



## CROP SCIENCE

# The irrigation effect on nuts' growth and yield of *Carya illinoensis*

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**Abstract:** Pecan trees require adequate soil moisture conditions to produce nuts with good yield and quality. Irrigation should be an important considered practice in orchards management plan, mainly in regions with water deficit periods. The objective of this research was to evaluate irrigation on pecan nuts growth and yield. This study was conducted in Uruguay, during the fast development phase until the pecan nuts harvest. Three irrigation treatments were used: 0 L (control), 70 L and 140 L plant<sup>-1</sup> of water every two days, applied via drip irrigation in seven-year-old pecan trees of the cultivar Success. The number of nuts per cluster and nuts' diameter and length were evaluated during nut growth. After harvesting, peeled nuts' width and length were measured, and after peeling, the kernel length, width, height and filling and the kernel and peel percentage were evaluated. According to the results, it was possible to identify that periods of water deficit during pecan nuts development affected the kernel filling stage and size. Irrigation in pecan plants provides greater dimensions and nuts mass. With the use of 140 L plant<sup>-1</sup>, there was an increase in the nut mass of more than 100% in relation to the nuts from plants without irrigation.

**Key words:** Kernel filling stage, pecan nut, pecan tree, water deficit.

## INTRODUCTION

The pecan [*Carya illinoensis* (Wangenh.) K. Koch (Grauke 1991)] is a nut native species of the United States and Mexico, but it has been commercially grown in several countries in South America, including Uruguay, Argentina, Chile, Peru and Brazil, and also countries in other continents, as China, Egypt, South Africa and Australia (Sparks 1991, Wells 2011, Thompson & Conner 2012, Castillo et al. 2013, Wells 2017a, Bilharva et al. 2018).

It is a species that does not tolerate soils with excess humidity for prolonged periods (Madero et al. 2017); however, it is sensitive to water deficit, especially in the reproduction period. Therefore, irrigation is one of the most

important management tool used in nuts production, which can result in increased nuts' size, productivity and quality (Worley 1982, Wells 2015, Ibraimo et al. 2016, Madero et al. 2017).

Water deficit is one of the main environmental stresses that affects agriculture (Colodetti et al. 2018), and water requirement depends on the region, soil, plant age, production load, among others (Wells 2015, Madero et al. 2017). According to Sierra et al. (2001), the minimum annual water amount required for the crop is about 750 mm, while the maximum estimated is about 2000 mm. During the growth period, water supply must be continuous to achieve good production and nuts quality.

In Uruguay, pecan is increasingly cultivated with a commercial interest (Varela et al. 2015);

however, although the annual rainfall is about 1200 mm, the average monthly rainfall in the summer is between 60 and 70 mm in the south of the country (Durán et al. 1999). According to Varela et al. (2015), pecan viability studies in Uruguay demonstrate that this culture is feasible and promising. Nevertheless, the same authors report a clear water deficit during the months of nuts development, for which the irrigation need is fundamental for commercial production.

In this context, the objective of this study was to evaluate the influence of irrigation use on pecan nuts' growth and yield.

## MATERIALS AND METHODS

The experiment was conducted at the «Wilson Ferreira Aldunate» experimental station, INIA - Las Brujas (National Institute for Agricultural Research), in the Department of Canelones, Uruguay (average altitude of 23 m 34°40'15" S and 56°20'27" O). According to Köppen classification, Uruguay climate is type Cfa, with average temperature of 17,5°C and annual rainfall mean of 1200 mm. The predominant soils in the region are a fine (mixed) smectitic thermic superactive Vertic Argiudolls (Durán et al. 1999).

The experiment was conducted from January (beginning of the phenological stage - fast nut development) until the first fortnight of May (harvest) 2018. The experimental design was completely randomized with three replicates, each being composed of one plant. Three irrigation treatments were used: 0 L (control), 70 L and 140 L plant<sup>-1</sup> of water applied by dripping in the crown projection (radius ± 1,5 meters) during a four-hour period (from 4pm to 8pm) every two days. Even when precipitation occurred, irrigation remained on. Seven-year-old pecan trees of cultivar Success were used for the study, planted on 10 x 10 m spacing.

During the period from January to April, the average temperature and monthly accumulated evaporation (Tank "A") and precipitation for the year 2018 and also the average from 2008 to 2018 (Figures 1a and b) were recorded, according to data obtained from the INIA meteorological station, located approximately 400 m from the orchard.

For the analyzes, 20 clusters per plant distributed in the four quadrants of the plant were selected and analyzed during the phenological stages 73 (fast nut growth) until the 79 (period in which the nuts reach the final dimension) of the BBCH coding scale (Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie) (Han et al. 2018). It was also determined the number of nuts per cluster, the nuts' diameter and length, in a period of 15 days, with the aid of a digital caliper.

In the first half of May, nuts were harvested, when they presented the capsule (epicarp) that surrounds the nut opening. The nuts' humidity and mass were obtained soon after harvesting and later dried in an air circulating oven with a temperature of 32°C (± 2°C) for 48 hours. After this, the nuts' weigh and humidity were again determined. The moisture was obtained with the aid of Dickey-John M-3G portable meter and the mass with a digital scale of two decimal places precision.

Three samples with 20 nuts each were used to measure the nuts' width and length with shell, and after peeling them off, it was evaluated the kernel length, width and height, percentage of kernel and shell and kernel filling stage (they were considered filled when fully occupied the shell cavity and little fulfilled when they occupied until  $\frac{3}{4}$  of the shell cavity). In addition, the amount of nuts to reach a kilogram was estimated.

The results were submitted to variance analysis, and the averages of the treatments

were compared through Tukey test at 5% probability, using the statistical program SISVAR (Ferreira 2011).

## RESULTS

According to the climatic data recorded during the research period (Figure 1a) and also based on the average of 10 years, between 2008 and 2018 (Figure 1b), it is possible to observe that during the period from January to April the accumulated evaporation is higher than the accumulated rainfall over the entire period. That is, the water loss by evaporation is greater than

the precipitation during the growth period of the pecan nuts in Uruguay.

During the period between the fast growing nut stage (BBCH 73) and the final dimension stage (BBCH 79), the number of nuts per cluster was reduced from the beginning to the end of the evaluations, however, there was no significant difference between irrigation treatments (Table I).

Fifteen days after the beginning of the evaluations (2018-01-23), the pecans' diameter and length were statistically superior on treatments in which 70 and 140 L of water per plant were applied, compared to no irrigated plants. The nuts' size stabilized on March 25, BBCH 79 stage (Table I, Figure 2).

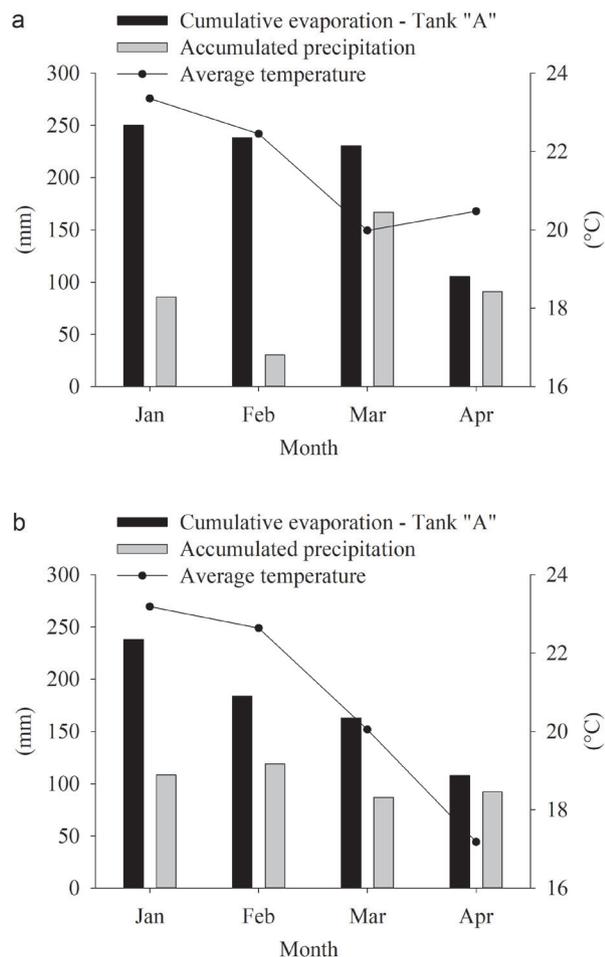
The average nuts' humidity at the harvest time ranged from 19.9 to 21.4% and after drying (48 hours at 32°C), a mean moisture content of 3.9% was obtained, showing no significant difference between treatments (Table II).

The amount of water applied influenced the nuts' width and length, and the use of 140 L per plant provided significantly larger nuts (Table II, Figure 3).

The nuts' width, length and height were also significantly higher when irrigation was used, with no difference between 70 and 140 L (Table II, Figure 3).

Another important aspect in nuts' quality and yield is the kernel filling stage. When no irrigation was used, 48% of the evaluated nuts had fillings classified as partially completed, that is, nuts with up to 75% of the filled shell cavity. However, plants irrigated with 70 L of water, only 5% of the nuts were partially filled, and, when 140 L, 100% of the nuts were completely filled.

Regarding fruit size, there was a significant difference between treatments. Those with 140 L of water per plant, fruit mass, kernels and shell were statistically superior. With the use of 70 L of water per plant, fruits (a) and kernels mass were,



**Figure 1. Cumulative evaporation - Tank «A» (mm), accumulated precipitation (mm) and average temperature (°C) monthly, in the year 2018 (a) and averages between the years 2008 to 2018 (b).**

**Table I.** Average number of nuts per cluster; pecan nuts' diameter and length during the nut growth stage under different amounts of water applied per plant.

Treatment (L) <sup>1</sup>	Date											
	09/01/18		23/01/18		09/02/18		22/02/18		12/03/18		25/03/18	
	<b>Average number of nuts per cluster</b>											
0	3.6	ns	3.6	ns	3.6	ns	3.6	ns	3.0	ns	2.9	ns
70	3.4	ns	3.4	ns	3.4	ns	3.4	ns	3.0	ns	2.7	ns
140	3.7	ns	3.7	ns	3.6	ns	3.6	ns	3.0	ns	2.9	ns
CV (%)	7.0		7.0		7.5		7.5		4.9		4.9	
	<b>Nuts' diameter (mm)</b>											
0	10.3	ns	12.1	b <sup>2</sup>	15.4	b	19.0	b	21.3	b	22.0	b
70	10.4	ns	14.2	a	18.3	a	23.4	a	26.6	a	28.0	a
140	10.6	ns	14.8	a	19.6	a	25.2	a	28.6	a	29.0	a
CV (%)	1.6		2.2		2.0		2.6		3.3		3.2	
	<b>Nuts' length (mm)</b>											
0	18,5	ns	20,1	b	24,3	b	27,3	b	29,0	b	29,0	b
70	19,0	ns	24,3	a	30,9	a	35,7	a	38,4	a	39,0	a
140	19,1	ns	24,9	a	31,8	a	37,3	a	40,2	a	40,0	a
CV (%)	6,1		4,9		2,6		4,1		4,9		4,5	

<sup>1</sup>Water quantity (in liters) applied per plant with interval between applications of two days. <sup>ns</sup>Not significant; <sup>2</sup>Means followed by the same letter in the column did not differ statistically by the Tukey test, at the level of 5% error probability.

respectively, 68.0 and 73.6% higher than fruits from plants without irrigation. At 140 L, the fruit mass was 102.8%, and the kernels mass 113.8% higher than fruits from plants which did not receive irrigation (Table III).

The percentage of kernel per fruit increased with irrigation, obtaining 3.8% and 5.6% increment, respectively with 70 and 140 L of water (Table III).

The required amount of nuts to reach one kilogram was significantly influenced by irrigation, requiring 281, 168 and 139 fruits to obtain one kilogram, respectively with 0 L; 70 L and 140 L of water (Table III). When transformed into percentage values, the number of fruits required to reach one kilogram was 41.2% (70

L) and 51.3% (140 L) lower than the number of fruits needed by plants without irrigation (0 L).



**Figure 2.** Nuts' size on March 25 (BBCH stage 79) under different amounts of water applied to the plants.

**Table II.** Nuts' moisture at harvest time and after drying; nuts' width (W) and length (L) nut with shell; almond width, length and height (H); almond filling (AF) - F = filled; PF = partially filled (3/4 of filled almond), under different amounts of water applied per plant.

Treatment (L) <sup>1</sup>	Nuts' moisture				Nuts in shell				Almond					AF		
	Harvest		Dry		W		L		W		L		H	F	PF	
	---- % ----				----- mm -----											--- % ---
0	19.9	ns	3.9	ns	18.2	c <sup>2</sup>	24.6	c	15.7	b	18.8	c	6.3	b	52	48
70	20.3	ns	3.9	ns	21.1	B	30.6	b	17.7	a	23.9	b	7.3	a	95	5
140	21.4	ns	3.9	ns	22.3	A	32.5	a	18.3	a	26.8	a	7.8	a	100	0
CV (%)	7.6		2.8		5.5		7.7		6.4		5.8		9.3		---	

<sup>1</sup>Water quantity (in liters) applied per plant with interval of two days among applications. <sup>2</sup>Means followed by the same letter in the column did not differ statistically by the Tukey test, at the level of 5% error probability. ns = Not significant. CV = coefficient of variation.



**Figure 3.** Dimensions of mature pecan nuts under different amounts of water applied to the plants.

**Table III.** Mass per fruit, almond and shell; almond and shell percentage and number of nuts per kilogram in different amounts of water applied per plant.

Treatment (L) <sup>1</sup>	Mass per fruit		Mass per almond		Mass per shell		Almond		Shell		Nuts per kilogram	
	---- g ----						%					
0	3.6	c <sup>2</sup>	1.6	c	2.0	c	44.5	b	55.5	a	281	a
70	6.0	b	2.8	b	3.2	b	46.2	ab	53.8	ab	168	b
140	7.2	a	3.4	a	3.8	a	47.0	a	53.0	b	139	b
CV (%)	8.98		10.12		8.55		2.88		2.44		17.46	
Percentage of increase or reduction under treatments												
70	68.0		73.6		64.3		3.8		-3.1		-41.2	
140	102.8		113.8		94.9		5.6		-4.5		-51.3	

<sup>1</sup>Water quantity (in liters) applied per plant with interval of two days among applications. <sup>2</sup>Means followed by the same letter in the column did not differ statistically by the Tukey test, at the level of 5% error probability. CV = coefficient of variation.

## DISCUSSION

Pecan nuts drop often occurs during the period of their development, and several factors may be involved. In this study, we evaluated the effect of growth on plants nutritional status and nutrient content (Sparks 1992, Wood et al. 2010, Wells 2017b, Bilharva et al. 2018). Madero et al. (2017) report that the reduction of pecan nuts percentage and nuts drop can occur if there is a lack of water in the soil. However, in this study, the water restriction (Figure 1) did not appear to be sufficient to cause greater fruit drop in 'Success' (Table I).

Large and well-formed kernel are desired by consumers and these characteristics are specific to each pecan cultivar, but they are also influenced by the orchard and crop management practices (Wells 2017b, Madero et al. 2017). Among them, irrigation has great influence in regions with water deficit, because, as observed in this study (Table II), water scarcity in the reproductive period reduces nuts and kernels size.

According to described data for cultivar Success in the United States, the average pecan nuts length is 36.8 mm and width 25.1 mm (USDA). It was found lower values in this study in which the average length and width obtained were 32.5 mm and 22.3 mm, respectively, when 140 L of irrigation water was used (Table II). The same happens for the nuts mass, where in the USA is 9.2 g and in this study, we obtained 7.2 g (Table III).

Even with the fruit size and mass increase obtained with irrigation, it is possible that more frequent irrigations or greater than 140 L per plant every two days would provide larger nuts. Therefore, studies which consider necessary water depth are still a need for the culture in Uruguay environmental conditions.

According to Avila (1996) and Avila et al. (2000), a water restriction period at any time during the

developmental phase is likely to affect the nuts' size or filling because moisture is withdrawn from the nuts and retained leaves during periods of excessive transpiration. Therefore, it is important that a uniform soil moisture supply is available to the plant during the nut growing and filling period so that it can reach the potential dimensions of each cultivar.

The kernels yield, that is, the kernel percentage in relation to the total nut mass, is an important criterion to be considered when choosing a cultivar (Wells 2017b). However, within the same cultivar, the percentage of kernel can undergo important changes through the different management used. For the cultivar Success, Wells (2017b) describes that in Georgia/USA there is a kernel yield of 50%, while information obtained from the United States Department of Agriculture (USDA) database yields 52.4%. When the irrigation effect was analyzed in this study, an increase of 5.6% in the kernels yield was observed with the use of 140 L of water (Table III), reaching 47% of kernel, lower value than those described for the cultivar in the USA.

According to Wells (2017b), the amount of nuts to reach a kilogram for the cultivar Success is 106. Smaller number compared to 139 required when used 140 L of water. However, when the plants were not irrigated, the difference is even greater, requiring 281 nuts. This is due to the nuts smaller dimensions with water restriction suffered by the plants during the period of fruit growth. In this way, it is important to consider irrigation as an indispensable practice in pecan orchards management, not only in arid regions, but also in places with periods of low precipitation during fruit growth.

With irrigation (140 L), it was possible to increase by 100% the nuts and kernels mass (Table III). Based on this, in an area of one hectare without irrigation, for example, with effective production of 1000 kg of nuts, an adequate water

management could produce approximately 1000 kg more in the same area.

It is important to emphasize that water application in orchards varies according to soil type, regional climate, management, irrigation method, production load, age and plants development stage (Worley 1982, Ibraimo et al. 2016, Madero et al. 2017). In this context, the actual water requirement must be considered and studied in each local situation, in order to adjust the real plants need. Although Uruguay does not have a defined dry season, there are often periods during the year with low rainfall, causing losses in agricultural production when irrigation is not used. For pecan, it is not different, because according to the results obtained in this study, it was possible to observe that this culture without irrigation can cause a reduction of more than 100% of the nuts yield, causing significant losses. Thus, we show the irrigation importance in the growth and yield of pecan nuts, so that producers and technicians involved in the crop consider this practice important, especially in regions and years with periods of more accentuated water deficit.

## CONCLUSIONS

Under the agroclimatic conditions of this study, with periods of water deficit during pecan nuts development, the nuts dimensions and the filling were affected.

The use of irrigation in pecan plants provides greater nuts' dimensions and mass.

With the irrigation of 140 L plant<sup>-1</sup>, every two days, the nuts and kernels mass reached an increase of more than 100% in relation to nuts from plants without irrigation.

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## REFERENCES

- AVILA CG. 1996. Crecimiento y desarrollo del fruto del pecanero (*Carya illinoensis* K) cv. Western y su relación con unidades calor, evapotranspiración y días. ITEA. Producción vegetal 92: 49-57.
- AVILA CG, JUÁREZ IR, ESTRADA CAT, RAMÍREZ MVH, CHIQUITO JC & VÁZQUEZ JM. 2000. Tecnología de Riego en Nogal Pecanero. Libro Científico N°. 1. 1° Ed., SAGAR. INIFAP. CIFAP-Comarca Lagunera, 113 p.
- BILHARVA MG, MARTINS CR, HAMANN JJ, FRONZA D, DE MARCO R & MALGARIM MB. 2018. Pecan: from Research to the Brazilian Reality. J Exp Agric Int 23: 1-16.
- CASTILLO IO, SANGERMAN-JARQUÍN D, HERNÁNDEZ MF, VÁZQUEZ CV & ROBLES MÁG. 2013. Producción y comercialización de nuez pecanera (*Carya illinoensis* Koch) en el norte de Coahuila, México. Rev Mex Cienc Agríc 4: 461-476.
- COLODETTI TV, RODRIGUES RR, RODRIGUES WN, MARTINS LD, BRINATE SVB, PIZETTA SC, REIS EF & TOMAZ MA. 2018. Rational management of water availability along the phenological stages of *Crambe abyssinica* Hochst. Aust J Agric Res 12: 350-356.
- DURÁN A, CALIFRA A & MOLFINO JH. 1999. Suelos del Uruguay según Soil Taxonomy. Uruguay. (en línea). Montevideo, Facultad de Agronomía, 14 p. Disponible em: [http://www.mgap.gub.uy/sites/default/files/multimedia/1615\\_suelos\\_del\\_uruguay\\_según\\_soil\\_taxonomy\\_0.pdf](http://www.mgap.gub.uy/sites/default/files/multimedia/1615_suelos_del_uruguay_según_soil_taxonomy_0.pdf). Acceso em: 13 jul. 2018.
- FERREIRA DF. 2011. SISVAR - A computer statistical analysis system. (UFLA) Ciênc Agrotec 35: 1039-1042.
- GRAUKE LJ. 1991. Appropriate Name for Pecan. HortSci 11: 1358.
- HAN M, PENG F & MARSHALL P. 2018. Pecan phenology in Southeastern China. Ann Appl Biol 172: 160-169.
- IBRAIMO NA, TAYLOR NJ, STEYN JM, GUSH MB & ANNANDALE JG. 2016. Estimating water use of mature pecan orchards: A six stage crop growth curve approach. Agr Water Manag 177: 359-368.
- MADERO ER, TRABICHET FC, PEPÉ F & D WRIGHT E. 2017. Manual de manejo del huerto de nogal pecan, Ediciones INTA, 94 p.

SIERRA EM, PEREZ SP, CASAGRANDE G & VERGARAR G. 2001. Efecto del ENSO sobre las precipitaciones del trimestre noviembre-enero (1981/1998) en el centro este de la provincia de la Pampa, Argentina. *Rev Bras Meteorol* 1: 83-87.

SPARKS DA. 1991. Geographical origin of pecan cultivars influences time required for fruit development and nut size. *J Am Soc Hortic Sci* 116: 627-631.

SPARKS DA. 1992. Pecan cultivars. *The orchard's foundation. Pecan Production Innovations*, 443 p.

THOMPSON TE & CONNER PJ. 2012. Chapter 20: Pecan. Lincoln, Nebraska: United States Department of Agriculture, p. 771-800. Available in: <http://digitalcommons.unl.edu/cgi/viewcontent.cgi?article=2327&context=usdaarsfacpub>. Accessed on July 17, 2018.

USDA - UNITED STATES DEPARTMENT OF AGRICULTURE. NCGR Pecan/Hickory Database (Pecan Cultivars). Available in [https://aggie-horticulture.tamu.edu/usda\\_pecan/plant\\_details.php?pid=Success&acno=518130](https://aggie-horticulture.tamu.edu/usda_pecan/plant_details.php?pid=Success&acno=518130). Accessed on July 14, 2018.

VARELA V, TAKATA V, CAMUSSI G & ZOPPOLO R. 2015. Pecan: Viability of a new crop in Uruguay. *Acta Hortic* 1070: 245-251.

WELLS ML. 2011. Response of pecan orchard soil chemical and biological quality indicators to poultry litter application and clover cover crops. *Hort Science* 46: 306-310.

WELLS ML. 2015. A reduced early-season irrigation schedule for pecans in the Southeastern United States. *Acta Hort* 50: 1070-1077.

WELLS ML. 2017a. Pecan America's Native NUT TREE. The University of Alabama Press, 264 p.

WELLS ML. 2017b. Southeastern Pecan Grower's Handbook. University of Georgia, 236p.

WOOD BW, WELLS ML & FUNDERBURKE F. 2010. Influence of Elevating Tree Potassium on Fruit Drop and Yield of Pecan. *Hort Science* 45: 911-917.

WORLEY RE. 1982. Tree yield and nut characteristics of pecan with drip irrigation under humid conditions. *J Amer Soc Hort Sci* 107: 30-34.

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#### Author contributions

Rudinei De Marco performed the experiment, analyzed samples, contributed to analyze the data, wrote the manuscript, reviewed and edited; Roberto J. Z. Goldschmidt and Flávio G. Herter acted as a PhD supervisor of Rudinei De Marco; Alison Uberti assisted in performing the experiment and revised the manuscript for intellectual content; Paulo C. Mello-Farias and Carlos R. Martins revised the manuscript for intellectual content. All authors discussed the results and approved the final version of the manuscript.

