

UNIVERSIDAD DE CÓRDOBA
DEPARTAMENTO DE INGENIERÍA FORESTAL

**Caracterización de la variabilidad, crecimiento y producción del
pino piñonero (*Pinus pinea* L.) en Chile en función del clima
y de algunas prácticas silviculturales**

**Characterization of variability, growth and production of
Stone pine (*Pinus pinea* L.) in Chile according
to climate and some silvicultural practices**

Tesis Doctoral / Doctoral Thesis
Verónica Francisca Loewe Muñoz

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Tesis Doctoral – Doctoral Thesis

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Programa de Doctorado en Biociencias y Ciencias Agroalimentarias

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TÍTULO DE LA TESIS: Caracterización de la variabilidad, crecimiento y producción del pino piñonero (*Pinus pinea* L.) en Chile en función del clima y de algunas prácticas silviculturales

DOCTORANDO/A: Verónica Francisca Loewe Muñoz

INFORME RAZONADO DEL DIRECTOR DE LA TESIS

El doctorando presenta y desarrolla un amplio estudio experimental a partir de plantaciones de *Pinus pinea* L. en Chile. La metodología empleada es correcta e incorpora técnicas novedosas de análisis estadístico y analítico, en particular orientadas a los estudios de producción y variabilidad. La tesis aporta una visión aplicada de la silvicultura y la producción de piñón en Chile.

El resultado es original en el contexto de los estudios de silvicultura intensiva de pino piñonero, al incorporar varios factores y las posibles relaciones en el éxito final de programas de domesticación de la especie. La tesis ha dado lugar a tres publicaciones en revistas indexadas de alto impacto:

Loewe M.V., Balzarini M., Álvarez A., Delard C., Navarro-Cerrillo R.M. 2016. Fruit productivity of Stone pine (*Pinus pinea* L.) along a climatic gradient in Chile. *Agricultural and Forest Meteorology* 223: 203-216. doi.org/10.1016/j.agrformet.2016.04.011. (Q1, IF 4.839, 2/127).

Loewe M.V., Balzarini M., Delard C., Álvarez A., Navarro-Cerrillo R.M. 2016. Growth of Stone pine (*Pinus pinea* L.) European provenances in central Chile. *iForests*. doi: 10.3832/ifor1984-009 (Q2, IF 1.110, 40/127).

Loewe M.V.; Delard R.C.; Balzarini M.; Álvarez C.A.; Navarro-Cerrillo R.M. 2015. Impact of climate and management variables on stone pine (*Pinus pinea* L.) growing in Chile. *Agricultural and Forest Meteorology*, 214-215: 106-116. Doi.org/10.1016/j.agrformet.2015.08.248. (Q1, IF 4.839, 2/127).

En la actualidad hay otros cinco trabajos en revisión:

Loewe V.; Alvarez, A., Balzarini, M., Delard, C., Navarro-Cerrillo, R.M., 2016. Fertilization and irrigation effects on growth and cone production in young and adult Stone pine (*Pinus pinea* L.) plantations in Chile. *Fruits Journal*.

Loewe MV, Alvarez K., Lutz M., Navarro-Cerrillo R, 2016. Pine nut morphometry and production in selected Stone pine (*Pinus pinea* L.) trees among Chilean macrozones. *Southern Journal of Forestry*.

Lutz M., Loewe V., Alvarez K., 2016. Chemical composition of pine nut (*Pinus pinea* L.) grown in three geographical macrozones in Chile. *Journal of Food CYTA*.

Loewe MV, Navarro-Cerrillo R, García-Olmo J, Riccioli C, Sánchez de la Cuesta R. 2016. Discriminant analysis of Mediterranean pine nuts (*Pinus pinea* L.) from Chilean plantations by Near Infrared Spectroscopy. *Food Control Journal*.

Zuleta A., Weisstaub A., Giacomino S., Dyner L., Loewe MV., Delard C., Lutz M. 2016. An ancient crop revisited: chemical composition of Mediterranean pine nuts (*Pinus pinea* L.) grown in Chile, Argentina, Italy, Spain, Turkey and Israel. *Journal of the Science of Food and Agriculture*.

Por otro lado, se han presentado 15 comunicaciones orales y posters en Congresos Nacionales e Internacionales, y 10 artículos de divulgación.

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 1° de Septiembre de 2016



Firma del director

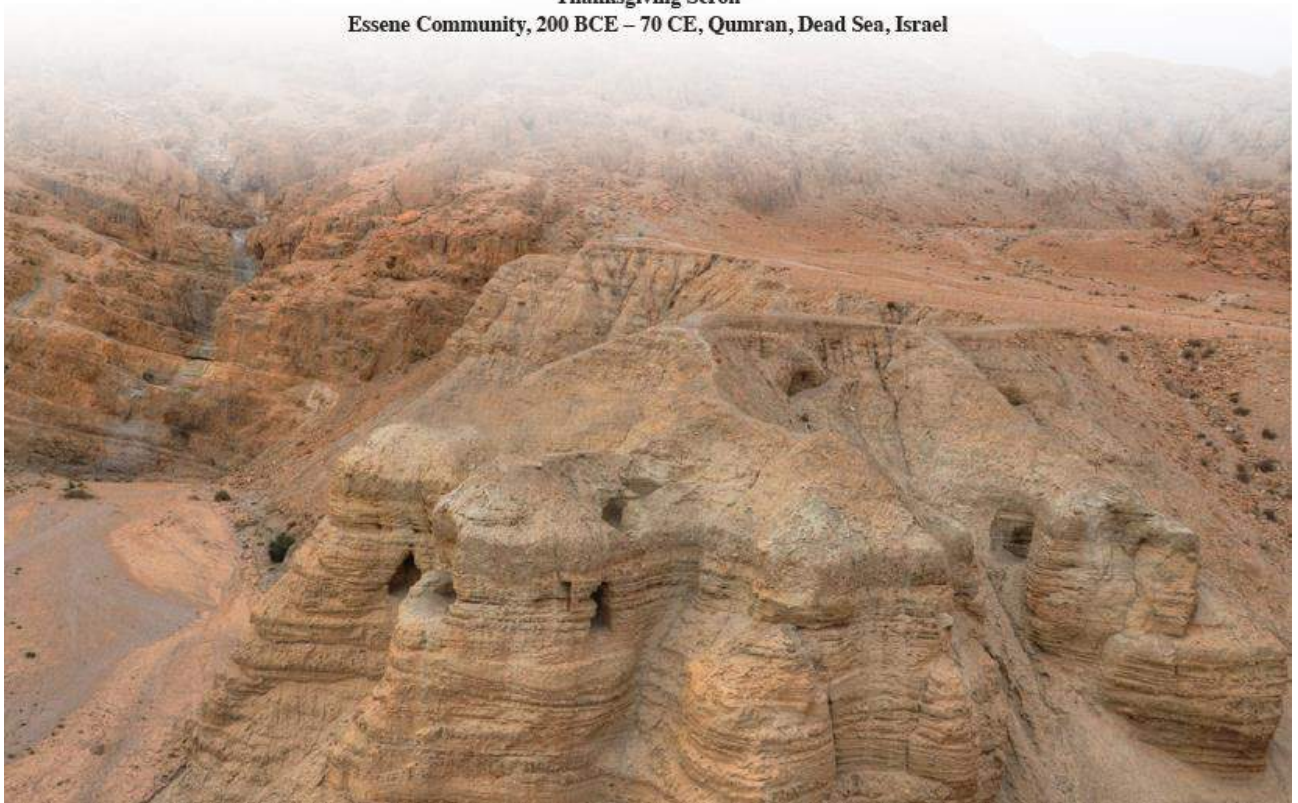
Fdo.: Rafael María Navarro Cerrillo

Esta tesis es un resultado del proyecto “Desarrollo de técnicas de manejo para producir piñones de pino piñonero (*Pinus pinea* L.), una opción comercial atractiva para Chile”, D11I1134, financiado por el Fondo de Fomento al Desarrollo Científico y Tecnológico FONDEF, perteneciente a la Comisión Nacional de Investigación Científica y Tecnológica CONICYT, Ministerio de Educación, Gobierno de Chile, y ejecutado por el Instituto Forestal de Chile (INFOR) y la Universidad de Valparaíso entre los años 2012 y 2016.

*By my hand Thou hast opened for them
a well-spring and ditches,
that all their channels may be laid out
according to a certain measuring-cord,
and the planting of their trees
according to the plumb-line of the sun,
that their boughs may become
a beautiful Branch of glory (Hymn 18, 20).*

*A plant of delight, a plant in His garden and in his vineyard.
its twigs will bear fruit and its branches will increase...
And its branches reaching above the elevated support of heaven;
And its splendor offers itself for everlasting generations,
producing fruit for all who are to taste it.
There will be no wild grapes among its fruits.
It will have foliage, leaves and blossoms.
None of its roots will be pulled up from its bed of balsam
(Hymnic Fragment, 4Q433a).*

Thanksgiving Scroll
Essene Community, 200 BCE – 70 CE, Qumran, Dead Sea, Israel



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Por la naturaleza toda y, en especial, por los árboles.

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Resumen

El pino piñonero (*Pinus pinea* L.) es una especie mediterránea caracterizada por una elevada plasticidad fenotípica, una alta tolerancia a un amplio rango de ambientes y una baja variabilidad genética; asimismo, es de gran importancia económica debido a sus semillas comestibles, los piñones, uno de los frutos secos más exclusivos y caros en el mundo. No obstante, los avances en las técnicas de manejo semi-intensivo o intensivo para su cultivo como árbol frutal son limitados, y la piña se cosecha principalmente de bosques naturales. Esta situación puede atribuirse a la extensión del ciclo de desarrollo del fruto (42 meses), con piñas de uno, dos y tres años creciendo de forma simultánea en el árbol, situación que hace más complejo el manejo hídrico y nutricional que en otras especies frutales y forestales productoras de fruto, entre otros factores.

En esta tesis se estudió el crecimiento y la fructificación del pino piñonero a lo largo de un gradiente climático en Chile; la variabilidad de la especie mediante análisis morfométricos, químicos, de espectroscopia del infra-rojo cercano (NIRS) y de biología molecular (proteómica); la adaptación de procedencias europeas en la zona central de Chile mediante la evaluación del crecimiento y producción; y los efectos de la fertilización y del riego en el crecimiento y fructificación de plantaciones intensivas de producción de fruto.

El crecimiento se examinó en 4.094 individuos distribuidos en 129 localidades entre las regiones de Coquimbo y Araucanía de Chile, en función de numerosas variables climáticas, cuya contribución se determinó mediante árboles de regresión y modelos lineales. Se definieron tres macrozonas (MZ) dentro del rango de distribución actual del pino piñonero en Chile que presentan diferencias significativas en el crecimiento: MZ Norte, Sur y Secano Costero. En la MZ Sur, las tasas de crecimiento fueron las más elevadas para la altura (35 cm año^{-1}) y el DAP ($1,5 \text{ cm año}^{-1}$), mientras que en la MZ del Secano Costero la especie mostró el menor crecimiento en altura y DAP (23 y $0,87 \text{ cm año}^{-1}$, respectivamente). El crecimiento en altura se vio favorecido por una oscilación térmica de invierno superior a 14°C , una precipitación anual superior a 1.400 mm , una temperatura media anual bajo 14°C y por un déficit hídrico de primavera inferior a 400 mm . El crecimiento en DAP también se vio favorecido por una temperatura media anual inferior a 14°C , y por una temperatura media mínima bajo $6,6^{\circ}\text{C}$.

La fructificación se analizó mediante el conteo del número de piñas por árbol (CN) en 3.464 árboles distribuidos a lo largo del gradiente climático de Chile. El peso de la piña en el momento de la cosecha (CW) y la cantidad de piñones con cáscara por piña (IS) se midieron en 76 árboles superiores. Se relacionaron variables climáticas y biometeorológicas -definidas en base a 11 fases reproductivas fisio-fenológicas- con caracteres de producción frutal. La fructificación mostró marcadas diferencias entre MZs. La mayor producción de piñas (32 kg árbol⁻¹) y CN (62 piñas árbol⁻¹) se registró en la MZ Sur. La producción de piñas a lo largo de Chile fue favorecida, en orden decreciente, por una oscilación térmica de otoño inferior a 12°C, un déficit hídrico de primavera menor a 345 mm, una precipitación de primavera superior a 65 mm y una oscilación térmica anual bajo 12°C; así como por una temperatura mínima superior a 7°C y un déficit hídrico inferior a 200 mm en el periodo de floración femenina, por una precipitación bajo 70 mm durante la floración masculina, y por una oscilación térmica inferior a 6°C durante la diferenciación de brotes reproductivos.

El peso de la piña se vio favorecido por una temperatura media anual sobre 14°C, por una precipitación anual superior a 507 mm y por un déficit hídrico anual inferior a 914 mm. Adicionalmente, una temperatura máxima superior a 23°C y un déficit hídrico inferior a 774 mm durante el periodo de desarrollo del embrión aumentó su peso, así como una precipitación acumulada sobre 14 mm durante la fase de crecimiento de la piña de 2 años.

La cantidad de piñones por piña (IS) aumentó significativamente con un déficit hídrico anual inferior a 914 mm y una temperatura máxima de otoño inferior a 19°C, junto a una temperatura máxima sobre 24°C durante la fase de desarrollo de la piña de 2 años y la precipitación acumulada en tres periodos clave (valores superiores a 53 mm durante la inducción de primordios masculinos, a 78 mm durante el crecimiento de la piña de 3 años y a 133 mm en la fase de maduración de la piña). Por ello, se recomienda el riego para mitigar el impacto negativo del déficit hídrico en la productividad de piñas.

La variabilidad de la especie se estudió mediante la caracterización morfológica, la composición química, y el análisis NIRS y molecular de piñones de la especie cosechados en las tres MZs de crecimiento de la especie en Chile. El análisis morfométrico mostró que individuos seleccionados de las macrozonas definidas se diferencian en tamaño de piña y morfometría del piñón, así como en el rendimiento de piña a piñón sin cáscara. La composición química realizada a nivel de MZs, y para 38 individuos seleccionados, fue similar a los valores reportados para la especie en los principales países productores (34,1-



35,5% proteína, 44,2-40,2% lípidos y 4,7-4,4% minerales, respectivamente). La composición química de individuos seleccionados fue homogénea entre MZs, con la excepción del ácido oleico, superior en la MZ Sur, y del potasio, superior en la MZ del Secano Costero. El análisis a nivel de macrozonas mostró que la Norte posee el mayor contenido de minerales y la del Secano Costero valores superiores de humedad, proteína y lípidos. Los contenidos medios de fitoesteroides alcanzaron 2.113 $\mu\text{g/g}$ aceite y de tocoferoles 113 $\mu\text{g/g}$ aceite. Mediciones independientes NIRS se usaron para identificar orígenes geográficos de piñones empleando diferentes tipos de muestras (piñones con y sin cáscara, y harina húmeda y seca). El análisis demostró el potencial de esta tecnología para el estudio de variabilidad, ya que es un método rápido y exacto para predecir el origen geográfico de piñones en Chile, mediante el cual se clasificaron correctamente 87,8% de muestras de las tres macrozonas en la fase de validación cruzada, y el mejor modelo se obtuvo con piñón intacto sin cáscara. La proteómica también permitió diferenciar poblaciones presentes en las tres macrozonas de la especie en Chile, y mostró ser una herramienta útil para detectar variabilidad entre poblaciones de diferentes ambientes, proponiéndose siete proteínas como posibles marcadores poblacionales. Se identificaron proteínas relacionadas principalmente con nutrientes de reserva, estrés de choque térmico, procesos glicolíticos, oxidoreductasas y peroxirredoxinas, y en menor medida, con la fijación del carbono y enlaces de ATP; 12% no pudo ser identificado. En síntesis, el análisis realizado sobre la variabilidad de poblaciones de pino piñonero en Chile, estudiada con diferentes técnicas, mostró que ésta varía con variables geográficas y climáticas probablemente como una expresión génica asociada con estrategias adaptativas.

El crecimiento y la fructificación de seis procedencias europeas de pino piñonero establecidas en la zona central de Chile se analizó a los 19 años de edad, así como su supervivencia durante los primeros 7 años. Las procedencias estudiadas mostraron diferencias significativas en CN y CW, así como en crecimiento en DAP, altura y diámetro de copa. Las procedencias se separaron en tres grupos de acuerdo con el crecimiento y variables productivas: las italianas y eslovena; Andalucía y Sierra Morena (España); y Meseta Castellana (España); esta última registró el mayor crecimiento y productividad. CN se correlacionó positivamente con CW y con variables de crecimiento.

Posteriormente, los efectos combinados e individuales de la fertilización y del riego en el crecimiento y fructificación, y su correlación, se evaluaron en plantaciones ubicadas en la

zona central de Chile. En una plantación de 30 años (Cáhuil) los tratamientos incluyeron control, fertilización de primavera y otoño, riego de primavera y verano, y fertilización y riego combinados; y en una plantación de 16 años (Toconey), los tratamientos fueron control y fertilización de primavera. En Cáhuil la fertilización tuvo un efecto positivo un año después de la aplicación (23,5% mayor en crecimiento en altura y 82,3% mayor producción de piñas de un año vs el control); después de dos años consecutivos de aplicación, se observó una influencia significativa respecto al control en crecimiento en DAP (17,5% superior), altura (20,4% mayor), y producción de piñas de un año (36,4% mayor), independientemente del status hídrico de los árboles. El riego estimuló la fructificación pero no afectó el crecimiento. La mayor cantidad de piñas de un año se observó en las parcelas fertilizadas y regadas (60% superior). En Toconey, el crecimiento en las parcelas fertilizadas fue mayor (41% mayor altura y 29% mayor diámetro de copa), y produjeron 94% y 2,4 veces más piñas que el control 3 y 4 años después de la fertilización. La fertilización también afectó el tamaño de las piñas, aumentando la cantidad de piñas de mayor tamaño las dos cosechas siguientes. Ambas prácticas culturales, aplicadas ya sea individualmente o en forma combinada, son herramientas útiles para estimular la producción frutal en plantaciones jóvenes y adultas, con una respuesta más pronunciada si se aplican a edades más tempranas.

Finalmente, en esta tesis se propone un modelo de silvicultura intensiva para la gestión de plantaciones de producción frutal de pino piñonero en Chile, incluyendo técnicas comúnmente usadas en fruticultura, tales como fertilización, riego, control de malezas, injerto y diferentes tipos de poda, incluyendo podas de raíz.

En síntesis, la hipótesis general de esta tesis doctoral -el pino piñonero es una especie adecuada a las condiciones edafoclimáticas de Chile en un amplio rango de condiciones ambientales, y presenta una alta capacidad productiva que permite prever niveles de producción de piñón similares o superiores a su región de origen, siendo la silvicultura del pino piñonero a una importante opción productiva para el país-, ha sido corroborada sobre la base de los resultados científicos obtenidos.

Esta tesis hace una contribución científica valiosa para la profundización del conocimiento de la especie en varios temas relevantes para su domesticación, abriendo nuevos temas de investigación que deberían ser tratados en el futuro.



Abstract

Stone pine (*Pinus pinea* L.) is a Mediterranean species characterized by great phenotypic plasticity, high tolerance to a wide range of environments and low genetic variability; it is also of great economic importance conferred by its edible seeds, the pine nuts, one of the most expensive and exclusive nuts worldwide. Advances in semi-intensive or intensive management for its cultivation as a fruit tree, however, have been limited, with fruits being harvested mostly from natural forests. This can be attributed to the long fruit development cycle (42 months), with one-, two- and three-year-old cones being present simultaneously on the tree, which makes nutritional and hydric management more challenging than in other fruit or forest trees, among other causes.

This thesis studied stone pine growth and fruiting along a climatic gradient in Chile; species variability through morphometric, chemical, Near Infrared Spectroscopy (NIRS) and molecular analyses (proteomic); species adaptation by assessing growth and fruiting response of European provenances in central Chile; and the effects of fertilization and irrigation on fruit production and growth.

Growth was examined in 4,094 trees located in 129 sites distributed across Coquimbo and Araucanía regions in Chile, via several climatic variables, whose relative contribution was assessed through regression trees and linear models. Three macrozones (MZs) showing marked growth differences were defined in the species distribution range: North, South and Dry Coast MZs. In the South MZ, growth rate was the highest for height (35 cm year^{-1}) and DBH ($1.50 \text{ cm year}^{-1}$), whereas in the Dry Coast MZ, the species showed the lowest growth rate in height and DBH (23 and $0.87 \text{ cm year}^{-1}$ respectively). Height growth was favored by winter thermal oscillation above 14°C , annual rainfall above $1,400 \text{ mm year}^{-1}$, an annual average temperature below 14°C and a spring water deficit below 400 mm . DBH growth was also favored by an average annual temperature below 14°C and by a minimum average temperature below 6.6°C .

Fruiting was analyzed by recording cone number per tree (CN) on 3,464 trees distributed along a climatic gradient in Chile. Cone weight at harvest (CW) and in-shell pine nut number per cone (IS) were measured on 76 superior trees. Climatic and biometeorological variables, defined based on 11 physio-phenological reproductive phases, were related to fruit production traits. Fruiting showed marked differences among MZs. The highest values of

cone productivity (32 kg tree⁻¹) and CN (62 cones tree⁻¹) were recorded in the South. Stone pine cone production throughout Chile was favored in decreasing importance by autumn thermal oscillation below 12°C, spring hydric deficit lower than 345 mm, spring rainfall above 65 mm and annual thermal oscillation below 12°C. It was also favored by a minimum temperature above 7°C and a hydric deficit below 200 mm in the female flowering period, by a rainfall below 70 mm during male flowering and a thermal oscillation below 6°C during differentiation of reproductive shoots.

Cone weight was favored by an annual average temperature above 14°C, annual rainfall above 507 mm and an annual hydric deficit below 914 mm; at a maximum temperature higher than 23°C and a hydric deficit lower than 774 mm during embryo development also increased cone weight, as well as an accumulated rainfall higher than 14 mm during the 2 year-old conelet growth period.

IS was significantly increased by an annual hydric deficit below 914 mm and a maximum autumn temperature lower than 19°C, along with a maximum temperature above 24°C during 2 year-old conelet growth, as well as by accumulated rainfall during three key periods (values above 53 mm in induction of male primordia, 78 mm in 3 year-old cone growth and 133 mm during cone ripening). Therefore, water supply is recommended to mitigate the negative impact of water deficit on fruit productivity.

Species variability was studied based on morphological, chemical, NIRS and molecular characterization of stone pine fruits harvested from the three growth MZs. The morphometric analysis showed that stone pine selected trees from the different MZs differed in cone size and pine nut morphometry, and cone to kernel yield. Chemical composition assessed at the MZ level and for 38 selected trees was similar to values reported for the species in the main producing countries, (34.1-35.5% protein, 44.2-40.2% lipids and 4.7-4.4% ash respectively). Chemical composition of selected trees was homogeneous among MZs, with the exception of oleic acid, which was higher in the South MZ, and potassium, which was higher in the Dry Coast MZ. The analysis by macrozones showed that the North MZ had the highest ash content and the Dry Coast MZ the highest values of moisture, protein and lipids. Average phytosterol content reached 2,113 µg/g oil and tocopherols 113 µg/g oil. Stand-alone NIRS measurements were employed for identifying pine nut geographical origin by testing different sample presentations (in-shell and shelled pine nuts, and humid and dried flour). This analysis demonstrated the potential of this technology, since it is proved to be a



rapid and accurate method for predicting the geographical origin of pine nuts in Chile; indeed, 87.8% of samples from the three MZs were correctly classified at the cross-validation stage, and the best model was obtained with shelled pine nuts. Proteomic analysis also allowed us to differentiate populations growing in the three macrozones of the species in Chile, and proved to be a useful tool to detect variability between populations of different environments, with seven proteins being identified as potential population markers. Proteins related principally to nutrient reserve, heat shock stress, glycolytic processes, oxidoreductase and peroxiredoxin activity, and in lower proportion, to carbon fixation and ATP binding, were identified, with 12% remaining unidentified. In summary, the present analysis of stone pine populations in Chile, involving different techniques, showed that the species variability is determined by geographical and climatic factors as a gene expression probably associated with adaptive strategies.

Growth and cone production of six European stone pine provenances established in central Chile were analyzed at the age of 19 years and survival during the first 7 years. The studied provenances significantly differed in cone number per tree and weight, and DBH, height and crown diameter growth. Provenances were separated into three groups, according to growth and productive variables: Italian and Slovenian; Andalucía and Sierra Morena (Spain); and Meseta Castellana (Spain), the latter showing the highest growth and productivity. Individual cone production was positively correlated with cone weight and growth variables.

Afterwards, individual and combined effects of fertilization and irrigation on growth and fruiting, and their correlation, were assessed in plantations located in central Chile. In a 30 year-old plantation (Cáhuil) treatments consisted of control, spring and autumn fertilization, spring and summer irrigation, and combined fertilization and irrigation; and in a 16 year-old plantation (Toconey), treatments included control and spring fertilization. In Cáhuil fertilization had a positive effect one year after application (23.5% superior height growth and 82.3% superior 1 year-old conelet production vs control); after two consecutive years of application, a significant influence on DBH (17.5% superior vs control), height (20.4% superior), and 1 year-old conelet production (36.4% superior vs control) was observed, independently of the hydric status of the trees. Irrigation enhanced fruiting but did not affect growth. The maximum 1 year-old conelet number was observed in fertilized and

irrigated plots (60% superior). In Toconey, tree growth in fertilized plots was higher (41% superior height and 29% crown diameter), and bore 94% and 2.4 times more cones vs the control 3 and 4 years after fertilization. Fertilization also affected cone size, increasing the quantity of heavier cones during the two following harvests. Both cultural practices, applied either individually or combined, are useful tools to enhance fruit production in young and adult stone pine plantations, with a stronger response if applied at younger ages.

Finally, this thesis proposes an intensive management model for stone pine cropping for fruit production in Chile, including techniques commonly used in horticulture, such as fertilization, irrigation, weed control, grafting and different pruning types, including root pruning.

Overall, the scientific results obtained have confirmed the general hypothesis of this doctoral thesis, i.e., stone pine is well adapted to soil and climate of Chile within a wide range of environmental conditions, and has a production capacity that allows us to predict productive levels similar to or higher than those in its native habitat, which makes the species cultivation an important productive alternative for the country.

This thesis makes a valuable scientific contribution to the advancement of stone pine knowledge in several aspects relevant for its domestication, opening new research topics that should be addressed in the future.



Capítulo 1. Introducción

Chapter 1. Introduction

Ejemplares de pino piñonero ubicados en la comuna de El Carmen, región del Bio Bio, Chile

Stone pine trees located in El Carmen municipality, Bio Bio region, Chile



1. Antecedentes generales de la especie

El *Pinus pinea* L. pertenece a la división Spermatophyta Gymnospermae, orden Coniferales, familia Pinaceae, subfamilia Pinoideae, género *Pinus*, sección Pinea (Gutiérrez, 2007), y su nombre común es pino piñonero o pino mediterráneo. Es un árbol majestuoso de hábito simpódico que alcanza 20-30 m de altura y hasta 1,5 m de diámetro (Fig. 1a), con copa globosa, característica compartida sólo con *Pinus nelsonii* Shaw de México. Sus ramas son curvadas hacia arriba cuando joven y en forma de paraguas cuando adulto (Peruzzi *et al.*, 1998) (Fig. 1b), debido a la baja dominancia apical de la especie (Mutke, 2004), que corresponde a una estrategia reproductiva orientada a maximizar la cantidad de puntos de fructificación (Mutke, 2005a). Desde el punto de vista productivo, esta baja dominancia apical es una ventaja respecto a los demás pinos productores de piñones que requieren de intervenciones frecuentes para mantener copas productivas bajas y amplias (Shen, 2003). La duración de su fase juvenil, de bajo consumo energético, depende más del tamaño de la planta que de la edad, pudiendo conservarla durante muchos años para sobrevivir en condiciones adversas (Mutke *et al.*, 2010); le sigue una fase de crecimiento rápido (Peruzzi *et al.*, 1998).

Posee dos hojas aciculares por braquiblasto, lo que junto a un elevado índice de metoxilación es considerado un rasgo de evolución dentro del ambiente mediterráneo en que se desarrolla (Shankar y Lebreton, 2010). Son más cortas, solitarias y de color verde azulado cuando jóvenes, mientras que las definitivas son de color verde, de 10 a 20 cm de longitud y 1,5 a 2 mm de grosor, levemente rígidas y puntiagudas (Castaño *et al.*, 2004), dispuestas alternadamente y en espiral, ligeramente onduladas; persisten 2-3 años, y hasta 4 o más si las condiciones del sitio son buenas. En particular Petisco *et al.* (2006) determinó que sus acículas presentan una longevidad de 2,9 años, lo que según Kikuzawak y Lechowicz (2011) entre especies del género *Pinus* es superior sólo a *P. taeda* L. (1,5 años), e inferior a *P. sylvestris* L. (6 años), *P. resinosa* Aiton (3,8 años), *P. contorta* Douglas (3,9 a 13,1 años) y *P. longaeva* D.K. Bailey (sobre 40 años), y también inferior a otras coníferas, incluidas varias nativas de Chile, tales como *Saxegothaea conspicua* Lindl. (4,2 años), *Podocarpus nubigenus* Lindl. (7,3 años), *Araucaria araucana* (Molina) K. Koch (23,9 años) y *P. salignus* D. Don (32 años). Las ramillas son de color café-amarillo, escamosas y ciliadas (Rodríguez y Rodríguez, 1984).

La corteza es gruesa, de color pardo grisáceo y fisurada en la juventud, más tarde de color canela y profundamente agrietada, desprendiéndose en gruesas placas irregulares

cuadrangulares, en cuyos bordes se aprecian los anillos de crecimiento (Sanz, 1990; Sabillón, 2001); su grosor y bajo contenido de resina respecto a otros pinos le confieren cierta resistencia al fuego.

Su sistema radicular se caracteriza por una raíz principal de rápido crecimiento juvenil (Geisler, 2008), que puede alcanzar más de un metro los dos primeros años, y raíces secundarias muy desarrolladas para extraer agua de napas profundas, casi horizontales, que se extienden en un área muy superior a la cubierta por la copa (Peruzzi *et al.*, 1998; Gutiérrez, 2007), hasta 30 veces mayor (Consejería de Medio Ambiente, 2004). Se han observado casos de anastomosis -unión de elementos anatómicos- entre raíces de diferentes individuos.

Es una especie monoica, presentando las flores de ambos sexos (Fig. 1c) en el mismo individuo, cuya aparición no es sincrónica (Rodríguez y Rodríguez, 1984; Castaño *et al.*, 2004). Las flores masculinas son oblongo-cilíndricas de 10-12 mm, agrupadas en espigas alargadas principalmente en las ramas inferiores, que aparecen antes que las femeninas (Gilman y Watson, 1994). Estas son ovoides, de 20 mm de largo, verdosas o rojizas con ombligos agudos, solitarias o en escaso número, sobre pedúnculos erectos, ubicadas al final de los brotes anuales de ramas situadas en la parte alta de la copa (Rodríguez y Rodríguez, 1984; Montoya, 1990; Gil y Prada, 1993; Borrero, 2004).

La reproducción sexual más frecuente es la alogamia, dado que la polinización es anemófila, estando los granos de polen dotados de estructuras que les permiten volar algunas semanas, pero también es posible la autogamia. Después de la polinización se inicia un ciclo que se prolonga por tres años (García de Pedraza y Pallares, 1989; Castaño *et al.*, 2004), siendo la característica más llamativa del ciclo reproductivo de la especie que la distingue de otras especies del género, en las que usualmente dura dos años. Durante los nueve meses siguientes a la polinización, la piña no sufre cambios visibles en forma externa, aunque en su interior ocurren procesos vitales para el embrión como son la vacuolización de la megaspora, el desarrollo de tejido esponjoso y la autonomía de la nucela (Abellanas, 1990), con un crecimiento limitado de la piña que, de acuerdo a Gordo *et al.* (2005), se expresa cerca de un año después de la polinización (piña de 2 años). Entre abril y agosto del año 3 en España (equivalentes a octubre y febrero en Chile), ocurre el crecimiento y desarrollo principal de la piña (Mutke *et al.*, 2005b; Gordo *et al.*, 2005). La fecundación se realiza en abril en el lugar de origen de la especie, dos años después de la polinización (Abellanas, 1990).

Los estróbilos, denominados piñas o conos, cuando maduros son ovoides, grandes, de



8-14 cm. de largo y 7-10 cm. de ancho, de forma globosa, lustrosos, de color pardo-rojizo, con escamas de consistencia leñosa y apófisis de forma variable, desde casi planas (Molina, 1991) a puntiagudas, ensanchadas hacia el ápice (Sabillón, 2001), recubiertas por una capa de resina. Son sésiles o con pedúnculos cortos, simétricos y gruesos (Fig. 1d). Se ubican en las ramas situadas en la parte alta de la copa (Castaño *et al.*, 2004), generalmente solitarias, ocasionalmente de a dos o tres (Crawford, 1995); persisten en el árbol durante varios años, aún después de la dispersión de las semillas. En cada bráctea normalmente se encuentran dos semillas. Los estróbilos femeninos de uno y dos años son globosos, erectos o casi horizontales (Borrero, 2004).

Las semillas son leñosas, ovaladas, de 17-18 mm de largo, 8-10 mm de ancho y 8 mm de espesor; poseen una testa gruesa –que les permite conservar por varios años su facultad germinativa-, de color café opaco con largo de alas variable (3-20 mm) (Crawford, 1995), cubiertas con un polvo negro comúnmente denominado cisco. En su interior se encuentra el piñón, de 15-16 mm de largo y 5 mm de espesor, de forma alargada, color blanco amarillo y consistencia harinosa (Carnevale, 1955), constituido por el endoderma primario y por el embrión, ambos comestibles. El embrión, y por lo tanto la nueva planta, presenta 10-12 cotiledones (Peruzzi *et al.*, 1998).

Produce una cantidad de piñas equivalente al tejido leñoso vegetativo (Mutke, 2005b), muy superior a lo verificado en otros pinos. La fructificación comienza entre los 15 y 18 años en el Hemisferio Norte, lográndose una producción plena pasados los 30-40 años (Castaño *et al.*, 2004), la que se mantiene hasta los 140-150 años (Pereira *et al.*, 2015). Según Crawford (1995), la producción de piñas inicia a los 10 años, siendo óptima alrededor de los 40 años; Ximénez de Embún (1959) afirma que a los 25 años comienza a producir con rendimientos interesantes cuando alcanza 20 cm de diámetro, concordando con Montoya y Mesón (2004b). Existen diversos antecedentes sobre la cantidad de semillas, variando entre 1.000 y 2.708 kg⁻¹ (Goor y Barney, 1976; Webb *et al.*, 1984; Gil y Prada, 1993; Gordo *et al.*, 1999; Consejería del Medio Ambiente, 2004).



Figura 1. a). Individuo de diámetro sobre 1,5 m; b). Forma típica de pino piñonero cuando adulto; c). Flores masculinas (izq.) y femeninas (der.); d). Piñas con pedúnculos simétricos y gruesos; e). Piñas de 1, 2 y 3 años en desarrollo simultáneo; f). Rodal de 30 años de variedad *Walker*, Australia.

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Normalmente se considera como una especie no longeva (Cutini, 2002), existiendo ejemplares aislados que viven 180-200 años (Gutiérrez, 2007), raramente superando los 200-250 años (Peruzzi *et al.*, 1998), aun cuando Montoya y Mesón (2004a) la consideran de longevidad media-larga, hasta 350-450 años, y ocasionalmente 600 años y más. Es una especie pionera y termófila; muy heliófila, lo que moldea su forma, aun cuando durante los primeros años se desarrolla mejor bajo una débil cubierta protectora que a plena luz (Pemán *et al.*, 2014); moderadamente resistente a vientos marinos; relativamente xerófila, soportando relativamente bien condiciones de aridez, con una resistencia a la sequía intermedia entre *P. halepensis* Mill. y *P. sylvestris* L. (El-Khorchani *et al.*, 2007); presenta una elevada plasticidad fenotípica y variación intraespecífica significativa respecto a varios rasgos en respuesta al déficit hídrico (Sánchez-Gómez *et al.*, 2011). Es resistente a la salinidad y alcalinidad (Trap, 1993; Antonellini y Mollema, 2010; Khaldi *et al.*, 2011).

Se caracteriza por una alta plasticidad, rusticidad y tolerancia a un amplio rango de suelos, con importantes variaciones en el crecimiento y desarrollo según las condiciones del sitio (Gordo *et al.*, 2009), pudiendo sobrevivir muchos años en condiciones adversas, característica que le confiere la habilidad de adaptarse a diferentes micrositos. Estas características hacen que la especie se emplee con variados usos (Pemán *et al.*, 2014), habiendo sido reconocida en Italia como un elemento del paisaje que contribuye al bienestar de la población (Tomao *et al.*, 2016). En forma natural se encuentra tanto en formaciones puras como mixtas (Bracciotti *et al.*, 2003), y es apta para su inclusión en sistemas agroforestales (Franco *et al.*, 2016; Dube *et al.*, 2016).

Su piñón es muy apreciado como fruto seco por su excelente calidad, estando entre las nueve especies productoras de frutos secos más importantes del mundo (Fady *et al.*, 2004). No obstante, los esfuerzos tendientes a su domesticación son bastante recientes, no existiendo variedades o cultivares definidos (Mutke *et al.*, 2007c) que se usen a nivel productivo. Existe sólo una variedad, denominada *fragilis* por la fácil apertura de su piñón con cáscara, pero no se cultiva debido al rápido deterioro de su piñón (Bussotti, 1997; Peruzzi *et al.*, 1998).

Existen varias razones que explican esta situación: es una especie de difícil propagación clonal, que inicia la etapa reproductiva tardíamente (15-20 años); de polinización anemófila, lo que dificulta la realización de cruzamientos controlados; la producción de piñas se produce en yemas preformadas subapicales, lo cual limita las opciones de manejo de la copa para mejorar la producción de frutos; y la maduración del fruto tarda 3,5 años,

encontrándose simultáneamente en el árbol piñas de 1, 2 y 3 años de edad (Fig. 1e), con vecería o añerismo.

Se considera una especie con baja variabilidad genética (Fallour *et al.*, 1997; Gómez *et al.*, 2002; Alía *et al.*, 2003; Nasri *et al.*, 2005a; Gordo *et al.*, 2007; Vendramin *et al.*, 2008; Mutke *et al.*, 2008; Sanchez-Gómez *et al.*, 2009), aislada en términos genéticos y con baja aptitud para hibridar con otros pinos (Vendramin *et al.*, 2008). Gordo (2004) determinó una amplia variabilidad ecológica y productiva, lo que permite seleccionar rodales y árboles por su producción de piñas, estando el 83% de la variabilidad productiva ligada a condiciones ambientales, atribuyéndose la diferencia a la genética. Mutke *et al.* (2005b) al analizar parcelas injertadas concluyeron que el efecto del genotipo presenta un coeficiente de determinación con la producción de piñas muy inferior a la correlación tamaño del árbol y producción, considerando relevante optimizar las condiciones de cultivo y favorecer el desarrollo vegetativo de los árboles para aumentar la producción.

La superficie de pino piñonero en el mundo alcanza 657.515 ha, de las cuales más del 70% se ubica en España, 10% en Portugal y 6% en Turquía e Italia; también existen poblaciones importantes en Francia, Norte de África e Israel (Martín y González, 2000; Mutke *et al.*, 2000).

Dado el uso que se ha dado a la especie, especialmente para sombra y producción de piñones, se ha difundido y cultivado en diversas zonas desde la antigüedad (Gil y Abellanas, 1989; Sanz, 1990; CABI, 2012), resultando difícil determinar con claridad dónde es autóctona y dónde ha sido introducida. Webb *et al.* (1984) consideran que su distribución natural corresponde a la Península Ibérica, en el norte y este del Mediterráneo (33-44°N); algunos autores señalan su origen en el oeste de la cuenca mediterránea (Eig, 1931; Agrimi y Ciancio, 1994), mientras que otros, como Feinbrun (1959) y Quezel (1980) cit. por Fallour *et al.* (1997) lo sitúan en el este, especialmente en Turquía y el Líbano. Según Sanz (1990), sería originaria de Creta, desde donde se habría extendido hacia el oeste, principalmente durante las colonizaciones de fenicios y griegos, y la expansión del imperio romano. Los bosques del sur de Francia y de la Península Ibérica se han estudiado paleobotánicamente, confirmándose como autóctona ya que su presencia data desde la prehistoria (Prada *et al.*, 1997; Martínez y Montero, 2004).

Estudios arqueológicos y paleobotánicos han datado restos de la especie en diferentes épocas históricas (Martínez *et al.*, 2003), incluyendo el pleistoceno superior en cronologías



cercanas a 50.000 años; el paleolítico superior, neolítico, edad de bronce y mesolítico (Salas-Salvadó *et al.*, 2005), demostrando que el hombre usó madera de pino piñonero como combustible y consumió sus piñones desde hace miles de años (Borrero, 2004).

Su amplia distribución ha llevado a priorizar la hipótesis de que la especie cuenta con una gran amplitud ecológica frente a aquella basada en la dispersión de la especie por causa de la acción antrópica. Dado que las zonas costeras del sur de Europa ejercieron como refugio para diversas especies durante la última glaciación, incluyendo los pinos mediterráneos, se considera que el pino piñonero pudo permanecer en reductos térmicos durante los periodos fríos del pleistoceno (Martínez *et al.*, 2003).

La especie se ha introducido en África (Libia, Túnez, Argelia, Marruecos, Zimbabue y Sudáfrica), Asia (Georgia, Rusia, Irak, Israel y China), Norteamérica (California, EE.UU.), Sudamérica (Argentina, Chile, Brasil y Uruguay), Europa (Albania, Rusia y Croacia) y Oceanía (Australia y Nueva Zelanda). A principios del siglo XX se estableció en Australia, donde se ha identificado una variedad muy vigorosa y altamente productiva denominada Walker (Fig. 1f), que tiene la particularidad de desprender las piñas en forma espontánea antes que se abran, lo que reduce las labores de la cosecha sólo a la recogida de las piñas desde el suelo, con una importante reducción de su costo.

Se ha detectado una reducción significativa de algunas poblaciones (Testi *et al.*, 2007), presentando muchos bosques de la especie síntomas de disminución y/o deterioro debido a problemas fitopatológicos, incendios acentuados en los últimos años por el cambio climático (Molina *et al.*, 2011), contaminación por *spray* marino, erosión costera generada por cambios en las corrientes marinas, estrés hídrico y daños derivados de fauna consumidora de piñones (Peruzzi *et al.*, 1998).

2. El piñón, características bromatológicas y usos

Los piñones de *P. pinea* se han utilizado históricamente como alimento, consumiéndose de diversas formas (crudos, secos, tostados, en salsas, en repostería y como condimento, entre otras), formando parte de la llamada Dieta Mediterránea (Lutz y Luna, 2016), aun cuando durante el imperio romano fue considerado un alimento de lujo que definía una identidad social en poblaciones no mediterráneas (van der Veen, 2003).

Se ha demostrado que su consumo, en ciertas dosis, disminuye el riesgo de accidentes vasculares (Segura *et al.*, 2006) y otras enfermedades crónicas no transmisibles, destacando por su contenido en ácido linoleico (omega 6) y linolénico (omega 3) (Costa y Evaristo, 2008; Sabaté y Ang, 2009). En particular, Bao *et al.* (2013) observaron una asociación inversa de su consumo con el riesgo de las mayores causas de muerte (cáncer, enfermedades cardíacas y respiratorias), así como con una reducción del riesgo de enfermedades coronarias e infarto no fatal del miocardio (Nergiz y Dönmez, 2004).

Es por ello que fueron incluidos, junto a la almendra, avellana, maní, pecana, pistacho y nuez, en un mensaje saludable o *health claim* aprobado por la U.S. Food and Drug Administration que indica que “estudios científicos sugieren, aunque no han demostrado, que el consumo de 1,5 onzas diarias de la mayoría de los frutos secos, como parte de una dieta baja en grasas saturadas y colesterol, puede reducir el riesgo de padecer una enfermedad cardíaca” (FDA, 2003). Por ello poseen un alto valor comercial, con un mercado caracterizado por una demanda insatisfecha creciente y una oferta en disminución (Ruitenberg, 2013).

Su composición química se ha caracterizado en Italia (Peruzzi *et al.*, 1998; Ruggeri *et al.*, 1998), Turquía (Bagci y Karaagacli, 2004; Ozcan, 2006), Túnez (Nasri *et al.*, 2005a,b), España (Cañellas *et al.*, 2005), Portugal (Carvalho, 2006; Costa y Evaristo, 2008; Evaristo *et al.*, 2008) y Chile (Escalona, 2005; González *et al.*, 2012), encontrándose que contienen cantidades elevadas de proteína vegetal (34%), con cerca de 20 aminoácidos, entre ellos la mayoría de los esenciales; y de grasas (48%), ricas en ácidos grasos insaturados, fibra (Salas-Salvadó *et al.*, 2005), vitaminas (ácido fólico o B9, niacina o B3, tocoferoles o E, B6 y B2), minerales (potasio, fósforo, calcio, hierro, cobre, zinc, magnesio y manganeso) (Castaño *et al.*, 2004) y bajo contenido de sodio, así como diversos compuestos bioactivos saludables, como fitoesteroles y polifenoles (Blomhoff *et al.*, 2006; Álvarez, 2015), con alto valor



nutricional (Segura *et al.*, 2006) (Tabla 1). En particular, poseen fitoesteroles en valores similares al pistacho y muy superiores a otros frutos secos (Escalona, 2005; Álvarez, 2015).

Tabla 1. Composición nutricional de piñón mediterráneo en varios países (g/100g)

Componente	Chile	España ²			Portugal		Turquía ⁴	Italia ⁵
	Mix país ¹	Andalucía	Meseta Norte	Sistema Central	Alcacer do Sal ²	Mezcla país ³	Kozak	Mezcla país
Humedad	4,42	4,66	4,88	5,18	7,70	5,90	5,10	5,51
Cenizas	4,53	4,45	4,60	4,96	4,43		4,50	4,14
Proteínas	35,93	36,37	36,99	37,44	36,52	33,85	31,60	39,45
Grasa total	46,02	49,26	48,15	47,36	46,17	44,71	44,90	42,50
Hidratos de Carbono	8,02	4,95	5,89	5,04	6,00		13,90	
Energía (Kcal)	590	609	605	596	586		580	

Fuente: 1: INFOR (2009); 2: Borrero (2004); 3: Costa y Evaristo (2008); 4: Nergiz y Dönmez (2004); 5: Peruzzi *et al.* (1998).

Los contenidos significativos de dichos compuestos bioactivos saludables le confieren a los piñones de *P. pinea* la característica de ser un alimento saludable per se, ya que su composición tiene una serie de efectos benéficos en la salud.

Estas características, junto a su sabor refinado y agradable, son responsables de los numerosos usos, principalmente alimentarios y medicinales, que se da a este interesante fruto (Loewe y Delard, 2012). A ello se suma el ser reconocido en forma creciente, sobre todo en países en que su consumo no ha sido tradicional, como un alimento de lujo que ofrece distinción y estilo debido a su calidad, énfasis característico de sociedades con marcada diferenciación de clases sociales, en los cuales puede contribuir a crear o a exaltar la exclusividad y diferenciación deseadas.

3. El pino piñonero en Chile

En Chile varias coníferas exóticas fueron introducidas por inmigrantes europeos desde el siglo XVII, inicialmente como especies ornamentales y más tarde para controlar la erosión y estabilizar dunas. El pino piñonero en particular fue introducido por colonizadores principalmente españoles e italianos como parte de su cultura y gastronomía, adaptándose muy bien, como ha sucedido en el país con numerosas especies, entre ellas el *Pinus radiata* D. Don. De hecho, en ciertas zonas se observa presencia de regeneración natural alrededor de las copas de grandes árboles, no siendo considerada una especie invasiva (Simberloff *et al.*,

2010).

Entre 1890 y 1920, fue empleada por Federico Albert, científico alemán que desempeñó un importante papel en el desarrollo de la ciencia forestal en Chile, para estabilizar dunas y dar sombra y protección al ganado en la región del Maule. Más tarde, entre 1930 y 1950 el Ministerio de Tierras y Colonización realizó acciones que se reflejan en la existencia de majestuosos individuos aislados de más de 1 m de diámetro (Fig. 1a). Posteriormente, en el marco del Plan Chillán (1955 a 1962) se establecieron rodales de numerosas especies forestales, entre ellas pino piñonero, plantadas en grupos de 25 árboles para proporcionar sombra a animales, lo que explica la existencia en las antiguas provincias de Maule, Ñuble y Concepción, de árboles aislados o pequeños bosquetes de más de 50 años, con interesante desarrollo (Fig. 2a). En 1967 el Departamento Forestal de la Dirección General de Agricultura y Pesca realizó una siembra aérea de la especie en la precordillera de Ñuble y Bio Bio.

Más tarde su presencia se amplió, sobre todo en zonas rurales de mayor fragmentación de la propiedad, por el interés que reviste para sus habitantes. La superficie cubierta por la especie en Chile, se estimaba en unas 100 hectáreas (Loewe *et al.*, 2011b; Loewe y Delard, 2012), habiéndose cortado numerosas plantaciones para elaborar chips dado el desconocimiento de la especie. Durante los últimos años se han realizado plantaciones orientadas a la producción de piñones (Fig. 2b), que superaron las 100 ha en 2014 (Loewe *et al.*, 2014a) y más de 350 ha en 2015 (Loewe, 2015), existiendo proyecciones de ulterior crecimiento en el corto y mediano plazo.

Adicionalmente a la producción de piñones, la especie reviste interés por su uso potencial de captura de carbono, lo que ha motivado la realización de estudios alométricos requeridos para su cuantificación (Correia *et al.*, 2010), y se prevé que en Chile se establezcan plantaciones con este objetivo adicional a la producción frutal (Unda¹, com. pers., 2016), representando el ingreso por la venta de créditos de carbono –cuyo precio ha venido aumentando– un interés especial, sobretudo en la fase inicial y hasta que se alcanza una producción de piñones en régimen. Otra opción en estudio corresponde a la asociación de la especie con la trufa *Tuber borchii* Vittad., para producir en forma conjunta piñones y trufas (Delard², com. pers., 2016), existiendo referencias sobre dicha micorrización (Bussotti, 1997; Cutini, 2002; Morcillo *et al.*, 2013, 2015; Micología y Forestal Aplicada, 2016).

¹ Alfredo Unda, 2016. Mikro-Tek Chile.

² Claudia Delard, 2016. INFOR.



4. Hipótesis de trabajo

El Instituto Forestal de Chile (INFOR) ha dedicado importantes esfuerzos de investigación durante los últimos veinticinco años al estudio del pino piñonero debido al enorme potencial de adaptación y producción que presenta la especie en Chile. La demanda creciente por el piñón, fruto de sus excelentes características organolépticas y nutricionales, ha motivado un aumento del interés por conocer la productividad de la especie en el país, y entender su grado de adaptación durante más de un siglo de presencia. Asimismo, es necesario validar y adaptar el conocimiento desarrollado desde hace décadas sobre su cultivo, principalmente en Europa, además de desarrollar nuevas técnicas silviculturales dadas las condiciones diversas en que se desarrolla la especie en Chile.

La potencialidad productiva y la viabilidad económica del cultivo han motivado a agricultores e inversionistas, tanto chilenos como extranjeros, a interesarse por el cultivo de la especie, lo que se ha traducido en varias acciones de divulgación/extensión (Anexo 1), y en el establecimiento de diferentes ensayos orientados a desarrollar sistemas productivos bajo una silvicultura adecuada a las condiciones de Chile. Las principales ventajas que tiene Chile para el establecimiento de esta especie son un clima adecuado, amplitud térmica día/noche en el periodo de maduración que origina un apreciado sabor tanto a la fruta fresca como a los frutos secos; ausencia de plagas y enfermedades; y la *contra estación*, que permitiría obtener piñón comercializable en el periodo de mayor consumo en el Hemisferio Norte.

La hipótesis de trabajo general de esta tesis doctoral parte de la adaptabilidad y la capacidad productiva que el pino piñonero ha mostrado en Chile, dada la similitud climática de la zona centro sur del país con la zona mediterránea, de donde se origina la especie, lo que permite prever niveles productivos similares e incluso superiores. La silvicultura del pino piñonero es una importante opción productiva para Chile, con potencialidad entre las regiones de Valparaíso y los Ríos. Sin embargo, esta opción debe validarse mediante el estudio de la variabilidad y adaptación de poblaciones introducidas, el análisis de la influencia del clima local en su crecimiento y productividad frutal, y la elaboración de una propuesta de cuidados culturales adecuados a las condiciones ecológicas en su área chilena de distribución.

Las características mencionadas la posicionan como una especie prioritaria para la diversificación productiva de los cultivos forestales en Chile, como una alternativa productiva para sectores rurales de menores ingresos que no pueden sustentar su economía en las especies forestales tradicionales –pino radiata y eucaliptos-, dado el largo periodo de

recuperación de la inversión y su rentabilidad, atractiva solamente a gran escala; y como una opción para la fruticultura, que debido a los efectos del cambio climático se enfrenta a un recambio de especies/variedades por otras con menores requerimientos hídricos, desplazando las tradicionalmente producidas hacia el sur.

La hipótesis general planteada en esta tesis doctoral se ha dividido en las siguientes hipótesis científico-tecnológicas:

- a) El material de base de la especie existente en Chile se estima tiene una base genética reducida, ya que fue introducido principalmente por inmigrantes italianos y españoles, habiéndose realizado también importaciones puntuales desde Portugal (Araneda, com. personal, 2013).

Es notable que existiendo una demanda insatisfecha creciente de piñón, y siendo un negocio atractivo por los altos precios que alcanza, caracterizados por su inelasticidad (Loewe y González, 2007), y correspondiendo al fruto seco más exclusivo y caro del mundo (€ 25-45 kg⁻¹ de piñón blanco a productor mayorista), esta especie, a diferencia de otros árboles frutales, no ha sido domesticada y carece de variedades (Mutke *et al.*, 2007c) que puedan emplearse a nivel productivo. Por ello, la caracterización del material de base de pino piñonero existente en Chile, desde el punto de vista productivo, morfométrico, químico y molecular, permitirá una mejor evaluación que facilite la determinación de acciones para seleccionar el material de base más adecuado para el desarrollo del cultivo en Chile.

- b) El piñón de pino es un fruto seco muy apreciado en las zonas productoras tradicionales (países mediterráneos) por sus destacadas propiedades nutricionales y sensoriales. Además de los nutrientes, contiene diversos compuestos bioactivos beneficiosos para la salud, como fitoesteroles, polifenoles, tocoferoles y otros (Nergiz y Dönmez, 2004; Gurí *et al.*, 2006; Bolling *et al.*, 2011; Evaristo *et al.*, 2013), habiéndose concluido que la calidad de piñones de pino cosechados en una amplia zona de Chile es comparable a la de piñones europeos (González *et al.*, 2011, 2012).

Es, por ello, que el ulterior análisis de la composición química permitirá caracterizar nutricionalmente el piñón de pino producido en Chile en distintas zonas geográficas, generando información que otorgue un valor agregado a este fruto seco producido en el país, potenciando su cultivo y comercialización.

- c) La producción mundial de piñones ha mostrado en la última década una disminución



progresiva muy importante debida al ataque del insecto *Leptoglossus occidentalis* Heidemann (Heteróptera, Coreidae) (Fig. 2c) (Mutke *et al.*, 2014), y se prevé que este problema se mantenga por un largo periodo, e incluso se agrave. Chile está en condiciones de aprovechar esta coyuntura de mercado a través del fomento de plantaciones y huertos injertados de la especie, dado que en el país, así como en todo el Hemisferio Sur, no está presente este agente de daño, lo que permitirá la intensificación del cultivo generando empleo, actividad económica e ingreso de divisas. El *L. occidentalis* es originario del oeste de EE.UU., desde donde se expandió hacia el este de ese país, a Canadá y a México. En Europa es citado por primera vez en Italia en 1999 y luego en España el 2003 (Domínguez, 2011), y más tarde en Portugal el 2010 (Calado, 2011), propagándose en forma progresiva, con un comportamiento invasor. Actualmente está presente en todos los principales países productores del Hemisferio Norte, relacionándose la significativa reducción de producción de piñones a la acción de este insecto que ataca las piñas de 1 año, la mayoría de las cuales posteriormente cae; aquellas que sobreviven y continúan su desarrollo tienen un alto porcentaje de piñones vanos, reduciéndose el rendimiento del 3,5-4% tradicional hasta menos de 1,8% (Calama *et al.*, 2014), e incluso hasta un 0,8%, es decir, de 100 kg de piña se obtienen 800 g de piñón blanco (Loewe y González, 2011, 2012).

El control de la plaga se está estudiando, existiendo los mayores avances en Italia dado el alto impacto que ha tenido sobre la producción de piñón, destacando la importación desde Canadá de un parasitoide de sus huevos, el himenóptero *Gryon pennsylvanicum* Ashmead (Hymenoptera, Platygasteridae) (Roversi *et al.*, 2011), como parte del programa de control nacional, encontrándose en etapa de cuarentena y reproducción.

La evaluación de la situación fitosanitaria de la especie en Chile realizada por González (2012) indica ausencia de plagas y enfermedades, lo que refuerza la potencialidad de Chile como productor potencial de este fruto.

- d) Chile presenta características edafoclimáticas que permiten el cultivo de *Pinus pinea* L. en importantes áreas, donde la especie ha mostrado adaptarse bien por más de 100 años, mostrando un crecimiento y una productividad comparables, e incluso superiores, a su hábitat nativo, lo que permite su establecimiento y aprovechamiento comercial. Esta importante potencialidad ya fue descrita por Albert (1909) hace más de 100 años, en uno de sus inspiradores libros.

Existen ejemplares de la especie entre las regiones de Coquimbo (clima árido seco) y Los

Lagos (clima templado húmedo), en forma de árboles aislados, bosquetes, cortinas cortaviento y plantaciones tradicionales, lo que ratifica la plasticidad de la especie para adaptarse a diferentes condiciones ambientales y de manejo. Específicamente, se ha constatado la presencia de la especie en más de 150 localidades, con gran diversidad de edades, desde plantaciones nuevas a otras que superan 100 años.

Ávila *et al.* (2012) determinaron las áreas potenciales para el cultivo de la especie en Chile entre las regiones de Coquimbo y la Araucanía, concluyendo que existe una superficie potencial de 8,7 millones de hectáreas que serían aptas para el cultivo de la especie sin riego. De ésta superficie, 1,2 millones de hectáreas serían de alta productividad frutal, concentradas en las regiones de Bio Bio y la Araucanía, y más de 3,6 millones de hectáreas de productividad media. Si se considera el uso de riego, la superficie potencial ascendería a 9,3 millones de hectáreas. Adicionalmente, la especie reviste gran interés desde el punto de vista de la protección ambiental, objetivo con el que podrían establecerse casi 2 millones de hectáreas en zonas con precipitación anual entre 250 y 350 mm. La evaluación económica privada considerando diferentes escenarios de precios, productividad, incentivos y productos, revela la conveniencia económica del cultivo para producir piñas y piñón con cáscara, así como significativos impactos socioeconómicos y ambientales que conllevaría su cultivo en Chile, especialmente en zonas deprimidas económicamente (Loewe y Delard, 2012).

Por otra parte, el Dr. Sven Mutke, durante la visita realizada a Chile el año 2009, indicó que *el potencial de crecimiento del pino piñonero en Chile parece elevado desde el punto de vista biológico-agronómico, comparable con las mejores situaciones de Portugal* (Mutke, 2009), indicando que Chile podría alcanzar un nivel productivo significativo. Esta aptitud del país al cultivo de árboles, en particular frutales, fue descrita hace más de un siglo por Espinoza (1897), al afirmar que en Chile “los árboles frutales crecen admirablemente y fructifican con mucha facilidad”.

Mediciones de crecimiento realizadas en Chile indican el potencial de la especie, mostrando un vigor elevado, aun cuando presenta diferencias importantes según las zonas en que se desarrolla, siendo superior en aquellas de mayor disponibilidad hídrica (Loewe *et al.*, 1998; Álvarez, 2010; Loewe *et al.*, 2011d). La productividad media en plantaciones es de 8 m³/ha/año en la zona centro norte, y 12,7 m³/ha/año en la zona centro sur, similares o superiores a las mejores situaciones de Europa, como Portugal, donde crece 4 m³/ha/año



(Vacas de Carvalho y Lopes, 1991) y de Túnez, donde alcanza 3 a 9 m³/ha/año (Ammari *et al.*, 2011).

Desde el punto de vista de la producción de piña, presenta una entrada en producción adelantada en relación a su área de distribución natural en el Mediterráneo europeo, donde comienza a fructificar alrededor de 15-20 años, encontrándose en Chile individuos en producción a partir de los 5-10 años (Loewe *et al.*, 2011a,d, 2012b; Loewe y Delard, 2012). En plantaciones forestales tradicionales, la producción de piña es reducida debido a la densidad elevada (Fig. 2d) y al manejo inadecuado, equivalente al aplicado al *P. radiata*; no obstante, la media alcanza cerca de 70 piñas por árbol en la clase diamétrica 41-60 cm (Loewe y Delard, 2012).

- e) El conocimiento y aplicación de técnicas apropiadas para el cultivo intensivo de la especie (Cutini, 2002), junto a una selección fenotípica de individuos con productividad superior a la media, permitirá una producción frutal sostenida y atractiva en términos económicos. La implementación de estas técnicas permitirá disminuir el *añerismo*, aumentar la producción de piñones y homogeneizar su calidad.

El ciclo de producción frutal de la especie se caracteriza por presentar años de grandes cosechas intercalados con otros de baja producción, fenómeno conocido como *añerismo* o *vecería* (Prades *et al.*, 2005), dependiendo la producción de piña de factores genéticos, edafoclimáticos, sanitarios, etarios y de densidad (Costa y Evaristo, 2008). Una alternativa para aumentar la producción de piñas, anticipar y homogeneizar la producción y facilitar su cosecha es mediante el uso de injertos (Cutini, 2002; Mutke *et al.*, 2007c), cuya aplicación a nivel de viveros permitiría producir gran cantidad de plantas de calidad.

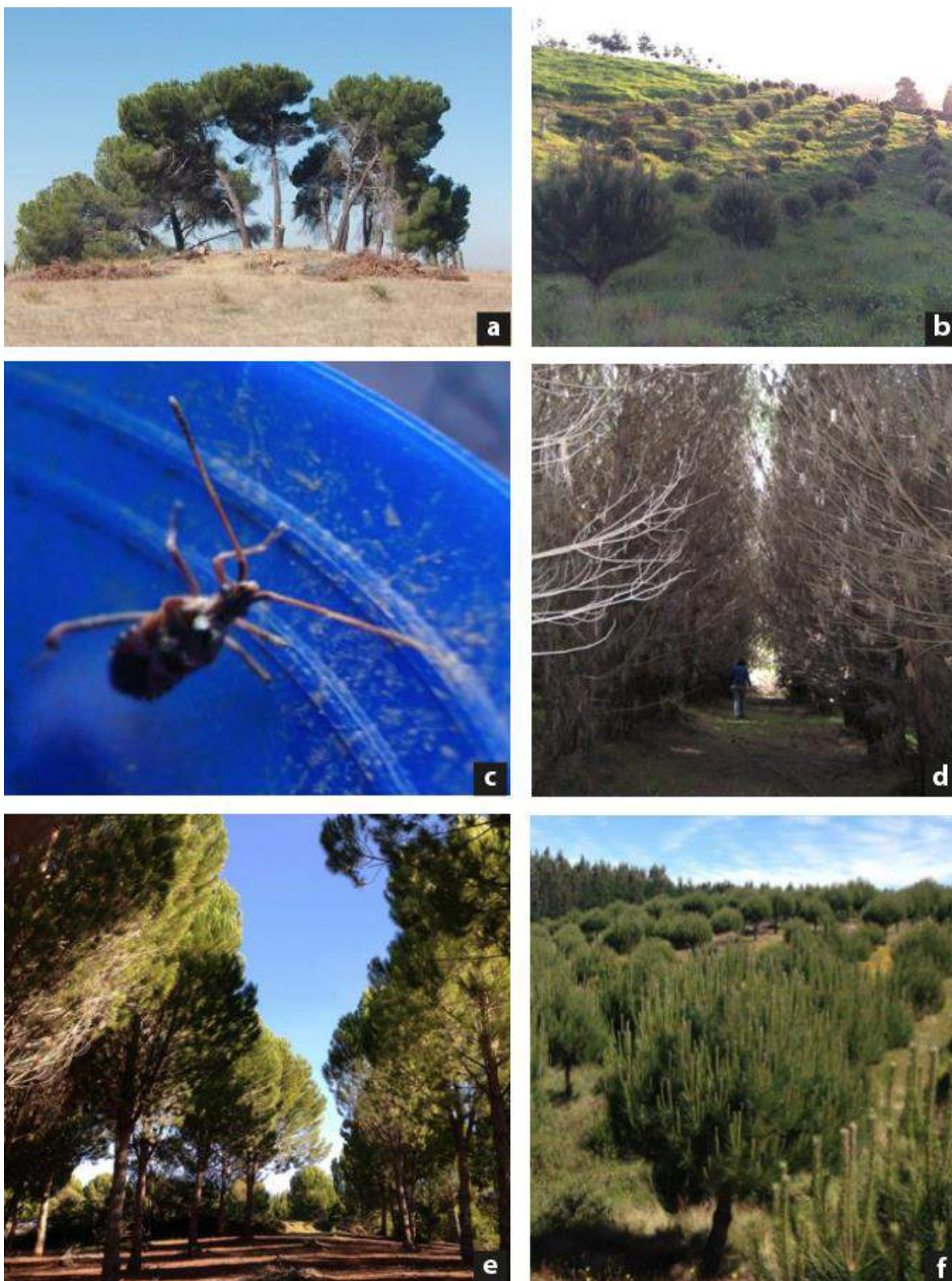


Figura 2. a). Bosquete de la zona central de Chile; b). Plantación de 5 años en región de la Araucanía; c). Ejemplar de *Leptoglossus occidentalis*; d). Plantación de 30 años establecida a alta densidad; e). Plantación de 19 años, raleada y podada a los 15 años; f). Plantación de 10 años podada.
 © a, b, c, e, f. V. Loewe; d: A. Salinas.



Las técnicas clásicas de manejo silvícola, como raleos y podas de fructificación, son poco usadas en los bosques de los países productores, donde se concentra la cosecha de piñas (Gordo *et al.*, 2009), pero en Chile se han probado con resultados interesantes en plantaciones jóvenes (Fig. 2e) (Loewe *et al.*, 2011c; Loewe y Delard, 2012). La disponibilidad hídrica también es un factor de gran incidencia en el crecimiento (Mazza *et al.*, 2014) y productividad. De hecho, el año 2008 se estableció en Cataluña (España), un ensayo con plantas injertadas para estudiar el efecto del riego, cuyos resultados han mostrado un aumento en la floración, con mayor inducción floral en los árboles regados (Aleta y Vilanova, 2014), a lo que se suma un mayor peso de las piñas (Bono y Aleta, 2011). Un estudio realizado en Chile (Loewe y Delard, 2014) mostró resultados coincidentes.

En Chile se han diseñado y establecido unidades experimentales en varias zonas del país con diferentes objetivos (estudio de densidad, origen de material colectado en Chile e importado, fertilización, manejo de plantaciones jóvenes y adultas, huertos clonales), obteniéndose resultados preliminares relacionados al manejo de formaciones adultas, y a técnicas de manejo tales como fertilización (Loewe *et al.*, 2012a), poda (Loewe y Delard, 2012) (Fig. 2f), raleo (Loewe *et al.*, 2011c, 2013), injerto (Loewe *et al.*, 2011a; Loewe y Delard, 2012), riego (Loewe y Delard, 2014) y manejo agroforestal (Loewe y Delard, 2015b).

El desarrollo de técnicas orientadas a la producción de piñones, unido al interés de inversionistas innovadores, incrementará la superficie de plantaciones de la especie en Chile, generando una nueva oferta que podrá insertarse en los nichos de mercado que comercializan el producto, tal como está sucediendo con Nueva Zelanda, donde se cultiva en suficiente cantidad para sustentar una actividad comercial, existiendo una industria de elaboración del piñón en expansión (Vanhanen y Savage, 2013). En particular destacan como de mayor interés los mercados de grandes dimensiones, como EE.UU., Alemania, China y Rusia, así como aquellos en donde el consumo *per capita* es mayor (España, Australia e Israel) (Evaristo, 2016).

5. Objetivo y estructura de la tesis

En función de las hipótesis descritas, se determinó como **objetivo general** de esta tesis caracterizar el crecimiento, la fructificación, la variabilidad y la adaptación de plantaciones de pino piñonero (*Pinus pinea* L.) en Chile, en función del gradiente climático del país y de los principales tratamientos silviculturales orientados a optimizar la producción de fruto.

Ese objetivo general se ha dividido en los siguientes objetivos específicos:

1. Evaluar el impacto de variables climáticas en el crecimiento de plantaciones de *Pinus pinea* L. distribuidas a lo largo de Chile.
2. Determinar la productividad frutal de *Pinus pinea* L. a través de un gradiente latitudinal en Chile y relacionarla con el clima.
3. Caracterizar la variabilidad morfométrica, química, de espectroscopía del infrarrojo cercano y molecular (-ómica) del piñón de *Pinus pinea* L. en Chile.
4. Evaluar el crecimiento y la producción de procedencias europeas de *Pinus pinea* L. establecidas en la zona central de Chile.
5. Analizar el efecto de la fertilización y del riego en el crecimiento y fructificación de plantaciones adultas jóvenes de *Pinus pinea* L.

Dichos objetivos se han estructurado en los siguientes capítulos principales de investigación de la Tesis:

En el **Capítulo 1** se presenta la introducción general, seguida del **Capítulo 2** que se centra en el análisis de la influencia del clima en el crecimiento a lo largo de la zona de distribución de la especie, a partir de la evaluación de 4.094 individuos distribuidos en siete regiones del norte, centro y centro-sur de Chile, en un gradiente latitudinal de unos 1.000 kilómetros.

El **Capítulo 3** evalúa la productividad frutal de *P. pinea* L. a lo largo del gradiente climático de su distribución en Chile, estudiando la producción de piñas de 3.464 árboles y analizando el impacto de variables climáticas y biometeorológicas en la producción frutal.

En el **Capítulo 4** se analiza la variabilidad de la morfometría del fruto (piña), de la semilla (piñón con cáscara) y de la almendra (piñón pelado), a partir del estudio de 76 árboles seleccionados por su mayor carga frutal distribuidos en 46 sitios en Chile; de la composición química y nutricional del piñón de *Pinus pinea* L. cosechado tanto de árboles seleccionados como de una muestra aleatoria y representativa obtenida en las tres macrozonas de Chile; y de la Espectroscopia del Infrarrojo Cercano (NIRS) evaluando, por primera vez, la aplicabilidad



de la técnica para autenticar orígenes geográficos del piñón mediterráneo.

Le sigue el **Capítulo 5**, en el que se estudió el perfil proteico de piñones de *P. pinea* empleando la proteómica como herramienta aplicada a semillas de 30 individuos seleccionados por su mayor producción frutal, distribuidos homogéneamente en las tres macrozonas.

El **Capítulo 6** se centró en la evaluación del crecimiento y producción de seis procedencias europeas de la especie establecidas el año 1994 en Casablanca, región de Valparaíso, a fin de estudiar la variabilidad entre genotipos (procedencias) de *P. pinea* como respuesta a una situación edafológica y climática difícil.

Posteriormente, en el **Capítulo 7** se analiza el efecto de la fertilización y del riego para proponer un modelo de manejo adecuado a las condiciones ecológicas en su área de distribución en Chile, proponiendo técnicas que mejoran el desarrollo y la producción de piñones bajo esquemas de manejo semi intensivo o intensivo aptos para las condiciones del país.

El **Capítulo 8** realiza una síntesis y discusión general de los resultados obtenidos en cada capítulo, revisando, analizando e integrando todo el trabajo desarrollado, y en el **Capítulo 9**, se exponen las principales conclusiones del trabajo.

El **Capítulo 10** presenta las referencias bibliográficas citadas, y el **Capítulo 11** los anexos a esta tesis, incluyendo los artículos divulgativos y científicos publicados.



Capítulo 2. Impacto del clima en el crecimiento de *Pinus pinea* L. a lo largo del área de distribución de la especie en Chile

Chapter 2. Impact of climate on stone pine (*Pinus pinea* L.) growing in Chile

Anillos de crecimiento de rama de ejemplar de pino piñonero en Purén, región de la Araucanía, Chile

Growth rings of a stone pine branch from a tree growing in Purén, Araucanía region, Chile

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Impact of climate and management variables on stone pine (*Pinus pinea* L.) growing in Chile

Abstract

Pinus pinea is an interesting species especially for its fruit production, which depends upon vegetative growth. Growth of this species was analyzed along a climatic gradient in Chile, in all sites where it was planted in the last century. Three macrozones (MZs) located in the north, south and coastal range were identified according to height and DBH growth rates. We also examined growth in relation to several climatic variables (annual and seasonal temperatures, thermal oscillation, rainfall and a hydric index measuring water stress) and two cultural practices (irrigation and pruning). The relative contribution of each variable on growth measurements was assessed through regression trees and linear models. Growth of stone pine showed marked differences among the three MZs. In the South MZ, growth rate was the highest for height (0.35 m year^{-1}) and DBH ($1.50 \text{ cm year}^{-1}$), whereas in the Dry Coast MZ, the species showed the lowest growth rate in height (0.23 m year^{-1}) and DBH ($0.87 \text{ cm year}^{-1}$). Temperature and rainfall had a high significant impact on height growth, which was favored by an annual average temperature below 14°C , high winter thermal oscillation ($>14^{\circ}\text{C}$), spring water deficit lower than 400 mm and annual rainfall over 1,400 mm year^{-1} . DBH growth was also favored by an average annual temperature below 14°C . Significant effects of pruning and irrigation were found. Stone pine growth throughout Chile was high compared to growth rates reported for other countries. However, in light of climate change, we should expect a reduction in growth rates especially in the North and Dry Coast MZs. Heat tolerance is proposed as a key breeding trait for increasing potential growth of stone pine.

Key words: climate impact, stone pine plantations, vegetative growth.

1. Introduction

Stone pine (*Pinus pinea* L.) is a species well known for its role as colonizer and eroded soil stabilizer, and for the economic use of its edible pine nuts. It is native to the European Mediterranean area, and has evidenced an expansion over the last century, especially in the southern and eastern Mediterranean basin, as well as a large increase of planted areas in its origin countries through forest restoration and farmland afforestation (Boutheina *et al.*, 2013). *P. pinea* is a highly interesting species given its multiple uses, and especially its fruit production, which depends upon vegetative growth. Pine nuts are the most expensive dried fruit in the world, with an increasing demand. However, production is undergoing a significant reduction worldwide caused by *Leptoglossus occidentalis* Heidemann (Heteróptera, Coreidae) an insect native to North America, which has affected the species forests across the northern hemisphere. Another factor that may be threatening production is climate change, which is also causing a decrease in growth rates in its native habitat (Mutke *et al.*, 2015).

The species was introduced in Chile by European immigrants (mainly Spaniards and Italians). Since the beginning of the 20th century, it has been used as a forest tree in a successful coastal sand dunes stabilization program (Albert, 1909) and was later included in other afforestation and rural development programs (Loewe and Delard, 2012).

As a result of these plantation activities, a total of 100 ha were established in Chile in different regions, settings (isolated trees, groves and forest plantations) and management conditions. Since the 1990's, a series of studies on stone pine were performed to assess its potential as an alternative crop to forest species (*Pinus radiata* D. Don or *Eucalyptus* sp.) or some fruit species (almonds or other higher water demanding species) for the Chilean Mediterranean zone, confirming its ecological adaptation expressed through a vegetative growth superior to the one presented in the main distribution areas across the world (Loewe *et al.*, 1998; Loewe and González, 2003; Loewe *et al.*, 2012b, 2014b). Potential areas for its cultivation in the country were studied by Ávila *et al.* (2012), showing that the species can grow in more than 8.6 million hectares and that there are over 4.8 million hectares suitable for medium and high pine nut production level.

The species has been found to have a low provenance differentiation in relation to growth parameters, high phenotypic plasticity, tolerance to poor soils and to adverse climatic



conditions such as drought and extreme or late frosts, and strong sensitivity to intra and interspecific competition (Thuiller *et al.*, 2003; Mutke *et al.*, 2008; Sánchez-Gómez *et al.*, 2009; Boutheina *et al.*, 2013). In particular, several authors (Gonçalves and Pommerening, 2012; Correia and Freire, 2014) have observed that the biggest trees produce more cones than smaller trees, being crown variables more related to cone production than age or tree density.

The study of growth dependence upon climatic and management variables is key for understanding the best conditions and practices for cultivation of stone pine with commercial purposes. More than one hundred years ago the species was characterized in several ecological aspects, including climatic characteristics, indicating that it prefers warm climates, with annual average temperature between 9 and 21°C, low valleys and altitude under 1,000 m a.s.l. (Romero and Gilsanz, 1888). Pardos *et al.* (2014) reported that, being a temperate pine, adult stone pine trees show a cyclic, preformed growth pattern occurring mainly in spring (in the northern hemisphere from April to early July). A correlation between tree-ring width and September-June rainfall was reported by Raventós *et al.* (2001), pointing out that the high correlation between tree growth and water availability is the main factor influencing the species growth under dune Mediterranean conditions. Accordingly, Gadbin (1994) indicated that spring rainfall favors diameter growth in the species, as well as autumn rainfall, but to a lower extent. In Turkey, Akkemik (2000) also reported a positive effect of the current annual rainfall and spring temperature on diameter growth. Other authors, such as Thuiller *et al.* (2003), stated the importance of extreme temperature and annual precipitations in the ability of this species, as well as other species, to survive and grow, defining its spatial distribution.

Despite the high growth reached by the species in Chile, no studies have addressed the relationship among growth performance and climatic and management variables; here, we make a contribution to the knowledge of these aspects. In this study, we addressed the following specific objectives: (i) to establish growth macrozones (MZs) for stone pine in Chile, (ii) to compare growth (height and DBH) responses to climate in relation to MZ climatic variability, and (iii) to assess growth response to management practices (irrigation and pruning) subjected to different climatic conditions.

2. Material and Methods

2.1 Study area

Over the past years, given the interest for stone pine cultivation, the Chilean Forest Institute (INFOR) has been focused on a long-term, large-scale national research effort to gather data from Chile, covering the area between Coquimbo (30.819817S°) and Araucanía (38.990393S°) regions. Inventories of stone pine populations were performed including plantations, windbreaks, groves and isolated trees established for commercial and environmental purposes. As a result of a census, 143 sites were visited and 4,094 trees of stone pine (*Pinus pinea*) were measured for diameter-at-breast-height (DBH) at 130 cm above the ground with a caliper, height with a hypsometer; vigor using a combination of foliage damage classes, including both needle losses and discoloration built based on the categories stated by Lakatos and Mirtchev (2014), as follows: (1) high, (2) medium, (3) low; straightness according to the methodology proposed by Toral *et al.* (2011) considering four categories: (1) straight, (2) curve < average, (3) curve > average, (4) very curved or double curved; biotic and abiotic damages through a visual observation following indications of Eichhorn *et al.* (2010); and age based on plantation data available from the owners. Plot establishment (100 tree plots whenever the formation contained more than 100 trees) and measurement methods followed those outlined by Prodan *et al.* (1997).

2.2 Identification of growth macrozones

For MZ identification study, a subset of 56 sites including 3,030 measured trees in groves and plantations were selected, excluding isolated trees. A multivariate classification analysis was performed on sites, including average height and DBH growth rates as well as site spatial coordinates. The analysis was performed with Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering (Johnson and Wichern, 2007) using Euclidean distance. UPGMA is used for the classification of sampling units on the basis of their pairwise similarities in the descriptor variables; the potential distortion produced in the clustering process is estimated by the cophenetic correlation (Sneath and Sokal, 1973).

In each cluster a spatial autocorrelation analysis was performed for DBH and height annual growth rates of stone pine trees growing in comparable conditions and management practices. Spatial correlation models, including Gaussian, Linear, Spherical and Exponential (Schabenberger and Gotway, 2004), were adjusted to model the spatial variability. Among



the adjusted models the exponential model was the best according to the Akaike Information Criteria (Anderson, 2007). Variability maps were constructed using Kriging interpolation for each variable according to the exponential model. Using the site classification as initial information, the total area with stone pine formations in Chile was divided into three MZs by elaborating convex polygon contour connecting the most distant sites inside each cluster. After drawing empirical polygons, we delimited the MZs with further details based on topography (water courses), proximity to the Andes Mountain chain and limits established in a previous study (Ávila *et al.*, 2012). Fig. 1S presents the MZs map overlapping administrative regions and agro-ecological zones previously defined by Hajek (1991).

2.3 Analysis of the impact of climatic variables and management practices on stone pine growth

To estimate the potential impact of climatic and management variables on stone pine growth, climatic information data were built based on the Chilean National Environmental Information System¹. Datasets containing relevant environmental predictors were available in GIS grid format at a resolution of 500 x 500 m and comprised 50 climate variables and altitude (ALT). All environmental variables were resampled according to the WGS 1984-UTM Zone 19S geographical coordinate system at a resolution of ~10 m. The recorded meteorological data were: annual average temperature (TAT); annual maximum average temperature (TXT); annual maximum absolute temperature (TXAT); annual minimum average temperature (TNT); annual minimum absolute temperature (TNAT); annual total rainfall (TAR); annual potential evapotranspiration (ETP); number of dry months; an annual hydric index (THI) as an indicator of water deficit (Rainfall-ETP); and the annual thermal oscillation (TTO). All climatic variables were also built by season (winter, spring, summer and autumn).

Climatic data were obtained from a 10-year climatic series from the meteorological station closest to the study site, 20 km being the average distance to the selected meteorological station. Other criteria, such as information on topography and bioclimatic areas (Uribe, 2012), were used to decide whether the climate at the weather station was representative of the site being evaluated. Site altitude was assigned to one of five classes: 0-400; 400-800; 800-1,200; 1,200-1,600; and over 1,600 m a.s.l.

¹ www.inia.cl; www.dga.cl

The surrounding vegetation was characterized using average values of four vegetation indices: Normalized Difference Vegetation Index (NDVI), Normalized Difference Water Index (NDWI), Normalized Canopy Index (NCI) and Green Normalized Difference Vegetative Index (GNDVI) (INEGI, 2013) calculated for a 30-m radius neighborhood from the evaluated stone pine trees, obtained from Landsat 5 TM and 7 ETM+ images for average seasonal values (winter, spring, summer and autumn) from a 7-year database (2006 to 2012).

Regarding management practices, we recorded presence/absence of irrigation (35% of trees) and pruning (traditional trunk cleaning in 39% of trees). The analysis did not consider fertilization or thinning, since only 3% of trees were fertilized and 1% thinned. Of the 143 sites, 14 sites were discarded after the identification of outliers (values outside of range of mean $\pm$ 3SD). The dropped sites showed data outside the expected range for density (over 2,500 trees ha⁻¹) or growth (over 2.5 cm year⁻¹ in DBH or 1 m year⁻¹ in height).

The relative variable contribution was estimated using CART (Classification and Regression Trees) algorithm (Breiman, 2001). CART is an analytic approach used to assess causes of variation for a response variable. It is named “classification tree” when the response variable is categorical and “regression tree” when the response to be explained is continuous, such as height and DBH annual growth. A regression tree can be built by using several variables as predictors which can be correlated, as is frequently the case when working with climatic variables. Moreover, in this method, the response variable is not necessarily modeled as a linear function of the predictors; on the contrary, the model is a piecewise constant function. Unlike regression models, CART allows us to work under multi-collinearity and non-linear relationships among variables. During data processing, the whole data (root node) are first split into two subsets based on the predictor variable and the value of that variable (threshold) that optimizes the explanation of the response variable. Each subset (daughter node), is then analyzed independently using the same binary partitioning procedure and recursively until a stop criterion is reached. The result of this recursive binary partitioning is a model whose structure can be displayed as a tree-like graph, with each split in the tree labeled according the variable and threshold used to define the split. CARTs have been successfully used to model growth and yield from highly correlated climatic variables (Lobell *et al.*, 2005; Heredia *et al.*, 2010).

For each variable identified as relevant to explain growth according to the regression trees, we performed an ANOVA to analyze the average growth differences between the two



conditions defined by the threshold of each explanatory variable using data from all sites and without conditioning upon other climatic variables. Categorical data were analyzed using a Chi square test. The analyses were performed using the software INFOSTAT and its interface with the software R (Di Rienzo *et al.*, 2013).

3. Results

3.1 Growth macrozones

Four groups of sites with stone pine formations were identified regarding DBH and height growth rates (Fig. 1). The cophenetic correlation coefficient was higher than 0.9. Considering geographical proximity of sites from clusters 3 and 4, and that cluster 4 sites correspond to special growth site conditions in the southern area, we defined three clusters of sites. Growth of stone pine showed marked differences among the three areas, one located in the north, another in the south and the remaining one in the coastal range, between the other two groups; they were named North, South and Dry Coast, respectively.

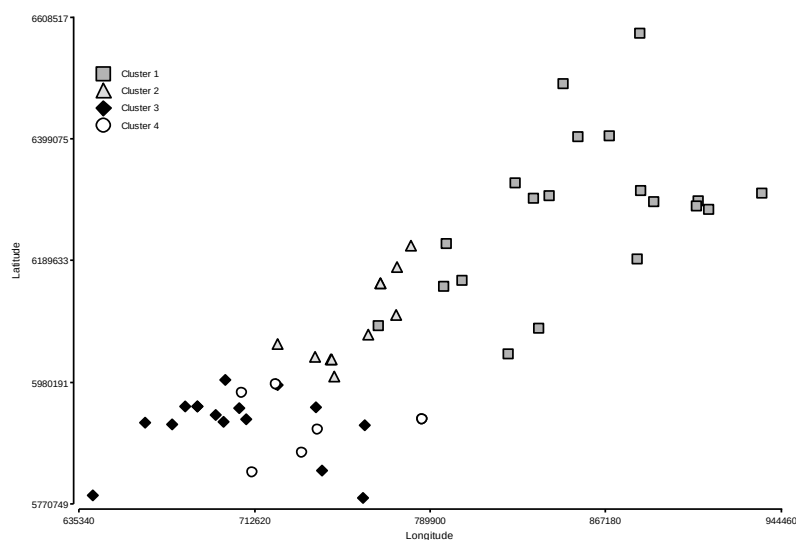


Figure 1. Spatial site dispersion. Sites are grouped according to pairwise similarities in DBH and height growth identified by cluster analysis.

Stone pine growth MZs total area reaches 11,505,611 ha, and is included in agro-ecological zones according to the following distribution: Desert (0%), Arid (12.2%), Mediterranean (64.4%), Humid temperate (23.4%) and Dry temperate (0%) (Fig. 1S). The North MZ is delimited between the IV and Metropolitan regions and in the central valley

between the VI and VII regions. The Dry Coast MZ is located between the VI and VII regions in areas with marine influence, including Cauquenes province, whereas the South MZ includes VIII and IX, and part of the southeastern VII (Fig. 2).

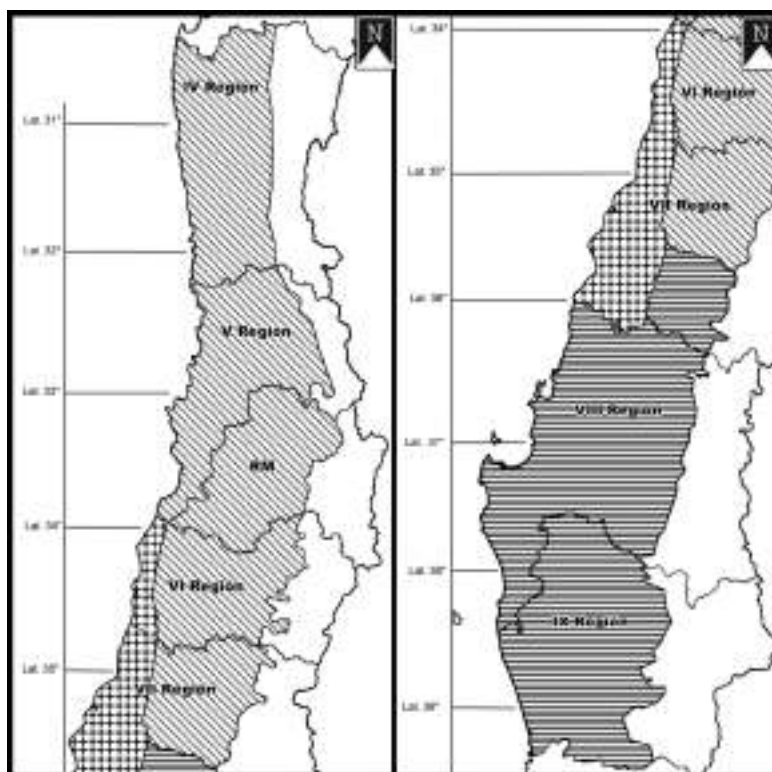


Figure 2. North (diagonal lines), Dry Coast (squared) and South (horizontal lines) macrozones for stone pine in Chile determined according to stone pine DBH and height growth rates. Total area of each MZ is 5,997,459; 911,351; and 4,596,801 hectares, respectively. IV Region: Coquimbo; V Region: Valparaíso; VI Region: O'Higgins; VII Region: Maule; VIII Region: Bio Bio.

Mapping of the spatial variability of DBH and height growth rate showed a higher spatial variability in DBH growth than in height growth. We did not observe spatially structured variability for annual height growth on the Dry Coast MZ (spatial variability model or spatial autocorrelation was not significant, $p > 0.05$).

The characterization of stone pine growth and the surrounding vegetation (NDVI) by MZs is presented in Table 1. Growth was higher in the South MZ, and lower in the intermediate Dry Coast MZ; high values in the North MZ can be explained by the wide latitudinal range of this MZ (including five regions, from Coquimbo to Maule) and by the presence of some plantations with irrigation.

**Table 1.** Characterization of stone pine growth and the surrounding vegetation index (NDVI) by macrozone

	North MZ n=48	Dry Coast MZ n=27	South MZ n=54	All sites n=129
Stone pine growth				
Height growth (m year⁻¹)	0.34 (55)	0.23 (61)	0.35 (35)	0.32 (49)
DBH growth (cm year⁻¹)	1.21 (49)	0.87 (45)	1.50 (29)	1.24 (43)
Crown area (m² tree⁻¹)	83.0 (116)	95.9 (105)	113.7 (95)	98.5 (104)
Vigor (% by category)	1: 60.4 2: 31.3 3: 8.3	1: 44.4 2: 37.0 3: 18.5	1: 74.1 2: 24.1 3: 1.9	1: 62.8 2: 29.5 3: 7.8
Straightness (% by category)	1: 8.3 2: 33.3 3: 56.3 4: 2.1	1: 0.0 2: 48.1 3: 40.7 4: 11.1	1: 1.9 2: 40.7 3: 42.6 4: 14.8	1: 3.9 2: 39.5 3: 47.3 4: 9.3
Trees with abiotic damage (%)	2.8	4.4	3.5	3.6
NDVI				
Autumn	0.11	0.20	0.22	0.17
Winter	0.18	0.30	0.32	0.26
Spring	0.15	0.25	0.33	0.25
Summer	0.16	0.19	0.27	0.21

Values in parentheses correspond to coefficient of variation (CV).

Vigor: (1) high, (2) medium, (3) low.

Straightness: (1) straight (2) curved < average, (3) curved > average, (4) very curved or double curved.

Season definitions: Autumn: March 21st to June 20th; Winter: June 21st to September 20th; Spring: September 21st to December 20th; and Summer: December 21st to March 20th.

In most of the plantations, independently of the MZ, damage by wind, frost and branch breaking derived from trunk or branch inclination for reaching light was observed. No damage by pests or diseases was detected. Regarding vigor, 62.8% of the studied trees presented high vigor. The highest vigor was found in the South MZ (74.1%), and the lowest (44.4%) in the Dry Coast MZ. Contrarily, straightness was higher in the Dry Coast MZ, where trees presented the lowest growth rates.

NDVI values represent the environment vegetation condition. We observed that NDVI recorded in the study areas, and independently of seasonal variations, always increased from north to south. Winter is the season with highest rainfall in the distribution area of the species in Chile. The observed winter NDVI values are relatively high in all

MZs; particularly in the South MZ, where NDVI values were also high in spring. Similar observations were derived from the other vegetation indices and for the stone pine crown area.

The climatic characterization by MZ (Table 2) shows a higher variability of climatic variables between sites of the North MZ. Annual average and annual maximum temperatures decreased from north to south. On the other hand, the annual minimal average temperature was lowest in the Dry Coast MZ. Rainfall increased from north to south, and simultaneously the hydric indices diminished from north to south, suggesting lower water deficit in the South MZ. Spatial variability of rainfall was higher than temperature variability.

Table 2. Climatic characterization of stone pine growth macrozones in Chile

Variable	North MZ n=48	Dry Coast MZ n=27	South MZ n=54	All sites n=129
Annual average temp (°C) (TAT)	14.1 (11.8; 16.7)	13.6 (12.5; 15.1)	13.2 (11.0; 14.2)	13.6 (11.0; 16.7)
Annual max average temp (°C) (TXT)	21.9 (17.6; 25.1)	21.0 (17.9; 23.0)	19.8 (16.3; 22.5)	20.8 (16.3; 25.1)
Annual min average temp (°C) (TNT)	7.5 (5.5; 9.9)	7.0 (5.5; 7.7)	7.5 (5.3; 9.2)	7.4 (5.3; 9.9)
Annual total rainfall (mm) (TAR)	383.7 (116.2; 1,260.1)	648.3 (416.3; 816.0)	1,047.0 (523.6; 1,666.9)	716.7 (116.2; 1,666.9)
Spring rainfall (mm) (PTR)	37.2 (1.5; 143.9)	61.8 (38.4; 89.8)	131.8 (80.8; 218.9)	81.9 (1.5; 218.9)
Summer rainfall (mm) (STR)	8.42 (0; 36.4)	29.1 (10.3; 46.3)	58.6 (31.7; 113.0)	33.8 (0.0; 113.0)
Annual hydric index (mm) (THI)	-995.4 (-1,349.7; -107.3)	-743.7 (-914.4; -474.2)	-244.1 (-736.4; -332.9)	-628.2 (-1,349.7; 332.9)
Spring hydric index (mm) (PHI)	-394.1 (-483.7; -269.5)	-376.9 (-439.5; -269.9)	-267.7 (-346.4; -121.3)	-337.1 (-483.8; -121.3)
Summer hydric index (mm) (SHI)	-485.8 (-563.1; -328.3)	-460.4 (-571.3; -332.6)	-399.9 (-517.7; -260.5)	-444.1 (-571.3; -260.4)
Annual thermal oscillation (°C) (TTO)	14.3 (9.2; 17.3)	14.0 (10.7; 17.0)	12.3 (8.2; 15.1)	13.4 (8.2; 17.3)

Values in parentheses correspond to minimum and maximum values recorded per variable.

Hydric index was calculated as accumulated rainfall minus potential evapotranspiration in the period.



3.2 Analysis of the impact of climate and management variables on stone pine growth

3.2.1 Height

Considering all sites (Fig. 3a), annual average temperature (TAT) was the variable with highest contribution in explaining height growth. Height growth diminished at TAT higher than 14.3°C, with the estimated annual average growth being 36 cm year⁻¹ and 21 cm year⁻¹ when TAT was lower and higher than this threshold, respectively. The second variable with explanatory potential of height growth was winter thermal oscillation (WTO), with a value above 14.3°C favoring growth (79 vs 35 cm year⁻¹ for higher or lower WTO than that threshold, respectively). The highest height growth was observed at winter thermal oscillation lower than 14.3°C and low values of annual hydric index (THI), meaning a lower water deficit, being 43 and 33 cm year⁻¹ for a lower and higher deficit, respectively. In sites with higher water deficit, rainfall over 1,398 mm year⁻¹ favored height growth, the average height growth in each node being 51 and 32 cm year⁻¹, respectively.

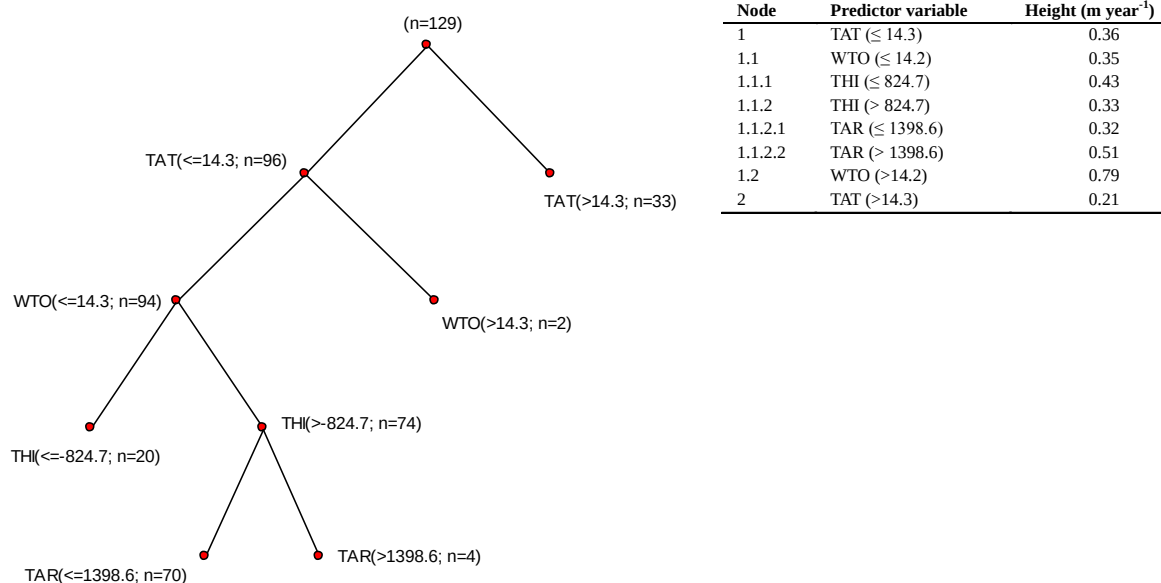


Figure 3a. Climatic variables that best explained height annual growth across the stone pine distribution in Chile (all sites). Total growth data were first split into two subsets based on the predictor variable (TAT) and its threshold (14.3). Each subset, or node, was then analyzed independently using the same procedure. Variables making top nodes are the most important to explain growth rates. Average height growth rates for each node are reported in the embedded table. TAT: annual average temperature; WTO: winter thermal oscillation; THI: annual hydric index; TAR: annual total rainfall.

The analysis by MZ (Fig. 3b) shows that the most relevant variable in the North MZ for height growth is the same as for all the study area, annual average temperature, with height growth values relatively low (22 cm year^{-1}) if TAT was higher than 14.3°C , a situation in which irrigation was favorable (16 vs 28 cm year^{-1} , without and with irrigation, respectively). When TAT was lower than 14.3°C , the average growth was 43 cm year^{-1} . In this situation, we found seven sites with extreme spring water deficit, but with high height growth (62 cm year^{-1}) due to the presence of irrigation. Sites with spring hydric indices above -400 mm (also indicating water deficit situations) were favored by higher accumulated annual rainfall (TAR) (above 468 mm year^{-1}), showing better height growth than those with lower TAR (46 vs 32 cm year^{-1}).

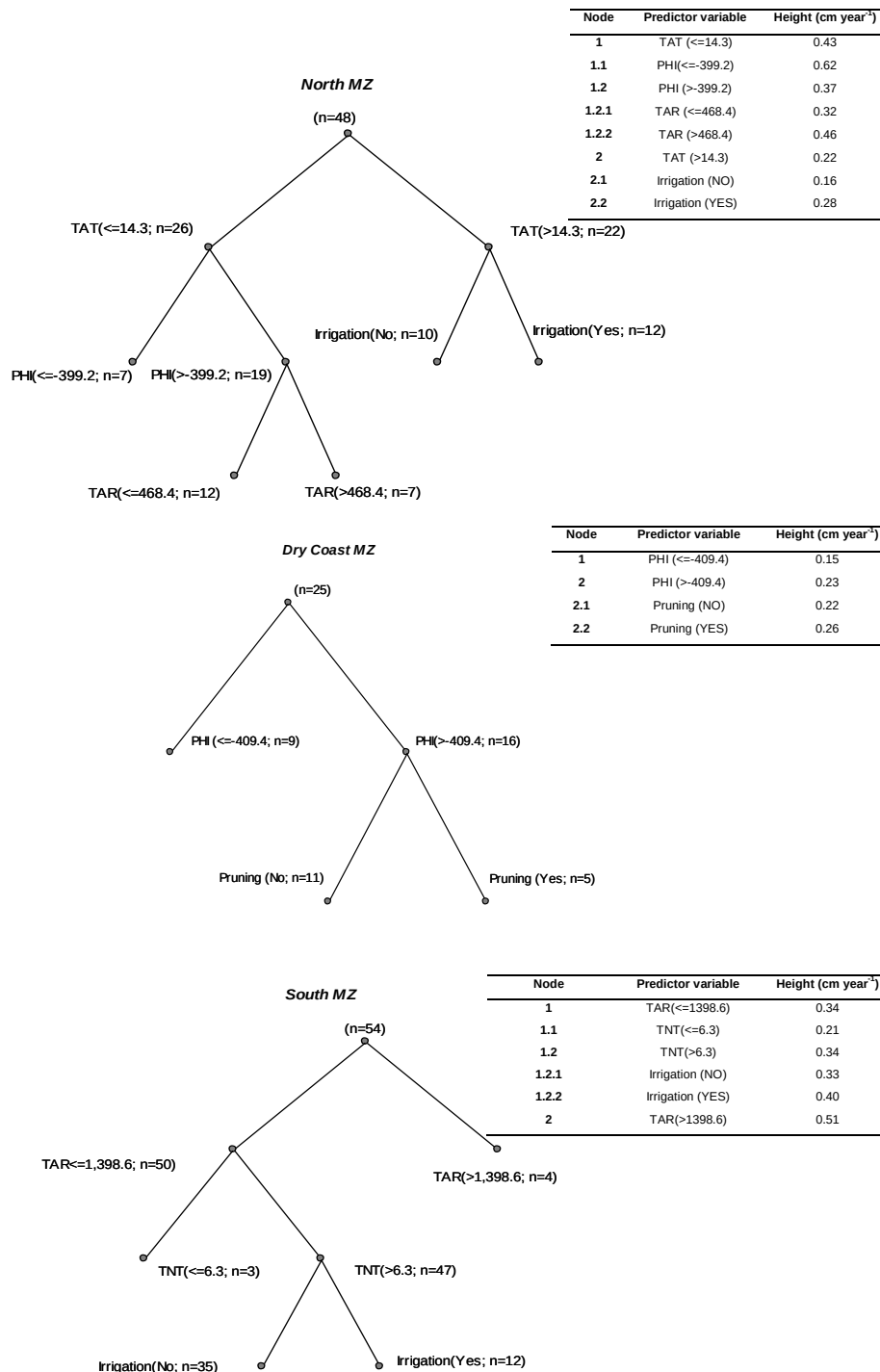


Figure 3b. Climatic variables that best explained height annual growth by stone pine macrozones in Chile. For each MZ, total growth data were first split into two subsets based on the predictor variable (TAT, PHI and TAR for North, Dry Coast and South MZs, respectively) and its thresholds (14.3°C; -409.4 mm and 1,398.6 mm, respectively). Each subset, or node, was then analyzed independently using the same procedure. Variables close to top nodes are the most important to explain growth rates. Average height growth rates for each node are reported in the embedded tables.

TAT: annual average temperature; PHI: spring hydric index; TAR: annual total rainfall; TNT: annual minimum average temperature.

Height growth in the Dry Coast MZ was most influenced by spring water deficit, being lower if it was higher than 409 mm (averages of 15 and 23 cm year⁻¹, respectively). When spring water deficit was under that threshold, we observed the effect of pruning (26 vs 22 cm year⁻¹ according to pruned or unpruned trees).

In the South MZ the accumulated annual rainfall (TAR) was an explanatory variable of height growth, striking positively when it was over 1,398 mm year⁻¹ (51 vs 34 cm year⁻¹). When TAR was lower than that threshold, height growth was favored by minimum annual average temperatures above 6.3°C (34 vs 21 cm year⁻¹), and by irrigation (40 vs 33 cm year⁻¹).

Overall, growth limiting factors were dependent on MZs. TAT was the most relevant factor in the North MZ, with irrigation being a recommended practice in sites with TAT above 14.3°C. In the Dry Coast and South MZs the water deficit index and the accumulated rainfall were the most important climatic variables to explain height growth, respectively. Management practices of pruning in the Dry Coast MZ and irrigation in the South MZ favored height growth.

The variables suggested as potential to explain height growth by the regression trees were further analyzed with ANOVA. The results (Fig. 4) showed significant differences in height growth between sites with values above and below 14.3°C for winter thermal oscillation, with height growth reached being under a high winter thermal oscillation (WTO), 2.2 times higher than that observed in sites with low WTO. Statistically significant differences were also found between sites with annual total rainfall above and below 1,398 mm year⁻¹, with growth being 70% higher in sites with rainfall above that value. Growth in sites with annual average temperature below 14.3°C was 67% higher. Differences of more than 30% in growth were observed between weather conditions defined by spring annual hydric index and annual minimum average temperature.

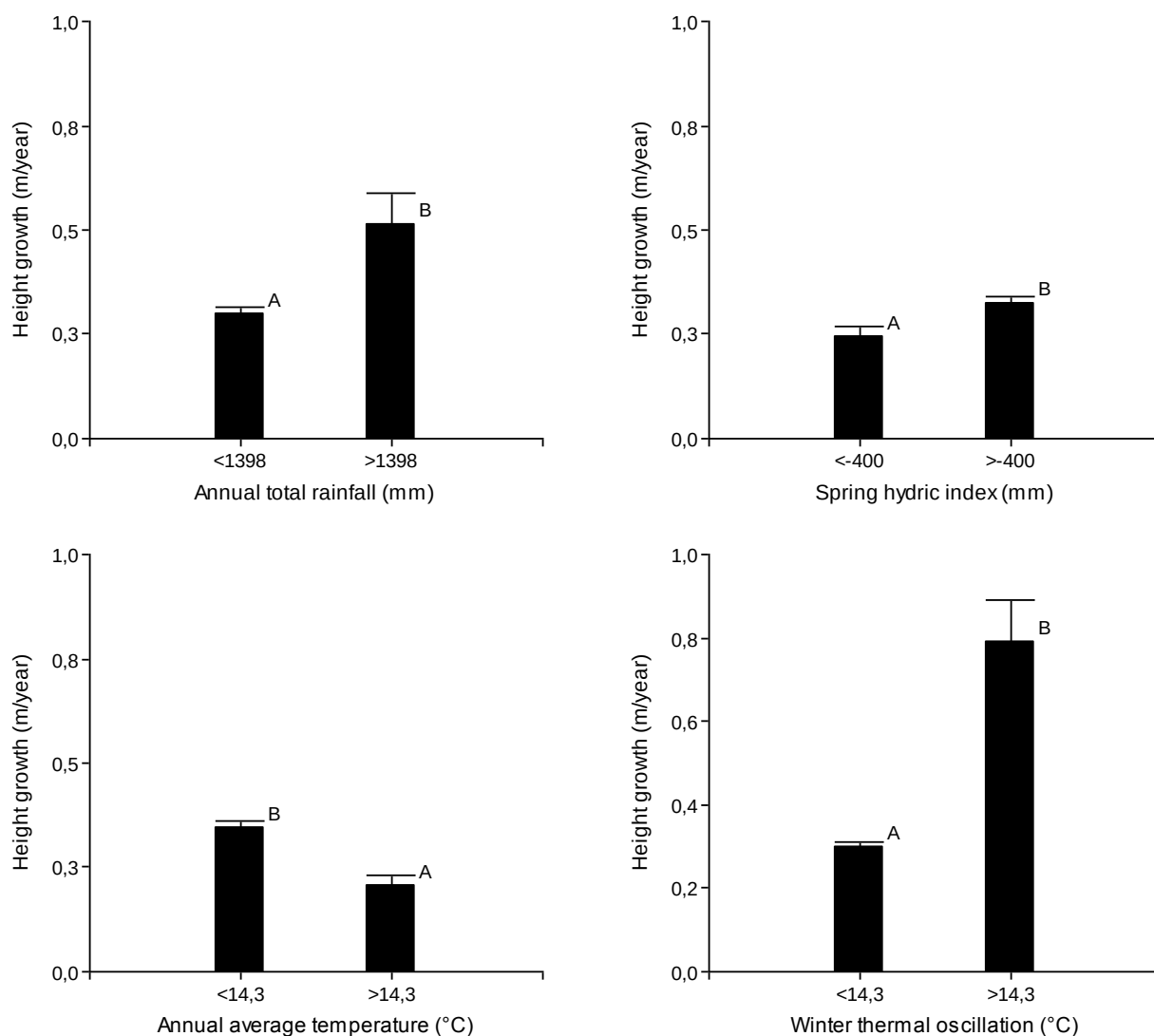


Figure 4. Climatic variables affecting height growth. Each threshold was detected by means of CART analyses using data from all sites without conditioning upon other climatic variables. Different letters indicate statistically significant differences ($p < 0.05$).

Height growth was reduced with altitude. Even though this reduction was one third when altitude was over 1,200 m a.s.l., differences in average growth rate among altitude categories were not statistically significant ($p > 0.05$).

3.2.2 DBH

Considering all the studied sites (Fig. 5a), the variable that best explained DBH growth was annual average temperature (TAT), with growth decreasing in sites with TAT above 14.3°C. Thus, sites favorable for height growth were also the most favorable for DBH growth in Chile. Average DBH growth values of 0.92 and 1.37 cm year⁻¹ were found above and below the TAT threshold value. In sites with TAT values below the threshold, DBH growth was higher when annual water deficit (THI) was lower than 1,100 mm (2.60 vs 1.34 cm year⁻¹, respectively). In sites with water deficit above that threshold, DBH growth was favored by irrigation, with averages of 1.56 and 1.25 cm year⁻¹ with and without irrigation, respectively. In plantations without irrigation, there was a positive impact of accumulated annual rainfall (1.43 vs 0.98 cm year⁻¹ above and below 781 mm year⁻¹, respectively).

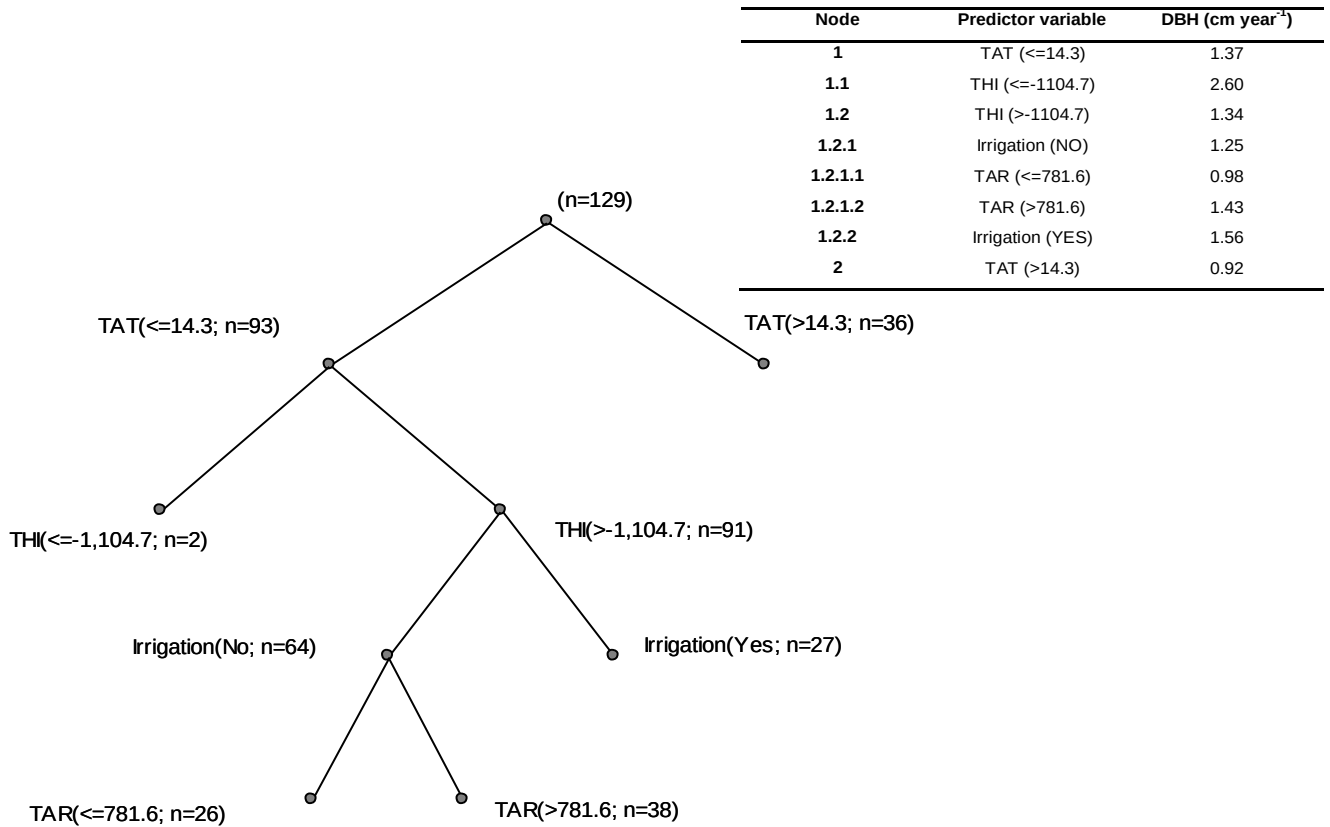


Figure 5a. Climatic variables that best explain DBH annual growth along the stone pine distribution in Chile (all sites). Total growth data were first split into two subsets based on the predictor variable (TAT) and its threshold (14.3). Each subset, or node, was then analyzed independently using the same procedure. Variables close to top nodes are the most important to explain growth rates. Average DBH growth rates for each node are reported in the embedded table.

TAT: annual average temperature; THI: annual hydric index; TAR: annual total rainfall.



The analysis by MZ (Fig. 5b) shows that for DBH growth the most relevant environmental variable in North MZ was annual minimal average temperature, with a threshold of 5.9°C, with DBH growth values of 1.97 and 1.10 cm year⁻¹ below or above that TNT threshold. DBH growth in the Dry Coast MZ was also influenced by annual average temperature, with higher growth at TAT below 12.8°C (0.99 vs 0.79 cm year⁻¹). In the South MZ maximum annual average temperature was important to explain growth. High DBH growth was recorded with relatively high temperatures; in fact, the highest growth took place with maximum temperatures above 17.8°C but below 19.6°C (1.81 cm year⁻¹).

Therefore, in all MZs the highest annual, minimum or maximum temperatures were found to be the most limiting DBH growth factors. At all sites, the impact of annual average temperature was indicated by a mean difference of 4.5 mm year⁻¹ between sites at above and below TAT of 14.3°C (Fig. 6).

DBH growth was reduced from an altitude of 800 m a.s.l. and higher, with statistically significant ($p \leq 0.05$) differences among categories 400-800 (1.43 cm year⁻¹) and over 1,600 m a.s.l. (0.60 cm year⁻¹).

Table 3 summarizes results regarding the influence of climatic variables on stone pine growth in Chile.

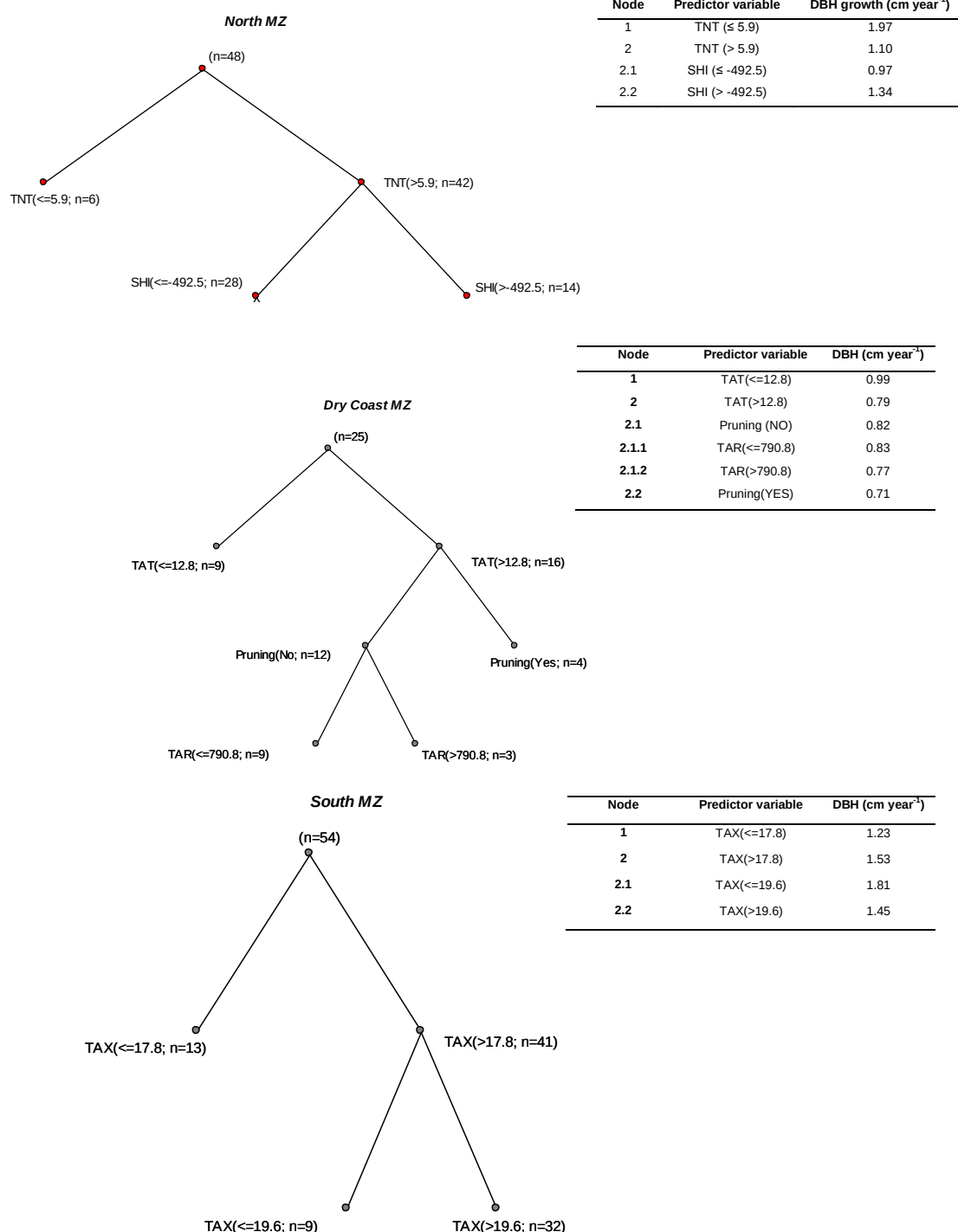


Figure 5b. Climatic variables that best explain DBH annual growth by stone pine macrozones in Chile. For each MZ, total growth data were first split into two subsets based on the predictor variable (TAT, TAT and TAX for North, Dry Coast and South MZs, respectively) and its thresholds (5.9; 12.8 and 17.8°C, respectively). Each subset, or node, was then analyzed independently using the same procedure. Variables close to top nodes are the most important to explain growth rates. Average DBH growth rates for each node are reported in the embedded tables.

TAT: annual average temperature; SHI: summer hydric index; TNT: annual minimum average temperature; TAR: annual total rainfall; TAX: annual average maximum temperature.

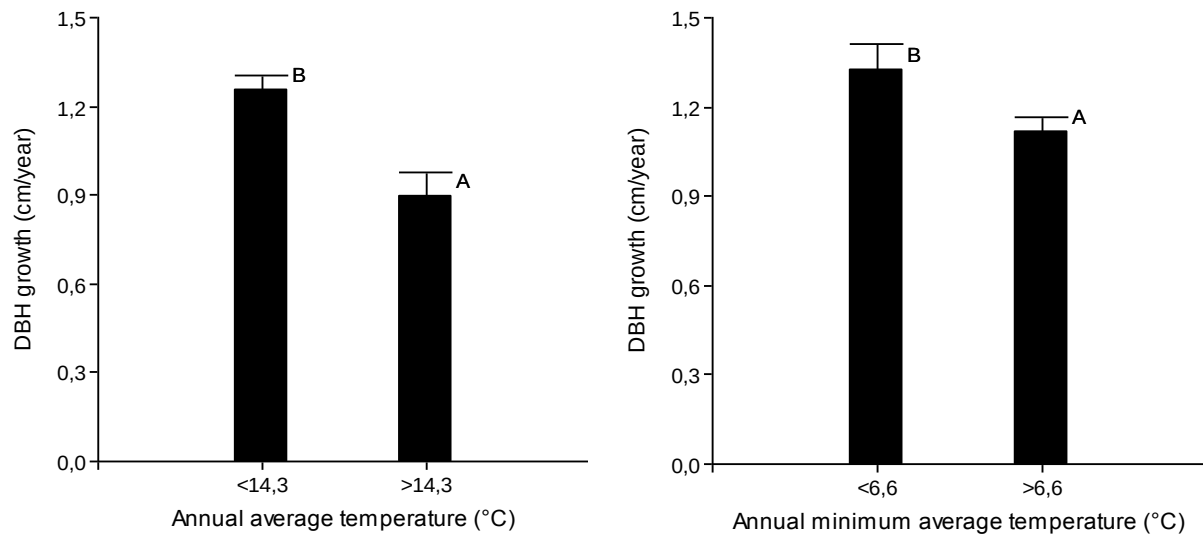


Figure 6. Climatic variables impacting DBH growth. Each threshold was detected by means of CART analyses using data from all sites without conditioning upon other climatic variables. Different letters indicate statistically significant differences ($p < 0.05$).

Table 3. Main climate variables that showed a significant influence on stone pine growth in Chile

Dependent variable	Winter thermal oscillation † (WTO)	Annual average temperature (TAT)	Minimum average temperature (TNT)	Annual Rainfall (TAR)	Spring hydric index †† (PHI)
Diameter growth		<14.3°C (49%)	<6.6°C (18%)		
Height growth	>14.3°C (2.2 times)	<14.3°C (67%)		≥ 1,398 mm (70%)	≥ - 400 mm (30%)

Values in parentheses correspond to the expected increase of the dependent variable under the identified key climatic conditions. †Winter thermal oscillation: average winter maximum absolute temperature, minus average winter minimum absolute temperature. ††Hydric index was calculated as accumulated rainfall in the period minus potential evapotranspiration of the same period.

4. Discussion

The stone pine growth rates observed in Chile were found to be higher than in other parts of the world, including its native habitat. In fact, the average DBH growth of 1.24 cm year⁻¹ is higher than the one reported in Spain (0.9 cm year⁻¹ at 40 years old in good sites) (Montero and Candela, 1998) and in plantations in Morocco (1 cm year⁻¹) (FAO, 2007) and Tunisia (0.8 cm year⁻¹ in coastal dunes) (Boutheina *et al.*, 2013), but lower than in Portugal (2 cm year⁻¹) (Guerra-Hernández *et al.*, 2016). Mean height growth in Chile reached 32 cm year⁻¹, which is similar to the best situations in Italy, where reported DBH growth values range between 19 and 35 cm year⁻¹ (Bianchi *et al.*, 2005), but lower than in some plantations in Portugal (49 cm year⁻¹) (Guerra-Hernández *et al.*, 2016).

In this study, we established three growth MZs for stone pine in Chile. The approach of establishing macrozones for the study of stone pine was also performed in Spain by Sánchez-Palomares *et al.* (2013), who classified the species as megamesothermic (potential evapotranspiration over 700 mm). However, the average annual temperature in Spain was higher than the values found in the current Chilean distribution area of stone pine. Annual average temperature in all Chilean MZs (14.1°C in the North, 13.6°C in the Dry Coast and 13.2°C in the South MZs) are within the temperature interval described by Guzmán *et al.* (2013) for stone pine in Spain (12.4-18.5°C). Regarding maximum temperatures, these authors define the average maximum temperature of the warmest month at between 26.4 and 37.3°C, and Manes *et al.* (1997) report significant depression in its photosynthetic capacity in response to hot summer days. In our study, however, we found that the maximum annual average temperature varied between 19.8 in the South MZ and 21.9°C in the North MZ.

For annual hydric deficits, Guzmán *et al.* (2013) set limits between 242 and 744 mm; however, in our study we found it to be 244 mm in the South MZ and 995 in the North MZ, being coincident in the south but much higher in the northern area where the species is also present. Those authors defined limits for accumulated annual rainfall between 284 and 1,327 mm; in our study we found this value to be 384 mm in the North MZ, 648 mm in the intermediate MZ (Dry Coast) and 1,047 in the South MZ. As for summer rainfall, they set limits between 15.6 and 64.4 mm, whereas in our study we found it to be considerably lower in the northern area (8.4 mm), 29.1 mm in the intermediate one, and 58.6 mm in the southern area.

We found different growth response patterns to climate for the three defined MZs,



with different effects for xeric, mesic and intermediate environments (Fig. 3a,b and 5a,b), as described by Pasho *et al.* (2012). According to Castillo *et al.* (2002), the fact that almost no impact of summer drought was detected in our study, with the exception of height growth in the North MZ at TAT above 14.3°C, can be due to the species adaptation to long drought periods in Mediterranean areas through a deep root system that allows it to reach deep water resources, as well as to some sites under irrigation in the northern area. Contrarily to our findings, Álvarez *et al.* (2013) found that maximum temperature in the growing season negatively affected *Pinus radiata* D. Don growth in Chile. They reported that sites with the highest productivities had the lowest annual water deficits, concluding that the most efficient use of water and light is found at sites with best rainfall distribution and highest soil water content, and that the most productive sites for this species are located on the western border of the coastal range, the piedmont of the Andes and the southernmost area, near 39-40° South latitude. Similar results were found for stone pine in our study.

Long and severe dry seasons and associated water shortages could seriously affect tree growth (Osborne *et al.*, 2000) and particularly stone pine, as pointed out by Bussotti *et al.* (1995). Our results showed that with annual total rainfall above 1,398 mm, height growth is 70% higher than that observed in sites with lower precipitation.

Accordingly, Mazza and Manetti (2013) reported the influence of low precipitation as the main factor driving tree diameter growth decline, an effect that was also found over longer periods, when stone pine can no longer obtain water from the soil by intensively exploring the ground at different levels. El-Khorchani *et al.* (2007) also stated the impact of hydric balance on diameter growth; and water stress can be intensified in sandy soils where water availability decreases drastically in dry years (Thabeet *et al.*, 2007). In Chile, this was the case of the Dry Coast MZ. Mazza and Manetti (2013) concluded that a higher water stress induced by an increase in air temperature and a decrease in rainfall for successive years could seriously affect the species tree growth. Those results are in agreement with our findings on height growth, which showed differences above and below a spring hydric deficit threshold of 400 mm. Bjorn *et al.* (1997) explain this effect, stating that plants respond to summer water stress with extensive needle loss and photosynthetic capacity of the remaining needles.

Our study showed that annual average temperature is a relevant variable for DBH growth, with a threshold of 14.3°C, being higher under that value. In Tunisia, Thabeet *et al.*

(2007) report a negative correlation between average temperature and growth, but with a superior threshold (16°C). In a study on the variation of stone pine productivity in relation to climate in Spain, Natalini *et al.* (2013) found that winter and spring rainfalls were positive to growth, whereas high spring temperature was negative. These authors found that stone pine growth is positively correlated to Palmer Drought Severity Index (PDSI) and with annual rainfall, and negatively correlated to maximum average annual temperature, which agrees with our findings for height growth in Chile. At a monthly and seasonal scale, they found a positive correlation of growth with winter, spring and October rainfall, as well as with winter average and minimum temperatures, and a negative correlation with spring and beginning of summer (June) temperatures. No significant correlations were found among growth and summer climatic variables, as in the present work. Similarly, Awada *et al.* (2003) found a significant height increase in response to shade in Greece.

Sánchez-Miranda *et al.* (2006) found in another conifer, *Abies alba* Mill., that diameter growth is more dependent upon temperatures than rainfall, which is in agreement with our results. We found that the effect of annual minimum average temperature was relevant for DBH growth, with a threshold of 6.6°C, with growth being significantly higher below that value. These results may exclude intensive frosts (-5 to -15°C), since they cause root damage in the species, as reported by Oliet *et al.* (2014). Similarly, Galli *et al.* (1992) reported a clear and consistent correlation among radial growth and winter severity (monthly minimum average temperature), establishing that a decrease of 1°C would cause a 13% decrease in ring width.

Even though these findings are relevant to decide where to cultivate the species, it should be considered that functional processes, including the response to climatic conditions, are closely related to tree dimensions (physiological control) and that they undergo changes as the tree ages (genetic control) (De Luis *et al.*, 2009). In fact, it has been shown that the size of the tree is more important than its age (Peñuelas, 2005).

This study did not include soil variables based on a previous study conducted in Chile (Ávila *et al.*, 2012), which showed that the species grows well in different soil types, including clay ones, contrarily to findings reported by several authors, which have indicated clay content as a limiting factor (Abellanas, 1990; Gandullo and Sánchez-Palomares, 1994; Gordo *et al.*, 2009). Overall, the lowest growth was observed in the Dry Coast MZ where most of the soils are sandy and granitic, with lower water availability than in the remaining



macrozones.

The comparison of the environmental characteristics of the species' native and exotic habitats helps us to gain insights about the species behavior outside its normal environmental range. This comparison shows that in Chile, annual average temperature is lower than in its native habitat, whereas annual rainfall is higher and spring and summer rainfalls are lower. Our results highlight the relevance of temperature for the species, in such a way that a cooler habitat allows it to live with a lower hydric availability, which would explain the interesting performance it presents in Chile. Therefore, in light of climate change scenario with increasing temperature and decreasing rainfall, we should expect a reduction in growth rates, especially in the North and Dry Coast MZs, in agreement with Natalini *et al.* (2013), who also reported the species vulnerability under increases of temperature due to climate change. Consequently, heat tolerance should be considered a key breeding trait for increasing potential growth of stone pine.

Both pruning and irrigation, the two management practices evaluated, affected stone pine growth under some conditions, but were not the most influencing variables (Figs. 3a,b and 5a,b). The irrigation effect was positive for both height and DBH, as was reported by Pestaña (2000) in the evaluation of the impact of fertirrigation in a 75 year-old plantation of limited growth and no management established in a clay-sand soil. The author used a sprinkler irrigation system with 35 L hour⁻¹ during 5 years, with variable and growing doses ranging between 2,460 and 5,480 m³ ha⁻¹; growth response was very positive, increasing from 2 to 15 mm diameter growth. Gordo (2010) stated that pruning negatively affects both height and diameter growth, whereas in our study we observed a positive effect on height growth, in agreement with findings reported by Loewe *et al.* (2012b).

Given the observational nature of our study, the results for these management practices should not be taken as conclusive, but as an indication that in a given site and under specific conditions, these interventions may be determinant for the species growth.

The results suggest an interaction between these management practices and the local climatic conditions. Irrigation effect can increase up to 75% height growth rates in the North MZ, where temperatures and hydric deficit are high; even in the South MZ, irrigation increased this rate by 21% when total annual rainfall was lower than 1,400 mm. Irrigation also increased DBH growth by 25%. For pruning, we observed height growth increases (18%) only in the Dry Coast MZ. Even though we do not have specific data on the costs of

these management practices for this study, according to Loewe and Delard (2012), irrigation represents over 30% of the establishment costs whereas pruning represents a low fraction of the total annual management costs (5%). A preliminary relationship between costs and benefits suggest that the implementation of irrigation, when necessary due to climatic conditions, should be considered when intending to maximize stone pine growth.

5. Conclusions

Stone pine growth throughout Chile is high compared to the growth rates reported for its native habitat. We observed average growth of 0.32 m year⁻¹ in height and 1.24 cm year⁻¹ in DBH, showing marked differences among the three identified MZs. The highest height and DBH annual growth were found in the South MZ, with 0.35 m year⁻¹ and 1.50 cm year⁻¹ respectively, whereas in the Dry Coast MZ the species showed the lowest growth rates in height (0.23 m year⁻¹) and DBH (0.87 cm year⁻¹).

Average annual temperature and rainfall had a significant impact on height growth rate, which was favored by an average annual temperature below 14°C, a high winter thermal oscillation above 14°C, a spring water deficit lower than 400 mm and annual rainfall over 1,400 mm year⁻¹. DBH growth was also favored by an average annual temperature below 14°C. In sites that reached this temperature requirement, DBH growth was higher when annual water deficit was lower than 1,100 mm year⁻¹.

The study showed that temperature is the variable with the highest impact on stone pine growth in Chile; particularly, height growth was more than double in sites with low annual average temperature and high winter thermal oscillations.

As practical applications of our results, we determined that a good selection of sites for the species cultivation in Chile should ensure annual average temperature below 14°C. Moreover, in light of climate change, we should expect a reduction in growth rates especially in the North and Dry Coast MZs, where temperature is superior; therefore, heat tolerance should be handled as a key breeding trait for increasing growth of stone pine.

Pruning and irrigation also affected stone pine growth in Chile, but its average influence was lower in magnitude than the climatic impact; irrigation effect was positive for both DBH and height growth, and pruning for height growth in the Dry Coast MZ. Management practices oriented to enhance vegetative growth will depend upon the macrozone where the site is located.



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Supplementary information



Figure 1S. Distribution of stone pine macrozones and its relationship with agro-ecological zones previously defined by Hajek (1991).



Capítulo 3. Productividad frutal de *Pinus pinea* L. a lo largo del gradiente climático de su distribución en Chile

Capítulo 3. Fruit productivity of Stone pine (*Pinus pinea* L.) along a climatic gradient in Chile

Copa productiva de ejemplar de pino piñonero y preparación de repostería con piñones mediterráneos

Stone pine productive crown and preparation of sweets with pine nuts

Published as:

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<http://dx.doi.org/10.1016/j.agrformet.2016.04.011.0168-1923>.



Fruit productivity of Stone pine (*Pinus pinea* L.) along a climatic gradient in Chile

Abstract

Pinus pinea is a Mediterranean species of economic importance due to its edible seeds, the pine nuts that have high market value. We analyzed fruit productivity by recording cone number per tree (CN) on 3,464 trees distributed along a climatic gradient in Chile. Cone weight at harvest (CW) and in-shell pine nut number per cone (IS) were measured on 76 superior trees. Climatic and biometeorological variables, defined based on 11 physio-phenological reproductive phases, were related to fruit production traits. Results showed marked differences among North, South and Dry Coast areas. The highest values of cone productivity (32 kg tree⁻¹) and CN (62 cones tree⁻¹) were recorded in the South. Stone pine cone production throughout Chile was favored in increasing importance by annual thermal oscillation <12°C, spring rainfall >65 mm, spring hydric deficit <345 mm and autumn thermal oscillation <12°C. It was also favored by a thermal oscillation <6°C during differentiation of reproductive shoots, by rainfall <70 mm during the male flowering period, and in the female flowering period by a minimum temperature >7°C and a hydric deficit <200 mm. Cone weight was favored by an average annual temperature >14°C, by annual rainfall >507 mm and by an annual hydric deficit <914 mm. Additionally it was determined that an accumulated rainfall >14 mm during the 2 year-old conelet growth period, as well as a maximum temperature >23°C and a hydric deficit <774 mm during embryo development, increased it. IS increased significantly with a maximum autumn temperature <19°C and an annual hydric deficit <914 mm, along with a maximum temperature >24°C during 2 year-old conelet growth, as well as by accumulated rainfall during three key periods (>53 mm in induction of male primordia, >78 mm in 3 year-old cone growth and >133 mm during cone ripening). Therefore, water supply is recommended to mitigate the negative impact of water deficit on fruit productivity.

Key words: biometeorological variables, climatic impact, cone production, cone weight, pine nut yield.

1. Introduction

Stone pine (*Pinus pinea* L.) is a well-known Mediterranean species of economic importance; its edible seed, the pine nut, has been used for human consumption since the Paleolithic period due to its high nutritional value (Mutke *et al.*, 2005a). It is characterized by high protein content (more than 32%) and fats (42%), mostly unsaturated, including several mono and poly-unsaturated fatty acids, such as linoleic (omega 6) and alfa-linolenic (omega 3) acids; over 11% of total fiber and low carbohydrate content (Zuleta *et al.*, 2013). Furthermore, protein quality is very high, because it is composed of 20 amino acids (Schröder *et al.*, 2014).

In Chile, the species exhibits vigorous growth and good phytosanitary condition, making its cultivation attractive. Production is focused on pine nuts due to the high prices they reach on international markets. In fact, pine nuts are among the nine most important dried fruits in the world (Soto *et al.*, 2008; Mutke *et al.*, 2005a; Gordo *et al.*, 2011) and have an increasing unsatisfied demand that has stricken the markets in the last decade (Schröder *et al.*, 2014) due to a drastically reduced production (close to 50%) since 2011 (International Nut and Dried Fruits, 2012), which has decreased even more lately.

Stone pine was introduced in Chile by European immigrants; later, early in 1900 it was used in sand dune stabilization programs (Albert, 1909) and in afforestation and rural development programs (Loewe and Delard, 2012). Up to 2010, stone pine was established in nearly 100 ha in different regions, settings (isolated trees, groves and plantations) and under different field managements. Those areas have been partially studied for assessing stone pine potential as a fruit tree, confirming its ecological adaptation and productive potential (Loewe *et al.*, 1998, Loewe and González, 2003).

Low levels of the species genetic diversity (Mutke *et al.*, 2012) and remarkable plasticity (Sánchez-Gómez *et al.*, 2009) have been reported, so a high contribution of genotype-by-environment interaction should be expected. Potential cultivation areas in Chile were studied by Ávila *et al.* (2012) considering four categories according to rainfall levels: environmental protection (driest conditions), and low, medium and high fruit production. The authors stated that the species can grow in more than 8.6 million ha; of these, over 4.8 million ha are suitable for medium and high fruit productivity. Loewe *et al.* (2015) determined three macrozones (MZs) according to height and DBH growth rates. South MZ growth rate was



superior for height (0.35 m year^{-1}) and DBH ($1.50 \text{ cm year}^{-1}$), whereas in the Dry Coast MZ, the species showed the lowest growth rate in height (0.23 m year^{-1}) and DBH ($0.87 \text{ cm year}^{-1}$).

The species shows a cone production masting habit, which seems to be related to climatic conditions (Mutke *et al.*, 2006; Calama *et al.*, 2011); this is even more relevant considering that, due to the long fruit cycle duration (3.5 years), a specific climatic event can impact three harvests that are simultaneously present in the tree. In Europe cone production starts when trees are between 10 (Crawford, 1995) and 20 years old (Goor and Barney, 1976), but commercial pine nut production involves individuals of about 25 years, with maximum production being attained with 40-50 year-old individuals (Peruzzi *et al.*, 1998) and being maintained for up to 100 years (García-Güemes *et al.*, 1997), and even up to 150 years (Pereira *et al.*, 2015).

Productivity is quite variable over time and regions, and in the main productive countries, it ranges from less than 200 kg ha^{-1} (Prades *et al.*, 2005; Pinea Project, 2011); between $200\text{-}600 \text{ kg ha}^{-1}$ (Calama *et al.*, 2005; Centre de la Propietat Forestal, 2009); $600\text{-}1,000 \text{ kg ha}^{-1}$ (Torres *et al.*, 2009; Centre de la Propietat Forestal, 2009); $1,000\text{-}5,000 \text{ kg ha}^{-1}$ (Gorrieri and Ceccini, 2010; Mutke, 2011b); to over $5,000 \text{ kg ha}^{-1}$ (Ottone, 1989; Federlegno-Arredo, 1992; FAO, 1995) in natural forests or plantations with densities ranging between 100 and 400 trees ha^{-1} (Borrero, 2004). In Chile, preliminary studies estimated interesting productive levels in plantations, with increasing values up to 70 cones tree^{-1} in diameter class 41-60 cm (Loewe and Delard, 2012); however, no study has been performed on variability of fruit productivity along the wide latitudinal range where the species has been planted in the country.

The need to contrast possible environmental correlations with the reproductive biology and phenology of the species has been indicated to identify causal mechanisms for differences in production among populations (Mutke *et al.*, 2005a), especially considering that environmental factors influence fruit production both in quantity and quality (Mutke *et al.*, 2005b). For example, in Tunisia, phenology of stone pine plantations was found to depend upon temperature increase in late winter (Schröder *et al.*, 2014); in Spain, Abellanas (1990) reported a temperature control over stone pine vegetative and reproductive development, with inter-annual differences of up to 20 days and growing degree days (GDD) of those years being similar (Mutke *et al.*, 2001). Models at different scales have been developed to establish relationships between productivity and several variables (Cantiani and Scotti, 1988; Cañadas, 2000; Gordo *et al.*, 2001; Piqué, 2003; Madrigal *et al.*, 2009), including climatic ones (Calama *et al.*, 2011).

Recently, Loewe *et al.* (2015) determined a climatic impact on stone pine growth across macrozones in Chile. However, these differences could not necessarily be translated to cone productivity; therefore, in order to contribute with knowledge on the dependence of stone pine fruit production upon climatic conditions, we studied this parameter along a 1,000 km latitudinal gradient, corresponding to its artificial distribution range in Chile. We aimed to unravel how cone number tree⁻¹ (CN), cone weight at harvest (CW) and in-shell pine nut number per cone (IS) were related to climatic factors across this wide range of environments.

We expected to find a lower cone production under drier conditions (Calama *et al.*, 2008a), thus declining from south to north. The knowledge of this species is reduced in relation to fruit production and one of the strengths of this work is to deepen in the relationships between fruit production and climatic, as well as biometeorological, variables that relate climate and physio-phenological phases involved in cone induction, formation and development.

2. Material and Methods

2.1 Study area and climatic variables

From 2008 to 2013, the Chilean Forest Institute (INFOR) conducted a long-term, large-scale national research effort to gather stone pine data in Chile, covering an area between Coquimbo-IV (30.819817°S) and Araucanía-IX (38.990393°S) regions (Fig. 1). Inventories of stone pine populations were performed including plantations, windbreaks, groves and isolated trees established for commercial and environmental purposes along the country.

To estimate the potential impact of climate on stone pine cone productivity, several environmental information layers were built based on the Chilean National Environmental Information System¹. Datasets containing relevant environmental predictors were available in a GIS grid format at a resolution of 500 x 500 m and comprised several climatic variables. All environmental variables were resampled according to the WGS 1984-UTM Zone 19S geographical coordinate system at a resolution of ~10 m.

¹www.inia.cl, www.dga.cl

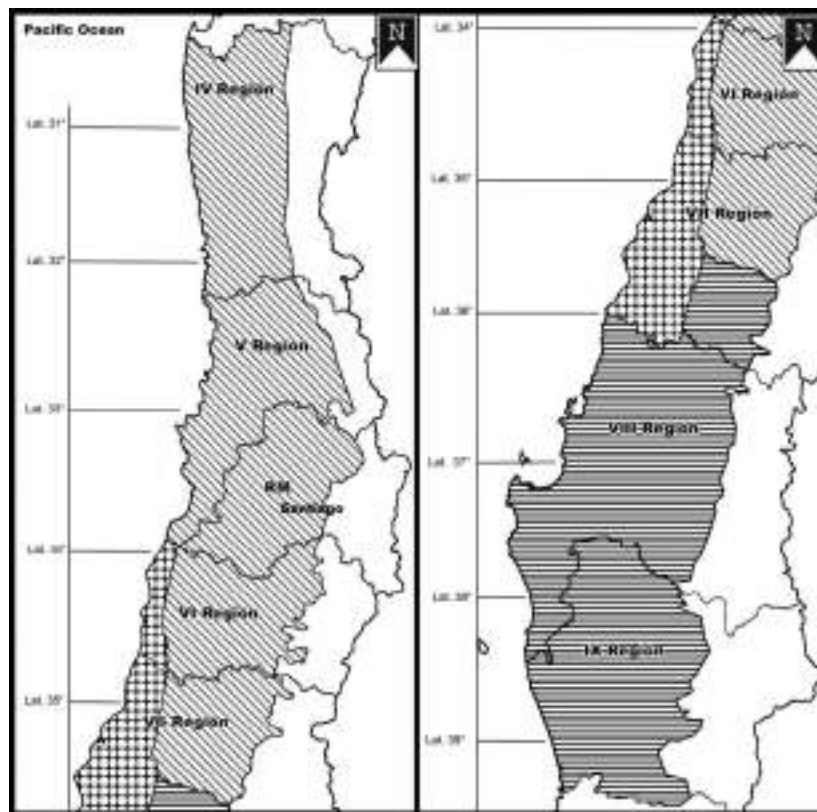


Figure 1. North (diagonal lines), Dry Coast (squared) and South (horizontal lines) macrozones for stone pine in Chile determined according to stone pine DBH and height growth rates. Total area of each MZ is 5,997,459; 911,351; and 4,596,801 hectares, respectively. IV Region: Coquimbo; V Region: Valparaíso; VI Region: O'Higgins; VII Region: Maule; VIII Region: Bio Bio. The Andes Mountain Range runs along the eastern limit of Chile.

Annual and seasonal (winter, spring, summer and autumn) climatic data were obtained from a 10-year climate series of the meteorological stations that were closest (average distance of 20 km) to the studied sites. If there were several stations available, we selected the most representative one based on topography and bioclimatic areas (Uribe, 2012). The following climatic data were recorded: average temperature (AT); maximum average temperature (MXT); maximum absolute temperature (MXBT); minimum average temperature (MNT); minimum absolute temperature (MNBT) (all temperature variables were calculated on a daily basis and are expressed in degrees Celsius); total annual rainfall (PP, expressed in mm); potential evapotranspiration as defined by Hargreaves and Samani (1985) (PET, expressed in mm); a hydric index (HI) as an indicator of water deficit ($HI = \text{Rainfall} - \text{Potential Evapotranspiration}$, expressed in mm); and thermal oscillation ($TO = \text{average maximum absolute temperature} - \text{average minimum absolute temperature}$ for a given

period, expressed in degrees Celsius).

Table 1 includes the climatic characterization of North, Dry Coast and South macrozones (MZs), as defined by Loewe *et al.* (2015), and includes annual climatic variables and others related to water availability in spring and summer, because central Chile has a Mediterranean climate, with rainfall concentrated in autumn and winter. Average annual and maximum annual temperatures decrease from north to south; on the other hand, the lowest minimal annual average temperature corresponds to the Dry Coast MZ. Rainfall increases from north to south, whereas the hydric index diminishes, suggesting lowest water deficit in the South MZ (Table 1). The highest variability of climatic variables among sites occurs in the North MZ.



Table 1. Climatic data of stone pine growth macrozones in Chile

Variable	North MZ n=42	Dry Coast MZ n=26	South MZ n=54	All sites n=122
Annual average temp (AT) (°C)	14.1 (11.8 ; 16.7)	13.6 (12.5 ; 15.1)	13.2 (11.0 ; 14.2)	13.6 (11.0 ; 16.7)
Annual maximum average temp (MXT) (°C)	21.9 (17.6 ; 25.1)	21.0 (17.9 ; 23.0)	19.8 (16.3 ; 22.5)	20.8 (16.3 ; 25.1)
Annual minimum average temp (MNT) (°C)	7.5 (5.5 ; 9.9)	7.0 (5.5 ; 7.7)	7.5 (5.3 ; 9.2)	7.4 (5.3 ; 9.9)
Annual thermal oscillation (TO) (°C)	14.3 (9.2 ; 17.3)	14.0 (10.7 ; 17.0)	12.3 (8.2 ; 15.1)	13.4 (8.2 ; 17.3)
Annual average rainfall (PP) (mm)	383.7 (116.2 ; 1,260.1)	648.3 (416.3 ; 816.0)	1,047.0 (523.6 ; 1,666.9)	716.7 (116.2 ; 1,666.9)
Annual hydric index (HI) (mm)	-995.4 (-1,349.7 ; -107.3)	-743.7 (-914.4 ; -474.2)	-244.1 (-736.4 ; -332.9)	-628.2 (-1,349.7 ; 332.9)
Spring average rainfall (PPsp) (mm)	37.2 (1.5 ; 143.9)	61.8 (38.4 ; 89.8)	131.8 (80.8 ; 218.9)	81.9 (1.5 ; 218.9)
Spring hydric index (HIsp) (mm)	-394.1 (-483.7 ; -269.5)	-376.9 (-439.5 ; -269.9)	-267.7 (-346.4 ; -121.3)	-337.1 (-483.8 ; -121.3)
Summer average rainfall (PPsu) (mm)	8.42 (0 ; 36.4)	29.1 (10.3 ; 46.3)	58.6 (31.7 ; 113.0)	33.8 (0.0 ; 113.0)
Summer hydric index (HIsu) (mm)	-485.8 (-563.1 ; -328.3)	-460.4 (-571.3 ; -332.6)	-399.9 (-517.7 ; -260.5)	-444.1 (-571.3 ; -260.4)

Values in parentheses correspond to minimal and maximum values recorded for each variable.

Hydric index was calculated as accumulated rainfall in the period minus potential evapotranspiration of the same period. n= number of sites. Source: www.dga.cl and www.inia.cl

2.2 Stone pine reproductive phases and biometeorological variables

Besides annual and seasonal meteorological variables, we built new climatic variables related to the species reproductive cycle, named biometeorological variables. These were calculated, for each site and year of measurement, by summarizing the above mentioned climatic variables for 11 reproductive physiological and phenological phases that are important for cone and seed productivity. The 11 phases used in this study were identified from several works (Abellanas and Pardos, 1989; Abellanas, 1990; Mutke *et al.*, 2005a) and adapted to the central area of Chile according to Venegas (2011) and to our field observations (Table 2 and Fig. 2).

Table 2. Reproductive cycle of stone pine in central Chile

Year	Physiologic or phenological phase	Month											
		A	S	O	N	D	J	F	M	A	M	J	J
0	P1. Induction of male primordia												
	P2. Induction of female primordia												
1	P3. Differentiation of reproductive shoots												
	P4. Male flowering												
	P5. Female flowering												
	P6. Pollination												
2	P7. Growth of 2 year-old conelet												
	P8. Growth of 3 year-old cone												
3	P9. Fecundation												
	P10. Embryo development												
	P11. Cone ripening												

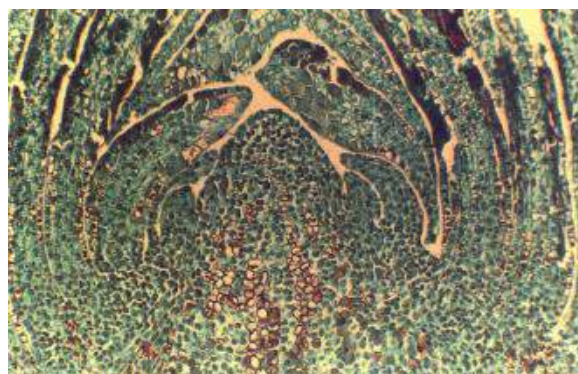
Source: built from Abellanas and Pardos (1989), Abellanas (1990) and Mutke *et al.* (2005a), and adapted to the central area of Chile according to Venegas (2011) and to our field observations.

The reproductive cycle lasts 3.5 years (42 months), and begins in year 0 with the induction of the primordia, first male primordia (P1), then female primordia (P2), in which axillary primordia begin to form secondary cataphylls. In year 1 four phases occur: differentiation of reproductive shoots (P3) when reproductive meristems differentiate in size from vegetative ones, female ones being larger and broader than vegetative ones, with male meristems having an intermediate size; male flowering (P4) when male flowers appear; female flowering (P5) when female strobili appear; and pollination (P6), when mature male flowers open and pollen is released and reaches receptive strobili. During year 2 only one, typically slow phase, occurs, 2 year-old conelet growth (P7). The reproductive cycle finishes



in year 3, with four phases: 3 year-old cone growth (P8), when cone and seeds develop; fecundation (P9), in which the pollen tube is formed and fertilization occurs; embryo development (P10); and cone ripening (P11) when seeds mature and later disseminate (Abellanas, 1990; Abellanas and Pardos, 1989; Mutke *et al.*, 2005a). To analyze CN, we considered the period from P1 to P7, when the number of cones is determined. To analyze CW and pine nut weight, we considered the period between P7 and P11, when fruit growth takes place; and to study IS, we considered the period from P1 to P11. Therefore, for each year of measurement and site, the biometerological variables summarized climatic records of specific periods during the reproductive cycle, comprising 42 months since induction to cone ripening.

Climatic characterization by physio-phenological phases for all sites is presented in Table 3; climatic characterization for each MZ is included as Supplementary Material (Tables 1S to 3S).



P1 (month 1) & P2 (month 7)



P3 (month 9)



P4 (month 11)



P5 (month 12)



P6 (month 13)



P7 (months 23-25)



P8 (months 35-38)



P9 (month 36)



P10 (months 37-40)



P11a (months 41-42)



P11b (months 43 onward)

Figure 2. Stone pine reproductive cycle phases: induction of male primordia (P1) and female primordia (P2); differentiation of reproductive shoots (P3), male flowering (P4), female flowering (P5), pollination (P6), growth of 2 year-old conelet (P7), growth of 3 year-old cone (P8), fecundation (P9), embryo development (P10), cone ripening (P11a) and seed dissemination (P11b). Pictures of phases P1, P2, P3 were taken from Abellanas (1990); designs were taken from Abellanas and Pardos (1989); other pictures belong to the authors of this work.

Table 3. Climatic characterization of physiological and phenological phases of stone pine across its distribution area in Chile

Phase	Average temperature (°C)	Maximum average temperature (°C)	Minimum average temperature (°C)	Rainfall (mm)	Hydric index (mm)	Thermal oscillation (°C)
Induction of male primordia (P1)	17.5 (13.2 ; 20.9)	25.1 (18.4 ; 30.0)	10.4 (6.6; 13.3)	44.5 (0 ; 212.1)	-294.4 (-392.4 ; -83.7)	5.2 (2.0 ; 7.4)
Induction of female primordia (P2)	8.9 (6.2 ; 14.6)	14.1 (10.9 ; 21.3)	4.5 (0.6 ; 9.1)	508.9 (0.8 ; 1.780.5)	386.1 (-143.6 ; 1691.3)	6.8 (3.1 ; 10.5)
Differentiation of reproductive shoots (P3)	9.3 (6.6 ; 14.7)	14.9 (11.8 ; 22.4)	4.4 (0.2 ; 8.1)	267.9 (37.4 ; 898.4)	135.5 (-184.5 ; 771.2)	6.2 (2.9 ; 8.6)
Male flowering (P4)	13.3 (10.0 ; 18.1)	20.4 (14.7 ; 27.2)	7.0 (3.8 ; 10.4)	68.1 (0 ; 272.6)	-167.4 (-298.7 ; 82.4)	5.5 (1.9 ; 8.3)
Female flowering (P5)	15.3 (11.4; 20.2)	22.6 (16.4 ; 28.7)	8.5 (5.3 ; 12.0)	43.6 (0 ; 203.1)	-252.1 (-373.6 ; -42.3)	5.4 (2.4 ; 10.2)
Pollination (P6)	17.3 (13.5 ; 21.6)	25.0 (18.5 ; 31.3)	10.3 (7.2 ; 13.7)	37.5 (0 ; 193.3)	-300.4 (-420.2 ; -58.5)	5.5 (1.8 ; 8.7)
Growth of 2 year-old conelet (P7)	15.7 (12.0 ; 19.3)	23.4 (17.7 ; 28.1)	8.7 (6.1; 12.8)	49.7 (0 ; 216.8)	-538.6 (-727.7 ; -231.3)	7.1 (3.8 ; 12.7)
Growth of 3 year-old cone (P8)	16.3 (12.4 ; 21.7)	23.8 (18.0 ; 30.3)	9.5 (5.7 ; 14.7)	95.7 (0 ; 323.8)	-651.9 (-955.6 ; -286.14)	7.8 (2.9 ; 13.1)
Fecundation (P9)	15.7 (11.6 ; 19.5)	23.3 (15.8 ; 28.6)	9.0 (4.8 ; 12.9)	39.8 (0 ; 189.9)	-260.1 (-380.7 ; -113.9)	5.5 (1.7 ; 10.1)
Embryo development (P10)	17.5 (13.6 ; 22.2)	26.0 (18.8 ; 32.2)	10.3 (6.7 ; 13.8)	56.1 (0 ; 247.7)	-699.9 (-996.8 ; -287.4)	7.3 (3.2 ; 13.4)
Cone ripening (P11)	12.7 (9.8 ; 18.3)	19.9 (15.2 ; 25.9)	6.8 (3.8 ; 11.2)	182.4 (9.5 ; 661.5)	-37.2 (-290.2 ; 464.9)	7.5 (3.5 ; 11.0)

Reported data correspond to average for the phase calculated with all sites. In parentheses, minimum and maximum values of the phase are presented.

2.3 Fruit production variables

We visited and measured all existing stone pine plantations recorded by INFOR in Chile, aged 8 years and over, which are considered productive in this country ($n=122$). For 3,464 stone pine trees distributed in a surveyed area of over 1 million hectares, all cones tree⁻¹ (CN) were counted from a standing position on the ground (estimation error was determined to be lower than 15%).

For a further detailed analysis of fruit characterization, we identified all superior trees for fruit production (76 individuals located in 46 sites distributed across MZs). Given the high environmental variability and different ages of the base populations, superior phenotypes were identified by applying the individual valuation method following Ipinza (1998). From each superior tree, a sample of 10 cones was randomly taken, cone weight at harvest (green) (CW) was recorded in the field and in-shell pine nut number cone⁻¹ (IS) as well as other fruit variables (size and weight of in-shell and shelled pine nuts) were measured in the laboratory. The proportion of shelled pine nuts in cones –known as pine nut yield (PNY)–, was calculated by drying the shelled pine nuts to 6% humidity content, weighing them and comparing that value with cone weight at harvest, using the following formula:

$$PNY(\%) = \frac{\text{Dried shelled pine nut weight}}{\text{Cone weight at harvest (green)}} * 100$$

Cone productivity (CP) was estimated by considering CN multiplied by CW and by 100, under the assumption that between-tree variability due to competition effects is controlled by plantation management. We estimated a density of 100 trees ha⁻¹ as close to a final density in productive plantations at an adult age (DBH over 30 cm), as indicated by Mutke *et al.* (2012) and by Consejería de Medio Ambiente (2004). Shelled pine nut production (SHP) was calculated based on PNY, also assuming plantations of 100 trees ha⁻¹. As covariates we recorded age and DBH of each tree and presence/absence of water supply.



2.4 Statistical analyses

The relative contribution of climatic and biometeorological variables to productivity measurements was estimated using CART (Classification and Regression Trees) algorithms (Breiman, 2001). The RT procedure creates a predictive model for a continuous response variable (CN, CW and IS) based on the recurrent classification of the studied cases into groups according to the values (threshold) of the predictor variables (climatic and biometereological variables). The result of this recursive binary partitioning is a model whose structure can be displayed as a tree-like graph, with each split in the tree labeled according to the variable and threshold used to define the split. Regression trees were used to explain variability of the selected response variables capturing nonlinear relationships and correlations between meteorological variables. The algorithm was not applied on productive variables showing low variability ($CV < 20\%$) or strongly correlated to the selected dependent variables. RT have successfully been used to model growth and yield from highly correlated climatic variables (Thuiller *et al.*, 2003; Lobell *et al.*, 2005; Heredia *et al.*, 2010).

A total of eight RTs, four using climatic variables (in all sites and by MZ) and four using biometeorological variables (in all sites and by MZ) as predictors were built for each of the stone pine productive variables. Boosting (Elith *et al.*, 2008) was used as a validation tool; only the first two nodes of each RT were used to identify the predictors that most strongly influenced the response variables. As confirmatory analysis we performed a non-parametric ANOVA to evaluate statistical significance of differences between yield mean groups suggested by the thresholds of the first RT node ($n > 15$). All ANOVAs included tree age and macrozone effects as controlling variables. We used the same non-parametric approach to evaluate water supply effects on cone production. The relationship between DBH and cone production was analyzed using a linear regression model. The analyses were performed using the software INFOSTAT and its interface with the software R (Di Rienzo *et al.*, 2013).

3. Results

3.1 Fruit productivity by stone pine macrozones in Chile

The description of stone pine cone productivity for all studied sites and per macrozone (MZ) is presented in Table 4. Average cone number tree⁻¹ (CN), cone weight at harvest (CW) and in-shell pine nut number per cone (IS) across sites were 57 cones tree⁻¹, 495 g and 100 in-shell pine nuts cone⁻¹, respectively, being the first two higher in the south. Pine nut yield (PNY) was in average 3.9%, with 3.5% in Dry Coast, 3.7% in the North MZ and 4.3% in the South MZ. In-shell pine nut weight (ISW) was the highest in the South MZ. The lowest value of shelled pine nut weight (SHW) was recorded in the Dry Coast MZ. Average CP reached 2,821 kg 100 trees⁻¹, increasing from north (2,355 kg 100 trees⁻¹) to south (3,183 kg 100 trees⁻¹); similarly, average shelled pine nut production amounted to 110 kg 100 trees⁻¹ across the country, increasing from north (87 kg 100 trees⁻¹) to south (137 kg 100 trees⁻¹).

Table 4. Characteristics of fruit production by stone pine macrozones in Chile

Production Variables	North MZ	Dry Coast MZ	South MZ	All sites
Cone tree ⁻¹ (#) (CN)	49 (111)	59 (150)	62 (122)	57 (126)
Weight of cone at harvest (green) (g) (CW)	480.7 (27)	489.5 (17)	513.4 (22)	495.0 (23)
Cone length (cm) (CL)	10.7 (17)	11.2 (14)	12.1 (18)	11.3 (18)
In-shell pine nut cone ⁻¹ (#) (IS)	93 (28)	107 (20)	106 (16)	100 (23)
Shelled pine nuts cone ⁻¹ (#) (SH)	87 (31)	94 (28)	101 (19)	94 (26)
In-shell pine nut weight (g) (ISW)	0.9 (16)	0.9 (19)	1.0 (13)	0.9 (15)
Shelled pine nut weight (g) (SHW)	0.22 (19)	0.18 (17)	0.21 (15)	0.21 (18)
Cone to shelled pine nut yield (%) (PNY)	3.7 (26)	3.5 (28)	4.3 (18)	3.9 (24)
Cone productivity (kg 100 trees ⁻¹) (CP)	2,355 (96)	2,888 (190)	3,183 (159)	2,821 (155)
Shelled pine nut productivity (kg 100 trees ⁻¹) (SHP)	87 (91)	101 (196)	137 (172)	110 (154)

Values in parentheses correspond to coefficient of variation (in percentage). n=46 for all variables, with the exception of CN (n=122). #=number. Mean values are adjusted by tree age.



3.2 Fruit productivity and climate

3.2.1 Cone number tree⁻¹ (CN)

The climatic variables that significantly influenced CN were annual and autumn thermal oscillation, and spring rainfall and water deficit (Fig. 3a). Accordingly, we detected three key physio-phenological phases that affected CN: differentiation of reproductive shoots (P3), and male (P4) and female flowering (P5), which were favored by a thermal oscillation below 6°C in P3, rainfall below 70 mm in P4, and minimum temperature above 7°C and a low water deficit in P5 (Fig. 3b).

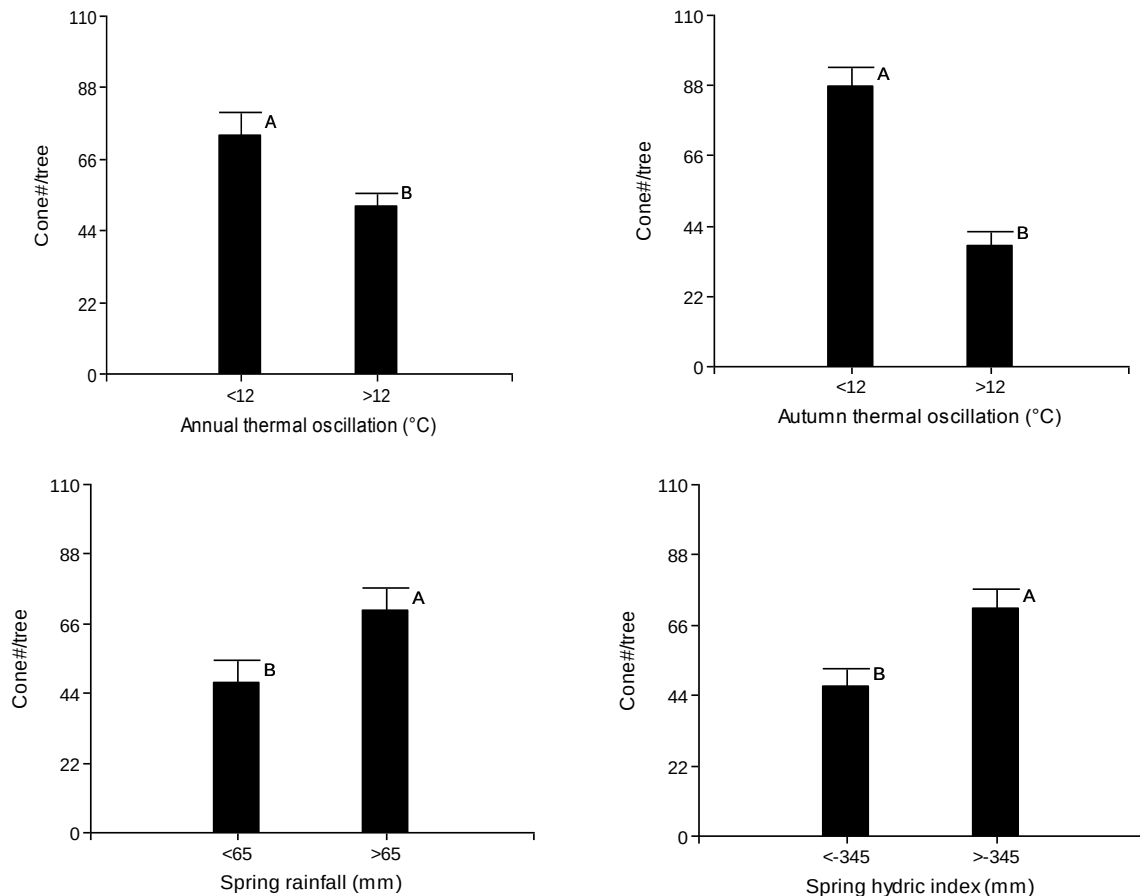


Figure 3a. Climatic variables that influence cone production in Chile. Each threshold was detected by CART analyses. Different letters indicate statistically significant differences ($p < 0.05$). Cone #: Cone number; Annual thermal oscillation: annual average maximum absolute temperature minus annual average minimum absolute temperature; Spring hydric index: spring accumulated rainfall minus spring potential evapotranspiration.

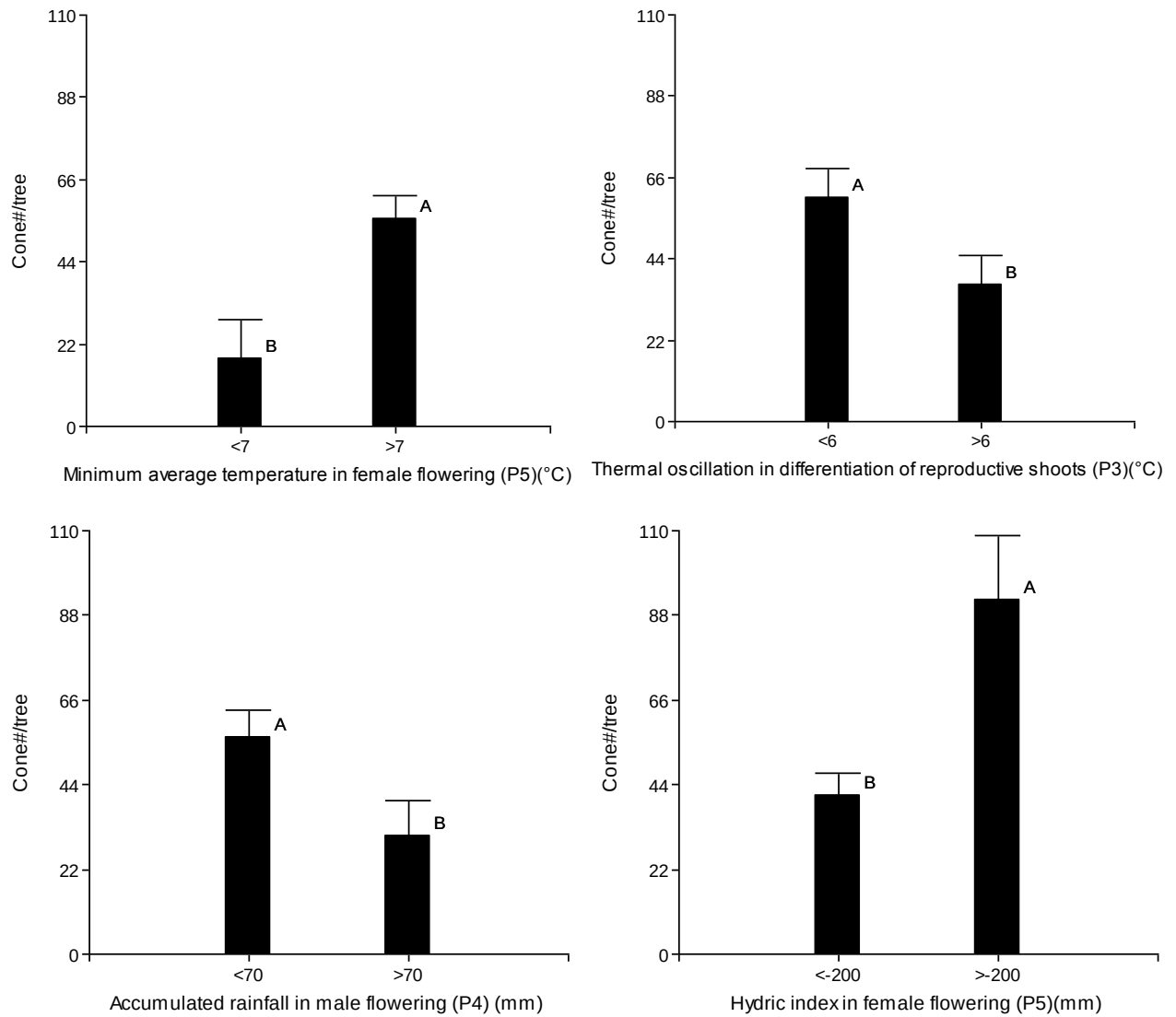


Figure 3b. Biometeorological variables that influence cone production in Chile. Each threshold was detected by CART analysis. Different letters indicate statistically significant differences ($p < 0.05$).

Cone #: Cone number; Thermal oscillation: average maximum absolute temperature minus average minimum absolute temperature; Hydric index: accumulated rainfall minus potential evapotranspiration.



Regression trees showed that CN (Fig. 1aS) was influenced by autumn thermal oscillation, with CN decreasing at ranges greater than 11.8°C and averages of 80 and 40 cones tree⁻¹ below and above that threshold. In sites with narrower autumn thermal oscillation, those with spring minimum temperature below 7.3°C presented a superior number of cones tree⁻¹ (154 vs 68). With autumn thermal oscillation above 11.8°C and summer maximum temperatures above 27.4°C, CN increased under a low spring water deficit (68 vs 41).

An average minimum temperature above 6.9°C during P5 favored CN (60 vs 20) (Fig. 1bS). On the other hand, when that minimum temperature was exceeded, accumulated rainfall of above 71.6 mm in P4 had a negative effect on CN (37 vs 75).

Besides being correlated with climatic variables, CN was significantly related to DBH and water supply. DBH was linearly related to CN, suggesting that for every cm of DBH growth, an increase in CN of half a cone can be expected ($CN = 19.7 + 0.45 * DBH$, $n=118$; $p=0.0066$). Statistically significant differences between irrigated and non-irrigated plantations were found for CN (60 vs 44 cones tree⁻¹), suggesting an increase of over 35% of tree cone production associated with this practice (Fig. 4).

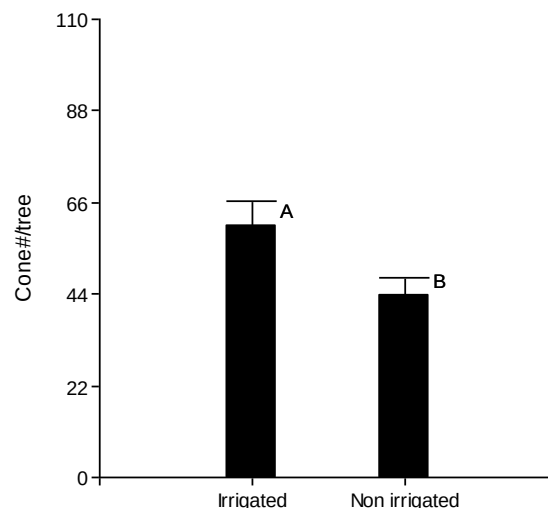


Figure 4. Water supply effect on cone production using data from all sites. Different letters indicate statistically significant differences ($p < 0.05$).

In the analysis focused on MZ (Figs. 2aS and 2bS), the variables related to temperature were also the main climatic variables affecting CN; autumn thermal oscillation (TOau) was the most relevant factor in North MZ, increasing CN in sites where it was below 11.6°C (105 vs 38). Low water deficit (less than 332 mm) during induction of male primordia (P1) was found to favor CN (80 vs 33). In the Dry Coast MZ, CN was negatively influenced by maximum autumn temperature above 17.9°C (105 vs 26) and positively affected by minimum average temperature above 12°C during induction of male primordia (P1) (66 vs 17). In the South MZ, sites with spring minimum average temperature below 6.9°C had greater fruit production (116 vs 50). CN was favored by a thermal oscillation below 6.2°C at differentiation of reproductive shoots (P3) (93 vs 38).

3.2.2 Cone weight (CW)

The climatic variables that significantly influenced CW along the climatic gradient in Chile were annual average temperature, annual water deficit and annual rainfall, and according to physio-phenological phases, maximum average temperature and water deficit during embryo development (P10), and accumulated rainfall in 2 year-old conelet growth (P7) (Fig. 5).

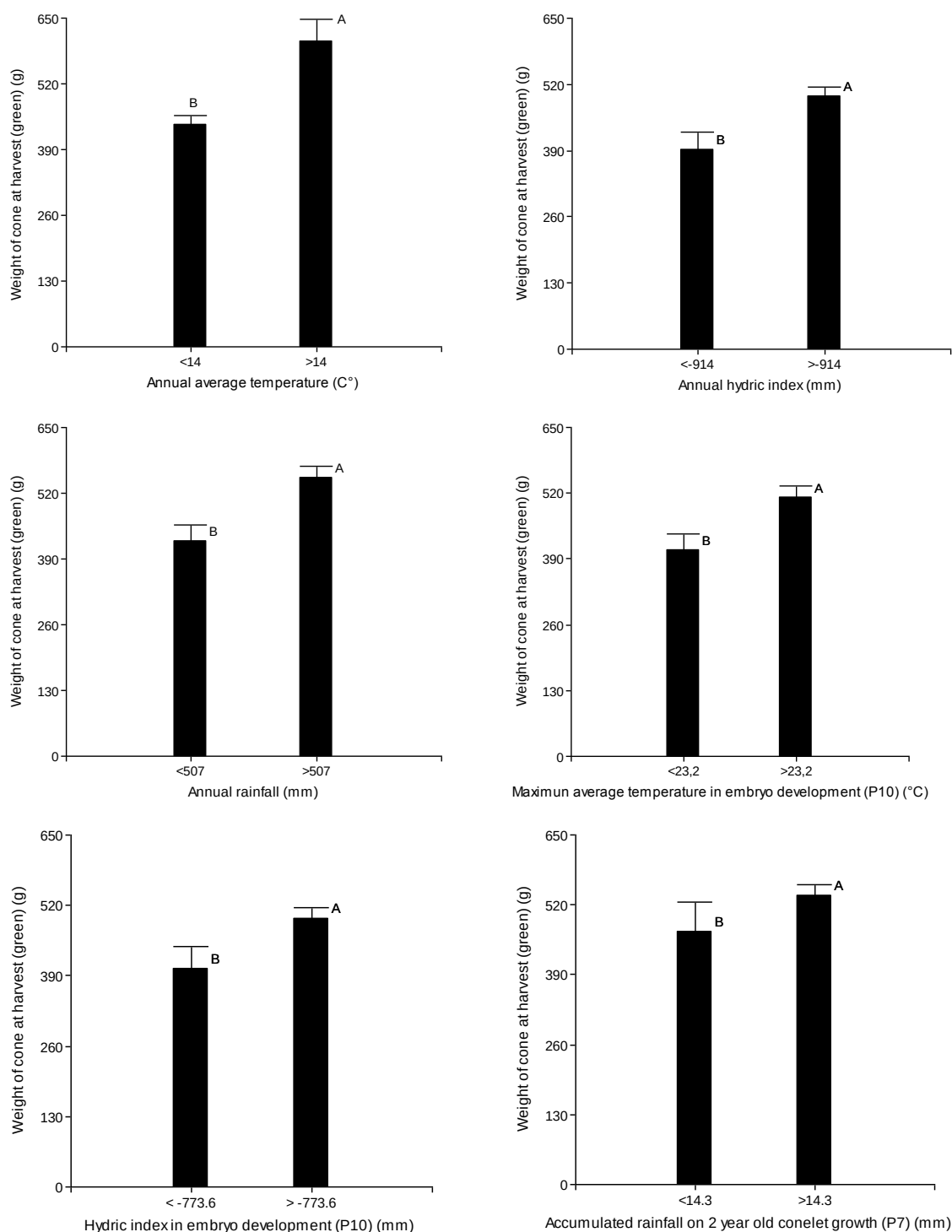


Figure 5. Variables that influence cone weight in Chile. Each threshold was detected by CART analysis. Different letters indicate statistically significant differences ($p < 0.05$). Annual hydric index: annual rainfall minus annual potential evapotranspiration.

The variable that best explained CW was annual average temperature (AT). Sites with higher AT ($>14.1^{\circ}\text{C}$) had a higher CW (438 vs 549 g), whereas in sites with AT smaller than $<14.1^{\circ}\text{C}$, CW was favored (334 vs 461 g) by a decrease of water deficit (Fig. 3aS). The analysis focusing on physio-phenological phases also evidenced the importance of temperature, showing that CW was favored by maximum temperature above 23.2°C during P10 (403 vs 519 g) and by water deficit during P10 (spring-autumn) below 774 mm (404 vs 568 g) (Fig. 3bS).

Considering macrozones, we observed (Figs. 4aS and 4bS) the effect of annual rainfall in the North MZ and of annual average temperature in the Dry Coast and South MZs. In the North MZ, sites with annual rainfall above 507 mm produced cones of 660 g, whereas below that threshold, CW decreased by nearly 35% (418 g). Below 507 mm, a winter average temperature below 8.3°C favored CW. Rainfall over 14.3 mm during growth of 2 year-old conelet (P7) represented a CW increase of over 35% (660 vs 419 g), as well as an average temperature in P8 below 13.9°C (631 vs 396 g). In the Dry Coast MZ, when average temperature was above 14.2°C , CW was 585 g, whereas at lower average temperature values it decreased approximately by 25%. Average temperature during 2 year-old conelet growth (P7) at above 18.8°C contributed to the production of heavier cones (628 vs 442 g). In the South MZ, where water is not generally a limiting factor for stone pine growth, annual average temperatures above 12.3°C produced heavier cones (512 vs 368 g), and with minimum average annual temperature below 7.6°C , cones had greater weight (586 vs 472 g). In the analysis focused on physio-phenological phases, we observed the positive effect of a maximum temperature above 22.5°C on CW (514 vs 385 g) during embryo development (P10).

3.2.3 *In-shell pine nut number (IS)*

Climatic variables that significantly influenced IS across Chile were annual water deficit and autumn maximum temperature; and accumulated rainfall during induction of male primordia (P1); maximum average temperature during 2 year-old conelet growth (P7), and rainfall during 3 year-old cone growth (P8) and cone ripening (P11) (Fig. 6). Hydric index (HI) was the variable that best explained IS. Sites with water deficit below 914 mm had higher IS (108 vs 79) than sites with water deficit above that value (Fig. 5aS). The analysis focused on physio-phenological phases also evidenced the importance of water availability, showing a positive effect of accumulated rainfall of over 133 mm on IS (111 vs 91) during cone ripening (P11) (Fig. 5bS).

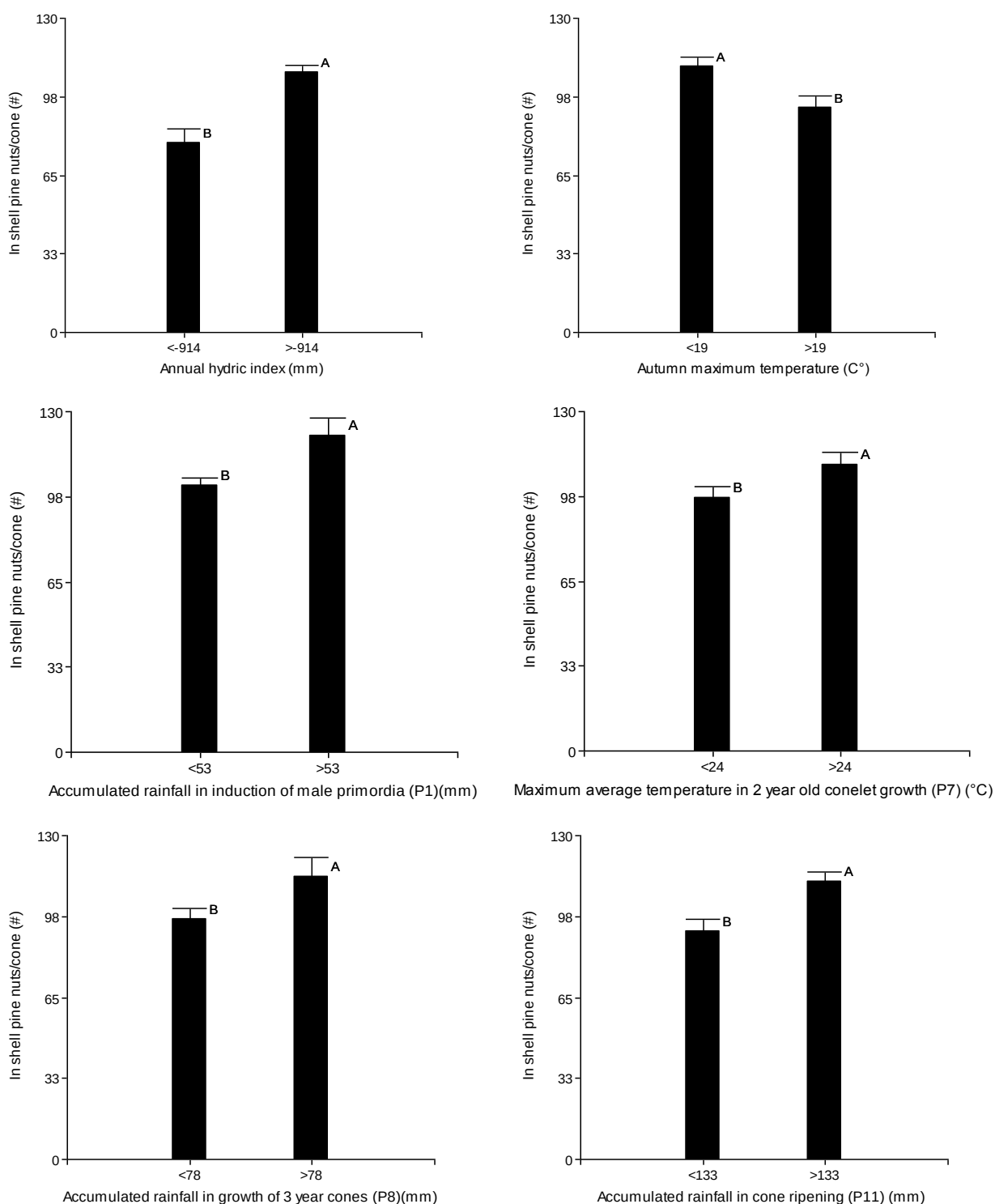


Figure 6. Variables that influence in-shell pine nut number per cone in Chile. Each threshold was detected by CART analysis. Different letters indicate statistically significant differences ($p < 0.05$). #: number; Annual hydric index: annual rainfall minus annual potential evapotranspiration.

The analysis focused on MZ (Figs. 6aS and 6bS) detected that in the North MZ, IS was significantly greater with water deficit below 914 mm (109 vs 79) and maximum average temperature during induction of male primordia (P1) below 27°C (109 vs 79). In the Dry Coast MZ, when autumn maximum temperature was below 19.1°C, IS reached 116, whereas at higher values, IS decreased over 25%. The analysis of the effect of bioclimatic variables on IS considering physio-phenological phases in this MZ showed that maximum temperature above 23.8°C during 2 year-old conelet growth (P7) had a positive influence of 38% (116 vs 84).

In the South MZ, when minimum summer temperature was above 12°C, IS reached 136, whereas at lower values, IS decreased to 105. In this MZ, accumulated rainfall during 3 year-old cone growth (P8) above 78.4 mm also favored IS (116 vs 93), as well as accumulated rainfall during induction of male primordia (P1) of above 52.4 mm (127 vs 109).

Tables 5 and 6 summarize the results regarding the influence of climatic and biometeorological variables on CN, CW and IS based upon the complete material from Chile.

Table 5. Main climate variables that showed a significant influence on stone pine cone production, cone weight and quantity of in-shell pine nut per cone in Chile

Dependent variable	Autumn maximum temperature (MXTau)	Annual average temperature (AT)	Rainfall (PP)	Spring rainfall (PPsp)	Hydric index† (HI)	Spring hydric index (HIsp)	Thermal oscillation† † (TO)	Autumn thermal oscillation (TOau)
Cone number tree ⁻¹ (CN)				≥65 mm (46%)		≥ - 345 mm (60%)	<12°C (43%)	<12°C (125%)
Cone weight (CW)		≥14°C (38%)	≥ 507 mm (29%)		≥ - 914 mm (27%)			
In-shell pine nut number cone ⁻¹ (IS)	≤ 19°C (18%)				≥ - 914 mm (37%)			

Values in parentheses correspond to the expected increase of the dependent variable under the identified key climatic conditions.

†Hydric index was calculated as accumulated rainfall in the period minus potential evapotranspiration of the same period; for the spring hydric index, spring data were considered.

††Thermal oscillation: average maximum absolute temperature, minus average minimum absolute temperature; for the autumn thermal oscillation, autumn data were considered.



Table 6. Main biometeorological variables that showed a significant influence on stone pine cone production, cone weight and quantity of in-shell pine nut per cone in Chile

	Induction	Differentiation	Flowering		Cone development			
	Male primordia	Reproductive shoots	Male	Female	Growth of 2 year-old conelet	Growth of 3 year-old cone	Embryo development	Cone ripening
Phase	P1	P3	P4	P5	P7	P8	P10	P11
Year	0	1			2	3		
Date								
From	Nov., 15	July, 15	Sep., 15	Oct., 15	Sep., 15	Sep., 15	Nov., 15	Mar., 15
To	Jan., 15	Sep., 15	Nov., 15	Dec., 15	Jan., 15	Feb., 15	Apr., 15	June, 15
Cone number tree⁻¹ (CN)		Thermal oscillation $\leq 6^{\circ}\text{C}$ (65%)	Rainfall $\leq 70\text{mm}$ (87%)	Min. temp $\geq 7^{\circ}\text{C}$ (205%) Hydric index [†] $\geq -200\text{mm}$ (130%)				
Cone weight (CW)					Rainfall $> 14\text{ mm}$ (14%)		Max. temp $\geq 23^{\circ}\text{C}$ (29%) Hydric index $\geq -774\text{ mm}$ (23%)	
In-shell pine nut number cone⁻¹ (IS)	Rainfall $> 53\text{mm}$ (19%)				Max. temp $\geq 24^{\circ}\text{C}$ (12%)	Rainfall $> 78\text{mm}$ (18%)		Rainfall $> 133\text{ mm}$ (22%)

Values in parentheses correspond to the expected increase of the dependent variable under the identified key biometeorological conditions.

[†] Hydric index was calculated as accumulated rainfall in the period minus potential evapotranspiration of the same period.

4. Discussion

This study has shown the relevance of climatic variables for stone pine fruit productivity, with increasing values along macrozones from north to south. Even though fruit productivity was different along the climatic gradient in Chile, all studied variables showed interesting values when compared with the native range of the species. The average cone production in Chile, of 57 cones tree⁻¹, represents a yield of 28 kg tree⁻¹, which is higher than data from its native habitat but lower than values reported for Lebanon (40 kg tree⁻¹) (Sfeir, 2011).

In Spain, stone pine production was found to be dependent upon climatic conditions (Gordo, 2004; Mutke *et al.*, 2005a), with production being related to rainfall and temperature during the earliest cone development phases. Our results showed that high spring rainfall and small thermal oscillation had a high positive effect on cone number tree⁻¹ (CN). Regarding temperature, we observed that autumn thermal oscillation smaller than 12°C favored cone production, precisely when induction of female primordia occurs. On the other hand, spring minimum temperature above 7°C had a positive effect on fruiting, when both male and female flowering and pollination take place.

Thermal fluctuations have been reported to influence cone initiation in several ways: by increasing cone differentiation when temperature is below average (Forcella, 1978) or accelerating it with increasing temperature (Lester, 1967). Accordingly, we found a negative effect of thermal oscillation greater than 6°C on CN at reproductive shoot differentiation (P3).

Higher than average temperatures during seed cone initiation in *P. ponderosa* Douglas ex C. Lawson have been associated with above-average cone production (Krannitz and Duralia, 2004); this effect was also demonstrated in other *Pinus* species, such as *P. sylvestris* L. (Karlsson, 2000). In our study, this relationship was observed in the Dry Coast MZ, where minimum average temperature above 12°C during induction of male primordia (P1) significantly favored cone production.

Other fruit species have been found to be negatively affected by the lack of chilling, with different consequences, such as delayed flowering, extended flowering period, weak vegetative growth and high heterogeneity in fruit size (Ghrab *et al.*, 2014). To our knowledge, this aspect has not been studied in stone pine; hence, we suggest addressing this issue in future studies, because we assume that this Mediterranean species may have such requirement. Elucidating this aspect would help to further define appropriate areas for the



species cultivation for fruit production under climate change, which would cause temperature increases and thermal oscillation decreases (Martelo, 2004; Del Río, 2005).

However, expected rainfall decreases would have a negative impact on the species' productivity. In fact, Fontes *et al.* (2013) reported that cone production depends highly and positively on accumulated rainfall in winter and spring, before the onset of pollination. Mutke *et al.* (2005a) found rainfall to be determinant for cone productivity at certain key periods during the 4 year-old cone development period. According to these authors, cone yield was favored by high water availability at the time of primordia formation, pollination and growth of 3 year-old cones up to cone ripening. High rainfall during flowering promotes high cone production (UNAC, 2014), and Calama *et al.* (2007b) also reported a positive effect of spring rainfall on female flowering and derived cone production. We quantified the effect of rainfall and water deficit on CN, with both variables confirming a positive relationship between CN and spring water availability. However, we also found a negative effect of high accumulated rainfall (>70 mm) during male flowering (P4). This finding is in agreement with records of Parlak *et al.* (2013), who pointed out the importance of the pollination period –influenced by altitude, rainfall, high relative humidity and extreme temperatures– on cone production. These authors found that non-productive areas located below 500 m a.s.l. were characterized by a thermal oscillation of 22°C and more frequent days with temperature below 10°C, early and late frosts, high relative humidity and fog.

Our results confirmed that rainfall and temperature were important climatic variables for stone pine fruiting across Chile. However, their relative importance varied among growth MZs, and this was observed for all fruit traits analyzed in this study. The highest fruit traits were recorded in the South MZ, where the highest vegetative growth was also observed (Loewe *et al.*, 2015). Here, gains in cone productivity reached 35% with respect to the North MZ.

Since annual rainfall and temperature fluctuations can induce changes during the cone induction period, more than one phenological calendar in the area cultivated with stone pine in Chile might be used for future studies.

This species lengthy (42 months) reproductive cycle of initiation, differentiation, pollination, fertilization, and embryo and seed development provides a large window for a complex variety of potentially interacting factors to play a role in the frequency of *P. pinea* cone production and in the quantity of seed produced. This is why in this long cycle

development of a seed-bearing cone species there are many opportunities for maternal regulation of seed and cone production via cone, ovule and embryo abortion (Krannitz and Duralia, 2004). This explains why, besides climate, management may also influence cone production.

The fact that irrigation had a significant effect on stone pine cone production, which is not in agreement with Aleta and Vilanova (2014), could be partially explained by the findings of Nilsson and Wiklund (1992), who reported dry mass increases due to irrigation. In that work, irrigation resulted in faster increment of needle dry mass because of an increased formation of needles and a reduced shedding of older ones.

In Catalonia, an irrigation test was established in 2008 to study the effect of different water regimes on cone induction and production (Bono and Aleta, 2011). For the 2014 production (IRTA, 2014) reported 18 and 23 strobili, conelets and cones per tree without and with irrigation, respectively, a difference of over 25% on average. In our study, higher differences in productivity were observed between irrigated and non-irrigated plantations due to the reported significant effect of irrigation on cone number.

Irrigation also results in increased DBH growth rates (Loewe *et al.*, 2015). Accordingly, we found a significant relationship between cone production and DBH. This is consistent with findings in *P. ponderosa* Douglas ex C. Lawson reported by Krannitz and Duralia (2004), who stated that tree diameter was a better predictor of cone production than age, and that trees of larger diameter also produced cones more frequently. Crown variables are also closely associated with cone production in *Pinus pinea* according to Correia and Freire (2014), but those variables were not considered in this study.

Cone quality is expressed through cone weight, which is assumed to control factors such as yield, empty/damaged pine nuts and pine nut size (Calama *et al.*, 2007b), varying from 50 to 800 g in Europe, and similarly in Chile (105 to 935 g cone⁻¹). According to these authors, weight can even determine the price paid per cone in the nut industry; in fact, heavier cones (up to 350 g) show higher yield in pine nuts and produce larger pine nuts, both shelled and in-shell. Furthermore, they indicate that even the rate of empty/damaged pine nuts is smaller in heavier cones.

Mutke (2005a) indicated that stone pine cones are usually about 250-350 g in weight, but weight can vary between 200 and 500 g (Mutke *et al.*, 2005a). In Tunisia, average cone weight is 252 g cone⁻¹ (Schröder *et al.*, 2014); in Portugal, this value ranges between 256 and



280 g cone⁻¹ (Gonçalves and Pommerening, 2012), and in Chile we found an average of 495 g cone⁻¹, with a low variability expressed as coefficient of variation equal to 23%.

A significant year effect on cone weight caused by climate has been reported (Calama *et al.*, 2008b). Our results showed that annual rainfall and annual water deficit significantly affected cone weight at harvest (CW), which could make these variables useful indicators for irrigation practices. In particular, we found that annual rainfall higher than 507 mm significantly increased CW, which is in agreement with several works, such as that of Mutke *et al.* (2006), who reported CW lower than 200 g with annual rainfall below 400 mm. Fontes *et al.* (2013) also reported a high dependence of cone production on water deficit, a great effect of late spring-summer rainfall on weight and size of pine nuts and cones; and Calama *et al.* (2007b) observed that CW of the next crop is largely affected by summer to winter rainfall. We found that accumulated rainfall during 2 year-old conelet growth (P7) significantly influenced CW and that water deficit below 774 mm during embryo development (P10) increased CW by 23%. We also observed a significant effect of temperature during P10, when maximum temperatures above 23°C increased CW by 29%.

We found that in-shell pine nut number per cone (IS) was positively benefited by an autumn maximum temperature below 19°C, but negatively affected by high water deficit (above 914 mm). We did not find any reference on the dependence of this variable on climatic factors. However, it has been established that IS is highly variable, depending upon production and cone size (Piqué, 2004). We observed that a small thermal oscillation favors cone production and that water availability is positive for CW; therefore, these variables are also expected to affect IS.

The fruit productivity variables CN and IS increased from north to south in Chile. Therefore, the average CP, estimated in 28 kg tree⁻¹, would also follow the same pattern along with an increase in annual rainfall. This assumption is in agreement with findings of Yagüe (1994), who found that in mature forests in Spain, productivity ranges between 54-325 kg ha⁻¹ with rainfall of 350-450 mm year⁻¹, and 113-433 kg ha⁻¹ with 700-800 mm year⁻¹. With 100 trees ha⁻¹ in Chile, which is a lower density than in the average Spain forests (Borrero, 2004), we would expect increasing cone production (CP) from 2,355 to 3,183 kg 100 tree⁻¹, and shelled pine nut production (SHP) from 87 to 137 kg 100 ha⁻¹ from north to south, where annual rainfall increases from 383 to 1,047 mm year⁻¹. Such increases represent gains of 35%

in CP and 57% in SHP. In Italy, CP values ranging between 500-1,500 kg ha⁻¹ were reported (Crawford, 1995); in Portugal, CP reached 700-900, and up to 2,000 kg ha⁻¹; in Tunisia, Schröder *et al.* (2014) reported an average productivity of 1,599 kg ha⁻¹, and in Argentina a production of 2,700 kg ha⁻¹ was reported by Ottone (1989). Even though the studied plantations were non-fertilized, our results suggest that the potential fruit productivity in Chile is high under favorable climate conditions, as those in the south. However, further studies focusing on the interaction of climatic influence with soil conditions, fertilization and other agronomic management practices are recommended in order to improve the species intensive cropping.

5. Conclusions

Tree cone production and cone productivity of stone pine showed marked differences along the climatic gradient in Chile, increasing from north to south. The lowest in-shell pine nut number cone⁻¹ was found in the North macrozone, while the lowest cone to shelled pine nut yield in the Dry Coast macrozone. For cone number, the most relevant climatic variables explaining fruit productivity were annual and autumn thermal oscillation, spring hydric index and spring rainfall. Accordingly, minimum average temperature and hydric index affected female flowering, accumulated rainfall affected male flowering, and thermal oscillation influenced differentiation of reproductive shoots. Cone weight at harvest and in-shell pine nut number cone⁻¹ increased with a low annual water deficit.

As practical applications of our results, we determined that a good selection of sites for fruiting of the species in Chile should ensure minimum average temperature above 7°C during female flowering (spring); annual and autumn thermal oscillation lower than 12°C; and a high spring rainfall, except during the male flowering period. Water supply proved to be a significant management practice for individual cone production (over 35% higher than in non-irrigated plantations). Moreover, in light of climate change, we consider that it represents a mitigation management practice to enhance fruiting, with requirements depending upon local water deficit. Further studies are needed to interpret these findings in relation to soil and other agronomic management practices.



Acknowledgements

The study was conducted as part of the R&D project “Development of techniques for producing Stone pine (*Pinus pinea* L.) pine nuts, an attractive commercial option for Chile” (D11I1134), funded by FONDEF (CONICYT), in execution between 2012 and 2016. We acknowledge the support from University of Córdoba-Campus de Excelencia CEIA3 (Spain), and Aldo Salinas and Luis Barrales for their significant contribution in field work activities.

Supplementary Material

Table 1S. Climatic characterization of physio-phenological phases in the North Chilean growth stone pine macrozone

Phase	Average temp (°C)	Maximum average temp (°C)	Minimum average temp (°C)	Accumulated rainfall (mm)	Hydric index (mm)	Thermal oscillation (°C)
P1	18.2 (14.1 ; 20.8)	26.6 (19.4 ; 30.0)	10.6 (6.6 ; 13.3)	11.6 (0 ; 185.5)	-348.0 (-392.4 ; -139.3)	5.0 (2.6 ; 7.4)
P2	9.7 (6.2 ; 14.6)	15.6 (11.5 ; 21.3)	4.7 (0.6 ; 9.1)	260.7 (0.8 ; 1,225.5)	117.8 (-143.6 ; 1,115.4)	6.3 (3.1 ; 8.9)
P3	9.9 (6.6 ; 14.7)	16.3 (12.5 ; 22.5)	4.5 (0.2 ; 8.1)	146.7 (37.4 ; 545.5)	-6.4 (-184.5 ; 440.1)	5.9 (3.3 ; 7.6)
P4	14.0 (11.2 ; 18.1)	21.7 (16.7 ; 27.2)	7.1 (3.8 ; 10.4)	47.4 (0 ; 181.0)	-205.5 (-298.7 ; -33.1)	5.6 (2.8 ; 7.9)
P5	15.8 (12.5 ; 19.4)	23.8 (17.7 ; 27.8)	8.5 (5.3 ; 11.9)	20.2 (0 ; 85.8)	-293.6 (-370.9 ; -199.2)	5.2 (3.2 ; 8.8)
P6	17.9 (13.9 ; 21.0)	26.13 (18.5 ; 31.3)	10.4 (7.9 ; 13.7)	2.7 (0 ; 24.0)	-351.7 (-414.4 ; -233.5)	5.1 (2.5 ; 7.4)
P7	16.3 (13.0 ; 18.2)	24.6 (18.2 ; 28.1)	9.1 (6.3 ; 12.8)	7.4 (0 ; 44.6)	-604.2 (-724.7 ; -407.4)	7.6 (3.9 ; 12.7)
P8	17.0 (13.2 ; 20.0)	24.7 (18.5 ; 29.4)	9.8 (5.7 ; 12.2)	52.4 (0 ; 223.7)	-716.6 (-952.5 ; -448.9)	8.2 (3.9 ; 10.9)
P9	16.3 (12.6 ; 19.5)	24.4 (18.3 ; 28.6)	9.2 (4.8 ; 12.9)	12.65 (0 ; 49.0)	-300.6 (-379.2 ; -209.7)	5.5 (3.1 ; 10.1)
P10	18.1 (14.3 ; 22.2)	26.8 (19.5 ; 32.2)	10.7 (7.2 ; 13.8)	21.2 (0 ; 130.2)	-755.7 (-988.0 ; -478.9)	6.2 (3.2 ; 10.1)
P11	13.4 (9.7 ; 18.3)	21.4 (17.0 ; 25.9)	7.0 (3.8 ; 11.2)	87.5 (9.5 ; 313.0)	-155.6 (-290.2 ; 94.5)	6.9 (3.9 ; 9.2)

In parentheses minimum and maximum values

**Table 2S.** Climatic characterization of physio-phenological phases in the Dry Coast Chilean growth stone pine macrozone

Phase	Average temp (°C)	Maximum average temp (°C)	Minimum average temp (°C)	Accumulated rainfall (mm)	Hydric index (mm)	Thermal oscillation (°C)
P1	18.0 (13.2 ; 20.9)	25.7 (19.3 ; 30.0)	10.8 (8.0 ; 13.3)	36.1 (4.0 ; 66.1)	-311.0 (-372.0 ; -217.0)	4.9 (2.0 ; 6.1)
P2	8.5 (6.8 ; 11.1)	14.2 (13.0 ; 15.5)	3.9 (1.4 ; 7.1)	500.0 (230.9 ; 729.6)	375.2 (107.4 ; 593.1)	6.5 (3.1 ; 8.9)
P3	9.4 (8.0 ; 11.4)	15.3 (13.2 ; 17.3)	4.2 (2.3 ; 6.3)	223.5 (50.6 ; 354.0)	90.7 (-79.2 ; 225.1)	5.6 (2.9 ; 7.5)
P4	13.8 (10.5 ; 17.1)	21.0 (15.2 ; 25.4)	7.0 (5.1 ; 9.4)	55.0 (1.2 ; 118.8)	-190.0 (-282.4 ; -96.0)	4.9 (1.9 ; 6.2)
P5	16.0 (12.1 ; 20.2)	23.6 (17.4 ; 28.7)	8.8 (6.7 ; 12.0)	22.6 (0.3 ; 73.5)	-287.0 (-373.6 ; -153.7)	5.0 (2.4 ; 5.7)
P6	17.8 (13.6 ; 21.6)	25.7 (19.0 ; 31.3)	10.5 (8.2 ; 13.5)	22.3 (0.3 ; 66.1)	-327.2 (-420.2 ; -217.0)	5.2 (1.8 ; 6.4)
P7	15.8 (12.5 ; 19.3)	24.0 (18.0 ; 28.0)	8.6 (6.5 ; 11.2)	31.7 (2.4 ; 88.6)	-581.1 (-727.7 ; -383.8)	6.6 (3.8 ; 8.9)
P8	16.7 (12.4 ; 20.3)	24.4 (18.4 ; 30.1)	9.5 (7.3 ; 13.9)	51.8 (2.0 ; 131.7)	-722.4 (-955.6 ; -489.9)	6.7 (2.9 ; 9.4)
P9	16.3 (11.6 ; 19.2)	24.1 (17.6 ; 28.6)	9.0 (6.0 ; 11.8)	23.0 (0.0 ; 87.0)	-290.5 (-380.7 ; -191.4)	4.8 (1.7 ; 5.5)
P10	17.6 (13.7 ; 21.2)	25.9 (20.0 ; 32.2)	9.8 (7.4 ; 12.8)	26.1 (0.0 ; 72.5)	-749.3 (-996.8 ; -526.8)	7.2 (5.0 ; 9.9)
P11	12.3 (10.4 ; 15.1)	19.8 (15.2 ; 22.7)	6.0 (4.0 ; 8.1)	139.8 (82.5 ; 219.5)	-83.5 (-171.3 ; 31.6)	6.8 (3.5 ; 9.7)

In parentheses minimum and maximum values

Table 3S. Climatic characterization of physio-phenological phases in the South Chilean growth stone pine macrozone

Phase	Average temp (°C)	Maximum average temp (°C)	Minimum average temp (°C)	Accumulated rainfall (mm)	Hydric index (mm)	Thermal oscillation (°C)
P1	16.6 (13.2 ; 20.9)	23.6 (18.4 ; 29.3)	10.1 (7.0 ; 13.3)	77.4 (24.5 ; 212.1)	-239.4 (-339.6 ; -83.7)	5.4 (2.8 ; 7.4)
P2	8.5 (6.2 ; 10.6)	12.8 (10.9 ; 14.4)	4.7 (1.1 ; 7.6)	729.5 (216.2 ; 1.780.5)	625.0 (102.6 ; 1691.3)	7.4 (4.5 ; 10.5)
P3	8.7 (6.7 ; 10.7)	13.5 (11.8 ; 16.3)	4.5 (1.5 ; 6.7)	395.6 (102.9 ; 898.4)	281.4 (-5.9 ; 771.2)	6.7 (4.0 ; 8.6)
P4	12.5 (10.0 ; 16.7)	18.9 (14.7 ; - 23.8)	6.8 (4.2 ; 10.3)	92.6 (11.2 ; 272.6)	-123.0 (-209.1 ; 82.4)	5.7 (3.6 ; 8.3)
P5	14.5 (11.4 ; 19.4)	21.1 (16.4 ; 27.0)	8.3 (5.8 ; 12.0)	74.4 (18.0 ; 203.1)	-198.4 (-316.3 ; -42.3)	5.7 (3.9 ; 10.2)
P6	16.6 (13.5 ; 19.9)	23.6 (18.6 ; 28.0)	10.1 (7.2 ; 12.5)	75.4 (17.8 ; 193.3)	-242.4 (-370.1 ; -58.5)	6.0 (4.2 ; 8.7)
P7	15.1 (12.0 ; 19.3)	22.1 (17.7 ; 27.7)	8.4 (6.1 ; 11.6)	95.4 (15.5 ; 216.8)	-460.4 (-652.9 ; -231.3)	7.0 (4.3 ; 10.2)
P8	15.6 (13.2 ; 21.7)	22.8 (18.0 ; 30.3)	9.2 (6.4 ; 14.7)	155.4 (21.0 ; 323.8)	-560.4 (-844.5 ; -286.1)	8.1 (4.6 ; 13.1)
P9	14.9 (11.6 ; 19.2)	21.9 (15.8 ; 27.2)	8.6 (5.4 ; 11.8)	71.9 (8.0 ; 189.9)	-209.7 (-344.5 ; -113.9)	5.8 (3.5 ; 9.5)
P10	17.0 (13.6 ; 20.0)	24.9 (18.8 ; 28.7)	10.2 (6.7 ; 13.4)	101.4 (6.0 ; 247.7)	-626.6 (-800.7 ; -287.4)	8.3 (4.2 ; 13.4)
P11	12.3 (10.3 ; 14.3)	18.8 (16.2 ; 21.7)	7.1 (4.2 ; 10.2)	286.3 (51.2 ; 661.5)	89.0 (-165.9 ; 464.9)	8.3 (5.3 ; 11.0)

In parentheses minimum and maximum values

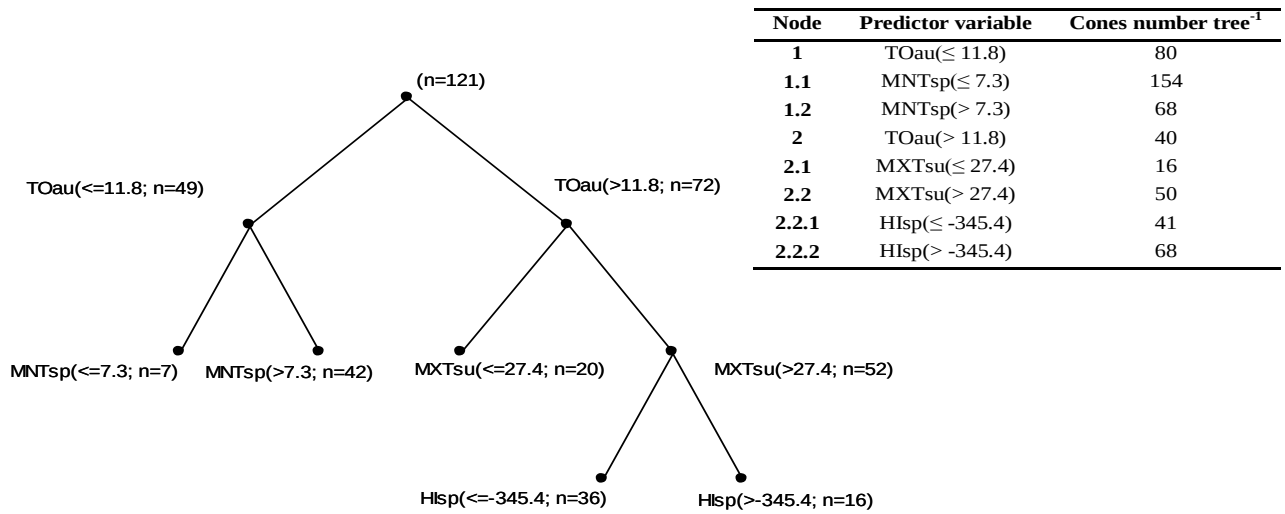


Figure 1aS. Climatic variables that best explain cone tree⁻¹ across stone pine plantations in Chile. Nodes represent variables identified as relevant to explain productivity. Total CN data were first split into two subsets based on the predictor variable (TOau) and its threshold (11.8°C). Each subset, or node, was then analyzed independently using the same procedure. Variables making top nodes are the most important to explain CN. Cone number at each node is reported in the embedded table.

TO_{au}: autumn thermal oscillation, *MNT_{sp}*: spring minimum average temperature, *MXT_{su}*: summer maximum average temperature, *HI_{sp}*: spring hydric index.

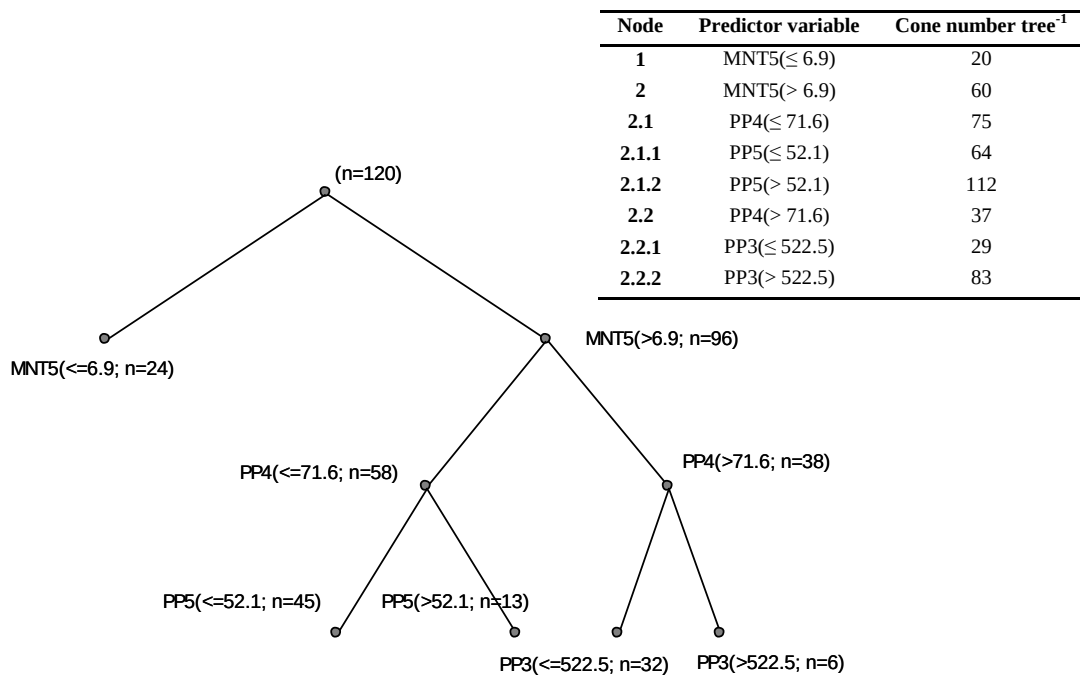
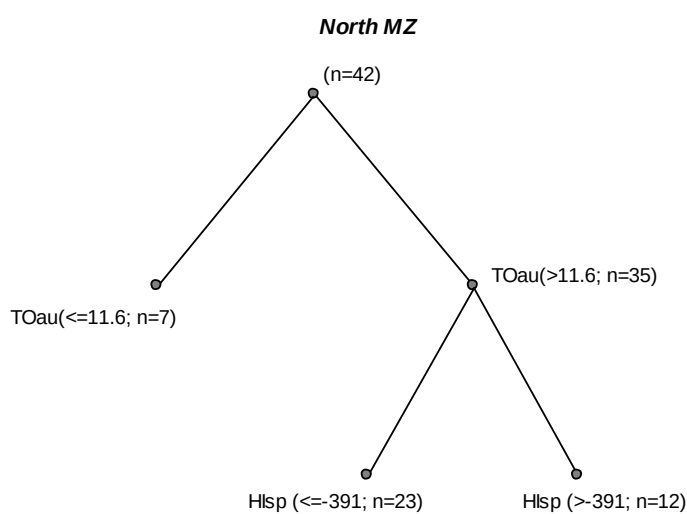
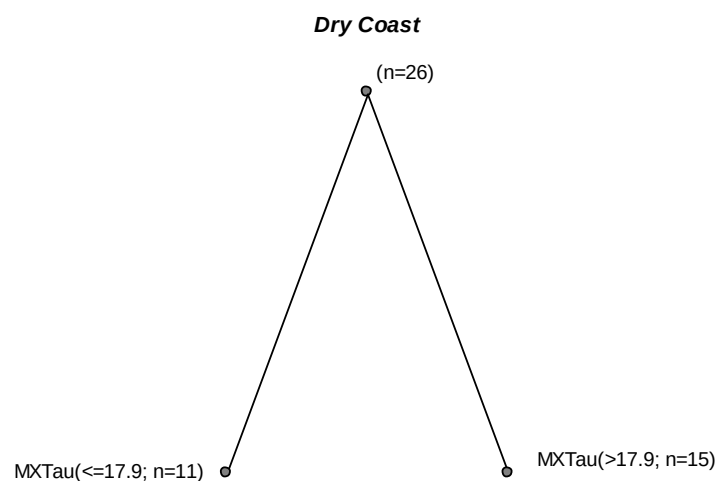


Figure 1bS. Climatic variables that best explain cone tree⁻¹ across stone pine plantations in Chile by physiophenological phases. Nodes represent variables identified as relevant to explain productivity. Total CN data were first split into two subsets based on the predictor variable (MNT5) and its threshold (6.9°C). Each subset, or node, was then analyzed independently using the same procedure. Variables making top nodes are the most important to explain CN.

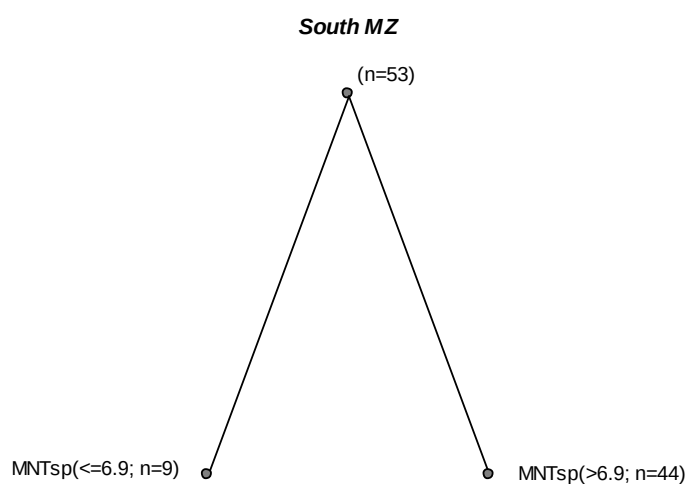
MNT5: minimum average temperature in P5, *PP3*: rainfall in P3, *PP4*: rainfall in P4, *PP5*: rainfall in P5.



Node	Predictor variable	Cone number tree ⁻¹
1	TOau (≤11.6)	105
2	TOau (>11.6)	38
2.1	Hlsp (≤-391)	49
2.2	Hlsp (>-391)	18



Node	Predictor variable	Cone number tree ⁻¹
1	MXTau (≤17.9)	105
2	MXTau (>17.9)	26



Node	Predictor variable	Cone number tree ⁻¹
1	MNTsp (≤6.9)	116
2	MNTsp (>6.9)	50

Figure 2aS. Climatic variables that best explain cone tree⁻¹ by stone pine macrozones in Chile. Nodes represent variables identified as relevant to explain productivity. Cone number at each node is reported in the embedded table.

TOau: autumn thermal oscillation, *MNTsp*: spring minimum average temperature, *MXTau*: autumn maximum average temperature, *Hlsp*: spring hydric index.

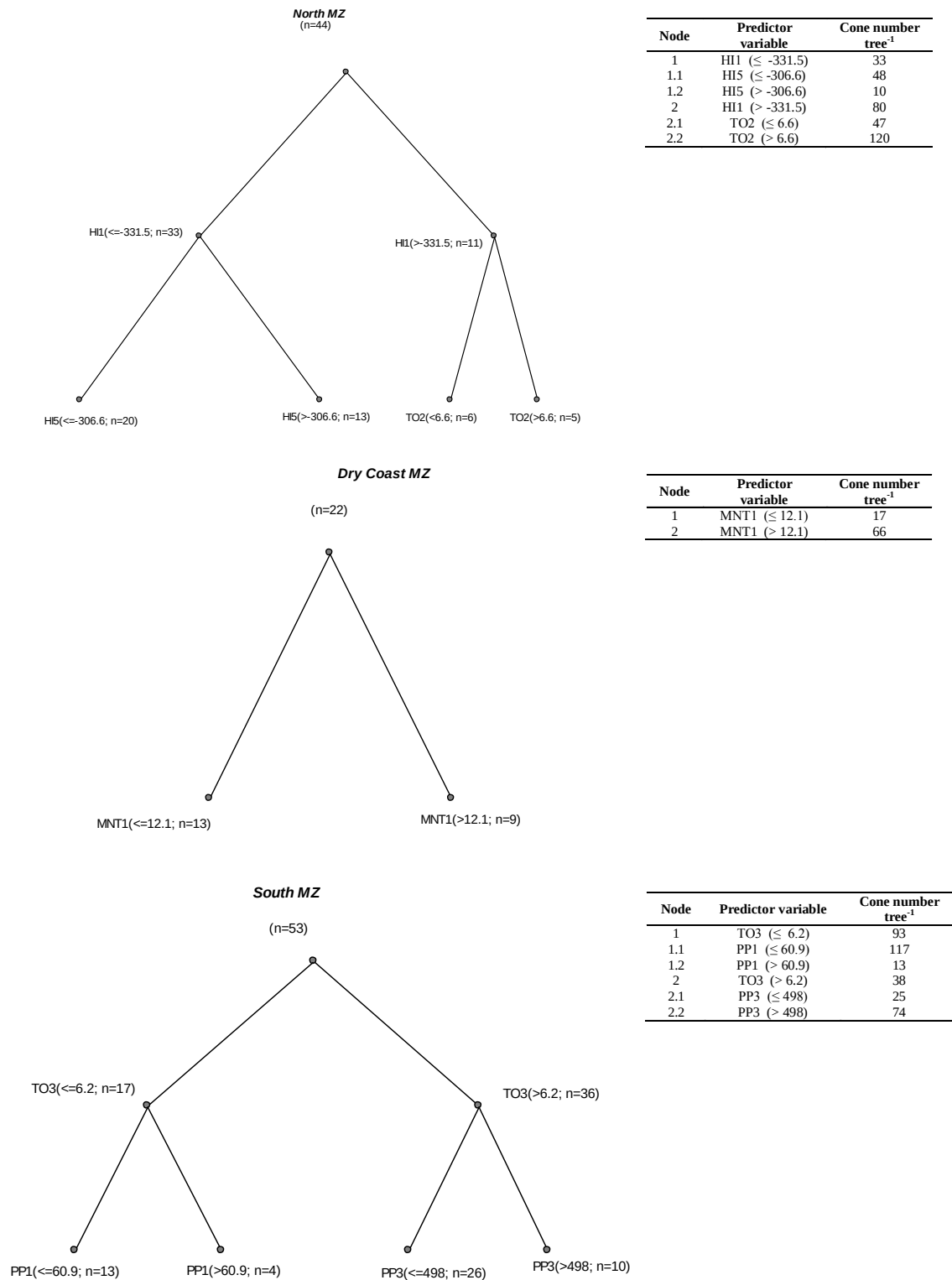


Figure 2bS. Climatic variables that best explain cone tree⁻¹ across Chilean stone pine macrozones according to physio-phenological phases. Nodes represent variables identified as relevant to explain CN. Cone number at each node is reported in the embedded table.

HI1: hydric index in P1, *HI5*: hydric index in P5, *TO2*: thermal oscillation in P2, *TO3*: thermal oscillation in P3, *MNT1*: minimum average temperature in P1, *PP1*: rainfall in P1, *PP3*: rainfall in P3.

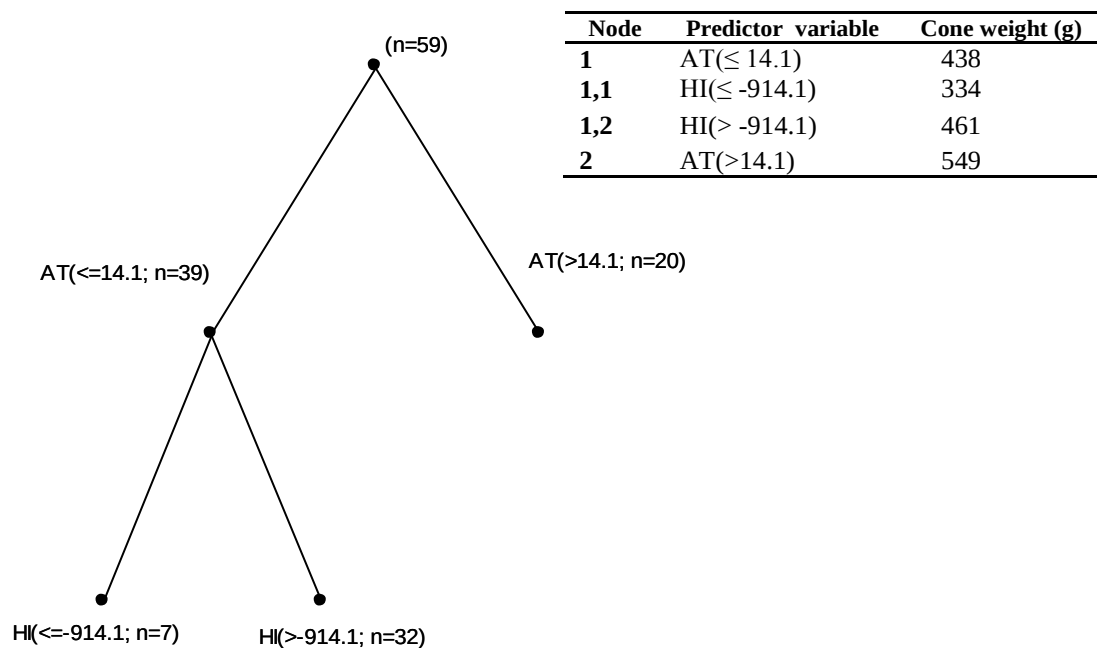


Figure 3aS. Climatic variables that best explain cone weight across stone pine plantations in Chile. Nodes represent variables identified as relevant to explain CW. Data were first split into two subsets based on the predictor variable (AT) and its threshold (14.1°C). Each subset, or node, was then analyzed independently using the same procedure. Variables making top nodes are the most important to explain CW. Cone weight at each node is reported in the embedded table.

AT: annual average temperature, HI: annual hydric index.

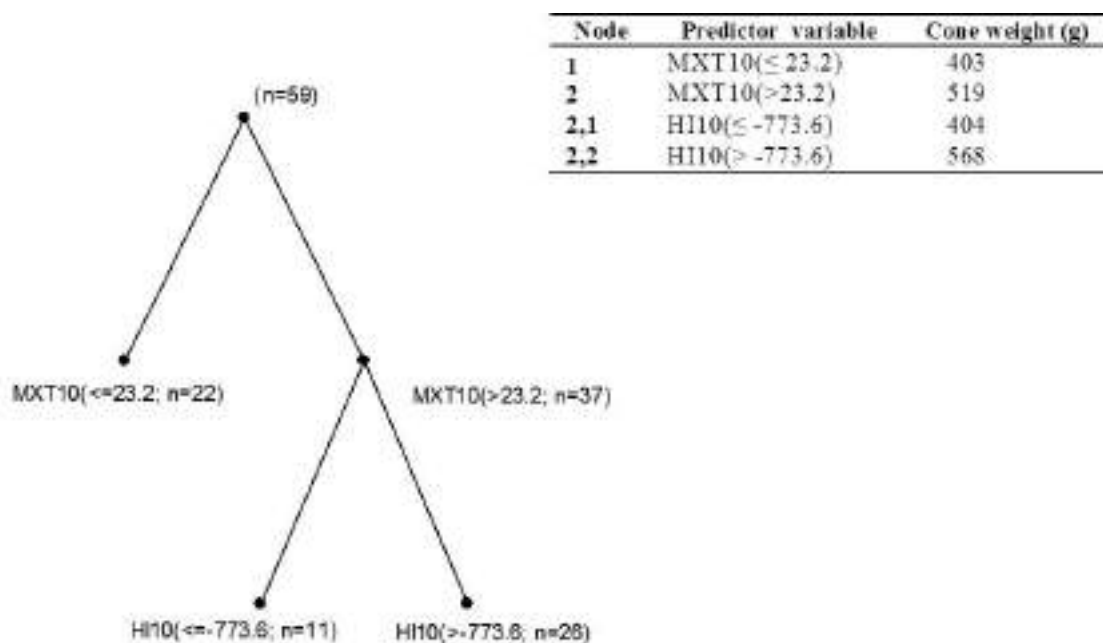
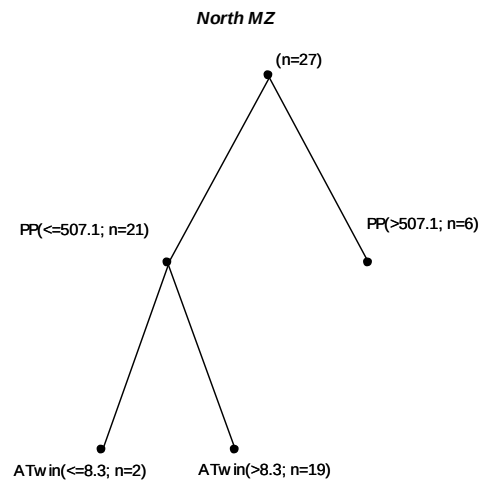
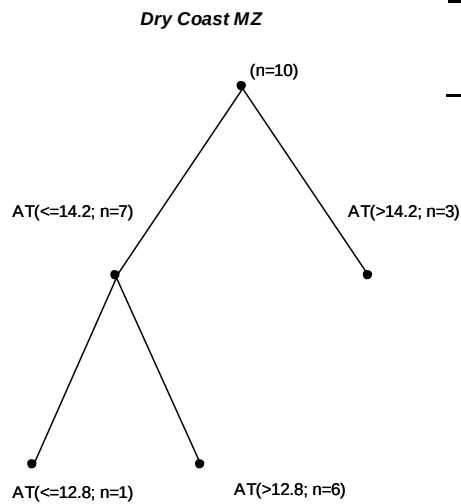


Figure 3bS. Climatic variables that best explain cone weight across stone pine plantations in Chile by physiophenological phases. Nodes represent variables identified as relevant to explain cone weight. Data were first split into two subsets based on the predictor variable (MXT10) and its threshold (23.2°C). Each subset, or node, was then analyzed independently using the same procedure. Variables making top nodes are the most important to explain CW.

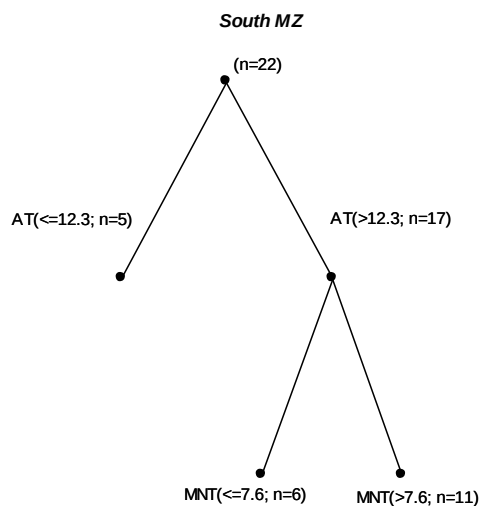
MXT10: maximum average temperature in P10, HI10: hydric index in P10.



Node	Predictor variable	Cone weight (g)
1	PP (≤ 507.1)	418
1,1	ATwin (≤ 8.3)	631
1,2	ATwin (> 8.3)	396
2	PP (> 507.1)	660



Node	Predictor variable	Cone weight (g)
1	AT (≤ 14.2)	433
1,1	AT (≤ 12.8)	480
1,2	AT (> 12.8)	425
2	AT (> 14.2)	585



Node	Predictor variable	Cone weight (g)
1	AT (≤ 12.3)	368
2	AT (> 12.3)	512
2,1	MNT (≤ 7.6)	586
2,2	MNT (> 7.6)	472

Figure 4aS. Climatic variables that best explain cone weight by stone pine macrozones in Chile. Nodes represent variables identified as relevant to explain CW. Cone weight at each node is reported in the embedded table.

AT: annual average temperature, ATwin: winter average temperature, PP: annual rainfall, MNT: minimum average temperature.

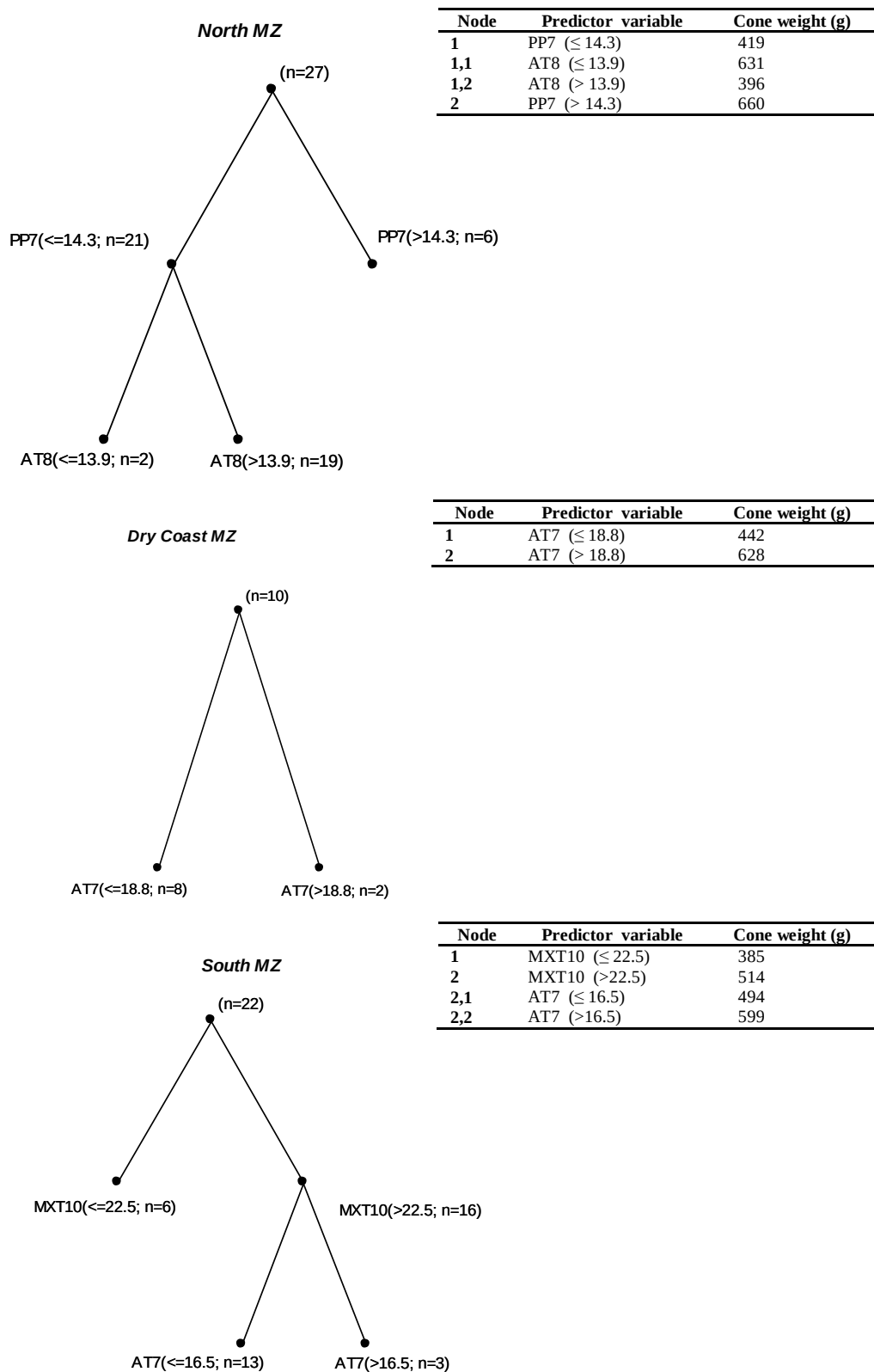


Figure 4bS. Climatic variables that best explain cone weight across Chilean stone pine macrozones according to physio-phenological phases. Nodes represent variables identified as relevant to explain cone weight. Cone weight at each node is reported in the embedded table.

PP7: accumulated rainfall in P7, *AT7*: average temperature in P7, *AT8*: average temperature in P8, *MXT10*: maximum average temperature in P10.

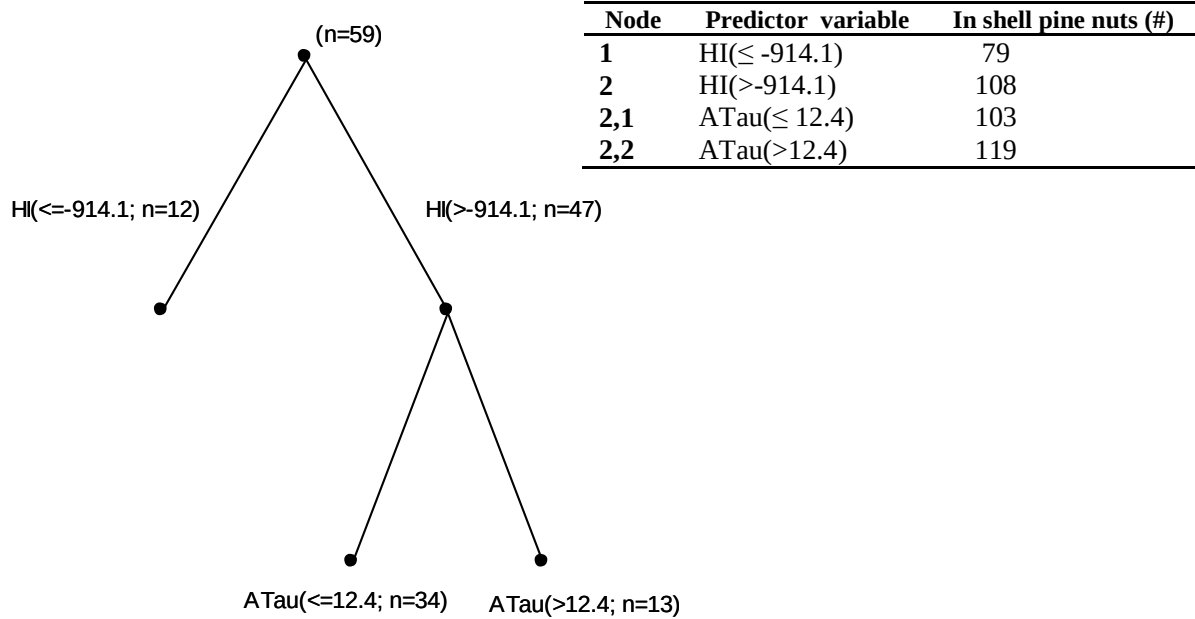


Figure 5aS. Climatic variables that best explain in-shell pine nut number cone⁻¹ across stone pine plantations in Chile. Nodes represent variables identified as relevant to explain IS. Data were first split into two subsets based on the predictor variable (HI) and its threshold (914.1 mm). Each subset, or node, was then analyzed independently using the same procedure. Variables making top nodes are the most important to explain IS. In-shell pine nut number at each node is reported in the embedded table.

HI: annual hydric index, AT_{au}: autumn average temperature.

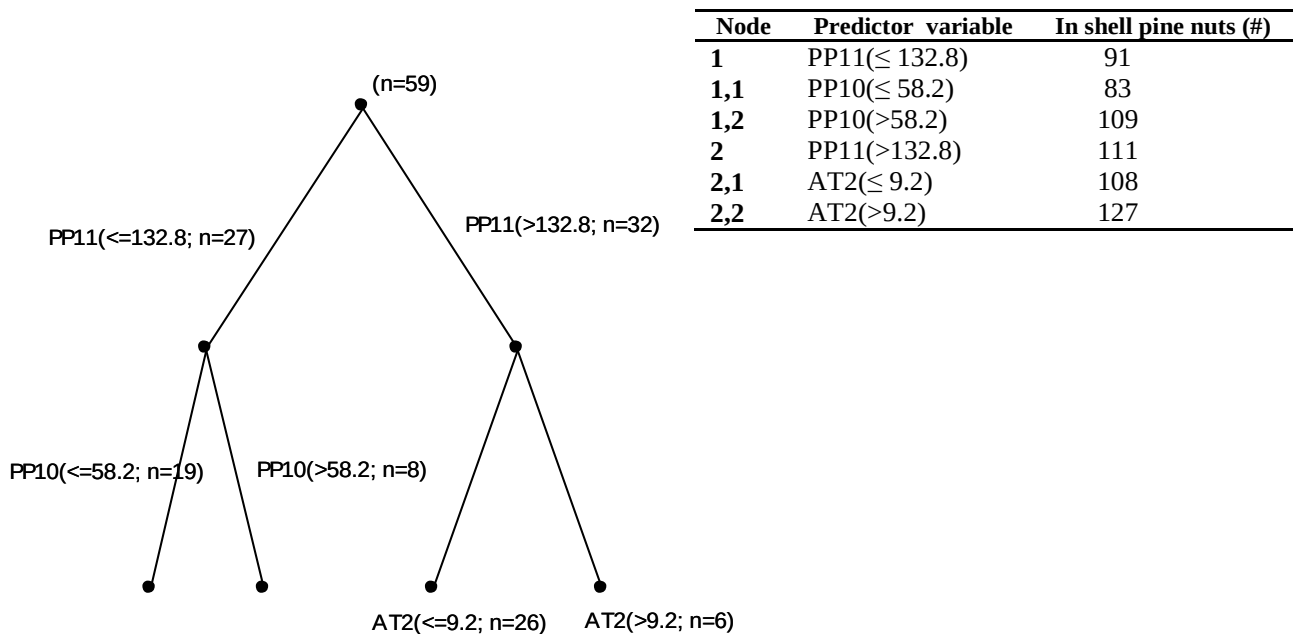


Figure 5bS. Climatic variables that best explain in-shell pine nut number cone⁻¹ across stone pine plantations in Chile by physio-phenological phases. Nodes represent variables identified as relevant to explain IS. Data were first split into two subsets based on the predictor variable (PP11) and its threshold (132.8 mm). Each subset, or node, was then analyzed independently using the same procedure. Variables making top nodes are the most important to explain IS.

PP10: rainfall in P10, PP11: rainfall in P11, AT2: average temperature in P2.

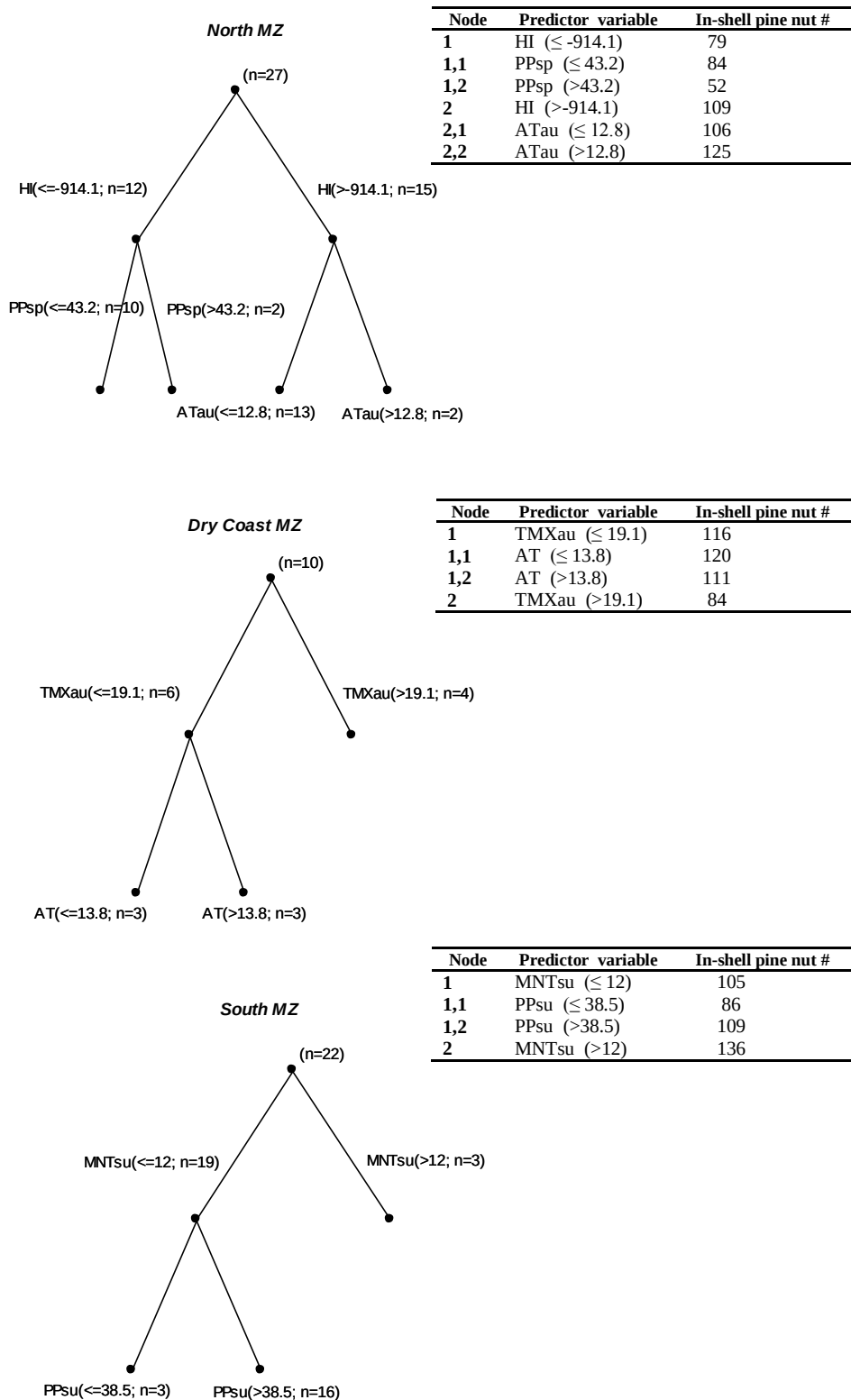


Figure 6aS. Climatic variables that best explain in-shell pine nut number cone⁻¹ across Chilean stone pine macrozones. Nodes represent variables identified as relevant to explain IS. In-shell pine nut number at each node is reported in the embedded table.

HI: annual hydric index; PPsp: rainfall in spring; ATau: autumn average temperature; TMXau: autumn maximum average temperature; AT: annual average temperature; MINTsu: summer minimum average temperature; PPsu: summer rainfall.

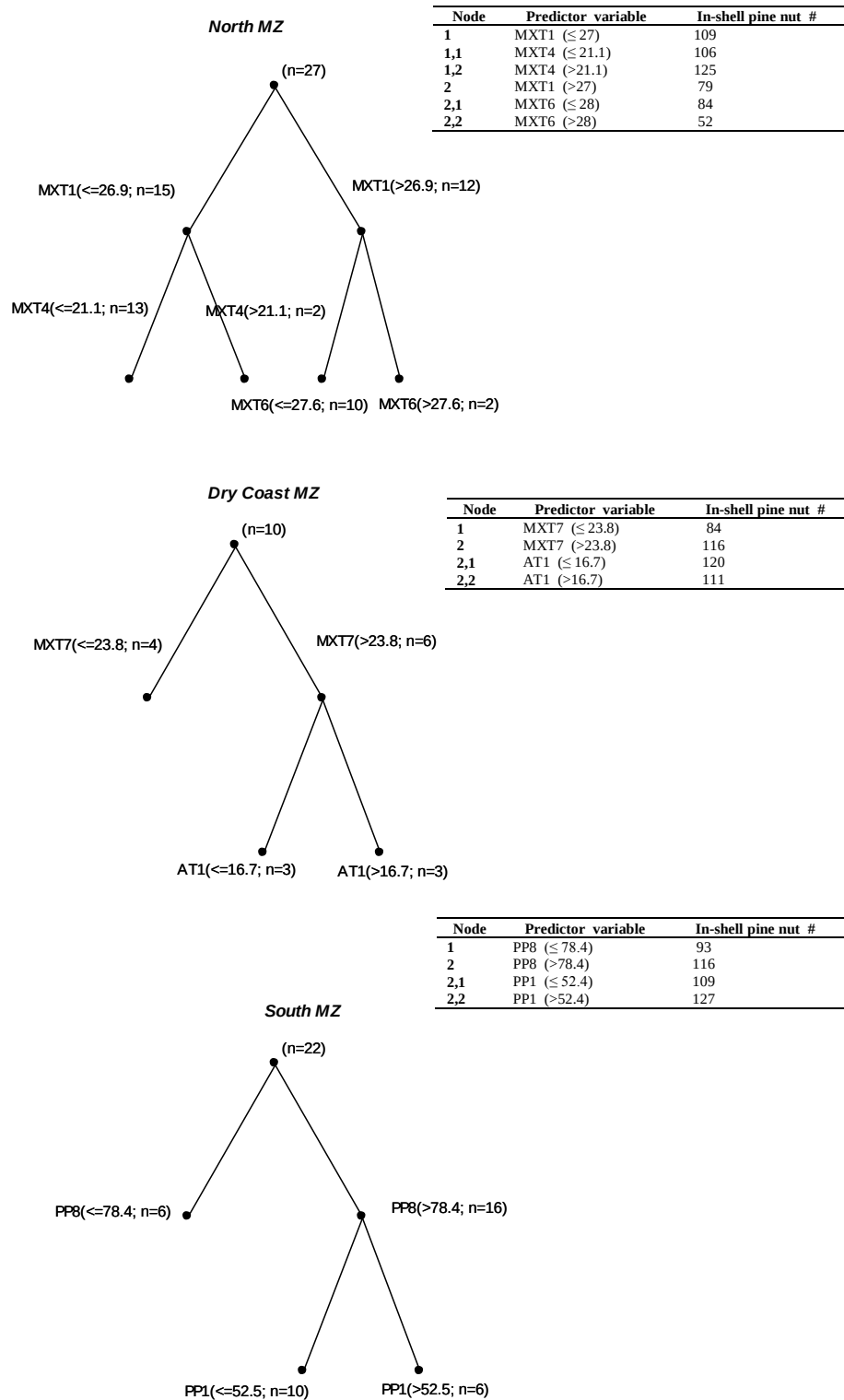


Figure 6bS. Climatic variables that best explain in-shell pine nut number cone⁻¹ across Chilean stone pine macrozones according to physio-phenological phases. Nodes represent variables identified as relevant to explain IS. In-shell pine nut number at each node is reported in the embedded table.

MXT1: maximum average temperature in P1; MXT4: maximum average temperature in P4; MXT6: maximum average temperature in P6; MXT7: maximum average temperature in P7; PP1: rainfall in P1; PP8: rainfall in P8; AT1: average temperature in P1.



Capítulo 4.- Variabilidad morfométrica, química y por espectroscopía del infrarrojo cercano (NIRS) de piñones de pino piñonero (*Pinus pinea* L.) en Chile

Chapter 4.- Morphometric, chemical and near infrared spectroscopy (NIRS) variability of Stone pine (*Pinus pinea* L.) nuts in Chile

Semillas y piña de *Pinus pinea* cosechados en Chile

Seeds and cone of *Pinus pinea* harvested in Chile

Based on the following articles:

Loewe MV, Navarro-Cerrillo R, García-Olmo J, Riccioli C, Sánchez de la Cuesta R. 2016. Discriminant analysis of Mediterranean pine nuts (*Pinus pinea* L.) from Chilean plantations by Near Infrared Spectroscopy. Food Control (in evaluation).

Loewe MV, Alvarez K., Lutz M., Navarro-Cerrillo R, 2016. Pine nut morphometry and production in selected Stone pine (*Pinus pinea* L.) trees among Chilean macrozones. Southern Forests: a Journal of Forest Science (in evaluation).

Lutz M., Alvarez K., Loewe V. 2016. Chemical composition of pine nut (*Pinus pinea* L.) grown in three geographical macrozones in Chile. Journal of Food CYTA (in evaluation).

Zuleta A., Weisstaub A., Giacomino S., Dyner L., Loewe MV., Delard C., Lutz M. 2016. An ancient crop revisited: chemical composition of Mediterranean pine nuts (*Pinus pinea* L.) grown in Chile, Argentina, Italy, Spain, Turkey and Israel. Journal of the Science of Food and Agriculture (in evaluation).



Abstract

Stone pine (*Pinus pinea* L.) is characterized by presenting phenotypic plasticity, tolerance to different environments and low genetic variability. To study the species variability in Chile, we performed a pine nut morphological, chemical and Near Infrared Spectroscopy (NIRS) characterization across three growth macrozones (MZs). For **selected stone pine trees**, we found differences in cone size and seed morphometry, and cone to kernel yield among macrozones; their composition was similar to values reported for the main producer countries and homogeneous among MZs, except for oleic acid and potassium, which were higher in the South and Dry Coast MZs, respectively. The analysis of chemical composition **in each MZs** was affected by environments: the South MZ, the coolest and most humid, showed the lowest mineral and protein contents; the North MZ, with the most adverse climate, had the lowest moisture and lipid contents, and the highest mineral contents; the Dry Coast MZ showed the highest contents of moisture, protein and lipids, suggesting that some stress would favor protein content. The most abundant fatty acids were polyunsaturated and monounsaturated; the most abundant phytosterol and tocopherol were β -sitosterol and γ -tocopherol, respectively. The comparison among **pine nuts grown in six countries** showed that the Italian ones had the highest TDF and moisture contents, those from Chile and Argentina had a similar composition, and the Turkish and Israeli ones had a similar composition, which was high in proteins. Significant differences were found among countries for protein, TDF, minerals and moisture, probably due to environmental conditions. The most relevant climate variables were thermal oscillation for moisture, dry months for minerals, and minimum and maximum average temperature for TDF. Stand-alone **NIRS measurements** were employed for identifying pine nut geographical origins by testing different sample presentations (in-shell and shelled pine nuts, and humid and dried flour). For the three previously identified macrozones, 87.8% of samples were correctly classified at the cross validation stage, with the best model obtained for shelled intact pine nuts spectra analysis. The results demonstrate the potential of this technique as a rapid and accurate method for predicting the geographical origin of Mediterranean pine nuts.

Keywords: absorption bands, chemical composition, morphometry, NIRS, geographical macrozones, pine nuts, traceability, yield.

1. Introduction

Stone pine (*Pinus pinea* L.) is one of the nine most important nut species in the world, and its pine nuts are regarded as a delicacy in many cultures (Geisler, 2008). The species is endemic to the Mediterranean Basin, and is mainly cultivated in Spain, Portugal, Italy, Turkey and Tunisia, where pine nuts have been part of the Mediterranean diet for millennia.

Unlike other nut-bearing trees, stone pine has not been domesticated, nor have varieties or cultivars been defined or used productively, with efforts to domesticate and manage the species being recent and limited (Mutke *et al.*, 2007c). The reasons that partially explain its lack of domestication include the difficulty of clonal reproduction through grafting, the long reproductive cycle (3.5 years), a late reproductive age (15-20 years in its area of origin), and wind pollination, which poses an additional challenge to controlled cross pollination. In addition, the location of cones in pre-formed sub-apical buds of the upper part of the tree crown limits crown management through pruning to improve seed production. Additionally, producing countries consider pine nuts as a non-wood forest product because *P. pinea* is a native species, a fact that has hindered its consideration as a nut tree and has limited the domestication and development of cultural techniques to obtain a high and constant nut production. Moreover, the species presents alternate bearing production between years, which is synchronized between individuals and even between regions (Mutke *et al.*, 2007a); this lack of fixed cycles does not allow harvest planning (Gordo, 2004). In Spain, Yagüe (1993) detected production cycles of five years, with low production and small-sized cones. Pine nut production in that country ranges between 40-120 kg ha⁻¹, which is equivalent to 10-30 kg of shelled pine nuts ha⁻¹ (Mutke *et al.*, 2007b).

Nuts are known for their contribution to a reduction of cardiovascular risk factors (Sabaté and Wien, 2010; Rees *et al.*, 2014; Ros, 2015), with unsaturated fatty acids, phytosterols, tocopherols and squalene being included as cardio-protective constituents (Wolff *et al.*, 1995; Maguire *et al.*, 2004). Tree nuts are also recognized as healthy foods because of their effect in reducing the risk of type-2 diabetes and some types of cancer (Feldman, 2002; Sabaté and Ang, 2009; Bolling *et al.*, 2010; Estruch *et al.*, 2013; Alasalvar and Bolling, 2015), and their tocopherol content confer nuts with antioxidant capacity (Espín *et al.*, 2000). Although nuts are high-fat and energy-dense foods, they have been recognized for their healthy fatty acid profile; additionally, nut consumption has been associated with reduced body mass index (BMI), being therefore advocated in weight-maintaining diets (Bes-



Rastrollo *et al.*, 2009; Kendall *et al.*, 2010; Mattes and Dreher, 2010; Flores-Mateo *et al.*, 2013; Ibarrola-Jurado *et al.*, 2013).

An interesting study conducted in the US (O'Neil *et al.*, 2010) concluded that even with a reduced daily dose of tree nuts (7 g), nutrient intake and diet quality was improved significantly, and suggested encouraging its consumption. Accordingly, health scientific societies and legislation agencies such as the US Food and Drug Administration (FDA, 2003) and the Canadian Cardiovascular Society (Anderson *et al.*, 2013) have recommended the regular consumption of nuts to prevent the risk of cardiovascular diseases.

In particular, pine nuts are a good sources of fats (48%), being rich in unsaturated fatty acids, protein (34%), vitamins (E, B6, niacin, folic acid), minerals (potassium and phosphorus are recognized as the most abundant, followed by magnesium, and zinc, iron and copper to a lesser degree) and various phytochemical components that include phytosterols and phenolic compounds (Nergiz and Dönmez, 2004; Bolling *et al.*, 2011; Evaristo *et al.*, 2013); this composition has interesting health benefits. In fact, there is growing evidence of the association of pine nut consumption with a wide range of health benefits (Sabaté and Ang, 2009; Jenkins *et al.*, 2011; Estruch *et al.*, 2013; Rees *et al.*, 2014). Bao *et al.* (2013) observed an inverse association of consumption with the risk of major death causes (cancer, heart disease and respiratory disease), as well as with a reduction in the risk of coronary heart disease and non-fatal myocardial infarction (Nergiz and Dönmez, 2004).

Given the high nutritional value and exquisite flavor of Mediterranean pine nuts, its demand is increasing worldwide, with the consequent increase in market price (Fady *et al.*, 2004; Barranco and Ortuño, 2004; International Nut and Dried Fruits, 2012). In turn, its offer has been seriously affected by the insect *Leptoglossus occidentalis* Heidemann (Sousa *et al.*, 2012), which has spread in most of stone pine forests in all major producing countries. As a consequence, efforts have been increasingly focused on morphometric studies because of the increasing presence of empty and damaged pine nuts associated with the insect (Mutke *et al.*, 2012, 2015), which has high economic impact on global markets (Ruitenbergh, 2013). This is so dramatic that expensive techniques have been tested to assess cone and pine nut quality, such as tomography, commonly used in clinical medicine (Nunes *et al.*, 2016).

In the last years, an alert has been issued in the international pine nut market due to the Pine Mouth Syndrome, or dysgeusia, a long-lasting taste alteration with an unpleasant bitter, metallic taste disturbance originated by the consumption of pine nuts from other pine species,

especially *P. koraiensis* Siebold & Zucc.; this phenomenon occurs because its pine nuts are often commercialized along with seeds of other pine species (Mutke *et al.*, 2013), with some of them even being non-edible, such as *P. armandii* Franch. and *P. massoniana* Lamb. (Destailats *et al.*, 2010; EC, 2011; Handy *et al.*, 2013). According to Zonneveld (2011), Ballin (2012) and Ballin and Mikkelsen (2016), this bitter aftertaste is definitely caused by *P. armandii*. To address this situation, Chinese authorities issued working instructions for pine nut inspections (STS, 2011) that exclude those species, which does not seem to be easily enforced. Some stone pine nut allergy cases have also been reported (García-Menaya *et al.*, 2000; Sathe *et al.*, 2005; Cabanillas *et al.*, 2012), and a series of nut allergens have been described, including seed storage proteins, such as vicilins, legumins and albumins (Cabanillas and Novak, 2015).

An important factor for conservation and breeding programs is that the species has been reported to have low genetic variability (Vendramin *et al.*, 2008; Sánchez-Gómez *et al.*, 2009), with limited genetic variability having been detected between species provenances (Fallour *et al.*, 1997; Gómez *et al.*, 2002; Evaristo *et al.*, 2002; Gordo *et al.*, 2007). By contrast, using mega gametophyte storage proteins as markers, Álvarez *et al.* (2004) reported a high degree of variation in populations of Andalucía, even higher than in maritime pine (*P. pinaster* Ait.) from Iberian peninsula populations, suggesting that this particular marker could be more appropriate for analyzing the genetic diversity of the species.

Qualitative analysis by NIRS chemometric techniques has been successfully used to distinguish the origin of agricultural and forest products with common chemical characteristics (Shenk and Westerhaus, 1995b; Williams and Norris, 2001; Roberts *et al.*, 2007; Shenk *et al.*, 2010), and is gaining strong acceptance in food science and technology, with many different applications. In the forest sector, the first results concerning NIRS spectra and wood chemical properties were presented 20 years ago, showing the suitability of this technique for the quantitative assessment of the physical, mechanical, and chemical properties of wood, i.e. longitudinal modulus of elasticity, lignin and cellulose content, wood density – even in *P. pinea* –, showing significant variation between trees (Fernandes *et al.*, 2013), moisture, wood age (Schimleck *et al.*, 2000; Via *et al.*, 2008), pulp yield (Labbé *et al.*, 2013) and other wood parameters (Tsuchikawa, 2007). This technique has also been applied to identify wood species of different genera, wood samples from species of the same genus, different origins of the same species (Brunner *et al.*, 1996; Tsuchikawa *et al.*, 2003), and



hybrids and purebreds in pine species with higher accuracy than that derived when using standard molecular techniques (Luan *et al.*, 2013), as well as to discriminate geographical origin of cork planks and stoppers (Prades *et al.*, 2012, 2013a) and to assess their quality (Prades *et al.*, 2013b).

In agriculture, NIRS has been used to discriminate fruits of different productive areas, breeds or genotypes, including protected geographical indication (Lin and Yin, 2009), and even between transgenic and non-transgenic wheats (García-Molina *et al.*, 2016). It has also been applied to predict the content of some minerals in leaves of different species, including *P. pinea* (Petisco *et al.*, 2005, 2006), and to assess seed vigor in *P. patula* Schiede ex Schltdl. & Cham. (Tigabu and Oden, 2003, 2004), as well as seed moisture content (Torbjörn and Geladi, 2003), seed water interactions (Torbjörn, 2003) and seed sources (Tigabu *et al.*, 2005) in *P. sylvestris* L. Moreover, NIRS has been used to determine leaf moisture content of *Quercus ilex* subsp. *ballota* [Desf.] Samp. (García *et al.*, 2013), and the species acorn chemical quality, with significant differences in fruit chemical composition between different populations being found (Valero *et al.*, 2012). Regarding Mediterranean pine nuts, to the best of our knowledge, qualitative analysis by NIRS has not been reported.

Pine nut characterization is important due to health, commercial, genetic and sanitary concerns. However, only few studies have been conducted on morphology, cone to kernel yield, and chemical composition variation of pine nuts in Chile, where there are suitable conditions for the species plantation and development (Loewe *et al.*, 2016a). Accordingly, the aim of this study was to assess the morphological, chemical and NIRS variability of stone pine nuts across the three macrozones described in Chile by Loewe *et al.* (2015). In particular, the research was focused on: (1) determining the morphometric characterization of stone pine fruits; (2) analyzing the chemical composition of pine nuts from *P. pinea* superior trees in the three growth macrozones across the species distribution in Chile, as well as in other five countries; and (3) assessing the use of NIRS in *P. pinea* nuts in order to provide a first approach to the analysis of chemical composition of nuts and prediction of seed-quality traceability in Chile.

2. Materials and Methods

2.1 Morphometric and chemical characterization of selected stone pine trees in Chile

2.1.1 Study area

The Chilean Forest Institute (INFOR) has gathered stone pine data in Chile, covering areas located between Coquimbo in the North (30.819817°S) and Araucanía in the South (38.990393°S), throughout 1,000 km, including populations established in plantations (regular plantations of over 100 trees), windbreaks (linear plantations), groves (irregular stands of up to 50 trees) and isolated trees, for different purposes. Three macrozones (North, Dry Coast and South) were previously identified according to height and DBH growth rates by Loewe *et al.* (2015). This study was performed in the 76 stone pine trees selected for high cone production, located in 46 sites distributed in the three studied macrozones (Fig.1). Average climatic variables of sites where those selected trees are located are summarized by macrozone in Table 1.

Table 1. Mean climatic variables of sites where selected stone pine trees are located by macrozone

Macrozone	Annual average temperature (°C)	Thermal oscillation (°C)	Autumn thermal oscillation (°C)	Annual rainfall (mm)	Spring hydric deficit (mm)	Annual hydric deficit (mm)	Summer hydric deficit (mm)
North n=17	13.7 (7)	13.2 (20)	12.9 (19)	430.2 (32)	-362.6 (15)	-856.0 (26)	-449.8 (17)
Dry Coast n=9	13.5 (6)	13.3 (11)	12.3 (11)	593.5 (24)	-356.8 (14)	-741.0 (20)	-439.1 (12)
South n=20	13.5 (6)	12.4 (17)	11.4 (18)	1,045.9 (20)	-273.0 (24)	-253.4 (117)	-410.2 (18)

Coefficients of variation are indicated in parentheses. Hydric deficit was calculated as accumulated rainfall in the period minus potential evapotranspiration of the same period; summer includes December 21 to March 20, spring includes September 21 to December 20. The number of sites where selected stone pine trees are located is indicated as *n*.

Climatic data were obtained from Chilean National Environmental Information System (www.inia.cl, www.dga.cl).



2.1.2 Superior tree selection

We identified all superior trees for fruit production from a group of 3,464 stone pine trees of 8 years and over –the age considered to be the beginning of the reproductive phase in Chile–, distributed in 122 sites. Given the high environmental variability and different ages (age data provided by owners) of those populations, superior phenotypes were identified by applying the individual valuation method, following Ipinza (1998).

Selection was performed considering production variables (over 30 cones tree⁻¹); tree vigor was estimated using a combination of foliage damage classes, including both needle losses and discoloration built based on the categories stated by Lakatos and Mirtchev (2014), as follows: (1) high, (2) medium, (3) low. Phytosanitary condition was determined by assessing biotic and abiotic damages through visual observation, following indications of Eichhorn *et al.* (2010). For each selected tree, dendrometric variables (DBH (measured at 1.3 m height), height, crown and trunk diameter) and productive variables (cone, number of in-shell and shelled pine nuts per cone, weight and dimensions) were measured and analyzed.

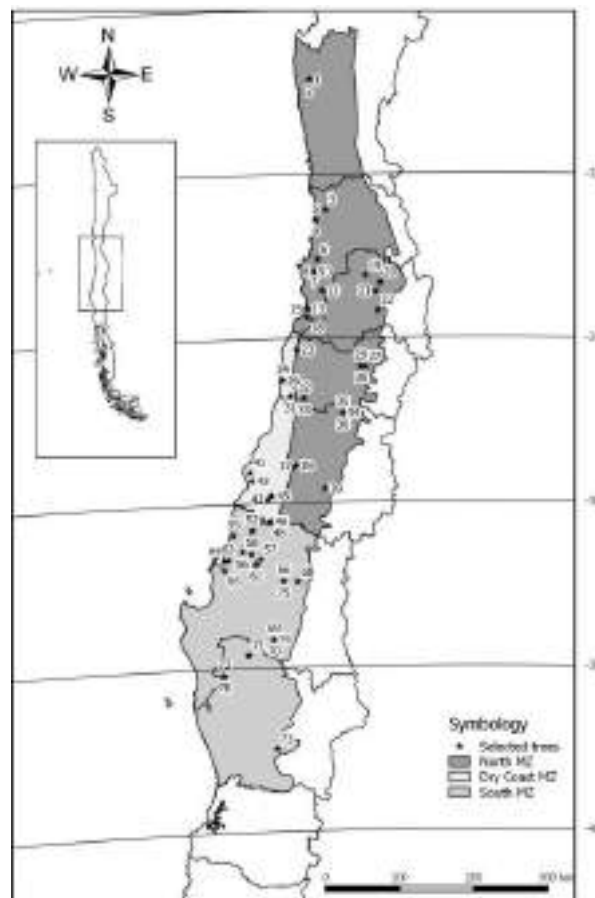


Figure 1. Distribution of selected stone pine trees in Chile by macrozones.

2.1.3 Measured variables

Cone and pine nut morphometry

Ten healthy 3 year-old cones were randomly harvested from each selected tree during winter 2013. Cone weight at harvest was measured using an analytical scale (Mettler AJ150) and in-shell pine nut number cone⁻¹ as well as size and weight of in-shell and shelled pine nuts were measured with a digital caliper and weighed in the laboratory. To determine cone to kernel yield (SHY), shelled pine nuts were dried to 6% moisture at 40°C in a Red Line Binger oven model RF115 and then weighed; weight was then compared with cone weight at harvest, using the following equation [1]:

$$SHY(\%) = \frac{\text{Dried kernel weight}}{\text{Cone weight at harvest}} * 100 \quad [1]$$

Cone productivity was calculated by multiplying cone weight by SHY.

Chemical and nutritional characterization of pine nuts from selected trees

In each macrozone, 50% of the selected trees (totaling 38) were randomly chosen to perform a chemical characterization. Ash, proteins, fat, lipid profile, energy and minerals (potassium and magnesium) were analyzed at ASISTEC lab (Universidad Católica de Valparaíso, Chile, www.asistec.ucv.cl).

For sample preparation, shelled pine nuts were dried at 40°C for 40 min until moisture reached 4%; then they were ground with a kitchen processor (Moulinex ®) and passed through a 0.5 mm mesh sieve, and finally frozen in sealed plastic bags at -20°C until analysis.

The following methods were used: protein content methodology corresponds to: protein content by Kjeldahl assay (AOAC 1996) in a Büchi 436 nitrogen digester and a Büchi 323 nitrogen distiller; lipids (AOAC 922.06/1998); ash (AOAC 938.06/1998) using an oven Labtech LDO150F and an analytical scale Precisa XT220A; lipid profile (liquid-liquid extraction SM 6410B, 5560D; gas chromatography (APHA-AWWA-WEF, 2005)); moisture (NCh841/1978) and minerals (acid digestion and quantification according to SM 3111B). All reagents and solvents were analytical grade chemicals from Merck (Germany).



2.2 Characterization of pine nut chemical composition by macrozone and country

2.2.1 Samples origin

Samples were collected from *P. pinea* planted trees distributed in the previously defined Chilean macrozones (MZs) (Loewe *et al.*, 2015) from June to November 2013. These MZs present a high climatic variability (Table 2), with average annual and maximum annual temperatures decreasing from north to south; the lowest minimum annual average temperature corresponds to the Dry Coast MZ. Rainfall increases from north to south, whereas hydric indices diminish, with the lowest water stress occurring in the South MZ.

Table 2. Climatic characterization of stone pine growth macrozones in Chile

Variable	North MZ n=48	Dry Coast MZ n=27	South MZ n=54	All sites n=129
Annual average temp. (°C)	14.1 (11.8; 16.7)	13.6 (12.5; 15.1)	13.2 (11.0; 14.2)	13.6 (11.0; 16.7)
Annual max average temp. (°C)	21.9 (17.6 ; 25.1)	21.0 (17.9; 23.0)	19.8 (16.3; 22.5)	20.8 (16.3; 25.1)
Annual min average temp. (°C)	7.5 (5.5; 9.9)	7.0 (5.5; 7.7)	7.5 (5.3; 9.2)	7.4 (5.3; 9.9)
Annual total rainfall (mm)	383.7 (116.2; 1,260.1)	648.3 (416.3; 816.0)	1,047.0 (523.6; 1,666.9)	716.7 (116.2; 1,666.9)
Annual hydric index (mm)	-995.4 (-1,349.7; -107.3)	-743.7 (-914.4; -474.2)	-244.1 (-736.4; - 332.9)	-628.2 (-1,349.7; 332.9)
Annual thermal oscillation (°C)	14.3 (9.2; 17.3)	14.0 (10.7; 17.0)	12.3 (8.2; 15.1)	13.4 (8.2; 17.3)

Values in parentheses correspond to minimum and maximum values recorded per variable.

Hydric index was calculated as accumulated rainfall minus potential evapotranspiration in the period.

The number of sites where selected stone pine trees are located is indicated as *n*.

Climatic data were obtained from Chilean National Environmental Information System (www.dga.cl, www.inia.cl).

In order to have a representative sample, the amount of trees sampled in each MZ was defined taking into account MZ size and variability of the most variable chemical contents, which according to international references (Loewe and Delard, 2012) corresponds to carbohydrates. The number of trees sampled were 42 in the North MZ (31° to 33° latitude), 89 in the Dry Coast MZ (34° to 35° latitude), and 36 in the South MZ (36° to 38° latitude). After cone harvest and extraction of in-shell pine nuts, these were kept in individually tagged plastic nets at room temperature until manually shelled. Chemical analyses were performed at Centro de Investigación y Desarrollo de Alimentos Funcionales (CIDAF), Universidad de

Valparaíso, Chile. For comparative purposes, a sample from Cádiz, Spain was used as reference (Álvarez, 2015).

Pine nuts from other 12 areas were collected from Turkey (n=3), Israel (n=3), Italy (n=3) and Spain (n=1) during winter 2013/14, and from Argentina (n=2) during winter 2013, and analyzed at the Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Argentina (Zuleta *et al.*, 2014, 2015; Lutz *et al.*, 2015). Average climatic variables of growing areas of the studied pine nuts are summarized in Table 3, and their distribution is presented in Fig. 2.

Table 3. Mean climatic variables of harvest sites of *Pinus pinea* nuts by location and country

Location	Country	Annual rainfall (mm)	Annual average temperature (°C)	Dry Month† (N°)	Maximum average temperature (°C)	Minimum average temperature (°C)	Thermal oscillation (°C)
Miramar	Argentina	660.7	15.1	5	22.1	8.0	14.1
Claromecó		758.4	14.5	4	21.2	7.9	13.3
Mean		709.6	14.8	5	21.7	8.0	13.7
North MZ		383.7	14.1	9	21.9	7.5	14.3
Dry Coast MZ	Chile	648.3	13.6	8	21.0	7.0	14.0
South MZ		1,047.0	13.2	7	19.8	7.5	12.3
Mean		693.0	13.6	8	20.9	7.3	13.5
Ortal		830.0	14.7	6	20.6	8.9	11.7
Bar'ham	Israel	682.1	16.2	7	20.4	12.0	8.4
Yatir		275.0	17.6	8	22.4	12.8	9.6
Mean		595.7	16.2	7	21.1	11.2	9.9
Cecina (LI)	Italy	833.6	14.5	4	19.8	9.1	10.7
Feniglia (GR)		455.1	13.2	4	16.0	10.3	5.7
Calabria (RC)		546.8	18.3	6	22.5	14.1	8.3
Mean		611.8	15.3	5	19.4	11.2	8.2
Cádiz	Spain	524.0	18.7	5	21.7	15.6	6.1
Balikesir	Turkey	576.8	14.6	6	20.5	8.9	11.5
Bagyuzu		743.2	13.1	5	18.9	8.8	10.1
Aydin		651.7	17.5	7	24.5	11.9	12.6
Mean		657.2	15.1	6	21.3	9.9	11.4

Climatic data source: Argentina, Argentine Meteorological Service (www.smn.gov.ar); Chile, Chilean Environmental Information System (www.inia.cl); Israel, The Israel Meteorological Service (www.ims.gov.il); Italy, Italian Army Aeronautic Meteorological Service (www.meteoam.it); Spain, State Meteorological Agency (www.aemet.es); Turkey, Turkish Meteorological Service (www.mgm.gov.tr).

†Dry Months were calculated as those months with monthly rainfall/monthly potential evapotranspiration lower than 0.5.

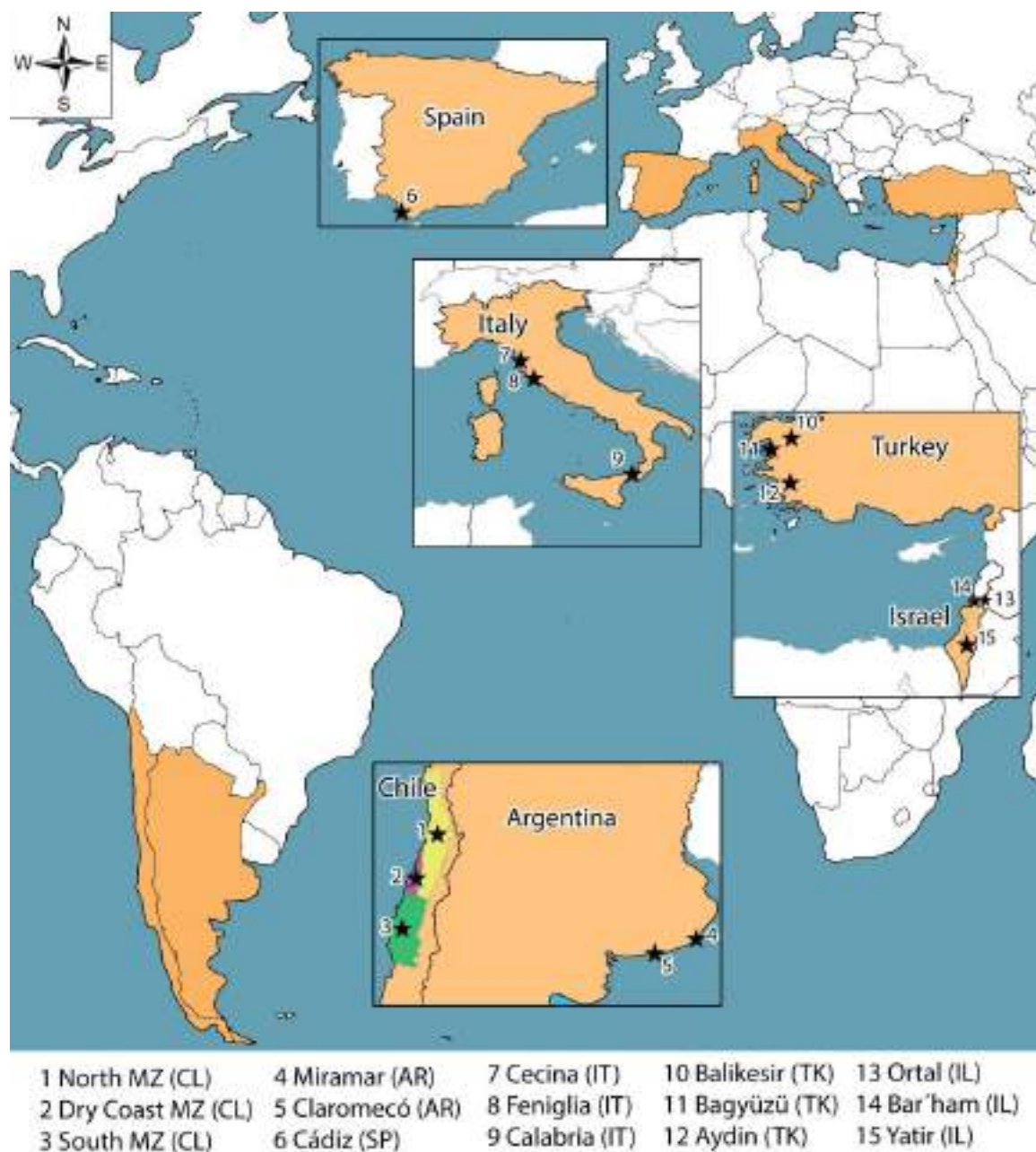


Figure 2. Distribution of locations in six countries where pine nuts were studied.

For sample preparation, shelled pine nuts were dried at 40°C for 40 min until moisture reached 4%, then ground with a kitchen processor (Moulinex ®), passed through a 0.5-mm mesh sieve, and frozen in sealed plastic bags at -20°C until analyses.

2.2.2 Chemical and nutritional characterization of pine nuts

Reagents and solvents were analytical grade chemicals from Merck (Germany), K-TDFR Megazyme, and the standards (gallic acid, catechin, caffeic acid, Trolox, β -sitosterol, campesterol, stigmasterol, α -, γ -tocopherol, ascorbic acid, dehydroascorbic acid) were Sigma Chemical (St Louis, MO, USA). For the fatty acid profile a standard of fatty acid methylesters Nu-Chek: FAME GLC 15A, Walnut Standard (Nu Check Prep Inc, MN, US) was used.

Proximate analysis methodology: protein content was determined by Kjeldahl assay (AOAC 920.54) in a nitrogen digester DK6 (VELP) and a nitrogen distiller UDK 129 (VELP), using a factor of 5.3 to convert nitrogen to proteins (Greenfield and Southgate, 1972). Moisture was determined using the AOAC method 945.15, and ash by AOAC method 942.05. Dietary fiber (total (TDF), soluble and insoluble fractions) was measured according to the AOAC enzymatic-gravimetric method 991.43 (AOAC, 2012). Lipids were extracted using the methods of Bligh and Dyer (1959) and Folch *et al.* (1957). In this fraction, fatty acid composition was determined by the Official Method ISO 5509:2000 for fatty acids in oils, animal and vegetable fats (ISO, 2000). Analysis of fatty acid methyl esters was carried out using an HP 5890 gas chromatograph with flame ionization detector and integrated software Young Lin Autochro-3000, version 2.0.

Phytosterols were analyzed by adapting the method of Maguire *et al.* (2004) in pine nut oil, after saponification with 3% methanolic NaOH. Phytosterols were extracted with hexane for their identification and quantification by HPLC. A Merck Hitachi Lachrom L-7455 liquid HPLC system with diode array detector was used at a wavelength of 210 nm, equipped with an RP-18 column, applying an isocratic mobile phase containing acetonitrile:methanol (70:30), according to Shah *et al.* (2010).

Tocopherols were measured by the IUPAC (1992) standard method; α - and γ -tocopherols in pine nut oil were determined after saponification with 50% KOH and 1% ethanolic pyrogallol. Both species were identified and quantified by HPLC in the same instrument described above, at a wavelength of 292 nm, using an isocratic mobile phase containing methanol:water (ultrapure) (99:1), as described by Maguire *et al.* (2004). Results were expressed as micrograms per gram oil ($\mu\text{g/g}$ oil).

Total phenol content (TPC) was determined by the method of Singleton and Rossi (1965) adjusted to a microplate reader (Synergy Multidetector HT, Biotek). A gallic acid standard solution series was prepared, ranging from 235 to 1,180 $\mu\text{mol/L}$. Extracts of the



samples were obtained using acetone:water (7:3). From each standard solution and sample, 50 μ L were mixed with 2.5 mL Folin-Ciocalteu reagent and 2.0 mL sodium carbonate solution. The samples were mixed and incubated at 20°C for 30 min and absorbance was read at 760 nm. Results were expressed as milligrams of gallic acid equivalents (mg GAE/g).

Antioxidant capacity ORAC (Oxygen Radical Absorbance Capacity) was determined as described by Ou *et al.* (2002) and Huang *et al.* (2005), adjusted for the microplate reader used (Synergy Multidetector HT, Biotek). The fluorescence of the probe decreases over time, and the decay kinetics is followed by reading every min, for 90 min, at 485 nm (excitation) and 528 nm (emission). Results are expressed as micromoles of Trolox equivalents (μ mol TE/g).

Vitamin C content (ascorbic and dehydroascorbic acids) was determined using the method of Gokmen *et al.* (2000), with modifications (50 mL 5% orthophosphoric acid containing 5 mM DTT (dithiotreitol) were added to 0.6 g pine nuts; this mixture was homogenized in an ultraturrax for 2 min, stirred in an orbital shaker in complete darkness for 30 min and centrifuged at 4,000 rpm at 25°C for 10 min and filtered) were analyzed by HPLC using the same instrument, measuring at a wavelength of 240 nm. The isocratic mobile phase was 25 mM KH_2PO_4 , pH 3.0, adjusted with H_3PO_4 .

2.3 Near-infrared spectroscopy (NIRS) technique

2.3.1 Sample origin

Pine nuts used for the NIRS study were harvested from the selected trees described in 2.1 (Fig. 1) in winter 2013.

2.3.2 Sample preparation

The training set used in this study includes four different levels of pine nut processing: in-shell pine nuts (IS), whole shelled pine nuts (SH), and humid (HF) and dried pine nut flour (DF) (Fig. 1S). In-shell pine nuts were obtained directly from the harvested cones and stored properly in dry conditions (4°C), protected from direct sunlight until analysis. Shelled whole pine nuts were obtained by manually cracking in-shell pine nuts, and separated from the external shell and from the inner seed coat. Immediately after the spectra were taken, to prepare the humid flour, pine nuts (including embryos) were ground in a blade mill (Moulinex AD56 42) until a homogeneous powder was obtained. Later, this flour was weighed and dried

in a forced-air drier at 45°C for 48 h, homogenized in a Waring Blender (LB20E, Waring Products, New Hartford, CT, USA), and sieved (1-mm mesh), to obtain a fine homogeneous flour. Samples were stored at 4°C until NIRS analysis.

2.3.3 Instrumentation and collection of spectra

The samples were scanned by reflectance using a circular cup in a transport module connected to a Foss-NIRSystems 6500 spectrophotometer equipped with auto gain detectors: one for 400-1,098 nm (known as the VIS region) and another for 1,100-2,500 nm (known as the NIR region).

Reflectance spectra were collected every 2 nm from 400 to 2,500 nm. The absorbance data were obtained and stored as $\log(1/R)$, where R is the reflectance. All data were stored using WinISI II software version 1.5 (Infrasoft International, Port Matilda, PA, USA). A reference spectrum was recorded before analyzing each sample. Three spectra for each tree were taken in the case of whole pine nuts, both in-shell and shelled, and two in the case of flour samples. These spectra were averaged to obtain the mean spectrum that was used to determine relationships with geographical origin.

2.4 Statistical analyses

2.4.1 Morphometric and chemical characterization of selected stone pine trees

A mixed linear model was used to test mean differences in pine nut morphometry, yield and chemical composition among macrozones, including macrozone, age and interaction effects MZ by age on the mean structure (fixed effects). Tree age was used as covariate to obtain adjusted means. Homogeneous and heteroscedastic models were fitted for each variable and compared using the Likelihood Ratio Test. Partial correlation was used to test for correlation between cone and pine nut morphometric data, chemical features and climate data, adjusting for macrozone effects. Further analyses of correlation were performed using canonical correlation coefficients between groups of traits. Analyses were performed using the software Infostat and its interface with the software R (Di Rienzo *et al.*, 2013).

2.4.2 Characterization of pine nut chemical composition by macrozone and country

For Chilean pine nuts, chemical analyses were performed in triplicate; each replicate was quantified in duplicate. A non-parametric Kruskal-Wallis test was performed to evaluate



macrozone effects. A $p < 0.05$ was considered significant. A principal component analysis (PCA) was also performed, generating a biplot for chemical composition of pine nuts and climate variables of the three macrozones.

Pine nuts collected from different locations of Italy, Turkey, Israel, Argentina and Chile were compared using heteroscedastic ANOVA, and statistical significance was determined with an LSD test ($p < 0.05$). The relative contribution of climatic variables to chemical components was estimated using CART (Classification and Regression Trees) algorithms (Breiman, 2001). As confirmatory analysis, the groups suggested by the identified climatic variables thresholds were also compared by ANOVA. Finally, a principal component analysis (PCA) was performed, generating a biplot for chemical composition of pine nuts and climate variables of different countries. Analyses were performed using the software Infostat and its interface with the software R (Di Rienzo *et al.*, 2013).

2.4.3 Near-infrared spectroscopy

The training set of pine nuts samples were qualitatively analyzed using Matlab R2016a (The Mathworks, MA). The total population of spectra was divided randomly and proportionally to geographical origin into a calibration training set.

The origin of samples was determined using classification models developed by discriminant partial least square (DPLS) (Kowalski, 1984). Briefly, DPLS correlates spectral variations (X) with defined classes (Y), and maximizes the covariance between the two types of variables, with the purpose of developing a classification model (Vandeginste *et al.*, 1998; Massart *et al.*, 1997). An unknown sample is classified according to the value predicted by the DPLS model, which ideally should be close to the values used to codify the class.

Before developing the classification models, the spectral data were subjected to different signal pre-treatments. Scatter correction was accomplished using the standard normal variate and detrend transformation (SNV+DT) algorithm (Barnes *et al.*, 1989). The spectra were transformed using different ranges of the 400–2,500 nm spectrum and different combinations of derivative math treatments applied to the spectral data. Matlab derivative math treatments are denoted by a four-digit notation (a, b, c, d), where *a* is the derivative order, *b* is the derivative gap, *c* is the smoothing segment, and *d* is the second smoothing segment (Shenk and Westerhaus, 1995a). Moreover, the influence of different wavelength regions was evaluated, and various qualitative models were applied successively on the full

spectrum (VIS+NIR; 400–2,500 nm), the NIR region (NIR; 1,100–2,500 nm), and the 400–2,200 region, as this is the region recommended by the manufacturer of the optic fiber probe to obtain spectra with a suitable signal-to-noise ratio.

The qualitative analysis methods were validated by studying the percentage of samples that were classified correctly or incorrectly (Massart *et al.*, 1997). A total of 76 samples were used to calibrate and full cross validate each model, with 34 belonging to the North MZ, 12 to the Dry Coast MZ and 30 to the South MZ.

Classification errors corresponding to the proportion of incorrectly classified samples (1-accuracy) were calculated according to the equation [2]:

$$\text{Classification error} = \frac{\text{FP} + \text{FN}}{\text{TP} + \text{TN} + \text{FP} + \text{FN}} \quad [2]$$

Considered rates correspond to: TP: True Positive, FP: False Positive, TN: True Negative, FN: False Negative.

3. Results

3.1 Morphometric and chemical characterization of selected stone pine trees

3.1.1 Pine nut morphometry

Morphometric traits and yield of pine nuts from the three macrozones are shown in Table 4. Statistical differences ($p < 0.05$) were observed in cone length and diameter, as well as in pine nut weight, diameter, cone to pine nut yield (ISY) and seed. Shelled pine nut morphometric traits showed differences among macrozones, with lowest mean trait values being generally found in the Dry Coast MZ. Mean cone number in selected trees was 105 cones tree⁻¹, cone weight was 495 g and in-shell pine nut number cone⁻¹ (IS) was 100.

SHY reached 3.9% across the whole area, ranging from 3.5% in the Dry Coast MZ to 4.3% in the South MZ. Pine nut weight was higher in the South MZ than in the Dry Coast MZ. The lowest value for kernel weight (0.18 g) was recorded in the Dry Coast MZ; SHY was superior in the South MZ. Average cone productivity was even among MZs, with an average of 53 kg tree⁻¹. Mean kernel production amounted to 2.0 kg tree⁻¹, ranging from 1.6 kg tree⁻¹ in the Dry Coast MZ to 2.4 kg tree⁻¹ in the South MZ, without statistical differences.

**Table 4.** Morphometric characterization of selected stone pine tree cones, in-shell and shelled pine nuts in three Chilean macrozones

Productive Variables		North n=34	Dry Coast n=12	South n=30	Adjusted mean
Cone					
CN	#	103.5 a (9.8)	97.4 a (16.9)	110.0 a (10.3)	105.1
CW	g	480.7 a (20.3)	489.5 a (34.9)	513.4 a (21.3)	494.9
DW	g	355.4 a (14.2)	352.6 a (24.5)	374.4 a (14.9)	362.5
CL	cm	10.7 b (0.3)	11.2 ab (0.6)	12.1 a (0.3)	11.3
CD	cm	9.3 b (0.1)	9.4 ab (0.2)	9.7 a (0.1)	9.4
CP	kg/tree	51.3 a (5.6)	47.4 a (9.6)	56.1 a (5.9)	52.6
In-shell pine nuts					
IS	#	92.6 a (3.7)	106.7 a (6.4)	106.2 a (3.9)	100.2
ISW	g	0.94 ab (0.02)	0.85 b (0.04)	0.97 a (0.03)	0.94
ISL	cm	2.18 a (0.1)	2.02 a (0.2)	1.91 a (0.1)	2.05
ISD	cm	1.07 a (0.0)	0.92 b (0.0)	0.97 b (0.1)	1.01
ISY	%	18.1 b (0.7)	18.6 ab (1.1)	20.4 a (0.7)	19.1
ISP	kg/tree	9.2 a (1.1)	8.7 a (1.9)	11.3 a (1.2)	9.9
Seed	#/kg	1.094.6 b (30.7)	1.218.9 a (52.9)	1.048.6 b (32.3)	1.096.1
Shelled pine nuts					
SH	#	86.6 b (4.1)	93.6 ab (7.1)	101.4 a (4.4)	93.6
SHW	g	0.22 a (0.01)	0.18 b (0.01)	0.21 a (0.01)	0.21
SHL	cm	1.39 a (0.0)	1.15 b (0.1)	1.26 b (0.0)	1.29
SHD	cm	0.59 a (0.0)	0.44 b (0.0)	0.49 b (0.0)	0.53
IS/SHY	%	20.8 ab (0.5)	18.7 b (0.9)	20.1 a (0.5)	20.5
SHY	%	3.7 b (0.15)	3.5 b (0.26)	4.3 a (0.16)	3.9
SHP	kg/tree	1.9 a (0.2)	1.6 a (0.4)	2.4 a (0.2)	2.0

Traits are expressed as means. MZ: macrozone; IS: in-shell pine nut number cone⁻¹; ISW: in-shell pine nut weight; ISL: in-shell pine nut length; ISD: in-shell pine nut diameter; ISY: cone to in-shell pine nut yield; ISP: in-shell pine nut production tree⁻¹; SH: shelled pine nut (kernel) number cone⁻¹; SHW: shelled pine nut (kernel) weight; SHL: shelled pine nut (kernel) length; SHD: shelled pine nut (kernel) diameter; IS/SHY: in-shell to shelled pine nut yield; SHY: cone to shelled pine nut (kernel) yield; SHP: shelled pine nut (kernel) production tree⁻¹. For each variable, mean values with different letters indicate statistical differences among macrozones ($p \leq 0.05$, LSD test). Values in parentheses correspond to standard error. n = number of sites within macrozone.

Cone length ranged from 10.7 to 12.1 cm; accordingly, diameter varied from 9.3 to 9.7 cm, with average values of 11.3 and 9.4 cm, respectively, increasing from north to south. Pine nut per cone quantity was not statistically different among macrozones, ranging from 93 to 107. In-shell pine nut diameter was highest in the North MZ (1.07 cm), and cone to pine nut yield increased from North MZ to South MZ (18.1 to 20.4%).

Shelled pine nut number cone⁻¹ (SH) also increased from North MZ to South MZ (87 to 101), with an average of 94; the highest length and diameter values were recorded in the North MZ (1.39 and 0.59 cm, respectively), and weight was lowest in the Dry Coast MZ (0.18 g). The highest value of cone to kernel yield was obtained in the South MZ (4.3%).

3.1.2 Pine nut chemical composition

The proximate composition of pine nuts had an average moisture content of 5.6%; protein content was in average of 35.5%, and lipids average reached 40.2%, with the highest value (42.2%) corresponding to the Dry Coast MZ. Mineral content was 4.4% and calories 555.9/100g.

Chemical composition varied for few components among pine nuts collected from selected trees distributed across macrozones (Table 5). Differences were statistically significant only for potassium content, with higher values in the Dry Coast MZ (9,193.3 mg kg⁻¹), and for oleic acid, with higher values in the South MZ (37.0 g 100 g⁻¹ oil).

Total saturated fatty acids (SFA) amounted to 16.5 g 100 g⁻¹ oil, total monounsaturated fatty acids (MUFA) to 37.8 g 100 g⁻¹ oil, and polyunsaturated (PUFA) to 40.8 g 100 g⁻¹oil. Oleic and linoleic fatty acids were the most abundant, totaling 74.3 g 100 g⁻¹oil.

Lineal correlations between morphometric, chemical, and climate variables from selected stone pine trees are presented in Table 6. Some positive correlations between morphometric and chemical variables were detected, especially between oleic acid and ISW and SHW, and between protein and ISW; a negative correlation was detected between protein and IS. ISW and SHW were positively correlated to CW and IS (Table 7).

**Table 5.** Chemical composition of selected stone pine tree pine nuts by macrozones in Chile

Macrozone	Moisture (g/100 g)	Protein (g/100 g)	Lipids (g/100 g)	Ashes (g/100 g)	NFE† (g/100 g)	Calories (100 g ⁻¹)	Palmitic acid C16:0 (g 100g oil ⁻¹)	Stearic acid C18:0 (g 100g oil ⁻¹)	Arachidic acid C20:0 (g 100g oil ⁻¹)	Total SFA (g 100g oil ⁻¹)	Oleic acid C18:1n-9 (g 100g oil ⁻¹)	Eicosenoic acid Cis-11 (g 100g oil ⁻¹)	Total MUFA (g 100g oil ⁻¹)	Linoleic acid C18:2n-6 (g 100g oil ⁻¹)	Linolenic acid C18:3n-3 (g 100g oil ⁻¹)	Eicosadienoic acid Cis-11.14 (g 100g oil ⁻¹)	Total PUFA (g 100g oil ⁻¹)	Potassium (mg kg ⁻¹)	Magnesium (mg kg ⁻¹)
North n=17	5.6 a (0.10)	35.8 a (0.40)	38.3 a (1.71)	4.4 a (0.05)	14.9 a (1.75)	546.8 a (8.58)	8.6 a (0.36)	6.8 a (0.36)	1.0 a (0.11)	16.4 a (0.73)	36.5 ab (0.41)	1.7 a (0.10)	38.2 a (0.35)	37.5 a (0.84)	1.4 a (0.05)	1.2 a (0.07)	40.1 a (0.77)	7,907.8 b (0.02)	3,010.9 a (0.02)
Dry Coast n=6	5.7 a (0.17)	35.8 a (0.69)	42.2 a (2.99)	4.4 a (0.09)	10.6 a (3.06)	565.3 a (14.96)	8.3 a (0.63)	6.8 a (0.62)	1.2 a (0.22)	16.3 a (1.56)	35.1 b (0.72)	1.5 a (0.18)	36.6 b (0.99)	39.9 a (1.46)	1.3 a (0.05)	1.2 a (0.13)	42.4 a (1.66)	9,193.3 a (0.02)	3,891.9 a (0.04)
South n=15	5.6 a (0.10)	35.0 a (0.42)	41.4 a (1.82)	4.3 a (0.05)	12.5 a (1.87)	562.5 a (9.14)	8.8 a (0.39)	7.1 a (0.38)	1.0 a (0.11)	16.9 a (0.53)	37.0 a (0.44)	1.7 a (0.11)	38.7 a (0.36)	37.4 a (0.89)	1.4 a (0.05)	1.1 a (0.08)	39.9 a (0.66)	8,037.2 b (0.03)	3,675.3 a (0.02)
Adjusted mean	5.6	35.5	40.2	4.4	13.3	555.9	8.6	6.9	1.0	16.5	36.5	1.7	37.8	37.8	1.4	1.2	40.8	8,695	3,845

†NFE: Nitrogen Free Extract.

SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids.

For each variable, mean values with different letters indicate statistical differences among macrozones ($p \leq 0.05$, LSD test). Values in parentheses correspond to standard error.

n= number of sites within macrozone.

3.1.3 Pine nut features and climate

The canonical correlation between climate and morphometric variables was statistically significant ($R=0.96$, $p<0.0001$), with the main climate variables being maximum average temperature, thermal oscillation, average summer temperature, average annual temperature, and summer hydric index.

Mean maximum temperature, summer average temperature, summer maximum temperature and thermal oscillation were directly correlated with in-shell pine nut and kernel weight. On the contrary, summer hydric index was inversely correlated with in-shell and shelled pine nut weight, and directly correlated with seed kg^{-1} .

Canonical correlation between climate and chemical composition variables was also statistically significant ($R=0.99$, $p=0.05$). The climate variables most related to chemical composition were average annual temperature, minimum average temperature, autumn average temperature, winter average temperature and winter maximum average temperature.

Average maximum temperature showed a direct correlation with protein and oleic acid, and summer maximum temperature and summer average temperature with protein content; summer hydric index had an inverse correlation with protein (Table 6).



Table 6. Lineal correlations between morphometric, chemical and climate variables in selected stone pine trees

Morphometric/Chemical Variable	Climatic data				
	ATsu	MXT	MXTsu	TO	HI _{su}
IS	-0.11	0.12	-0.11	-0.11	0.11
ISW	0.65*	0.62*	0.67*	0.50*	-0.59*
SHW	0.52*	0.53*	0.58*	0.51*	-0.61*
Seed	-0.59*	-0.59*	-0.62*	-0.47*	0.55*
Protein (%)	0.55*	0.40*	0.51*	0.34	-0.39*
Lipids (%)	0.21	0.13	0.19	0.06	-0.05
Oleic acid	0.30	0.38*	0.37	0.33	-0.35
Linoleic acid	-0.33	0.25	-0.29	-0.16	0.25
Potassium (mg kg⁻¹)	0.11	-0.01	-0.03	-0.09	0.04
Magnesium (%)	-0.28	-0.30	-0.26	-0.21	0.28

Asterisks indicate statistical significance (p value ≤ 0.005). Correlation significance was adjusted for multiplicity using Bonferroni criterion. IS: in-shell pine nut cone⁻¹; ISW: IS weight; SHW: shelled pine nut weight; ATsu: summer average temperature; MXT: maximum average temperature; MXTsu: summer maximum average temperature; TO: thermal oscillation; HI_{su}: summer hydric index.

Table 7. Lineal correlations between morphometric and chemical variables in selected stone pine trees

	CW	IS	ISW	SHW
IS	0.29			
ISW	0.71*	0.65*		
SHW	0.66*	0.83*	0.76*	
Seed	-0.69	-0.09	-0.99	-0.74
Protein (%)	0.09	-0.43*	0.42*	-0.32
Lipids (%)	0.28	0.24	0.21	0.14
Oleic acid	0.18	-0.24	0.38*	0.38*
Linoleic acid	0.17	0.12	-0.14	0.38
Potassium (mg kg⁻¹)	0.05	0.25	-0.05	-0.04
Magnesium (%)	-0.18	-0.06	-0.27	-0.10

Asterisks indicate statistical significance (p value ≤ 0.005). Correlation significance adjusted for multiplicity using Bonferroni criterion. CW: cone weight; IS: in-shell pine nut number cone⁻¹, ISW: in-shell weight; SHW: shelled pine nut weight.

3.2 Characterization of pine nut chemical composition by Chilean macrozones and countries

3.2.1 Characterization at the macrozone level

Chemical composition of Chilean pine nuts is presented in Tables 8 to 11 and Fig. 3. The most abundant component was fat (42.2 to 46.9 g/100 g), which was highest in the Dry Coast MZ and lowest in the North MZ; followed by protein (32.1 to 35.2 g/100 g), which was significantly higher in the Dry Coast MZ and lower in the South MZ.

Table 8. Chemical composition of pine nuts by Chilean stone pine macrozone (g/100 g)

Macrozone	Moisture	Ashes	Protein	Lipids	Total DF	Insoluble DF	Soluble DF
North	4.09±0.15 b	4.74±0.01 a	34.93±0.10 ab	42.25±0.33 b	11.60±0.14 a	9.23±0.11 a	2.37±0.03 a
Dry Coast	4.48±0.01 a	4.71±0.02 ab	35.25±0.10 a	46.92±0.07 a	11.61±0.31 a	9.79±0.10 a	2.00±0.04 a
South	4.34±0.01 ab	4.58±0.03 b	32.09±0.14 b	43.58±0.06 ab	11.83±0.12 a	9.80±0.13 a	2.04±0.01 a
Chile average	4.30±0.07	4.67±0.03	34.09±0.51	44.25±0.70	11.68±0.10	9.61±0.13	2.14±0.08
Spanish sample	4.77±0.04	4.14±0.03	34.82±0.08	47.67±0.37	12.37±0.33	9.95±0.01	2.11±0.11

n=3. Mean±SE. Results are expressed as dry weight. Different letters within a column indicate significant differences ($p<0.05$, Kruskal-Wallis test). Source: based on Álvarez (2015).

The lowest mineral (ash) content was recorded in the South MZ (4.6 g/100 g). Total DF was even across macrozones (average 11.7 g/100g), with the insoluble fraction being the most abundant (9.6 g/100g). The Spanish sample showed higher total DF values than the three MZs (12.4 g/100g).

Total phenolic content, antioxidant capacity (ORAC), and total vitamin C content (Table 9), and phytosterol and tocopherol contents (Table 10), were homogeneous among macrozones. Average total phenolic content was 0.34 mg GAE/g, average ORAC capacity was 8.6 $\mu\text{mol TE/g}$ and average Vitamin C was 2.8 mg/100 g. The most abundant vitamin E was γ -tocopherol –which is increasingly recognized as a relevant anti-atherogenic molecule (Sabaté and Wien, 2010)–, ranging from 75.6 (South MZ) to 134.7 (Dry Coast MZ) $\mu\text{g/100 g oil}$.

**Table 9.** Phenolic, antioxidant capacity and vitamin C content in pine nuts by macrozone

Macrozone	TPC mg GAE/g	ORAC μmol TE/g	Vitamin C mg/100 g
North	0.27 ± 0.01 a	8.60 ± 0.03 a	2.37 ± 0.05 a
Dry Coast	0.39 ± 0.00 a	8.44 ± 0.02 a	2.84 ± 0.03 a
South	0.35 ± 0.01 a	8.59 ± 0.02 a	3.10 ± 0.10 a
Chile average	0.34 ± 0.05	8.54 ± 0.03	2.77 ± 0.14

TPC: total phenolic compounds, ORAC: Oxygen Radical Absorbance Capacity. n=3. Mean±SE.

Results are expressed as dry weight. Different letters within a column indicate significant differences (p<0.05, Kruskal-Wallis test). Source: based on Álvarez (2015).

β-sitosterol was the main phytosterol, which increased from north to south (1,576.6 to 1,948.8 μg 100 g⁻¹ oil). Stigmasterol content ranged from 208.0 μg 100 g⁻¹ in the North MZ to 356.5 μg 100 g⁻¹ in the Dry Coast MZ.

Table 10. Main phytosterols and tocopherols of pine nuts by stone pine macrozone (μg/100 g oil)

Macrozone	Stigmasterol	β-sitosterol	α-tocopherol	γ-tocopherol
North	208.00 ± 0.08 a	1,576.55 ± 0.05 a	9.44 ± 0.01 a	111.05 ± 3.17 a
Dry Coast	356.52 ± 0.15 a	1,927.96 ± 0.02 a	5.74 ± 0.12 a	134.69 ± 4.58 a
South	320.89 ± 0.03 a	1,948.81 ± 0.03 a	2.53 ± 0.03 a	75.61 ± 2.92 a
Chile average	295.13 ± 28.31	1,817.77 ± 76.38	5.90 ± 1.26	107.11 ± 10.98

n=2. Mean±SE. Results are expressed as dry weight. Different letters indicate significant differences (p<0.05, Kruskal-Wallis test). Source: based on Álvarez (2015).

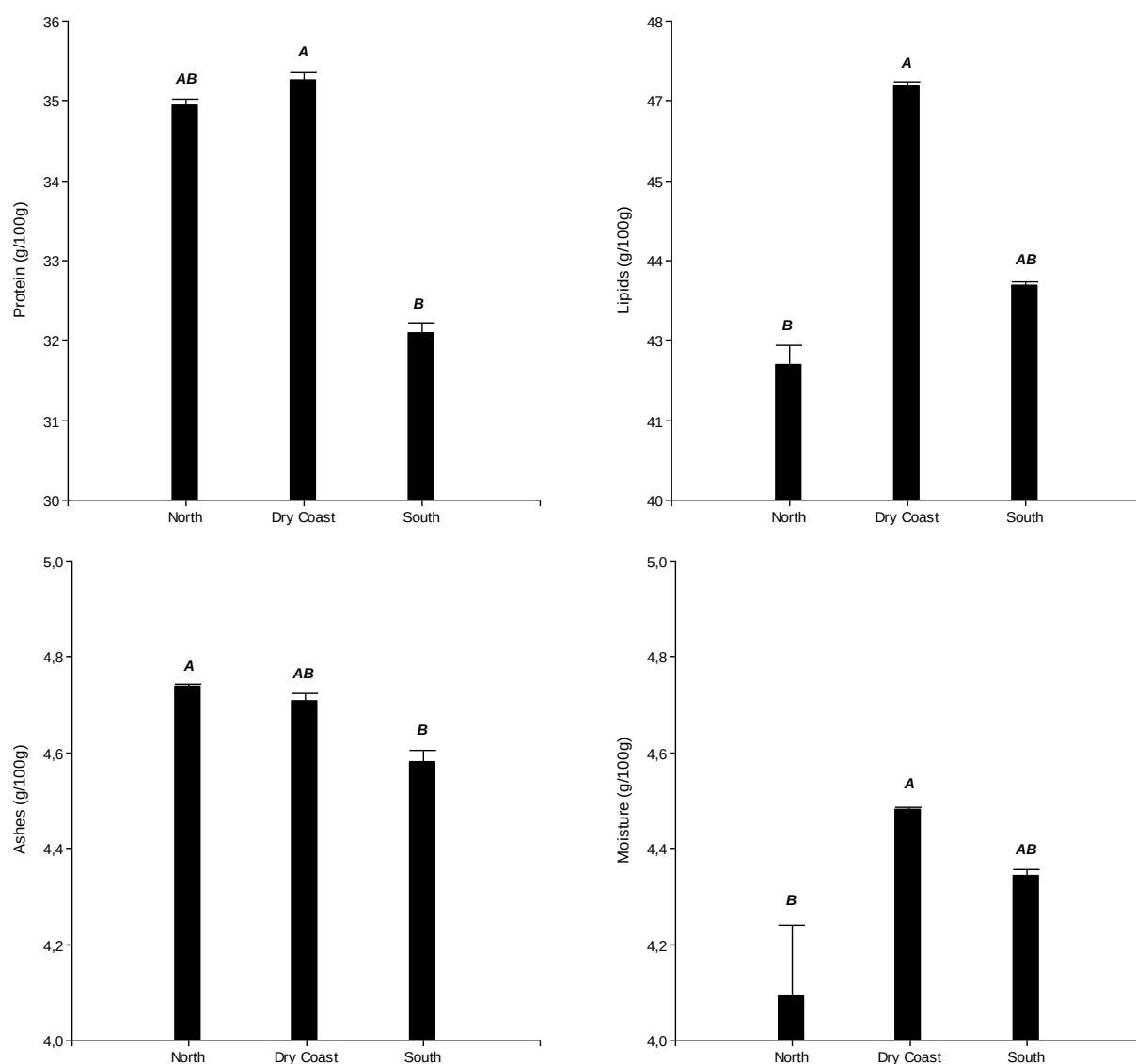
Table 11 shows the fatty acid profile of pine nuts by macrozone; the values did not show any statistical differences among MZs. Polyunsaturated fatty acids were the most abundant (average of 49.2 g/100 g oil) being highest in the Dry Coast MZ (51.1 g/100 g oil), with linoleic acid being the most important (48.8 g/100 g oil on average). Monounsaturated fatty acids were the second most abundant (average of 39.4 g/100 g oil), being lowest in the Dry Coast MZ (37.1 g/100 g oil, mostly oleic acid). Saturated fatty acids accounted for 11.4 g/100 g oil, with similar content among MZs.

Table 11. Fatty acid composition of pine nuts by Chilean stone pine macrozone (g/100 g oil)

Fatty acids	North	Dry Coast	South	Chile average
C16:0 Palmitic	6.16 ± 0.02 a	6.50 ± 0.03 a	6.19 ± 0.05 a	6.28 ± 0.07
C18:0 Stearic	4.17 ± 0.03 a	4.49 ± 0.04 a	4.25 ± 0.09 a	4.30 ± 0.07
C20:0 Arachidic	0.77 ± 0.01 a	0.80 ± 0.00 a	0.90 ± 0.01 a	0.82 ± 0.03
Total saturated	11.09 ± 0.03 a	11.79 ± 0.07 a	11.34 ± 0.03 a	11.40 ± 0.13
C18:1n-9 Oleic	40.92 ± 0.05 a	37.13 ± 0.03 a	40.28 ± 0.07 a	39.44 ± 0.74
Total monounsaturated	40.92 ± 0.05 a	37.13 ± 0.03 a	40.28 ± 0.07 a	39.44 ± 0.74
C18:2n-6 Linoleic	47.64 ± 0.09 a	50.72 ± 0.07 a	48.01 ± 0.04 a	48.79 ± 0.61
C18:3n-3 Linolenic	0.37 ± 0.00 a	0.37 ± 0.04 a	0.39 ± 0.01 a	0.37 ± 0.01
Total polyunsaturated	48.01 ± 0.09 a	51.08 ± 0.04 a	48.39 ± 0.03 a	49.16 ± 0.61

n=2. Mean±SE. By row, different letters indicate statistical differences (p<0.05, Kruskal-Wallis test).

Source: based on Álvarez (2015).

**Figure 3.** Chemical composition of Chilean pine nuts by stone pine macrozones. Different letters indicate statistically significant differences (p<0.05).



The biplot of the two principal components (Fig. 4) explained 100% of the variability. Pine nuts differed among the three Chilean macrozones. Pine nuts grown in the South MZ, where rainfall is highest, have a higher TDF content; those harvested in the North MZ –where average temperature, maximum average temperature, number of dry months and thermal oscillation are highest– showed a high mineral and protein content. Pine nuts collected in the Dry Coast MZ showed a high content of lipids, moisture and protein. Finally, lipids seem to increase with decreasing minimum average temperature.

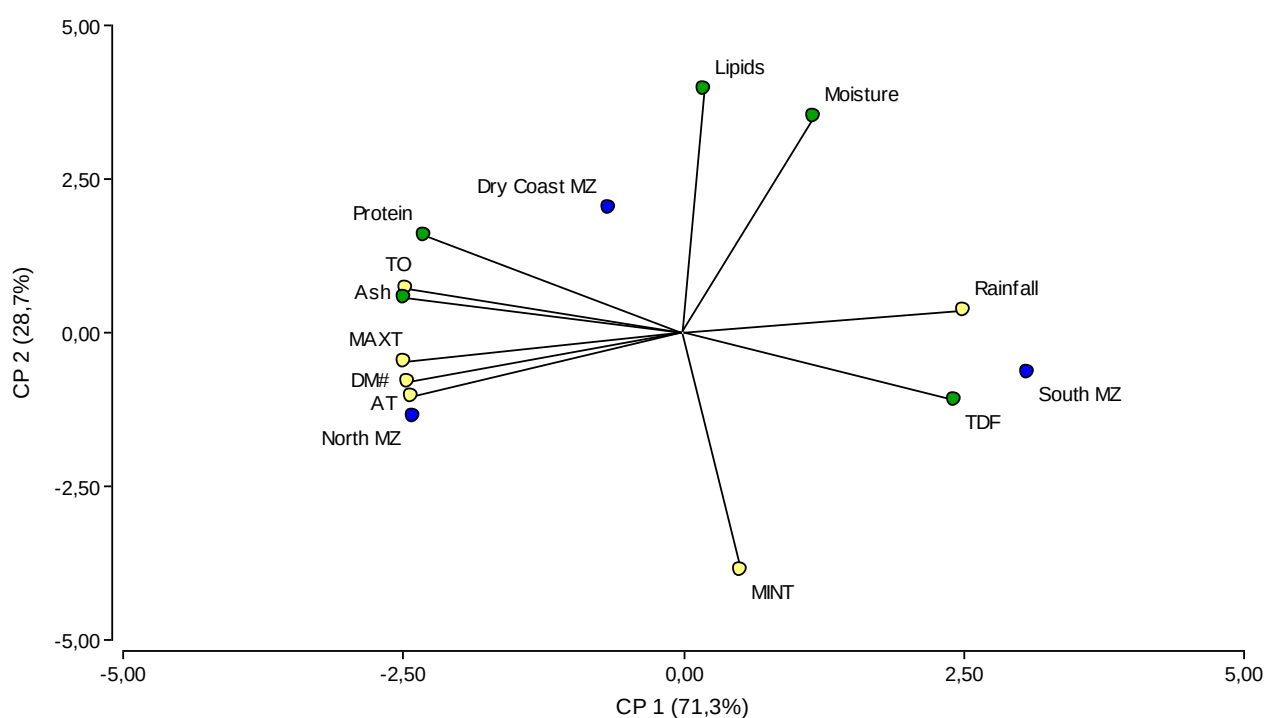


Figure 4. Biplot for the chemical composition of pine nuts and climate variables by macrozone. MAXT: maximum average temperature, AT: average temperature, MINT: minimum average temperature, DM#: number of dry months, TO: thermal oscillation, TDF: total dietary fiber.

3.2.2 Characterization at the country level

Table 12 shows the chemical composition of pine nuts collected from six countries -including the Chilean mixes-, with statistical differences for some components being indicated. The highest moisture value was recorded in the Italian sample (5.1%) and the lowest one in the Israeli sample (3.7%); protein content ranged between 32.1% (Italy and Argentina) and 36.6% (Israel), lipids between 34.7% (Israel) and 45.3% (Spain), minerals between 4.1% (Spain and Turkey) and 4.7% (Israel and Chile), and total dietary fiber between 9.8% (Argentina) and 14.6% (Italy).

Table 12. Chemical composition of pine nuts by location and country (g/100 g)

Location/Country	Total Dietary Fiber	Protein	Lipids	Ashes	Moisture
Miramar	10.50	33.06	42.00	4.20	4.55
Claromecó	9.08	31.22	41.61	4.60	3.00
Argentina	9.79 ± 0.71 c	32.14 ± 0.92 b	41.81 ± 0.20 a	4.40 ± 0.20 ab	3.78 ± 0.78 ab
North MZ	11.60	34.93	42.25	4.74	4.09
Dry Coast MZ	11.61	35.25	46.92	4.71	4.48
South MZ	11.83	32.09	43.58	4.58	4.34
Chile	11.68 ± 0.10 b	34.09 ± 0.51 b	44.25 ± 0.70 a	4.67 ± 0.03 a	4.30 ± 0.07 a
Ortal	13.00	35.53	31.00	4.82	3.50
Bar'ham	12.25	37.19	30.15	4.66	3.70
Yatir	11.90	37.03	42.94	4.63	4.01
Israel	12.38 ± 0.32 b	36.58 ± 0.53 a	34.70 ± 4.13 a	4.70 ± 0.06 a	3.74 ± 0.15 b
Cecina (LI)	14.65	33.23	36.96	4.17	5.16
Feniglia (GR)	13.90	32.83	37.94	3.93	4.87
Calabria (RC)	15.22	30.32	36.39	4.65	5.18
Italy	14.59 ± 0.38 a	32.13 ± 0.91 b	37.10 ± 0.45 a	4.25 ± 0.21 ab	5.07 ± 0.10 a
Spain (Cádiz)	12.37	33.80	45.29	4.14	4.77
Balikesir	12.22	33.32	45.00	4.25	3.74
Bgyuzu	12.60	34.04	43.47	4.01	3.98
Aydin	14.41	36.96	40.12	4.12	4.02
Turkey	13.08 ± 0.68 ab	34.77 ± 1.11 ab	42.86 ± 1.44 a	4.13 ± 0.07 b	3.91 ± 0.09 ab

Mean±SE. Different letters indicate statistically significant differences (p<0.05).



The biplot of the two principal components (Fig. 5) explained 66% of the variability. Differences were observed between pine nuts grown in Chile and Argentina, in Turkey and Israel, which form two groups, differing from the ones from Italy and from Spain. Pine nuts from South America, which are grown in areas with the highest thermal oscillation and rainfall, showed a similar chemical composition. Italian pine nuts are characterized by the highest total dietary fiber and moisture contents. Spanish pine nuts showed a different composition, which was related to the minimum average temperature and average temperature. Israel and Turkey pine nuts showed a similar chemical composition, exhibiting a high protein content. Mineral content is related to the number of dry months, and lipids to the maximum average temperature. Lipids content was superior when maximum average temperature was high.

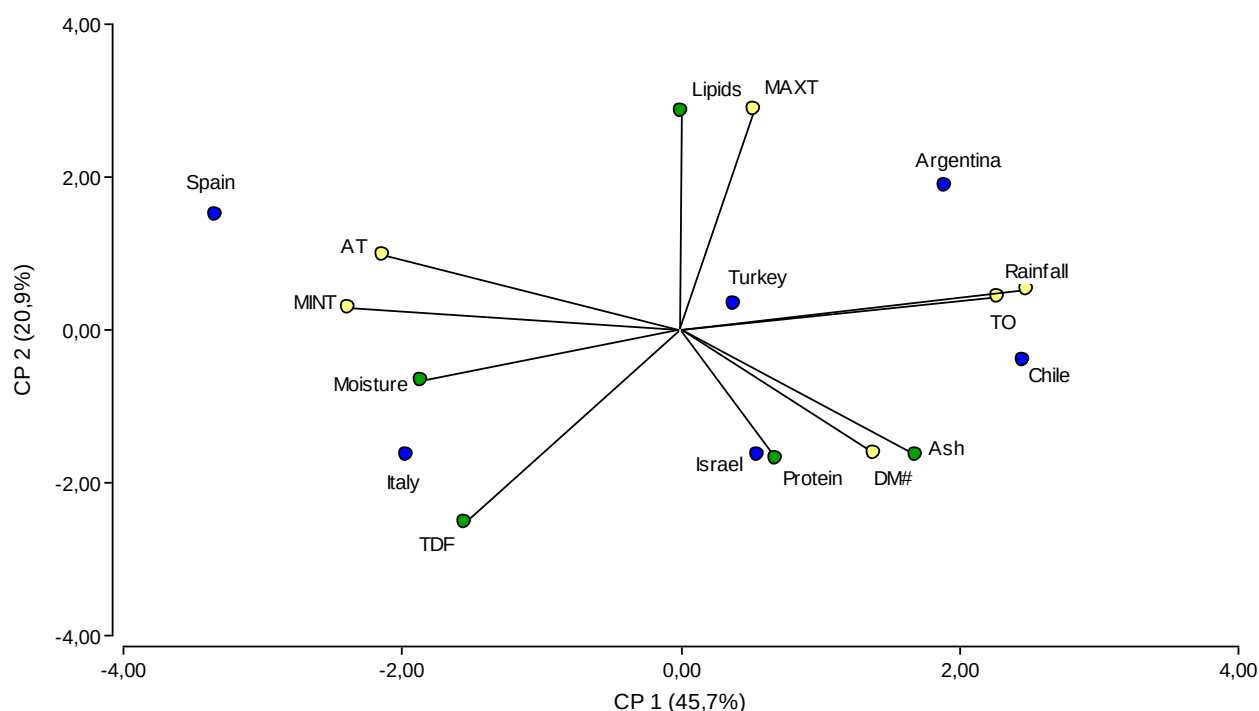


Figure 5. Biplot for chemical composition of pine nuts and climate variables according to country. MAXT: maximum average temperature, AT: average temperature, MINT: minimum average temperature, DM#: dry month number, TO: thermal oscillation, TDF: total dietary fiber.

Significant differences were found among countries for protein ($p=0.0053$), total dietary fiber ($p=0.0003$), ash ($p=0.0008$) and moisture ($p=0.0001$) (Fig. 6, Table 12). Across locations, TDF was significantly influenced by climatic variables, with an increase of 21.1% at a minimum average temperature above 8.4°C (13.2 vs 10.9 g/100g) ($p=0.0032$), and an increase of 22.3% at a maximum average temperature above 22.4°C (14.8 vs 12.1 g/100g) ($p=0.0203$). Minerals were influenced by the number of dry months ($p=0.0063$), with an increase of 9.5% when dry months exceeded 5.5 (4.6 vs 4.2 g/100g). Moisture was influenced ($p=0.0101$) by thermal oscillation, being 22.5% higher when thermal oscillation was below 8.4°C (4.9 vs 4.0) (Fig. 7).

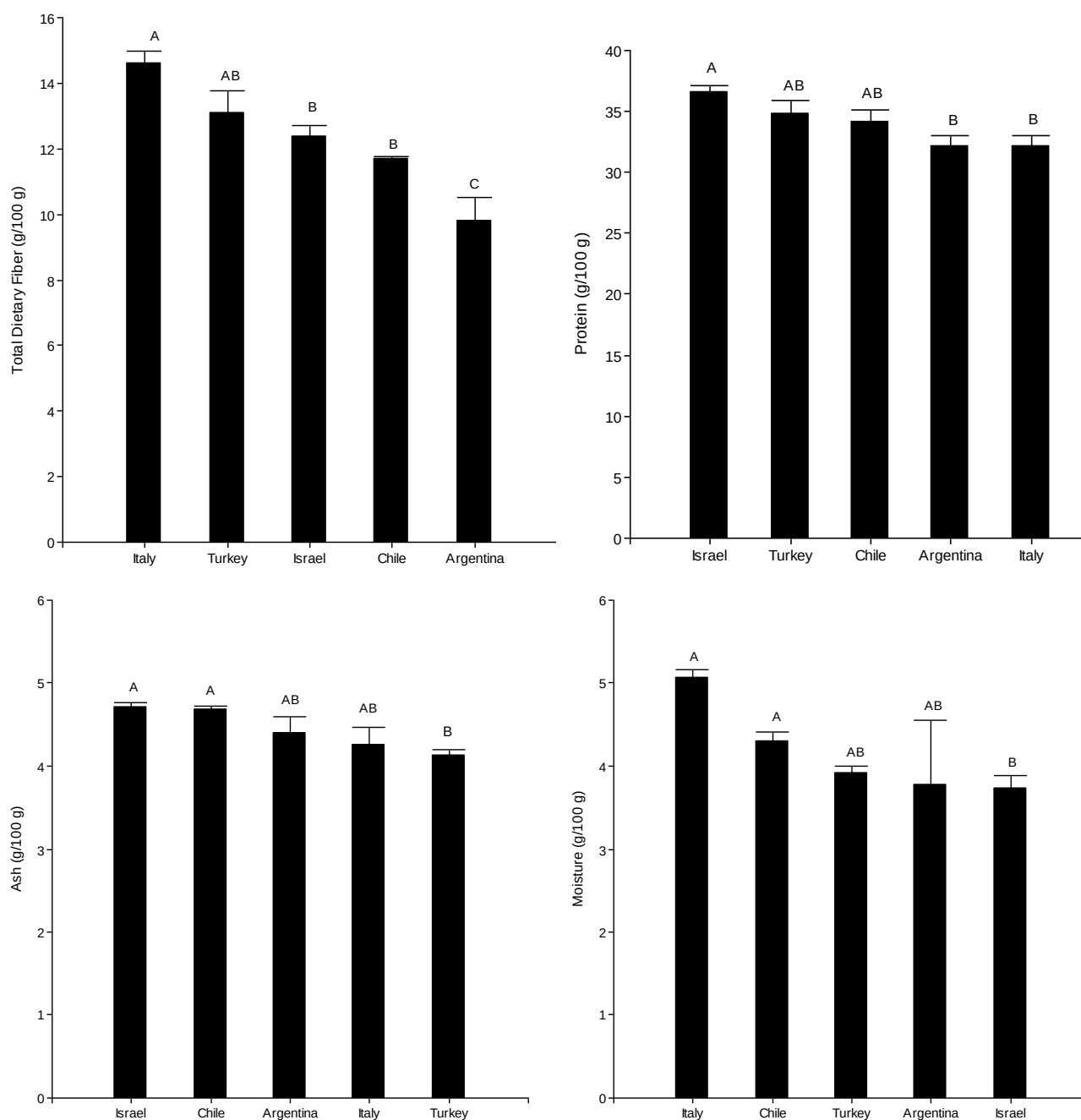


Figure 6. Chemical composition of pine nuts by country. Different letters indicate statistically significant differences ($p<0.05$).

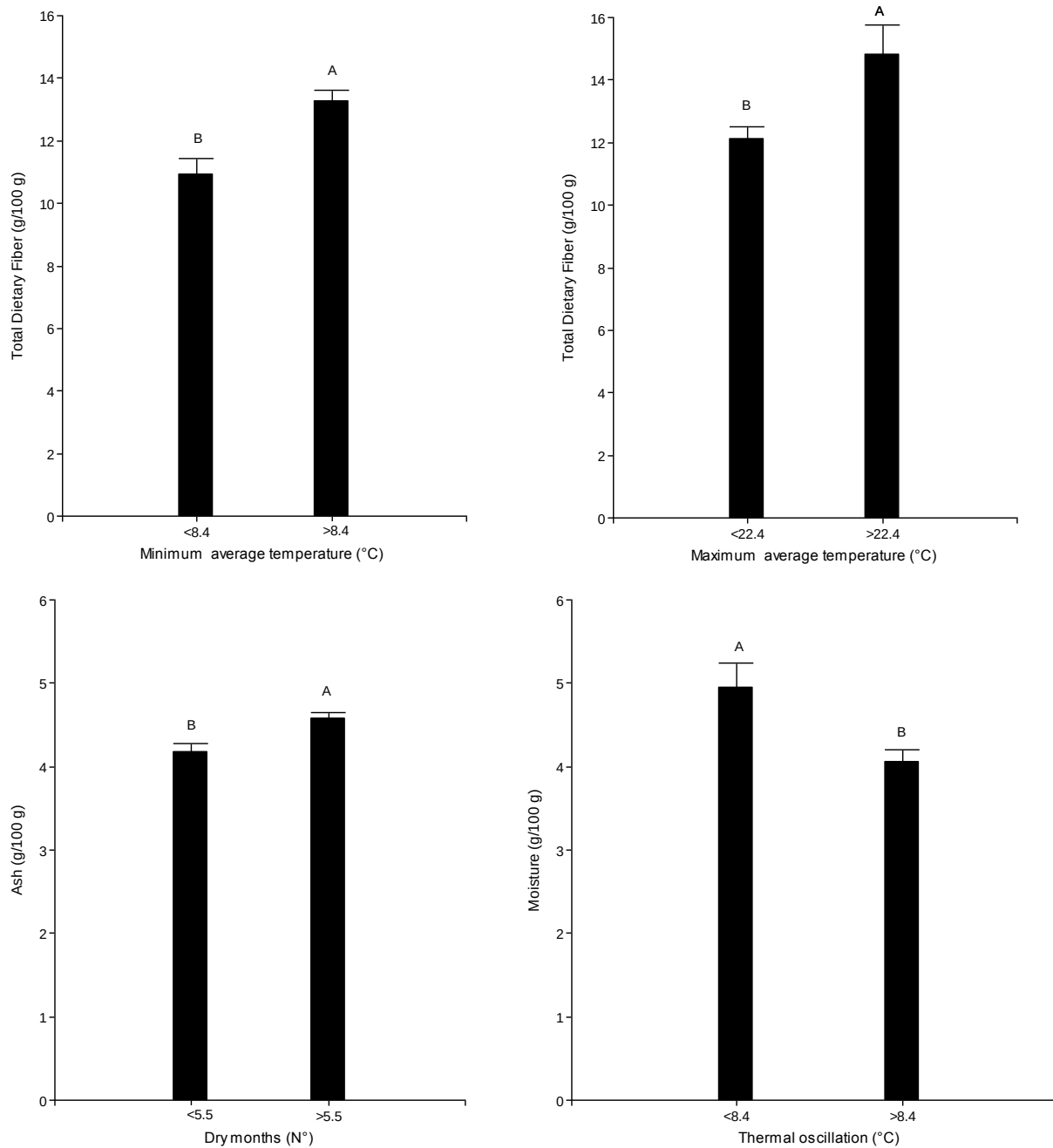


Figure 7. Climatic variables that influence chemical composition across fifteen localities distributed in six countries. Each threshold was detected by CART analysis. Different letters indicate statistically significant differences ($p < 0.05$).

Thermal oscillation: annual average maximum absolute temperature minus annual average minimum absolute temperature.



3.3 Near-infrared spectroscopy (NIRS) technique

3.3.1 Spectral analysis according to sample presentation

Fig. 8 shows the mean spectra obtained for the four different sample presentations in the visible and NIR regions by macrozone. The mean spectrum of the whole pine nuts (SH) is very close to the mean spectrum of the two different flour samples (HF and DF). The greatest differences were found between the IS spectra and the other sample types in the visible region and over 1,900 nm.

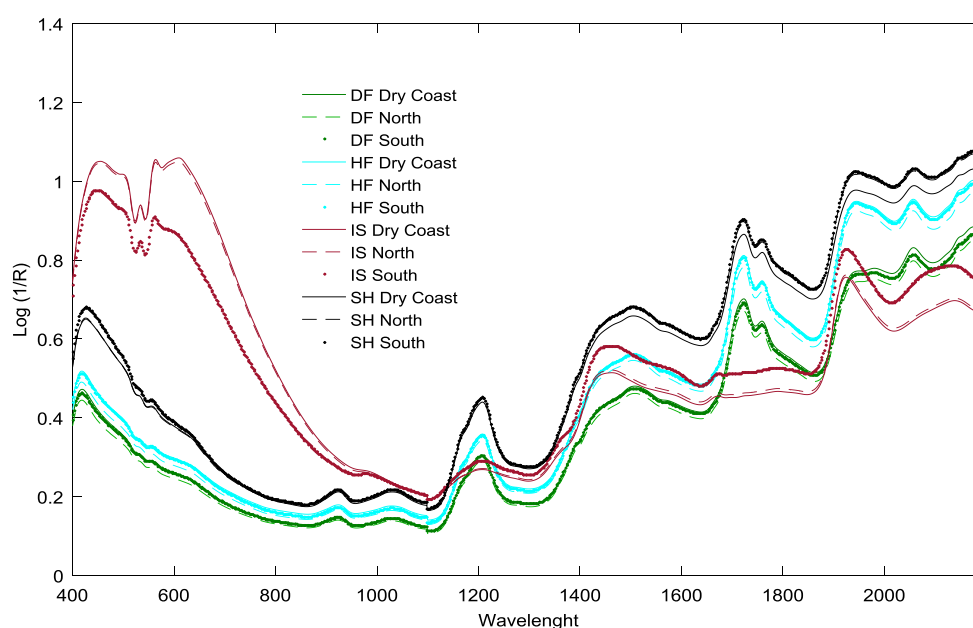


Figure 8. Mean spectra of the three Chilean stone pine macrozones obtained for in-shell (IS), shelled pine nuts (SH), humid (HF) and dried (DF) pine nut flour samples.

In-shell pine nuts spectra showed differences among South MZ and the other two, mainly from 400 to 900 nm (visible range) and 1,100 nm up. Shelled whole pine nuts spectra showed a slightly different pattern for samples from the Dry Coast MZ from 1,100 nm onwards. The two flour samples did not differ among MZs, with a clear superposition of spectra. In the visible region (400–1,100 nm), the differences between the mean spectra showed the same patterns for all type samples, with the exception of in-shell pine nut, given its darker color. Absorption peaks were observed at 550; 1,200; 1,500; 1,720; 1,760; 1,940 and 2,350 nm. The bonds in the main compounds of pine nuts were identified in the absorption peaks corresponding to fatty acids (1,200 nm) and proteins (1,520 nm).

3.3.2 Discriminant models

Models were developed for the entire spectral range (400–2,500 nm) and using only the NIRS region (1,100–2,500 nm). Although the results were similar, the best models were obtained in the 400 to 2,500 nm region for all cases. With just one exception, the best pre-treatment was (SNV+DT), and the best mathematical treatment was the second derivative. The average classification errors in cross validation were 21% for IS, being 14% with the most processed samples (HF and DF). SH showed a 12.2% error, being the best preparation for discriminating among the three macrozones.

Fig. 9 shows the two-dimensional distribution of the spectral populations of pine nuts in the best-selected model at the country scale, as well as the previously defined separation into three growth macrozones; the best models for each case are described in detail in Table 13. In all cases, an overlap of the Dry Coast MZ with the other two is evidenced, which is expected given its size and intermediate location.

Fig. 10 shows the predicted vs the actual MZ origin for the preparation of SH sample, the one that obtained the best results in terms of classification error.



Table 13. Models for the classification of different pine nut presentations for three Chilean growth macrozones; spectral range 400–2,500 nm

Sample type/ Geographical origin	In-shell (IS)		Shelled pine nuts (SH)		Humid flour (HF)		Dried flour (DF)	
	Number of misclassified samples	Partial classification error (%)	Number of misclassified samples	Partial classification error (%)	Number of misclassified samples	Partial classification error (%)	Number of misclassified samples	Partial classification error (%)
North MZ	15/34	21.1	7/34	18.4	5/34	18.4	8/34	19.7
Dry Coast MZ	5/12	28.9	2/12	6.5	6/12	7.9	5/12	14.5
South MZ	4/30	13.1	5/30	11.8	5/30	15.8	3/30	7.9
Total Classification error (%)	21.0		12.2		14.0		14.0	
Best Model	1441		0011		0011		1441	
Latent variables	12		9		10		13	

Table 14. Models for the classification of pine nuts in Chile by two macrozones; spectral range: 400–2,500 nm

Sample type Geographical origin	In-shell (IS)	Shelled pine nuts (SH)	Humid flour (HF)	Dried flour (DF)
	Number of misclassified samples	Number of misclassified samples	Number of misclassified samples	Number of misclassified samples
North' MZ	6/38	7/38	2/38	4/38
South' MZ	8/38	7/38	5/38	3/38
Total Classification error (%)	18.9	18.4	9.2	9.2
Best Model	1441	2105	1441	2105
Latent variables	8	8	8	8

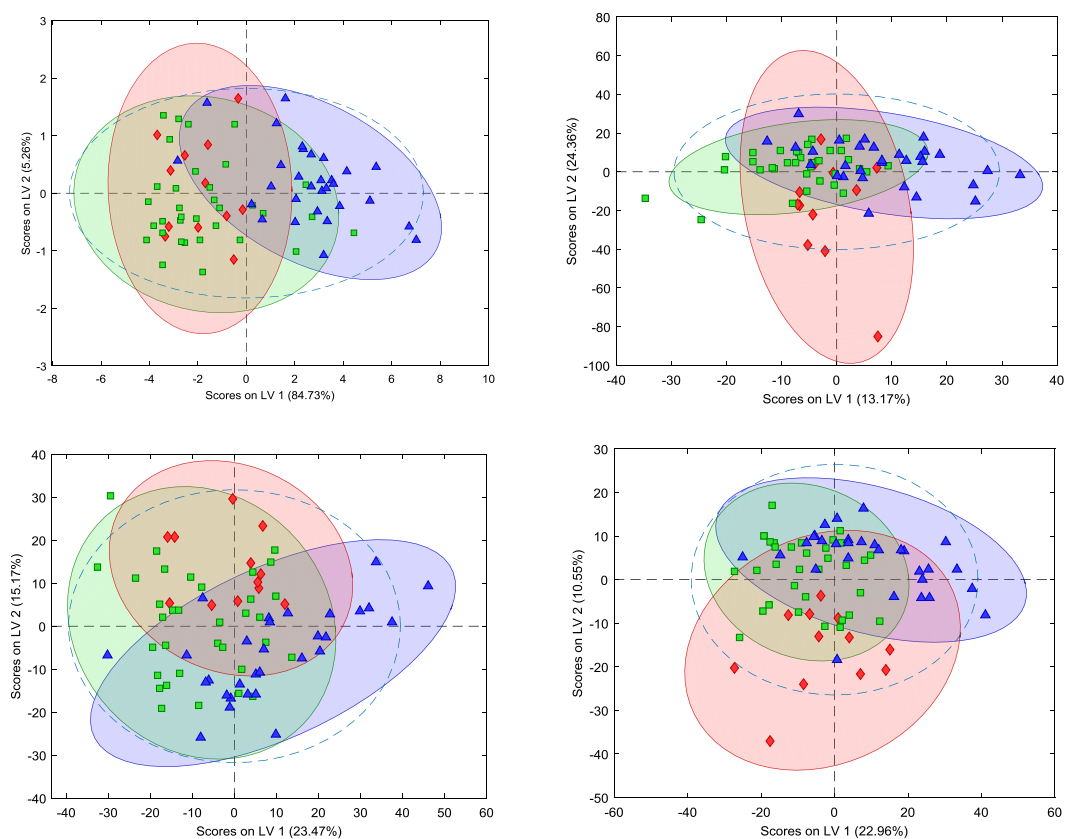


Figure 9. Discriminant PLS plot of spectra of pine nuts from three Chilean growth macrozones in the selected models for in-shell pine nuts (IS) (top left), shelled whole pine nuts (SH) (top right), humid pine nut flour (HF) (bottom left) and dried pine nut flour (DF) (bottom right). Red diamonds: Dry Coast MZ, Green squares: South MZ, Blue triangles: South MZ; dotted line: 95% confidence level.

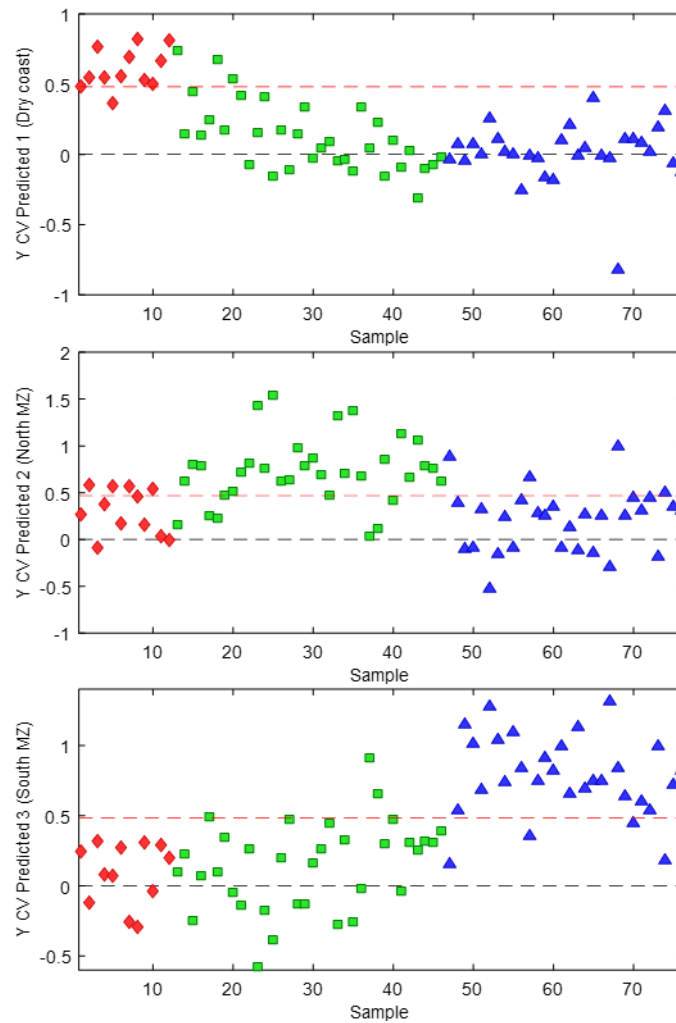


Figure 10. Predicted vs measured cases in cross validation for shelled pine nut (SH) by macrozone (red: Dry Coast MZ, green: North MZ, blue: South MZ). A value of 0.5 distinguishes between classes (ordinate axis).

The small-sized Dry Coast MZ is located at an intermediate position between the North and South MZs, with intermediate climate characteristics (Table 2), with the exception of annual minimum average temperature, which is 0.5°C lower than in the other two macrozones. Based on this fact, we assigned Dry Coast MZ trees to either the North or South MZs, which were extended. To reassign the trees, we used the Maule River as criterion; this influential geographical feature determines the limit between these two MZs in the area where they meet. The two new built macrozones were named North' and South', and each contained 38 samples (Fig. 11).

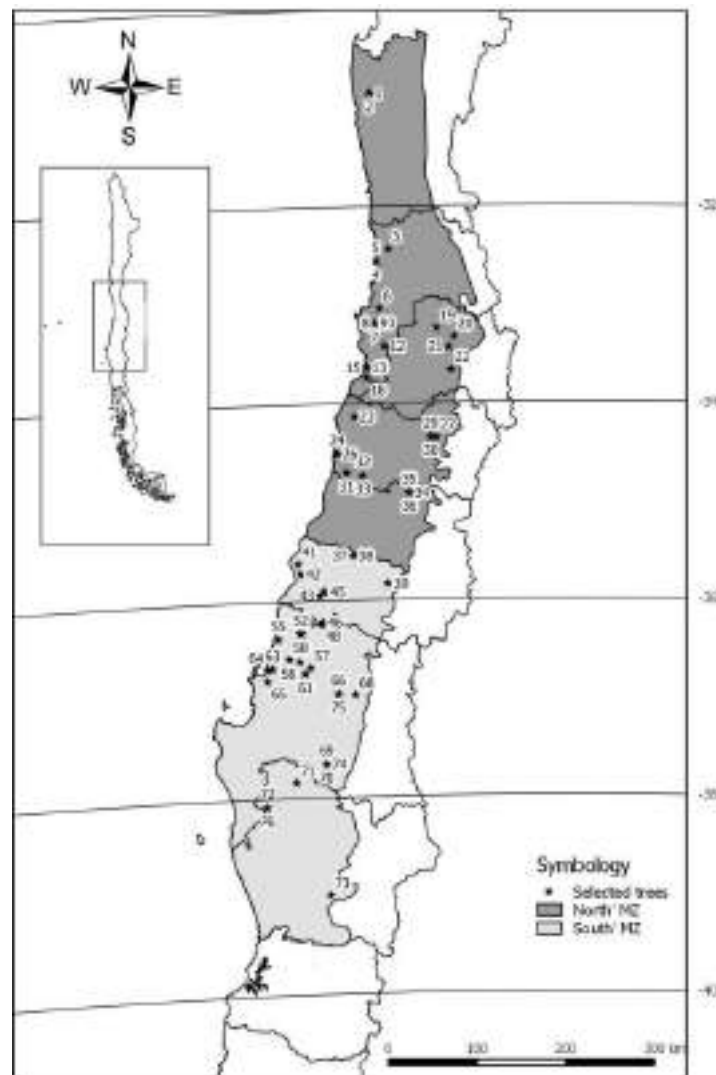


Figure 11. Distribution of selected stone pine trees in Chile according to two newly defined macrozones, named North' and South'.

Fig. 12 shows the two-dimensional distribution of the different samples of spectral populations of pine nuts in the best selected model at the country scale, as well as the separation between the two new stone pine macrozones, with the best models being described in Table 14. As predicted, classification errors decreased in all cases.

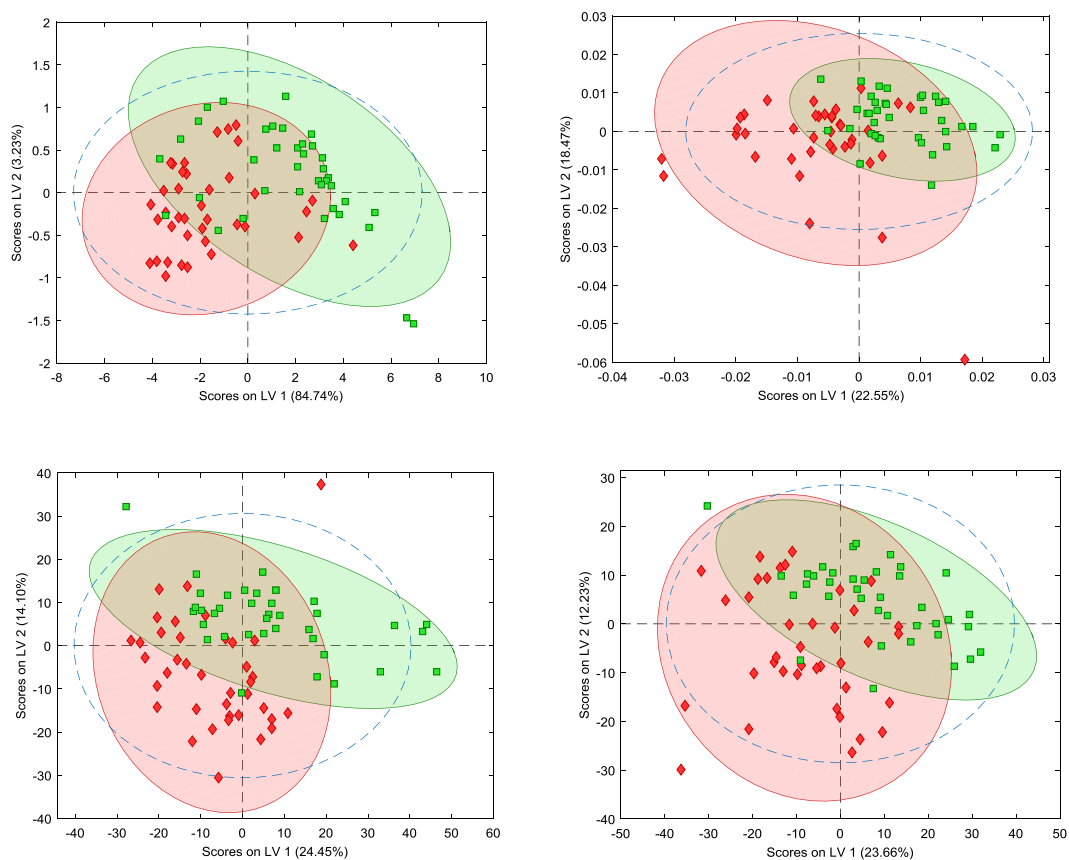


Figure 12. Discriminant PLS plot of spectra of pine nuts from two macrozones in the selected models for in-shell pine nuts (IS) (top left), shelled whole pine nuts (SH) (top right), humid pine nut flour (HF) (bottom left) and dried pine nut flour (DF) (bottom right). Red diamonds: North' MZ, Green squares: South' MZ, dotted line: 95% confidence level.

3.3.3 Country-scale models to discriminate among pine nuts from Chilean macrozones

The best country-scale classification model considering the three macrozones was obtained with shelled pine nuts (SH), with 87.8% of the samples being correctly classified, followed by humid (HF) and dried flour (DF) samples, each with 86.0%. In a second analysis, when discriminating between the two further defined extended macrozones, HF and DF were equally useful, with 90.8% of samples correctly classified in both cases. Fig. 13 evidences that only two samples belonging to the North' MZ were misclassified by the discriminant model in the cross validation.

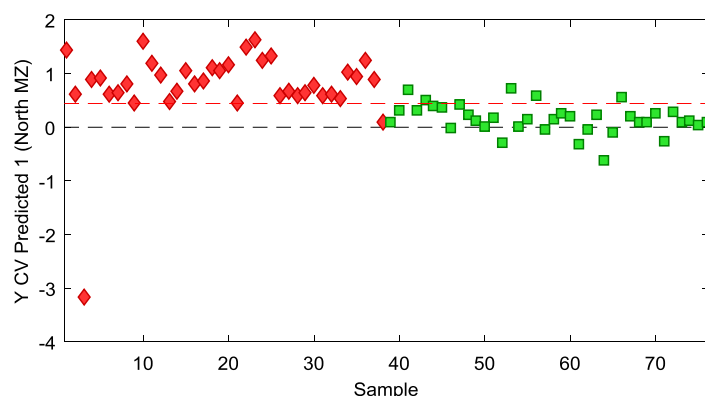


Figure 13. Predicted vs measured cases in cross validation for humid flour (HF) in two macrozones (red diamonds: North' MZ, green squares: South' MZ). A value of 0.5 distinguishes between classes (ordinate axis).

At the most important wavelength for the MZs discrimination, the coefficients of the discriminant models on the visible region (Fig. 14) were close to zero. In the NIR region, the most important absorption bands with the highest variability for pine nuts specifically were bands 1,200; 1,500; 1,720; 1,760; 1,940 and 2,350 nm.

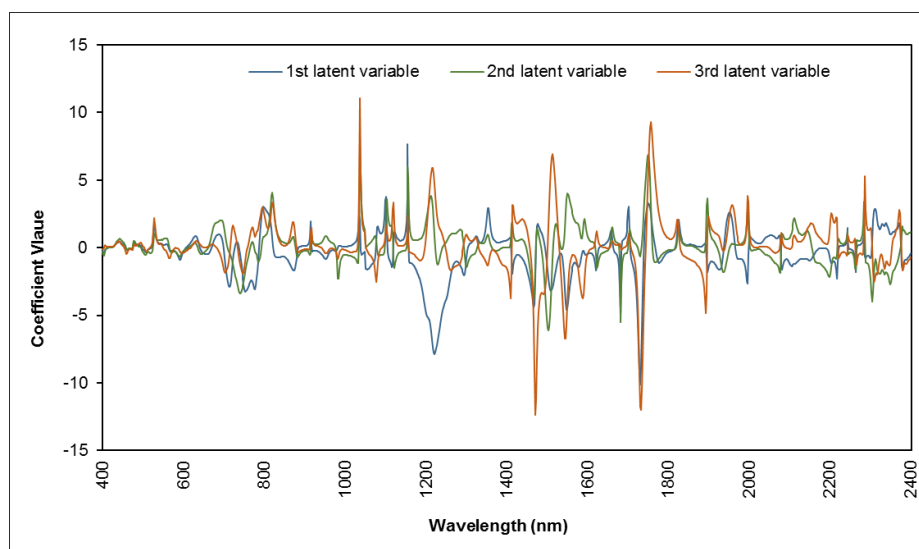


Figure 14. Weights of the bands for the visible and NIR region of the models obtained for pine nut dried flour at a Chilean scale with two macrozones (derivative 2, 10, 5, and 1 with an error of 9.2%).



4. Discussion

4.1 Morphometric and chemical characterization of selected stone pine trees

Some morphometric characteristics measured in selected stone pines cones (length and diameter), in-shell pine nuts (weight and diameter) and shelled pine nuts (size and weight), as well as yield of cone to in-shell pine nuts and to kernel varied among macrozones, as previously reported for other forest species (Agundez *et al.*, 1992; Valero *et al.*, 2012). Selected trees from the South MZ had the largest cone size and the highest yield of cones to kernels (4.3%), which is close to the average value reported for Portugal (4.5%) (Barriguinha *et al.*, 2009), and trees from the North MZ had the smallest cones and pine nut number cone⁻¹.

Even though cone size was different among macrozones in Chile, all studied cone traits showed values comparable to the native range of the species.

The average cone number observed for selected trees (105 cones tree⁻¹) is higher than mean inventory plot values reported in Portugal, that range from 29 to 70 cones tree⁻¹ (Pinheiro *et al.*, 2003; Gonçalves and Pommerening, 2012), almost doubled the mean cone number tree⁻¹ found in Chile when considering all trees that bore cones (57 cones tree⁻¹) (Loewe *et al.*, 2016a) and was also higher than productive levels of 70 cones tree⁻¹ in Chilean plantations of 41-60 cm DBH class (Loewe and Delard, 2012). This difference may be due to the climate at the sites where selected trees grow, where some important biometeorological variables comply with thresholds identified by Loewe *et al.* (2016a) as critical for cone production (rainfall during male flowering below 70 mm and minimum temperature during female flowering above 7°C).

The average cone weight (495 g) represents a production of 53 kg tree⁻¹, which is higher than values reported for the main producing countries (Spain, Portugal, Turkey, Italy and Lebanon), with mean inventory plot values ranging from 29 to 70 cones tree⁻¹ in Portugal (Pinheiro *et al.*, 2003; Gonçalves and Pommerening, 2012), and 40 kg tree⁻¹ in Lebanon (Sfeir, 2011). In Spain, according to Calama *et al.* (2011), the most productive trees are the least frequent; the authors reported a high frequency of trees bearing 0-1 kg cones tree⁻¹, a medium frequency with 2-6 kg, a low frequency with 7-25 kg, and almost null above that quantity, with 54% corresponding to null observations, and most of the production being limited to few trees.

In Tunisia, where stone pine plantations cover over 20,000 ha, Boutheina *et al.* (2013) reported 7 cones tree⁻¹, each weighing in average 201.7 g, with 65.2 seeds cone⁻¹ of which 80.5% filled.

No lineal correlation between pine nut number cone⁻¹ and cone weight was observed; this finding is in contrast with results of Calama and Montero (2007) and Boutheina *et al.* (2013), who found a positive correlation of pine nuts and cone dry weight, with heavier cones containing more seeds. However, cone weight was lineally correlated to both in-shell and shelled pine nut weight, as stated by Álvarez (2010).

Our results indicate that cones of selected Chilean stone pine trees are heavier than cones from Portugal, where average cone weight ranges from 227 to 304 g (Gonçalves and Pommerening, 2012) and also heavier than in Spain, where cone weight average reaches 250-350 g (Mutke *et al.*, 2012).

The high cone weight identified in cones of selected trees along Chile may be due to the climate at the sites where these trees grow, where most of the main climate variables (annual average temperature, annual rainfall, annual water deficit) and biometeorological variables (rainfall during growth of 2 year-old conelet and maximum temperature and water deficit during embryo development) comply or are close to thresholds identified as critical for cone weight by Loewe *et al.* (2016a).

Mean cone size was 11.3 cm in length and 9.4 cm in the widest diameter, which is larger than the average 8 cm in length and 5.6 cm in diameter observed by Bilir (2009) in Turkey. Average number of pine nuts cone⁻¹ (100) is higher than the values reported for Turkey (93 pine nuts cone⁻¹) (Bilir, 2009) and Portugal (78 pine nuts cone⁻¹) (Evaristo *et al.*, 2008). In-shell pine nuts cone⁻¹ values of selected trees in Chile may be due to the climate at the sites where these trees grow, where most of the main climate variables (autumn maximum temperature and annual water deficit) and biometeorological variables (rainfall during growth of 3 year-old cone and cone ripening) comply or are close to thresholds identified as critical for IS by Loewe *et al.* (2016a).

The average pine nut weight (0.94 g) was different across macrozones, but in all areas higher than the value reported by Mutke *et al.* (2012) (0.6 g). In-shell pine nut diameter and length were 1.0 and 2.0 cm, respectively, with the greatest diameter being observed in the North MZ; similar values (0.9 and 1.9 cm, respectively) were reported by Álvarez (2010). In Spain, Cárcel *et al.* (2012) reported an average of 1.7 cm in length and 0.8 cm in width (at



9.8% moisture), which increased by 3.5 and 5.8%, respectively, at 25% moisture. The pine nut shell is a rigid woody material in which dimensional changes caused by moisture are negligible; therefore, for the industrial cracking an excess of water during the soaking period does not reduce rupture energy, but increases drying costs.

Mean shelled pine nut number per cone (94) was lower than the value of 101 reported by Álvarez (2010). The lower kernel number cone⁻¹ observed in isolated trees from the North MZ may be due to a lack of pollen during the pollination period, being stone pine absent in most of the area. Average kernel weight was 0.21 g across the country, which is similar to the value of 0.23 g reported by Álvarez (2010). In Turkey, Bilir (2009) reported a total weight of 7.4 g cone⁻¹, whereas in this study it was 2.6-fold higher. Kernel diameter and length values were similar to values previously reported (Álvarez, 2010).

Observed average cone to in-shell pine nut yield reached 19.1%, similar to the one reported for Portugal (19.2%) by Silveira (2012).

Total non-viable pine nuts reached on average 6.6%, with this value being lower than the one reported in Croatia, where 10.8% of pines nuts were empty and 4.6% were rotten or decayed (Jakovljevic *et al.*, 2009); our value was also lower than the one found in Tunisia (Boutheina *et al.*, 2013) where 19.3% are empty seeds, a fact attributed to the attack of *Dioryctria mendacella* Staudinger, and in Spain, where empty seeds have increased from below 10% to 39% (Sousa *et al.*, 2012), and up to 50% (Mutke *et al.*, 2016).

In Chile, of the total of non-viable pine nuts, 16% were damaged, 29% empty and 55% undeveloped (Loewe *et al.*, unpublished data), with the last two categories being most probably due to the lack of pollen observed in many plantations. No damages to the endosperm with developed embryo, classified as type II by Calama *et al.* (2015), were observed.

Bigger seeds lead to increased seedling growth, and higher root and shoot ratios, which can improve seedling performance by developing and maintaining a deep and extensive root system that can capture water from deep within the soil profile (Ramírez-Valiente *et al.*, 2009; Singh *et al.*, 2010). In this study a significantly higher seed diameter was detected in the North MZ, the most arid zone; this finding is in agreement with results reported by Gómez (2004), who stated that selection for big seeds in *Q. ilex* -as a consequence of selection for tall seedlings- is likely to be stronger in water-limited environments.

Our results showed that temperature -especially summer average temperature, maximum summer temperature, maximum average temperature and thermal oscillation- was the most important climatic variable for cone, in-shell pine nut and kernel weight across Chilean macrozones, followed by summer water deficit. This result partially agrees with findings reported by Calama and Montero (2007), who stated that mean cone weight is largely related to weather conditions during the spring, immediately before cone maturation.

The analysis of the chemical composition of pine nut kernels from selected trees showed an average lipid content of 40.2%, which is lower than values reported by Ruggeri *et al.* (1998) for Italian pine nuts (50.3%), by Nergiz and Dönmez (2004) and Salgin and Salgin (2013) for Turkish pine nuts (47.4% and 44.9% respectively), and by Venkatachalam and Sathe (2006) in the US (61.7%), but higher than in Algeria (19.8%) (Kadri *et al.*, 2015); this variability could be explained by differences in soil composition among regions.

Protein content (35.5%) was higher than the value reported for most consumed nuts (Sabaté and Wien, 2010) and for nuts from other pines, including *P. cembra* L. (17.5%), *P. cembroides* Zucc. (19.0%), *P. edulis* Engelm. (14.3%), *P. koraiensis* Siebold & Zucc. (17.5%), *P. maximartinezii* Rzedowski (31.3%), *P. monophylla* Torr. & Frém (9.5%), *P. quadrifolia* Parlato ex Sudworth (11.0%), *P. sabiniana* Dougl. (29.0%) and *P. sibirica* Du Tour (17.0%) (López-Mata, 2001), as well as than values reported for *P. pinea* nuts by Kadri *et al.* (2015) for Algeria (14.3%), by Ruggeri *et al.* (1998) for Italy (32.9%), by Evaristo *et al.* (2013) for Portugal (33.8%), and by Nergiz and Dönmez (2004) for Turkey (31.1%).

Mineral content reached 4.4%, which is similar to the value of 4.3% identified by Nergiz and Dönmez (2004), and higher than values reported by Venkatachalam and Sathe (2006) and Kadri *et al.* (2015) (2.5 and 1.9%, respectively).

Most nuts show a higher content of monounsaturated fatty acids (MUFA), mainly oleic acid, but pine nuts exhibit a fatty acid profile in which polyunsaturated (PUFA) are more abundant, with a prevalence of linoleic acid (Nergiz and Dönmez, 2004; Nasri *et al.*, 2005b; Ros and Mataix, 2006; Ryan *et al.*, 2006; Venkatachalam and Sathe, 2006; Ros, 2009; Evaristo *et al.*, 2010; Kornsteiner-Krenn *et al.*, 2013), as also happens in *P. maximartinezii* pine nuts (López-Mata, 2001). MUFA and PUFA are considered more desirable than saturated fatty acids (Sabaté and Wien, 2010).

Oleic and linoleic acids amounted to 74.3% (contributing similar amounts: 36.5 and 37.8% respectively), being the most important contributors to MUFAs and PUFAs, respectively,



as well as to total lipids in seeds. In this study, both acids showed a similar content, differing from data reported by Venkatachalam and Sathe (2006) (27.44 and 46.84% respectively), Evaristo *et al.* (2013) (38.36 and 46.40% respectively), and Salgin and Salgin (2013) (38.60 and 47.60%, respectively), and closer to the 34.63 and 30.67%, respectively, reported by Kadri *et al.* (2015), as well as for the oleic acid value of 39.1% reported by Ruggeri *et al.* (1998).

Saturated fatty acids (palmitic, stearic and arachidic fatty acids) amounted to 16.5%, a value higher than that of 10.9% reported by Evaristo *et al.* (2013) and Salgin and Salgin (2013). Salgin and Salgin (2013) indicated that Turkish pine nut lipid is a rich source of biologically active compounds, consisting of 92% of PUFAs and MUFAs, whereas in the selected Chilean trees that value was lower (78.6%). According to those authors, these differences can be explained by different extractions used and growth conditions.

The mineral profile of *P. pinea* nuts has been reported for several countries, such as for Portugal (Evaristo *et al.*, 2013), Spain (Gómez-Ariza *et al.*, 2006) and Turkey (Nergiz and Dönmez, 2004). Gómez-Ariza *et al.* (2006) and Evaristo *et al.* (2013) observed significant differences in the mineral profile and other chemical components of pine nuts grown in different regions, suggesting that environment and soil types have an important influence, and that this variation in mineral composition may be useful to discriminate between Mediterranean populations of *P. pinea*.

Magnesium is involved in many vital metabolic reactions, and its deficiency is becoming common in Western populations; interestingly, nuts contain more magnesium than any other common edible plant. Potassium intake is beneficial for reducing blood pressure and stroke risk (Segura *et al.*, 2006). Mineral contents of *P. pinea* nuts grown in Chile, which have not been reported previously, account for 3,845 mg kg⁻¹ of magnesium and 8,695 mg kg⁻¹ of potassium. These values are lower than those reported for pine nuts by Vanhanen and Savage (2013) (14,538.7 mg kg⁻¹ of potassium and 7,880.7 mg kg⁻¹ of magnesium) and by Evaristo *et al.* (2013) (8,920 mg kg⁻¹ of potassium and 5,332 mg kg⁻¹ of magnesium), but higher than values reported by Kadri *et al.* (2015) in Algerian seeds (2,700 mg kg⁻¹ of potassium and 3,386 mg kg⁻¹ of magnesium). Magnesium content is considerably higher than those reported for most consumed nuts, which range from 1,210 to 2,920 mg kg⁻¹ (Sabaté and Wien, 2010).

In this study, selected trees located in the three studied macrozones did not differ in chemical components, with the exception of oleic acid, which was higher in the South MZ, and potassium, which was higher in the Dry Coast MZ. This is in agreement with results reported by

Dodd *et al.* (1993) and Rafii *et al.* (1991), who found differences in fatty acids in different *Quercus* spp. populations, and attributed variations in the chemical composition content of oak seeds to possible factors associated with climatic conditions, such as precipitation, temperature and thermal oscillation (Vázquez, 1998).

Our analyses confirmed that *P. pinea* pine nuts grown in Chile possess a wide range of beneficial nutrients to human health, being a good source of proteins, fatty acids and some minerals compared to other seeds.

4.2 Pine nut chemical composition characterization by Chilean macrozones

The contents of ashes, moisture, lipids and proteins exhibited significant differences between macrozones, being a good source of proteins and fats as previously reported by several authors (Ruggeri *et al.*, 1998; Nergiz and Dönmez, 2004; Evaristo *et al.*, 2010). The quantified protein content (34.1%) is higher than a previous determination in the country of 29% (Escalona, 2005) while fat content was lower (44.2 vs 47.7 g 100 g⁻¹).

The total dietary fiber content was even across macrozones (average 11.7 g/100 g), with a predominance of the insoluble fraction, with these values being similar to those reported for pistachio (Lintas and Capelloni, 1992). These results indicate that pine nut kernels grown along Chile have higher contents of proteins, lipids, and dietary fiber than walnuts, almonds and hazelnuts, the most frequently consumed nuts in the country.

Average mineral content was 4.7 g 100 g⁻¹, similar to data reported by Nergiz and Dönmez (2004) (4.5 to 4.9 g 100 g⁻¹), and higher than values reported by Escalona (2005) in Chile (4.3 g 100 g⁻¹) and by Venkatachalam and Sathe (2006) (2.5 g 100 g⁻¹) in the US, showing that pine nuts are a good source of minerals.

Moisture content, an important factor with great influence on pine nut quality that needs to be measured and controlled at the different steps of the production and commercialization chain, was in all cases under 6%, which is the recommended value for a better preservation of kernel quality, and in agreement with the European standard for *P. pinea* nuts (6%±10%) (UNECE, 2013).

Phenolic compounds are beneficial to health due to their anti-inflammatory, anti-cancer, and immune system activator effects, among others (Vislocky and Fernández, 2010; Lutz *et al.*, 2011), and are concentrated in the thin skin that covers dried fruits. Pine nuts are not a good source of phenolic compounds, unlike other tree nuts such as walnuts (Pellegrini *et*



al., 2006), which are consumed with the peel. Average phenolic content observed was 0.34 mg GAE/g, which is lower than values reported by Alasalvar and Shahidi (2008) and Alasalvar and Bolling (2015).

The ORAC values –which quantify the global capacity of antioxidants to inhibit peroxide radicals- observed in Chile (8.5 $\mu\text{mol TE/g}$ in average) are similar to those reported by Alasalvar and Bolling (2015) (7.2 $\mu\text{mol TE/g}$) and others (Wu *et al.*, 2004; Kornsteiner *et al.*, 2006). Even though pine nuts are not a good source of vitamin C, its content is relatively high among dried fruits (Farris, 1983).

Pine nuts are an excellent source of phytosterols, being β -sitosterol the most abundant, accounting for over 60% of total free sterols/stanols, as well as in other edible nuts such as almond, hazelnut and walnut (Segura *et al.*, 2006; Esche *et al.*, 2013). Nasri *et al.* (2007) analyzed seven populations of pine nuts from different Mediterranean countries and reported very high levels of sterols (4,376 mg/kg TL), with β -sitosterol being the most abundant (74%) in all *P. pinea* populations (3,208 mg/kg TL), followed by campesterol (661 mg/kg TL), Δ^5 -avenasterol (296 mg/kg TL), and $\Delta^5,24$ -stigmastadienol (21 mg/kg TL). In Chile, an average value of β -sitosterol of 1,817.8 g/100 g was found, almost three times higher than the value reported by Escalona (2005).

In an analysis of 10 different tree nuts, Esche *et al.* (2013) observed that the levels of tocopherols were the highest in pine nuts (0.33 mg/g nut), which is in agreement with Nasri *et al.* (2009) who reported the presence of various tocopherols in pine nuts at remarkably high levels. We found that γ -tocopherol content, a very good source of vitamin E, is higher than α -tocopherol content, which is in agreement with results from Kornsteiner *et al.* (2006) for most dried fruits.

Shahidi and Miraliakbari (2005) reported that stone pine nut oil contained predominantly linoleic (46%) and oleic (38%) acids, whereas Maritime pine nut (*Pinus pinaster* Ait.) oil contain two special fatty acids: pinoleic and sciadonic. Pinolenic acid, very interesting for its role in enhancing cell proliferation, hypertension prevention, blood lipid and sugar level reduction, reduction of allergic reactions and weight control (Sharashkin and Gold, 2004), has been reported in levels ranging from 0.0 to 3.70 g/100 g oil in pine nuts from other pine species (Wolff *et al.*, 1995; Bagci and Karaagaccli, 2004; Nasri *et al.*, 2005a), especially *Pinus sibirica* Du Tour. However, this fatty acid was not detected in this analysis,

even though it has been previously detected in pine nuts grown in Chile (1.03% of methyl esters) (Escalona, 2005), which is higher than the 0.35% reported by Wolff *et al.* (1995).

Results of these analyses are similar to the ones reported by Reza *et al.* (2012) for palmitic acid (6.28 vs 6.20 g/100 g oil), stearic acid (4.30 vs 3.64 g/100 g oil) and arachidic acid (0.82 vs 0.60 g/100 g oil) and higher than values reported by Destailats *et al.* (2010) (5.55; 3.2 and 0.4 g/100 g oil respectively).

Our results are also interesting considering that human populations of several countries are ageing but not enjoying extra years of good health, and there is emerging evidence that omega-3 fatty acids, vitamins B and D and calcium are the most promising nutrients for a healthy ageing, the former for cognitive health and the latter for a better bone health (Ruxton *et al.*, 2016); chemical composition of pine nuts fits most of these needs, confirming their valuable characteristics for dietary nutrition.

In general, the obtained results indicate that *P. pinea* nuts grown in three geographical macrozones in Chile, differing in climatic and growth conditions, exhibit good nutritional and healthy properties. In particular, the South MZ, the coolest and most humid, presented the lowest protein and mineral values. The North MZ, characterized by the most adverse climate variables, showed the lowest moisture and lipids, and the highest of minerals. The transition Dry Coast MZ, characterized by intermediate climate variables, showed the highest values of moisture, protein and lipids, suggesting that the composition of pine nuts is affected by geographical and climatic conditions, as stated by Sagrero-Nieves (1992) and Savage (2001).

All pine nuts grown in Chile have a chemical composition similar to those produced in the Mediterranean zone, in most of their components. Our results are comparable to values of the Spanish reference sample. This information allows us to conclude that the nutritional and health properties of Chilean pine nuts are comparable to those of Spain, the main producer country in the world. These results are complementary to those that report good productive indices of *P. pinea* in the country (Loewe *et al.*, 2016a), and reveal that Chile possess an adequate environment to produce this highly appreciated nut, representing an opportunity for increasing fruit crop diversification.

The chemical composition of pine nuts from selected trees showed lower lipid and mineral and higher moisture and protein contents than the analysis performed by macrozones, which can be attributed to differences among laboratories and methodologies used in both cases, besides biological variability.



4.3 Pine nut chemical composition by country

We studied pine nut chemical composition across six countries, three located in the traditional growing areas (Italy, Spain and Turkey) and three in which there is no commercialization of the seeds (Israel, Argentina, and Chile). The comparison of Chilean samples with pine nuts grown in five other countries, showed that in Chile, protein content (34.1%) is lower than in Israel (36.6%) and Turkey (34.8%), and higher than in Spain (33.8%), Argentina and Italy (32.1% in both countries); lipid content (44.2%) was lower than in Spain (45.3%), and higher than in Turkey (42.9%), Argentina (41.8%), Italy (37.1%) and Israel (34.7%). These results are in agreement with findings of Wolff *et al.* (1997) who stated that fat content varies with sites and environmental factors. TDF values (11.7%) were lower than in all countries with the exception of Argentina (9.8%), with both South American countries showing a lower content.

Here, we characterized pine nuts grown in Israel for the first time; these nuts showed the highest protein content (36.6%), being the location with one of the highest annual average temperature and lowest rainfall, along with Cádiz. Israeli pine nuts also showed interesting values of TDF (12.4%) and minerals (4.7%), and the lowest content of lipids (34.7%) and moisture (3.7%).

Italian pine nuts had the highest total dietary fiber (14.6%) and moisture (5.1%) content, the latter being similar to the value reported by Peruzzi *et al.* (1998) (5.5%); and 37.1% of lipids, which is lower than the value reported by Ruggeri *et al.* (1998) (50.3%) and Peruzzi *et al.* (1998) (42.5%). Proteins reached 32.1% and minerals 4.2%, the former being lower than and the second similar to the ones reported by Peruzzi *et al.* (1998) for that country (39.5 and 4.1%, respectively).

The comparison of our results with those from pine nuts from Bgyuzu (Kozak basin), located in the same area in Turkey reported by Nergiz and Dönmez (2004), showed a higher protein content (34.0 vs 31.6%) and lipids (43.5 vs 42.9%) and similar content of minerals (4.0 vs 4.1%) and moisture (4.0 vs 3.9%). Our results for lipids in Turkish pine nuts (42.9%) are also lower than the 44.9% reported by Salgin and Salgin (2013).

Pine nuts grown in Argentina had 9.8% TDF, 32.1% of proteins, 41.8% of lipids, 4.4% of minerals and 3.8% moisture. Chilean pine nuts showed a similar profile, with lower values of proteins (34.1%) and lipids (44.2%) than previous determinations by INFOR (2009) (35.9 and 46% respectively), higher proteins content than reported by Escalona (2005) (29.0%), and

similar mineral content (4.7%). This similar composition among Chilean and Argentine pine nuts could be explained by similar values of thermal oscillation and rainfall, which are higher than in the the other examined countries.

These differences in the chemical composition of pine nuts grown in different regions would be explained by the environment and soil type variability between regions, as stated by several authors (Gómez-Ariza *et al.*, 2006; Evaristo *et al.*, 2013; Kadri *et al.*, 2015). Interestingly, the influence of climate was observed in some chemical components. In particular, a significant negative influence of high thermal oscillation on moisture was detected, as well as significant positive effects of a long dry period on minerals, and of high minimum and maximum temperatures on TDF.

4.4 Near-infrared spectroscopy (NIRS) technique

Given the precision, versatility and simultaneity characteristics of NIRS technique, it has been widely used to assess fruit quality expressed as different components and attributes (sugar and soluble solids content, acidity, firmness, etc.) (Lin and Yin, 2009), including several dried fruits; Mediterranean pine nuts, however, have not been qualitative analyzed using NIRS.

The technique has been applied in hazelnut to detect kernel flaws (Moscetti *et al.*, 2013; Pannico *et al.*, 2015) and contaminated kernels (Kalkan *et al.*, 2011), and to authenticate Protected Designation of Origin (PDO) fruits. In almonds, NIRS has been applied to detect fungal infection (Liang *et al.*, 2015) and seed damage (Nakariyakul, 2014), and to assess bitterness (Borràs *et al.*, 2014). In walnut, NIRS has been used to monitor kernel quality changes (Jensen *et al.*, 2001); in shea nut, to characterize fat profiles and to segregate different geographic origins (Davrieux *et al.*, 2010); and in peanut, to determine protein and amino acids (Wang *et al.*, 2012), moisture content (Govindarajan *et al.*, 2009) and oil acid value (Rao *et al.*, 2009). In *Quercus ilex* acorn, it was used to assess chemical quality (Valero *et al.*, 2012).

Absorption spectra were characterized for the first time for Mediterranean pine nuts, since the assignments of absorption bands and functional grouping to main compounds of *P. pinea* nuts has still not been reported. The coefficients of the discriminant models on the visible region close to zero confirm that the spectral variability observed in the color does not contribute to the discriminant power of the models. We initially assumed it to be similar to



other nuts, based on the similar patterns described for several dry fruits such as peanut (Govindarajan *et al.*, 2009), walnut (Jensen *et al.*, 2001), shea nut (Davrieux *et al.*, 2010) and others; our results confirm this hypothesis since we observed a correspondence among patterns. In particular, we found records for other dry fruits in 6 out of the 7 observed peaks: 550 nm for hazelnut, 1,200 nm for peanut, almond and shea nut; 1,720 nm for peanut, almond, hazelnut and shea nut; 1,760 nm for peanut and almond; 1,940 nm for hazelnut and shea nut; and 2,350 nm for peanut, hazelnut and shea nut. No coincidence was found for the band 1,500 nm.

In fact, according to the literature, over 950-1,650 nm the peanut spectra ranges contain information on the relative proportions of C-H bonds (usually from fats and oil), O-H bonds -found in water- and N-H, bonds found in protein (Wang *et al.*, 2012). In particular, peaks at 950; 1,120; 1,180; 1,230 and 1,410 nm were identified, concluding that protein and amino acid contents of peanuts can be determined through this technique. For peanut oil, bands 1,726; 1,761; 2,303 and 2,346 nm, associated with CH stretching vibration (Rao *et al.*, 2009) were reported. Govindarajan *et al.* (2009) reported peaks at 1,033; 1,159 and 1,358 nm for whole peanut kernels. For almonds, Liang *et al.* (2015) reported bands 1,210; 1,720 and 1,760 nm as associated with lipid absorbance, and 1,160 and 1,220 nm with cellulose. Nakariyakul (2014) described peaks at 710, 930 and 1,120 nm, being absorbance peaks at 930 and 1,120 nm attributed to oil.

For hazelnuts, Moscetti *et al.* (2014) reported bands 1,120; 1,720; 1,920; 2,120 and 2,250 nm, whose interpretation is complicated because specific bands in this region contain information of various molecular vibration and generic functional groups. Bands at 1,120 and 1,200 nm can be related to the second overtone of C-H stretching vibrations, normally associated with fatty acid content. These authors stated that bands 1,620 and 1,720 nm can be assigned to the first overtone of C-H, which is generally related to unsaturated fatty acids with *cis* double bonds; bands at 1,920 and 1,950 nm are mainly associated with the second overtone of C=O due to protein acid and ester stretching vibrations; and bands at 2,120 and 2,198 nm involve polysaccharides, carbohydrates and lipids. Moscetti *et al.* (2013) identified bands 550-600 nm and 1,150-1,650 nm to be important for classifying hazelnut flaws, with 1,250-1,650 nm being the predominant band, which is related to oil oxidation. According to Pannico *et al.* (2015), the raw adsorption spectra of hazelnut kernels showed peaks at 1,700; 1,900; 2,300 and 2,400, with wavelengths at about 1,400 and 1,900 nm being related to O-H

and C-H tones (i.e. carbohydrates), and between 2,100 and 2,300 nm, being related to double C bond stretching and deformation band of lipids. Davrieux *et al.* (2010) characterized shea nut fat profile, describing wavelength peaks corresponding to absorption bands of chemical bonds specific to fat, such as C-H (1,160; 1,172; 1,214; 1,724 and 2,308 nm), O-H (1,450; 1,916 nm), CH₂ (1,396 nm) and C=C (2,100-2,200 nm). To sum up, most important bands for discriminating the origin of pine nuts from Chile are related to proteins (1,200 and 1,940 nm), lipids (1,500; 1,720; 1,760 and 2,350 nm) and polysaccharides and carbohydrates (2,350 nm).

The results obtained for pine nut classification according to Chilean geographical origins, considering the three macrozones previously described, showed a classification error of 12.2% for whole shelled pine nuts. The chemical composition of the studied pine nuts grown in the three MZs is similar, differed only in oleic acid (higher in the South MZ) and potassium (higher in the Dry Coast MZ). Potassium, as well as other minerals, protein and moisture, were determined with NIRS in alfalfa (González-Martín *et al.*, 2007), and oleic acid was found useful to segregate peanut cultivars (Shin *et al.*, 2010) and to differentiate shea nut populations according to geographical origin (Davrieux *et al.*, 2010).

Accordingly, considering that oleic acid should be at the base of the classification ability of the models, we regrouped trees in two MZs, obtaining lower errors, which amounted to 9.2% for pine nut flour, humid or dried. In fact, the comparison of the model errors for 3 and 2 macrozones (Tables 13 and 14 respectively) showed a decrease in total errors for IS, HF and DF (2.1; 4.8 and 4.8% respectively), but an increase of 6.2% in the case of SH. Our results are in line with results from Tigabu (2003), who identified *Pinus sylvestris* seed sources with 71, 93 and 100% accuracy; but of lower precision than results reported by Prades *et al.* (2012) when discriminating geographical origin of cork planks and stoppers from the most representative cork-producing areas in the world, with 98% of the international cork plank samples and 95% of the Spanish being correctly classified in the calibration and validation stages. This difference could be explained by the exotic origin of stone pine in Chile. The technique use for discriminating cultivation areas is in agreement with findings of Lin and Ying (2009) and with Fu *et al.* (2007), who pointed out that even fruits of the same variety planted in different areas display distinguishable qualities.

Other factors that undoubtedly influenced discrimination have to do with the environmental conditions in which stone pine grows. It is difficult to explain exhaustively the results obtained for geographical origin discrimination, but they are most likely due to a



combination of factors that require further investigation. In fact, Stellacci *et al.* (2012) indicated that growth under different conditions might affect discrimination due to differences in water and nutrient availability and temperature regimes leading to variations in seed composition.

For sample typology, the best-tested sample with the NIRS technique in the analysis with three macrozones was shelled pine nuts (SH), but the error with this sample increased when considering two wider macrozones. This can be attributed to the large and heterogeneous kernel sizes since whole shelled pine nuts sit in only 1 to 2 layers in the measuring cell of the NIRS instrument, generating air gaps between kernels. The size of these air gaps varies from one filling to another, yielding variable transmission or absorbance values for the same sample when emptied and refilled, as pointed out by Govindarajan *et al.* (2009) in peanuts. Even if results in flour can be better than in whole kernels, given the relevance of shelled whole pine nut commercialization, SH presentation would be the easiest to work with sample because it is cost and time-effective. Accordingly, we suggest using a broader sample size in the future to improve predictions with this easy to work presentation, which has been found to be satisfactory for several whole grains and seeds (Williams and Sobering, 1993), almonds (Borràs *et al.*, 2014) and even whole apples (Paz *et al.*, 2009).

Finally, given the interest of growers, processors and sellers for developing a rapid, precise, robust, non-destructive and cost-effective method to evaluate the quality of the components, we built preliminary calibration equations to determine several chemical components of pine nuts (protein, total fat, moisture, ash, potassium, magnesium, as well as some fatty acids, such as palmitic, stearic, oleic, linoleic, linolenic, arachidic, cis-11 eicosenoic and cis-11.14 eicosadienoic). However, given the low quantity of available samples (38) up to date, these equations are not ready for use, with the inclusion of more samples from different origins being among the future challenges.

5. Conclusions

Our results showed that *P. pinea* selected trees from the Chilean macrozones differ in cone and in-shell pine nut size, kernel morphometry and cone to kernel yield. Trees from the Dry Coast display an intermediate position, with lighter kernels; cone to kernel yield was higher than plot average values in Europe.

Chemical composition of shelled pine nuts from selected trees was similar to values reported for the species in the main producer countries, showing several beneficial nutrients to human health; these nutrients were found to be homogeneous among MZs, with the exception of oleic acid, which was higher in the South MZ, and potassium, which was higher in the Dry Coast MZ. Despite the observed differences, and given the outstanding characteristics of the seeds, all selected trees could be a suitable source of reproductive material for afforestation in the country.

Pine nuts grown across the three Chilean macrozones exhibited good nutritional properties, including several bioactive compounds that are important for human health, confirming the advantages of stone pine cropping. Chemical composition of pine nuts was affected by environmental variables, with the South MZ, the coolest and most humid MZ, showing the lowest protein and mineral values. The North MZ with the most adverse climatic conditions for stone pine growth, showed the lowest value of lipids and moisture and the highest of minerals. Finally, the transition Dry Coast MZ, characterized by intermediate climate variables, showed the highest values for moisture, protein and lipids, suggesting that some degree of environmental stress would favor protein content, as also observed in the three locations of Israeli pine nuts.

The comparison among pine nuts grown in six countries showed geographical differences, with four identified groups: Chile and Argentina; Turkey and Israel; and Italy and Spain. Pine nuts from South America showed a similar chemical composition, related to high thermal oscillation and rainfall. Israel and Turkey pine nuts displayed a similar chemical composition, with a high protein content. Italian pine nuts had the highest total dietary fiber and moisture content, while Spanish pine nuts had the highest lipid content, related to the minimum and average temperatures. Mineral content was related to the length of the dry period, and lipids to the maximum average temperature.

Significant differences were found among countries for protein, total dietary fiber, minerals and moisture, variability probably related to climate and environmental conditions of the growing areas. Relevant climatic variables were thermal oscillation for moisture, dry months for minerals, and minimum and maximum average temperature for TDF.

Overall, lipid content was enhanced in Chile by low minimum temperature, and across countries by high maximum temperatures.



NIRS measurements were employed for identifying geographical origins of *P. pinea* nuts according to environmental variables and nut quality. Different sample presentations of pine nut (in-shell and shelled whole pine nuts, and humid and dried pine nut flour) were tested, and multivariate classification models at the country scale were developed using a training set composed of pine nuts harvested from different macrozones where stone pine is growing in Chile. The results indicate that NIRS technology is capable of differentiating between pine nut samples of different geographical origins with errors ranging between 12.2 and 9.2%. The best sample for geographical discrimination among the three Chilean macrozones previously described was shelled pine nuts. Considering the relevance of the shelled whole pine nut market, and the different results that this presentation can show, sample size should be increased to improve predictions with this easy to work presentation, which has been found to be satisfactory for several whole grains, seeds and fruits, including dried fruits.

We suggest examining pine nut quality and origin of a greater number of samples as part of the traceability process using the NIRS analytical approach. Accordingly, the main application of this technique aims to improve traceability, which is essential for controlling quality and origin in the pine nut industry, and is relevant considering the increasing number of allergy cases reported for pine nuts, mostly from other pine species. Implementing this technique at an industrial scale will require the use of training sets tailored to a specific aim, as well as deeper knowledge of the chemical and structural characteristics related to the spectral ranges on which classification models rely. These results should be enriched by developing more robust models that include pine nuts of other geographical origins and of samples collected during several years.

According to the recorded restricted origins of the stone pine genetic material introduced to Chile, it is most likely that the observed differences respond to the environment and to the interaction genotype by environment, rather than to genetics, which would be translated into phenotypic responses to different environments and management practices, confirming the species adaptability. Additional studies on genetic diversity in *P. pinea* would help to clarify this issue and design effective conservation, breeding and productive strategies.

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Supplementary Material



Figure 1S. Samples used in the study. a) Shelled whole pine nuts (SH), b) Humid pine nut flour (HF).

