

Effect of nitrogen and boron fertilisation on chemical composition of *Pinus pinea* L. nut kernels

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Abstract. The Mediterranean pine nut is the seed of the stone pine tree (*Pinus pinea* L.). The kernel primarily comprises protein and fat, being an excellent source of mono and polyunsaturated fatty acids. It is also rich in vitamin B complex, vitamin C, and various minerals. The present study investigated the effects of N and B fertilization on the chemical composition of pine nut kernels. The trial was established in 2018 in a stone pine stand of Ribatejo, Portugal, and four levels of N (0, 30, 60 and 90 kg/ha annually) and two levels of B (0 and 2 kg/ha every two years) were tested. The results obtained from 2018/2019 to 2020/2021 show that fertilization with N and B did not significantly influence the levels of moisture, ash, protein, and total fat, the fatty acid composition (except for myristic acid), as well as the concentrations of P, K, Ca, Mg, Zn, and Cu in pine nut kernels. However, Fe, Mn, and B concentrations decreased with N application. Overall, kernel composition varied over the three years.

Key words: ash, fatty acids, minerals, protein, stone pine

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Efeito da fertilização com azoto e boro na composição química do miolo de pinhão de *Pinus pinea* L.

Sumário. O pinhão Mediterrânico é a semente do pinheiro-manso (*Pinus pinea* L.). O miolo de pinhão é composto maioritariamente por proteína e gordura, sendo uma excelente fonte de ácidos gordos mono e polinsaturados. É, também, rico em vitaminas do complexo B, vitamina C e diversos minerais. No presente trabalho estudou-se o efeito da fertilização com N e B na composição química do pinhão. O ensaio foi instalado em 2018, num povoamento de pinheiro-manso do Ribatejo, em Portugal, e foram testados quatro níveis de N (0, 30, 60 e 90 kg/ha anualmente) e dois níveis de B (0 e 2 kg/ha a cada 2 anos). Os resultados obtidos de 2018/2019 a 2020/2021 mostram que as fertilizações com N e B não influenciaram significativamente os teores de humidade, cinza, proteína e gordura, a composição em ácidos gordos (exceto em ácido mirístico), bem como as concentrações de P, K, Ca, Mg, Zn e Cu do pinhão. Por outro lado, as concentrações de Fe, Mn e B diminuíram com a aplicação de N. De uma forma geral, a composição do pinhão variou ao longo dos três anos.

Palavras-chave: ácidos gordos, cinza, minerais, proteína, pinheiro-manso

Introduction

Pinus pinea L., stone pine or umbrella pine, is a medium-sized evergreen tree. It is distributed throughout the Mediterranean region from Portugal to Syria and it is more present in south-western Europe (ABAD VIÑAS *et al.*, 2016). It occurs mainly in coastal areas, except in Spain and Portugal, where it can also grow at some distance from the sea (ABAD VIÑAS *et al.*, 2016). In Portugal, stone pine occupies approximately 193 thousand hectares (ICNF, 2019), making it the country with the second largest area, after Spain (AGRI-CIÊNCIA, 2014).

Pine nuts have always been part of the Mediterranean diet, along with other nuts. The positive nutritional effects of pine nuts are well known, being rich in healthy fat, protein, vitamins, and minerals and containing a low-glycaemic profile (ALASALVAR & BOLLING, 2015). Additionally, eating pine nuts contributes to higher antioxidant levels, having a beneficial effect on inflammation and lowering the risk of thrombosis and cardiovascular disease (ROS & MATAIX, 2006). The monounsaturated fat in nuts raises HDL (good cholesterol) levels and lowers LDL (bad cholesterol) levels (POONAN, 2022). Pine nuts, along with other seeds and nuts, may help keep blood sugar levels stable because of the balance of fats, fibre, and protein. Furthermore, the magnesium in pine nuts may help improve insulin's ability to uptake glucose (POONAN, 2022).

The growing evidence of the association between the consumption of pine nuts and health benefits has resulted in an increased demand for pine nuts (LUTZ *et al.*, 2017). This rising interest has further highlighted the economic and cultural importance of the stone pine, currently one of the most valuable forest species in Mediterranean agroforestry. In Portugal, the cultivation of stone pine is expanding and, although it can be found across various regions, its presence is particularly strong in the south of the country (ICNF, 2019). The promotion of a product with such good nutritional characteristics can also become an important asset for rural development, as it plays a crucial role in the cultural and gastronomic traditions of these regions. Moreover, their distinctive flavour and versatile culinary applications make pine nuts highly prized by the gourmet food industry.

A balanced fertilisation, combined with other appropriate practices, is now recognized as a management strategy that can enhance pine cone and pine nut production. Despite existing studies focusing on the fertilization of stone pine across various regions, including Portugal (MARCELO *et al.*, 2022; CORREIA *et al.*, 2024), Spain (CALAMA *et al.*, 2007; PIQUÉ & MARTÍN, 2007), and Chile (LOEWE-MUÑOZ *et al.*, 2020; LOEWE-MUÑOZ *et al.*, 2023), further research is

required to enhance our understanding of its impacts. This necessity arises from the variability of results under different experimental conditions, such as dryland and irrigated systems, plant age, and applied nutrients, among other factors.

EVARISTO *et al.* (2010) determined the nut chemical composition and nutritive value of distinct populations of *P. pinea* grown in 27 locations in Portugal and observed significant variability in nut composition, especially in moisture and mineral contents.

Given the importance of N and B for plant growth and development, and the fact that the soils where stone pine typically grows are particularly poor in these nutrients (MARCELO *et al.*, 2022), these elements were selected to evaluate the effects of their application under both field conditions and controlled environments. In a pot experiment conducted over three years, the response to four B levels, with and without N, showed that N fertilization led to an increase in all growth variables (height growth, root collar diameter, total branch length, and terminal bud length), as well as aboveground and root biomass production. Conversely, the application of B had no significant effect on the evaluated parameters (SEMPITERNO *et al.*, 2023).

In order to examine the effect of N and B fertilization on different variables, namely cone production, pine nut yield, and pine nut kernel characteristics, under field conditions, a study was conducted in a *P. pinea* L. stand located in Ribatejo, Portugal. The data presented in this paper including the composition of pine nut kernels (moisture, ash, protein, total fat, fatty acids composition - saturated, monounsaturated, and polyunsaturated -, and minerals) and were obtained in three consecutive years of production (2018/2019 to 2020/2021).

Materials and Methods

Location and soil characteristics

The field experiment was established in a pure stone pine stand planted in 1994 (blocks 1 and 2) and 2004 (block 3). It is located at Vale Montinho, municipality of Coruche, in the Portuguese Region of Ribatejo. The trees were not grafted.

The soil was classified as an *Arenosol Oligoetric (Aric, Ochric)* (WRB, 2015). It had a sandy and loamy sand texture, with a slightly to moderately acidic reaction, low levels of organic matter, extractable phosphorus and extractable magnesium,

low to moderate levels of extractable potassium, and very low levels of extractable boron and potential cation exchange capacity.

Experimental design and treatments

The experiment was arranged into complete randomized blocks with three replications and eight experimental treatments (4 nitrogen levels x 2 boron levels). Each experimental plot was composed of four (blocks 1 and 2) or five trees (block 3). The trial was installed in Spring 2018.

The experimental treatments were as follows: T1 - N0B0 (without fertilization); T2 - N1B0; T3 - N2B0; T4 - N3B0; T5 - N0B1; T6 - N1B1; T7 - N2B1; T8 - N3B1. The nutrient levels used are: N0 - 0 kg/ha; N1 - 30 kg/ha; N2 - 60 kg/ha; N3 - 90 kg/ha N annually; B0 - 0 kg/ha and B1 - 2 kg/ha B every two years. Nitrogen was applied as urea (46 % N) and boron as Tecnifol (11 % B). The fertilizers were applied to the soil, but Tecnifol was previously diluted in 5 L of water to ensure the homogeneous distribution of the product around each tree.

Pine nut samples and chemical analysis

Cone yield per tree was evaluated in winter 2018/2019, 2019/2020 and 2020/2021 in each one of the 24 plots of the experimental site. The annual mean values obtained were 1.2, 1.8, and 5.1 kg per tree, respectively. Ten healthy pine cones were then sampled from each experimental plot in order to evaluate the kernel yield and its chemical composition.

These pine cones were weighed, dried at approximately 50 °C in a ventilated oven for about a week, and then the pine nuts in the shell were removed. These were opened and the kernels separated.

The average kernel yields observed were 2.9 %, 3.7 %, and 2.8 % over the three years of the experimental period, with an overall mean of 3.2 %.

For all chemical analysis, pine nut kernels were ground and homogenized in an electric coffee grinder. The chemical parameters evaluated were moisture, ash, protein, total fat, fatty acids composition, and minerals.

Moisture and ash

Moisture was determined gravimetrically after drying of the homogenised sample in an oven at 103 °C for 4 hours, according to ISO 6496 (1999). Ash was

determined gravimetrically after calcination of the sample in a muffle at 525 °C, according to ISO 5985 (2002).

Protein

Protein content was determined from total nitrogen by applying the conversion factor of 5.3 (MARIOTTI *et al.*, 2008).

In 2018/2019 total nitrogen concentration was determined using the Kjeldahl method: approximately 500 mg of ground material were digested in tubes with Kjeldahl catalyst tablets and 10 ml concentrate sulphuric acid, thus converting nitrogen compounds present to ammonium sulphate. The temperature program followed in the digestion block was 30 minutes at 185 °C and 60 minutes at 410 °C. After adding 50 ml of 40 % sodium hydroxide to the digestion solution, the produced ammonium is evaporated by distillation as ammonia. This was condensed in the cooling system and flowed into a conical flask containing 20 ml of 2 % boric acid solution. This solution is analysed for ammonia by titration with 0.025 M hydrochloric acid.

In 2019/2020 and 2020/2021 total nitrogen determination was performed using an Elemental Analyser based on dynamic flash combustion. A precisely weighed amount of vegetal sample (approximately 6 mg) was introduced into a combustion reactor under a pulse of O₂ flow at 950 °C. The resulting gases were carried by a flow of helium, separated in a Gas Chromatography column (GC), and quantified using a Thermal Conductivity Detector (TCD).

This new equipment began operating in 2019 and was chosen for its ability to provide faster analyses. In a specific year, different experimental treatments were compared using the same analytical method.

Total fat

For fat determination, 2 g of the homogenized samples were placed in a Soxhlet apparatus and lipids were extracted with petroleum ether, under reflux, for 4 hours. The extract was evaporated in a rotavapor at a temperature of 40 °C and placed in an oven at 80 °C for a period of 45 minutes. After cooling, the extracted fat was weighed to calculate the fat content.

Additionally, an aliquot of the extracted fat was used for fatty acid composition analysis.

The results of moisture, ash, protein, and total fat are referred to as g per 100 g of dry matter (DM).

Fatty acids

For fatty acid profile determination by gas chromatography, fatty acid methyl esters (FAME) were prepared by transmethylation with KOH in a methanol solution 2 N. Separation and quantification of FAME was performed in a gas chromatograph with a flame ionization detector (GC-FID), Trace GC 2000, from Thermo Unicam. The chromatographic column was a DB 23 (J & W, USA) with 60 m length, 0,25 mm I.D. and 0,25 µm phase thickness. The oven temperature was raised from 70 °C up to 195 °C (isotherm of 30 minutes) at a rate of 5 °C/minute. The injector temperature was set at 220 °C and the detector temperature at 280 °C. Helium was used as a carrier gas, at a constant pressure of 70 kPa. Quantification was made after converting the relative areas percentages (% area) into weight percentages of total fatty acids (g/100 g), by multiplying % area with the correction factors, calculated from the analysis, of a standard mixture of known composition, in the same conditions (52 FAME-Nu-Chek-Prep Inc., USA).

The following fatty acids were quantified: myristic (C14:0), palmitic (C16:0), hexadecenoic (C16:1 c7), palmitoleic (C16:1 c9), margaric (C17:0), stearic (C18:0), oleic (C18:1 c9), taxoleic (C18:2 c5,9), linoleic (C18:2 c9,12), pinolenic (C18:3 c5,9,12), linolenic (C18:3 c9,12,15), arachidic (C20:0), gadoleic (C20:1 c11), eicosadienoic (C20:2), sciadonic (C20:3 c5,11,14), and behenic (C22:0). From these results, the following sums were calculated: SFA - saturated fatty acids, MUFA - monounsaturated fatty acids and PUFA - polyunsaturated fatty acids.

The Diagnostic Index (DI) was calculated with a formula proposed by DESTAILLATS *et al.* (2011): $DI = [(C18:2\ c5,9 + C18:3\ c5,9,12 + C20:3\ c5,11,14) / (C18:1\ c9\ and\ c11 + C18:2\ c9,12 + C20:2\ c11,14)] \times 10$.

Minerals

For the analysis of total phosphorus (P), potassium (K), magnesium (Mg), calcium (Ca), iron (Fe), manganese (Mn), zinc (Zn), copper (Cu) and boron (B), approximately 500 mg of ground material was reduced to ash in a muffle furnace at 500±10 °C for four hours. The ash was dissolved in a 3 M hydrochloric acid solution. The nutrients were determined in the resulting solution by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) (DUARTE *et al.*, 1998).

The results are referred as mg per 100 g of DM.

Statistical analysis

The statistical analysis was performed through a two-way analysis of variance (ANOVA) to evaluate the effects of the experimental treatments on the variables under study. When the ANOVA results indicate a significant effect ($p \leq 0.05$), Tukey's HSD test ($p = 0.05$) was employed to established differences among means. Statistical analysis was conducted using STATISTICA 12 software.

Results and discussion

The results for moisture, ash, protein, total fat, fatty acids, and minerals are presented in Tables 1, 3, and 5 (mean values for each experimental treatments) and in Tables 2, 4, and 6 (mean values for each year).

Moisture, ash, protein and total fat

The results obtained showed that no significant differences ($p > 0.05$) were observed in moisture, ash, protein and total fat due to N and B fertilization.

The mean values of moisture and ash obtained in the experimental treatment are uniform, varying, respectively, between 6.7 to 6.9 g/100 g DM and 4.8 to 4.9 g/100 g DM (Table 1).

Table 1 – Mean values of moisture, ash, protein, and total fat (g/100 g DM) for each experimental treatment obtained over three years

Experimental treatment	Moisture	Ash	Protein	Total fat
T1 – N0B0	6.7a	4.8a	36.8a	47.7a
T2 – N1B0	6.7a	4.9a	36.9a	46.5a
T3 – N2B0	6.7a	4.8a	36.1a	47.8a
T4 – N3B0	6.7a	4.9a	35.6a	47.1a
T5 – N0B1	6.7a	4.9a	37.4a	46.0a
T6 – N1B1	6.9a	4.9a	38.6a	46.4a
T7 – N2B1	6.8a	4.9a	40.1a	46.3a
T8 – N3B1	6.8a	4.8a	37.1a	46.9a
n	68	68	67	68
Mean	6.7	4.9	37.3	46.9
sm (\pm)	0.14	0.062	1.09	0.46

n - number of observations; sm (\pm) - standard error; mean values followed by the same letter, in the column, do not differ significantly ($p = 0.05$) by Tukey's HSD test.

The protein content for each experimental treatment varied between 35.6 and 40.1 g/100 g DM. These mean values are similar or higher with the values reported by other authors for the protein content for pine nuts of *P. pinea*, namely, 31.6 (NERGIZ & DÖNMEZ, 2004), 26.5-37.4 (EVARISTO *et al.*, 2010), 32.1-35.3 (LUTZ *et al.*, 2017), and 32.1-36.6 g/100 g DM (ZULETA *et al.*, 2018).

The higher mean is associated with the elevated values observed in the last two campaigns (2019/2020 and 2020/2021) compared to the first (Table 2), and this difference could be partially attributed to the distinct analytical methods used for nitrogen determination. In the latter campaigns, nitrogen was quantified using an Elemental Analyzer, whereas in the first year, the Kjeldahl method was employed. According to some authors (THOMPSON *et al.*, 2002; JUNG *et al.*, 2003) the values obtained using the Kjeldahl method are generally lower than those obtained with the Elemental Analyzer.

Table 2 - Mean values of moisture, ash, protein, and total fat (g/100 g DM) obtained each year for the set of eight experimental treatments

Year of production	Moisture	Ash	Protein	Total fat
2018/2019	6.9a	4.9b	33.0b	48.3a
2019/2020	6.8a	4.6c	39.2a	47.0b
2020/2021	6.5b	5.0a	39.8a	45.2c
sm (\pm)	0.085	0.038	0.67	0.28

sm (\pm) - standard error; mean values followed by the same letter, in the column, do not differ significantly ($p=0.05$) by Tukey's HSD test.

The pine nut kernels had a mean value for total fat of 46.9 g/100 g DM, ranging between 46.0 g/100 g DM in T5-N0B1 and 47.8 g/100 g DM in T3-N2B0 (Table 1), values that are not significantly different ($p>0.05$) from each other. Throughout the experimental period, the mean values of total fat decreased (Table 2), in contrast to what happened with the pine cone yield, that increased.

Total fat is the most abundant nutrient in pine nuts, followed by protein, as had already been observed by other authors (EVARISTO *et al.*, 2010; LUTZ *et al.*, 2017). However, oil content in pine seeds may vary with the species and environmental factors (NERGIZ & DÖNMEZ, 2004). VALERO-GALVÁN *et al.* (2019) indicates values as high as 58 % for fat in *Pinus cembroides*, specie with great distribution in Mexico, and ZULETA *et al.* (2018) refers different values related to the origin, such as 31 % for fat in *P. pinea* from Israel and 44 % in samples from Chile.

When comparing results for the three years of production, the content obtained for moisture, ash, and total fat are significantly different ($p \leq 0.05$) over this period (Table 2), which probably reflects the influence of climatic conditions.

Fatty acid composition

The proportions of fatty acids in pine nut kernels were not influenced by N and B fertilization ($p > 0.05$), with exception for myristic acid (C14). The highest mean percentage of this fatty acid was obtained in T1-N0B0, without N and B application (Table 3).

The levels of other fatty acids remained approximately constant and are consistent with those reported in the literature for *P. pinea* (WOLFF *et al.*, 1997; NASRI *et al.*, 2005; VANHANEN *et al.*, 2017).

In tables 3 and 4, values for the sum of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), and Diagnostic Index (DI) are also presented.

Table 3 – Mean values of fatty acid composition (g/100 g of total fatty acids) for each experimental treatment obtained over three years

Experimental treatment	C14:0*	C16:0	C16:1 (c7)*	C16:1 (c9)	C17:0	C18:0	C18:1 (c9)	C18:2 (c5,9)	C18:2 (c9,12)	C18:3 (c5,9,12)	C18:3 (c9,12,15)
T1 - N0B0	0.161a	6.22a	0.059a	0.106a	0.085a	4.02a	39.66a	0.143a	44.46a	0.37a	0.65a
T2 - N1B0	0.057b	5.79a	0.104a	0.116a	0.075a	4.06a	39.91a	0.080a	44.55a	0.28a	0.62a
T3 - N2B0	0.061b	6.67a	0.092a	0.116a	0.075a	4.09a	39.82a	0.076a	43.74a	0.27a	0.59a
T4 - N3B0	0.075b	6.69a	0.097a	0.105a	0.074a	4.17a	40.57a	0.112a	43.15a	0.27a	0.57a
T5 - N0B1	0.061b	5.87a	0.107a	0.096a	0.065a	3.96a	39.50a	0.085a	44.83a	0.31a	0.66a
T6 - N1B1	0.056b	6.37a	0.098a	0.121a	0.090a	4.25a	40.51a	0.186a	42.81a	0.27a	0.57a
T7 - N2B1	0.064b	6.17a	0.120a	0.099a	0.079a	4.07a	39.93a	0.072a	44.01a	0.29a	0.62a
T8 - N3B1	0.052b	6.38a	0.104a	0.121a	0.077a	3.97a	40.62a	0.089a	43.37a	0.30a	0.62a
n	45	66	38	63	59	66	66	63	66	66	66
Mean	0.073	6.27	0.097	0.110	0.078	4.07	40.07	0.105	43.86	0.30	0.61
sm (\pm)	0.0097	0.300	0.0164	0.0114	0.0078	0.126	0.490	0.0287	0.669	0.028	0.022

* Two years; n - number of observations; sm (\pm) - standard error; mean values followed by the same letter in the column, do not differ significantly ($p=0.05$) by Tukey's HSD test.

Table 3 - continued

Experimental treatment	C20:0	C20:1 (c11)	C20:2	C20:3 (c5,11,14)	C22:0	SFA	MUFA	PUFA	Diagnostic Index
T1 - N0B0	0.68a	0.86a	0.58a	1.68a	0.17a	11.16a	40.67a	47.82a	0.26a
T2 - N1B0	0.69a	0.90a	0.55a	1.68a	0.17a	10.78a	41.03a	47.77a	0.24a
T3 - N2B0	0.68a	0.86a	0.54a	1.56a	0.17a	11.72a	40.90a	46.79a	0.22a
T4 - N3B0	0.67a	0.87a	0.49a	1.58a	0.18a	11.84a	41.64a	46.20a	0.23a
T5 - N0B1	0.69a	0.88a	0.51a	1.72a	0.15a	10.78a	40.59a	48.13a	0.25a
T6 - N1B1	0.68 a	0.92a	0.48a	1.52a	0.15a	11.58a	41.70a	45.86a	0.24a
T7 - N2B1	0.69a	0.88a	0.53a	1.68a	0.18a	11.23a	41.06a	47.20a	0.24a
T8 - N3B1	0.65a	0.91a	0.51a	1.65a	0.18a	11.28a	41.74a	46.54a	0.24a
n	66	66	65	66	55	66	66	66	66
Mean	0.68	0.89	0.52	1.63	0.17	11.30	41.17	47.04	0.24
sm (\pm)	0.047	0.053	0.030	0.094	0.025	0.321	0.511	0.724	0.013

SFA - saturated fatty acids; MUFA - monounsaturated fatty acids; PUFA - polyunsaturated fatty acids
 Diagnostic Index = $[(C18:2\ c5,9 + C18:3\ c5,9,12 + C20:3\ c5,11,14)/(C18:1\ c9 + C18:2\ c9,12 + C20:2)] \times 10$
 n - number of observations; sm (\pm) - standard error; mean values followed by the same letter, in the column, do not differ significantly ($p=0.05$) by Tukey's HSD test.

Fatty acids were mainly unsaturated, with polyunsaturated (PUFA) mean values always above 45 g/100 g of total fatty acids and monounsaturated (MUFA) mean values above 40 g/100 g of total fatty acids. The two main unsaturated fatty acids are linoleic (C18:2 c9,12) and oleic (C18:1 c9) acids, accounting for more than 83 % of the total fatty acids (Table 3).

The mean value of saturated fat (SFA) was 11.30 g/100 g of total fatty acids. The main saturated acids are palmitic (C16:0) and stearic (C18:0). Being a low saturated fat, its regular consumption helps to reduce the level of cholesterol circulating in the blood and has beneficial effects on inflammation, thrombosis, and vascular reactivity (ROS & MATAIX, 2006). Furthermore, the high levels of oleic acid (approximately 40 g/100 g of the total fatty acids) contribute to a decrease in the susceptibility to oxidation of lipoproteins and, consequently, to a reduction in the inflammatory and degenerative processes. Also, linoleic acid represents about 44 g/100 g of the total fatty acids in pine nuts. This essential fatty acid is associated with a decreased risk of cardiovascular disease mortality (DJOUSSÉ *et al.*, 2003) and can be converted into n-6 eicosanoids, which play an important role in the process of blood coagulation (ROS & MATAIX, 2006).

Pine seed contains several unusual polymethylene-interrupted unsaturated fatty acids with a *cis*-5 ethylenic bond. These are taxoleic acid (C18:2 c5,9), pinolenic acid (C18:3 c5,9,12), keteleeronic acid (C20:2 c5,11) and sciadonic acid

(C20:3 c5,11,14) (WOLFF *et al.*, 1997). The sum of their percentages, relative to total fatty acids, varies from values of 3.1 % (*P. pinea*) to 30.3 % (*P. sylvestris*), according NO & KIM (2013). These characteristic fatty acids enable the calculation of the Diagnostic Index (DI), with a formula proposed by DESTAILLATS *et al.* (2011). This index is a tool that contributes to the taxonomic identification of species in the *Pinus* genus. Stone pine (*P. pinea*) may have DI values of 0.35 and Chinese white pine (*P. armandii*) values ten times higher (VANHANEN *et al.*, 2017). Elevated values of DI have been associated with taste disturbances referred to as “pine mouth” (FARDIN-KIA *et al.*, 2012).

The values of DI obtained for the samples from the experimental treatments in the present study ranged between 0.22 and 0.26, confirming the botanical identification as *P. pinea*.

Table 4 – Mean values of fatty acid composition (g/100 g of total fatty acids) obtained each year for the set of eight experimental treatments

Year of production	C14:0	C16:0	C16:1 (c7)	C16:1 (c9)	C17:0	C18:0	C18:1 (c9)	C18:2 (c5,9)	C18:2 (c9,12)	C18:3 (c5,9,12)	C18:3 (c9,12,15)
2018/2019	-	6.06a	-	0.098b	0.070a	3.60c	40.49a	0.166a	44.31a	0.27a	0.64a
2019/2020	0.064a	6.65a	0.097a	0.122a	0.080a	4.55a	40.65a	0.070b	42.35b	0.30a	0.52b
2020/2021	0.083a	6.10a	0.098a	0.111ab	0.080a	4.07b	39.05b	0.080b	44.94a	0.32a	0.68a
sm (±)	0.0050	0.184	0.0082	0.0070	0.0048	0.077	0.299	0.0176	0.409	0.017	0.013

sm (±) - standard error; mean values followed by the same letter, in the column, do not differ significantly (p=0.05) by Tukey's HSD test.

Table 4 - continued

Year of production	C20:0	C20:1 (c11)	C20:2	C20:3 (c5,11,14)	C22:0	SFA	MUFA	PUFA	Diagnostic Index
2018/2019	0.53b	0.80b	0.47b	1.53a	0.13b	10.43c	41.47a	47.43a	0.23a
2019/2020	0.77a	0.95a	0.52ab	1.72a	0.19a	12.29a	41.84a	45.45b	0.25a
2020/2021	0.74a	0.91ab	0.57a	1.65a	0.19a	11.17b	40.19b	48.23a	0.24a
sm (±)	0.029	0.032	0.018	0.057	0.016	0.196	0.312	0.443	0.0078

SFA - saturated fatty acids; MUFA - monounsaturated fatty acids; PUFA - polyunsaturated fatty acids
 Diagnostic Index = [(C18:2 c5,9 + C18:3 c5,9,12 + C20:3 c5,11,14)/(C18:1 c9 + C18:2 c9,12 + C20:2)]x10
 sm (±) - standard error; mean values followed by the same letter, in the column, do not differ significantly (p=0.05) by Tukey's HSD test.

Throughout the experimental period, significant differences (p≤0.05) were observed in the quantities of some fatty acids: C16:1(c9), C18:0, C18:1(c9), C18:2(c5,9) C18:2(c9,12), C18:3(c9,12,15), C20:0, C20:1(c11), C20:2, and C22:0

(Table 4), revealing the likely influence of variations in climatic conditions during the three years of observation.

Minerals

No significant mean effects of the N and B fertilization ($p > 0.05$) were found regarding P, K, Ca, Mg, Zn, and Cu concentrations. On the contrary, the concentrations of Fe, Mn and B were affected ($p \leq 0.05$) by fertilization (Table 5). The highest Mn concentration was observed when N was not applied (N0) and the highest Fe concentration was observed at the N1 level (30 kg/ha N), followed by N0 (0 kg/ha N). In the case of B, a reduction in its concentration was also observed with N application. The highest B concentration was recorded in the treatment where N was not applied, but B was (T5-N0B1). These results suggest that N fertilization may hinder the translocation of those nutrients to the pine cone seed.

Table 5 – Mean values of P, K, Ca, Mg, Fe, Mn, Zn, Cu, and B (mg/100 g DM) for each experimental treatment obtained over three years

Experimental treatment	P	K	Ca	Mg	Fe	Mn	Zn	Cu	B
T1 - N0B0	1111a	896a	33.3a	474a	10.1ab	16.3a	11.1a	2.64a	2.12ab
T2 - N1B0	1102a	914a	30.9a	482a	10.2a	10.8b	11.3a	2.90a	2.08 ab
T3 - N2B0	1110a	829a	32.3a	465a	9.2b	10.5b	10.4a	2.76a	1.92b
T4 - N3B0	1094a	866a	32.1a	467a	9.5ab	10.2b	11.1a	2.84a	2.20ab
T5 - N0B1	1112a	873a	32.4a	477a	9.5ab	14.4ab	10.9a	2.85a	2.31a
T6 - N1B1	1094a	849a	34.3a	467a	10.3a	11.6ab	11.3a	2.67a	2.01b
T7 - N2B1	1102a	870a	35.4a	464a	9.8ab	10.9b	11.1a	2.96a	2.08ab
T8 - N3B1	1086a	832a	33.5a	467a	9.5ab	12.3ab	10.8a	2.71a	1.96b
n	67	67	67	67	67	67	67	67	67
Mean	1101	866	33.0	470	9.8	12.1	11.0	2.79	2.09
sm (\pm)	14.9	46.1	3.95	6.8	0.20	1.40	0.27	0.197	0.064

n - number of observations; sm (\pm) - standard error; mean values followed by the same letter, in the column, do not differ significantly ($p = 0.05$) by Tukey's HSD test.

Phosphorus was the most abundant element contained in pine nut kernels (1101 mg/100g DM), followed by K (866 mg/100g DM), and Mg (470 mg/100g DM). Subsequently, in descending order, were Ca, Mn, Zn, Fe, Cu, and B, as observed also by EVARISTO *et al.* (2010).

Manganese and Zn were the most important microelements, with mean values of 12.1 and 11.0 mg/100g DM, respectively, while Cu and B had the lowest mean concentrations (2.8 and 2.1 mg/100g DM, respectively).

Minerals are important for several body functions, namely regulating blood pressure, influencing muscle and nerve function, contributing to bone formation and regulating body's water balance (WEYH *et al.*, 2022).

Being a good source of minerals, mainly P, K, Ca, and Mg, pine nuts are an excellent food for a balanced diet. They can present variations in mineral composition depending on different soil and climatic conditions, and values in literature show some differences (YALIM *et al.*, 2022). Higher mineral contents (P, K, Mg, Ca, Fe, Zn, and B) were observed for *P. pinea* nuts by VANHANEN & SAVAGE (2013) in New Zealand, and lower values were obtained by NERGIZ & DÖNMEZ (2004), in Turkey. EVARISTO *et al.* (2010) found variability in the composition of pine nut kernels, especially in mineral contents, from different *P. pinea* populations in Portugal, reflecting the importance of environmental factors.

The values for all the studied minerals, except for Mn, fluctuated during the experimental period, justifying the significant differences observed between the three years of production (Table 6).

Table 6 – Mean values of P, K, Ca, Mg, Fe, Mn, Zn, Cu, and B (mg/100 g DM) obtained each year for the set of eight experimental treatments

Year of production	P	K	Ca	Mg	Fe	Mn	Zn	Cu	B
2018/2019	1153a	896a	27.7b	514a	9.3b	12.9a	11.6a	3.12a	2.23a
2019/2020	1053c	951a	32.4a b	431c	10.2a	11.1a	11.2a	2.69b	2.11b
2020/2021	1098b	752b	38.9a	467b	9.8ab	12.4a	10.3b	2.55b	1.92c
sm (±)	9.1	28.3	2.42	4.2	0.12	0.86	0.17	0.121	0.039

sm (±) - standard error; mean values followed by the same letter, in the column, do not differ significantly ($p=0.05$) by Tukey's HSD test.

For most of the evaluated variables, the effect of the "year of production" was more pronounced than that of the different fertilization treatments, revealing the expected influence of variations in climatic conditions (humidity, temperature, radiation, among others) over the three years of observation. Indeed, based on data from the meteorological station located at Quinta Grande, in Coruche (located 4 km away from the experimental plot), precipitation values varied considerably, with lower levels recorded in 2019 (340 mm) and 2020 (494 mm), and higher values in 2018 (599 mm). Precipitation was particularly low during

the cone growth period (from March to October) of 2019 and 2020. Furthermore, the annual mean temperature increased progressively from 2018 to 2020, reaching 15.5 °C, 15.8 °C, and 16.3 °C, respectively. The average monthly temperature from March to December 2020 was 0.4 °C higher compared to the 2012-2024 time series.

These and other climatic variables, particularly high temperatures and reduced precipitation, induced water stress that affected physiological processes such as metabolite translocation, photosynthetic activity, water-use efficiency, and, ultimately, carbon allocation and reproductive development. In addition to these, other factors, such as biotic constraints like the presence of pests and diseases, may affect the development and composition of pine nuts, and as such, the results obtained.

Conclusions

The data obtained for moisture, ash, protein, and total fat for *P. pinea* nut kernels, with different levels of N and B applied to the soil, across three years of production, did not reveal differences between fertilizations. Total fat was the predominant component (46.9 g/100 g DM), closely followed by protein (44.0 g/100 g). The application of N and B also did not affect fatty acid composition, except for the percentage of myristic acid (C14), which was higher without fertilization.

The low values for Diagnostic Index obtained are a guarantee that disturbances in taste are not expected for *P. pinea* nut kernels. This index may also be a valuable tool to assure that commercial pine nuts are not adulterated with non-edible or with other species of lower economic or gastronomic value.

The concentrations of P, K, Ca, Mg, Zn, and Cu in pine nut kernels were not affected by N and B fertilization, unlike the concentrations of Fe, Mn, and B. The most abundant minerals were P (1101 mg/100 g DM) and K (866 mg/100 g DM).

During the three years of this study, the mean values of most of the evaluated variables fluctuated, revealing that the climatic and, possibly, other factors observed throughout the experimental period affect the composition of pine nut kernels. Nevertheless, further investigations, carried out under diverse environmental conditions, are needed to validate these results.

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