic desiccation [52]. This is also consistent with what was demonstrated in a grapefruit orchard, where irrigation dose produced changes in the relative abundance of soil microbial populations at the phylum level, where irrigation at 100% ET<sub>0</sub> increased the relative abundance of Firmicutes, while an irrigation corresponding to 50% ET<sub>0</sub> increased the abundance of Proteobacteria [24]. The lower increases in FDA by irrigation at 150% ET<sub>0</sub> indicate that this irrigation dose probably reduces soil aeration [53], since it was shown that soil moisture (%) of the treatment with 150% of ET<sub>0</sub> (Table 2) in the months of maximum demand was up to 35% after 24 h past one irrigation, which, corresponded to half the soil porosity of the study site, which was 0.67% (data not shown). However, the high percentage of organic matter present in the study site soil (9.7%), led to better structural conditions for air circulation and water drainage, reaching soil microbial respiration values up to 50% higher than the non-irrigated treatment [53]. Although our finding has not been previously described in cultivated and wild calafate, we believe it is necessary to evaluate the effects of irrigation dose by analyzing the relative abundance of soil microorganism communities in future research.

Soil microorganisms synthesize and secrete extracellular enzymes, which play an important role in soil nutrient cycling [54]. In our study, the irrigation rates evaluated affected differentially the activity of the quantified enzymes. The observed increase in urease activity with water replenishment of 50% ET<sub>0</sub> could be attributed to a higher soil microbial mass produced under this treatment capable of hydrolyzing soil

organic matter (SOM) (9.7%) containing N [55], with the site soil having ideal conditions by presenting high SOM content with 9.7% and low available N content (19 mg kg<sup>-1</sup>). These results agree with those reported in a Mediterranean forest of *Quercus ilex* L., where increasing the intermediate soil moisture by 10% and 21%, also increased the activity of this enzyme up to 10% and 42%, respectively [56]. We can also infer that the irrigation dose with 150% ET<sub>0</sub> probably led to reduced high soil temperatures during the time of irrigation application, since it has been shown that urease activity not only depends on soil fertility and water availability, but climatic factors such as soil temperature determine the phenology of enzymes such as urease [57]. Dehydrogenase is an extracellular enzyme that plays an important role in the oxidation of organic matter and the incorporation of soil organic C [58]. In our study, dehydrogenase activity was 86% higher with respect to the non-irrigated treatment under 50% ET<sub>0</sub> irrigation. In agreement with the present results, previous studies have shown that soil drought significantly decreases the action of this enzyme [59], resulting in a slowing down of SOM decomposition [60]. In contrast, adequate soil moisture through irrigation significantly increases its activity [60]. Irrigations with higher doses (100 and 150% ET<sub>0</sub>), followed a pattern similar to that of FDA and soil microbial respiration, which is explained by the fact that this enzyme only exists in viable microbial cells and is a good indicator of the global metabolic activity of the soil [59]. Acid phosphatase can hydrolyze organic P to inorganic P compounds [61]. The results obtained show that acid phosphatase activity was slightly higher (14% and 9%) with irrigations of 50% and 100% ET<sub>0</sub> with respect to the treatment without irrigation. It has been shown that a drought condition decreases the activity of this enzyme due to factors such as, lower secretion by soil microbes, lower stabilization efficiency by the smaller SOM, and even, high temperature conditions can denature this enzyme [59]. Strangely, the 150% ET<sub>0</sub> decreased and inhibited the activity of this enzyme. Recently, it has been shown that high-dose irrigation was able to decrease the activity of this enzyme in an almond orchard [25]. However, further work is needed to clarify the dynamics of this enzyme by irrigation dose effect in fruit orchards, especially in recently established species such as calafate.

Associations between roots of dicotyledonous plants and AMF are more common than in the roots of non-mycorrhizal plants. This association has already been reported in some species of the family Berberidaceae, for example, in the species *Berberis vulgaris* L. [62]. This association has also been reported in *Berberis* species from southern Chile, being the percentage of colonization similar to that reported in our study [63]. Although there were nonsignificant differences between irrigation treatments, a higher colonization percentage (20%) was determined for the irrigation treatment with 50% ET0 compared to the non-irrigation treatment. It has been shown that AMF colonization in Mediterranean climates is influenced by soil nutrient fertility such as P [64]. Therefore, we believe that the 50% ET0 irrigation dose, would increase somewhat the colonization capacity of these microorganisms by presenting a soil with better structural conditions [53] and P fertility mediated by acid phosphatase [61], leading to competitive exclusion of other soil microorganisms [64]. However, we recognize that our results may be somewhat limited by measuring only a single parameter corresponding to AMF colonization. Further work is needed to taxonomically characterize AMF using molecular techniques to identify their diversity and population structure in order to better understand the ecological impacts of irrigation dose on the calafate crop.

#### 4.2. Plant Physiological Parameters

Irrigation dose influenced the indirect physiological parameters measured in this study. The maximum quantum yield of photosystem II had a decreasing trend as the day progressed. This is consistent with that reported in a calafate orchard in south-central Chile under the same radiation conditions, which reached maximum direct photosynthetically active radiation values close to  $2000 \mu\text{mol m}^{-2} \text{s}^{-1}$ . Research has also shown that the irrigation treatment with 50% ET0 recovers earlier and with higher values of Fv/Fm its photosynthetic apparatus with respect to the treatment without irrigation. This increase could be attributed to better physiological and nutritional conditions of the plant to cope with abiotic stress factors such as high levels of radiation and temperature that can generate photooxidative damage to the leaves

[65]. These results also corroborate the findings of Retamal-Salgado et al. [38], who demonstrated that high ambient radiation and temperature can negatively affect the efficiency of the photosynthetic apparatus in blueberries. Another important finding in the present investigation is that 50% and 100% ET<sub>0</sub> treatments positively influenced leaf stomatal conductance, presenting higher values at the beginning of the day. Plant irrigation has been shown to drastically influence growth, development, and physiological characteristics of photosynthesis [66]. Unlike these treatments, the 150% ET<sub>0</sub> treatment did not respond in the same way, which may be due to oxygen depletion in the root zone affecting physiological functions of the plants, since, as shown in Table 2, this treatment after 24 and 48 h of irrigation showed higher soil moisture than the other treatments. In blueberries of the cultivars highbush and rabbiteye, it was shown that irrigation doses above 100% of ET<sub>0</sub> are capable of producing oxygen depletion in the root zone, leading to a decrease in stomatal conductance and photosynthesis, in addition, they determined, for both processes to recover to optimal conditions reached prior to irrigation, long periods of up to 18 days or more were required [67] which does not occur in our study, being the period between irrigations of two days. Therefore, we believe that in responses to stress conditions (Treatments 0% and 150% ET<sub>0</sub>) gas exchange may decrease and even close their stomata to avoid water loss [68]. This is consistent with that reported in a rabbit's eye blueberry orchard [68].

Irrigation rate affected leaf chlorophyll content ( $p < 0.05$ ). Irrigation treatments increased chlorophyll levels in leaves compared to the non-irrigation treatment. Irrigation of 50% ET<sub>0</sub>, had greater increases in leaf chlorophyll. Soil moisture has been shown to alter plant physiological response by improving soil physicochemical conditions for plant nutrient transport and utilization and increasing chlorophyll content to promote photosynthesis and respiration [68]. We can also infer that stressed plants with lower chlorophyll values have less photosynthesis and respiration, which is corroborated by the previously discussed data of photosystem II maximum quantum yield and stomatal conductance. The leaf area index is an indirect indicator of the physiological state of the plant; which in our case did not respond to

the irrigation dose. These results corroborate, but extend, the findings of much of the previous work on wild calafate [65,69,70] that demonstrate the hardiness of the plant and its high degree of phenotypic adaptation. Consequently, this is the first time that this approach has been proposed to determine the influence of irrigation dose on the canopy of domesticated calafate. This can be considered a significant advance in its level of adaptation to drought conditions and excessive soil moisture.

#### 4.3. Fruit Yield and Chemical Parameters

The effect of irrigation dose has been studied in different fruit species because of its importance in plant productivity [14]. Irrigation at 50% ET<sub>0</sub> produced the highest fruit yield compared to the other treatments ( $p < 0.05$ ). This is consistent with the literature, which reported similar yields under the same temperature conditions, mean 14.5 °C and maximum 31 °C, and Andisol origin, as well as the same harvest date defined at 130 days after full flowering [6]. In blueberries, when evaluating different irrigation doses, it was shown that an intermediate irrigation dose of 100% ET<sub>0</sub> produced significant increases in different productive parameters such as fruit weight, number of fruits per plant and fruit yield, because the plants presented less stress [67], a situation similar to that of the 50% ET<sub>0</sub> treatment in our research. In our study, the yield of plants irrigated with a dose of 0%, 100% and 150% ET<sub>0</sub> was lower than the treatment of 50% ET<sub>0</sub> with nonsignificant differences between them (Figure 6a). Consistent with the present results, previous studies have shown that there are significant reductions in fruit yield in some berries due to severe drought conditions [71] and excessive irrigation [72] that cause stress on the plant by inducing stomatal closure, decreased CO<sub>2</sub> assimilation, decreased root activity and increased prevalence to fungal diseases [70]. However, our results show that calafate plants under different degrees of water stress with doses of 0% and 150% ET<sub>0</sub>, are able to fruit similarly, which corroborates its high hardiness described for different climatic conditions with contrasting rainfall levels [7]. According to our results (Figure 6a), it was shown that the irrigation treatment with 0% of ET<sub>0</sub> did not present significant statistical differences with the treatments 100% and 150% of ET<sub>0</sub>, which

demonstrates that the water requirements of calafate to generate its physiological functions and fruiting are low. Our results are corroborated by Radice et al. [7] who demonstrated that non-irrigated cauliflower in the region of Moreno, Argentina, with warm temperate climates similar to those of our study were able to fruit, however, the lower water regimes of Moreno compared to Ushuaia, Argentina, produced smaller fruit size. Therefore, we emphasize the importance of evaluating physical parameters of the fruit in future research. Furthermore, to our knowledge, this is the first study published to date that demonstrates the effects of irrigation dose on the productivity of cultivated calafate. However, we recognize that our results may be somewhat limited by seasonality, because only one season was evaluated and a single productivity parameter was determined.

Phenolic compounds confer the particular characteristics of color and aroma in calafate and other berries such as blueberries, raspberries, and strawberries [67], with anthocyanins being responsible for defining color during the veraison stage until harvest [8]. Higher concentrations of polyphenols and anthocyanins were observed in the most stressed treatments of the present study, both due to water deficiency and excess water replenishment. Previous studies have shown a high accumulation of anthocyanins influenced by abiotic factors such as water stress [73], which is part of the protective mechanism of plants to cope with oxidative stress through the uptake of reactive oxygen species [74]. For their part, Bryla et al. [72] demonstrated similar stress effects in blueberry plants by high doses of water replenishment. The decrease in the concentrations of polyphenolic compounds observed in the 50% ET<sub>0</sub> treatment could be attributed to the higher fruit yield, which may result in larger caliber and delayed physiological maturity of the fruit [71], which could have diluted the content of bioactive compounds in the fruit due to increased water content. In this sense, a study on *Vitis vinifera* L. showed that the irrigation dose increased fruit yield but decreased the content of polyphenolic compounds [75].

Research has also shown that the predominant anthocyanins were delphinidin, petunidin and malvidin and their derivatives, similar to that reported by Romero-Román et al. [8] in wild calafate from southern Chile. This is also consistent with that

reported in grapes, where the three glycoside-conjugated anthocyanins are the majority due to the temperature and pH of the berry extract [76]. Delphinidin represented between 35% and 41% of the total anthocyanins, being lower with the treatment without water replenishment. This decrease could be attributed to the low stability of anthocyanins to external factors such as light and temperature, which were probably more intense when there was no irrigation, a situation similar to that reported by Torres et al. [77], who demonstrated in grapes that water stress decreased pH of the fruit and, as a result, the fruit turned reddish instead of purple. The results of total delphinidins and anthocyanins reported in this study are lower than those previously reported in wild calafate [8]. This could be explained by differences in the quality of solar radiation in both agroclimatic regions [78], since in the study region it reaches a value of photosynthetically active radiation 26% higher in southern Chile [6]. Samkumar et al. [79] found that red light produced a higher induction of specific anthocyanin and delphinidin biosynthesis genes. We can also infer that the time of harvest is a determining factor in the accumulation of anthocyanins by fruit ripening [80] being in our case at 130 days after full flowering during December. Our delphinidin results are lower than those (80%) reported in maqui (*Aristotelia chilensis* (Mol.) Stuntz), which is another Chilean native berry species with high anthocyanin content in fruit [80] but which are similar to the delphinidin accumulation of 40% reported in blueberries [81]. Another important finding in the present investigation is that total antioxidant activity was influenced by irrigation dose, being higher in treatments of 0% and 150% ET<sub>0</sub>. These values are similar to those reported in previous research under wild conditions [8,82]. However, irrigation doses with 50% and 100% ET<sub>0</sub> showed lower values of fruit antioxidant capacity but similar to those reported in cultivated calafate from central-southern Chile [6]. The anthocyanin content may explain the total antioxidant activity observed in the fruit ( $r = 0.89$ ), a situation similar to that previously reported in maqui from central-southern Chile [80]. We can highlight that the irrigation treatment of 50% ET<sub>0</sub>, although it presented a fruit yield 86% higher than the irrigation treatment of 100% ET<sub>0</sub>, it maintained its quality, by presenting a significantly similar value of total

anthocyanins and ORAC antioxidant capacity. To our knowledge, this is the first report on the effects of irrigation on anthocyanins and their antioxidant capacity in cultivated calafate fruit.

#### 4.4. Influence of Variables

The correlation matrix reaffirmed the importance of evaluating water replenishment management in a soil-plant system and chemical compound content of calafate fruit. Although moderate correlations were found between soil microbiological parameters and two plant physiological parameters measured in the correlation matrix, a more significant positive correlation ( $r = 0.78$ ) was found between dehydrogenase and plant chlorophyll index (Figure 7). It has been previously reported that dehydrogenase plays an important role in the incorporation of soil organic C under soil water replenishment treatments that directly affects the physiological state of the plant through its nutrition [58]. Likewise, dehydrogenase was one of the most sensitive enzymes to irrigation on fruit chemical composition, demonstrated by its high negative correlation with parameters such as total anthocyanins ( $r = -0.79$ ), total polyphenols ( $r = -0.69$ ), antioxidant capacity DPPH ( $r = -0.78$ ) and ORAC ( $r = -0.71$ ). On the other hand, FDA and AMF colonization were directly and negatively correlated with the evaluated fruit chemical parameters (Figure 7). Recently, a study in almond trees showed that the higher soil microbial activity produced by an optimal irrigation dose negatively affects fruit chemical compounds [24], which could be explained by the higher yield and fruit size resulting from higher values of measured soil microbial activity and plant physiological parameters. Another important finding is the fact that irrigation directly influences the content of bioactive compounds in the fruit. The high relationship between the bioactive compounds of the fruit corroborate the above, for example, there was a high positive correlation between total polyphenols and antioxidant capacity measured through different modes of action, DPPH ( $r = 0.89$ ) and ORAC ( $r = 0.83$ ), which agrees with what has been previously reported in maqui from central-southern Chile [80]. The principal component analysis of the variables

(Figure 8a) corroborates the above, since there is a greater closeness in distance and colors between the soil, plant and fruit variables mentioned above.

Through the principal component analysis of the variables (Figure 8a,b), the research shows that the most stressed treatments (0% and 150% ET<sub>0</sub>) are the ones that presented a positive correlation with the bioactive compounds of the fruit, but not the treatments of 50% and 100% ET<sub>0</sub>, which presented a positive correlation with the microbiological and enzymatic activities of the soil and physiological and productive activities of the plant. These results can be considered a significant advance in the domestication of calafate through the irrigation dose, capable of influencing the soil microbiological communities, which are closely related to the plant physiology and its productivity of fruits and/or bioactive compounds. Furthermore, to our knowledge, this is the first study published to date that demonstrates the influence of irrigation on soil microbiology, the calafate plant, and its productivity.

## **5. CONCLUSIONS**

Our study was able to demonstrate that irrigation of 50% ET<sub>0</sub> significantly increased urease, dehydrogenase and soil acid phosphatase, which led to greater physiological response of the plant with higher values of stomatal conductance and chlorophyll index. This also led to a significant increase in fruit production, which maintained an adequate level of total anthocyanins and ORAC antioxidant capacity, being similar to the 100% ET<sub>0</sub> irrigation. On the contrary, 0% and 150% ET<sub>0</sub> treatments showed the lowest values in the mentioned parameters, but higher values on total anthocyanins and antioxidant capacity of the fruit. Based on these results, we can conclude that although the calafate plant has a high adaptability to extreme conditions of water replenishment such as drought and over-watering, it shows better results at soil-plant and fruit level with an irrigation of 50% ET<sub>0</sub>, allowing the optimization of water resources and thus contributing efficiently to food security. On the other hand, as calafate is a very rustic species, we propose to evaluate in future research suboptimal irrigation doses between 0% and 50% ET<sub>0</sub>, to further improve water efficiency and

evaluate the chemical composition of the fruit. We emphasize soil microbiological evaluations should not be neglected, to better understand their contribution to the adaptability of this species under commercial orchards.

### **Author Contributions**

Conceptualization, J.R.-S. and M.S.; methodology, J.R.-S., M.S., M.B. and R.V.-R.; software, M.B. and M.D.L.; validation, J.R.-S., M.S. and M.D.L.; formal analysis, J.R.-S. and M.B.; investigation, M.B., M.S. and J.R.-S.; resources, M.S., M.D.L. and J.R.-S.; data curation, M.D.L., J.R.-S. and M.B.; writing—original draft preparation, M.B., M.S., J.R.-S., R.V.-R. and M.D.L.; writing—review and editing, J.R.-S., M.S., M.D.L. and R.V.-R.; project administration, M.S. and J.R.-S.; funding acquisition, M.S., J.R.-S., R.V.-R. and M.D.L. All authors have read and agreed to the published version of the manuscript.

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### **Institutional Review Board Statement**

Not applicable.

### **Informed Consent Statement**

Not applicable.

### **Data Availability Statement**

The data presented in this study are available in the article.

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## Conflicts of Interest

The authors declare no conflict of interest.

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### III. CAPÍTULO II: NOVEL APPROACH TO ORGANIC MULCHING FROM NATURAL-BASED SOLUTIONS TO ENHANCE SOIL HEALTH AND FUNCTIONAL VALUE OF CALAFATE FRUIT

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#### **ABSTRACT**

Mulching suppresses weeds, improves soil biology, and increases physical or bioactive fruit yield in fruit orchards. However, there is no information on its impact on calafate (*Berberis microphylla* G. Forst.) orchards, which produce berries with high antioxidant content. To address this gap, in 2021, an experiment was conducted to evaluate the effect of 5 years of mulching on soil, plants, and calafate fruit. Four mulching treatments were established: no mulch (control), geotextile, oat straw, and hazelnut shell. All mulches suppressed weeds (43%) and maintained more soil

moisture (5%) than the control. Soil microbial activity increased only with hazelnut shell compared with the control, up to 46%. Only oat straw and hazelnut shell increased basal respiration and urease up to 31% and 15% more than the control. Oat straw produced the highest fruit yield with 0.44 t ha<sup>-1</sup>, while the lowest yield was produced by the control and hazelnut shell with 0.1 and 0.15 t ha<sup>-1</sup>, respectively. The geotextile with 0.35 t ha<sup>-1</sup> of fruit produced no differences between treatments. The ORAC antioxidant capacity was only higher in the control and hazelnut shell, with a mean of 3272 μmol TE 100 g<sup>-1</sup>. Hazelnut shell mulch is recommended to improve the biological functions of the soil and the antioxidant capacity of the calafate fruit.

**Keywords:** Berberis; soil respiration; polyphenols; antioxidants; urease

## 1. INTRODUCTION

A widely accepted practice in fruit orchards is the use of ground covers for their efficient physical suppression of weeds and inhibition of seed germination [1] and for their ability to replace herbicides [2]. In soil, the use of certain types of mulch favors biodiversity and the activity of microbial communities, which contribute to plant nutrition and productivity [3]. However, the composition of the mulch must be carefully evaluated. Plastic mulch significantly enhances the enzymatic activities of alkaline phosphatase, invertase, catalase, and urease, as well as the carbon (C) and nitrogen (N) contents of soil microbial biomass [4]. Synthetic mulches of polyethylene and other black synthetic polymers can absorb solar radiation by emitting energy in the form of heat into the soil [5], which also leads to a decrease in soil biological activity [6].

Mulching, implemented in berry species such as grapevine, blueberries, or strawberries, modifies the soil environment, influencing plant growth, development, and aspects of berry quality, such as concentrations of sugars, anthocyanins, and total polyphenols [7,8]. Taparauskienė and Miseckaitė [9] showed that black plastic mulch improved fruit yield by 60% compared with soil without mulch and by 56%

compared with soil with wheat straw mulch in a strawberry orchard due to an increase in soil temperature. In contrast, when a combination of rice straw mulching with an irrigation strategy was applied in a grapevine orchard, there was an increase in berry diameter, fruit yield, water use efficiency, and berry sugar concentration by 2.8 mm, 271.5 g tree<sup>-1</sup>, 33% and 15%, respectively, compared with treatment without mulching and irrigation [10]. In another case, compost mulching increased soil C and N content by 50%, and straw mulching suppressed weeds by 90% and increased soil moisture by 5% compared with soil without mulching but induced smaller changes in fruit yield in an apple orchard [11].

Chen et al. [12] demonstrated that mulching with corn stover significantly increased soil bacterial communities because of the contribution of organic matter (OM) and improvements in soil physicochemical conditions caused by this treatment in an apple orchard. Similarly, mulching with sawdust, corn straw, and wild grass improved the richness and diversity of soil bacterial and fungal communities, as well as the stability of the edaphic ecosystem, in response to changes in soil pH, phosphorus (P), C, and C:N ratios [13]. For this reason, organic mulches are often preferred, innovating with materials high in lignin and of greater durability, coming from industrial wastes, such as the timber industry [14]. In this context, the nut industry in Chile, such as hazelnut production for export, is increasing and currently has 36,000 ha planted [15], which produces high amounts of hazelnut shells as waste, with potential use as mulch due to its woody composition.

In the last decade, it has been determined that the calafate (*Berberis microphylla* G. Forst.) berry has a high concentration of phenols, higher than fruits such as oranges, blueberries, and strawberries [16,17] and that it substantially reduces the presence of degenerative, cardiovascular and carcinogenic diseases [18]. In addition, it has a higher sugar content (14.6%) compared with other fruits, such as cherry (9.9%), strawberry (5.5%), blueberry (6%), and raspberry (4.8%) [19]. The production of calafate fruit in Chile is mainly wild, having a cultivated area in Southern Chile of only 0.24 ha [15]. However, in the Ñuble Region, Central Southern Chile, an orchard was established in 2017 with the purpose of evaluating its response

to different agronomic managements, such as organic fertilization and hydric replenishment. Pinto-Morales et al. [20] demonstrated that fertilization with compost at a dose of 10 t ha<sup>-1</sup> significantly improved the yield and DPPH antioxidant capacity of the fruit by 100% and 20%, respectively, compared with a plant without fertilization. On the other hand, Betancur et al. [21] demonstrated that it is possible to reduce water replenishment from 100% to 50% of the reference evapotranspiration (ET<sub>0</sub>), improving soil microbial activity by 38% and fruit production by 86%. Despite these initial advances, other aspects of agronomic management—such as sustainable weed control, which reduces environmental pollution and contributes to better soil health—require further research in calafate orchards.

Despite this background, there is no evidence of the effects of mulching on the soil, plant, and fruit of the calafate orchard. Therefore, the purpose of this study is to investigate the impacts on soil microbiological parameters, physiological aspects of the plant, and physicochemical aspects of the calafate fruit using different types of mulches, highlighting the geotextile, widely used in agriculture, as well as organic mulches from organic residues, such as hazelnut shells and oat straw. This research contributes to the development of sustainable management practices by using agricultural residues, with the purpose of establishing future organic calafate orchards.

## **2. MATERIALS AND METHODS**

### **2.1. Agronomic Management of the Orchard and Soil and Climatic Conditions**

The establishment of the orchard was carried out in 2017 at the experimental station of the Adventist University of Chile located in the Ñuble Region, Chile (36°31' S; 71°54' W). Agronomic management was equally standardized for all treatments, according to Pinto-Morales et al. [20]. Water replenishment was carried out annually from October to April according to the criteria proposed by Betancur et al. [21]. The orchard (Figure 1a) has 352 plants (Figure 1b) that are established 1 m above the row and 3 m between rows.



(a) (b)  
Figure 1. (a) Establishment of different mulch treatments in the calafate orchard, 2017; (b) flowering calafate plant with oat straw mulch, 2019.

The soil of the calafate orchard was Andisol order (Melanoxerand) [22] and was characterized by chemical soil analysis at the time of establishment. The results of soil chemical analysis (0–20 cm) are shown in Supplementary S1.

The climate of the Ñuble Region is temperate Mediterranean. For the growing seasons 2020–2021 and 2021–2022, the mean annual temperatures were 13.5 and 13.2 °C, respectively, and accumulated precipitation was 920 mm and 650 mm, respectively (accumulated monthly temperatures and precipitation for 2021 and 2022 in the Ñuble Region can be found in Supplementary S2).

## 2.2. Experimental Setup

In 2017, four treatments were established: a control treatment without mulch (CK), geotextile mulch (GEO), oat straw mulch (OAT), and hazelnut shell mulch (HAZ). The mulches of organic origin, oat straw and hazelnut shell (chemical analysis of oat straw and hazelnut shell mulches, shown in Supplementary S3), were applied to the soil with a thickness of 10 cm and a coverage area of 1 m radius around each plant. HAZ

was applied only once at initiation, GEO was installed only once at initiation, and OAT was renewed on an annual basis.

The ground cover model geotextile mulch (Polytex, Santiago, Chile) was composed of black polypropylene with 98% absence of light and 100  $\mu\text{m}$  thickness. The statistical design used was a randomized complete block design with four treatments and four replicates ( $n = 16$ ). In turn, each replicate consisted of the average of two plants that were evaluated independently. The results obtained from the two plants were averaged to obtain each replicate for each treatment.

### 2.3. Weeds, Soil Moisture, Temperature of Mulch, and Leaves

The dry weight of weeds was determined by weighing those growing at a 1 m radius from each plant, and the weeds were subsequently dried in a BOV-VF Series forced air oven (BIOBASE, Jinan, China) at 105  $^{\circ}\text{C}$  for 72 h.

Volumetric soil water content (%) was determined by averaging three measurements taken 1 d after each irrigation during December 2021, at a depth of 0.2 m with a Diviner 2000 portable soil moisture probe (Sentek, Stepney, Australia).

Leaf and mulch surface temperature ( $^{\circ}\text{C}$ ) was determined with a portable infrared thermometer CTR1000 (Instrumentos WIKA S.A.U., Barcelona, Spain) at four times of the day (09:00, 12:00, 15:00, and 18:00 h) in December 2021.

### 2.4. Microbiological and Chemical Soil Analysis

Soil samples for microbiological and chemical analyses were collected on 21 October at a depth of 0–20 cm [21]. Samples were stored at  $-20^{\circ}\text{C}$  for analysis and were subsequently sieved at 2 mm and conditioned to 60% field capacity. For soil microbiological analyses, four technical replicates per treatment and replicate were used.

Fluorescein diacetate (FDA) activity [23] and basal soil respiration [24] were estimated, expressed as  $\mu\text{g FDA g}^{-1}$  and  $\mu\text{g CO}_2 \text{ g}^{-1} \text{ h}^{-1}$ , respectively. Urease [25], dehydrogenase [26], and acid phosphatase [27] enzyme activities were estimated. Urease and acid phosphatase were expressed as  $\mu\text{mol ammonium (NH}_4^+)$  and p-

nitrophenol (PNP) per gram of soil (dry weight) per hour (h), respectively. In contrast, dehydrogenase was expressed as  $\mu\text{g}$  idonitrotetrazoliumformazan (INTF) per gram of soil (dry weight).

## 2.5. Plant Physiological Measurements

Physiological measurements were made on 12 January 2021, using as criteria plants and leaves exposed to the sun and from the second third of the season's shoot.

Leaf area index (LAI;  $\text{m}^2 \text{m}^{-2}$ ) was measured at 12:00 h, with an AccuPAR LP-80 ceptometer (Decagon Devices Inc., Washington, DC, USA).

The maximum quantum yield of photosystem II (Fv/Fm) and stomatal conductance ( $g_s$ ,  $\text{mmol m}^{-2} \text{s}^{-1}$ ) were measured at four times of the day (09:00, 12:00, 15:00, and 18:00 h), with a portable fluorometer OS-5p (Opti-Sciences, Hudson, NH, USA), adapting the leaves to darkness for 30 min and with a portable porometer equipment model SC-1 (Decagon Devices, Washington, DC, USA), respectively [28,29].

## 2.6. Yield and Physical Fruit Measurements

Yield and physical parameters of fruit were measured immediately after harvest on 30 December 2021, by weighing total fruit per plant and individually ( $n = 10$ ) with a Precisa Gravimetrics 360 ES Series analytical balance (Precisa Gravimetrics AG, Dietikon, Switzerland) and measuring polar and equatorial diameter (mm) ( $n = 10$ ) with a digital meter foot model E5001002  $\pm 0.003$  mm (Veto Y Cia Ltd., Santiago, Chile).

## 2.7. Soluble Solids and Antioxidant Properties of the Fruit

The concentration of soluble solids ( $^{\circ}\text{Brix}$ ) and antioxidant properties of the fruit were evaluated after harvest [21]. For the chemical analysis of the fruit, four technical replicates per treatment and repetition were used.

Soluble solids concentration ( $^{\circ}\text{Brix}$ ) was measured by extracting a random sample of fresh fruit ( $n = 4$ ) from each total yield per plant, which was squeezed to

obtain two drops of liquid. The liquid was measured in a digital refractometer model HI96801  $\pm$  0.2% (Hanna Instruments S.R.L., Woonsocket, RI, USA). Total polyphenol content was measured using the Folin–Ciocalteu reagent, as described by [30]. The absorbance was measured at 760 nm and the results were expressed as mg gallic acid 100 g<sup>-1</sup> FW [31]. The antioxidant capacity of DPPH was performed as suggested by Romero-Román et al. [32]. The absorbance readings were performed at 515 nm. ORAC antioxidant capacity was determined as suggested by Romero-Román et al. [33] with excitation and emission wavelengths of 485 and 520 nm. The results of fruit antioxidant capacity were expressed as  $\mu$ mol Trolox equivalent (TE) per 100 g fresh fruit (FW) [33,34]. Individual anthocyanins were quantified with high-performance liquid chromatography (HPLC-DAD), as suggested by Romero-Román et al. [33], and the results were expressed as mg 100 g<sup>-1</sup> FW [34].

## 2.8. Statistical Analysis

The results were subjected to analysis of variance (ANOVA), where the comparison of means was performed with Fisher's least significant difference (LSD) test with a significance of 0.05. Principal component analysis (PCA) and analysis of correlations between soil–plant–fruit variables were performed, and the data were processed with R Studio software 4.2.1 [35] using the packages FactoMineR and ggplot2 [36], focusing on the mean based on eigenvalues.

## 3. RESULTS

### 3.1. Weeds, Soil Moisture and Temperature, and Leaf Temperature

Weed weight (Figure 2a) was 43% higher ( $p < 0.05$ ) in the control treatment (CK) with respect to the other treatments, with no differences between them ( $p > 0.05$ ). The main weeds were monocotyledonous weeds of the Poaceae family, such as *Paspalum vaginatum* Sw. and *Avena fatua* L., and, to a lesser extent, dicotyledonous weeds of the Asteraceae and Boraginaceae families, such as *Centaurea solstitialis* L. and *Echium vulgare* L., respectively.

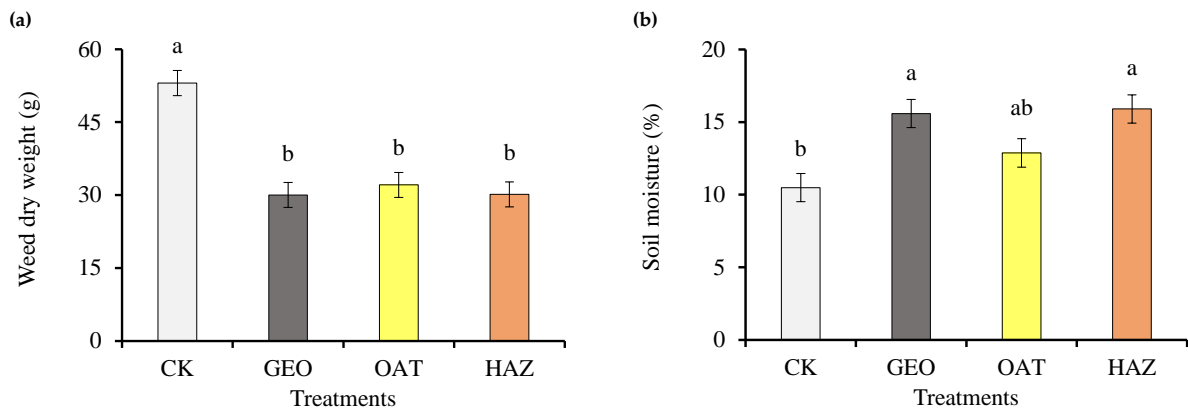


Figure 2. (a) Measurement of weed dry weight (g) harvested from different mulch treatments. (b) Measurement of soil moisture (%) from different mulch treatments. Treatments: CK: no mulch, GEO: geotextile mulch, OAT: oat straw mulch, and HAZ: hazelnut shell mulch. Different lowercase letters indicate significant differences between treatments according to Fischer's LSD test ( $p < 0.05$ ). Mean  $\pm$  standard error ( $n = 4$ ). Bars correspond to experimental error for each treatment.

Soil moisture (Figure 2b) had a significant effect ( $p < 0.05$ ) with GEO and HAZ treatments at 15.8%, compared with CK at 10.5%. However, no significant difference was observed in OAT treatments.

Leaf temperature (Figure 3a) did not show significant differences among treatments ( $p > 0.05$ ) at different times of the day but increased to 40 °C at 15:00 h and decreased slightly to 38 °C at 18:00 h. Mulch temperature (Figure 3b) increased significantly ( $p < 0.05$ ) with GEO treatment compared with the other treatments, reaching 54 and 61 °C at 12:00 and 18:00 h, respectively. On the other hand, the CK treatment generally presented the lowest temperature at different measurement hours with a minimum value of 26 °C at 09:00 h.

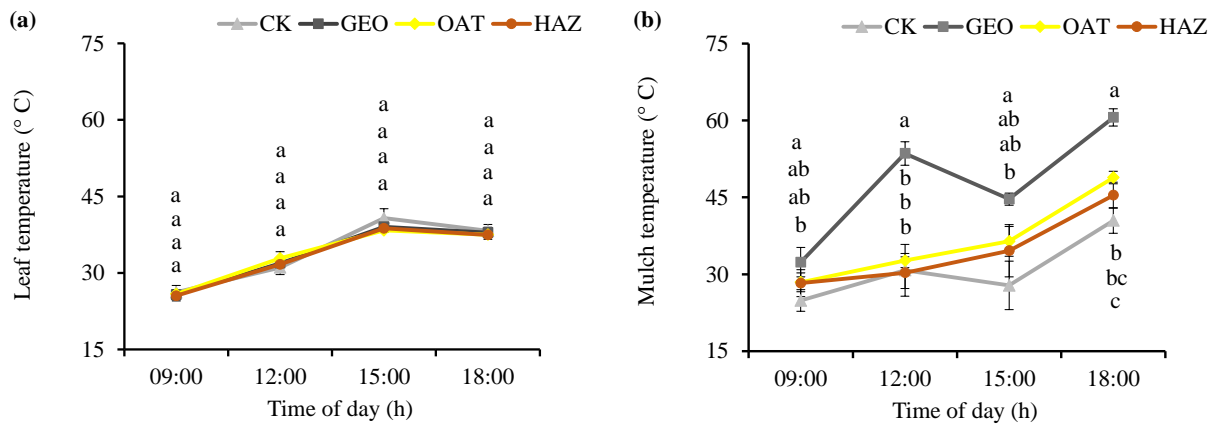


Figure 3. Measurement of calafate leaf temperature (°C) at four times of the day: 09:00, 12:00, 15:00, and 18:00 h. (a) Measurement of mulch surface temperature (°C) at four times of the day: 09:00, 12:00, 15:00, and 18:00 h. (b) Measurement of mulch surface temperature (°C) at four times of the day: 09:00, 12:00, 15:00, and 18:00 h. Treatments: CK: no mulch, GEO: geotextile mulch, OAT: oat straw mulch, and HAZ: hazelnut shell mulch. Different lowercase letters indicate significant differences between treatments according to Fischer's LSD test ( $p < 0.05$ ). Mean  $\pm$  standard error ( $n = 4$ ). The bars correspond to the experimental error for each treatment.

### 3.2. Soil Microbiological and Chemical Parameters

Soil microbial activity (Table 1) increased significantly ( $p < 0.05$ ) with HAZ treatment, and was 40% higher than that of CK. Likewise, basal soil respiration (Table 1) increased significantly with HAZ with respect to the other treatments, reaching  $1.9 \mu\text{g CO}_2 \text{g}^{-1} \text{h}^{-1}$ . GEO presented the lowest respiration with  $1.0 \mu\text{g CO}_2 \text{g}^{-1} \text{h}^{-1}$ .

Table 1. Soil microbiological properties and enzyme activity in response to mulch.

Treatments	Microbial activity ( $\mu\text{g FDA g}^{-1}$ )	Soil basal respiration ( $\mu\text{g CO}_2 \text{ g}^{-1} \text{ h}^{-1}$ )	Urease activity ( $\mu\text{mol NH}_4^+ \text{ g}^{-1} \text{ h}^{-1}$ )	Dehydrogenase activity ( $\mu\text{g INTF g}^{-1}$ )	Acid phosphatase activity ( $\mu\text{mol PNP g}^{-1} \text{ h}^{-1}$ )
<b>CK</b>	33.38 $\pm$ 3.20 b	1.32 $\pm$ 0.10 bc	1.25 $\pm$ 0.04 c	36.36 $\pm$ 6.10 a	27.29 $\pm$ 2.36 a
<b>GEO</b>	41.42 $\pm$ 7.31 ab	0.98 $\pm$ 0.16 c	1.34 $\pm$ 0.01 bc	38.96 $\pm$ 3.95 a	17.57 $\pm$ 2.03 b
<b>OAT</b>	49.75 $\pm$ 3.71 ab	1.46 $\pm$ 0.15 b	1.44 $\pm$ 0.04 ab	32.36 $\pm$ 2.60 a	17.28 $\pm$ 1.89 b
<b>HAZ</b>	55.75 $\pm$ 7.99 a	1.90 $\pm$ 0.15 a	1.48 $\pm$ 0.05 a	29.16 $\pm$ 1.39 a	19.66 $\pm$ 2.10 b
<b>Anova p values</b>	0.0095	0.0053	0.0063	0.3462	0.0181

Treatments: CK: no mulch, GEO: geotextile mulch, OAT: oat straw mulch, and HAZ: hazelnut shell mulch. Different lowercase letters indicate significant differences between treatments according to Fischer's LSD test ( $p < 0.05$ ). Mean  $\pm$  standard error ( $n = 4$ ). FDA: Fluorescein diacetate activity, INFT: Iodonitrotetrazoliumformazan, PNP: p-nitrophenol.

Soil enzyme activity showed different responses to mulching treatments. In this sense, urease activity increased significantly in HAZ treatment by 20% and 13% compared with CK and GEO, respectively. Dehydrogenase activity with a mean of 34.21  $\mu\text{g INTF g}^{-1}$  showed no differences among treatments. Acid phosphatase activity was significantly higher in the CK treatment and was 33% higher than that of the mulch treatments.

Chemical analysis of the soils at the end of the experiment compared with the beginning of the study indicated that MO increased by 2.8% with the OAT treatment. Chemical analysis of the soil (0–20 cm) affected by the compost treatments at the end of the study is shown in Supplementary S4. The pH with mulch treatments (GEO, OAT, and HAZ) increased by an average of 2.3% compared with CK. There was 46% higher N availability in the CK treatment compared with the GEO and HAZ treatments. The availability of S and exchangeable Ca reached the highest value in GEO with 34.5  $\text{mg kg}^{-1}$  and 9.19  $\text{cmol}^+ \text{kg}^{-1}$ , respectively. The K availability was 31.9% higher in OAT than in CK. In all treatments, the availability of micronutrients such as Zn, Fe, and Mn tended to decrease toward the end of the study compared

with the beginning of the study. There was, on average, 31.5% and 28.5% higher availability of Cu and Mn, respectively, with the mulched treatments (GEO, OAT, and HAZ) compared with the control.

### 3.3. Plant Physiological Parameters

The leaf area index (LAI) (Figure 4) with a mean value of  $1.6 \text{ m}^2 \text{ m}^{-2}$  did not show significant differences between treatments ( $p > 0.05$ ).

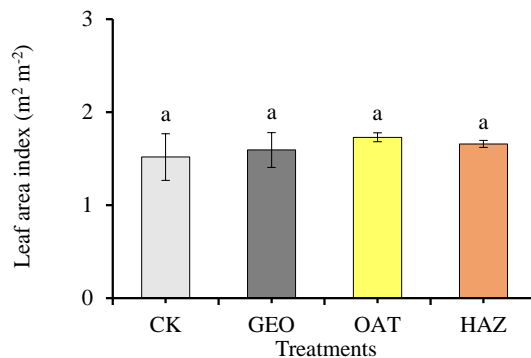


Figure 4. Leaf area index in calafate plants. Treatments: CK: no mulch, GEO: geotextile mulch, OAT: oat straw mulch, and HAZ: hazelnut shell mulch. Different lowercase letters indicate significant differences between treatments according to Fischer's LSD test ( $p < 0.05$ ). Mean  $\pm$  standard error ( $n = 4$ ). The bars correspond to the experimental error for each treatment.

The maximum quantum yield of photosystem II (Fv/Fm) (Figure 5a) did not show significant differences ( $p > 0.05$ ) among treatments, with a decreasing trend as the day progressed until an average value of 0.75 at 18:00 h. Stomatal conductance (Figure 5b) did not show significant differences between treatments. The average values of stomatal conductance were 180, 139, 128, and 117  $\text{mmol m}^{-2} \text{ s}^{-1}$  at 09:00, 12:00, 15:00, and 18:00 h, respectively.

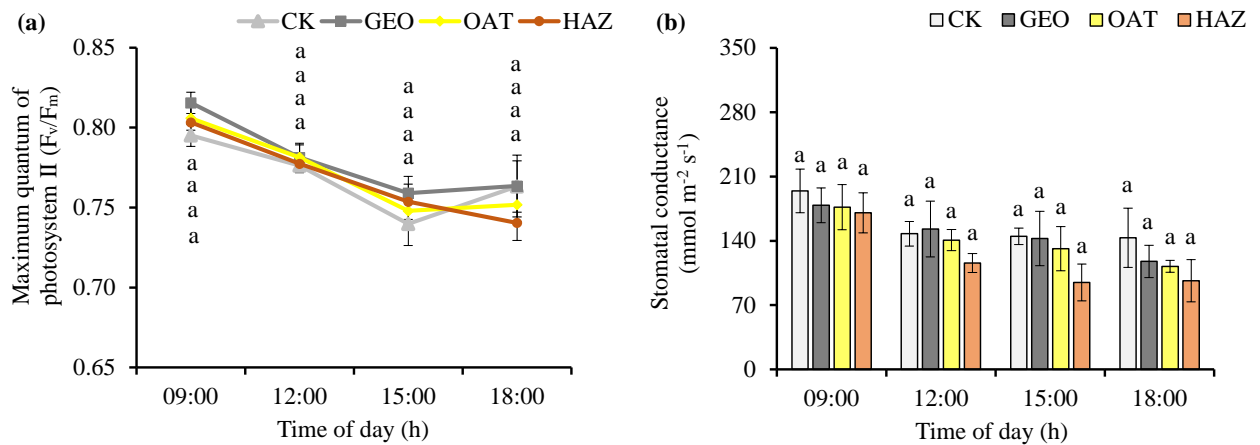


Figure 5. Variation of maximum quantum yield of photosystem II ( $F_v/F_m$ ) (a) and values recorded for stomatal conductance ( $\text{mmol m}^{-2} \text{s}^{-1}$ ) in calafate plants (b); evaluated at different times of the day: 09:00, 12:00, 15:00, and 18:00 h. Treatments: CK: no mulch, GEO: geotextile mulch, OAT: oat straw mulch, and HAZ: hazelnut shell mulch. Different lowercase letters indicate significant differences between treatments according to Fisch-er's LSD test ( $p < 0.05$ ). Mean  $\pm$  standard error ( $n = 4$ ). The bars correspond to the experimental error for each treatment.

### 3.4. Yield and Physical Parameters of the Fruit

Fruit yield (Figure 6a) increased significantly ( $p < 0.05$ ) with the OAT treatment, which presented  $0.4 \text{ t ha}^{-1}$  compared with the CK and HAZ treatments, which presented  $0.1$  and  $0.2 \text{ t ha}^{-1}$ , respectively. Likewise, OAT presented a higher fruit weight (Figure 6b) of  $1.89 \text{ g}$  and an equatorial diameter (Figure 6c) of  $6.2 \text{ mm}$  compared with the control, but there were no significant changes ( $p > 0.05$ ) in the polar diameter of the fruit (Figure 6d).

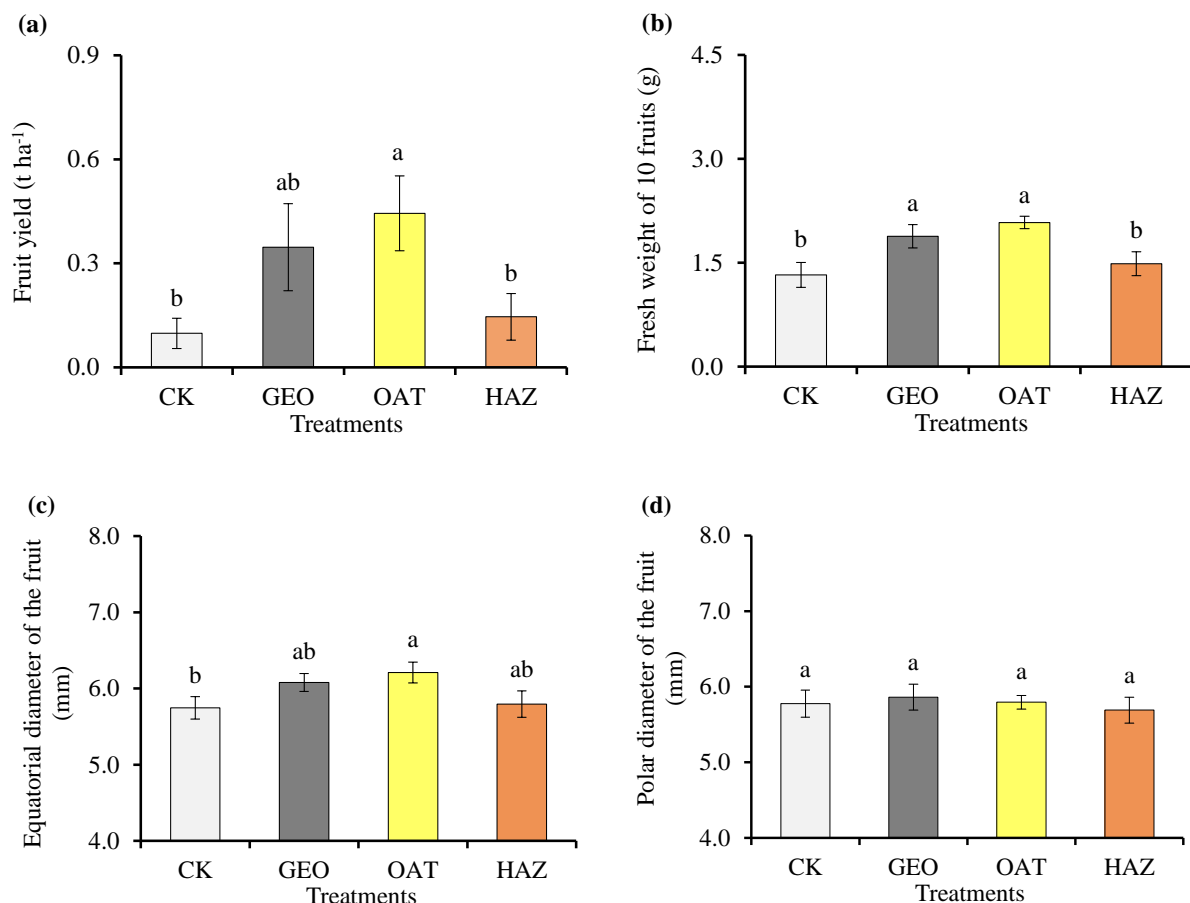


Figure 6. Average fresh fruit yield of calafate (a); average weight of 10 fresh fruits (b); average equatorial diameter (c); average polar diameter of fresh fruit (d). Treatments: CK: no mulch, GEO: geotextile mulch, OAT: oat straw mulch, and HAZ: hazelnut shell mulch. Different lowercase letters indicate significant differences between treatments according to Fischer's LSD test ( $p < 0.05$ ). Mean  $\pm$  standard error ( $n = 4$ ). The bars correspond to the experimental error for each treatment.

### 3.5. Soluble Solids and Antioxidant Parameters of the Fruit

Soluble solids (Figure 7a) increased significantly ( $p < 0.05$ ) with the CK treatment, which presented 40 °Brix, compared with 31 °Brix in OAT. Total polyphenols (Figure 7b) were significantly higher in the CK and HAZ treatments with values 741 and 760 mg gallic acid 100 g<sup>-1</sup> FW, respectively. Likewise, the antioxidant activity DPPH (Figure 7c) and ORAC (Figure 7d) were significantly higher ( $p < 0.05$ ) in the CK and

HAZ treatments, with values of 4021 and 3981  $\mu\text{mol TE } 100 \text{ g}^{-1} \text{ FW}$ , respectively, for DPPH, and with values of 3245 and 3299  $\mu\text{mol TE } 100 \text{ g}^{-1}$ , respectively, for ORAC.

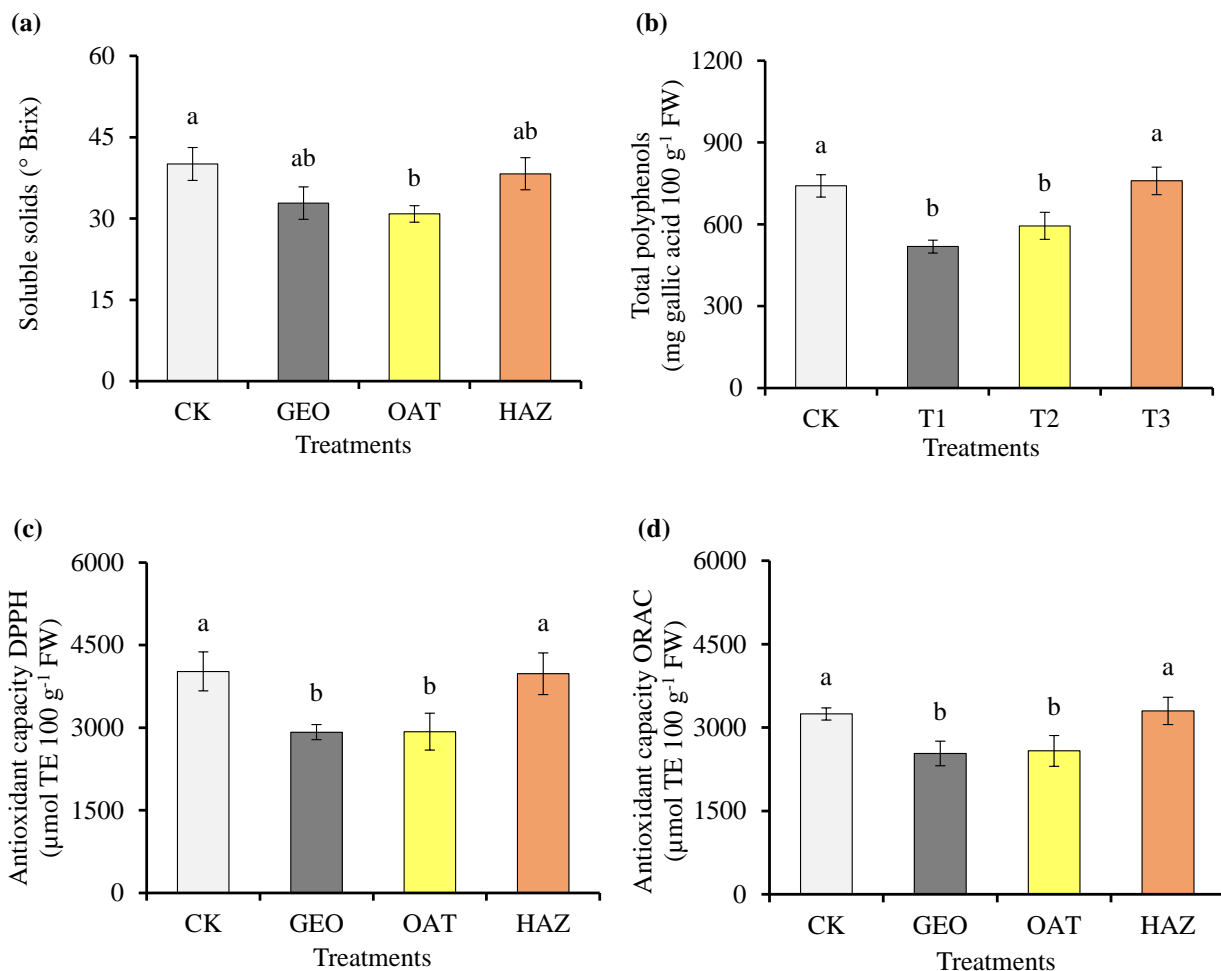


Figure 7. Soluble solids (a); total polyphenols (b); DPPH antioxidant capacity (c); and ORAC antioxidant capacity (d) determined from fresh fruit of calafate plants. Treatments: CK: no mulch, GEO: geotextile mulch, OAT: oat straw mulch, and HAZ: hazelnut shell mulch. Different lowercase letters indicate significant differences between treatments according to Fischer's LSD test (p < 0.05). Mean  $\pm$  standard error (n = 4). The bars correspond to the experimental error for each treatment.

The total anthocyanin concentration (Table 2) was significantly higher (p < 0.05) for the CK treatment with 461.2 mg  $100 \text{ g}^{-1} \text{ FW}$  compared with the OAT treatment that

presented 319.1 mg 100 g<sup>-1</sup> FW. Total anthocyanins were 86%, 82%, 83%, and 85% in the CK, GEO, OAT, and HAZ treatments, respectively, represented by the anthocyanins delphinidin, petunidin, and malvidin 3-glucoside.

Table 2. Anthocyanins (mg 100 g<sup>-1</sup>) of fresh calafate fruit by HPLC.

Anthocyanins	Treatments				Anova p values
	CK	GEO	OAT	HAZ	
Delphinidin 3,3-dihexoside	2.4 ± 0.6 a	1.8 ± 0.4 a	2.2 ± 0.2 a	2.3 ± 0.4 a	0.8147
Petunidin 3,5-dihexoside	9.4 ± 0.4 a	9.8 ± 2.1 a	10.4 ± 3.0 a	8.6 ± 1.5 a	0.9253
Malvidin 3,5-dihexoside	5.8 ± 0.2 b	8.7 ± 1.1ab	8.2 ± 2.3 ab	11.5 ± 0.8 a	0.0446
Delphinidin 3-glucoside	190.6 ± 28.8 a	109.3 ± 14.9 b	105.6 ± 20.5 b	137.3 ± 11.9 ab	0.0395
Delphinidin 3-rutinoside	1.7 ± 0.3 a	1.5 ± 0.3 ab	1.0 ± 0.2 b	1.7 ± 0.3 ab	0.0371
Cyanidin 3-glucoside	29.7 ± 1.3 a	20.5 ± 2.7 a	20.5 ± 3.3 a	22.4 ± 4.6 a	0.1939
Petunidin 3-glucoside	133.9 ± 12.6 a	99.3 ± 8.2 bc	92.0 ± 12.0 c	124.6 ± 6.9 ab	0.0380
Petunidin 3-rutinoside	3.1 ± 0.2 a	2.1 ± 0.6 ab	1.1 ± 0.1 b	2.1 ± 0.4 ab	0.0239
Peonidin 3-glucoside	11.9 ± 0.7 a	20.9 ± 3.7 a	10.2 ± 1.2 a	13.2 ± 1.7 a	0.2730
Malvidin 3-glucoside	72.7 ± 3.8 a	79.4 ± 18.8 a	67.8 ± 1.7 a	98.5 ± 9.9 a	0.2553
Total anthocyanins	461.2 ± 46.0 a	353.3 ± 27.8 ab	319.1 ± 39.0 b	422.2 ± 28.3 ab	0.0439

Treatments: CK: no mulch, GEO: geotextile mulch, OAT: oat straw mulch, and HAZ: hazelnut shell mulch. Different lowercase letters indicate significant differences between treatments according to Fischer's LSD test ( $p < 0.05$ ). Mean ± standard error (n = 4).

### 3.6. Soil–Plant–Fruit Parameter Interaction

Of the 20 parameters evaluated (Figure 8), the principal components PC1 and PC2 retained 34.66% and 16.18% of the variance, respectively. In the biplot, each parameter is represented as a vector whose length indicates its importance in the analysis (Figure 8a). Treatments are represented by numbers in the PCA (Figure 8b): 1–4 for CK, 5–8 for GEO, 9–12 for OAT, and 13–16 for HAZ.

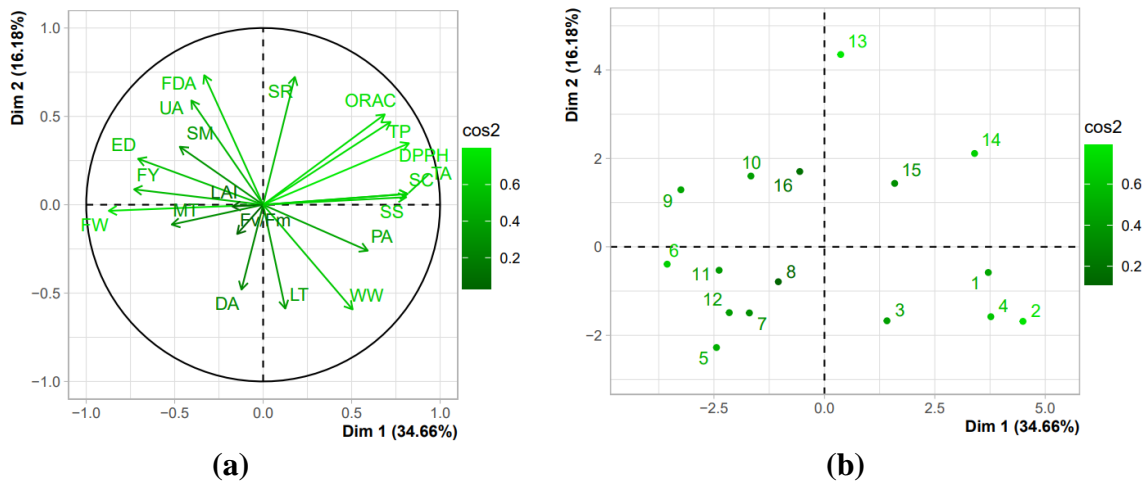


Figure 8. Principal component analysis (PCA) of variables (a) and individuals (b) of soil microbiological, plant physiological, and physicochemical variables of fruit. Soil microbiological activity (FDA); soil respiration (SR); urease activity (UA), dehydrogenase activity (DA); acid phosphatase activity (PA); leaf temperature (LT); leaf area index (LAI); stomatal conductance (SC); maximum photosystem II efficiency (Fv/Fm); fruit productivity (FY); fruit equatorial diameter (ED); fruit fresh weight (FW); soluble solids (SS); total polyphenols (TP); DPPH antioxidant activity (DPPH); ORAC antioxidant activity (ORAC); total anthocyanins (TA); soil moisture (SM); mulch temperature (MT); weed dry weight (WW).

Correlations among soil, plant, and fruit (Figure 9) were analyzed using Pearson's correlation coefficients ( $r$ ). Moderate correlations ( $r > 0.59$  or  $r < -0.59$ ) were observed among soil biological indicators. There were moderate relationships between environmental and soil biological variables ( $r > 0.5$  or  $r < -0.5$ ). Plant physiological variables interacted moderately with plant yield ( $r < -0.5$ ), but there was a higher relationship with soluble solids ( $r > 0.7$ ) and DPPH antioxidant activity ( $r > 0.6$ ). Fruit physical parameters had a high relationship with chemical attributes ( $r > -0.8$ ) and also between the latter ( $r > 0.5$  or  $r < 0.8$ ). In addition, the correlation matrix corroborated the closeness between variables demonstrated in the PCAs (Figure 8a,b).

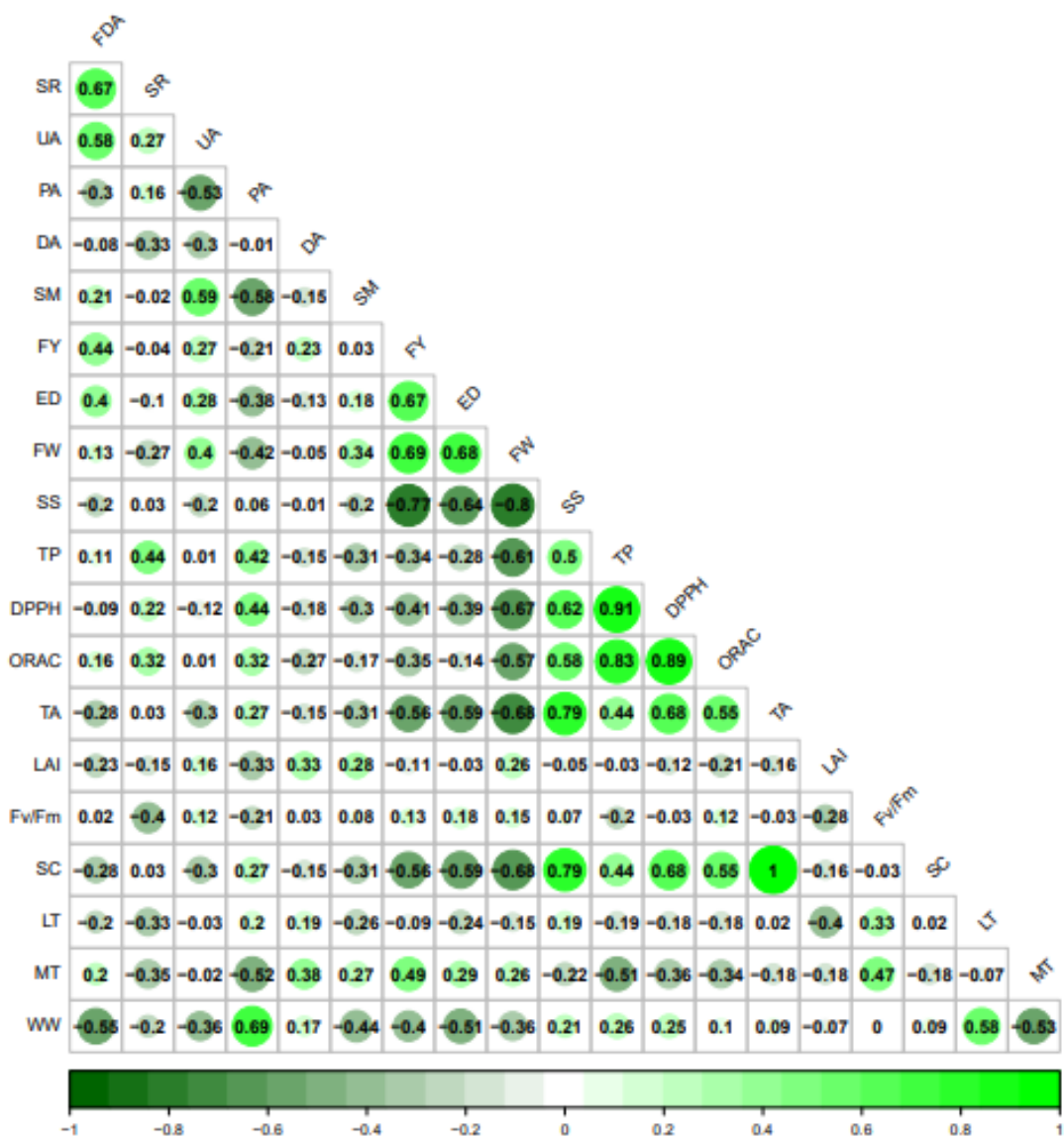


Figure 9. Correlation matrix for soil variables, plant physiological parameters, yield parameters and fruit bioactive compounds. Soil microbiological activity (FDA); soil respiration (SR); urease activity (UA), dehydrogenase activity (DA); acid phosphatase activity (PA); leaf temperature (LT); leaf area index (LAI);; stomatal conductance (SC); maximum photosystem II efficiency (Fv/Fm); fruit productivity (FY); fruit equatorial diameter (ED); fruit fresh weight (FW); soluble solids (SS); total polyphenols (TP); DPPH antioxidant activity (DPPH); ORAC antioxidant activity (ORAC); total anthocyanins (TA); soil moisture (SM); mulch temperature (MT); weed dry weight (WW).

## 4. DISCUSSION

### 4.1. Weeds, Soil Moisture and Temperature, and Leaf Temperature

In our study, different types of mulches showed a significant effect on weed suppression compared with the control. This reaffirms the ability of mulches, regardless of the material used, to exert a physical effect on weed suppression and weed seed germination [37]. It is important to note that both GEO and HAZ mulches showed greater efficacy in soil moisture retention. This retention capacity is directly related to the less permeable composition of these materials, which act as an effective barrier to soil water vapor losses [38]. Also, it is likely that HAZ mulching led to increased soil aggregation, reduced bulk density, and the appearance of larger pore spaces, increasing water retention [39]. In contrast, the CK and OAT treatments, which did not show significant differences between them, showed a lower capacity to retain moisture. This may be attributed to the higher permeability and degradability of OAT [11], as well as the relatively lower capacity of weeds to retain moisture in the case of CK [40].

As for leaf temperature, the results indicate that the different mulches did not have a significant effect. This suggests that the ambient temperature in the study area, with maximums of up to 30 °C at the time of the measurements, may not have been a determining factor in this parameter [41]. However, when analyzing the soil surface temperature, it was observed that the GEO mulch showed a higher temperature compared with the control, while it presented similarity in this aspect with the other mulching treatments. These findings support the inherent ability of mulches to absorb a wide range of visible and infrared wavelengths of solar radiation, leading to an increase in soil surface temperature due to heat emission [8]. Importantly, despite the similarities in solar radiation absorption capacity among the different mulches, the GEO mulch showed a tendency to record higher temperatures than the OAT and HAZ mulches, especially during midday hours and at the end of the evaluation period. This pattern suggests that the black color of the GEO mulch plays a key role in absorbing solar radiation and, thus, heat emission [42,43].

Our results demonstrate that HAZ mulch emerges as an effective option for weed control, maintaining greater moisture reserves and moderate soil temperatures compared with soil without mulch. Furthermore, these comparative benefits highlight it in relation to the other mulches evaluated in this study. We emphasize the need to evaluate other types of low-degradability agricultural residue mulches that contribute to maintaining adequate soil temperature and moisture levels in calafate orchards.

#### 4.2. Soil Microbiological and Chemical Parameters

The results of our investigation show that there was higher fluorescein diacetate (FDA) activity in HAZ. However, the other mulches did not generate significant changes in FDA activity. It is important to mention that the FDA is considered an indicator of hydrolysis carried out by living microorganisms in the soil [24]. It is plausible that after a period of 5 years since the implementation of HAZ, the convergence of environmental factors such as soil moisture and temperature may have contributed to the increase in live microbial biomass and, therefore, higher microbial activity observed. Similar findings were presented by Huang et al. [14] who evidenced an increase in soil microbial activity due to higher soil aeration, temperature, and moisture, after the application of woody mulch from Acacia trees. Likewise, it was indicated in a peach orchard (*Prunus persica* L. Batsch) that organic pine needle mulch, which regulated soil moisture and temperature regimes, significantly improved microbial activity and microbial biomass carbon [44]. It is likely that the use of organic mulch increased soil water content by reducing soil evaporation and, in turn, by increasing water storage and infiltration capacity [45]. Given the increase in FDA activity observed in the HAZ treatment after a 5-year period, it would be beneficial to conduct longer-term studies to understand how the effects of different mulch types on soil microbial activity evolve over time.

On the other hand, in our study, OAT, despite having induced an adequate soil temperature, caused greater soil moisture loss. It is likely that this reduction in moisture in turn decreased the abundance, distribution, and activity of soil microorganisms [46]. Meanwhile, GEO, despite increasing soil moisture, generated

increased temperatures above the ambient temperature, which negatively affects microbial growth [6]. This condition could also explain the lower basal soil respiration rate observed in this treatment. In contrast, higher basal respiration was observed in HAZ, which is congruent with the previously mentioned results. Basal respiration in OAT was significantly higher compared with GEO. This suggests that mulches derived from readily decomposable materials, such as oat straw, may promote a higher basal respiration rate. This could be attributed to the contribution of new microbial communities from OAT that enrich native soil bacterial diversity [13], as well as soil structural improvements in bulk density and proportion of larger soil aggregates that favor soil microclimate and microbial action [47]. However, we emphasize the need to investigate alterations in microbial diversity and composition, which would provide more background on the mechanisms involved in the response to the types of mulches applied.

Urease activity increased significantly with HAZ and OAT compared with CK. This increase could be explained by the strong correlation of this enzyme with soil microbial biomass content [48]. It is important to highlight that the activity of this enzyme may also be related to the activity of extracellular enzyme-organic complexes, which are influenced by the organic amendments applied [49]. On the other hand, the lower urease activity in CK is consistent with the higher nitrate levels present toward the end of the study, which may inhibit the action of this enzyme through repression of microbial synthesis [50]. It should be noted that urease is involved in urea hydrolysis and would not explain nitrogen mineralization [49], suggesting that its understanding requires further investigation. The mulches used in our study showed a nonsignificant impact on dehydrogenase activity, which is responsible for the oxidation of organic carbon in the soil [51]. According to the literature, the incorporation of organic compounds into the soil generates a high response due to the formation of organo-enzyme complexes, which improve their stability [49]. However, this response could be more pronounced in soils with low fertility, as previously observed [52]. Therefore, by presenting considerable levels of organic matter from the orchard establishment to the end of the study (9.7% at the

time of orchard establishment and an average between treatments of 9.2% at the end of the study), our results suggest that the treatments with mulch and without mulch have reached a threshold in which the action of this enzyme is not significantly affected. On the other hand, acid phosphatase activity increased significantly with CK. This finding may be explained by the decrease in pH treatment, which may stimulate a higher activity of this enzyme [27]. It is likely that the soil under CK, lacking a physical barrier limiting exposure to precipitation in winter and constant irrigations in summer, experienced a reduction in pH due to a decrease in bases and an increase in anions such as nitrate [53].

In relation to the chemical composition of the soil, it is relevant to highlight that the HAZ treatment resulted in a decrease in OM after a period of 5 years from its incorporation, without it having been replenished during this period. This decrease could be related to the environmental conditions favored by this treatment, which favored an increase in active microbial biomass. As a consequence, it is possible that this increased biomass consumed more efficiently the C readily available in the soil, using this component as an energy substrate [54]. In contrast, the OAT treatment, due to its high degradability, experienced a more frequent release to the soil, requiring replenishment annually over the same 5-year period. This trend could explain the observed increase in MO in the OAT treatment. These findings are consistent with the observations of Von Lützow and Kögel-Knabner [55] who emphasize that the impact of temperature on the degradation rate of organic compounds may vary according to the type of material applied. It is worth mentioning that the OAT treatment also showed a positive effect on soil fertility, particularly on the availability of elements such as S, Ca, sum of bases, and CEC. A similar situation was demonstrated in a vineyard with straw mulch, in which the OM content and available P, K, Na, Mg, and S increased after 4 years [45]. It is important to note that the control treatment showed a significant accumulation of available nutrients, such as N and P, during the same evaluation period. This accumulation can be attributed to the annual recycling of the most abundant weeds in this treatment, which can contribute significantly to the improvement of soil fertility [5]. Our results demonstrate

that the favorable soil microclimate provided by organic mulch covers, especially HAZ, dampens elevated temperatures and improves soil moisture, which slows the decline of the bacterial population in the soil. In addition, our results are consistent with Micallef et al. [56] who indicated that organic mulch covers have the ability to modify the carbon and nitrogen fractions in the soil, which increases soil respiration and leads to changes in fungal and bacterial diversity.

Furthermore, our results support the benefits of the application of organic mulches such as oat straw despite its high degradability and lower efficacy in improving the evaluated soil biological properties, proved to be more beneficial than no mulching and geotextile mulching in terms of these properties. However, we highlight the need for longer-term research to better understand the sustainability of organic mulches on the stability and persistence of soil organic matter and nutrients.

#### 4.3. Plant Physiological Parameters

Mulches did not affect the leaf area. These results contrast with previous studies in pear and apple trees where polypropylene geotextile mulches for 4 years in pear trees and organic mulches of straw and pine bark for 3 years in apple trees positively impacted photosynthetic rate, stomatal conductance, and stimulated plant growth, due to improvements in environmental conditions, such as soil moisture and soil nutrition [57,58]. However, it has been shown that the leaf area index of locally cultivated calafate plants is favored by applications of organic, highly decomposable, and N-rich components, such as compost, at doses higher than 10 t ha<sup>-1</sup> [20]. A similar situation occurred in an olive orchard where the easily decomposable animal manure mulch, high in N, had the greatest influence on plant morphophysiological traits [59]. It has also been shown that leaf area index can be negatively affected by extreme soil moisture deficiency (0% ET<sub>0</sub> replenishment) [21], which did not occur in our study where irrigations were constant with 100% ET<sub>0</sub> replenishment.

Regarding the maximum photosystem II yield and stomatal conductance, there were no differences with mulch applications. This suggests that initial soil fertility is adequate for the physiological functions of this species, which exhibits a high level of

hardiness [60]. However, the general tendency to decrease as the day progressed indicates that the plant, when subjected to a higher intensity of environmental factors such as light or temperature, presents resistance strategies such as decreasing the maximum photosystem yield [28] or reducing the flow of gas exchange through the stomata [61].

These results indicate that the different types of mulches did not have a significant impact on the leaf area index and physiological parameters in the calafate plant. However, it is essential to emphasize the importance of conducting research over several years to achieve a more comprehensive understanding of their effects.

#### 4.4. Physicochemical Parameters of Fruits

The OAT treatment had a higher fruit yield compared with the HAZ and CK treatments. These results are in agreement with the higher fertility levels observed in the OAT treatment and the lower levels in HAZ. Soil fertility has been documented to be closely related to crop productivity, with K, which exhibited a higher presence in OAT, being one of the main components of calafate fruit [62]. Soil-available K that can be extracted by the plant improves fruit firmness, caliber, flavor, and aroma [62]. Our results are in agreement with previous literature, with cereal straw mulch being more effective in contributing to soil fertility and producing increases in yield, fruit size, and weight [63,64].

On the other hand, the HAZ treatment, despite showing higher soil biological activity, did not show a significant increase in fruit production. This could be related to the lower fertility and availability of nutrients in HAZ, which are essential factors for the production of wild and cultivated calafate [19,20]. In fact, this treatment reached a production level similar to the control (CK). On the other hand, fruit weight in the GEO treatment was higher than the control despite the suppression of soil biological activity caused by high temperature. However, it is relevant to mention that in the case of calafate, temperature accumulation has been pointed out as a requirement for the adequate growth of its roots [62]. It is known that roots play a crucial role in the absorption of nutrients and translocation of organic compounds for fruit formation.

This increase in yield due to the use of synthetic black mulch covers has been documented in other berries, such as grape, strawberry, raspberry, and blueberry [8]. In all treatments, a close correlation was observed between yield, fruit number, and equatorial diameter, which may be inherent to calafate since the same relationship was found in wild calafate from Central Southern Chile [65]. However, it is important to consider that this study covered only 1 year, so longer-term research is needed to obtain a deeper understanding of the effects of different covers on the yield of calafate.

#### 4.5. Soluble Solids and Antioxidant Parameters of the Fruit

The study revealed an inverse relationship between the soluble solid concentration and yield. Smaller fruit treatments, HAZ and CK, exhibited higher soluble solids or sugar concentrations due to reduced water content. In contrast, the larger fruit treatments, OAT and GEO, with higher yields showed lower sugar concentrations, similar to those reported in strawberries [66]. This trend was also reflected in the phenolic compounds of the fruit and their antioxidant capacity as has been previously reported in calafate berries [64]. Although OAT and GEO had lower phenolic values and antioxidant activity compared with HAZ and CK, they proved superior to berries such as blueberries and strawberries with a high potential to counteract oxidative stress [17].

Anthocyanin content was significantly lower with OAT compared with CK, but no significant differences were observed among other mulching treatments. This suggests that calafate plants, both with HAZ and GEO and with CK, produce fruit with high levels of anthocyanins, which contribute to intense tones in the fruit [67], relevant to the natural pigment industry [32]. All treatments showed three predominant anthocyanins: delphinidin, petunidin, and malvidin, which is in agreement with previous studies in cultivated [21] and wild [33] calafate. The prevalence of delphinidin, which represented 40% of total anthocyanins, was similar to that reported in blueberries [68], gaining relevance for its potent antioxidant properties attributed to a higher number of hydroxyl groups on the B-ring [69].

Our study provides evidence that the use of HAZ mulching significantly increases bioactive compounds, especially anthocyanins such as delphinidin (Table 2), and these higher levels of delphinidin generate darker fruits, which better counteracts cellular oxidative stress. Therefore, these findings are valuable for their benefits to health and the natural pigment industry. It is important to take into consideration that more years of study are required to address in depth the physical and chemical productivity of calafate as an excessive increase in yield may compromise its phenolic composition and antioxidant capacity for which it is renowned.

#### 4.6. Soil–Plant–Fruit Parameter Interaction

The moderate retentions in the principal components PC1 with 34.66% and PC2 with 16.18% contribute to improving the understanding of the interaction of soil, plant, and fruit variables. The moderate correlations between environmental and soil microbiological parameters demonstrate that the use of mulch promotes soil microbial and enzymatic activity through improvements in environmental conditions, which is consistent with previously reported [4,12]. Moderate correlations between plant parameters with fruit yield demonstrate that mulching contributes to fruit yield increases without affecting plant morphophysiology as has been demonstrated in other berry species, such as grapevine [10]. However, the highest correlations between physical parameters and fruit bioactive compounds demonstrate that increases in fruit yield due to mulching lead to a decrease in phenolic and antioxidant content of the fruit, a situation that has been corroborated in berries such as grapes and strawberries [7,9]. This study represents a significant advance in the evaluation of mulch used in calafate orchards, analyzing in an integrative manner a total of 20 variables, which is higher than the 13 in previous research in cultivated calafate [21]; however, due to their moderate interrelationship, they should be analyzed in depth.

## 5. CONCLUSIONS

Our research highlights the benefits of mulch for the soil, plant, and fruit of cultivated calafate. The results show that mulch, regardless of the material, has high effectiveness in the control of weeds, up to 43%, compared with the soil without mulch. In particular, hazelnut shell (HAZ) mulch emerges as a low degradable soil microbiological enhancer, significantly increasing soil microbial activity, basal respiration, and urease enzyme activity by 40%, 31%, and 20%, respectively, compared with the control (CK). However, we see the need to investigate alterations in microbial diversity and composition. On the other hand, the highly degradable oat straw (OAT) mulch generates increases of 8% in OM and 31% in P available compared with CK. Long-term research would help to better understand the sustainability of mulches in changing soil OM and nutrient levels. Despite these results, the physiology and morphology of the calafate plant were not affected. The calafate without mulch, in itself, is a plant with a high phenolic content, but with the use of OAT and geotextile (GEO) mulches, this decreases significantly. However, HAZ mulch is capable of maintaining adequate levels of total polyphenols, anthocyanins, and antioxidant capacity with values of 760 mg gallic acid 100 g<sup>-1</sup> FW, 422 mg 100 g<sup>-1</sup> FW, and 3299 μmol TE 100 g<sup>-1</sup>, respectively. We highlight the need to evaluate these parameters in a comprehensive way for a longer time since the changes in yield caused by this type of management can compromise the bioactive composition of the calafate fruit. On the other hand, we recommend the use of HAZ mulch, as an innovative strategy to enhance the bioactive compounds of the calafate fruit through efficient, environmentally conscious soil management.

### Supplementary Materials

The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/horticulturae9111202/s1>, S1: Soil chemical analysis (0–40 cm) of the study site in the Ñuble Region of Chile; S2: Monthly accumulated temperatures and precipitation during the evaluation seasons (2021 and

2022) in the Ñuble Region, Chile; S3: Chemical analysis of oat straw and hazelnut shell mulches; S4: Soil chemical analysis at end of study.

### **Author Contributions**

Conceptualization, M.S., J.R.-S. and M.D.L.; methodology, R.V.-R., M.S., J.R.-S., M.B. and M.D.L.; software, M.B. and M.D.L.; validation, M.S., J.R.-S. and M.D.L.; formal analysis, M.S., J.R.-S. and M.B.; investigation, R.V.-R., M.B., M.S., J.R.-S. and M.D.L.; resources, M.S., M.D.L. and J.R.-S.; data curation, M.B., M.S., J.R.-S. and M.D.L.; writing—original draft preparation, M.B., M.S., J.R.-S. and M.D.L.; writing—review and editing, J.R.-S., M.S. and M.D.L.; project administration, M.S. and J.R.-S.; funding acquisition, R.V.-R., M.S., J.R.-S. and M.D.L. All authors have read and agreed to the published version of the manuscript.

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### **Data Availability Statement**

The data presented in this study are available in the article.

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### **Conflicts of Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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#### **IV. CAPÍTULO III: ENHANCING SOIL HEALTH AND FRUIT QUALITY IN CALAFATE ORCHARDS THROUGH SUSTAINABLE AMENDMENTS**

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#### **ABSTRACT**

Calafate (*Berberis microphylla* G. Forst.) produces fruits with a high antioxidant activity. There is limited information on the effect of compost application on the soil properties and physicochemical parameters of fruits. A 2-year compost fertilization experiment was conducted in a calafate orchard established in 2017. The effects of compost fertilization (0, 5, 10, and 15 t ha<sup>-1</sup> of compost) on soil, plant, and fruit were evaluated. The treatments with 5 and 10 t ha<sup>-1</sup> compost, without significant differences between them, significantly improved soil fluorescein diacetate activity

(FDA) by 18% and basal respiration by 43% compared to the control. The 10 t ha<sup>-1</sup> treatment resulted in a 42% increase in leaf area index and a 68% increase in chlorophyll compared to the other treatments including the control. Likewise, the 10 t ha<sup>-1</sup> treatment also had a 200% higher fruit yield than the control and achieved a fruit oxygen radical absorbance antioxidant capacity (ORAC) of 3029 μmol TE 100 g<sup>-1</sup> FW. The 15 t ha<sup>-1</sup> treatment had the highest fruit production 3.8 t ha<sup>-1</sup>, but it decreased soluble solid concentration by 28% and ORAC to 2526 μmol TE 100 g<sup>-1</sup> FW. Multivariate analysis revealed a positive correlation between FDAse activity and fruit antioxidant activity (r=0.61). Compost application at varying doses enhances FDAse activity and basal soil respiration. Notably, a dose of 10 t ha<sup>-1</sup> is recommended to boost FDAse activity and basal soil respiration. This, in turn, results in a substantial increase in the antioxidant activity of the calafate fruit after a 2-year application period.

**Keywords:** Berries; Anthocyanins; Antioxidant capacity; Enzymes activities; Polyphenols

## 1. INTRODUCTION

The calafate (*Berberis microphylla* G. Forst.) is a native species of Chile, which grows in the wild from the Metropolitan to Magallanes y la Antártica Chilena Region, Chile (Pinto-Morales et al. 2022). The shrub can grow up to 2 m tall in adult stage and produces a dark blue berry with a high content of phenolic compounds, such as anthocyanins, which confer antioxidant capacity up to four times higher than those of blueberry (Rodoni et al. 2014). The antioxidant capacity of calafate has been highlighted in different studies as it has been shown to be effective in prevention of cancer, cardiovascular, and degenerative diseases (Fredes et al. 2020). In recent decades, the food trend has been to prefer nutritious foods with bioactive potential (Ruiz et al. 2014), which has led to a significant increase in demand and scarcity of the wild fruit, raising its value (Tacón 2004). These factors collectively render the

calafate an enticing subject for the exploration of management practices in its cultivation, with the aim of enhancing its bioactive properties.

The edaphoclimatic adaptability of calafate is another of its outstanding characteristics, since it can grow and fructify under extreme climatic conditions of drought, wind, rain, and snow (Radice et al. 2018). In this context, López et al. (2018) pointed out that the south-central zone of Chile presents edaphoclimatic characteristics suitable for the development of species such as calafate, which was later supported by different works that established calafate orchards in this zone, improving the fruit supply and its bioactive properties (Betancur et al. 2022; Pinto-Morales et al. 2022). Specifically, a study that evaluated water replenishment of reference evapotranspiration (ET<sub>0</sub>) in a calafate orchard in the Ñuble Region showed that plants subjected to severe water stress (0% ET<sub>0</sub>) during the months of highest water demand, managed to produce yields of 0.8 t ha<sup>-1</sup>, and also increased by 48% the phenolic compounds of the fruit; on the other hand, an adequate management in water replenishment (50% ET<sub>0</sub>) improved the yield up to 1.2 t ha<sup>-1</sup> maintaining a content of 620 mg gallic acid 100 g<sup>-1</sup> FW (Betancur et al. 2022). In spite of these advances, there are still uncertainties about the influence of other fundamental managements in calafate orchards, such as soil fertilization.

Organic fertilization with composted biowaste is a recurrent practice in commercial orchards due to the benefits to soil fertility, which translate into increased yields and crop quality (Chang et al. 2007). In the case of grapevine, fertilization with compost increased N mineralization and availability, producing average yields of 171 kg ha<sup>-1</sup>, compared to an inorganic fertilization that obtained 164 kg ha<sup>-1</sup> (Elzobair et al. 2016). In the case of blackberry, compost significantly increases plant height and dry matter (Vásquez and Maraví, 2017); similarly, in nectarines, it was observed that compost increases leaf photosynthetic efficiency (Sorrenti et al. 2019). Besides, compost significantly improved the quality of strawberry by increasing the concentration of anthocyanins, phenolic compounds and antioxidant capacity of the fruit (Wang and Lin 2003). Pinto-Morales et al. (2022) demonstrated that compost significantly improved calafate yield, reaching values of up to 3.1 t ha<sup>-1</sup>, and fruit total

polyphenol content reached 764 mg gallic acid 100 g<sup>-1</sup> FW. Despite these encouraging results, there is an important gap in describing the chemical components and physical parameters of calafate fruit as influenced by compost. Therefore, further studies are needed to fully determine the potential of compost in the production of high quality calafate berries.

Soil health encompasses a range of biological, chemical, and physical properties, including microbial communities, inorganic nutrients, soil aggregates, among others, that enhance the value of agricultural systems (Wilhelm et al. 2023). Soil biology has become an important indicator of overall soil quality in commercial orchards, as the biodiversity and activity of soil microbial communities are responsible for plant nutrition and productivity (Wang et al. 2019b). In this regard, compost fertilization amendment has a significant impact on soil microbial community structure, abundance, and activity (Wang et al. 2019a). Likewise, microbes, through decomposition of organic matter affect soil enzyme activities (Zhao et al. 2016). This is particularly relevant, since research has shown that in calafate orchard, soil microbiological and enzymatic activity affects plant production parameters, and phenolic compounds in the fruit (Betancur et al. 2022). However, there is still uncertainty about how compost affects soil microbiological and enzymatic activities and its repercussion on plant physiology and physicochemical performance of cultivated calafate. Building upon this, it is anticipated that the application of varying compost dosages will exert a substantial influence on the microbiological, physiological, and physicochemical parameters of calafate cultivated in central-southern Chile. As part of this hypothesis, we propose that compost fertilization will elevate soil microbiological and enzymatic activity, subsequently exerting a positive influence on plant physiology. This, in turn, will enhance the physicochemical attributes of the fruit, particularly the concentration of phenolic compounds and antioxidant capacity. Therefore, the objective of this research was to determine the effect of different doses of compost on soil microbiological, plant physiological, and physicochemical parameters of the fruit, in calafate grown in central-southern Chile.

The insights garnered from this study will guide us in determining the optimal compost dosage for enhancing soil health and antioxidant activity of calafate fruits.

## 2. MATERIALS AND METHODS

### 2.1 Orchard Establishment and Edaphoclimatic Characteristics of the Study Site

The first calafate orchard was established at the experimental station of the Adventist University of Chile, Chillán, Ñuble Region, Chile (Fig. 1a). The orchard was established in 2017 by transplanting 2-year-old plants previously obtained by seed from the Perquenco nursery, Valdivia, Los Ríos Region, Chile. The orchard soil is an Andisol (Melanoxerand) and was characterized and analyzed (Table 1).

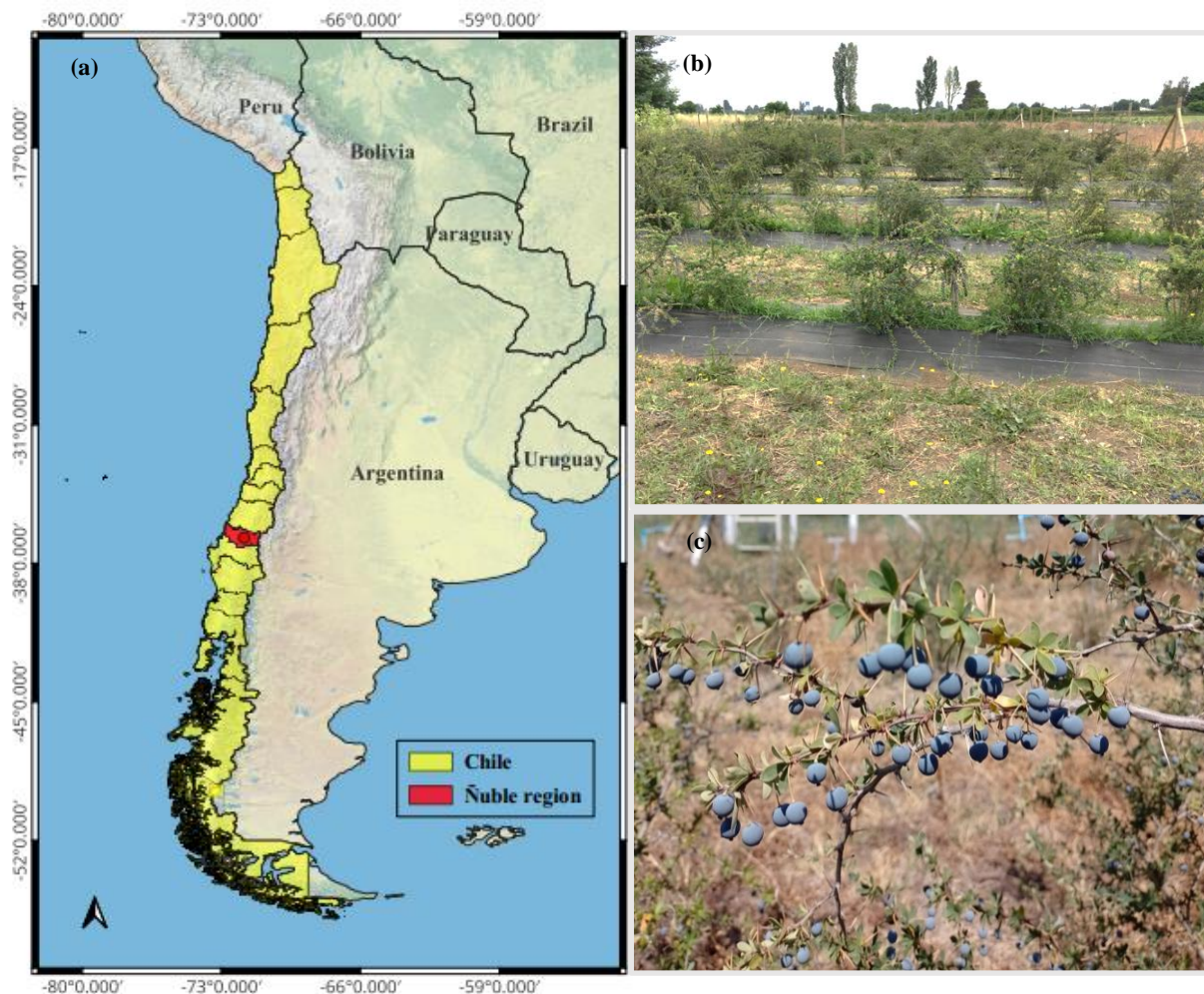


Figure 1. a Ñuble Region in Chile where the calafate orchard is located. b Calafate orchard where compost treatments were applied, year 2021; c fruiting branch of calafate, year 2022.

Table 1. Soil chemical analysis (0–40 cm) of the study site in the Ñuble Region of Chile

<b>Analysis</b>	<b>Unit</b>	<b>Result</b>
OM	%	9.70
pH (water)		6.40
N availability	mg kg <sup>-1</sup>	19.00
Olsen P	mg kg <sup>-1</sup>	15.30
K availability	mg kg <sup>-1</sup>	496.00
S availability	mg kg <sup>-1</sup>	24.00
Exchangeable Ca	cmol <sub>c</sub> kg <sup>-1</sup>	8.70
Exchangeable Mg	cmol <sub>c</sub> kg <sup>-1</sup>	1.60
Exchangeable K	cmol <sub>c</sub> kg <sup>-1</sup>	1.30
Exchangeable Na	cmol <sub>c</sub> kg <sup>-1</sup>	0.01
Sum of bases	cmol <sub>c</sub> kg <sup>-1</sup>	11.60
Interchangeable Al	cmol <sub>c</sub> kg <sup>-1</sup>	0.02
CEC	cmol <sub>c</sub> kg <sup>-1</sup>	11.60
Al saturation	%	0.10

CEC: cation exchange capacity

The calafate orchard (Fig. 1b) has a total of 352 plants in full fruit production (Fig. 1c), distributed in 22 rows, each with 16 plants. The planting density was established at 1 m above the row and 3 m between rows. Inorganic fertilization and phytosanitary management were carried out in the first year of establishment according to Pinto-Morales et al. (2022), and irrigation according to Betancur et al. (2022).

The climate of the area is temperate Mediterranean, with mean annual temperatures and accumulated rainfall of 13.5 °C and 649 mm, respectively, for the season 2021/2022, and 13.2 °C and 920 mm, respectively, for the season 2022/2023 (Fig. 2).

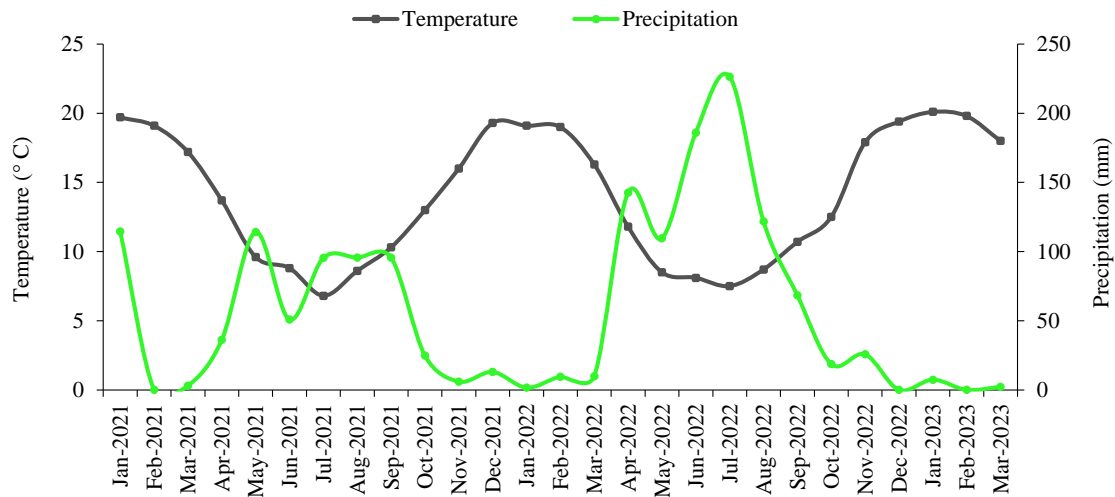


Figure 2. Ambient temperature (°C) and accumulated precipitation (mm) of the study locality according to information from the agroclimatic station of the Instituto de Investigaciones Agropecuarias (Agrometeorología, 2023).

## 2.2 Experimental Trial Setup

Four compost treatments were applied in August 2021 and 2022; no compost application ( $0 \text{ t ha}^{-1}$ ) and applications of 5, 10, and  $15 \text{ t ha}^{-1} \text{ year}^{-1}$ . The applications were made manually in August of each year. The compost used in the study was obtained from the composting and recycling center of the Adventist University of Chile where a composting process was carried out that lasted 5 months, controlling during that time temperature, humidity and ventilation (Sayara et al. 2020). The materials used for the manufacture of the compost were chicken manure and oat straw obtained from the same center, mixed in a ratio of 3:1 (v/v). The compost was chemically characterized (Table 2) at the soil laboratory of the Instituto de Investigaciones Agropecuarias (INIA Quilamapu), located in Chillán. The experimental design was a randomized complete block with four treatments and four replicates ( $n = 16$ ). Each replicate consisted of four plants, considering as experimental unit the two central plants that were evaluated independently. The results obtained from both central plants were averaged in each treatment and replicate.

Table 2. Chemical analysis of the compost used for the assay

Analysis	Unit	Result
Humidity (dry basis)	%	23.1
pH in water 1:5		6.9
Electric conductivity 1:5	dS m <sup>-1</sup>	0.5
OM	%	21.3
Organic C	%	11.8
Total N	%	0.7
Ammonium (N-NH <sub>4</sub> <sup>+</sup> )	mg kg <sup>-1</sup>	0.09
Nitrate (N-NO <sub>3</sub> <sup>-</sup> )	mg kg <sup>-1</sup>	39.1
NH <sub>4</sub> <sup>+</sup> /NO <sub>3</sub> <sup>-</sup> ratio	-	0.002
C/N ratio	-	16.6

### 2.3 Microbiological and Chemical Soil Analysis

Soil samples were collected for microbiological analysis prior to harvest, on October 10, 2021, at a depth of 0–30 cm from the soil adhered to the calafate roots (Betancur et al. 2022). Immediately afterwards, soil samples were kept at – 20 °C for further soil analysis. For microbiological soil analyses, the homogenized soil samples were sieved at 2 mm, removing roots and other debris before being conditioned to 60% of their field capacity. At the end of the study, on May 16, 2023, a chemical soil analysis was performed.

Microbial activity was estimated by fluorescein diacetate (FDA) hydrolysis as described by Alef and Nannipieri (1995), and basal respiration as described by Joergensen (1995), expressed as  $\mu\text{g FDA g}^{-1}$  and  $\mu\text{g CO}_2 \text{ g}^{-1} \text{ h}^{-1}$ , respectively. The following enzymatic activities were determined: urease (Nannipieri et al. 1980), dehydrogenase (Garcia et al. 1997), and acid phosphatase (Tabatabai and Bremner 1969). Urease and phosphatase were expressed as ammonium-N and p-nitrophenol units, respectively, per gram of soil (dry weight) and hour ( $\mu\text{mol NH}_4^+ \text{ g}^{-1} \text{ h}^{-1}$  and  $\text{PNP g}^{-1} \text{ h}^{-1}$ ), whereas dehydrogenase was expressed as micrograms of iodonitrotetrazolium formazan per gram of soil (dry weight) ( $\mu\text{g INTF g}^{-1}$ ).

### 2.4 Plant Physiological Analysis

For physiological measurements, the selection criteria were leaves exposed to the sun, in the second third of the shoot of the season. Physiological measurements were made after harvest on January 10, 2022 and 2023.

Leaf area index (LAI;  $\text{m}^2 \text{m}^{-2}$ ) was determined with an AccuPAR LP-80 ceptometer (Decagon Devices Inc., Washington D.C., USA) that averages the reading of 80 quantum sensors to determine direct and residual photosynthetically active radiation from beneath the plant canopy (Sonnentag et al. 2007). The SPAD (Soil Plant Analysis Development) index was measured with a portable chlorophyll meter kit MC-100 (Apogee Instruments, Logan, Utah, USA) (Ribeiro Da Cunha et al. 2015). Leaf indices were measured at midday.

Maximum quantum yield of photosystem II ( $F_v/F_m$ ) was measured with a portable OS-5p fluorometer (Opti-Sciences, Hudson, New Hampshire, USA) by dark adapting leaves for 30 min with a movable shutter (Maxwell and Johnson 2000). Stomatal conductance ( $g_s$ ,  $\text{mmol m}^{-2} \text{s}^{-1}$ ) was measured using a portable porometer equipment model SC-1 (Decagon Devices, Washington D.C., USA). Both parameters were measured at four times of the day, at 09:00, 12:00, 15:00, and 18:00 h (Retamal-Salgado et al. 2017).

## 2.5 Yield and Physical Parameters of the Fruit

A manual harvest was performed 130 d after full flowering (Betancur et al. 2022). Fruit physical parameters were measured immediately after harvest for the 2021/2022 and 2022/2023 seasons. For the determination of chemical parameters, fruit samples from the 2021/2022 season were transported cold in a 35 L Tavarua Cooler (Reyplastic Spa, Santiago, Chile).

Fruit productivity ( $\text{t ha}^{-1}$ ) was determined by weighing the total fruit of each plant using an analytical balance (360 ES Series, Precisa Gravimetrics AG, Dietikon, Switzerland). For the fresh weight of 10 fruits, 10 fruits were randomly selected from the total production per plant ( $n = 4$ ) and weighed on the same analytical balance. The polar diameter and equatorial diameter (mm) of each fruit ( $n = 10$ ) were measured

using a digital meter foot model E5001002  $\pm$  0.003 mm (Veto y Cia Ltda., Santiago, Chile).

## 2.6 Soluble Solids and Antioxidant Properties of Fruit

The concentration of soluble solids ( $^{\circ}$  Brix) was measured by extracting a random sample of fresh fruit ( $n = 4$ ) from each total yield per plant, which was squeezed to obtain juice. This juice was measured using a digital refractometer model HI96801  $\pm$  0.2% (Hanna Instruments S.R.L., Woonsocket, Rhode Island, USA).

Total polyphenol concentration (Singleton and Rossi 1965), 2,2-diphenyl-picrylhydrazyl (DPPH) antioxidant capacity (Romero Román et al. 2019), and ORAC (Romero-Román et al. 2021) were determined. Polyphenols were expressed as mg gallic acid in fresh fruit (mg gallic acid 100  $g^{-1}$  FW) (Radice and Arena 2015), and both antioxidant activities were expressed as  $\mu$ mol Trolox equivalents in fresh fruit ( $\mu$ mol TE 100  $g^{-1}$  FW) (Pinto-Morales et al. 2022; Mena et al. 2011). Individual anthocyanins were identified using high-performance liquid chromatography (HPLC) (Romero-Román et al. 2021) and expressed as mg of fresh fruit (mg 100  $g^{-1}$  FW) (Mena et al. 2011).

## 2.7 Statistical Analysis

The data were subjected to analysis of variance (ANOVA). For the comparison of means, Fisher's least significant difference (LSD) test was applied at a significance level of 0.05. On the other hand, a principal component analysis (PCA) and a correlation analysis were performed to determine the relationship between soil-plant-fruit variables. Data were processed by R software (RStudio 2015) using the FactoMineR and ggplot2 packages (Kahle and Wickham 2013), focusing on the mean based on eigenvalues.

# 3. RESULTS

## 3.1 Microbiological and Chemical Soil Parameters

In the 2021/2022 evaluation season, the treatments with compost applications (5, 10, and 15 t ha<sup>-1</sup>) presented significant difference for FDA activity and basal soil respiration ( $p > 0.05$ ) with respect to the control, increasing 30.6% and 40% respectively (Table 3). Despite these results, there were nonsignificant differences in urease and dehydrogenase enzyme activities with or without compost applications, their average values being 1.14  $\mu\text{mol NH}_4^+ \text{g}^{-1} \text{h}^{-1}$  and 39.32  $\mu\text{g INTF g}^{-1}$ , respectively (Table 3). Acid phosphatase activity was significantly higher at 5 t ha<sup>-1</sup> with respect to 0 and 10 t ha<sup>-1</sup> reaching 22.68  $\mu\text{mol PNP g}^{-1} \text{h}^{-1}$  (Table 3).

Table 3. Soil microbiological properties and enzyme activity in response to compost dosing for the 2021/2022 season

Treatments (t ha <sup>-1</sup> )	Microbial activity ( $\mu\text{g FDA g}^{-1}$ )	Soil basal respiration ( $\mu\text{g CO}_2 \text{g}^{-1} \text{h}^{-1}$ )	Urease activity ( $\mu\text{mol NH}_4^+ \text{g}^{-1} \text{h}^{-1}$ )	Dehydrogen ase activity ( $\mu\text{g INTF g}^{-1}$ )	Acid phosphatase activity ( $\mu\text{mol PNP g}^{-1} \text{h}^{-1}$ )
0	31.7 ± 1.94 b*	0.6 ± 0.05 b	1.2 ± 0.04 a	37.5 ± 6.67 a	16.4 ± 1.33 b
5	49.8 ± 5.45 a	1.0 ± 0.09 a	1.1 ± 0.04 a	39.8 ± 2.61 a	22.7 ± 1.84 a
10	47.5 ± 3.17 a	1.0 ± 0.10 a	1.1 ± 0.03 a	37.9 ± 4.30 a	16.0 ± 1.68 b
15	39.9 ± 3.45 ab	1.0 ± 0.11 a	1.2 ± 0.05 a	42.2 ± 1.38 a	18.0 ± 1.18 ab
<b>Anova p values</b>	0.0193	0.009	0.203	0.6589	0.0335

Different letters indicate significant differences between treatments according to Fischer's LSD test ( $p < 0.05$ ). FDA, fluorescein diacetate activity. Mean ± standard error (n = 4)

All the compost treatments generated higher availability of macronutrients such as N, P, and K, with respect to the control, likewise, they generated higher cation exchange capacity (CEC) with respect to the control. Soil organic matter (OM) increased 3% with 5 and 10 t ha<sup>-1</sup> compost treatments respect to the control and decreased 3% with 15 t ha<sup>-1</sup> respect to the control. On the other hand, when no compost was applied to the soil (control), it had a lower pH compared to the compost-treated soil (Table 4).

Table 4. Chemical analysis of the soil (0-20 cm) affected by the compost treatments at the end of the study (2022/2023)

Analysis	Unit	Treatments (t ha <sup>-1</sup> )			
		0	5	10	15
OM	%	9.32	9.67	9.61	9.06
pH (water)		6.46	6.55	6.73	6.71
Nitrate (N-NO <sub>3</sub> <sup>-</sup> )	mg kg <sup>-1</sup>	6.80	10.20	14.50	8.10
Ammonium (N-NH <sub>4</sub> <sup>+</sup> )	mg kg <sup>-1</sup>	4.60	6.10	7.30	7.80
N availability	mg kg <sup>-1</sup>	11.30	16.30	21.80	16.00
Olsen P	mg kg <sup>-1</sup>	8.40	14.50	17.10	17.60
K availability	mg kg <sup>-1</sup>	223.60	309.50	226.60	272.60
S availability	mg kg <sup>-1</sup>	29.70	42.60	24.10	31.00
Exchangeable Ca	cmol <sub>c</sub> kg <sup>-1</sup>	9.23	10.48	11.52	10.01
Exchangeable Mg	cmol <sub>c</sub> kg <sup>-1</sup>	3.81	3.33	3.99	4.33
Exchangeable K	cmol <sub>c</sub> kg <sup>-1</sup>	0.57	0.79	0.58	0.70
Exchangeable Na	cmol <sub>c</sub> kg <sup>-1</sup>	0.34	0.40	0.35	0.44
Sum of bases	cmol <sub>c</sub> kg <sup>-1</sup>	13.96	15.00	16.44	15.48
Interchangeable Al	cmol <sub>c</sub> kg <sup>-1</sup>	0.02	0.02	0.01	0.01
CEC	cmol <sub>c</sub> kg <sup>-1</sup>	13.97	15.02	16.45	15.49
Al saturation	%	0.11	0.10	0.09	0.09
K saturation	%	4.10	5.28	3.53	4.51
Ca saturation	%	66.06	69.78	70.01	64.59
Mg saturation	%	27.30	22.17	24.23	27.97

CEC: cation exchange capacity

### 3.2 Plant Physiological Parameters

In the 2021/2022 season, leaf indices, leaf area (Fig. 3a) and chlorophyll (Fig. 3b) did not differ among treatments ( $p > 0.05$ ), with an average value of 2 m<sup>2</sup> m<sup>-2</sup> and 4.6 SPAD, respectively. However, in the 2022/2023 season, 10 t ha<sup>-1</sup> showed an increase of 27.8% and 41.5% in leaf area and chlorophyll indices, respectively, compared to the other treatments (including control).

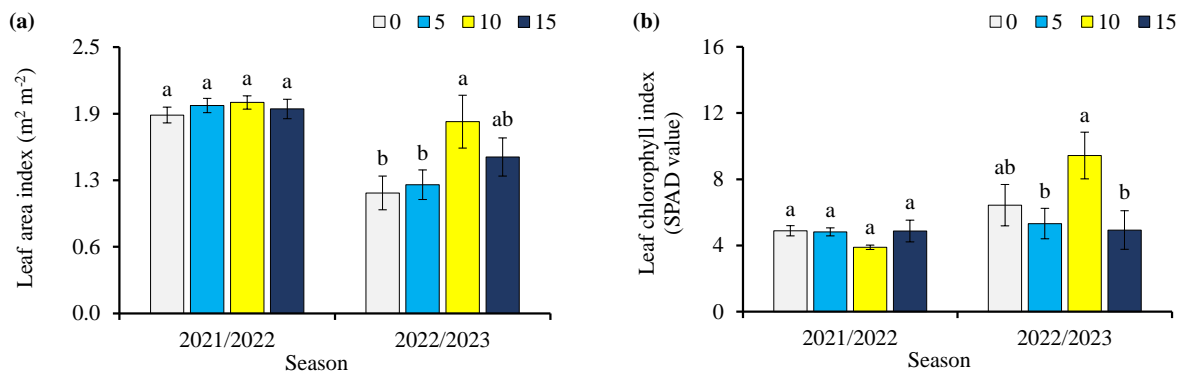


Figure 3. Leaf area index (a) and leaf chlorophyll index (b) in calafate plants evaluated in the 2021/2022 and 2022/2023 seasons. Compost treatments: 0, 5, 10, and 15 t ha<sup>-1</sup>. Different letters indicate significant differences between treatments according to Fischer's LSD test ( $p < 0.05$ ). Mean  $\pm$  standard error ( $n = 4$ ). The bars correspond to the experimental error for each treatment

In both evaluation seasons (2021/2022 and 2022/2023), Fv/Fm values were not affected by the compost dose (Fig. 4a, b). In all treatments, there was a decreasing trend as the hours of measurement progressed, which was more noticeable in the 2021/2022 season, when the average values at 09:00, 12:00, 15:00, and 18:00 h were 0.81; 0.77; 0.74, and 0.73, respectively.

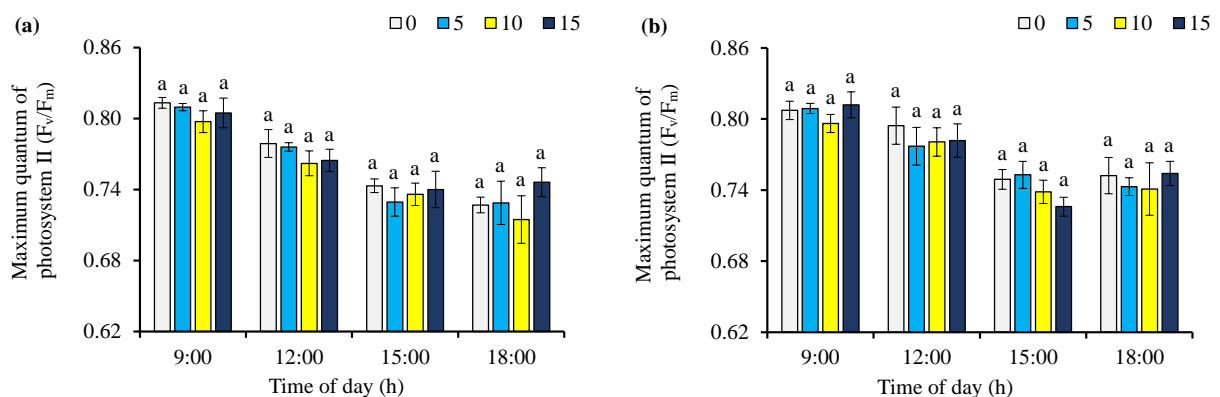


Figure 4. Variation of maximum quantum yield of photosystem II (Fv/Fm) in calafate plants seasons 2021/2022 (a) and 2022/2023 (b); evaluated at different times of the day: 09:00, 12:00, 15:00, and 18:00 h. Compost treatments: 0, 5, 10, and 15 t ha<sup>-1</sup>. Different letters indicate significant differences between treatments according to

Fischer's LSD test ( $p < 0.05$ ). Mean  $\pm$  standard error ( $n = 4$ ). The bars correspond to the experimental error for each treatment

In the 2021/2022 season, there were significant differences only at 12:00 h, when 15 t  $\text{ha}^{-1}$  produced the highest stomatal conductance (gS) with  $177 \text{ mmol m}^{-2} \text{ s}^{-1}$  and 10 t  $\text{ha}^{-1}$  treatment the lowest gS with  $96 \text{ mmol m}^{-2} \text{ s}^{-1}$  (Fig. 5a). In the 2022/2023 season, compost dose did not affect gS ( $p > 0.05$ ) (Fig. 5b). During the 2021/2022 season, a decrease in gS was observed as measurement hours progressed, where at 09:00, 12:00, 15:00, and 18:00 h, gS values were 191, 134, 126, and  $100 \text{ mmol m}^{-2} \text{ s}^{-1}$ , respectively.

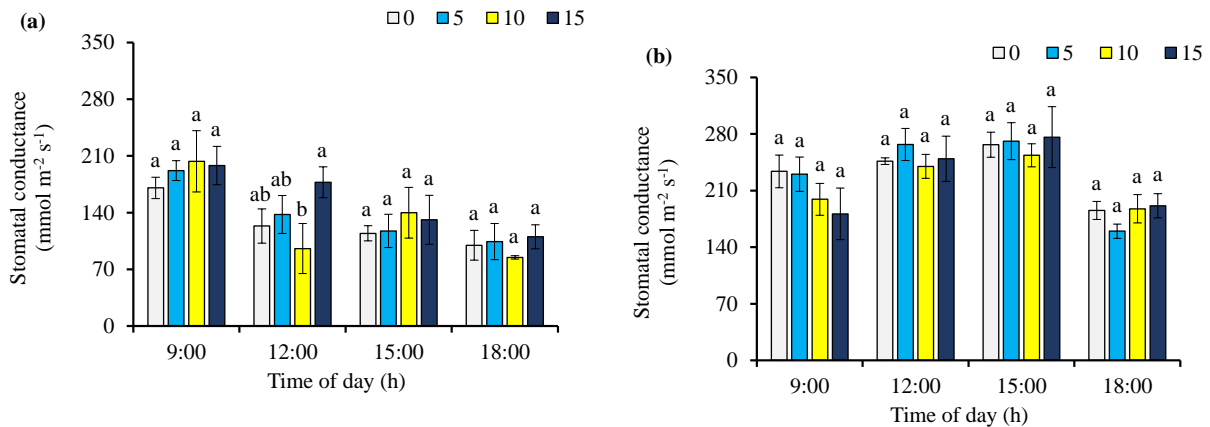


Figure 5. Values recorded for stomatal conductance ( $\text{mmol m}^{-2} \text{ s}^{-1}$ ) in calafate plants seasons 2021/2022 (a) and 2022/2023 (b); evaluated at different times of the day: 09:00, 12:00, 15:00, and 18:00 h. Compost treatments: 0, 5, 10, and 15 t  $\text{ha}^{-1}$ . Different letters indicate significant differences between treatments according to Fischer's LSD test ( $p < 0.05$ ). Mean  $\pm$  standard error ( $n = 4$ ). The bars correspond to the experimental error for each treatment

### 3.3 Yield and Fruit Physical Parameters

In the 2021/2022 season, 15 t  $\text{ha}^{-1}$  treatment generated the highest yield with  $0.93 \text{ t ha}^{-1}$  and the control produced the lowest yield with  $0.31 \text{ t ha}^{-1}$  (Fig. 6a). The fruit physical parameters evaluated, fruit weight (Fig. 6b), equatorial diameter (Fig. 6c),

and polar diameter (Fig. 6d), did not show significant differences with compost dose. In the 2022/2023 season, the highest yields were for 15 and 10 t ha<sup>-1</sup> treatments, with values of 3.8 and 3.6 t ha<sup>-1</sup>, respectively. The control with 1.2 t ha<sup>-1</sup> was the lowest yielding (Fig. 6a). The 15 t ha<sup>-1</sup> treatment produced heavier fruit (Fig. 6b) and larger equatorial diameter (Fig. 6c) than the other treatments. Likewise, it presented greater polar diameter with respect to the 5 t ha<sup>-1</sup> treatment (Fig. 6d).

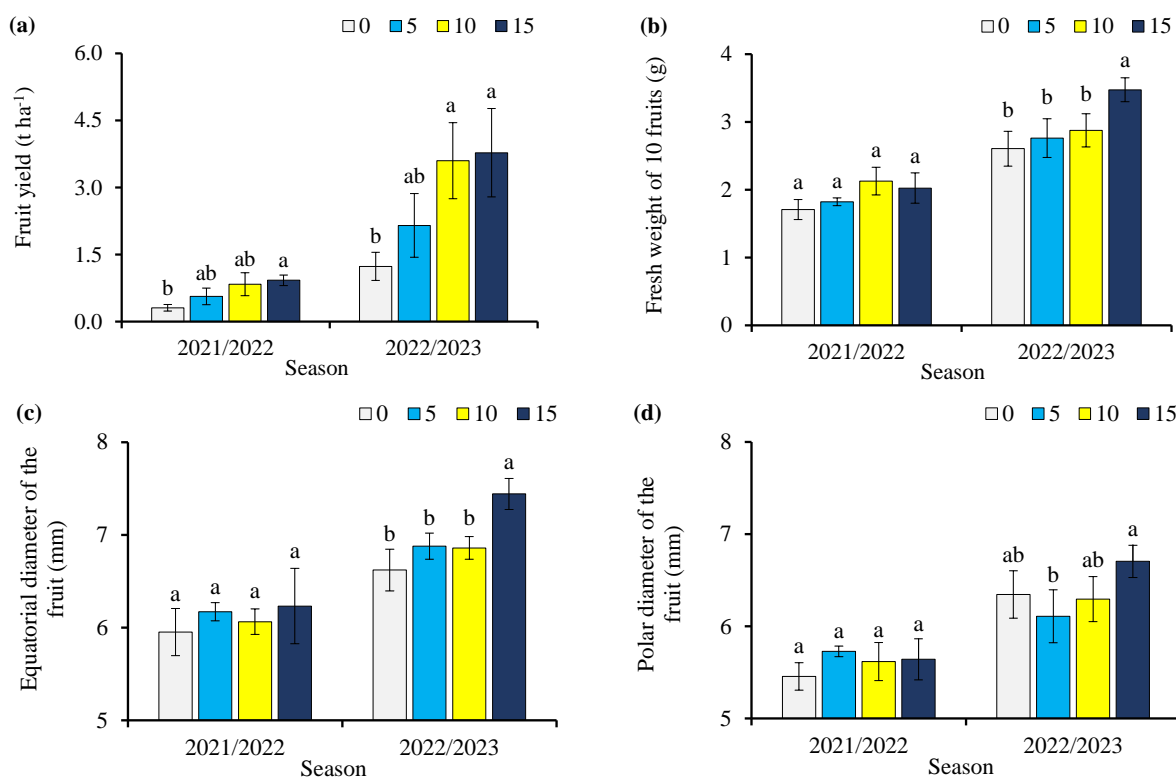


Figure 6. a Average fresh fruit yield of calafate for 2021/2022 and 2022/2023 seasons; b average weight of 10 fresh fruit for 2021/2022 and 2022/2023 seasons; c average equatorial diameter of fresh fruit for 2021/2022 and 2022/2023 seasons; d average polar diameter of fresh fruit for 2021/2022 and 2022/2023 seasons. Compost treatments: 0, 5, 10, and 15 t ha<sup>-1</sup>. Different letters indicate significant differences between treatments according to Fischer's LSD test (p < 0.05). Mean ± standard error (n = 4). The bars correspond to the experimental error for each treatment

### 3.4 Soluble Solids and Antioxidant Properties of Fruit

The chemical results of the fruit in the first evaluation season (2021/2022) showed that there was a higher soluble solids content with the control treatment (41°Brix), compared to the treatments of 10 and 15 t ha<sup>-1</sup> with 33 and 32°Brix, respectively (Fig. 7a).

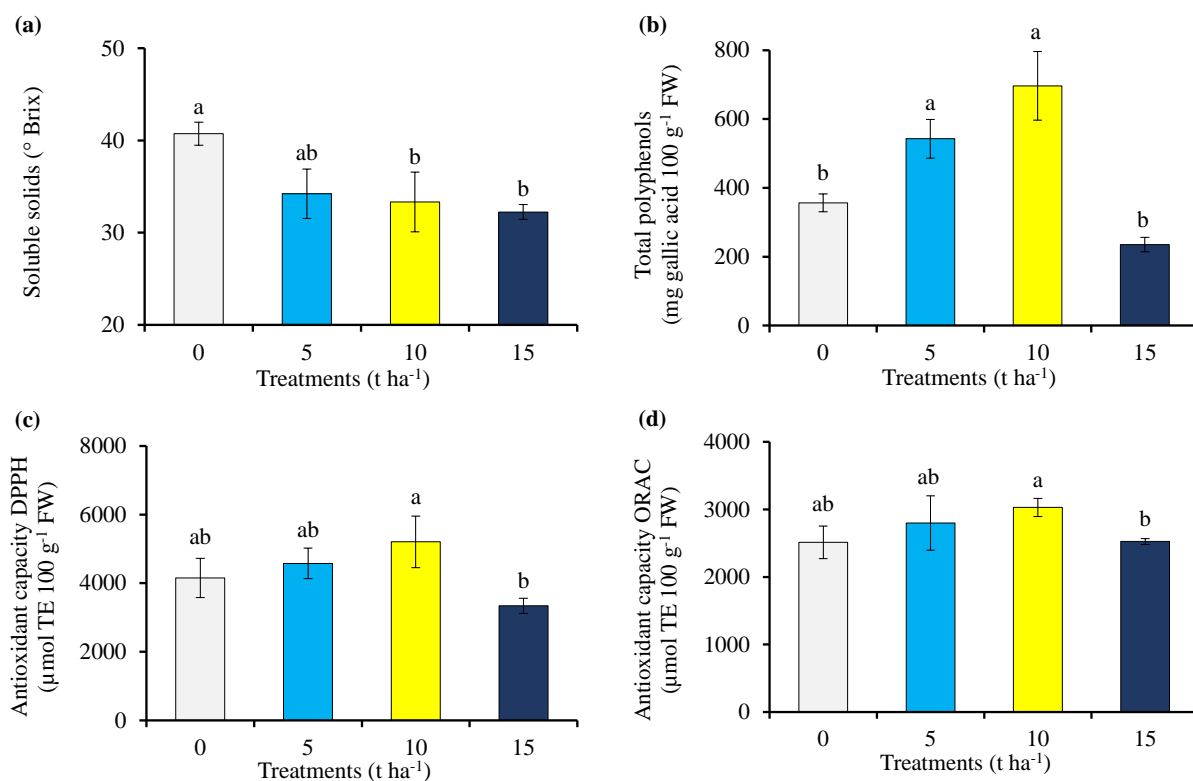


Figure 7. a Soluble solids (°Brix); b total polyphenols; c DPPH antioxidant capacity; and d ORAC antioxidant capacity determined from fresh fruit of calafate plants for the 2021/2022 season. Compost treatments: 0, 5, 10, and 15 t ha<sup>-1</sup>. Different letters indicate significant differences between treatments according to Fischer's LSD test ( $p < 0.05$ ). Mean  $\pm$  standard error ( $n = 4$ ). The bars correspond to the experimental error for each treatment

Total polyphenols were significantly higher for treatments 5 and 10 t ha<sup>-1</sup> with 543 and 697 mg gallic acid 100 g<sup>-1</sup> FW, respectively, and lower with treatments 0 and 15 t ha<sup>-1</sup> with 356 and 235 mg gallic acid 100 g<sup>-1</sup> FW, respectively (Fig. 7b). The

antioxidant capacity DPPH and ORAC was significantly higher with 10 t ha<sup>-1</sup> treatment with 5207 and 3029 µmol TE 100 g<sup>-1</sup> FW, respectively, and lower with 15 t ha<sup>-1</sup> with 3342 and 2526 µmol TE 100 g<sup>-1</sup> FW, respectively. The antioxidant capacity in the 0 and 5 t ha<sup>-1</sup> treatments did not present significant differences with the other treatments (Fig. 7c, d).

Total anthocyanin content (Table 5) was significantly superior ( $p < 0.05$ ) with 5 t ha<sup>-1</sup> compost with 862 mg 100 g<sup>-1</sup> FW. The 10 t ha<sup>-1</sup> compost treatment was significantly superior to the no compost treatment with 479.6 mg 100 g<sup>-1</sup> FW. The 15 t ha<sup>-1</sup> compost treatment, with 253.8 mg 100 g<sup>-1</sup> FW, showed nonsignificant differences with the 0 and 10 t ha<sup>-1</sup> treatments. Regarding the anthocyanin composition, it was observed that the anthocyanins delphinidin, petunidin and malvidin 3-glucoside represented 86%, 84%, 85%, and 85%, respectively, in compost treatments 0, 5, 10, and 15 t ha<sup>-1</sup>.

Table 5. Anthocyanins (mg 100 g<sup>-1</sup>) of fresh calafate fruit by HPLC according to compost dose for the 2021/2022 season

Anthocyanins	Treatments (t ha <sup>-1</sup> )				Anova <i>p</i> values
	0	5	10	15	
Delphinidin 3,3-dihexoside	2.2 ± 0.6 b*	6.6 ± 2.2 a	4.2 ± 0.8 ab	2.0 ± 0.6 b	0.0080
Petunidin 3,5-dihexoside	6.8 ± 1.4 b	21.4 ± 4.0 a	12.0 ± 2.6 b	5.4 ± 0.8 b	0.0038
Malvidin 3,5-dihexoside	4.8 ± 0.2 c	16.6 ± 3.2 a	11.0 ± 1.4 b	5.8 ± 1.0 c	0.0021
Delphinidin 3-glucoside	79.8 ± 17.8 b	329.6 ± 73.6 a	188.2 ± 26.8 b	95.4 ± 15.2 b	0.0037
Delphinidin 3-rutinoside	0.2 ± 0.02 b	6.8 ± 1.6 a	4.6 ± 1.2 a	1.0 ± 0.2 b	0.0032
Cyanidin 3-glucoside	9.0 ± 1.2 c	53.0 ± 7.6 a	24.2 ± 4.4 b	15.8 ± 2.8 bc	0.0002
Petunidin 3-glucoside	49.0 ± 7.8 c	227.6 ± 43.0 a	128.2 ± 12.8 b	71.4 ± 6.6 bc	0.0006
Petunidin 3-rutinoside	0.6 ± 0.2 c	8.0 ± 2.2 a	4.4 ± 0.8 ab	1.6 ± 0.4 bc	0.0039
Peonidin 3-glucoside	2.0 ± 0.4 c	26.0 ± 1.6 a	12.6 ± 2.6 b	7.2 ± 2.0 bc	0.0001
Malvidin 3-glucoside	25.6 ± 4.4 c	166.4 ± 33.2 a	90.2 ± 13.4 b	48.2 ± 3.2 bc	0.0008
Total anthocyanins	180.0 ± 34.0 c	862.0 ± 172.2 a	479.6 ± 66.8 b	253.8 ± 32.8 bc	0.0005

Different letters indicate significant differences between treatments according to Fischer's LSD test ( $p < 0.05$ ). Mean ± standard error (n = 4)

### 3.5 Soil–Plant–Fruit Parameter Interaction

PCA was carried out on 19 variables from soil, plant, and fruit (Fig. 8). The principal components PC1 and PC2 retained 20.89% and 17.96% of the variance, respectively. In the biplot, each parameter was represented as a vector, the length of which indicates its importance in the analysis (Fig. 8a). Treatments were represented by numbers in the PCA (Fig. 8b): 1–4 for 0 t ha<sup>-1</sup> treatment, 5–8 for 5 t ha<sup>-1</sup>, 9–12 for 10 t ha<sup>-1</sup> and 13–16 for 15 t ha<sup>-1</sup>.

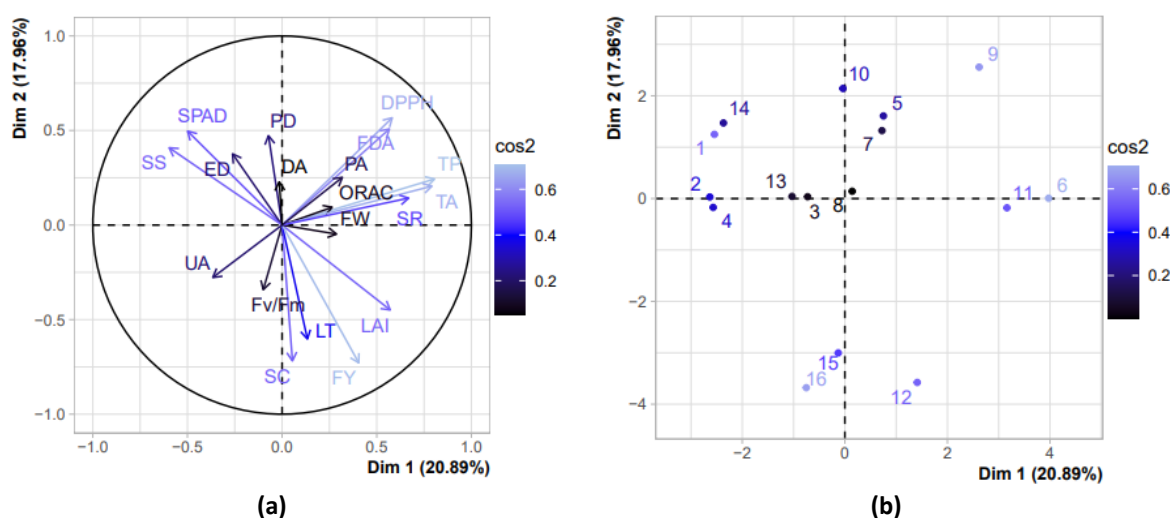


Figure 8. PCA of soil microbiological, plant physiological and physicochemical variables of fruit for the 2021/2022 season. Soil microbiological activity: fluorescein diacetate activity (FDA); soil respiration (SR); urease activity (UA), dehydrogenase activity (DA); acid phosphatase activity (PA); leaf temperature (LT); leaf area index (LAI); chlorophyll index (SPAD); stomatal conductance (SC); maximum quantum yield of photosystem II (Fv/Fm); fruit productivity (FY); fruit equatorial diameter (ED); fruit polar diameter (PD); fruit fresh weight (FW); soluble solids (SS); total polyphenols (TP); DPPH antioxidant activity (DPPH); ORAC antioxidant activity (ORAC); total anthocyanins (TA). a PCA of variables; b PCA of individuals

To obtain a complete picture of soil biological parameters, plant physiological parameters and bioactive compounds of the calafate fruit, correlations between the different parameters were analyzed and the obtained Pearson correlation coefficients

(r) were used to plot the correlation (Fig. 9). Moderate correlations ( $r > 0.6$  or  $r < -0.6$ ) were observed between soil biological indicators and fruit chemical attributes. There were moderate correlations between plant physiological parameters and fruit yield ( $r > 0.6$  or  $r < -0.6$ ). Likewise, moderate correlations ( $r > 0.6$  or  $r < -0.6$ ) were observed between physical and chemical parameters of the fruit.

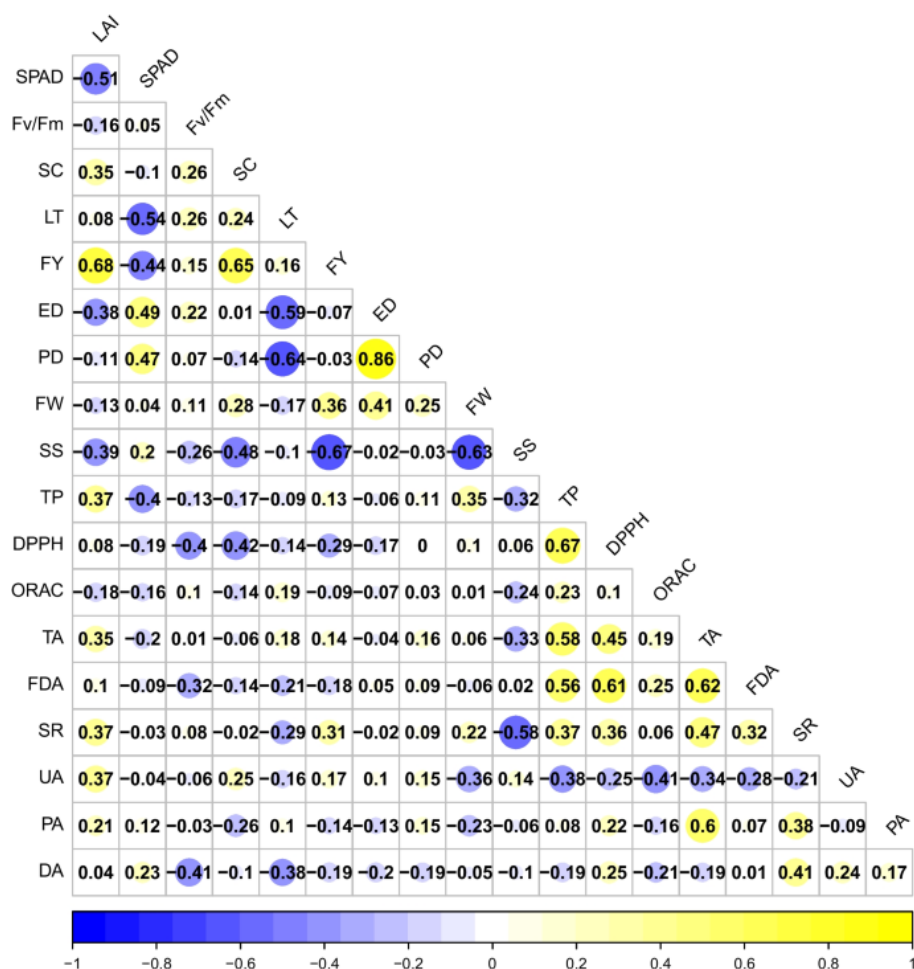


Figure 9. Correlation matrix for soil variables, plant physiological parameters, yield parameters and fruit bioactive compounds for the 2021/2022 season. Leaf area index (LAI); chlorophyll index (SPAD); maximum photosystem II efficiency (Fv/Fm); stomatal conductance (SC); leaf temperature (LT); fruit productivity (FY); fruit equatorial diameter (ED); fruit polar diameter (PD); fruit fresh weight (FW); soluble solids (SS); total polyphenols (TP); DPPH antioxidant activity (DPPH); ORAC

antioxidant activity (ORAC); total anthocyanins (TA); soil microbiological activity: fluorescein diacetate activity (FDA); soil respiration (SR); urease activity (UA), acid phosphatase activity (PA); dehydrogenase activity (DA). The size and color of the circles represent the correlation coefficients of the indicators

#### **4. DISCUSSION**

The addition of compost increased soil microbial activity and basal respiration and was dependent on compost dose applied to the soil. Earlier research has proposed that these enhancements arise from compost amendments, which stimulate the introduction of active microbial communities while simultaneously encourage the biostimulation of native microorganisms (Adejoro et al. 2018; Azizah et al. 2023; Heisey et al. 2022). Similar results have been reported in avocado and apple orchards, where compost favored the growth of soil bacterial communities (Bonilla et al. 2012; Peck et al. 2011). In addition, a similar trend was evidenced in potted blueberry with the addition of compost, although with higher FDAse values, due to lower compost losses and greater homogenization of the compost with the soil in the pots compared to our field study (Montalba et al. 2010). The notably higher basal respiration values observed in the compost-treated samples, in comparison to the control, imply a significant contribution by soil microorganisms to the decomposition of organic matter (OM) through mineralization, utilizing C as an energy source and thereby increasing biomass (Luu et al. 2022; Rastogi et al. 2020). Although our research did not encompass an evaluation of soil physical parameters, it was evident that there was reduced OM loss in the 5 and 10 t ha<sup>-1</sup> compost treatments right from the establishment phase. This could have concurrently fostered improvements in soil structure (Sayara et al. 2020), thus creating a microenvironment favorable for microbial activity. This is critical because soil OM content is determined not only by biological factors but also by physical and chemical factors (Sayara et al. 2020).

In our study, the evaluation of enzymatic activities revealed that compost application did not significantly impact urease and dehydrogenase enzyme activity.

These results differ from previous studies in which an increase in enzyme activity was observed with the addition of compost (Ouyang et al. 2018; Zhou et al. 2022). It is worth noting that the elevated levels of ammonium and nitrate found in the compost treatments might have repressed the microbial synthesis required for the formation of these enzymes (Bohacz 2019). The addition of compost had no influence on the activity of dehydrogenase, which is responsible for the oxidation of organic C in the soil (Sun et al. 2017). This suggests that the high levels of organic matter present in all treatments are sufficient to maintain the activity of this enzyme in the soil, compared to soils with low fertility or degraded soils that show a high response of this enzyme to the addition of organic matter through compost (Ouni et al. 2013; Panettieri et al. 2022). We observed changes in acid phosphatase activity, which is an essential enzyme in the mineralization of organic P, which was higher in the treatment with 5 t ha<sup>-1</sup> that produced the most acidic pH, which plays an important role in the response of this enzyme (Nannipieri et al. 2012). Based on these results, these findings suggest that, in the short term, the overall soil microbiological responses can be enhanced by applying 5 and 10 t ha<sup>-1</sup> compost doses. This was because soil microbial activity did not systematically coincide with enzyme activity, as evidenced by the weak correlations observed in our study.

In the assessment of foliar indices, it was evident that fertilizing with compost at a rate of 10 t ha<sup>-1</sup> elicited a notably heightened response, particularly during the second season. This response could be attributed to the heightened availability of essential nutrients, namely, N and P, within the soil. It should be noted that N and P are constituents of important organs such as stems and leaves in the calafate plant (Ojeda et al. 2017), which would be favoring the vegetative development of the plant. In the soil, N availability, besides being fundamental for plant growth, is relevant for physiological processes such as photosynthesis (Guo et al. 2019). Coinciding with these findings, Arena et al. (2020) demonstrated that increasing the availability of nutrients in the soil through fertilization increased the uptake by the calafate plant, which led to a greater synthesis of photosynthetic pigments such as chlorophylls and carotenoids, which supports the results obtained in this study. Furthermore, our

results are in agreement with other studies indicating that compost application promotes plant vegetative development and increases total chlorophyll content in leaves (Hasnain et al. 2020). For example, in an apple orchard, compost increased tree biomass compared to a soil without compost and even compared to a soil fertilized with N (Thompson et al. 2019). Negative yet moderately significant correlations between the chlorophyll index and leaf temperature suggest that environmental conditions play a pivotal role in shaping structural and physiological alterations within plants (Arena et al. 2020). Within the scope of our study, the application of 10 t ha<sup>-1</sup> of compost triggered a substantial upswing in plant growth. This can be attributed to the enhanced soil fertility fostered by the favorable microbiological conditions created by the compost. However, it is worth noting that this increase in compost dosage did not induce significant alterations in physiological parameters, as exemplified by the nonsignificant changes in maximum quantum yield of photosystem II and stomatal conductance. In both cases, a decreasing trend was observed as the hour of measurement progressed, which is in agreement with previous findings reported by Betancur et al. (2022) in cultivated calafate and with other species such as blueberry (Retamal-Salgado et al. 2023). This decrease can be attributed to the increase of climatic factors such as solar radiation and daytime temperature, which are more intense during the summer, which corresponded to the time of measurement (January). In our study, the maximum quantum yield of photosystem II values recorded, which would demonstrate that the plants show a low degree of stress and that at their maximum point of light saturation photosynthetic rates would remain constant (Betancur et al. 2022).

As for stomatal conductance, it only showed significant variations at 12:00 h during the first season. However, no consistent trend was observed at different measurement times or between seasons. In species such as blueberries, it has been determined that stomatal conductance depends not only on the fertilization dose but also on other factors such as water replenishment rates (Guo et al. 2021), which was not a limiting factor in our study, constant irrigations were applied for all treatments. It should be noted that stomatal conductance varied between seasons, with higher

average values recorded in the second season, which would indicate a greater acclimatization capacity of this species over time, through changes in leaf composition and structure (Radice et al. 2018). The findings indicate that the application of 10 t ha<sup>-1</sup> of compost appears sufficient to enhance plant growth, photosynthesis, and transpiration processes in calafate plants, which exhibit remarkable adaptation to the local climate.

At the fruit level, compost doses of 10 and 15 t ha<sup>-1</sup> significantly improved performance. During the two evaluation seasons, 15 t ha<sup>-1</sup> dose of compost resulted in higher fruit yield, possibly due to higher N extraction by the plant, as soil N levels decreased in this treatment at the end of the study. It has been evidenced that short-term fruit yield can increase significantly due to higher uptake of available soil N, which is also reflected in higher fruit N concentrations (Carranca et al. 2018). Notably, during the second evaluation season, the compost dose of 10 t ha<sup>-1</sup>, which presented a greater leaf area, produced a yield similar to that obtained with 15 t ha<sup>-1</sup>, mainly due to a greater number of fruits and not necessarily to fruits of greater size or weight. These results clearly demonstrate that organic amendments can improve tree vigor and consequently fruit yield, either through improved nutrient availability and access, or through beneficial changes in soil biology and root-microbe interactions (Chatzistathis et al. 2021). Therefore, soil fertility plays a crucial role in plant productivity (Mohamed et al. 2021; Wen et al. 2021). In our study, the doses with the highest productive response (10 and 15 t ha<sup>-1</sup>) increased the availability of bases such as Na, Ca, and Mg, as well as CEC in the soil, fundamental elements for the formation of calafate fruit (Ojeda et al. 2017). On the other hand, the dose of 5 t ha<sup>-1</sup> did not improve fruit yield compared to the control, despite improvements in soil fertility. These results are in agreement with previous studies that have indicated that the use of organic amendments in young orchards, while it can improve soil quality, does not always increase productivity, although it can increase long-term productivity potential (Chatzistathis et al. 2021; Sas-Paszt et al. 2014). It is noteworthy to highlight that, in our study, a significant increase in yield was observed across all treatments as the second season approached. This increase suggests an enhancement in the

plant's physiological functions, possibly attributed to its adaptability, as indicated in the research by Arena et al. (2020). Our results indicate that regardless of the evaluation season, the application of compost to the soil at doses of 10 and 15 t ha<sup>-1</sup> significantly improves the yield of calafate compared to a soil without compost.

In relation to fruit soluble solids, a reduction was noted with compost doses of 10 and 15 t ha<sup>-1</sup>. This decline can be attributed to the increased fruit production, which, in turn, leads to sugar dilution (Chen et al. 2020). However, under these same doses we found contrasting effects on phenolic compounds and antioxidant activity. In other species such as strawberries and rhubarb compost has been observed to improve fruit bioactive compounds compared to unfertilized soil (Cojocarú et al. 2020; Mohamed et al. 2021). However, the decrease observed with the 15 t ha<sup>-1</sup> dose would indicate a dilution of its compounds due to the higher yield obtained in the first season of evaluation and even higher in the second season. Despite the fact that the treatment with 15 t ha<sup>-1</sup> compost produces fruit with a lower antioxidant capacity, it is still superior to that of other berries such as blueberry (Rodoni et al. 2014). In our study, total anthocyanin contents were higher than those previously reported in calafate grown on plants of the same planting year and vegetative development, even though the yields obtained in this study were 200% higher than the yields of the aforementioned study; this shows that in the calafate species evaluated in this study, there is a greater impact of the soil nutrient rate than the water replenishment rate (species tolerant to water deficit), both in plant development and fruit quality (Betancur et al. 2022). The three predominant glycoside-conjugated anthocyanins were delphinidin, petunidin, and malvidin, which is consistent with that reported in other berries such as grapes (De Oliveira et al. 2015). Specifically, delphinidin, the anthocyanin with the highest antioxidant action, had a lower content in the treatment with 15 t ha<sup>-1</sup> compost. This is possibly due to the low stability of anthocyanins to external factors such as light and temperature, which were probably more intense when the fruits had a higher water content, resulting in a decrease in pH and the production of lighter colored fruits (Torres et al. 2022).

Our results show that the compost doses 10 and 15 t ha<sup>-1</sup>, although decreasing the anthocyanin content, could prolong the fruit maturity period and its harvest window. In addition, the treatment 5 t ha<sup>-1</sup> improves anthocyanin content, which are responsible for sensory characteristics such as color in the calafate fruit (Romero-Román et al. 2021) and used as natural colorants. However, it is important to note that calafate grown without compost produces fruit with an antioxidant capacity equal to plants treated with compost, which provides health benefits by helping to scavenge reactive oxygen radicals and counteract oxidative stress (Fredes et al. 2020).

Our study revealed significant correlations between soil microbiological parameters and fruit chemistry. Specifically, we found a notable correlation between soil microbial biomass and fruit DPPH antioxidant capacity. This correlation confirms that soil microorganisms play a pivotal role in enhancing plant productivity and influencing the phenolic composition of the fruit (Betancur et al. 2023). Consequently, our results underscore the importance of evaluating sustainable agriculture strategies and their impact on chemical and biological soil properties and the production of bioactive compounds in calafate fruit.

## **5. CONCLUSIONS**

Compost amendment improved soil health and phenolic compounds in calafate fruit. The dose of 10 t ha<sup>-1</sup> of compost was the most effective in enhancing basal respiration and soil microbial activity when compared to the control. It also resulted in notable enhancements in leaf area and chlorophyll indices of the plants, consequently leading to a higher fruit yield and antioxidant capacity compared to the control. Our study also revealed correlations between soil and fruit biological parameters, highlighting that the yield of calafate is intricately linked to improvements in soil biology resulting from compost application. We recommend that future studies on calafate consider the integration of soil biological parameters for a more comprehensive understanding of soil health.

## **Contributions**

Conceptualization, M.S., J.R.-S., and M.D.L.; methodology, R.V-R., M.S., J.R.-S., M.B., and M.D.L.; software, M.B. and M.D.L.; validation, M.S., J.R.-S., and M.D.L.; formal analysis, M.S., J.R.-S., and M.B.; investigation, R.V-R., M.B., M.S., J.R.-S., and M.D.L.; resources, M.S., M.D.L., and J.R.-S.; data curation, M.B., M.S., J.R.-S., and M.D.L.; writing—original draft preparation, M.B., M.S., J.R.-S., and M.D.L.; writing—review and editing, J.R.-S., M.S., and M.D.L.; project administration, M.S. and J.R.-S.; funding acquisition, R.V-R., M.S., J.R.-S., and M.D.L. All authors have read and agreed to the published version of the manuscript.

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## **Data Availability**

The data presented in this study are available in the article.

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### **Competing Interests**

The authors declare no competing interests.

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## V. CAPÍTULO IV: PLANTING DENSITY: KEY STRATEGY FOR OPTIMIZING SOIL HEALTH AND FRUIT ANTIOXIDANT ACTIVITY IN A CALAFATE ORCHARD

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### ABSTRACT

Limited information exists regarding the impact of planting density on soil fertility, plant productivity, and the phenolic content of calafate (*Berberis microphylla* G. Forst.) fruits. This study aimed to assess the effects of planting density on the soil, plants, and fruits in a calafate orchard. Four treatments were employed: High-density (HD; 6667 plants ha<sup>-1</sup>), traditional density (TD; 3333 plants ha<sup>-1</sup>), medium density (MD; 2222 plants ha<sup>-1</sup>), and low density (LD; 1667 plants ha<sup>-1</sup>). In the soil, TD exhibited a 28% increase in basal respiration compared with MD, and a 29% increase in urease compared with HD. Additionally, TD enhanced soil N availability by 57%,

ammonium availability by 58%, and Mn availability by 33% compared to LD. Photosystem II experienced an increase with MD and LD ( $p > 0.05$ ), surpassing TD and HD by 3%. The LD significantly outperformed the other treatments in terms of yield per plant, reaching up to 873 g. Conversely, despite its lower yield per plant, HD produced larger and heavier fruits, albeit at the expense of phenolic content. However, HD and MD, averaging 2.1 t ha<sup>-1</sup>, generated more fruit per unit area than LD and TD did. Notably, planting density did not affect the fruit antioxidant capacity. These findings suggest that TD planting density in calafate preserves the biological and chemical functions of the soil, while maintaining the antioxidant capacity of calafate fruit.

**Keywords:** Basal respiration; *Berberis microphylla*; berberis; berries; polyphenols; stomatal conductance

## 1. INTRODUCTION

The calafate (*Berberis microphylla* G. Forst.) is a shrub belonging to the genus *Berberis* and the family Berberidaceae, flourishing in the wild landscapes of Chile and Argentina. Purplish-black berries, utilized since ancient times, play a crucial role in the crafting of diverse food products. In particular, they are distinguished by their sweetness and phenols with antioxidant properties that exceed up to four times those of blueberries (Rodoni et al., 2014). Recent studies have highlighted the pharmacological and nutritional potential of calafate berries in vivo, with substantial improvements in blood glucose tolerance (Soto-Covasich et al., 2020).

In recent years, the scarcity of calafate has spurred the nascent cultivation in southern Chile, encompassing an area of 0.24 ha (CIREN-ODEPA, 2022). Additionally, an organic orchard dedicated to research and focused on the domestication of calafate was established in south-central Chile, covering an area of 0.15 ha (Pinto-Morales et al., 2022). These emerging orchards, managed by both conventional and organic practices, have embraced conventional planting densities.

This density, similar to that used for berries, such as blueberries, implies a 3 m row spacing and a plant spacing of approximately 1 m. However, the optimum planting density that improves biological and chemical soil conditions, encourages plant development, and amplifies the distinctive phenolic content of the fruit remains unknown.

Establishing an optimal density in orchards is crucial for fostering soil health, encompassing both chemical and biological properties, such as inorganic nutrients and microbial communities that enhance agricultural systems (Wang et al., 2019). This is achieved by optimizing resource distribution and minimizing interplant competition, thereby amplifying the performance of each individual plant. However, the appropriateness of density requires tailored evaluation for each fruit species, as exemplified in olive orchards, where, higher densities (401-1500 plants ha<sup>-1</sup>) have demonstrated an increase in soil biodiversity and an enhancement of N balance compared to lower densities (201-400 plants ha<sup>-1</sup>) (Sobreiro et al., 2023). Furthermore, a high-density of 1850 plants ha<sup>-1</sup> resulted in a remarkable 29% increase in soil organic C (SOC) and root biomass compared to a lower density of 300 plants ha<sup>-1</sup> (Gómez et al., 2022). Similarly, in blueberries, high densities result in greater efficiency in the use of water and soil nutrients (Fang et al., 2020).

In recent decades, there has been a trend to establish high-density orchards to optimize early-stage performance and achieve favorable profitability. However, the implications of high planting density require thorough evaluation given its potential adverse impact on plant development. This effect is evident in specific leaf area requirements, for example, in a coffee (*Coffea arabica* L.) orchard planted at a density of 10 000 plants ha<sup>-1</sup>, the leaf area index (LAI) per plant decreased by 9% compared to a density of 6000 plants ha<sup>-1</sup>, with no increase in yield, which remained at 5.7 t ha<sup>-1</sup> (Rakocevic et al., 2021). Furthermore, because yield is closely related to the concentration of soluble solids and phenolic compounds, planting density can affect fruit quality, as happened in olive trees, where yield reduction attributable to increased density increased relative oil content from 16.8% to 18% when the density increased from 179 to 286 plants ha<sup>-1</sup> (Lavee et al., 2012).

In cultivated calafate, soil disturbance alters plant growth and physiology, influencing the productivity and phenolic content of the fruit (Betancur et al., 2023). Consequently, this study was devoted to investigating the impact of different planting densities on the soil, plant, and fruit parameters of calafate grown in south-central Chile. The main objective was to establish a baseline for identifying the most appropriate planting density to ensure the soil health and phenolic content of the calafate. The knowledge acquired in this study can serve as a reference for the strategic establishment of new calafate orchards under sustainable productivity.

## **2. MATERIALS AND METHODS**

### **2.1 Edaphoclimatic characteristics and agronomic management of orchards**

The calafate orchard is located in the Chillán (36°31' S; 71°54' W), Ñuble Region, Chile. The soil of the study site was classified as Melanoxerands and was chemically characterized at the Soil Laboratory of the Instituto de Investigaciones Agropecuarias (INIA): pH 6.4, 9.7% organic matter (OM), 19 mg kg<sup>-1</sup> available N, 15.3 mg kg<sup>-1</sup> available P; 496 mg kg<sup>-1</sup> available K; 8.7 cmol kg<sup>-1</sup> exchangeable Ca; 1.6 cmol kg<sup>-1</sup> exchangeable Mg, 1.3 cmol kg<sup>-1</sup> exchangeable K; 0.01 cmol kg<sup>-1</sup> exchangeable Na and 0.02 cmol kg<sup>-1</sup> exchangeable Al. The climate is temperate Mediterranean, with mean temperatures and cumulative annual precipitation of 13.5 °C and 649 mm by 2021 and, 13.2 °C, and 920 mm by 2022. The orchard was established in 2017 using 2-yr-old plants obtained from seeds from Valdivia, Los Rios Region, Chile. Weed control was performed over the row using a 100 µm thick black polypropylene geotextile mulch (Protekta, Santiago, Chile). Irrigation was carried out annually from August to March, replenishing 100% of the daily potential evapotranspiration (ET<sub>0</sub>) of the crop, according to Betancur et al. (2022).

### **2.2 Experimental trial setup**

In the orchard, which had a total area of 1.5 ha, four planting density treatments were established: High density (HD) with 0.5 m plant spacing and 6667 plants ha<sup>-1</sup>;

traditional density (TD) with 1 m plant spacing and 3333 plants ha<sup>-1</sup>; medium density (MD) with 1.5 m plant spacing and 2222 plants ha<sup>-1</sup>; and low density (LD) with 2 m plant spacing and 1667 plants ha<sup>-1</sup>. An inter-row distance of 3 m was maintained for all the treatments. The statistical design used was a randomized complete block with four treatments and four replicates (n = 16). Each replicate consisted of four plants, with the two central plants that were evaluated independently as the experimental unit. The results obtained from both central plants were averaged for each treatment and replicate.

### 2.3 Chemical and microbiological soil analysis

Soil samples for microbiological and chemical analyses were collected at the end of the study on 10 October 2023, at a depth of 0-30 cm. Soil chemical analysis was performed at the Laboratory of Chemical Analysis of Soils and Plants of the University of Concepción, Chillán, Chile, as described by Sadzawka et al. (2006). For microbiological analyses, the soil adhering to the roots was collected. Subsequently, the samples were homogenized and sieved at 2 mm before being conditioned to 60% of their field capacity.

Soil microbial activity was estimated by fluorescein diacetate (FDAse) activity, as described by Alef and Nannipieri (1995), and basal respiration, as described by Joergensen (1995), expressed as  $\mu\text{g FDA g}^{-1}$  and  $\mu\text{g CO}_2 \text{ g}^{-1} \text{ h}^{-1}$ , respectively. The following enzymatic activities were determined: Urease, acid phosphatase (Nannipieri et al., 1980), and dehydrogenase (Garcia et al., 1997). Urease and phosphatase were expressed as ammonium-N and p-nitrophenol (PNP) units, respectively, per gram of soil (dry weight) and hour ( $\mu\text{mol NH}_4^+ \text{ g}^{-1} \text{ h}^{-1}$  and  $\text{PNP g}^{-1} \text{ h}^{-1}$ ), whereas dehydrogenase was expressed as micrograms of idonitrotetrazolium formazan (INTF) per gram of soil (dry weight) ( $\mu\text{g INTF g}^{-1}$ ).

### 2.4 Foliar and physiological plant measurements

Plant foliar and physiological measurements were made on leaves exposed to the sun and from the second third of the shoot during the season. On each plant, four

subsamples were taken from each of the cardinal points at an average plant height of 1.2 m. Measurements were performed after harvest in December 2021 and 2022.

Leaf temperature (°C) was measured using a portable infrared thermometer CTR1000 (Instrumentos WIKA S.A.U., Barcelona, Spain) four times of the day (09:00, 12:00, 15:00, and 18:00 h). The leaf area index (LAI; m<sup>2</sup> m<sup>-2</sup>) was determined at 12:00 h using a portable ceptometer (AccuPAR LP-80, Decagon Devices, Washington D.C., USA), which averages the readings of 80 quantum sensors to determine direct and residual photosynthetically active radiation from below the plant canopy (Sonnentag et al., 2007). The soil plant analysis development (SPAD) chlorophyll index was measured at 12:00 h using the portable chlorophyll meter kit MC-100 (Apogee Instruments, Logan, Utah, USA) (Cunha et al., 2015).

The maximum photochemical efficiency of photosystem II (Fv/Fm) was calculated using the following relationship:  $Fv/Fm = (Fm - F0)/Fm$ , where, the maximum fluorescence intensity (Fm) as well as the minimum chlorophyll fluorescence intensity (F0) was measured with a portable fluorimeter model OS-5p (Opti-Sciences, Hudson, New Hampshire, USA). Stomatic conductance (mmol m<sup>-2</sup> s<sup>-1</sup>), was measured using portable porometer equipment (SC-1 Decagon Devices). Stomatal conductance and Fv/Fm measurements were performed four times a day (09:00, 12:00, 15:00, and 18:00 h) (Retamal-Salgado et al., 2017).

## 2.5 Physical-chemical measurements of the fruit

The physicochemical parameters of the fruit were measured in December, immediately after manual harvest, 130 d after full bloom (Pinto-Morales et al., 2022). Physical and solid parameters were determined in the 2021 and 2022 seasons, and chemical parameters were determined only in the 2022 season.

Fruit productivity (g plant<sup>-1</sup>) was determined by weighing the total fruit of each plant using an analytical balance (Series 360 ES; Precisa Gravimetrics AG, Dietikon, Switzerland). For the fresh weight of 10 fruits, 10 fruits were randomly selected from the total production per plant (n = 4) on the same analytical balance. The polar and equatorial diameters (mm) of each fruit (n = 10) were measured using a digital meter

foot model E5001002  $\pm$  0.003 mm (Veto y Cia Ltda., Santiago, Chile). The concentration of soluble solids ( $^{\circ}$ Brix) was measured in the sample juice using a digital refractometer (model HI96801  $\pm$  0.2%; Hanna Instruments S.R.L., Woonsocket, Rhode Island, USA) from a random sample of fresh fruit ( $n = 4$ ) from each total yield per plant.

Total polyphenol concentration was determined using the Folin-Ciocalteu method (Singleton and Rossi, 1965) and expressed as mg gallic acid in fresh fruit (mg gallic acid 100  $g^{-1}$  FW). Antioxidant capacity was determined using 2,2-diphenyl-picryl-hydrazyl (DPPH) and oxygen radical absorbance antioxidant capacity (ORAC) (Romero-Román et al., 2021), which were expressed as  $\mu$ mol Trolox equivalents in fresh fruit ( $\mu$ mol TE 100  $g^{-1}$  FW). Individual anthocyanins were identified using high-performance liquid chromatography (HPLC) (Romero-Román et al., 2021) and expressed as mg of fresh fruit (mg 100  $g^{-1}$  FW).

## 2.6 Statistical analysis

The data obtained were analyzed (ANOVA) and compared using Fisher's least significant difference (LSD) test at a significance level of 0.05. The relationship between soil, plant, and fruit variables was analyzed by principal component analysis (PCA) and correlation analysis with R software (Allaire, 2011) using the FactoMineR and ggplot2 packages, focusing on the mean based on eigenvalues.

## 3. RESULTS

### 3.1 Chemical and microbiological soil parameters

The activity of FDAse showed nonsignificant response to planting density, with an average of 42.33  $\mu$ g FDA  $g^{-1}$ . The same lack of response was evidenced in dehydrogenase and acid phosphatase enzymes, which presented an average activity of 85.36  $\mu$ g INTF  $g^{-1}$  and 92.10  $\mu$ mol PNP  $g^{-1} h^{-1}$  respectively. In contrast, basal respiration was 28% higher ( $p < 0.05$ ) in TD and LD than in MD. The HD with a

respiration of  $0.59 \mu\text{g CO}_2 \text{ g}^{-1} \text{ h}^{-1}$  did not present significant differences between treatments. Urease responded to planting density, being favored ( $p < 0.05$ ) by 29% in TD compared to HD (Table 1).

Chemical analysis of the soil at the end of the experiment indicated that planting density significantly influenced soil fertility (Table 2). Specifically, there was a significant increase of 10% in OM with HD compared to LD. Similarly, HD significantly improved Zn by more than 21% with respect to MD and LD. In contrast, TD significantly improved the availability of N (57%), ammonium (58%), and Mn (33%) with respect to LD, and even with this treatment, ammonium was higher than MD (43%) and HD (50%). The other parameters were not influenced ( $p > 0.05$ ) by planting density.

Table 1. Soil microbiological properties and enzyme activity in response to planting density at the end of the study (2023).

Treatments	FDase activity ( $\mu\text{g FDA g}^{-1}$ )	Soil basal respiration ( $\mu\text{g CO}_2 \text{ g}^{-1} \text{ h}^{-1}$ )	Urease activity ( $\mu\text{mol NH}_4^+ \text{ g}^{-1} \text{ h}^{-1}$ )	Dehydrogenase activity ( $\mu\text{g INTF g}^{-1}$ )	Acid phosphatase activity ( $\mu\text{mol PNP g}^{-1} \text{ h}^{-1}$ )
HD	$40.68 \pm 4.63$ a	$0.59 \pm 0.03$ ab	$0.47 \pm 0.03$ b	$95.59 \pm 4.61$ a	$88.73 \pm 9.72$ a
TD	$46.24 \pm 3.54$ a	$0.68 \pm 0.02$ a	$0.66 \pm 0.04$ a	$83.09 \pm 11.72$ a	$97.07 \pm 12.39$ a
MD	$40.19 \pm 1.33$ a	$0.53 \pm 0.03$ b	$0.55 \pm 0.03$ ab	$74.30 \pm 11.90$ a	$90.29 \pm 1.76$ a
LD	$42.20 \pm 3.99$ a	$0.67 \pm 0.06$ a	$0.55 \pm 0.06$ ab	$88.46 \pm 6.18$ a	$92.32 \pm 6.42$ a
Anova <i>p</i> values	0.6400	0.0357	0.0359	0.4462	0.9088

Different letters indicate significant differences between treatments according to Fischer's LSD test ( $p < 0.05$ ). Mean  $\pm$  standard error ( $n = 4$ ). FDase: Fluorescein diacetate; INTF: idonitrotetrazolium formazan; PNP: p-nitrophenol; HD: high density ( $6667 \text{ plants ha}^{-1}$ ); TD: traditional density ( $3333 \text{ plants ha}^{-1}$ ); MD: medium density ( $2222 \text{ plants ha}^{-1}$ ); LD: low density ( $1667 \text{ plants ha}^{-1}$ ).

Table 2. Chemical analysis of the soil in response to planting density at the end of the study (2023).

Analysis	Unit	Trataments				P-value
		HD	TD	MD	LD	
Organic matter	%	11.64 ± 0.37 a	11.04 ± 0.07 ab	11.10 ± 0.19 ab	10.46 ± 0.29 b	0.0367
pH (water)		6.52 ± 0.05 a	6.47 ± 0.03 a	6.43 ± 0.07 a	6.45 ± 0.04 a	0.6066
Nitrate (N-NO <sub>3</sub> <sup>-</sup> )	mg kg <sup>-1</sup>	5.77 ± 2.15 a	9.97 ± 2.94 a	5.17 ± 1.16 a	4.47 ± 1.96 a	0.3282
Ammonium (N-NH <sub>4</sub> <sup>+</sup> )	mg kg <sup>-1</sup>	5.00 ± 0.17 b	9.97 ± 2.53 a	4.33 ± 0.29 b	4.17 ± 0.22 b	0.0366
N availability	mg kg <sup>-1</sup>	10.77 ± 2.32 ab	19.90 ± 5.31 a	9.53 ± 0.99 b	8.63 ± 1.73 b	0.0281
Olsen P	mg kg <sup>-1</sup>	26.07 ± 9.25 a	17.73 ± 4.31 a	26.80 ± 13.76 a	12.27 ± 2.66 a	0.6062
K availability	mg kg <sup>-1</sup>	357.73 ± 48.88 a	411.33 ± 75.63 a	375.10 ± 57.10 a	285.90 ± 46.91 a	0.5189
S availability	mg kg <sup>-1</sup>	12.87 ± 2.27 a	14.83 ± 1.34 a	13.13 ± 1.43 a	14.27 ± 0.56 a	0.7758
Exchangeable Ca	cmol+ kg <sup>-1</sup>	9.06 ± 0.98 a	8.03 ± 0.36 a	8.12 ± 0.61 a	7.44 ± 0.35 a	0.3926
Exchangeable Mg	cmol+ kg <sup>-1</sup>	2.50 ± 0.24 a	2.30 ± 0.21 a	1.99 ± 0.30 a	2.16 ± 0.43 a	0.6952
Exchangeable K	cmol+ kg <sup>-1</sup>	0.92 ± 0.12 a	1.05 ± 0.19 a	0.96 ± 0.15 a	0.73 ± 0.12 a	0.5179
Exchangeable Na	cmol+ kg <sup>-1</sup>	0.15 ± 0.02 a	0.11 ± 0.02 a	0.10 ± 0.03 a	0.11 ± 0.02 a	0.3935
Sum of bases	cmol+ kg <sup>-1</sup>	12.63 ± 1.15 a	11.50 ± 0.54 a	11.16 ± 0.86 a	10.44 ± 0.82 a	0.4037
CEC*	cmol+ kg <sup>-1</sup>	12.65 ± 1.15 a	11.51 ± 0.54 a	11.18 ± 0.86 a	10.46 ± 0.82 a	0.4053
Al saturation	%	0.09 ± 0.04 a	0.13 ± 0.01 a	0.13 ± 0.01 a	0.14 ± 0.01 a	0.3984
K saturation	%	7.51 ± 1.65 a	9.18 ± 1.60 a	8.73 ± 1.51 a	7.03 ± 1.02 a	0.7095
Ca saturation	%	71.45 ± 1.47 a	69.76 ± 0.27 a	72.63 ± 0.58 a	71.57 ± 2.48 a	0.6069
Mg saturation	%	19.72 ± 0.16 a	19.94 ± 1.26 a	17.65 ± 1.85 a	20.26 ± 2.82 a	0.7367
B	mg kg <sup>-1</sup>	0.47 ± 0.09 a	0.50 ± 0.06 a	0.37 ± 0.03 a	0.40 ± 0.01 a	0.3662
Cu	mg kg <sup>-1</sup>	1.83 ± 0.15 a	1.90 ± 0.15 a	1.70 ± 0.10 a	1.57 ± 0.09 a	0.3106
Zn	mg kg <sup>-1</sup>	0.80 ± 0.01 a	0.73 ± 0.03 ab	0.63 ± 0.07 bc	0.57 ± 0.07 c	0.0440
Fe	mg kg <sup>-1</sup>	37.13 ± 3.38 a	40.40 ± 7.41 a	32.87 ± 2.34 a	32.07 ± 2.89 a	0.5498
Mn	mg kg <sup>-1</sup>	2.67 ± 0.32 ab	2.80 ± 0.31 a	2.20 ± 0.23 ab	1.87 ± 0.22 b	0.0334

Different letters indicate significant differences between treatments according to Fischer's LSD test ( $p < 0.05$ ). Mean ± standard error ( $n = 4$ ). HD: High density (6667 plants ha<sup>-1</sup>); TD: traditional density (3333 plants ha<sup>-1</sup>); MD: medium density (2222 plants ha<sup>-1</sup>); LD: low density (1667 plants ha<sup>-1</sup>). CEC: Cation exchange capacity.

### 3.2 Foliar and physiological plant parameters

Leaf temperatures in 2021 (Figure 1a) and 2022 (Figure 1b) did not show significant differences between treatments ( $p > 0.05$ ). However, in both seasons, an increasing trend was observed as the measurement period progressed during the day, increasing by 26.8% during the 2021 season and by 29% during the 2022 season, from 09:00 to 18:00 h.

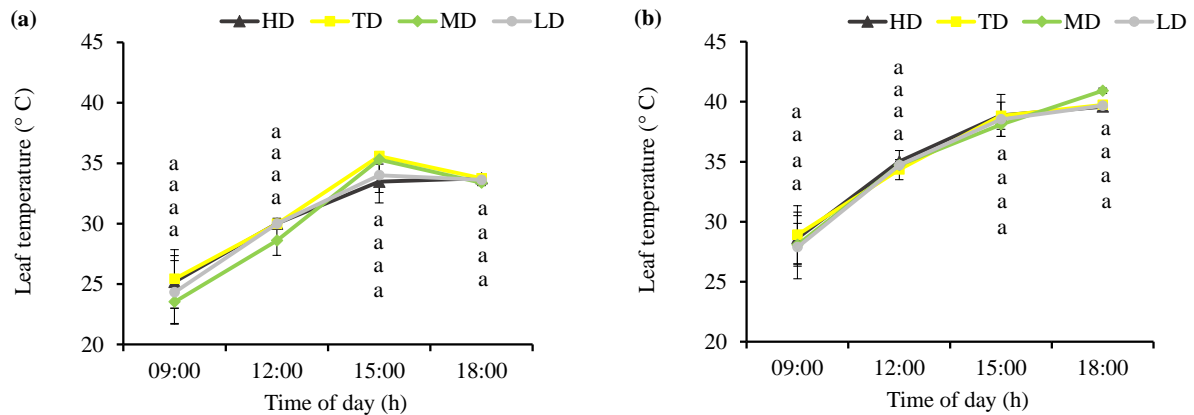


Figure 1. Leaf temperature in calafate plants in 2021 (a) and 2022 season (b), at different times of the day: 09:00, 12:00, 15:00 and 18:00 h. HD: High density (6667 plants ha<sup>-1</sup>); TD: traditional density (3333 plants ha<sup>-1</sup>); MD: medium density (2222 plants ha<sup>-1</sup>); LD: low density (1667 plants ha<sup>-1</sup>). Mean  $\pm$  standard error (n = 4). The bars correspond to the experimental error for each treatment.

The LAI in 2021 (Figure 2a) was significantly higher ( $p < 0.05$ ) with LD than with MD (27%), TD (42%), and HD (71%). In the same period, MD and TD, without differences between them, were significantly higher than HD (56%). In the 2022 season, nonsignificant variations ( $p > 0.05$ ) were observed in the LAI, with an average of 2.8 m<sup>2</sup> m<sup>-2</sup>.

The SPAD index in 2021 (Figure 2b) was not influenced by the planting density, with an average SPAD value of 9. However, in 2022, an increase of 31% was observed in the MD and LD treatments with respect to TD and HD ( $p > 0.05$ ).

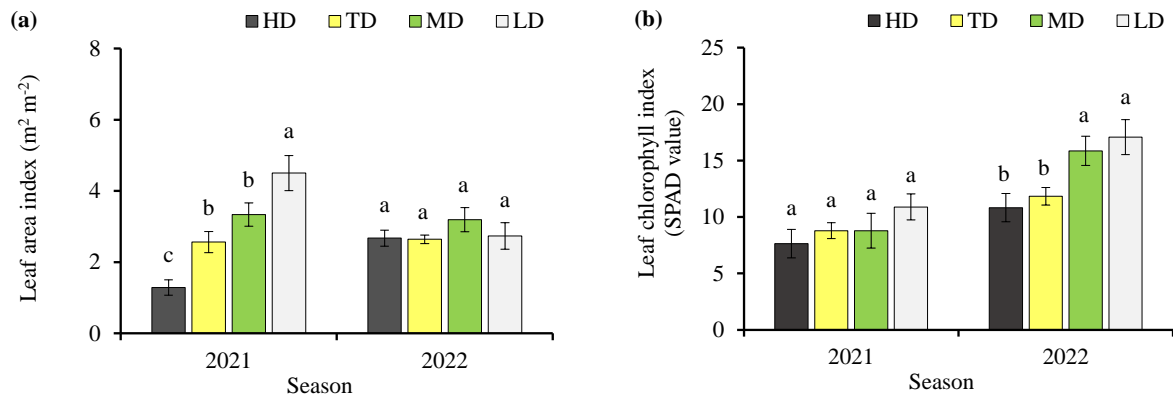


Figure 2. Leaf area index (LAI) (a) and leaf chlorophyll index (b) in calafate plants, evaluated in the 2021 and 2022 seasons. HD: High density (6667 plants ha<sup>-1</sup>); TD: traditional density (3333 plants ha<sup>-1</sup>); MD: medium density (2222 plants ha<sup>-1</sup>); LD: low density (1667 plants ha<sup>-1</sup>). Different letters indicate significant differences between treatments according to Fischer's LSD test ( $p < 0.05$ ). Mean  $\pm$  standard error ( $n = 4$ ). The bars correspond to the experimental error for each treatment.

The maximum quantum yield of photosystem II in 2021 (Figure 3a) showed nonsignificant differences between treatments. However, a slight decreasing trend was observed during the last hour of the measurement. In 2022 (Figure 3b), the MD and LD treatments were significantly higher by 3% than TD and HD at 15:00 h and 4% at 18:00 h. In general, all treatments showed a decreasing trend with advancing measurement time, with a decrease of up to 14% from 09:00 to 18:00 h.

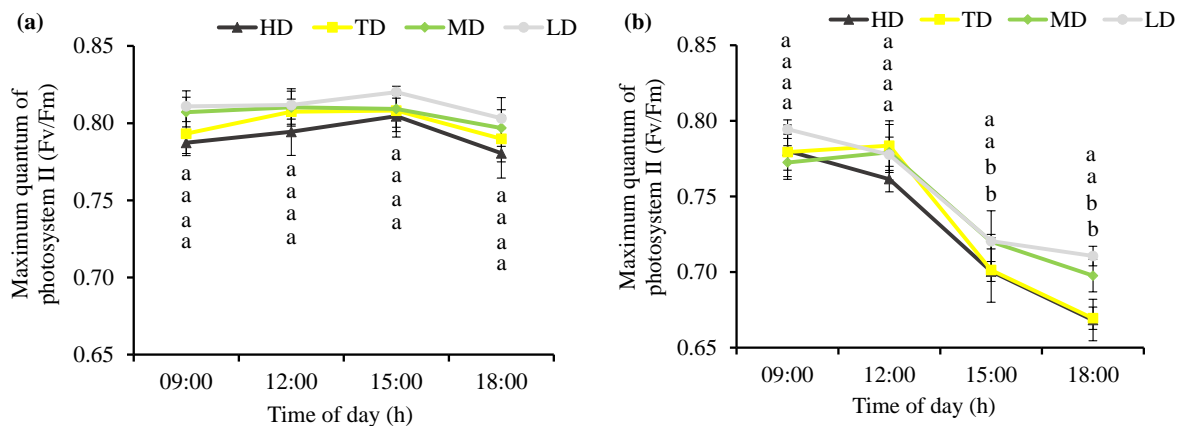


Figure 3. Maximum quantum yield of photosystem II (Fv/Fm) in calafate plants season 2021 (a) and 2022(b); evaluated at different times of the day: 09:00, 12:00, 15:00 and 18:00 h. HD: High density (6667 plants ha<sup>-1</sup>); TD: traditional density (3333 plants ha<sup>-1</sup>); MD: medium density (2222 plants ha<sup>-1</sup>); LD: low density (1667 plants ha<sup>-1</sup>). Different letters indicate significant differences between treatments according to Fischer's LSD test (p < 0.05). Mean ± standard error (n = 4). The bars correspond to the experimental error for each treatment.

The stomatal conductance in 2021 (Figure 4a) significantly increased with LD to 25.7% and 30.5% at 12:00 and 18:00 h, respectively, compared to HD. In 2022 (Figure 4b), stomatal conductance was significantly higher at 09:00 h with LD than with TD (27%) and HD (40%). In both seasons, a decreasing trend in stomatal conductance was observed, with a 17% decrease in 2021 and 23.5% decrease in 2022 from 09:00 to 18:00 h.

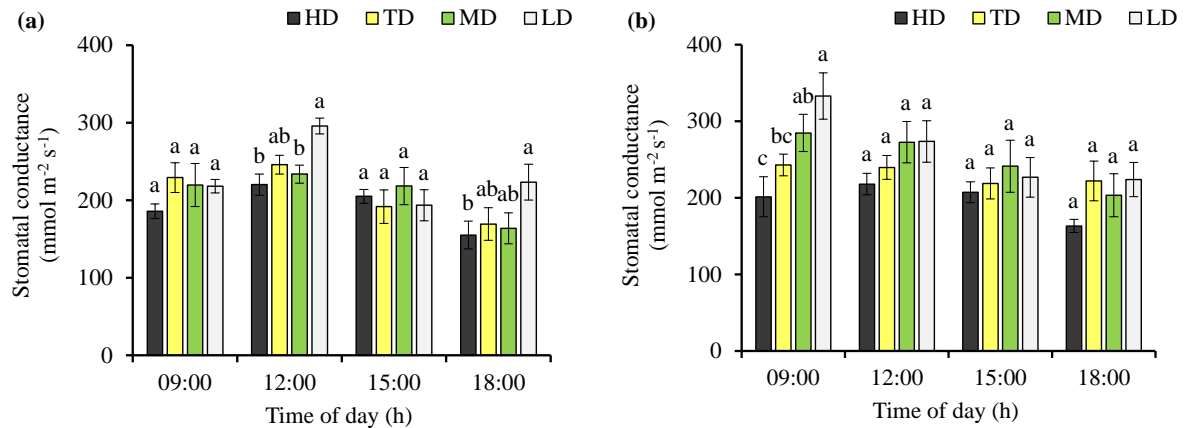


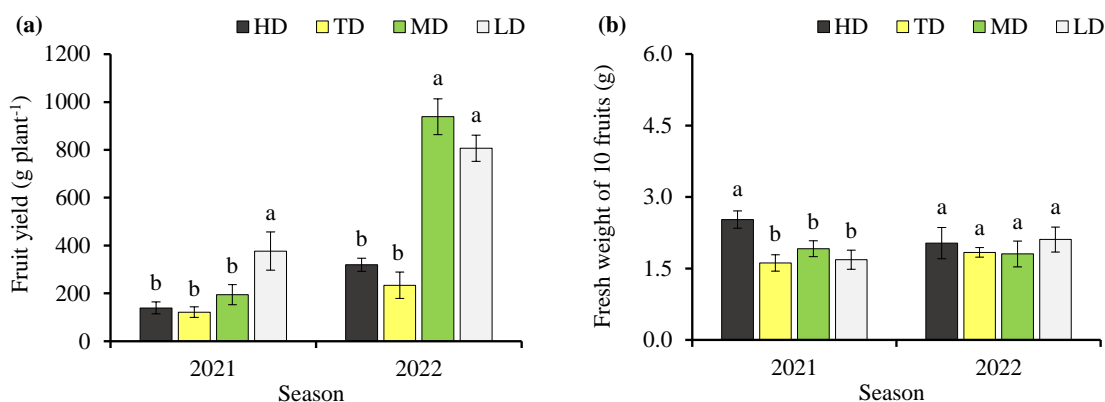
Figure 4. Recorded values of stomatal conductance in calafate plants season 2021 (a) and season 2022 (b); evaluated at different times of the day: 09:00, 12:00, 15:00 and 18:00 h. HD: High density (6667 plants ha<sup>-1</sup>); TD: traditional density (3333 plants ha<sup>-1</sup>); MD: medium density (2222 plants ha<sup>-1</sup>); LD: low density (1667 plants ha<sup>-1</sup>). Different letters indicate significant differences between treatments according to

Fischer's LSD test ( $p < 0.05$ ). Mean  $\pm$  standard error ( $n = 4$ ). The bars correspond to the experimental error for each treatment.

### 3.3 Physicochemical fruit parameters

Fruit yield in 2021 (Figure 5a) increased significantly with LD, reaching  $377 \text{ g plant}^{-1}$ , compared with the other treatments, with an average of  $151 \text{ g plant}^{-1}$  in all other treatments. In 2022, yield ( $\text{g plant}^{-1}$ ) significantly increased in the LD and MD treatments, with an average of  $873 \text{ g plant}^{-1}$ , compared to the HD and TD treatments, with an average of  $277 \text{ g plant}^{-1}$ . In terms of fruit yield per hectare ( $\text{t ha}^{-1}$ ), in 2021, HD presented  $0.9 \text{ t ha}^{-1}$  being significantly superior to the TD and MD treatments, which averaged  $0.4 \text{ t ha}^{-1}$ . In 2022, HD and MD treatments, with an average yield of  $2.1 \text{ t ha}^{-1}$  were significantly superior to LD and TD. During that period, LD, with a yield of  $1.3 \text{ t ha}^{-1}$ , was superior to TD, which had  $0.8 \text{ t ha}^{-1}$ .

Regarding the 10-fruit weight (Figure 5b), in 2021, HD was 32% higher than the other treatments. However, in 2022, the 10-fruit weight did not change significantly with planting density. The equatorial diameter of fruits (Figure 5c) in 2021 was significantly greater with HD than with LD (6%). In the same period, the TD and MD treatments averaged 6.9 mm. In the 2022 season, nonsignificant differences were found in equatorial diameter among the treatments. As for the polar diameter (Figure 5d), similar patterns were observed with respect to the equatorial diameter in both measurement seasons, but with slightly lower values.



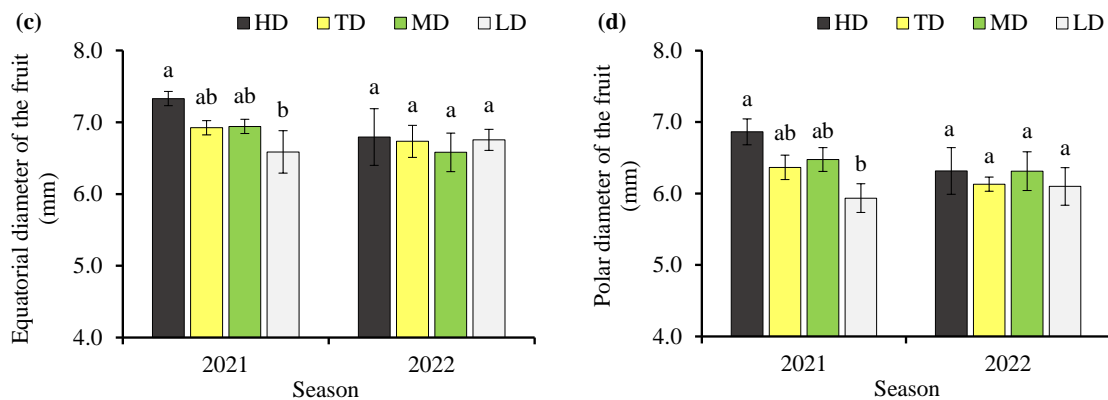


Figure 5. Fruit yield (a), weight of 10 fruits(b), fruit equatorial diameter (c), and fruit polar diameter (d) for the 2021 and 2022 seasons. HD: High density (6667 plants ha<sup>-1</sup>); TD: traditional density (3333 plants ha<sup>-1</sup>); MD: medium density (2222 plants ha<sup>-1</sup>); LD: low density (1667 plants ha<sup>-1</sup>). Different letters indicate significant differences between treatments according to Fischer's LSD test ( $p < 0.05$ ). Mean  $\pm$  standard error ( $n = 4$ ). The bars correspond to the experimental error for each treatment.

Fruit soluble solids (Figure 6a) were not influenced by planting density, with an average of 40 °Brix. The same response was evident in the antioxidant capacities DPPH (Figure 6c) and ORAC (Figure 6d), with average values of 6066 and 12998  $\mu\text{mol TE } 100 \text{ g}^{-1} \text{ FW}$ , respectively, which were not significantly different from each other. However, there was a 36% higher total phenolic content in fruits under TD and MD than under HD. The phenolic content of fruits in LD with an average of 407 mg gallic acid  $100 \text{ g}^{-1} \text{ FW}$  did not show significant differences with respect to the other treatments.

### 3.4 Parameter interaction soil-plant-fruit

Principal component analysis (PCA) of the variables (Figure 7a) and individuals (Figure 7b) was performed for 31 soil, plant, and fruit parameters. The principal components PC1 and PC2 retained a low percentage of variance, corresponding to 23.9% and 20.6%, respectively. In the biplot, each parameter is represented as a vector, and the vector length shows how well the variables are represented in the

plot. The treatments in the PCA of individuals (Figure 7b) were represented by the numbers 1-3 for HD, 4-6 for TD, 7-9 for MD and 10-12 for LD, in the 2022 season.

The PCA results confirmed what was indicated in the correlation matrix described in Figure 8 and were analyzed using Pearson's correlation coefficient ( $r$ ). Moderate correlations were observed between soil indicators, such as N ( $N$ ,  $NH_4^+$ , and  $NO_3^-$ ), FDAse, basal soil respiration, and urease. Likewise, there were higher correlations between soil S and K availability and FDAse ( $r = 0.78$ ) and acid phosphatase ( $r = 0.74$ ), respectively. Moderate correlations were observed between the soil and plant indicators. The OM and soil Fe, Mn, Zn and Cu availability were moderately correlated ( $r > -0.51$  and  $r < -0.72$ ) with chlorophyll index, Fv/Fm and plant stomatal conductance. However, there were high correlations between fruit parameters. Polyphenols were positively correlated with the antioxidant activities of DPPH ( $r = 0.81$ ) and ORAC ( $r = 0.72$ ).

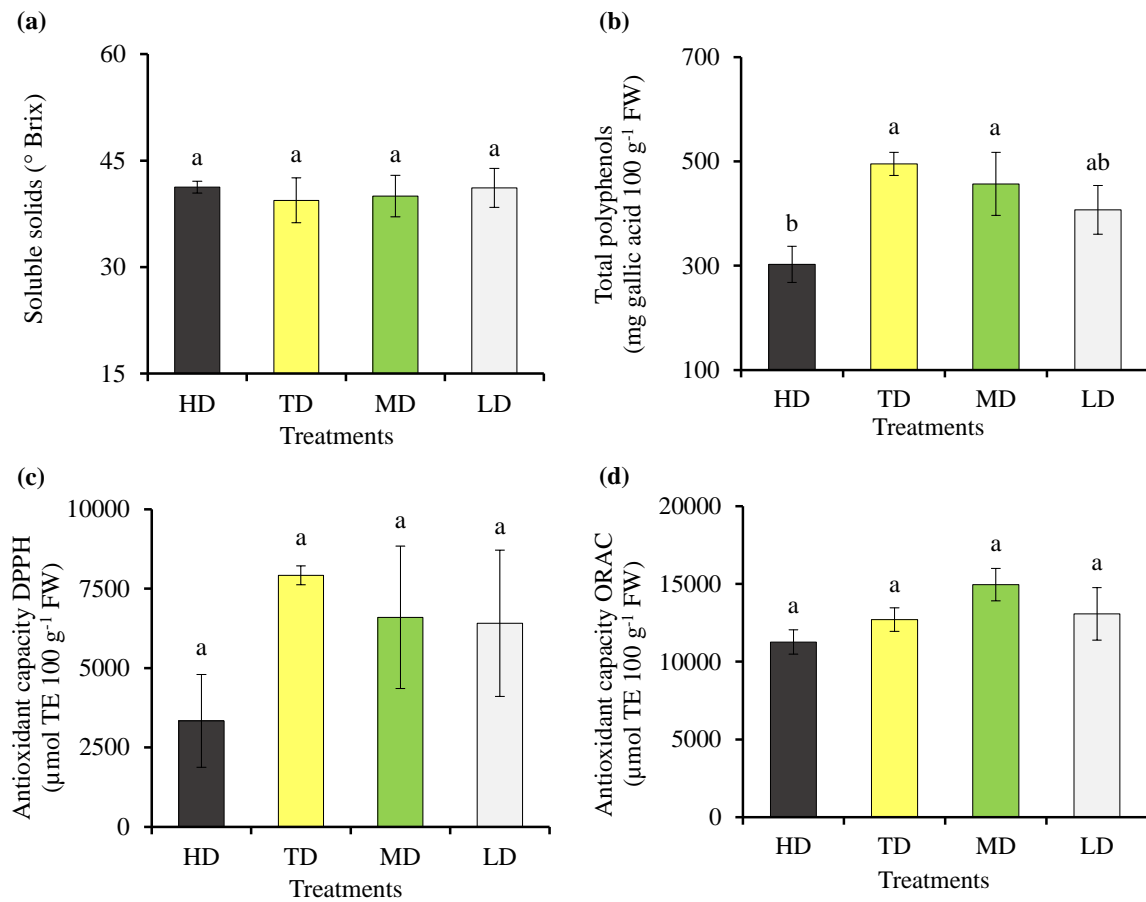


Figure 6. Soluble solids (a), total polyphenols (b), DPPH antioxidant capacity (c), and ORAC antioxidant capacity (d) of calafate fruit in 2022. HD: High density (6667 plants ha<sup>-1</sup>); TD: traditional density (3333 plants ha<sup>-1</sup>); MD: medium density (2222 plants ha<sup>-1</sup>); LD: low density (1667 plants ha<sup>-1</sup>). Different letters indicate significant differences between treatments according to Fischer's LSD test ( $p < 0.05$ ). Mean  $\pm$  standard error ( $n = 4$ ). The bars correspond to the experimental error for each treatment.

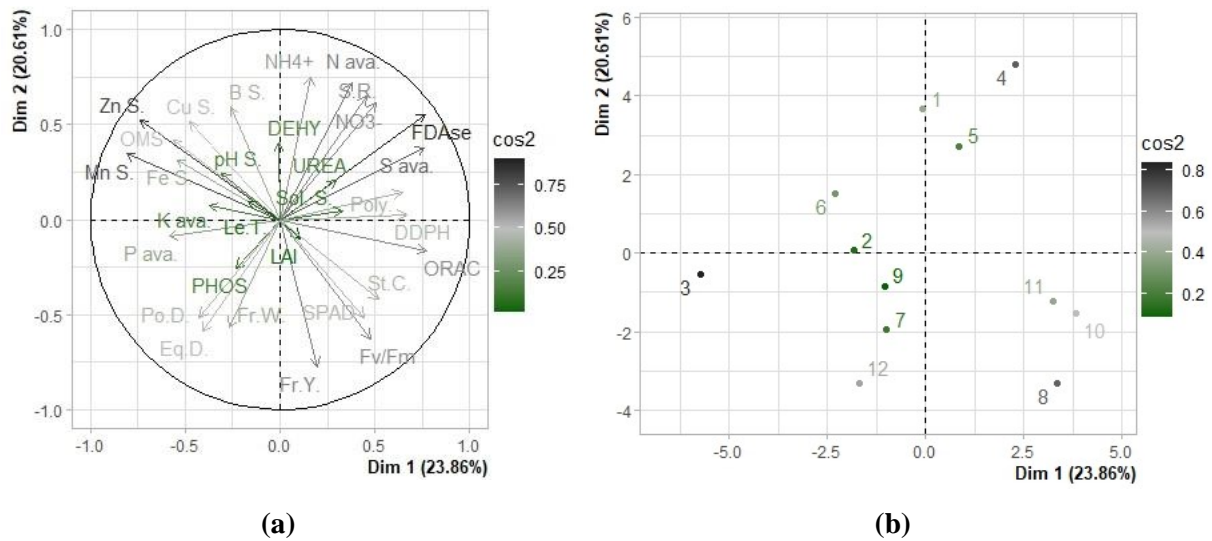


Figure 7. Principal component analysis (PCA) for soil, plant and fruit variables in 2022; PCA of variables (a) and PCA of individuals (b). Soil variables: pH: pH; OM: organic matter; NO<sub>3</sub><sup>-</sup>: nitrate; NH<sub>4</sub><sup>+</sup>: ammonium; N: available N; P: available P; K: available K; S: available S; FDAse: microbial activity; SR: basal soil respiration; UREA: urease; PHOS: acid phosphatase; DEHY: dehydrogenase. Plant variables: LAI: leaf area index; SPAD: chlorophyll index; Fv/Fm: photosystem II maximum quantum yield; StC: stomatal conductance; LeT: leaf temperature. Fruit variables: SS: soluble solids; Poly: total polyphenols; DPPH: DPPH antioxidant activity; ORAC: ORAC antioxidant activity; FrY: yield; EqD: equatorial diameter per fruit; PoD: polar diameter per fruit; FrW: 10 fruit weight.

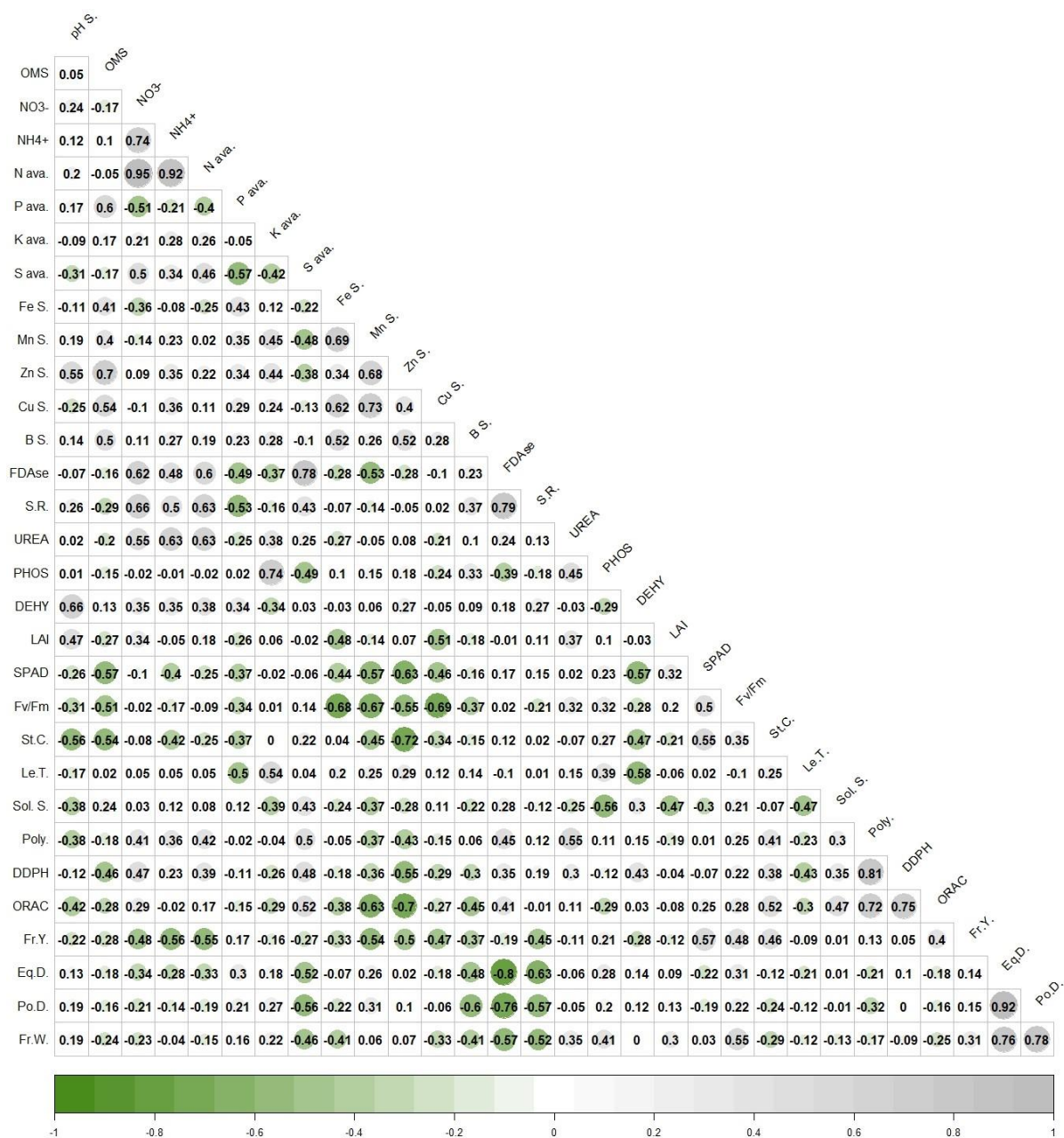


Figure 8. Correlation matrix for soil, plant and fruit variables in 2022. Soil variables: pH: pH; OM: organic matter; NO<sub>3</sub><sup>-</sup>: nitrate; NH<sub>4</sub><sup>+</sup>: ammonium; N: available N; P: available P; K: available K; S: available S; FDase: microbial activity; SR: basal soil respiration; UREA: urease; PHOS: acid phosphatase; DEHY: dehydrogenase. Plant variables: LAI: leaf area index; SPAD: chlorophyll index; Fv/Fm: photosystem II maximum quantum yield; StC: stomatal conductance; LeT: leaf temperature. Fruit

variables: SS: soluble solids; Poly: total polyphenols; DPPH: DDPH antioxidant activity; ORAC: ORAC antioxidant activity; FrY: yield; EqD: equatorial diameter per fruit; PoD: polar diameter per fruit; FrW: 10 fruit weight.

## 4. DISCUSSION

### 4.1 Chemical and microbiological soil responses

The different planting densities did not affect FDAse, considered as an indicator of microbial activity, dehydrogenase involved in the C cycle through the oxidation of soil organic C, or phosphatase that catalyzes the mineralization of organic P. It is likely that the Andisol soil type, due to its high OM content, as well as the agronomic practices, irrigation and mulching, used throughout the study attenuated this response by modulating the stabilization of the soil microclimate, in particular, soil moisture, which is crucial for the activity of FDA and these enzymes. The FDAse increases its sensitivity to changes in soil moisture in the short term (Gutiérrez et al., 2013). Although lower planting densities may result in lower evaporative soil moisture, it is likely that constant water replenishment and summer ground cover mitigated these losses in the short term (Betancur et al., 2022; 2023). However, the lack of response in acid phosphatase between planting density treatments can be attributed to the high OM content of the soil in all treatments, with high buffer capacity and alkaline mineral content, such as Ca, Mg, and K, which maintained a neutral pH (Yang et al., 2022), with a lower response than in acidic conditions, where this enzyme improves its action. The lack of dehydrogenase response could also be explained by the high OM content in the soil at the beginning of the study. Although there was a higher OM content in HD, previous studies in the same area indicated that OM levels higher than 9% reached a threshold where there was no change in the activity of this enzyme (Betancur et al., 2022; 2023). However, it is important to note that the response of these enzymes is more pronounced with fertilization treatments in soils with low fertility because it promotes the formation of organo enzyme complexes that improve their stability (Nannipieri et al., 2012).

Despite this, TD led to higher basal soil respiration, which is another indicator of soil microbial community activity with respect to MD. This could be due to the fact that TD probably generated a greater aerial biomass and a greater number of roots per surface area, which contributed more OM to the soil, thus favoring microbial activity (Gómez et al., 2022; Hou et al., 2023). It is important to note that HD did not show differences in soil respiration with respect to the other treatments, despite having a high OM content. A similar situation was demonstrated by Rui et al. (2016), where soils, despite having a high OM content, presented a higher stable proportion of OM that generated slow decomposition and activity by microorganisms. Similarly, in our study, TD generated a regulated extraction of soil nutrients, such as N, leaving a stock available as a substrate source for microbial and enzymatic growth and activity (Selvalakshmi et al., 2022). However, HD increased the soil N uptake and decreased the available N at the end of the study. It should be noted that in this study, the disparity in the response of basal respiration and soil FDAse could be explained because the former measures the overall release of CO<sub>2</sub> from all microbial processes in the long term, which in our study corresponded to 7 yr, while FDAse would indicate an effect in the short term.

As expected, there was a change in soil chemical properties under the different planting densities, HD improved the availability of Zn, an element that is mobilized in the soil by root exudates, and was probably higher in this treatment (Hamzah Saleem et al., 2022). The TD significantly improved the availability of N, ammonium and Mn with respect to HD, MD and LD. Therefore, we highlight that TD treatment, to some extent, improved the biological activity of the soil and maintained greater chemical fertility towards the end of the study. However, it is important to note that more research is needed on the cycling and movement of nutrients in the soil, as well as on their uptake by calafate plants.

#### 4.2 Foliar and physiological plant responses

In terms of foliar indices, LD led to a greater leaf area and chlorophyll, which is due to less competition between plants, and even stress, as there is more availability of

fundamental resources for vegetative development, such as nutrients and water (Huang et al., 2021). Similar results were reported by Casanova-Gascón et al. (2019), who demonstrated lower SPAD values in less vigorous trees.

Also, in the research it is noteworthy that there was lower LAI in HD with respect to TD and MD, probably due to the shading which affects photosynthetic activity that depends on the light energy disappointed by chlorophylls, so that a lower amount limits photosynthesis and growth (Yan et al., 2019). Likewise, it has been indicated that a decrease in the availability of photosynthetically active radiation (PAR) induces an altered phytochrome response, which alters shoot elongation and leaf area (Retamal-Salgado et al., 2017).

The HD and TD treatments resulted in a lower maximum quantum yield of photosystem II, and HD led to lower stomatal conductance. Similar cases have been documented in species such as apples, where a reduction in tree planting distance to four times (from 1 to 0.25 m) produced more stress, decreasing photosynthetic rate by 39% and up to 2.5 times stomatal conductance (Laužiké et al., 2020). Therefore, HD, being a plant that is more repressed in size and leaf pigments, is more affected by abiotic stress, which would explain the decrease in photosynthesis and gas exchange. It is important to mention that there was a greater effect on the chlorophyll index, maximum quantum yield of photosystem II, and stomatal conductance in the second evaluation season than in the first, which could be explained by climatic conditions such as temperature and more intense precipitation during the evaluation season. Likewise, the lack of change in LAI in the second season with respect to the first could be due to the higher environmental temperature of that season, which could have influenced the development of the crop, as was the case for blueberries (Retamal-Salgado et al., 2017). However, the need for long-term monitoring to verify the continuity of these effects on the physiological parameters of calafate has been highlighted.

### 4.3 Physicochemical fruit response

In the present study, HD and TD led to lower fruit production per plant. These fruit yield results agree with the moderate correlations found in our study, where this productive parameter was positively correlated with physiological parameters, such as SPAD or maximum quantum yield of photosystem II. Therefore, it was confirmed that plants in a better physiological state were more productive, but not HD or TD. Moreover, these results agree with those reported in species such as *Citrus aurantifolia* Swingle, where the yield per plant was always lower at a high-density of 1600 plants ha<sup>-1</sup> compared to 800 and 400 plants ha<sup>-1</sup>, respectively (Ladaniya et al., 2020).

However, it should be noted that in terms of cumulative production (t ha<sup>-1</sup>) HD always produced higher productivity, which would be due to the greater number of trees per surface area compared to TD (2 times more), MD (3 times more) and LD (4 times more), which has also been reported in species such as apple (Lordan et al., 2018) and olive (Díez et al., 2016), which is also indicated as an advantage in productive terms by conferring precocity of young orchards and reducing cultural costs associated with harvesting and pruning (Lavee et al., 2012). Despite these results, we highlight that fruit production per tree and area with MD was high in the second season, reaching the cumulative production of HD.

In addition, in this study, it was demonstrated that higher fruit production per plant was associated with a greater number of fruits and not necessarily with larger fruits, as we found that the plants with lower individual productivity (HD) were those with higher fruit weight and size. These results are in agreement with those reported for blueberries, where plants with lower fruit production showed better quality attributes such as fruit size and weight (Hirzel et al., 2023). This could be attributed to the fact that a lower amount of fruit per plant reduces competition for resources such as sunlight, water, and nutrients, which allows individual fruits to develop more fully (Lordan et al., 2018), which was also corroborated by the moderate correlations in this study between soil nutrients such as N, Mn, and Zn, and fruit yield. On the other hand, in coffee plants, it has been shown that yield decreases at high density

because of decreased light and branching frequency, which has a strong photomorphogenetic effect on sprouting (Rakocevic et al., 2021). Therefore, self-shading is a fundamental factor affecting berry quantity and quality (Cheng et al., 2020).

The HD led to a decrease in the total polyphenol content, but not enough to affect the antioxidant capacity measured using different methodologies. In addition, the higher correlation found in this study corroborates that the antioxidant capacity of calafate is conferred by phenolic compounds, among which anthocyanins (Romero-Román et al., 2021) would stand out. Also, in this study, it was possible to demonstrate that planting density did not generate differences in the soluble solid content of the fruit, which is fundamental because it can be used as an indicator to plan the ripening period of the fruit as the soluble solid determinants of the sugar content in the pulp and its state of maturity (Arena et al., 2003). In addition, despite the lower phenolic content of HD, it produced a higher antioxidant activity than other berries such as blueberry and strawberry, which is beneficial for scavenging reactive oxygen radicals and counteracting oxidative stress (Rodoni et al., 2014). This is interesting for the natural pigment industry because these phenolic compounds provide sensory attributes such as berry pigmentation (Romero Román et al., 2020) and their characteristic antioxidant capacity. However, it is noteworthy that any density generates a significantly higher antioxidant capacity than wild calafate (Romero-Román et al., 2021), probably because of the edaphoclimatic conditions of our study, such as high summer temperatures capable of generating abiotic stress in the plant, inducing changes in secondary metabolism and its accumulation in the fruit (Mariangel et al., 2013).

The results of this study indicate that HD improves calafate productivity per unit area, but at the expense of phenolic content. In contrast, a moderate density, such as MD, which in turn reduces the cost of plants per area, can produce high individual and area productivity, as well as fruits with adequate phenolic content and antioxidant capacity.

This study signifies a notable progression in the assessment of planting density in calafate orchards, as it illustrates the role of planting density in enhancing soil microbial and enzymatic activity, thereby influencing the antioxidant activity of calafate fruit (Betancur et al., 2022; 2023). Nevertheless, due to their moderate interrelationships, a comprehensive analysis of these factors is warranted.

## **5. CONCLUSIONS**

The impact of planting density on soil health and phenolic compounds in calafate fruits was investigated in this study. The results revealed that traditional density (TD, 3333 plants ha<sup>-1</sup>) was the most effective in enhancing basal respiration, urease activity, and the availability of essential nutrients such as soil N, Zn, and Mn compared to the other planting density treatments. Moreover, medium density (MD, 2222 plants ha<sup>-1</sup>) and low density (LD, 1667 plants ha<sup>-1</sup>) demonstrated a significant increase in leaf area index, chlorophyll content, photosystem II maximum quantum yield, and stomatal conductance relative to the other treatments. Additionally, MD and LD resulted in higher fruit yield per plant, while MD and high density (HD, 6667 plants ha<sup>-1</sup>) exhibited the highest fruit yield per hectare. The study also found that planting density influenced the phenolic content, with HD showing lower phenolic content compared to the other treatments. Based on the findings, it is recommended that TD be considered due to its positive impact on microbial activity, soil nutrient availability, and the production of fruits with high phenolic content and antioxidant activity. However, in terms of productivity per surface area, MD was observed to generate a high fruit content per plant and surface area with high antioxidant capacity. The study also suggests that future research on calafate should incorporate soil biological parameters to gain a more comprehensive understanding of soil health.

### **Author contribution**

Conceptualization: M.S. J.R.-S., M.D.L. Methodology: M.S., J.R.-S., M.B., R.V-R., M.D.L. Software: M.B., M.D.L. Validation: M.S., J.R.-S., M.D.L. Formal analysis: M.S.,

J.R.-S., M.B. Investigation: M.B., M.S., J.R.-S., R.V-R., M.D.L. Resources: M.S., M.D.L., J.R.-S., R.V-R., M.B. Data curation: M.B., M.S., J.R.-S., M.D.L. Writing-original draft preparation: M.B., M.S., J.R.-S., M.D.L. Writing-review & editing: M.B., J.R.-S., M.S., M.D.L. Visualization: M.B., M.S., J.R.-S., M.D.L. Supervision: M.S., J.R.-S., R.V-R., M.D.L. Project administration: M.S., J.R.-S. Funding acquisition: M.S., J.R.-S., R.V-R., M.D.L. All co-authors reviewed the final version and approved the manuscript before submission.

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## VI. CONCLUSIÓN GENERAL

Este estudio fue eficiente en seleccionar las mejores prácticas de manejo agronómico para una producción sostenible de calafate que mejoren el suelo, la planta y fruto. En cuanto a la reposición hídrica, al igual que se planteó en la hipótesis, un riego moderado entre 0% y 150% de la evapotranspiración de referencia (ET<sub>0</sub>), optimizó la respuesta microbiana del suelo (biomasa microbiana, respiración basal, deshidrogenasa y fosfatasa ácida), respuesta de la planta (conductancia estomática e índice de clorofila) y fruta de calafate (rendimiento, antocianinas y capacidad antioxidante). Se seleccionó una dosis de riego de 50% de la ET<sub>0</sub> porque optimiza los recursos hídricos en comparación a dosis más altas. Respecto a la aplicación de mulch, tal como se planteó en la hipótesis, los mulch orgánicos de paja de avena y cascara de avellano, demostraron ser efectivos en mejorar la salud del suelo (actividad microbiana, respiración basal y ureasa) y la calidad de los frutos (antocianinas y capacidad antioxidante), que suelo sin mulch y geotextil. Se seleccionó la cáscara de avellana por ser un desecho orgánico con baja degradabilidad y mayor permanencia para el cultivo. En cuanto a la fertilización orgánica, la incorporación de compost mostró mayor respuesta en suelo, planta y fruto que sin compost corroborando la hipótesis inicial. Particularmente, mayores dosis (10 y 15 ton ha<sup>-1</sup>) mejoraron parámetros microbiológicos del suelo (biomasa microbiana y respiración basal), vegetativos de planta (índice de área foliar e índice de clorofila), rendimiento y calidad de fruta (contenido de antocianinas y capacidad antioxidante) que dosis más bajas. Se escogió como mejor tratamiento 10 ton ha<sup>-1</sup> de compost, porque reduce el uso del fertilizante orgánico que dosis más altas. Respecto a la densidad de plantación, destacó la densidad de plantación tradicional de 3333 plantas ha<sup>-1</sup> mostrando mayor respuesta microbiológica del suelo (respiración basal y ureasa), desarrollo vegetativo y fisiológico de la planta (índice de área foliar y conductancia estomática) y rendimiento de fruto respecto a alta densidad, confirmando la hipótesis inicial. Se seleccionó la densidad de 3333 plantas ha<sup>-1</sup> respecto a alta densidad, permitiendo ahorro en el número de plantas por

hectárea al establecimiento del huerto. Por lo tanto, los manejos agronómicos de mejor respuesta del suelo, planta y fruto de calafate son, riego con 50% de la ET<sub>0</sub>, uso de mulch de cascara de avellano, fertilización con compost a dosis de 10 ton ha<sup>-1</sup> y densidad de plantación con 3333 plantas ha<sup>-1</sup>. Esto promueve la salud del suelo y la sostenibilidad del sistema productivo. No obstante, queda abordar otras preguntas por el uso de manejo agronómico como son, su comportamiento a lo largo de varias temporadas, bajo distintas condiciones edafoclimáticas y otros manejos agronómicos como, el control fitosanitario, poda y cosecha.

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