The effects of fertirrigation and Azospirillum brasilense inoculation on photosynthetic compounds of Agave angustifolia

Preprint *in* Australian Journal of Crop Science · January 2022

DOI: 10.21475/ajcs.22.16.01.p3280

CITATIONS
2

READS
80

6 authors, including:

D. Jarquín-Rosales
3 PUBLICATIONS **6** CITATIONS
SEE PROFILE

José Raymundo Enríquez-del Valle
Instituto Tecnológico del Valle de Oaxaca
77 PUBLICATIONS **343** CITATIONS
SEE PROFILE

Juan Alpuche
Universidad Autónoma Benito Juárez de Oaxaca
29 PUBLICATIONS **722** CITATIONS
SEE PROFILE

Gerardo Rodríguez-Ortiz
Instituto Tecnológico del Valle de Oaxaca
98 PUBLICATIONS **402** CITATIONS
SEE PROFILE

Australian Journal of Crop Science AJCS

AJCS 16(01):162-168 (2022) ISSN:1835-2707
doi: 10.21475/ajcs.22.16.01.p3280

The effects of fertirrigation and *Azospirillum brasilense* inoculation on photosynthetic compounds of *Agave angustifolia*

Domitila Jarquín-Rosales[1], José Raymundo Enríquez-del Valle[1], Juan José Alpuche-Osorno[2], Gerardo Rodríguez-Ortiz[1*], Meredith P Martin[3], Gisela Virginia Campos-Ángeles[1]

[1]Division of Graduate Studies and Research. TecNM / Technological Institute of the Valley of Oaxaca, Mexico. Ex-Hacienda de Nazareno, Xoxocotlán, Oaxaca. Z. C. 71233.
[2]Research Center, Faculty of Medicine, National Autonomous University of Mexico- Benito Juárez Autonomous University of Oaxaca, Mexico.
[3]Department of Forestry and Environmental Resources, North Carolina State University, Raleigh, USA

*** Corresponding author: gerardo.rodriguez@voaxaca.tecnm.mx**

Abstract

Agave angustifolia is the most important species of the genus *Agave* in Oaxaca, Mexico for its use as raw material for the production of mescal. However, research is lacking on the best agricultural methods for growing this species, including on the most effective fertilizer rates and on the use on bacterial inoculation. Our objective was to evaluate the production of photosynthetic compounds in *A. angustifolia* plants originating from seeds when they were fertilized by irrigation and inoculated with *Azospirillum brasilense*. An experiment was carried out in a completely randomized design with a 3×2 factorial arrangement, with the type of irrigation factor at three levels: 1) water; 2) a Steiner nutrient solution composed of N, P, K and micronutrients at 50%; and 3) the nutrient solution at 100%. Subsequently, each irrigation group was separated into two subgroups to evaluate them under the effect of the second factor, inoculation with *A. brasilense*: 1) inoculated plants; 2) control plants not inoculated for a total of six treatments with 20 plants (replications) per treatment. In each treatment we quantified Chlorophyll *a*, *b* and total, Rubisco, and sugars content in foliar samples, as well as the acidity attributed to malic acid at 8:00 and 16:00 hours. We found that plants fertilized and inoculated had bigger leaves with higher amount of chlorophyll *a*, *b* and total and higher amounts of sugars and Rubisco compared to both plants unfertilized and without inoculation and to plants with just fertilization or just inoculation alone. Furthermore, the highest acidity value attributed to malic acid occurred during the first hours of the morning, in the plants of all treatments, but more noticeable in those plants fertirrigated with more nutrient solution (100%) and inoculated with *A. brasilense*. We therefore advise using both fertilization and inoculation for greater growth and accumulation of photosynthetic compounds in *A. angustifolia*.

Keywords: biofertilizer; chlorophyll; malic acid; nutrition; photosynthesis CAM; mescal.
Abbreviations: Ab_ *Azospirillum brasilense*; Cl*a*_chlorophyll a; Cl*b*_chlorophyll b; Cl*t*_total chlorophyll; FR_fertilization in irrigation; LFW_leaf fresh weight; NS_Nutritive solution; Trat_treatments.

Introduction

Agaves are ecologically, socially, and economically important species that have been widely used since pre-Hispanic times for food, living fences, fiber extraction, medicines and distilled beverages (García-Mendoza, 2007; Barrientos et al., 2019). In particular, tequila and mescal are economically and culturally important Mexican beverages distilled from a range of *Agave* species. Tequila uses the *Agave tequilana Weber* var. Azul as raw material, while mescal is a spirit that can be made from a number of different *Agave* species (Lachenmeier et al., 2005). Most commercial mescal is produced in Oaxaca state (90.1% of national production in 2019), and most of this mescal is made from *Agave angustifolia*, which represented 86% of mescal production in Oaxaca state in 2019 (Consejo Regulador del Mezcal_CRM, 2020). Furthermore, the mescal industry is increasing in breadth and economic importance; mescal is currently exported to 64 countries, and during the period from 2011 to 2019 there was a 40% increase in the production of this drink (CRM, 2020). As the economic importance of mezcal increases, more research is needed on the best and most efficient growing techniques for producing increased quantities of *A. angusitfolia* raw material.

Agave angustifolia presents morphological adaptations that allow them to survive in environments with long periods of drought, high temperature and light intensity. These adaptations include the development of thick cuticles, wax deposition in the epidermis, sunken stomata, and succulent

tissues for water storage, abundant fibers in the leaves that maintain their rigidity and superficial roots that facilitate the absorption of rainwater when it only moistens the surface of the soil (García-Mendoza, 2007: Heyduk et al., 2016). Agaves also perform Crassulacean acid metabolism (CAM) as an adaptation mechanism that only 7% of vascular plants carry out (Cushman, 2001). CAM plants keep the stomata open at night to capture carbon dioxide (CO_2) from the environment and accumulate it in the vacuoles in the form of malic acid (Gilman and Edwards, 2019). The availability of nutrients is key to the success and rates at which these physiological processes function. Young *A. angustifolia* plants have shown superior growth in direct relation to the amount of nutrients supplied to them, within a certain optimal range (Enríquez-del Valle et al., 2012; Enríquez-del Valle et al., 2018). In other species such as sorghum (Díaz et al., 2016), corn (Castellanos et al., 2017) and olive (Tejada and González, 2004; Boussadia et al., 2010), leaf area, the amount of chlorophyll, net photosynthesis and biomass accumulation are positively related to the level of nutritional supply. One way to supply essential mineral nutrients to plants is through the application of fertilizer. For example, Arreola-Tostado et al. (2020) reported that in a six-year-old *Agave tequilana* crop, fertilized plants produced from 73 to 104% more raw material compared to unfertilized plants, and also had up to 100% higher sugar yield (which is important for the production of mescal and tequila, since sugars are the substance converted into alcohol through fermentation).

Another way to improve plant nutrition is the application of commercially available nitrogen-fixing bacteria such as *Azospirillum brasilense*, which has been shown to increase the nitrogen assimilation and growth in corn and other plants (Zeffa et al., 2019). In *Prosopis sp., A. brasilense* inoculation improved growth parameters such as biomass, aerial volume, chlorophyll content in leaves and root system size (González et al., 2018). Furthermore, *Urochloa brizantha* plants inoculated with the bacteria required 20% less nitrogen fertilizer and reached greater height, number of tillers and forage production (Leite et al., 2019). The use of this bacterial inoculatant has been tested in *Agave americana* plants, which had a higher concentration of sugars compared to those plants not inoculated (Torre-Ruiz et al., 2016), and other phosphorous solubilizing bacteria have been tested on *A. angustifolia* (Bautista-Cruz et al., 2015). However, the use of *A. brasilense* has not yet been tested on the growth of *A. angustifolia*. Furthermore, the effect of this bacterial inoculation in combination with fertilization on *A. angustifolia* also remains unknown. The aim of this study was therefore to evaluate the effect of fertigation and inoculation with *Azospirillum brasilense* on the content of important biological compounds in *Agave angustifolia* originated from seeds. We compare the effects of fertilizer application alone, bacterial inoculation alone, and a combination of the two treatments in order to assess the most efficient and effective way to increase nutrient content and therefore plant productivity in this species.

Results

Effect of fertilization on the accumulation of photosynthetic compounds

We found that fertilization in irrigation caused highly significant differences in chlorophyll *a* and total content, Rubisco, the amount of malic acid of *A. angustifolia* plants, and the foliar fresh weight (FFW) (ANOVA, P ≤0.001), as well as the content of sugars between treatment types (Table 1; ANOVA, F = 0.0069, df=2, P≤0.05). When comparing means, plants supplied with the highest dose of fertilizer had significantly higher amounts of chlorophylls *a*, *b* and *total* per gram of fresh weight, malic acid and sugars, but lower amounts of Rubisco per gram of fresh weight of leaf tissue compared to those with the medium fertilizer dose, which in turn had significantly higher amounts compared to plants in the control group (Tukey pair-wise comparison, p<0.05; Table 2). The results showed that the plants fertirrigated with NS-100 % and those that received only water had g^{-1} of foliar tissues: 5.29 and 0.59 mg of Cl*a*; 2.70 and 1.73 mg of Cl*b*; 7.99 and 2.32 mg of total Cl; 0.96 and 2.36 mg of Rubisco; 343.57 and 225.31 µg of malic acid, 54.91 and 48.31 µg of sugars, respectively, quantities that in each case were significantly different (Tukey, 0.05).

Effect of the inoculation of A. brasilense on the accumulation of photosynthetic compounds

Inoculation with *A. brasilense* caused highly significant statistical differences (Table 1; ANOVA, F= 0.0001, df=1, P≤0.001) in the variables amount of chlorophyll *a*, rubisco and malic acid. Pair-wise comparisons found that plants inoculated with *A. brasilense* had significantly higher quantities of compounds involved in the photosynthesis process compared to non-inoculated plants (Tukey's pair-wise comparisons, p<0.05; Table 2). The plants inoculated with *A. brasilense* and the non-inoculated plants had g^{-1}: 3.17 and 2.57 mg of Cl*a*, 2.38 and 1.35 mg of Cl*b*, 5.55 and 3.92 mg of total Cl, 1.83 and 1.45 mg of Rubisco; 348.0 and 251.91 µg of malic acid, respectively, amounts that in each case were significantly different (Tukey, 0.05). Despite this, inoculation did not cause differences in the sugar variable.

Effect of fertilization and inoculation on the accumulation of photosynthetic compounds

The interaction of fertilization in irrigation with inoculation with *A. brasilense* caused highly significant statistical effects for *total* chlorophyll and Rubisco (Table 1; ANOVA, F= <0.0001, df=2, P <0.001). We found that the plants fertirrigated with NS-100% and inoculated with *A. brasilense* had 15.3 g of fresh foliar weight, an amount that was 4.3 times the 3.6 g of foliar fresh weight of the unfertilized and non-inoculated plants (Table 3). Plants fertilized with NS-100% and inoculated with *A. brasilense* had higher means of all variables, at 103.51 mg of Cl*a* $plant^{-1}$, 61.62 mg of Cl*b* $plant^{-1}$ and 165.13 mg of Cl*t* $plant^{-1}$, 20.95 mg of Rubisco $plant^{-1}$, 6127.62 µg of malic acid $plant^{-1}$ and 839.57 µg of sugars $plant^{-1}$. In comparison, the total of the foliar tissues of the plants that were only irrigated with water and were not inoculated had 11.21 mg of Cl*a* $plant^{-1}$, 3.51 mg of Cl*b* plant-1, 14.71 mg of Cl*t* $plant^{-1}$, 9.34 mg of Rubisco plant-1, 670.75 µg of malic acid $plant^{-1}$ and 172.95 µg of sugars $plant^{-1}$ (Figure 1).

Discussion

We found that both fertilization alone and inoculation with *A. brasilense* alone generated significantly higher fresh leaf weight, as well as significantly higher concentrations of chlorophyll compounds, malic acid and rubisco compared to

Table 1. Summary of the analysis of variance of biochemical variables of *Agave angustifolia* that during four months received different nutritional fertilization in irrigation (FR) and inoculation with *Azospirillum brasilense* (Ab).

Source of variation	DF	Mean Square and Significance						
		Cl*a*	Cl*b*	Cl*t*	Rubisco	Malic Acid	Sugars	FFW
FR	2	13.3163**	0.2563	0.6196**	2.9498**	25310.6655**	0.0980*	222.61**
Ab	1	2.9798**	0.3360	0.0069	0.6645**	41547.2795**	0.0233	40.20*
FR×Ab	2	0.7052*	0.5803	2.5395**	0.8723**	559.2048	0.0023	6.59
Error	12	0.1037	0.02	0.0170	0.0104	147.71836	3.589344	3.03
Total	17							

Biochemical variables include chlorophyll a (Cl*a*), chlorophyll b (Cl*b*), total chlorophyll (Cl*t*), rubisco, malic acid, sugars, and foliar fresh weight (FFW). DF = degrees of freedom; * F values significant at (p≤ 0.05), ** F values significant at (p≤ 0.01).

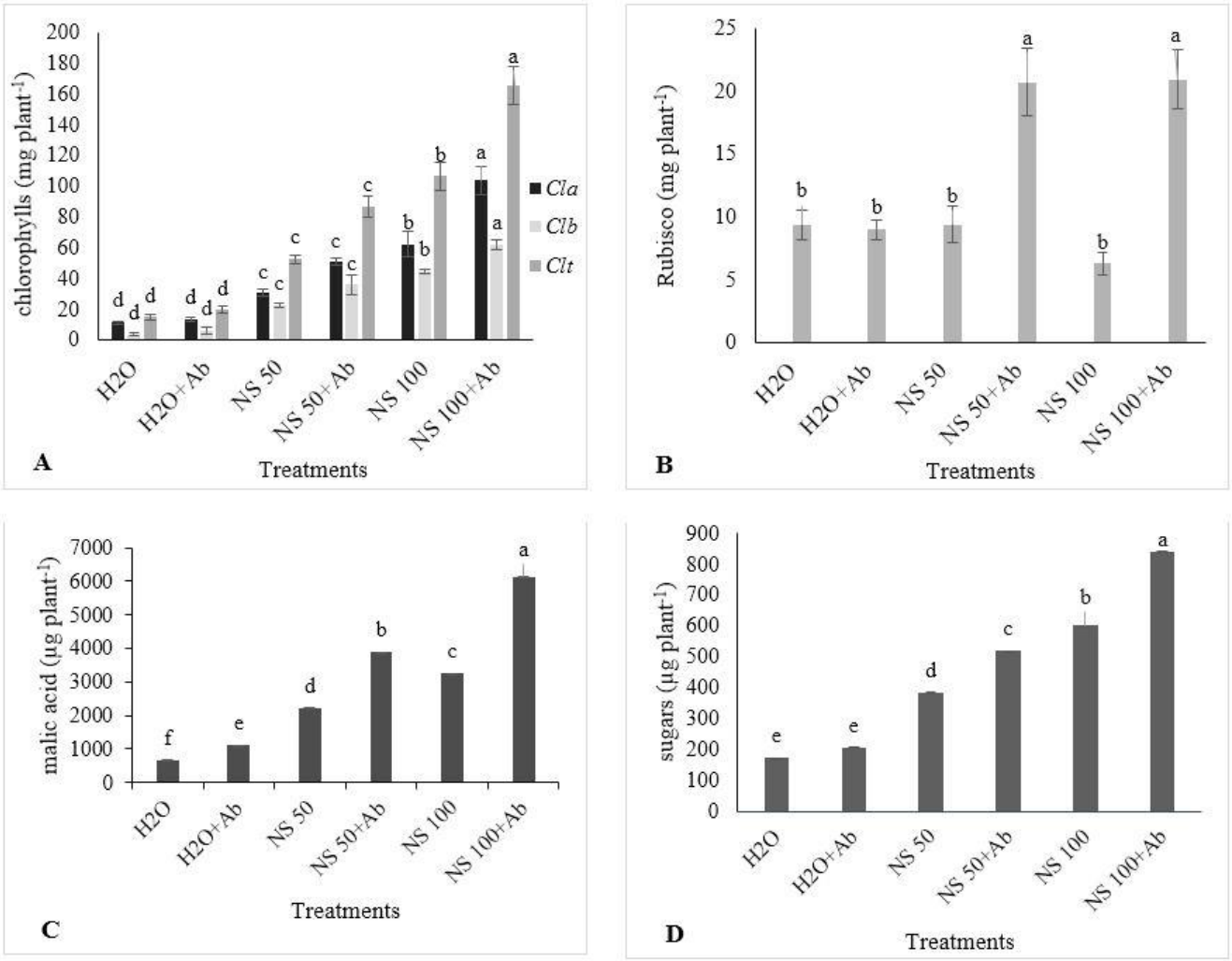

Figure 1. Concentration of A) chlorophylls (mg plant^{-1}), B) Rubisco (mg plant^{-1}), C) malic acid (µg plant^{-1}) and D) sugars (µg plant^{-1}) in foliar tissues of *Agave angustifolia* plants that for four months received different nutritional supplies and inoculation with *Azospirillum brasilense*. NS 0 = water, NS = nutrient solution, Ab = *A. brasilense*, Cl*a* = chlorophyll *a*, Cl*b* = chlorophyll *b*, Cl*t* = total chlorophyll. Values with different letters are significantly different (Tukey HSD, p<0.05).

Table 2. Substances involved in photosynthesis in foliar tissues of *Agave angustifolia* plants, which for four months were under the influence of two factors (FA), which were fertilization in irrigation (FR) and inoculation with *Azospirillum brasilense* (Ab).

FA	Cl*a*	Cl*b*	Cl*t*	Rubisco	_Malic acid µg g^{-1}__		Sugars	FFW
	(mg g^{-1})	(mg g^{-1})	(mg g^{-1})	(mg g^{-1})	8:00 h	16:00 h	(mg g^{-1})	(g)
Fertilization in irrigation								
H_2O	0.59±0.29^{c}	1.73±0.18^{b}	2.32±0.31^{c}	2.36±0.28^{a}	225.31±43.61^{b}	0±0^{a}	48.58±0.96^{b}	3.91±0.85^{c}
NS 50	2.74±0.31^{b}	1.15±0.20^{b}	3.89±0.32^{b}	1.60±0.48^{b}	330.98±54.52^{a}	0±0^{a}	49.76±2.48^{b}	9.07±1.86^{b}
NS 100	5.29±0.72^{a}	2.70±1.82^{a}	7.99±2.31^{a}	0.96±0.45^{c}	343.57±63.35^{a}	0±0^{a}	53.86±2.00^{a}	13.33±3.13^{a}
Inoculation with Ab								
Ab0	2.57±1.96^{b}	1.35±0.35^{b}	3.921.74^{b}	1.45±0.92^{b}	251.91±49.01^{b}	0±0^{a}	49.98±2.62^{a}	7.62±3.63^{b}
Ab1	3.17±2.17^{a}	2.38±1.52^{a}	5.55±350^{a}	1.83±0.36^{a}	348.00±65.47^{a}	0±0^{a}	51.48±3.22^{a}	9.93±4.98^{a}

The variables analyzed are chlorophyll a (Cl*a*), chlorophyll b (Cl*b*), total chlorophyll (Cl*t*), rubisco, malic acid, sugars and foliar fresh weight (FFW). NS= nutrient solution, Ab0= non-inoculated, Ab1= inoculated. In each column and factor levels, values with different letters present statistically significant differences (Tukey HSD, p<0.05). The mean is accompanied ± the standard deviation.

Table 3. Substances involved in photosynthesis (chlorophyll a (Cl*a*), chlorophyll b (Cl*b*), total chlorophyll (Cl*t*), rubisco, malic acid, sugars) in foliar tissues of *Agave angustifolia* that received different treatments for 120 days (Trat) with different doses of nutritive solution (NS) and inoculation with *Azospirillum brasilense* (Ab) and fresh foliar weight (FFW).

Trat	Cl*a*	Cl*b*	Cl*t*	Rubisco	Malic acid µg g^{-1}		Sugars	FFW
	(mg g^{-1})	(mg g^{-1})	(mg g^{-1})	(mg g^{-1})	8:00 h	16:00 h	(mg g^{-1})	(g)
H_2O	3.1±0.2^{c}	1.0±0.1^{c}	4.1±0.2^{e}	2.6±0.0^{a}	187.4±14.8^{c}	0.0±0.0	48.3±0.8^{b}	3.6±0.9^{d}
H_2O+Ab	3.2±0.2^{c}	1.4±0.2^{c}	4.5±0.2^{e}	2.1±0.0^{b}	263.3±14.7^{b}	0.0±0.0	48.8±1.2^{b}	4.3±0.7^{d}
NS 50	3.9±0.2^{c}	2.8±0.2^{b}	6.7±0.2^{d}	1.2±0.2^{c}	282.0±0.0^{b}	0.0±0.0	48.8±1.7^{b}	7.9±1.3^{c}
NS 50+Ab	4.9±0.7^{b}	3.5±0.4^{ab}	8.4±0.3^{c}	2.0±0.1^{b}	380.0±15.3^{a}	0.0±0.0	50.7±3.2^{ab}	10.3±1.6^{bc}
NS 100	5.5±0.2^{b}	3.9±0.3^{a}	9.3±0.1^{b}	0.6±0.1^{d}	286.4±0.0^{b}	0.0±0.0	52.8±2.4^{ab}	11.4±2.3^{b}
NS 100+Ab	6.8±0.1^{a}	4.0±0.2^{a}	10.8±0.0^{a}	1.4±0.1^{c}	400.8±14.8^{a}	0.0±0.0	54.9±0.9^{a}	15.3±2.7^{a}

In each column and factor levels, values with different letters present statistically significant differences (Tukey HSD, p<0.05). The mean is accompanied ± the standard deviation.

controls in *Agave angustifolia* plants. Fertilization of *A. angustifolia* also generated significantly higher sugar concentrations in comparison to control plants. In addition, the interaction of fertilization and inoculation had significant effects on the concentration of *total* chlorophylls, rubisco, and malic acid, such that plants receiving both fertilization and inoculation with bacteria had higher concentrations compared to plants receiving fertilizers alone.

The size of the leaf area and the amount of chlorophyll are important characteristics determining photosynthetic efficiency and which influence the growth and accumulation of biomass by plants (Melis, 1991). Nutritional supply, particularly nitrogen, is essential in the formation of chlorophyll molecules (Evans and Clarke 2019), so the addition of nitrogen either directly through fertilization or indirectly through inoculation with nitrogen-fixing bacteria should lead to both the increased production of chlorophyll and increased leaf weight that was found here. Thus, i). These results are supported by experiments on other species, including sorghum plants (Díaz-Franco et al., 2018) and corn plants (Zambrano et al., 2019), which both had more chlorophyll in leaves and accumulated more biomass when inoculated with *A. brasilense* compared to non- inoculated plants.

In the case of sorghum, the plants inoculated with nitrogen-fixing bacteria also showed increased sugar content (Díaz-Franco et al., 2018). In our study, we found that sugar content was not significantly higher for plants inoculated with bacteria overall, but that the combination of inoculation and the highest level of fertilization led to significantly higher sugar content compared to all other treatments. Sugar production in plants is linked to the amount of Rubisco, a protein is crucial in photosynthesis that is responsible for the assimilation of most of the CO_2 (Raven 2009; Kubis and Bar-Even, 2019). This protein depends on the supply of nitrogen to maintain its carboxylase activity (Cheng and Fuchigami, 2000). In our study, we found that plants that were inoculated with *A. brasilense* had a greater amount of Rubisco both in the total plant and per unit of fresh weight of leaves. Interestingly, plants treated with fertilizers alone generated significantly less Rubisco per unit of fresh weight of leaves, but those treated with both fertilizer and inoculated with bacteria had the highest amounts of Rubisco. Sugar content, and therefore Rubisco, is particularly important for agaves used to produce mescal, as sugars are converted to alcohol during the fermentation process.

The final part of the CAM photosynthetic stage in Agaves is the generation of sugars, which are then stored in tissues either in the succulent stems or leaves. When an *Agave* plant is close to the sexual reproductive stage, it presents the maximum accumulation of sugars in its stem and leaves, and then the plant uses all its reserves of sugars and other nutrients to support the development of its inflorescence and fruit with seeds. For this reason, farmers prevent inflorescence development by cutting the flower stalk early in its development, thereby enabling the harvest of a sugar-rich raw material to use for fermentation and then distillation of mescal (Zuñiga-Estrada et al., 2018; Davis et al., 2019). For *Agave angustifolia,* the most commonly used in the mescal industry, plants with higher amounts of sugar generate better quality mescal (Pérez et al., 2016). Similarly, a study of another *Agave*

used for mescal (*Agave potatorum*) found that fertilized plants had accumulated higher sugar quantities compared to unfertilized plants (Martínez et al., 2012). We also found that fertilization significantly increased sugar content of *A. angustifolia*, and furthermore, that the plants subjected to the combination of fertilization and inoculation with *A. brasilense* generated the highest sugar concentrations.

Finally, we found that the acidic condition of the foliar tissues both varied in the diurnal rhythm expected for CAM photosynthesis (Dodd et al.,2002; Winter, 2019), and that the amount of malic acid that accumulated was directly related to the nutritional condition of the plant. Maxwell et al. (2002) showed that during the night and until the first hours of dawn, the fixation of CO_2 and the accumulation of malic acid occurs both in well-irrigated plants and in plants with a water deficit. Later during the day and until dusk, malic acid is decarboxylated and CO_2 is again fixed by the Calvin–Benson cycle. In this study, the data show that the acidity condition of the foliar tissues varies in the diurnal rhythm aligning with research by Casierra-Posada and González (2009) in the CAM metabolism species, *Furcraea macrophylla* and *F. castilla*, and Romero-H et al. (2017) in *Ferocactus histrix* and *F.* pilosus, in which the acid content in the leaves begins to decrease during the day from 8:00 am to around 7:00 pm, and then the acidity of the tissues gradually increases as a consequence of the fixation of CO_2 and the production of organic acids during the night. We found the highest values of malic acid were recorded at 8:00 hours in the plants that were fertirrigated with NS-100% and inoculated with *A. brasilense*, and were significantly greater than the acidity of foliar tissues of plants irrigated with only water and not inoculated. Because malic acid accumulation is linked to CO_2 and carbon-fixation, this provides further evidence that the combination of fertilization and bacterial inoculation generated greater photosynthetic production and biomass accumulation compared to control treatments.

Materials and methods

Experimental design and treatment types

We grew one hundred and twenty plants of *Agave angustifolia* from seed in the nursery for three months from March – June, 2019. The experiment was established according to a completely randomized design with a 3 × 2 factorial arrangement based on three levels of the fertilization factor in irrigation and two levels of the inoculation factor for a total of six treatments. Each treatment had 20 plants as replicates, and all plants were grown for 120 days.

The three fertilization treatments used the following doses: 1) only water (i.e. the control treatment); 2) nutrient solution (NS) at 50%, or 3) NS at 100% concentration of nutrients. The NS 100% of the Steiner (1984) formulation contained in mg L^{-1}: 166.42 N, 30.68 P, 276.44 K, 182.34 Ca, 49.09 Mg, 111.15 S, 1.25 Fe, 0.21 Mn, 0.025 Zn, 0.076 B, 0.005 Cu and 0.021 Mo, which were supplied using calcium nitrate, potassium nitrate, magnesium sulfate, potassium sulfate, monopotassium phosphate, potassium hydroxide, zinc sulfate, boric acid, manganese sulfate, sodium molybdate, copper sulphate, ferrous sulfate, EDTA-disodium salt, hydrochloric acid. Fertilization treatments began when plants were three months old, and were applied through irrigation once a week.

Each fertilizer treatment group of plants was divided into two sub-groups for inoculation with *A. brasilense*: 1) non-inoculated plants (control), and 2) inoculated plants. Inoculation was done by applying 10 mL of a suspension of *A. brasilense* per plant, containing 202,200 colony-forming units (CFU) which was obtained by adding 1 g of the trade name product Azofer (Biofactory, XXI century) in one liter of water. Four inoculations were applied at intervals of each month for those in the inoculation sub-goups.

Plant traits measured

After 120 days, five plants of each of the six treatments were harvested, the roots were separated and the leaf fresh weight (PFF) was recorded using an analytical balance with a precision of 0.1 mg (Shimadzo brand model AY224y). Chlorophylls *a*, *b* and *total* were quantified in an unfolded leaf of each plant using the standard protocols described by Richardson et al. (2002) and using the formula of Arnon (1949).

We collected 20 g of foliar tissue from another five plants from each of the six treatments to quantify Rubisco following the standard protocol described by Geada et al. (2011). Electrophoresis was used to assess the purity of the Rubisco protein following standard protocols described by Laemmli (1970). To examine the integrity of the Rubisco enzyme, it was observed in the 12% polyacrylamide gel showing the subunit greater than 56 kDa (Osborne et al., 1998) and the subunit less than 16 kDa (Ishida et al., 1997). Similar results were obtained when purifying Rubisco from tobacco using this same method (Geada et al., 2011; Carmo-Silva et al., 2011), which turned out to be potential and scalable for the purification of Rubisco from foliar samples, without the use of chromatographic steps.

To estimate sugar content, we collected two leaves from the middle part of the rosette in an additional five plants per treatment. Each sample was filtered through a funnel with Whatman™ filter paper number 1, and determined the Brix degrees from the filtrate with a portable refractometer Model 32 br, ATC Brand that was calibrated with distilled water prior to reading each sample. Finally, we used one undamaged leaf from the middle of the rosette in five more plants from each of the six treatments to assess acidity attributable to malic acid. We measured malic acid two times a day, at 8 am and 4 pm, because during the night and until the first hours of the morning there is a greater accumulation of acidity while it decreases later during the afternoon. We analyze each sample following standard protocols and calculated the percent acidity attributed to malic acid according to the formula described by Romero-H et al. (2017).

Statistical analysis

To test whether inoculation and fertilizer treatments had an impact on chlorophyll, rubisco, sugar, and acid contents, we ran a series of two-way ANOVA tests with inoculation levels and fertilizer levels and the interaction effect between inoculation and fertilizer levels as predictors for each of these response variables. For each ANOVA, we checked the assumptions of normality using the Shapiro-Wilk test and homogeneity of variances using Bartlett's test. The data for chlorophyll *b*, *total* chlorophyll and sugars did not meet the normality assumptions, so these data were transformed using logarithm (for chlorophyll *b*), a cosine transformation (the amount of total chlorophyll) and a cosine transformation with

logarithm (for the sugar content). Later multiple range tests were performed for the mean separation (Tukey p <0.05) for each of the untransformed variables, in all cases the statistical package SAS 9.0 (Statistical Analysis System, 2004) was used.

Conclusions

Fertilization in irrigation and inoculation with *Azospirillum brasilense* favored the synthesis of compounds essential to the photosynthesis process in *Agave angustifolia* plants originating from seed. The foliar fresh weight of the plants, as well as their content of chlorophylls, Rubisco, malic acid and sugars increased as their nutritional condition improved. The plants that received a combination of fertigation with 100% Steiner solution and inoculation with *Azospirillum brasilense*, showed a better response in fresh leaf weight and sugar content, both essential for the production of mezcal. Therefore, we recommend that Agaves grown for mescal be treated with both fertilizer and inoculated with nitrogen-fixing bacteria to maximize production of this culturally and economically important spirit.

Acknowledgements

We thank the National Council of Science and Technology (CONACYT-Mexico) for the support provided with a research grant. The research was funded by the National Technological Institute of Mexico, with the project key 6617.18-P, entitled "Asexual propagation of two endemic species of Oaxaca."

References

Arnon D (1949) Copper enzymes in isolated chloroplasts polyphenoloxidase in *Beta vulgaris.* Plant Physiol. 24(1): 1-15.

Arreola-Tostado JM, Montoya-Jasso VM, Arreola-Nava JM, Castillo-Valdez X, Olivares-Arreola EA, Báez-Pérez A (2020) Efecto de la aplicación de levasa (mosto de caña de azúcar) en la producción y calidad de *Agave tequilana* Weber. Rev Mex Cienc Agríc. 11(6): 1311- 1324.

Bautista-Cruz A, Martínez-Gallegos V, Martínez- Martínez L, Martínez GG (2015) Effect of phosphate solubilizing bacteria on the growth of *Agave angustifolia* Haw. (maguey espadín). Pak. J. Bot. 47(3): 1033-1038.

Barrientos RG, Esperanza IEL, Segura PHR, Talabera MO, Sampedro RML, Hernandez CE (2019) Caracterización morfológica de *Agave angustifolia* y su conservación en Guerrero, México. Rev Mex Cienc Agríc. 10(3):655-668.

Boussadia O, Steppe K, Zgallai H, Ben El Hadj S, Braham M, Lemeur R, Labeke MCV (2010) Effects of nitrogen deficiency on leaf photosynthesis, carbohydrate status and biomass production in two olive cultivars 'Meski' and 'Koroneiki'. Sci Hort. 123:336-342.

Carmo-Silva AE, Berta CV, Salvucci ME (2011) Isolation of Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase from Leaves. Methods Mol Biol. 684:339-347.

Casierra-Posada F, González DM (2009) Cambio circadiano de pH y acidez titulable en la savia de fique (*Furcraea castilla y F. macrophylla*). Orinoquia. 13(1):5-13.

Castellanos RMA, Valdés CR, López GA, Guridi IF (2017) Mediciones de índices de verdor relacionadas con área foliar y productividad de híbrido de maíz. Cult Trop. 38(3):112-166.

Consejo Regulador del Mezcal (2020) Informe estadístico. Disponible en <http://www.crm.org.mx/PDF/INF_ACTIVIDADES/INFORME2019.pdf>. acceso en: agosto 2020.

Cushman J (2001) Crassulacean Acid Metabolism. A Plastic Photosynthetic Adaptation to Arid Environments. Plant Physiol. 127:1439-1448.

Cheng L and Fuchigami LH (2000) Rubisco activation state decreases with increasing nitrogen content in apple leaves. J Exp Bot. 51(365):1687–1694.

Davis CS, Simpson J, Gil-Vega del CK, Niechayev AN, Tongerlo VE, Hurtado-Castano N, Dever VL, Búrquez A (2019) Undervalued potential of crassulacean acid metabolism for current and future agricultural production. J Exp Bot. 70(22):6521-6537.

Díaz FA, Espinosa RM, Ortiz CFE (2016) Promoción de biomasa y contenido de azúcares en sorgo dulce mediante abonos orgánicos y micorriza arbuscular. Rev Int Contam Ambie. 32(3):353-360.

Díaz-Franco A, Aguado-Santacruz A, Rosas-Quijano R, Vázquez-Ovado A, Gálvez-López D (2018) Cepas microbianas con potencial para sustituir la fertilización inorgánica de sorgo dulce (*Sorghum bicolor*). Rev. Int. Contam. Ambie. 34(3) 405-416.

Dodd NA, Borland MA, Haslam PR, Griffiths H, Maxwell K (2002) Crassulacean acid metabolism: plastic, fantastic. J Exp Bot. 53(369): 569–580.

Enríquez-del Valle JR, Cruz-Valdez I, Carrillo-Castañeda G (2012) Acclimatization of *Agave angustifolia* Haw. Vitroplants in Inert Substrates and Fertigated with Different Nutrimental Dose. Acta Hort. 947: 101- 104.

Enríquez-del Valle JR, Rodríguez-Ortiz G, Ruiz-Luna J, Pacheco-Ramírez AJ, Vásquez-Vásquez L (2018) Crecimiento y condición nutrimental de plantas micropropagadas de *Agave angustifolia* abonadas y fertirrigadas en vivero. Rev Mex Agroecos. 5(2): 28-38.

Evans RJ and Clarke CV (2019) The nitrogen cost of photosynthesis. J Exp Bot. 70(1):7 -15.

García-Mendoza AJ (2007) Los agaves de México. Ciencias. 87: 14-23.

Geada D, Alvarez A, Alonso A, García H, Valdés R, Geada G, Fierro W, Maestre D, Cuza M, Vargas S (2011) Purificación de la Rubisco de tabaco sin pasos cromatográficos: evaluación genotóxica y citotóxica en células de escherichia coli. Cuba Tab. 12(1):50-62.

González EJ, Hernandez JP, De-Bashana le, Basha Y (2018) Dry micro-polymeric inoculant of *Azospirillum brasilense* is useful for producing mesquite transplants for reforestation of degraded arid zones. App Soil Ecol. 129: 84-93.

Gilman IS and Edwards EJ (2019) Crassulacean acid metabolism. Curr Biol. 30:51-63.

Heyduk K, McKain MR, Lalani F, Leebens-Mack J (2016) Evolution of a CAM anatomy predates the origins of Crassulacean acid metabolism in the Agavoideae (Asparagaceae). Mol Phylogenetics Evol. 105:102-113.

Ishida H, Nishimori Y, Sugisawa M, Makino A, Mae T (1997) The Large Subunit of Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase is Fragmented into 37-kDa and 16-kDa Polypeptides by Active Oxygen in the Lysates of Chloroplasts from Primary Leaves of Wheat. Plant Cell Physiol. 38(4): 471-479.

Kubis A and Bar-Even A (2019) Synthetic biology approaches for improving photosynthesis. J Exp Bot. 70(5):1425-1433.

Laemmli UK (1970) Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. Nature. 227:680–685.

Lachenmeier DW, Richling E, L MG, Frank W, Schreier P (2005) Multivariate analysis of FTIR and ion chromatographic data for the quality control of tequila. J Agric Food Che. 53(6): 2151–2157.

Leite R da C, Dos Santos JGD, Silva EL, Alves CR, Hongria M, Leite RC, Dos Santos AC (2019) Productivity increase, reduction of nitrogen fertiliser use and drought-stress mitigation by inoculation of Marandu grass (*Urochloa brizantha*) with *Azospirillum brasilense*. Crop Pasture Sci. 70:61-67.

Martínez RS, Trinidad SA, Robles C, Galvis SA, Hernández MT, Santizo RJ, Bautista SG, Pedro SE (2012) Crecimiento y sólidos solubles de *Agave potatorum Zucc*. inducidos por riego y fertilización. Rev Fitotec Mex. 35(1): 61-68.

Melis A (1991) Dynamics of photosynthetic membrane composition and function. Biochimica et Biophys Acta. 1058 (2):87-106.

Maxwell K, Griffiths H, Helliker B, Roberts A, Haslam PR, Girnus J, Robe EW, Borland MA (2002) Regulation of Rubisco activity in crassulacean acid metabolism plants: better late than never. Funct Plant Biol. 29(6):689-696

Osborne CP, LaRoche J, Garcia RL, Kimball BA, Wall GW, Pinter PJ, LaMorte RL, Hendrey GR, Long SO (1998) Does Leaf Position within a Canopy Affect Acclimation of Photosynthesis to Elevated CO_2?. Plant Physiol. 117: 1037-1045.

Pérez HE, Chávez PMC, González HJC (2016) Review of agave and mezcal. Rev Colomb Biotecnol. 18(1):148-164.

Raven J (2009) Contributions of anoxygenic and oxygenic phototrophy and chemolithotrophy to carbon and oxygen fluxes in aquatic environments. Aquat Microb Ecol. 56: 177-192.

Richardson AD, Duiga SP, Berlin GP (2002) An evaluatíon of non invasive methods to estimate foliar chlorophyll content. New Phytol. 153:185-194.

Romero-H ME, Peña-Vladivia CB, García-N R, Campo H (2017) Efecto de la restricción de agua edáfica en el potencial de agua y acidez diurna y nocturna de *Ferocactus histrix y F. pilosus.* Polibotanica. 44 :167-183.

Statistical Analysis System (SAS) (2004) User's guide. SAS/ETS® 9.1. SAS Institute Inc. Cary, NC. USA. s/p.

Steiner AA (1984) The universal nutrient solution. *In*: Proceedings of ISOSC. Sixth International Congress on Soilless Culture. April 29-May 5, 1984. Wageningen, The Netherlands. pp: 633-650.

Tejada M and Gonzalez JL (2004) Effects of foliar application of a byproduct of the two-step olive oil mill process on rice yield. Europ. J. Agronomy. 21:31-40.

Torre-Ruiz N, Ruiz-Valdiviezo VM, Rincón-Molina CI, Rodríguez-Mendiola M, Arias-Castro C, Gutiérrez-Maceli FA, Palomeque-Domínguez H, Rincón-Rosales H (2016) Effect of plant growth-promoting bacteria on the growth and fructan production of *Agave americana* L. Braz J Microbiol. 47(3):587-596.

Winter K (2019) Ecophysiology of constitutive and facultative CAM photosynthesis. J Exp Bot. 70(22):6495-6508.

Zambrano GF, Souza AD, Zucareli C, Sarkis YJ, Amaral H, Matias da Costa R, Raia D, Garcia M, Guimaraes MF (2019) Efecto de la inoculación con cianobacterias y coinoculación con *Azospirillum brasilense* sobre características fitométricas en maíz. Bioagro. 31(3): 193-202.

Zeffa DM, Perini LJ, Silva MB, De Sousa NV, Scapim CA, Oliveira ALM, Júnior ATA, Goncalves LSA (2019) *Azospirillum brasilense* promueve aumentos en el crecimiento y la eficiencia en el uso de nitrógeno de los genotipos de maíz. PLoS ONE. 14(4): e0215332.

Zuñiga-Estrada L, Rosales-Robles E, Yáñez-Morales MJ, Jacques-Hernández C (2018) Características y productividad de una planta MAC, *Agave tequilana* desarrollada con fertigación en Tamaulipas, México. Rev Mex Cienc Agríc 9(3):553–564.