Temperature and Precipitation Effect on the Production of Flavonoids in Wild Populations of Stevia Salicifolia in Queretaro.

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***Abstract*— Plants respond physiologically to the meteorological variations of the area where they grow and develop. Temperature and precipitation are two of the main meteorological conditions that affect plant's metabolism, mainly the secondary metabolism, in which substances such as flavonoids are produced. These allow the cell to be protected from oxidative stress caused by both, biotic and abiotic factors. The objective of this work was to analyze the meteorological conditions that affects the physiology that synthetize total flavonoids in wild populations of *Stevia salicifolia*. Four populations were established, two in the municipality of Amealco, one in the municipality of Huimilpan and the last in the municipality of Cadereyta. The highest concentration of total flavonoids was obtained from Huimilpan's population, followed by Amealco's populations and the lowest concentration was obtained from Cadereyta's population. Based on the statistical analysis of multivariate correlations, it was established that temperature is the main factor for flavonoids synthesis in *S. salicifolia*, since the highest concentration of total flavonoids was obtained in a temperature range of 27.20-28.60 °C, while that the lowest concentration was obtained in a range of 19.20 - 21.1 °C.**

Keywords— temperature, precipitation, *S. salicifolia*, flavonoids.

I. INTRODUCTION

Plants carry out a different kind of metabolism where compounds that do not have any apparent application are produced, these compounds are known as secondary metabolites and together with their route of synthesis and functions, they constitute the secondary metabolism [1]. Flavonoids are a group of phenolic compounds that derive from this metabolism, due to their wide diversity in phenolic structures they are classified in different subgroups such as Flavones, Flavonols, Flavanones, Flavanonols, Flavanols or Catechins, Anthocyanins, and Chalcones [2]. These compounds are mainly distributed in the aerial part of the plant and are responsible for the color in leaves, flowers, and fruits [3]; in addition to their ecologic and biologic functions [4].

These compounds synthesis is affected by the climate conditions where the plant grows and develops. Being different in each species, this synthesis depends on the requirements and exposure to meteorological factors as a measure of acclimatization for the plant [5]. For instance, temperature is one of the main meteorological factors affecting the biosynthetic pathway of flavonoids [6]. As an example, when herbaceous plants like *Nicotiana tabacum* are exposed to 4 °C, the genes responsible for the synthesis of anthocyanins is activated, resulting in the accumulation of this compound [7]. On the other hand, *Árnica montanica,* activates a secondary metabolism synthesis route that will generate another kind of phenolic compound useful for the plant when submitted to a variation in temperature greater than 5 °C [8].

It has been shown that in *Arabidopsis thaliana* the synthesis of flavonoids (anthocyanins) increases when exposed to temperatures of 4 °C. Another study states that, depending on the temperature at which strawberry plants are exposed, the amount of phenolic compounds produced is different, with lower concentrations at temperatures between 18 °C and 22 °C [10].

Like temperature, precipitation in wild populations is of great importance, since water deficit causes the synthesis and accumulation of flavonoids, which are mainly used as a protection against oxidative stress [11]. In *Triticum aestivum* species, gene expression for flavonoids synthesis begins when in presence of hydric stress as a protective measure against oxidative stress due to drought [12].

There is a lack of information regarding the biology, ecology, and biochemistry of Stevia spp. This genus is composed by at least 150 species, all originated in the American Continent. *S. salicifolia* is a shrub used in ethnobotany due to the medicinal potential of its leaves [13] and roots [14].

However, there is not enough information about the relationship between climate, soil, ecological and meteorological conditions with plant´s primary and secondary metabolisms. This information is necessary for the controlled production of this species, since the plants used by the communities are extracted directly from wild populations.

Therefore, the objective of this work is to identify and establish the temperature and precipitation conditions where wild populations of *Stevia salicifolia* yield the highest concentration of flavonoids in its leaves. Thus, determining the conditions needed to grow controlled crops of wild species and obtaining secondary metabolites with industrial applications.

II. METHODS

A. Location and stablishment of S. salicifolia populations in the state of Queretaro.

The Jersey Rzedowski herbarium database from the Autonomous University of Querétaro ("QMEX") and the World Biodiversity Information Network (REMIB) were consulted to collect georeferenced data on wild populations of *S. salicifolia* in the state of Queretaro. Selection of the populations for the study was determined by the different distribution points of the same species, as well as different meteorological conditions in those zones, that is, populations of the same species distributed in different municipalities.

B. Metereologic conditions of the populations of interest.

Weather stations databases were consulted on the website of the Secretary of Agricultural Development (Secretaría de Desarrollo Agropecuario, SEDEA), in the climate section. Temperature and precipitation data were collected for each of the three municipalities with the following station keys: CEA-AMEALCO IAMEALCO2 in Amealco, CEA-CONCYTEQ IQUERETA15 in Cadereyta and CEA-HUIMILPAN IQUERETA19 for the municipality of Huimilpan.

C. Data analysis and ombrothermic diagrams of temperature and precipitation elaboration.

Temperature and precipitation data were analyzed monthly. The maximum, minimum and average monthly, weekly and collection day temperatures were used. While annual, monthly, and collection day accumulation data were used for precipitation. The ombrothermic diagrams were elaborated in the Microsoft Office 2010 Excel tool. In the ombrothermic diagram, the maximum and minimum monthly temperatures were plotted on the first axis and the accumulated monthly precipitation on the second axis.

D. Plant material and treatment.

Leaves were collected during the plant’s reproductive phenological state in different localities of the three municipalities. They were stored in brown paper bags to avoid contact with light; next, leaves were dried in an oven at 37 °C for 8 days. After removing them from the dehydration process, the leaves that had any signs of microorganisms infection or did not dehydrated properly were discarded. Finally, the leaves were pulverized and stored in amber bottles in a dry and dark place for further analysis.

E. Extraction and quantification of total flavonoids.

Extraction and quantification of total flavonoids were carried out in triplicate using the spectrophotometric method [15] with some modifications [16]. For the extraction, a water: ethanol: acetone (80: 18: 2 v / v) extracting solution was used. Then, 0.5 g of powdered sample was weighed and added to 5 mL of the extracting solution while stirring for 30 seconds. Next, the solution was sonicated for 30 minutes at room temperature, after which it was centrifuged at 8500 rpm (revolutions per minute) for 15 minutes at 4 °C. The same procedure was applied to the resulting pellet, and the final volume was measured after filtering with a 2 µm pore acrodisc. Finally, the solution was refrigerated for further analysis.

For the calibration curve, the flavonoid catechin standard, with concentrations ranging from 0.0 to 0.6 mg / mL. Readings were taken in triplicate at a wavelength of 510 nm, same as the total flavonoid samples.

F. Extraction of phenolic compounds for HPLC analysis.

For the analysis of phenolic compounds, 200 mg of dry and finely ground sample was weighed. 1 mL of methanol (HPLC grade) was added to each sample, it was then vortexed for 30 seconds. Subsequently, the samples were placed in an ultrasonic bath for 30 minutes at room temperature, while protected from light. After this, the samples were centrifuged at 9500 rpm for 5 minutes. The supernatant was recovered and the solid residue was subjected to the same extraction procedure for four consecutive times. The supernatants were combined and diluted to 5 mL. The resulting extract was filtered with an acrodisk (0.45 µm) and stored in amber colored vials (at -20 °C) until further analysis.

G. Analysis of phenolic compounds by HPLC.

The analysis of phenolic compounds was carried out by convergence chromatography (UPC^2: Ultra-performance convergence chromatography). In order to know their chromatographic profile, the previously prepared samples were injected in the UPC^2, according to the analysis conditions shown in Table 1. Afterwards, each of the standards was injected to determine its retention time and obtain its UV spectrum. The retention times and the UV spectra from the different peaks in the samples were compared with the standards to determine if they matched. Matching peaks were subjected to a co-elution (by adding the standard until three different concentrations were reached) in order to confirm the correspondence of the compounds. The compounds that were identified were quantified using calibration curves of the corresponding standard.

Table 1. Conditions of UPC[2] for the detection of flavonoids.

Time (minutes)	*CO_2 (%)	Methanol (%)
0	95	5
8	70	30
9	70	30
10	95	5
11	95	5
Injection volume: **10 µL** Flow: **1.5 mL/min** Column: **Viridis BEH 5µm, 4.6 X 100 mm** Column temperature: **40 °C** ABPR: **1500 psi**		
*CO_2 coleman grade		

H. Statistical analysis.

Stat Graphics Centurion XV program was used to carry out ANOVA tests of the meteorological data, as well as for the concentration of total flavonoids' concentration. The analysis was followed by a Tukey test and, finally, by a multivariable correlation test in order to observe the effect of the meteorological conditions in these compounds synthesis.

III. RESULTS AND DISCUSSION.

A. Database consultation and population stablishment.

Nine data were obtained from the World Biodiversity Information Network and eleven from the Jersey Rzedowski herbarium database of the Autonomous University of Querétaro. From these, four populations were located in different municipalities. Two of them were located in the municipality of Amealco, one in Huimilpan and the last one in Cadereyta de Montes (Fig. 1).

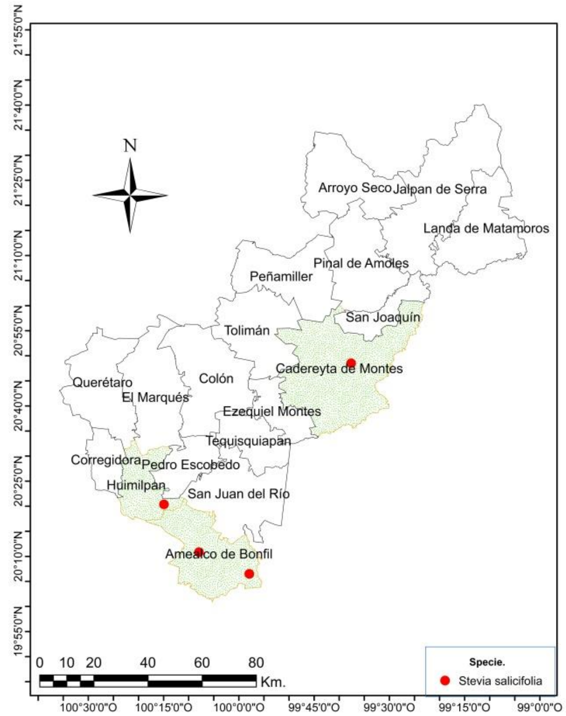

Fig.1. Location of the populations of *S. salicifolia* in the three municipalities.

B. Metereological conditions and ombrothermic diagrams.

The monthly meteorological conditions by municipality are presented in table 2. The collection of vegetal material from the municipalities of Amealco and Huimilpan were made in the month of August, and in the month of September for the municipality of Cadereyta. The latter presented the highest monthly temperature with 27 °C, followed by the municipality of Huimilpan with 25.6 °C and finally with 23.2 °C in the municipality of Amealco. The minimum monthly temperature behaved differently, the lowest was recorded in the municipality of Amealco with 8.3 °C while the highest was for the municipality of Huimilpan with 10.8 °C.

Table 2. Monthly weather conditions by municipality.

Municipality	Max. monthly temp. (°C)	Min. monthly temp.(°C)	Average month temp. (°C)	Accumulated monthly precipitation (mm)
Amealco	23.2	15.1	8.3	83.1
Huimilpan	25.6	10.8	18.2	11.7
Cadereyta	27	9	18.3	23.4

The weekly meteorological conditions, by municipality, are presented in table 3. The highest and lowest minimum temperatures were registered in the municipality of Huimilpan, in contrast to the precipitation, which recorded the lowest accumulation in this municipality.

Table 3. Weekly weather conditions by municipality.

Municipality	Max. weekly temp. (°C)	Min. weekly temp.(°C)	Average weekly temp. (°C)	Accumulated week precipitation (mm)
Amealco	25.6	10.8	18.9	29.2
Huimilpan	25.6	10.8	18.9	11.2
Cadereyta	24.8	9	17.5	22.4

For the municipality of Amealco, the maximum temperature recorded during collection was 22.2 °C, the minimum of 10.7 °C, while the accumulated rainfall was 24.4 mm, being the municipality with the highest accumulated precipitation (Fig. 2).

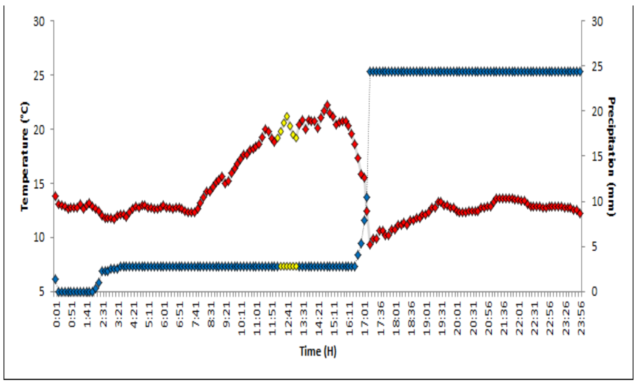

Fig. 2: Ombrothermic diagram for collection day in the municipality of Amealco. Yellow markers indicate collection time of the leaves.

In the municipality of Cadereyta, the following meteorological measurements were obtained: maximum temperature was 21.8 °C, minimum of 10.7 °C and 0.00 mm of accumulated precipitation (Fig. 3).

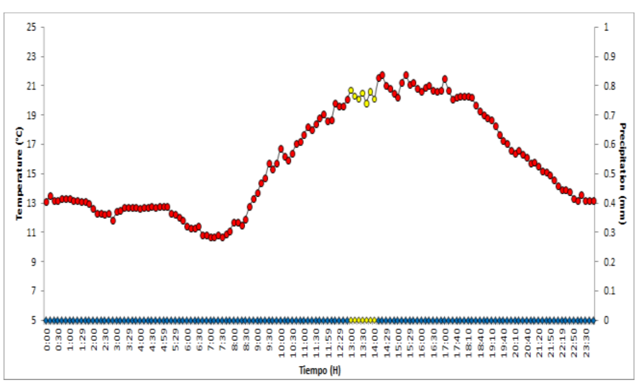

Fig. 3: Ombrothermic diagram for collection day in the municipality of Cadereyta. Yellow markers indicate collection time of the leaves.

Lastly, for the municipality of Huimilpan, the following meteorological conditions were recorded: 24.0 °C for the maximum temperature, 12.3 °C for the minimum and 2.3 mm of accumulated precipitation, this being the municipality with the maximum temperature of the day recorded (Fig. 4).

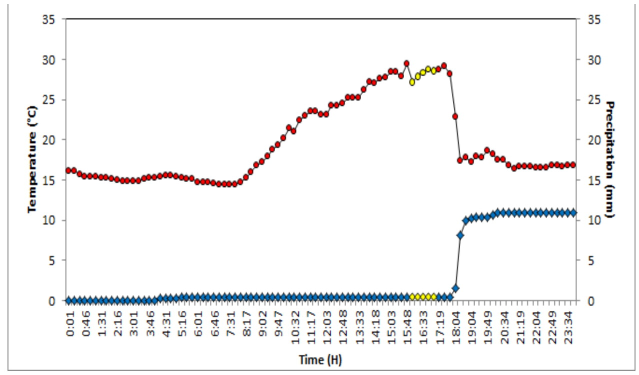

Fig. 4: Ombrothermic diagram for collection day in the municipality of Huimilpan. Yellow markers indicate collection time of the leaves.

C. Total flavonoids.

Different concentrations of flavonoids were obtained in the wild populations of *S. salicifolia*. Obtaining the highest concentration in the population from Amealco, while the lowest concentration was obtained from the population of Tenasda in the municipality of Amealco (Fig. 5).

The amount of 69.99 mg / g ce (catechin equivalents) of total flavonoids was obtained for the population of Huimilpan. This might be due to the combination of environmental factors since the time of collection of the plant material was at a temperature peak, close to the maximum temperature of the day and with only 2.3 mm of precipitation.

The following concentrations of total flavonoids correspond to the populations of Amealco, with 42.35 mg / g ce and 40.68 mg / g ce for Amealco and Tenasda, respectively. The time of collection in these populations was before the peak of temperature and precipitation so that their degree of physiological stress could be lower

Finally, lowest total flavonoid concentration was obtained from the populations in the municipality of Cadereyta, with 39.61 mg / g ce, said population recorded the lowest maximum temperature, and with total lack of precipitation.

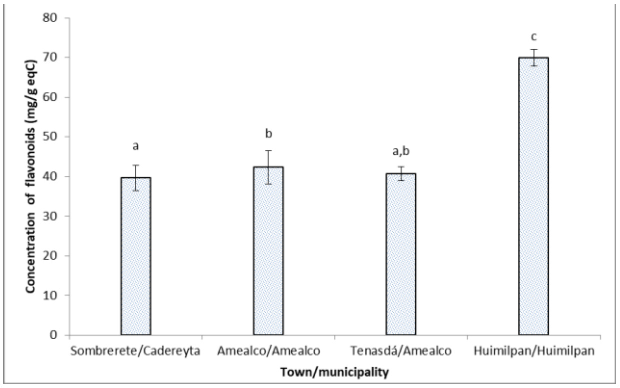

Fig. 5. Concentration of flavonoids obtained from the four wild populations of *S. salicifolia*. a, b, and c, data differ statistically in the Fisher LSD test with 95% of confidence.

D. Phenolic compounds.

In *S. salicifolia* three phenolic compounds were detected as it can be observed in (Fig. 6). For the population of Huimilpan, a concentration of 7.36 mg / g of *p-coumaric* acid was obtained, and only the routine compound was detectable. For the population of Cadereyta, a concentration of 21.68 mg / g of Kaempferol was obtained, in addition to the detection of Rutin. In the Amealco population, the only compound detected was the rutine. Finally, in the population of Tenasdá, Amealco, the highest concentration of Kaempferol was recorded, higher than the population of Cadereyta, with 22.09 mg / g ps sample, and rutine was detected as well.

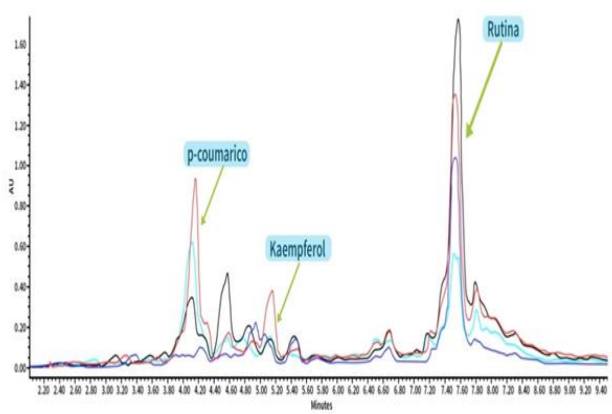

Fig. 6. Chromatographic profile of *S. salicifolia*. Blue: Tenasdá, Amealco. Green: Cadereyta. Red: Huimilpan. Black: Amealco, Amealco.

E. Correlation between the weather conditions and the concentration of flavonoids.

According to the statistical analysis of the effect of meteorological conditions on the production of total and specific flavonoids in the species *S. salicifolia*, the maximum temperature had the greatest effect on the synthesis of these compounds, since their correlation value is of 0.9662, which is close to the value 1, this being the maximum value and most important in a correlation. Another factor that had a strong correlation according to the statistical analysis, was the minimum temperature since its value was 0.8349, which indicates that it also had an effect on the synthesis of flavonoids.

On the other hand, precipitation had no influence on the synthesis of the flavonoids. The correlation value is -0.4535, which indicates that between close to the value -1 their effect is much smaller than those positive values (Table 4).

Table 4. Product moment correlations of Pearson between each pair of variables.

	Accumulated precipitation (mm)	Maximum temperature (°C)	Minimum temperature (°C)
Accumulated precipitation (mm)		-0.3457 (48) 0.0161	
Maximum temperature (°C)	-0.3457 (48) 0.0161		
Minimum temperature (°C)	-0.8552 (48) 0	0.782 (48) 0	
Flavonoids	-0.4535 (48) 0.0012	0.9662 (48) 0	0.8349 (48) 0

The correlation coefficients ranges from -1 to +1, and they measure the strength of the linear relationship between the variables. Additionally, the number of pairs of data used to calculate each coefficient is also shown in parentheses. In red, is a P-value that tests the statistical significance of the estimated correlations. P-Values below 0.05 indicate correlations significantly different from zero, with a confidence level of 95.0%.

IV. Conclusion.

The response of *S. salicifolia* was different to the conditions suggested for other species in terms of temperature and precipitation, in this study the greatest effect on the synthesis of total flavonoids in wild populations was obtained by the maximum temperature since the highest concentration was obtained at a temperature range of 27.20-28.60 °C, while the lowest concentration was obtained in the range of 20.7 - 19.80 °C.

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