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Nitrogen fertilization and collection periods of pecan tree for cloning by minicutting

Abstract – The objective of this work was to evaluate the effect of nitrogen fertilization doses and propagule collection periods on the cloning of pecan tree (*Carya illinoinensis*) through minicutting. The experiment was conducted in a greenhouse, in collection periods summer 2020/2021, autumn 2021, spring 2021, summer 2021/2022, and autumn 2022, combined with weekly fertilizations with doses of 0, 7.5, 14.5, 19.5, 27.5, and 33.5 mg nitrogen per ministump. The experimental design was in randomized complete blocks, with four replicates of ten ministumps per plot. Considering collection period, higher values were observed for the variables callus formation in summer 2021/2022 (90.17%) and rooting percentage in summer 2020/2021 (83.25%) and summer 2021/2022 (84.72%). Superior results were obtained for the variables minicutting production, number of roots, and length of the longest root in summer 2020/2021 and summer 2021/2022, with a linear increase up to a nitrogen dose of 33.5 mg per ministump. Collection periods with high temperatures and higher doses of nitrogen fertilization result in a better rooting and root development for the cloning of pecan tree by minicutting.

Index terms: *Carya illinoinensis*, callus, rooting, seedlings.

Adubação nitrogenada e épocas de coleta de nogueira-pecã para clonagem por miniestaquia

Resumo – O objetivo deste trabalho foi avaliar o efeito de doses de adubação nitrogenada e de épocas de coleta de propágulos na clonagem de nogueira-pecã (*Carya illinoinensis*) por miniestaquia. O experimento foi conduzido em casa de vegetação, nas épocas de coleta verão 2020/2021, outono 2021, primavera 2021, verão 2021/2022 e outono 2022, combinadas com adubações semanais de 0, 7,5, 14,5, 19,5, 27,5 e 33,5 mg de nitrogênio por minicepa. O delineamento experimental foi em blocos ao acaso, com quatro repetições de dez minicepas por parcela. Ao se considerar as épocas de coleta, foram observados valores mais altos para as variáveis formação de calo no verão 2021/2022 (90,17%) e percentual de enraizamento no verão 2020/2021 (83,25%) e no verão 2021/2022 (84,72%). Resultados superiores foram obtidos para as variáveis produção de miniestacas, número de raízes e comprimento da maior raiz no verão 2020/2021 e no verão 2021/2022, com aumento linear até a dose de nitrogênio de 33,5 mg por minicepa. Épocas de coleta com temperaturas altas e maiores doses de adubação nitrogenada resultam em melhor enraizamento e desenvolvimento da raiz para a clonagem de nogueira-pecã por miniestaquia.

Termos para indexação: *Carya illinoinensis*, calo, enraizamento, mudas.

Márcio Alberto Hilgert[(1 ✉)],
Lucas Ludwig Hoerlle[(1)],
Claudimar Sidnei Fior[(1)],
Marília Lazarotto[(2)] and
Caciara Gonzatto Maciel[(1)]

[(1)] Universidade Federal do Rio Grande do Sul, Faculdade de Agronomia, Avenida Bento Gonçalves, nº 7.712, CEP 91540-000 Porto Alegre, RS, Brazil.
E-mail: marcioahilgert@yahoo.com.br, lucashludwig@outlook.com, csfior@ufrgs.br, caciaragonzatto@gmail.com

[(2)] Universidade Federal de Pelotas, Rua Benjamin Constant, nº 989, Porto, CEP 96010-020 Pelotas, RS, Brazil.
E-mail: marilia.lazarotto@ufpel.edu.br

✉ Corresponding author

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Introduction

Among the species introduced in Brazil for fruit production, pecan tree [*Carya illinoinensis* (Wangenh) K.Koch] is economically important for the production of nuts with a high market value (Boscardin & Costa, 2018; Fronza et al., 2018). In addition, the species presents qualities related to its wood, used mostly for the manufacture of furniture and floors, and can establish a symbiotic relationship with fungi for the production of truffles (Ferrara et al., 2023). Currently, the commercial production of pecan tree seedlings occurs through grafting using rootstocks from seeds, which causes a consequent unevenness in germination, variability between rootstocks, and differences in growth between plants and orcharding (Casales et al., 2018; Vahdati et al., 2020).

An alternative to seedling production through grafting are minicuttings, a propagation technique aimed at forming individuals for clonal plantations using small propagules from mother plants of seminal origin or clones of selected individuals (Joaquini et al., 2021). To obtain these minicuttings, the new shoots of ministumps, which, together, form a clonal mini-garden, are collected and rooted (Lattuada et al., 2016). Several factors contribute to the success of a minicutting, such as the used species, use of growth regulators, type of minicutting, presence of leaves, the environment where the minicutting is performed, substrate composition, nutrition of the mother plant, and collection time (Hartmann et al., 2014).

There is a strong correlation between the success of the propagation of a minicutting and the nutritional status of the ministumps, whose state influences the production and reserve of carbohydrates, hormones, and other metabolic compounds that contribute to plant growth and rhizogenesis (Guirra et al., 2021). Therefore, mineral nutrition influences the development of ministumps, which, subsequently, influences the root formation of minicuttings, with variations across species. Among the nutrients most required by plants, nitrogen stands out due to its participation in the chemical composition of cell elements, such as nucleotides and amino acids, as well as in the formation of nucleic acids and proteins (Emer et al., 2019).

Another determining factor for the success of a minicutting is the season in which it is produced and collected, which is related to the physiological activities of the ministump and, subsequently, influences rooting. The differences due to collection time can be explained by the variation in the rate of meristematic activity and the lignification of propagation-material tissues (Hilgert et al., 2020). Furthermore, there is a complex interaction of temperature and photoperiod with the production of hormones such as auxins and cytokinins, which are directly related to the growth behavior of propagule-supplying plants and the rhizogenesis of minicuttings (Hartmann et al., 2014). Evaluating cuttings from 38-year-old pecan trees, Hilgert et al. (2020) reported callus formation and rooting of 35 and 30%, respectively, which could be higher if minicuttings from younger pecan mother plants were used.

The objective of this work was to evaluate the effect of nitrogen fertilization doses and propagule collection periods on the cloning of pecan tree through minicutting.

Materials and Methods

The study was conducted from 12/29/2020 to 7/20/2022 in a greenhouse at the premises of the Horticulture and Forestry Department of Universidade Federal do Rio Grande do Sul, located in the municipality of Porto Alegre, in the state of Rio Grande do Sul, Brazil (30º29'S, 51º06'W).

The seeds used for the production of the ministumps were collected from six plants of the Barton cultivar from a 12-year-old commercial orchard belonging to the Paralelo 30 company (Cachoeira do Sul, RS, Brazil). The seeds were collected before the natural fall of nuts in April 2020 and, then, were homogenized to form a single batch and subjected to natural drying to 6% moisture.

After drying, the seeds were stratified in vermiculite, with a 60% water holding capacity of the substrate, and stored in a cold chamber, at 4°C, for 90 days (Poletto et al., 2015). After the stratification period, sowing was carried out using collective trays with a volume of 18 L (40x33x16 cm), filled with a 15 cm high layer of medium sand. Forty-five days after sowing, the ministumps were transplanted into individual 1.7 L containers (12 cm diameter and 20 cm height), filled with medium sand, and were placed on benches in the greenhouse, where they were spaced at 15 cm from each other in the row and at 20 cm between rows, totaling 33 ministumps per square meter. To control the growth of the ministumps, pruning started 30 days after transplanting.

The minicuttings were collected every 14 days, always in the morning, from 7 a.m. to 10 a.m. Through a bevel-shaped cut in their lower portion, the minicuttings were removed from the apical part of the branches of the ministumps, with a length of 6.0 to 8.0 cm and a diameter of 4.0 to 6.0 mm. The minicuttings were maintained with two leaves cut in half to reduce transpiration.

The minicuttings were placed in 55 mL tubes, filled with carbonized rice husk substrate, and kept in the greenhouse for 60 days. Irrigation consisted of an intermittent nebulization system, with 15 s of activation every 5 min, from 7 a.m. to 7 p.m., and with 15 s every 15 min, from 7 p.m. to 7 a.m. The temperature inside the greenhouse was monitored on a daily basis using a digital thermo-hygrometer (Figure 1).

The experimental design consisted of randomized complete blocks, in a 6x5 factorial arrangement, with six nitrogen doses x five collection periods, with four blocks of 10 ministumps per plot, totaling 40 ministumps per treatment. The collection periods were: summer 2020/2021, autumn 2021, spring 2021, summer 2021/2022, and autumn 2022. In total, six collections were performed per season; no collection was carried in winter due to the vegetative rest of the species.

The seedlings were fertilized weekly through fertigation, with the application of 50 mL of a nutrient solution per ministump including the treatment substances (Table 1). In the first two weeks after transplantation, the ministumps were irrigated by sprinkling, six times a day, and then by dripping, twice a day at 8:00 a.m. and 5:00 p.m., for 2 min, resulting in a daily water depth of 6.36 mm. The irrigation water used was analyzed at the beginning of the experiment, indicating an average pH of 5.5 and electrical conductivity of 131 µS cm^{-1}.

Budding in the ministumps was evaluated by determining the number of minicuttings per ministump and of minicuttings per collection time. After 60 days, the minicuttings that were removed and planted in the substrates were analyzed for the following variables: callus formation (%), rooting (%), survival (%),

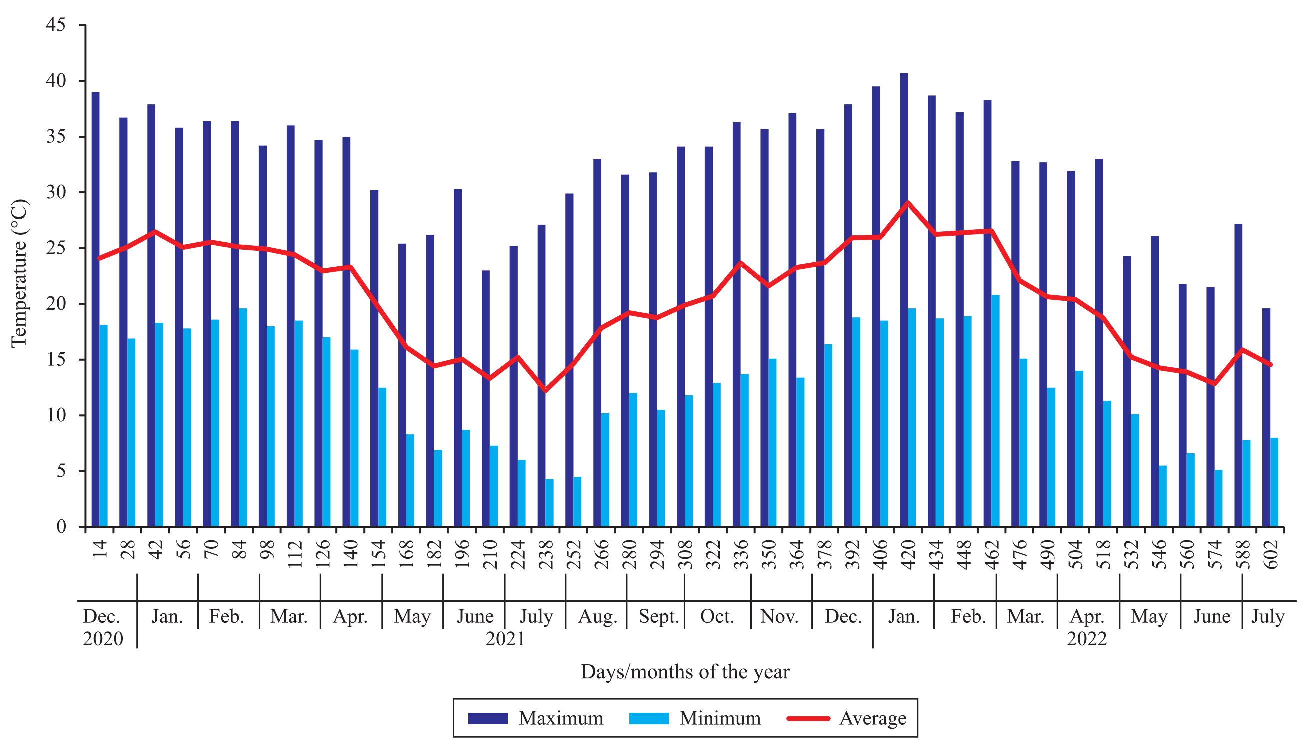

Figure 1. Maximum, minimum, and average temperatures inside the greenhouse with intermittent nebulization applied every 14 days and monthly from December 2020 to July 2022.

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average number of roots, average length of the longest root (cm), and average dry and fresh mass of roots (g).

At the end of the experiment, a destructive analysis was performed to determine the volume of ministump roots and the buildup of mineral nitrogen in the substrate. Root volume was obtained through water displacement in a graduated beaker, whereas the buildup of mineral nitrogen was determined according to Tedesco et al. (1995), as follows:

$$\text{N mineral (mg kg}^{-1}) = \frac{\left(\text{mL H}^{+}\text{ am} + \text{mL H}^{+}\text{ br}\right) \times 70 \times 2.5}{5}$$

where mL H^+ am is the sample titration value, and mL H^+ br is the blank titration value.

The obtained data were subjected to Shapiro-Wilk's normality test and Bartlett's test for homogeneity of variances. Subsequently, a parametric (two-way) analysis of variance was carried out. Data with significance for qualitative factors were subjected to Tukey's test for mean comparisons, at 5% probability, using the R statistical software, version 4.2.1 (R Core Team, 2022), and quantitative factors were subjected to the polynomial regression analysis using the SigmaPlot software, version 10.0 (Systat Software Inc., Chicago, IL, USA).

Results and Discussion

There was a significant interaction between collection periods and nitrogen fertilization doses for the variables minicutting production per ministump, average length of the longest root, number of roots, and average dry and fresh mass of roots. However, for the variables callus formation, rooting, and survival, there was significance only for collection periods.

For the variable survival of minicuttings, better results were obtained for the collection periods summer 2020/2021, spring 2021, and summer 2021/2022 (Table 2). For callus formation, a better result of 90.17% was observed in summer 2021/2022, followed by summer 2020/2021. A similar result was obtained for rooting, with better results in summer 2020/2021 and summer 2021/2022.

The values obtained for callus formation and rooting were high, especially in summer. Similarly, Hilgert et al. (2020) found better results for season of the year (summer) when evaluating cuttings collected from 38-year-old adult pecan trees; however, the obtained rates of callus formation and rooting were lower, i.e., 35 and 30%, respectively. The better results for these latter variables in the present study may have been due to the evaluation of younger pecan mother plants from a 12-year-old commercial orchard.

The superior results observed for several variables in the summer collection periods can be attributed to the highest temperatures of this season. According to Rasmussen et al. (2015), high temperatures influence the rooting of minicuttings, increasing their metabolic activity, which leads to a greater synthesis of auxins, adventitious rooting, and shoot development. A similar behavior was observed in different periods for clones of *Eucalyptus benthamii* Maiden & Cambage × *Eucalyptus dunnii* Maiden (Brondani et al., 2012) and in the propagation of *Campomanesia aurea* O. Berg by minicuttings (Emer et al., 2019). Hartmann et al. (2014) explained that high temperatures favor cell division, in

Table 1. Doses of the fertilizers applied by weekly fertigation through a 50 mL nutrient solution per ministump of pecan (*Carya illinoinensis*).

Fertilizer dose (mg per ministump)	NPK ratio	Total nitrogen dose (mg per ministump)	Nitrogen availability
Without fertilization	0:0:0	0	0
SF[(1)]	4:1:4	7.5	100% NO_3
SF + 15 mg urea	7:1:4	14.5	51% NO_3 + 49% $Co(NH_2)_2$
SF + 26.5 mg urea	10:1:4	19.5	38% NO_3 + 62% $Co(NH_2)_2$
SF + 45 mg urea	13:1:4	27.5	26% NO_3 + 74% $Co(NH_2)_2$
SF + 57.5 mg urea	16:1:4	33.5	22% NO_3 + 78% $Co(NH_2)_2$

[(1)]Standard fertilization per ministump: 12.5 mg potassium chloride (58% K_2O), 50 mg calcium nitrate (15% NO_3 + 19% Ca), 50 mg magnesium sulfate (16% MgO + 32% SO_3), and 4.0 mg MKP (52% P_2O_5 + 34 % K_2O).

addition to a higher production of hormones, such as cytokinins, resulting in the appearance of new shoots.

For the autumn collection period, poorer results were obtained for the variables callus formation and rooting due to the low temperatures of this season, in addition to the fact that pecan trees are deciduous species and enter a period of dormancy. Low temperatures have the effect of delaying plant metabolism, consequently reducing the rhizogenic capacity of minicuttings (Hartmann et al., 2014). According to Kaur et al. (2020), the gradual decrease in temperature initially triggers the process of leaf senescence and, subsequently, the period of endodormancy and pause in the physiological processes of the pecan tree. From the beginning of the dormancy period in deciduous species, there is an accumulation of phenols and inhibitors that cause a reduction in the growth of shoots and in the activity of young tissues and vascular cambium, restricting propagation through the minicutting method, due mostly to a decreased rhizogenesis (Hartmann et al., 2014).

The number of daily sunlight hours in the collection periods varied. A higher number was observed during summer, with 8 hours and 16 min in summer 2021/2022 and 8 hours and 2 min in summer 2020/2021; spring 2021 followed with 7 hours and 41 min. A lower number of daily sunlight hours was verified in autumn, with 5 hours and 33 min in autumn 2021 and 5 hours and 11 min in autumn 2022 (Inmet, 2022). These differences can be explained by the reduction in daily sunlight, combined with colder periods, which can affect the variables related to the rhizogenesis of a species.

According to Petri et al. (2021), the reduction in sunlight is associated with the photoperiod and, consequently, signals the beginning of the dormancy period in deciduous species. Strimbeck et al. (2015) added that the photoperiod triggers several biochemical and physiological alterations in the plant, with changes in the concentrations of carbohydrates and compatible solutes, lipid composition of the membrane, and production of proteins, mainly dehydrins, increasing plant tolerance to periods of cold in the dormancy stage. Leal et al. (2015) concluded that daily sunlight can interfere with the morphophysiological responses of plants, directly affecting the photochemical processes and the biochemical reactions of photosynthesis, plant growth, and the appearance of new shoots. Therefore, the perception and amount of sunlight received by the plants can induce changes in gene expression and hormonal regulation, resulting in varied morphological

Table 2. Survival (S), callus formation (CF), and rooting (R) of minicuttings of pecan (*Carya illinoinensis*) 60 days after collection in five periods (seasons)(1).

Period	S (%)	CF (%)	R (%)
Summer 2020/2021	92.73a	88.32b	83.25a
Autumn 2021	85.69b	57.17d	53.39c
Spring 2021	93.16a	81.37c	78.06b
Summer 2021/2022	94.53a	90.17a	84.72a
Autumn 2022	83.22b	53.84e	49.84d

(1)Means followed by equal letters, in the column, do not differ by Tukey's test, at 5% probability.

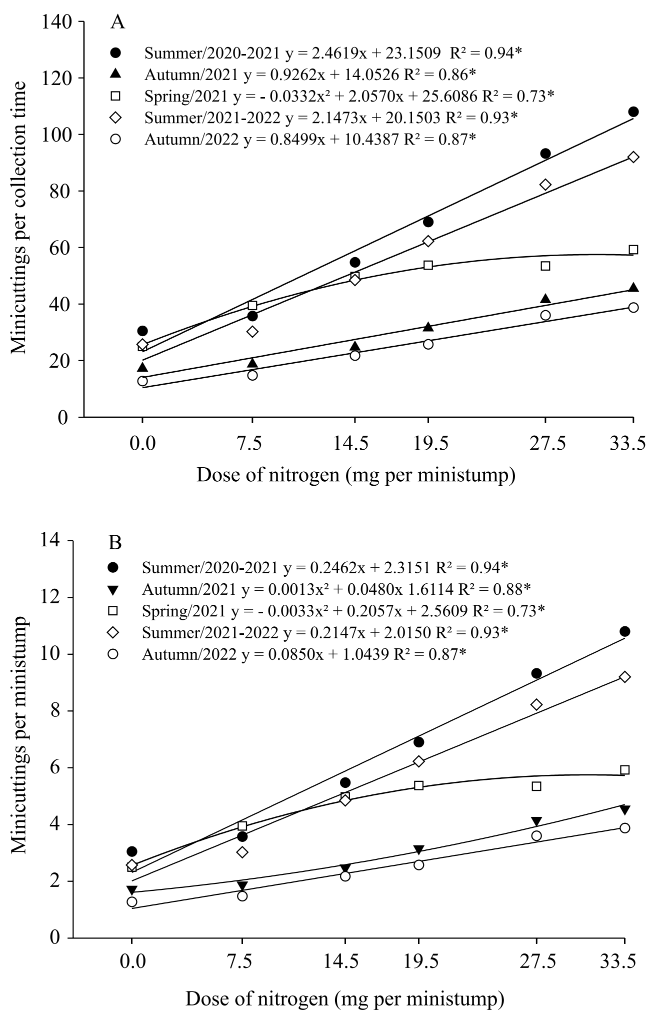

Figure 2. Production of total minicuttings per collection time (A) and production per ministump (B) of pecan (*Carya illinoinensis*), subjected to six nitrogen doses and five collection times. *Significant at 1% probability.

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developments, which are improved in periods with more sunlight (Cho et al., 2019).

For the variables total production of minicuttings in different collection periods and production of minicuttings per ministump, a better result was obtained for summer 2020/2021, followed by summer 2021/2022 (Figure 2). In both collection periods, there was an increase in the studied variables with the increase of nitrogen doses.

For the variables number of roots per minicutting and average length of the longest root, better results were also obtained for the summer collection periods, combined with a higher nitrogen dose. For number of roots, a linear growth was observed for summer 2020/2021 (Figure 3 A), differing from summer 2021/2022, in which there was adherence between the data and the parabola of a quadratic equation, indicating a maximum point for nitrogen doses. A similar effect occurred for the variable length of the longest root for both summer collection periods (Figure 3 B).

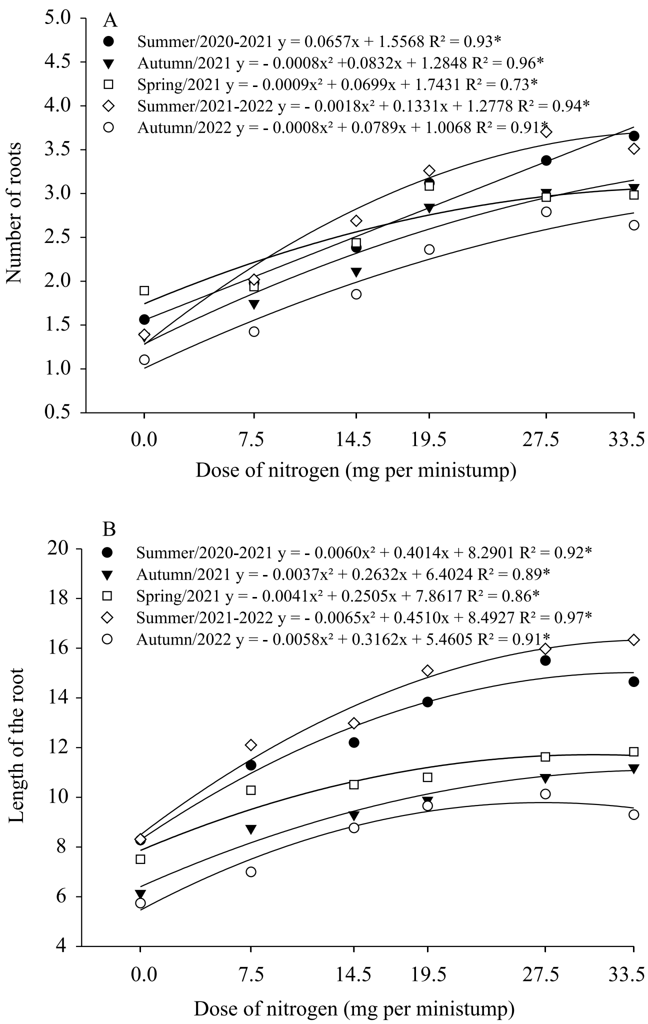

Figure 3. Number of roots (A) and length of the longest root (B) of minicuttings from ministumps of pecan (*Carya illinoinensis*), subjected to six nitrogen doses and five collection times. *Significant at 1% probability.

According to Guirra et al. (2021), nitrogen fertilization favors a greater production of the cytokinin hormone, which is responsible for the formation and development of lateral buds and the growth of new shoots and leaves. Cunha et al. (2008) concluded that the positive effect of nitrogen on the increase in minicutting productivity is related to the function of this nutrient in metabolic pathways and vegetative growth. These same authors reported a linear increase in the production of minicuttings per ministump with the applied nitrogen doses, when propagating the 1128 *Eucalyptus* (*Eucalyptus grandis* W.Hill x *Eucalyptus urophylla* S.T.Blake) clone; however, the optimal point of the nitrogen dose was not reached. In the case of the present study, it was not possible to obtain the point of maximum response of the nitrogen dose for the minicutting productivity variables by collection period and by ministump, as the highest tested dose showed better responses for these variables.

Although nitrogen doses higher than 33.5 mg per ministump can improve the production of minicuttings due to their linear behavior, caution should be taken when increasing the applied amounts of this element. Carvalho et al. (2011), for example, evaluating different doses of nitrogen in the form of urea in the propagation of *E. urophylla*, observed a decline in minicutting production with increasing doses of this nutrient. In the present work, a similar effect was verified for number of roots in summer 2020/2021 and root length in all studied seasons of the year, in which the estimated maximum point was obtained with the highest dose. Zhang & Forde (2000) found that high nitrogen doses, both in nitric and ammonia form, stimulate the formation and elongation of roots, favoring their development process, but are inhibitory at higher concentrations.

The minicuttings showed better results for the variables fresh and dry mass of roots in summer 2020/2021 and, subsequently, in summer 2021/2022, combined with the highest nitrogen fertilization dose of 33.5 mg per ministump, presenting an increasing linear behavior through the polynomial regression, following an increase in nitrogen doses (Figure 4). The increase in these variables is mainly linked to the other evaluated variables related to rooting (number of roots and root length), which showed a similar increase in the same periods, also combined with higher nitrogen doses.

Until the last collection in summer, temperatures favored budding and rhizogenesis. In autumn and at 60 days inside the greenhouse with intermittent nebulization, there was a gradual reduction in temperature (Figure 1), which reduced rhizogenesis and the development of the root system of the minicuttings, as well as the weight of the fresh and dry mass of roots.

For the variables ministump root volume and nitrogen buildup in the substrate, an increasing effect was observed with increased nitrogen doses (Figure 5). At higher doses, the regression line showed a decreasing behavior for ministump root volume; however, the saturation point could not be estimated, differing from the nitrogen buildup variable, which allowed the maximum point of saturation to be determined, at a dose of 27.5 mg per ministump.

The greater root development observed for the ministumps that received the highest nitrogen dose is also linked to the higher accumulation of this nutrient

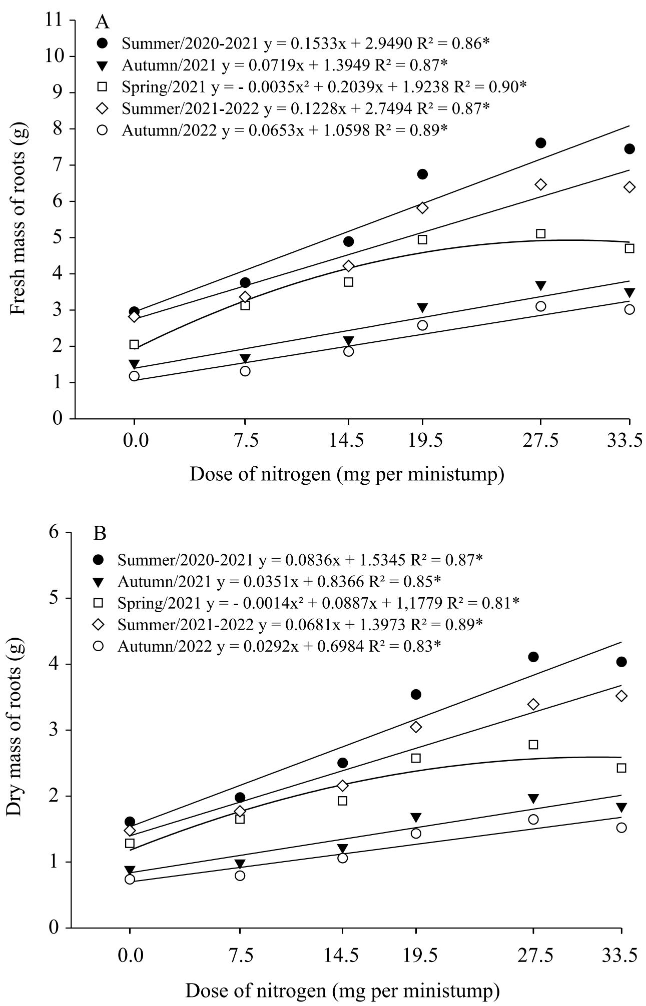

Figure 4. Fresh mass (A) and dry mass (B) of roots of minicuttings from ministumps of pecan (*Carya illinoinensis*), subjected to six nitrogen doses and five collection times. *Significant at 1% probability.

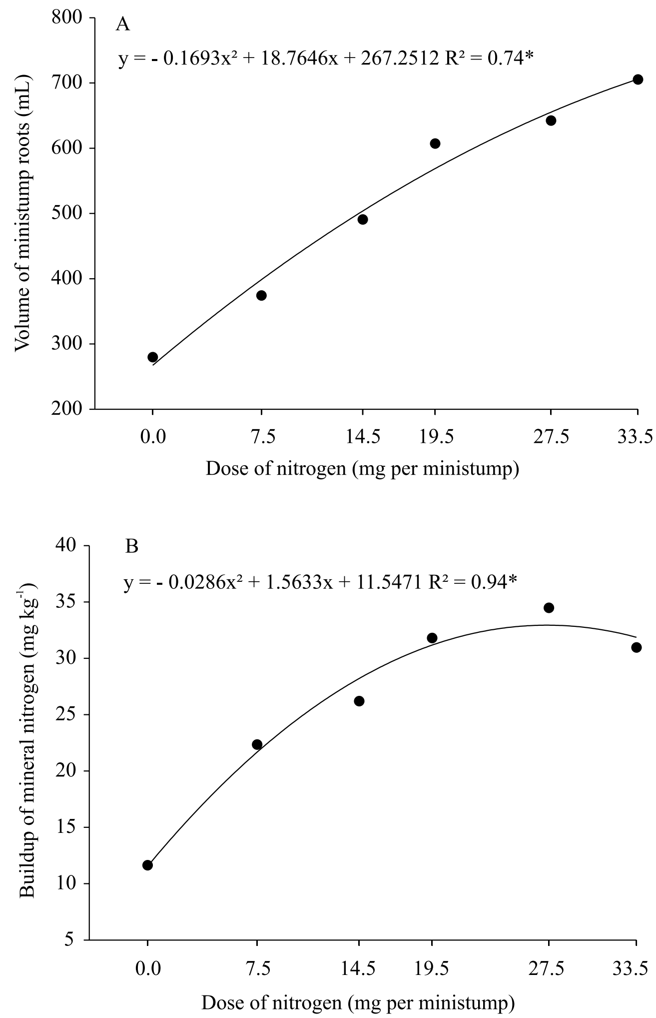

Figure 5. Root volume of pecan (*Carya illinoinensis*) ministumps (A) and accumulated nitrogen in the sand substrate (B) at the end of five collection times under fertilization using six nitrogen doses. *Significant at 1% probability.

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in the substrate throughout and, consequently, at the end of the experiment. Because nitrogen is an essential nutrient associated with metabolic activities of differentiation, its greater availability for ministumps contributes to the process of root development (Picolotto et al., 2015).

The ministumps derived from seminal materials produced high-quality minicuttings and increased the propagation rate of the studied species, compared with that of plants derived from seeds that present unevenness in germination and in seedling formation, resulting in an uneven orchard (Vahdati et al., 2020). Therefore, the minicutting technique represents an important advance for the propagation of pecan, with the future possibility of being used in the selection of superior genotypes from seeds and the retrieval of rejuvenated materials from adult plants or from micropropagation for forming clonal mini-gardens.

The results obtained in the present study for the fertilization of pecan ministumps evidenced the feasibility of producing quality minicuttings with satisfactory rooting responses. However, it is important to conduct additional studies in order to investigate the possibility of reaching the maximum production of minicuttings per ministump using a higher nitrogen dose, which may further favor this method of vegetative propagation.

Conclusions

1. Clonal propagation by pecan (*Carya illinoinensis*) minicuttings is feasible, and propagules (minicuttings) can be produced from young seedlings (ministumps) of a clonal mini-garden.
2. The nitrogen fertilization of ministumps has a positive effect on the rooting of the minicuttings of the studied species.
3. Summer is the best season of the year for producing pecan minicuttings and is also the most favorable for rooting and root development.

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