KINETICS OF THE PHYSIOLOGICAL AND ANTIOXIDANT RESPONSE TO WATER STRESS IN LETTUCE

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SUMMARY

*Fresh foods with functional properties such as their antioxidant content are in increasing demand for their benefits to human health. The agronomic management of specific conditions as water stress could permit to obtain fresh foods with a high antioxidant content based on the plants response. In this project, the kinetics of the physiological and antioxidant responses to water stress were studied in lettuce (*Lactuca sativa *L.) to identify the appropriate conditions to obtain plants with a higher content of antioxidant phytochemical compounds. The response in terms of mineral composition was also studied. Before harvest, the plants were subjected to a water stress treatment and evaluations were performed on the days 0, 2, 4, 6, 8 and 10. The photosynthesis rate increased in response to water stress, reaching its highest values on day 4, while the highest content of phenolic compounds was determined on day 6. A physiological and non-destructive determination such as the photosynthesis rate could be used as an indicator of a high antioxidant content prior to harvest. The mineral composition and the plant morphology remained without important variations on day 6. The experimental evidence suggests that it would be possible to obtain lettuce plants as fresh food with antioxidant proprieties through the agronomic management of the water stress; however, further studies are needed in post-harvest and regarding human health benefits.*

Introduction

Exposure of plants to unfavorable environmental conditions such as water stress increases the production of reactive oxygen species (ROS), and a large accumulation of ROS affects the cellular functions causing significant production losses (Foyer and Noctor, 2005; Khan and Singh, 2008). As protection against ROS plant cells and organelles have developed a defense system formed by enzymes and antioxidant phytochemical compounds derived from the secondary metabolism (Gill and Tuteja, 2010). The latter are of special interest due to their contribution in prevention and reduction of risk factors of many chronic degenerative diseases in humans, such as cancer, cardiovascular problems and aging (Pandey and Rizvi, 2009). The antioxidant content in plants varies considerably as a function of the agronomic management and the crop conditions. It has been demonstrated that these compounds are part of a complex plant defense mechanism in response to different types of stress, and this represents an opportunity to improve the health benefits based on food of plant products (Oh *et al.*, 2009). Some studies have evaluated the possibility of increasing the amount of phytochemical antioxidants by subjecting the plants to stress conditions, mainly in species where the economic interest lies in foliage consumption, as in *Lactuca sativa* L. (Oh *et al.*, 2009; Chisari *et al.*, 2010; Boo *et al.*, 2011; Galieni *et al.*, 2015). Lettuce is the most popular leaf vegetable in the world, appreciated for its versatility in the elaboration of salads and high content of phytochemical compounds that are health promoters (Durazzo *et al.*, 2014). However, the response to stress in plants depends on the type of the stress factor applied as well as its intensity and duration, generating expression patterns and protein synthesis that lead to the production of metabolites and to different physiological states (Moreno, 2009). Water stress severely limits the crop yield (Farooq *et al.*, 2009); nevertheless, it is a source of variation that is easy to evaluate experimentally and, in consequence, generate oxidative stress in plants (Beck *et al.*, 2007). Plants must be subjected to water stress carefully because it might compromise the final quality in terms of yield, since one of the most crucial factors during the lettuce cultivation is the availability of water. The aim of this project was to study the kinetics of the physiological and antioxidant responses to water stress of *Lactuca sativa* L. in order to identify conditions to obtain plants with a higher content of antioxidant phytoche-

KEYWORDS / Functional Fresh Food / *Lactuca sativa* L. / Minerals / Morphology / Phytochemical Compounds /

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CINÉTICA DE LAS RESPUESTAS FISIOLÓGICA Y ANTIOXIDANTE AL ESTRÉS HÍDRICO EN LECHUGA
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RESUMEN

*Los alimentos frescos con propiedades funcionales tales como contenido de antioxidantes son cada vez más demandados por sus beneficios para la salud humana. El manejo agronómico de condiciones específicas como el estrés hídrico podría permitir obtener alimentos frescos con un alto contenido de antioxidantes con base en la respuesta de las plantas. En el presente proyecto se estudió la cinética de la respuesta fisiológica y antioxidante al estrés hídrico en lechuga (*Lactuca sativa *L.) para identificar las condiciones apropiadas para obtener plantas con un alto contenido de componente fitoquímicos antioxidantes. Igualmente, la composición mineral de la respuesta al estrés fue estudiada. Antes de la cosecha, las plantas fueron sometidas a tratamientos de estrés hídrico y las evaluaciones se realizaron los dias 0, 2, 4, 6, 8 y 10. La tasa fotosintética incrementó en respuesta al estrés hídrico alcanzando su valor mas alto el dia 4, mientras que el mayor contenido de compuestos fenólicos se determinó en el dia 6. Una determinación fisiologica y no destructiva tal como la tasa de fotosíntesis podría ser utilizada como indicador de un alto contenido de compuestos fenólicos antes de la cosecha. La composición mineral y la morfología de las plantas permaneció sin variación importante en el dia 6. Las evidencias experimentales sugieren que podría ser posible obtener plantas de lechuga como alimento fresco con propiedades antioxidantes mediante el manejo agronómico del estrés hídrico; sin embargo, se necesitan estudios adicionales como en la postcosecha y los beneficios para la salud humana.*

CINÉTICA DA RESPOSTA FISIOLÓGICA E ANTIOXIDANTE AO ESTRESSE DA ÁGUA EM ALFACE
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RESUMO

*Alimentos frescos com propriedades funcionais, como seu conteúdo antioxidante, estão cada vez mais em demanda por seus benefícios à saúde humana. O manejo agronômico de condições específicas como o estresse hídrico pode permitir a obtenção de alimentos frescos com alto teor antioxidante baseado na resposta das plantas. Neste projeto, a cinética das respostas fisiológicas e antioxidantes ao estresse hídrico foi estudada em alface (*Lactuca sativa *L.) para identificar as condições adequadas para obtenção de plantas com maior teor de compostos fitoquímicos antioxidantes, tambiem, a resposta da composição mineral foi estudada. Antes da colheita, as plantas foram submetidas a um tratamento de estresse hídrico e as avaliações foram realizadas nos dias 0, 2, 4, 6, 8 e 10. A taxa de fotossíntese aumentou em resposta ao estresse hídrico atingindo seus maiores valores no dia 4, enquanto o maior teor de compostos fenólicos foi determinado no dia 6. Uma determinação fisiológica e não destrutiva como a taxa de fotossíntese poderia ser usada como um indicador de um alto teor de antioxidantes antes da colheita. A composição mineral e a morfologia das plantas permaneceram sem uma variação importante no dia 6. As evidências experimentais sugeriram que seria possível obter plantas de alface como alimento fresco com propriedades antioxidantes através do manejo agronômico do estresse hídrico; contudo, mais estudos são necessários como na pós-colheita e benefícios para a saúde humana.*

mical compounds. The mineral composition was also studied during the observed chnges.

Materials and Methods

Plant material and experimental conditions

The 'Chavela' cultivar of iceberg type lettuce was used because it is lartgely produced in the region where the experiment was performed and it is cultivated under different systems. The plants were kept under greenhouse in pots with peat substrate Sunshine® fine mix Nº 3. Constant irrigation was maintained until they reached the stage of head formation (60 days). At that time, to ensure that the water loss was similar in each repetition as the intensity of the water stress increased, the weight of the pots was normalized to 1.1kg, considering plant development, amount of substrate, water volume and pot weight. Evaluations were performed on the days 0, 2, 4, 6, 8 and 10 from the beginning of the water stress treatment, thus generating the kinetics of the response. The experiment was carrie out at the facilities of the Life Sciences Division, Universidad de Guanajuato, Mexico, located at 20º44'35''N and 100º19'50''W, at 1743masl. Average maximum and minimum temperatures were 29.4 and 14.5ºC, and average maximum and minimum relative humidity were 87.5 and 57.4%, according to the weather station Copal of the Fundación Guanajuato Produce A.C.

Determination of the antioxidant response

The determination of phenolic compounds was performed according to the procedure reported by Zin *et al*. (2006) with some modifications: 3g of leaf tissue were grinded during 1h at room temperature with 10ml of 50% methanol/water solution and the solution filtered. To determine phenolic compounds, 250µl of the extract were mixed with 250µl of Foli-Ciocalteu reagent (1:1). After 5min, 500µl of 20% Na_2CO_3 solution were added to the mixture and kept at 40ºC for 25min, after which the concentration of phenolic compounds (PC) was determined by spectrophotometry as absorbance (ABS) at 750nm. The results were expressed as gallic acid equivalent (mg GAE/gfw). The antioxidant activity (AA) was measured according to the methodology proposed by Awad *et al*. (2011) with some modifications, through the inactivation of the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical. An 3.9ml aliquot of 0.1 mM DPPH was added into 100µl of the previously obtained extract. The mixture was incubated for 30min at 40ºC in darkness and ABS measured at 515nm. The antioxidant activity was expressed in

chelation percentage terms by using the formula:

$$AA\% = \frac{ABS_{control} - ABS_{sample}}{ABS_{control}}$$

Determination of minerals

N (%) was determined by acid digestion of H_2SO_4 and the semi-micro Kjeldjal method (Yoshida *et al.*, 1976). To determine P (%), K (%), Ca (%), Mg (%), Na (%), Fe (ppm), Cu (ppm), Zn (ppm) and Mn (ppm), digestions were performed with 0.5g of dry foliage in 30ml of acid mixture (20ml of 70% HNO_3 and 10ml of 65% $HClO_4$) for 12h at room temperature. The digestion mixture was warmed to 150ºC for 40min in a rotary evaporator with glass beads. Afterwards, the temperature was increased at 210°C until an azeotropic mixture of $HClO_4$ was obtained, and the mixture was solubilized in 50ml of deionized water. The concentration of P was calorimetrically determined, while those of K and Na were obtained by flame spectrophotometry and the contents of the other minerals by atomic absorption.

Evaluation of physiological and morphological variables

The plant height (PH; cm) and total biomass (TB; g) were determined, the latter being separated into foliage (FB) and root (RB) biomass. The water relative content in the foliage (WRCF; %) was measured by the procedure described by Bernacchia *et al.,* (1996); likewise, this determination was performed in the substrate (WRCS; %) as an indicator of the increasing in-tensity of the water stress. Photosynthesis (A; µmol $CO_2 \cdot m^{-2} \cdot s^{-1}$), transpiration (E) and stomatal conductance (g_s) were measured with an infrared gas analyzer LI-6400 (LICOR); the last two variables were expressed as mmol $H_2O \cdot m^{-2} \cdot s^{-1}$.

Statistical analysis

The results were subjected to analysis of variance by a completely randomized design with five repetitions and performing mean separation tests of Tukey, using the statistical software Minitab ® 16.2.3. The days after the water stress was initiated were considered as treatments. Complementary information of the statistical analysis performed is presented in the Appendix.

Results and Discussion

The water relative content in the substrate (WRCS) gradually decreased ($p<0.01$) from the detention of irrigation and therefore the intensity of the water stress increased proportionally (Table I). The somatal conductal (g_s), transpiration (E) and water relative content in the foliage (WRCF) were reduced ($p<0.01$) as the water availability in the substrate was limited. The stomatal closure is a physiological response to avoid the water stress and to keep the water relative content in the tissues (Nunes *et al.*, 2008); in fact, the capacity to keep a high WRCF during water stress is an indicator of tolerance (Silva *et al.*, 2007), even if the leaves partially wither (Rivero *et al.*, 2007). In our experiment, the Chavela cultivar did not keep its WRCF despite of the stomatal closure and the E reduction, indicating that this cultivar is highly affected by the water deficiency. On the contrary, photosynthesis (A) increased ($p<0.01$) when the water stress was moderate and reached its highest magnitude on day 4; later, stress intensity became too severe and photosynthesis decreased. The increase in A under moderate water stress has been reported in other species with C3 metabolism, as *Triticum durum* (Abbad *et al.*, 2004), *Phaseolus vulgaris* (Ruiz-Nieto *et al.*, 2015) and *Gossypium hirsutum* (Massacci *et al.*, 2008). The last author explained such increase as due to the quantum efficiency of the photosystem II, which indicatea less photorespiration, since photosynthesis is barely affected by the water limitation; later, when the stress is severe and prolonged, photosynthesis is inhibited (Muller *et al.*, 2011). Even under optimal water conditions, the formation rate of hydrogen peroxide by the electron transport chain during photosynthesis in C3 species is as high as 4µmol·m^{-2}·s^{-1} (Foyer and Noctor, 2003), while under stress conditions the accumulation of such oxidant might increase several times (Queval *et al.*, 2008).

The accumulation of phenolic compounds (PC) increased ($p<0.01$) in proportion to the water stress intensity, it being on day 6 when the greatest content of PC was found (Figure 1a). These results were similar to those previously reported by Oh *et al.* (2009) in lettuce plants subjected to stress by temperature and light. However, the antioxidant activity (AA) remained relatively stable ($p<0.01$) from the first until the sixth day of evaluation (Figure 1b). There are several compounds with important antioxidant proprieties in plants, but their chelating capacities differ depending on the chemical structure and amount in the plant; therefore, a high amount of PC is not always related to a high AA, especially for the

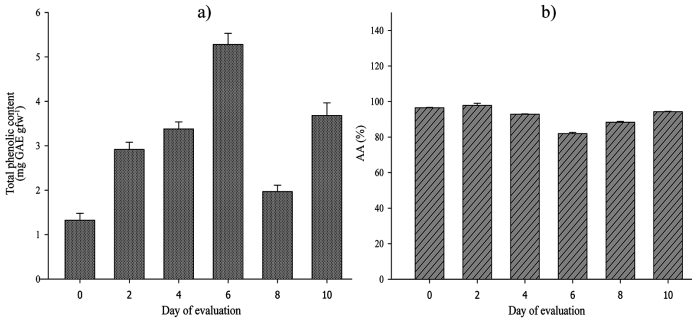

Figure 1. Kinetics of the antioxidant response to water stress. a: Phenolic compounds, b: antioxidant activity**. * Significant differences ($p<0.05$); ** highly significant differences ($p<0.01$).

TABLE I
PHYSIOLOGICAL AND MORPHOLOGICAL VARIABLES EVALUATED DURING THE KINETIC STUDY OF THE RESPONSE TO WATER STRESS

Variable	Day 0	Day 2	Day 4	Day 6	Day 8	Day 10
PH	20.10 a A	23.46 a A	23.80 a A	23.36 a A	23.30 a A	20.50 a A
FB	1.86 b A	2.30 ab A	2.82 ab A	2.40 ab A	3.42 a A	2.77 ab A
RB	1.64 a A	1.26 a A	1.48 a A	1.14 a A	1.42 a A	1.06 a A
TB	3.50 a A	3.58 a A	4.16 a A	3.54 a A	4.48 a A	3.64 a A
WRCF**	92.06 a A	87.99 a A	57.82 ab AB	44.53 a AB	40.26 b AB	23.65 b B
WRCS**	47.97 a AB	53.88 a A	31.28 bc CD	34.30 b BC	16.26 d D	21.51 cd CD
A**	1.14 d DE	5.13 b B	6.48 a A	2.06 c C	1.52 d CD	0.65 e E
E**	2.38 a A	2.39 a A	1.63 b B	0.52 c C	0.32 c C	0.55 c C
g_s**	0.07 a A	0.07 ab A	0.05 b A	0.01 c B	0.01 c B	0.01 c B

PH: plant height (cm), FB: foliage biomass (g), RB: root biomass (g), TB: total biomass (g), WRCF: water relative content in the foliage (%), WRCD: water relative content in the substrate (%), A: photosynthesis (µmol CO_2 m^{-2}·s^{-1}), E: transpiration (mol H_2O m^{-2}·s^{-1}), g_s: stomatal conductance (mol H_2O m^{-2}·s^{-1}). Values with the same lowercase or capital letter within averages rows are statically equal according to the Tukey test ($p<0.05$ and $p<0.01$, respectively). ** Highly significant differences ($p<0.01$).

DPPH radical that sometimes reacts after longer periods (Sanchez-Moreno *et al*., 1998). Indeed, due to the different pathways of antioxidant activity and the extreme dependency of this method on the employed condition, not all the PC present in nature could have the same AA against the DPPH radical (Peña-Cerda *et al*., 2017). It was remarkable that the highest A was determined on day 4 and the highest accumulation of PC was determined two days later. Considering that the determination of such antioxidant compounds is destructive, and based on the experimental evidence, a physiological and non-destructive measurement such as the A could be used to infer that when this variable reaches its highest value, two days later the maximum accumulation of phenolic compounds will be also reached.

In relation to the determination of minerals, the concentration of N, P, K and Zn in the tissue was reduced ($p<0.01$) as the intensity of the water stress increased (Table II). According to Khasanova *et al*. (2013), under water stress N cannot be mobilized from the senescent leaves and be internally stored. The water stress reduces the contents of P and/or K , limiting them as the tissue develops, due to the importance of these elements in the plant biology (Sardans and Peñuelas, 2007). If the plants would have been harvested on day 6 after beginning the water stress, the P, K, Ca, Mg and Cu contents would have been the same as on day 0; while those of Mn and Na would have been higher. Mn is an essential element in the human diet and has a key role in the defense against free radicals (Lobo *et al*., 2010; Valko *et al.,* 2007). In relation to Fe, a tendency to increase its concentration in the tissue was observed. It is well known that the Fe plays a fundamental role in human health (Abbaspour *et al*., 2014). There were no differences ($p>0.05$) in the plant height and total biomass, which might indicate that the plant size was the same between the days 0 and 6; therefore, the market value of the plant would not decrease because of subjecting the plant to water stress on any of these days, but the accumulation of PC and potentially of Fe would provide a high added value. One of the better known morphological responses to water stress is the increase in the development of a radical system (Jaleel *et al*., 2009). However, we found no difference ($p>0.05$) between the biomass destined to the root and foliage, which ensures that there would be no significant differences in the plant size if harvested on the recommended day after the beginning of the water stress. The studies about antioxidants in plants and food have become one of the most popular topics today in the agri-food area, and it has been proposed that compounds as polyphenols might contribute to improve health through the consumption of fruits and vegetables (Herrera *et al*., 2009).

Conclusions

The lettuce plants of the Chavela cultivar subjected to water stress at the end of their productive cycle show an increase of their photosynthesis rate on the day 4, and two days later the highest increase of the phenolic compounds was determined, while their morphology and mineral content remained without important variation. Through the management of the water stress it would be possible to harvest plants with antioxidant proprieties by using physiological non-destructive responses as indicators. However, it is necessary to evaluate the management in experimental field plots, different stress sources, perform deeper antioxidant and enzymatical essays, and evaluate the post-harvest management and the human health benefits.

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TABLE II
MINERALS DETERMINED DURING THE KINETIC STUDY OF THE RESPONSE TO WATER STRESS

Mineral	Day 0	Day 2	Day 4	Day 6	Day 8	Day 10
N (%) **	3.94 ab AB	4.03 a A	3.78 abc ABC	3.62 cd BC	3.74 bcd ABC	3.49 d C
P (%) **	0.48 a A	0.45 a A	0.47 a A	0.44 a A	0.48 a A	0.34 b B
K (%) **	5.43 b B	5.77 a A	4.87 c C	5.76 a A	3.68 e E	4.41 d D
Ca (%) **	2.23 b B	3.36 b B	2.54 b B	2.71 b B	3.41 b B	5.27 a A
Mg (%) **	0.17 c B	0.19 bc B	0.20 ab AB	0.19 bc B	0.19 bc B	0.22 a A
Na (%) *	1.56 d D	2.52 b B	2.49 b B	2.83 a A	2.52 b B	2.19 c C
Fe (ppm)	193.33 a A	216.00 a A	208.33 a A	217.00 a A	213.33 a A	222.33 a A
Cu (ppm)	2.00 a A	3.00 a A	1.33 a A	1.33 a A	1.33 a A	2.00 a A
Zn (ppm) **	67.00 abc AB	68.33 a A	67.67 ab AB	62.00 bcd AB	60.33 d B	61.00 cd AB
Mn (ppm) **	154.33 d D	178.33 bc BC	172.33 c C	213.33 a A	191.00 b B	225.00 a A

Values with the same lowercase or capital letter within averages rows are statically equal according to the Tukey test ($p<0.05$ and $p<0.01$, respectively).
* Significant differences ($p<0.05$).
** Highly significant differences ($p<0.01$).

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APPENDIX
COMPLEMENTARY INFORMATION OF THE STATISTICAL ANALYSIS

Variable	MST	MSE	F	P	R^2	CV	HSD
PH	13.36	7.37	1.81	0.149	12.28	11.68	2.716
FB	1.42	0.58	2.44	0.064	19.85	27.76	0.765
RB	0.24	0.28	0.83	0.540	0.0	33.47	0.537
TB	1.41	2.01	0.70	0.629	0.0	32.96	1.419
WRCF	3.200	469	6.82	< 0.01	50.07	38.88	21.67
WRCS	1.068	41.2	25.93	< 0.01	81.13	22.84	6.420
A	28.52	0.05	543.17	< 0.01	98.94	12.08	0.229
E	4.59	0.06	70.69	< 0.01	92.32	19.08	0.254
g_s	< 0.01	< 0.01	47.28	< 0.01	88.86	24.53	0.010
PC	< 0.01	< 0.01	143.66	< 0.01	97.67	7.00	0.007
AA	104.31	0.39	267.25	< 0.01	98.74	0.54	0.624
N	0.11	< 0.01	13.27	< 0.01	78.31	2.11	0.093
P	< 0.01	< 0.01	12.11	< 0.01	76.57	4.81	0.025
K	2.08	< 0.01	279.50	< 0.01	98.79	1.55	0.086
Ca	3.56	0.20	17.88	< 0.01	83.23	10.77	0.446
Mg	< 0.01	< 0.01	9.75	< 0.01	72.02	4.80	0.009
Na	0.57	< 0.01	114.38	< 0.01	97.09	2.32	0.070
Fe	307	255	1.20	0.365	5.63	5.15	15.97
Cu	1.30	0.66	1.95	0.159	21.84	43.87	0.816
Zn	40.06	4.83	8.29	< 0.01	68.19	3.01	2.198
Mn	2091.3	34.4	60.81	< 0.01	94.62	2.62	5.864

For symbols and units of the variables see Tables I and II. PC: phenolic compounds, AA: antioxidant activity, MST: mean square of the treatments, MSE: mean square of the error, CV: coefficient of variation, HSD: honestly significant difference of Tukey (0.05).