

Article

# Effect of Cropping System and Humidity Level on Nitrate Content and Tipburn Incidence in Endive

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**Abstract:** Tipburn is an important disorder caused by a calcium deficiency that affects the marketability of endives. Genotype, air relative humidity, and temperature are directly involved in tipburn occurrence. Our study aimed to investigate the effect of cropping systems and relative humidity on the marketable yield, nitrate accumulation, and incidence of tipburn in endives. Two cultivars were evaluated in pots ('Cuartana' and 'Natacha'), two cropping systems (greenhouse and open-field), and in two different air humidity levels (high level: plants under a plastic tunnel with an extra supply of humidity with micro-sprinklers, and low level: plants without an extra supply of humidity and outside of a plastic tunnel) during two years (2013 and 2014) in different growing seasons. Nitrate content was determined by reflectometry, and tipburn was evaluated using a qualitative scale. Results showed that tipburn was favored under greenhouse with low humidity levels, with 40–60% plants affected. 'Natacha' was more susceptible to tipburn (>20% plants affected) than 'Cuartana' (<20% plants affected). Leaf nitrate accumulation was favored by the highest temperatures (greenhouse). It is concluded that in our conditions, tipburn incidence in endives depends on the interaction of genotype and the environmental conditions. Nitrate content was more influenced by the temperature than by the cultivars used.

**Keywords:** *Cichorium endivia*; chlorophyll; control; greenhouse; nitrate; open-field; tipburn; relative humidity; tunnel

### 1. Introduction

In Spain, the annual consumption of fresh vegetables for prepared salads is about 6.67 kg person [1]. The per capita consumption in the United Kingdom, France and Italy is 12, 6, and 4 kg, respectively; however, consumption in Belgium or Croatia is 4–10 times higher. Sales of fresh vegetables for prepared salads in Spain increased 57.8% in 2018 compared to the previous year, indicating its importance as a specialty crop [2].

Vegetables are the most important source of nitrate ( $NO_3^-$ ) intake for humans, but the consumption of these compounds in high concentrations can be toxic. Leafy vegetables are especially prone to nitrate accumulation [3]. Although nitrate is relatively non-toxic, it is converted to nitrites in the digestive system, which are associated with methaemoglobinemia, and the development of stomach cancer [4,5].

The maximum allowable concentration of nitrate specified in the Official Journal of the European Union [6] is 5000 (in greenhouse) and 4000 mg  $NO_3$  kg<sup>-1</sup> (in open-field), when the vegetables



are harvested between October and March, and from 4000 (in greenhouse) to 3000 mg  $NO_3$  kg<sup>-1</sup> (in open-field) when harvested between April and September [6].

Nitrate accumulation is the result of excessive uptake, and is affected by environmental factors and genotype [7]. Solar radiation has been shown as one of the key factors [8]. Its importance is related to the role of nitrate in regulating the osmotic pressure. When light levels are not optimal for photosynthesis, carbohydrate concentration in the vacuoles decreases, and nitrate accumulates in the vacuoles for maintaining cell turgor [8]. On the other hand, some authors have suggested that high levels of solar radiation could stimulate the activity of nitrate reductase, favoring the conversion of nitrate in ammonium [9,10].

Related to the genotype, there are several studies in lettuce and endive (*Cichorium endivia* L.) that found differences in nitrate accumulation between cultivars [11–13]. These differences are attributed to variable photosynthetic rates [12].

Leaf tipburn is a physiological disorder that frequently affects some leafy vegetables produced all year round for the fresh market, like lettuce and endives, and it is characterized by necrosis associated with a localized deficiency of calcium that appears in the apex and margins of young leaves, reducing the marketability of these crops [14,15].

Calcium movement in plants occurs in the xylem and its transport to the different parts of the plants is directed correlated with transpiration during the day and with water moved by root pressure flow during the night [16–18]. The development of tipburn disorder has been studied by several authors. They describe that tipburn apparition is associated to the genotype and can be modified by environment conditions including relative humidity and temperature [14,19,20].

Low humidity can increase tipburn during the day because it favors the transpiration of outer leaves in comparison to inner head leaves, consequently reducing the flow of calcium. At night, low humidity may decrease plant turgor potential, and as a result less calcium ascends by guttation [16,19,21].

The effect of the temperature is related to plant growth. One of the principal functions of calcium concerns its role in structural organization during cell division and enlargement, and it is one of the principal compounds of cell walls and membranes [22–24]. High temperature encourages rapid growth and increases the demand for calcium in expanding leaf tissues [18].

The aim of this work was to evaluate the effect of environment conditions, especially relative humidity, using two humidity treatments (low and high) and two crops systems (greenhouse and open-field) to demonstrate whether humidity level can influence tipburn and the nitrate content of leaves. We evaluated the marketable yield, nitrate accumulation, tipburn incidence, and chlorophyll, obtained of two commercial endive cultivars ('Cuartana' and 'Natacha') grown in two crop systems (greenhouse and open-field) under two humidity levels (high and low).

#### 2. Materials and Methods

Field trials were conducted during two growing seasons (Table 1) in a Venlo-type greenhouse and in an open-field, both located at the *Universitat Politècnica de València* (U.P.V.) in eastern Spain (latitude: 39°380′ N, longitude: 0°220′ W) during two years (2013 and 2014).

The venlo-type greenhouse had a glass cover with automatic zenith windows and a cooling-system inside that evaporate water to plants when temperatures were higher than 28 °C or lower than 14 °C.

The experiment was arranged in a factorial split-split-plot. The main-plot was the cropping system and the subplots were the humidity treatment and cultivar. Cropping system treatments included greenhouse and open-field. The greenhouse cropping system was a glass covered venlo-type greenhouse with automatic zenith window and an evaporative cooling- system (setpoints 28 °C and 14 °C). The humidity level included low level (plants grown in greenhouse and open-field without an extra supply of humidity) and high level (plants grow in the greenhouse and open-field under a plastic tunnel with supplemental humidity supplied by micro-sprinklers located at the top of the tunnel). The supplemental humidity supplied by the micro-sprinklers did not result in additional irrigation water for plants, as this water was used for evaporative cooling. Cultivars included 'Cuartana' and

'Natacha'. All main-plots and subplots treatments were assigned by complete block randomization. Each treatment combination (2 cropping system  $\times$  2 humidity levels  $\times$  2 cultivars) was replicated three times with 10 plants in each replicate for a total of 240 plants.

Year	Seasons	Seeding Date	Transplanting Date	Harvest Date	
2013 -	Winter	29/01/13	06/03/13	Greenhouse Open-field	26/04/13 08/05/13
	Spring	03/05/13	27/05/13	Greenhouse Open-field	16/07/13 22/07/13
2014 -	Winter	04/12/13	10/01/14	Greenhouse Open-field	10/03/14 to 20/03/14 27/03/14 to 01/04/14
	Spring	30/03/14	24/04/14	Greenhouse Open-field	02/06/14 to 03/06/14 12/06/14 to 13/06/14

**Table 1.** Seeding, transplanting, and harvest dates for endives planted in greenhouse and open-field.

Seeds of two cultivars (Enza Zaden, Almería, Spain) were sown in polystyrene trays (Herku Ref HPE 66; Projar, Valencia, Spain) filled with a mixture (6:3:1) of coconut coir (Cocopeat fine<sup>®</sup>; Projar, Valencia, Spain), sphagnum peat (Kekkilä LSM 2W R8264; Projar, Valencia, Spain), and black peat (Kekkilä DSM W NR1 KSON R7753; Projar, Valencia, Spain). Seedlings with 3 to 4 leaves were transplanted to the greenhouse and open-field in 8-L pots (Serial CD; Projar, Valencia, Spain) filled with a mixture of coconut coir: perlite (1:1) (Cocopeat<sup>®</sup> P10; Projar, Valencia, Spain). We used a balanced nutrient solution composed of (mmol L<sup>-1</sup>): NO<sub>3</sub><sup>-</sup> = 12.41, H<sub>2</sub>PO<sub>4</sub><sup>-</sup> = 1.20, SO<sub>4</sub><sup>2-</sup> = 2.45, NH<sub>4</sub><sup>+</sup> = 0.50, K<sup>+</sup> = 5.00, Ca<sup>2+</sup> = 3.95, and Mg<sup>2+</sup> = 2.96, and CE (dS·m<sup>-1</sup>) = 2.01 applied by a drip system (two 2.2 L·h<sup>-1</sup> per plant) (Navya; Azud, Murcia, Spain). Drip irrigation was supplied based on estimations of the weekly crop evapotranspiration (ETc). The volume of each irrigation and the number of irrigations were scheduled to maintain drainage between 20% and 30%.

The evaluation of tipburn incidence and the determination of nitrate content were performed immediately after harvest. Tipburn was evaluated using a qualitative scale from 0 to 3 (0 = no damage, 3 = high level of damage). Only plants in the 0–1 value range were considered marketable. After harvesting, four fully expanded leaves were collected from two plants in each replication. A solution of midrib sap and distilled water (1:25) was used to determine nitrate content by reflectometry using a commercial kit with nitrate-sensitive test strips containing two reactive zone pads that turn red–violet when exposed to nitrate solution. The color intensity is proportional to the concentration of nitrate in the solution. Nitrate concentration was determined quantitatively by comparing the color of test strip reactive zones using a hand held reflectometer (RQflex <sup>®</sup> KGaA; Merck, Darmstadt, Germany).

To determine the chlorophyll content by spectrophotometry, a 0.5-g sample of the leaf blade (was taken. Samples were ground in a mortar with acetone (90%) and centrifuged for 10 min at 4000 rpm. The supernatant was poured into volumetric flask and diluted with acetone (90%) to 50 mL. The absorbance of this solution was measured at the wavelengths A470, A645, and A663 nm. The chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids were determined according to the following equations:

Chla (mg/g FW) =  $[(11.75 \times A663 - 2.35 \times A645) \times 50]/500$ Chlb (mg/g FW) =  $[(18.61 A645 - 3.96A663) \times 50]/500$ 

Chlt (mg/g FW) = Chla + Chlb

FW: fresh weight

Where: A663, A645, A470: Absorbance at 663, 645, and 470 nm

Air humidity and temperature were measured over time in the two humidity levels. In the conditions of high humidity, two data loggers (Testo Logger 177-H1; Testo SE & Co, Lenzkirch, Germany) were installed under the plastic tunnels. For the plants grown without an extra supply of water, we used a weather station (Weather Monitor II<sup>®</sup>; Davis Instruments, Hayward, CA, USA)

(open-field) and a datalogger connected to climatic sensors (Climatec Agro, Climatec, Almería, Spain) (greenhouse) with a logging interval of ten minutes.

The variation of temperature and humidity obtained in the conditions of low humidity and high humidity in two years were assessed by the mean, standard error, and confidence intervals using a statistical program (Statgraphics Centurion XV; Statgraphics Tecnologies Inc, Lawrence, NJ, USA) (Table 2).

**Table 2.** Ranges of temperature and humidity relative to the different conditions of humidity tested (low humidity and high humidity) and in the two cropping systems (greenhouse and open-field).

Greenhouse26Open-field23	.91± 0.53 [26.38; 27.73] .75± 1.08 [22.67; 24.83]	57.44± 2.57 [57.87; 60.01] 67.62± 2.11 [65.51; 69.73]
Low humidity 20 High humidityH 25	.22± 0.42 [19.80; 20.64] .29± 0.55 [24.70; 25.84]	55.37± 1.49 [53.88; 56.86] 68.49± 1.23 [67.26; 69.72]

Means  $\pm$  standard error; [confidence intervals] with  $p \le 0.05$ .

For each year and season, the significance of the effects was determined by means of ANOVA for the biomass of marketable plants, tipburn incidence, nitrate accumulation, and chlorophyll content, considering the cultivar (Cv), humidity level (HL), and crop system (CS) and their interactions using a statistical software (Statgraphics Centurion XV; Statgraphics Tecnologies, Inc., Lawrence, NJ, USA). In order to determine tipburn incidence, an angular transformation was performed: arcosin  $\sqrt{x}$ , x = percentage of plants with tipburn. Mean separation was performed using least significant differences (LSD) test ( $p \le 0.05$ ).

#### 3. Results

The biomass of marketable plants was influenced by the crop system in winter of 2013 and both seasons of 2014 ( $p \le 0.01$ ). In general, plants grown in the open field obtain higher values of marketable biomass ( $p \le 0.05$ ); but, when the interaction HL x CS is taking into consideration ( $p \le 0.01$ ), these differences are only obtained under low humidity conditions. The marketable biomass is 68.2% higher in the open-field system compared to the greenhouse system in the winter of 2013 and both seasons of 2014.

No statistical significance was observed for cultivar nor in the interactions (double or triple) with the cultivar. The only difference in biomass between cultivars are observed in spring of 2014, with 'Natacha' showing the highest values ( $p \le 0.01$ ) (Table 3). We found significant interaction of Cv × CS in open-field ( $p \le 0.05$ ). The effect of humidity on yield is variable between years and seasons.

Results show a statistical influence of the cultivar in the tipburn incidence ( $p \le 0.01$ ) (Table 4). Generally, 'Natacha' presents significantly higher values of tipburn ( $p \le 0.01$ ) (Table 4). An interaction is detected with Cv x HL (2013  $p \le 0.01$ ; 2014  $p \le 0.05$ ) to influence tipburn where differences between cultivars are found in low humidity conditions, while no differences are detected in high humidity treatment (Figure 1).

**Table 3.** Biomass of marketable plants of two endive cultivars under different humidity and cropping systems cultivated in two growing seasons.

	Biomass of Marketable Plants (g·Plant <sup>-1</sup> )			
	201	13	2014	
	Winter	Spring	Winter	Spring
Cultivar (Cv)				
'Cuartana'	$669.3 \pm 58.2$	$317.4 \pm 28.7$	$769.7 \pm 27.9$	694.4 ± 32.5 b
'Natacha' Humidity level (HL)	$650.7 \pm 58.2$	$365.7 \pm 49.7$	755.4 ± 27.9	811.0 ± 40.7 a

	<b>Biomass of Marketable Plants (g.Plant<sup>-1</sup>)</b>				
	203	13	2014		
Low Humidity	$621.1 \pm 58.2$	422.8 ± 49.7 a	694.2 ± 28.8 b	$629.8 \pm 35.5$	
High Humidity	$698.9 \pm 58.2$	$260.3 \pm 28.7 \text{ b}$	830.9 ± 29.4 a	$709.6 \pm 44.5$	
Cropping system (CS)					
Greenhouse	539.4 ± 58.2 b	$377.7 \pm 49.7$	477.2 ± 26.3 b	543.6 ± 31.8 b	
Open-field	$780.7 \pm 58.2 \text{ a}$	$305.7\pm28.7$	$1047.9 \pm 26.3 a$	795.6 ± 52.1 a	
Analysis of variance Parameters (degrees of freedom)		Significance level			
Cv ( <i>n</i> = 1)	ns	ns	ns	**	
HL $(n = 1)$	ns	**	**	ns	
CS(n=1)	**	ns	**	**	
$Cv \times HL (n = 1)$	ns	ns	ns	ns	
$Cv \times CS (n = 1)$	ns	ns	ns	*	
$HL \times CS (n = 1)$	ns	**	**	ns	
$Cv \times HL \times CS (n = 1)$	ns	ns	ns	ns	

Table 3. Cont.

Significance level: \*\*  $p \le 0.01$ , \*  $p \le 0.05$  and ns not significant. Mean  $\pm$  SD followed by different letters are statistically different at  $p \le 0.05$  (LSD test).

**Table 4.** Tipburn incidence of two endive cultivars under different humidity and cropping systems cultivated in two growing seasons.

	Tipburn Incidence (%)			
	2013		20	14
	Winter	Spring	Winter	Spring
Cultivar (Cv)				
'Cuartana'	$6.7 \pm 8.4$	$10.8 \pm 7.9$ b	$1.6 \pm 2.1 \text{ b}$	18.3 ± 7.3 b
'Natacha'	$20.2 \pm 25.2$	46.9 ± 7.9 a	$16.6 \pm 20.9$ a	48.3 ± 7.3 a
Humidity level (HL)				
Low Humidity	25.0 ± 31.5 a	$29.4 \pm 7.9$	16.6 ± 25.2 a	57.7 ± 6.0 a
High Humidity	1.7 ± 2.1 b	$28.3 \pm 7.9$	$1.6 \pm 1.3  \text{b}$	21.1 ± 6.0 b
Cropping system (CS)				
Greenhouse	26.4 ±33.5 a	47.1 ± 7.9 a	16.6 ± 25.2 a	31.1 ± 6.0 b
Open-field	$0.0 \pm 0.0 \text{ b}$	$10.7 \pm 7.9$ b	$1.6 \pm 1.3$ b	$47.7 \pm 6.0 a$
Analysis of variance Parameters (degrees of freedom)	Significance level			
Cv(n = 1)	ns	**	**	**
HL $(n = 1)$	**	ns	**	**
CS(n=1)	**	**	**	*
$Cv \times HL$ ( <i>n</i> = 1)	*	*	**	**
$Cv \times CS (n = 1)$	*	*	**	ns
$HL \times CS (n = 1)$	**	ns	**	**
$Cv \times HL \times CS (n = 1)$	*	*	**	**

Significance level: \*\*  $p \le 0.01$ , \*  $p \le 0.05$  and ns not significant. Mean ± SE followed by different letters are statistically different at  $p \le 0.05$  (LSD test).

The performance of both cultivars was similar across the experiment, with 'Natacha' presenting higher tipburn incidence than 'Cuartana' under greenhouse conditions and no differences between cultivars in open-field (Cv x Cs interaction  $p \le 0.01$ ) (Figure 2).



**Figure 1.** Interaction effect between cultivar x humidity level on percent of tipburned plants. Incidence of tipburn' in plants across different humidity levels during 2013 (**A**. Winter; **B**. Spring) and 2014 (**C**. Winter; **D**. Spring). Columns followed by different letters indicate mean  $\pm$  SE and are statistically different at  $p \le 0.05$  (LSD test). Vertical bars indicate interval least significant differences (LSD).

The influence of the crop system is statistically significant in tipburn incidence ( $p \le 0.01$ ;  $p \le 0.05$ ) (Table 4). Tipburn incidence is higher in the greenhouse ( $p \le 0.05$ ), but this significant difference is only found in cultivar 'Natacha' (interaction Cv × CS) (Figure 2).

Humidity level influenced the tipburn incidence ( $p \le 0.05$ ) (Table 4) with higher tipburn incidence in plants grown under low humidity ( $p \le 0.05$ ), this is further confirmed with the interaction HL × CS ( $p \le 0.01$ ) (Figure 3).

Generally, nitrate content is not affected by cultivar. 'Cuartana' grown in greenhouses during winter of 2014, accumulated more nitrates than 'Natacha' ( $p \le 0.05$ ) (Table 5) (Figure 4).

	Nitrate Content (mg/kg Fresh Product)			
	2013		2014	
	Winter	Spring	Winter	Spring
Cultivar (Cv)				
'Cuartana'	2253.8 ± 206.2 a	$3690.4 \pm 412.8$	$2172.9 \pm 159.4$	$2733.7 \pm 283.2$
'Natacha'	1656.3 ± 206.2 b	$3595.1 \pm 412.8$	$1847.0 \pm 159.4$	$2849.1 \pm 283.2$
Humidity level (HL)				
Low Humidity	$2019.3 \pm 206.2$	$3260.9 \pm 412.8$	$2178.0 \pm 130.2$	3061.8 ± 231.3 a
High Humidity	$1890.7 \pm 206.2$	$4020.6 \pm 412.8$	$1935.9 \pm 130.2$	2443.5 ± 231.3 b
Cropping system (CS)				
Greenhouse	2411.1 ± 206.2 a	$3567.9 \pm 412.8$	2634.2 ± 130.2 a	3130.8 ± 231.3 a
Open-field	$1498.8 \pm 206.2 \text{ b}$	$3713.5 \pm 412.8$	1479.8 ± 130.2 b	2374.4 ± 231.3 b

**Table 5.** Nitrate content of two endive cultivars under different humidity and cropping systems cultivated in two growing seasons.

	I	Nitrate Content (mg/kg Fresh Product)				
-	2013		2014			
Analysis of variance Parameters (degrees of freedom)	Significance level					
$\operatorname{Cv}(n=1)$	*	ns	ns	ns		
HL $(n = 1)$	ns	ns	ns	**		
CS(n=1)	*	ns	**	**		
$Cv \times HL (n = 1)$	ns	ns	ns	ns		
$Cv \times CS (n = 1)$	ns	ns	*	ns		
$HL \times CS (n = 1)$	ns	ns	ns	ns		
$Cv \times HL \times CS (n = 1)$	ns	ns	ns	ns		

Table 5. Cont.

Significance level: \*\*  $p \le 0.01$ , \*  $p \le 0.05$  and ns not significant. Mean ± SE followed by different letters are statistically different at  $p \le 0.05$  (Fisher's LSD test).



**Figure 2.** Interaction effect between cultivar x crop system on percent of tipburned plants. Incidence of tipburn plants across different crop systems during 2013 (**A**. Winter; **B**. Spring) and 2014 (**C**. Winter). Columns followed by different letters indicate mean  $\pm$  SE and are statistically different at  $p \le 0.05$  verticals bars indicate LSD test). Vertical bars indicate interval LSD.



**Figure 3.** Interaction effect between humidity level x crop system on percent of tipburned plants. Incidence of tipburn plants across different humidity levels in greenhouse and open-field growth systems during 2013 (**A**. Winter) and 2014 (**B**. Winter, **C**. Spring). Columns followed by different letters indicate mean  $\pm$  SE and are statistically different at  $p \le 0.05$  (LSD test). Vertical bars indicate interval LSD.

In addition, it was observed that the crop system had a significant effect on nitrate content (2013  $p \le 0.01$ ; 2014  $p \le 0.05$ ), except in one season (Spring 2013), which presented higher nitrate content in greenhouse plants ( $p \le 0.05$ ) (Table 5). Humidity did not significantly affect leaf nitrate concentration for most of the trials, except that low humidity favored nitrate accumulation in plants in the spring of 2014 ( $p \le 0.05$ ) (Table 5). No interactions between humidity level and cultivar or crop system are found for nitrate content.

In relation to the chlorophyll content in plants, cultivar and crop system are statistically significant ( $p \le 0.05$ ). In both years, higher values of chlorophyll are obtained in 'Cuartana' ( $p \le 0.05$ ) and in the open-field system( $p \le 0.05$ ) (Table 6).



**Figure 4.** Interaction effect between cultivar x crop system on leaf nitrate concentration. Leaf nitrate concentration in the different crop systems during winter of 2014. Columns followed by different letters indicate mean  $\pm$  SE and are statistically different at  $p \le 0.05$  (LSD test). Vertical bars indicate interval LSD.

**Table 6.** Chlorophyll content (mg/g fresh product) of two endive cultivars under different humidity and cropping systems cultivated in two growing seasons.

	Chlorophyll Content (mg/g Fresh Product)				
	20	13	2014		
	Winter	Spring	Winter	Spring	
Cultivar (Cv)					
'Cuartana'	$1.07 \pm 0.009$ a	$1.17 \pm 0.01$ a	$1.01 \pm 0.006$ a	$1.02 \pm 0.01$ a	
'Natacha'	$1.00 \pm 0.009 \text{ b}$	$1.12 \pm 0.01 \text{ b}$	$0.96 \pm 0.006 \text{ b}$	$0.99 \pm 0.01 \text{ b}$	
Humidity level (HL)					
Low Humidity	$1.06 \pm 0.009$ a	$1.14\pm0.01$	$0.99 \pm 0.006$	$1.01\pm0.01$	
High Humidity	$1.01 \pm 0.009 \text{ b}$	$1.15\pm0.01$	$0.98 \pm 0.006$	$1.00\pm0.01$	
Cropping system (CS)					
Greenhouse	$1.01 \pm 0.009 \text{ b}$	$1.08 \pm 0.01 \text{ b}$	$0.96 \pm 0.006 \mathrm{b}$	$0.96 \pm 0.01 \text{ b}$	
Open-field	$1.06 \pm 0.009$ a	$1.21 \pm 0.01$ a	$1.01 \pm 0.006$ a	$1.05 \pm 0.01$ a	
Analysis of variance Parameters (degrees of freedom)	Significance level				
$\operatorname{Cv}(n=1)$	*	*	**	*	
HL(n=1)	*	ns	ns	ns	
CS(n=1)	**	**	**	**	
$Cv \times HL (n = 1)$	ns	ns	ns	ns	
$Cv \times CS (n = 1)$	ns	ns	ns	ns	
$HL \times Cv (n = 1)$	ns	ns	ns	ns	
$Cv \times HL \times CS (n = 1)$	ns	ns	ns	ns	

Significance level: \*\*  $p \le 0.01$ , \*  $p \le 0.05$  and ns not significant. Mean  $\pm$  SE followed by different letters are statistically different at  $p \le 0.05$  (Fisher's LSD test).

Humidity level was significant ( $p \le 0.05$ ) in spring 2013, resulting in higher chlorophyll content in plants under low humidity ( $p \le 0.05$ ) (Table 6). No interactions are found for cultivar, humidity level, and cropping system (Table 6).

#### 4. Discussion

Plant growth is promoted by open-field conditions, with higher marketable biomass compared to the greenhouse system. Warmer greenhouse temperatures do not favor the accumulation of biomass. Wurr and Fellows [25], and Wheeler et al. [26], also reported a negative relationship between temperature and yield in lettuce.

The response of the cultivars to tipburn incidence varies over time depending on environmental conditions and the cultivar; 'Natacha' shows more susceptibility to tipburn disorder than 'Cuartana'. The different susceptibility of cultivars in conditions that favored tipburn incidence was observed in other studies with lettuce. These studies found that the incidence of tipburn disorder is genetically determined but highly influenced by environmental conditions [27,28].

The low humidity treatment (55.37%  $\pm$  1.49%) favors tipburn occurrence. On the other hand, the increase of the humidity level artificially (high humidity, 68.49%  $\pm$  1.23%), decreases the severity of tipburn and the performance of the two cultivars is similar.

This adverse effect of low humidity on the incidence tipburn has been previously reported since low humidity causes an increase in transpiration [29,30]. These conditions may induce leaf water stress which leads to stomatal closure. Therefore, the water stops flowing from the xylem to leaves, and the consequently calcium transport is reduced, favoring tipburn incidence [31–33]. In addition, during the day, when the transpiration increases by the low levels of humidity, the outer leaves transpire more rapidly than inner leaves and can limit the concentration of transported calcium [34]. At night, the low humidity could decrease the turgor of leaves and root pressure, causing a loss of calcium that is ascended to the tissues by guttation [35].

On the other hand, less incidence of tipburn with the increase of the humidity level artificially (high humidity,  $68.49\% \pm 1.23\%$ ) occurs as high humidity can promote the transport of calcium (by the xylem) to the leaves during expansion. Similarly, according to Kuronuma et al., [36], total calcium concentrations in six of seven Lisianthus cultivars were significantly higher under the high humidity treatment ( $70\% \pm 5\%$ ) than under a low humidity treatment ( $50\% \pm 5\%$ ). Two of seven cultivars showed tipburn under high humidity environments [36].

In addition to humidity, nitrogen and calcium are known to influencing the incidence of tipburn. [37] reported an increase in tipburn when the nitrogen was supplied in excess, and [16,18] agree that calcium deficiency is the cause of tipburn. Different studies have shown that calcium is closely associated with nitrogen metabolism [38,39]. Calcium is essential for the reduction of nitrate to nitrite and plants grown without calcium accumulated nitrate in leaves [38,39]. The leaf calcium concentration was closely related to nitrogen levels in plants, and both concentrations were affected by nutrient solution composition [40]. Our results indicate higher nitrate concentration in plants with higher incidence of tipburn grown in greenhouses systems.

The values of nitrate obtained in endive plants never exceeded the standards set by the European Union for the lettuce [6]. Nitrate accumulation has been shown to differ among cultivars of spinach, rocket salad, and lettuce [13,41,42]. Differences are not observed in this experiment between 'Natacha' and 'Cuartana'.

In general, studies about the accumulation of nitrate in plant tissues have shown that in conditions of low temperature and light (autumn and winter), the activity of the enzyme nitrate reductase (the enzyme involved in the reduction of nitrate) is favored, and the content of this compound in plants increases [43,44]. To the contrary, our results indicate that nitrate accumulation was favored in the crop system with the higher temperatures (greenhouse). These results are consistent with the finding of studies that reported nitrogen concentration in endive was higher in high temperature seasons [45].

In the winter of 2013 and in the two seasons of 2014, the nitrate content was higher under greenhouse conditions; in addition, leaf chlorophyll content was low in all seasons. According to several authors nitrate accumulation is favored in seasons with higher temperatures [46,47]. In lettuce (crop with similar requirements to endive), a decline in photosynthesis was observed, causing a reduction in the concentration of accumulated carbohydrates in the vacuoles that were replaced by nitrates to keep the osmotic potential with temperatures over 22 °C [10,48,49]. Additionally, differences in the nitrogen absorption have been observed as a function of temperature. Therefore, the low temperatures in open-field system in comparison to the greenhouse could limit its absorption, especially in nitric form [50].

# 5. Conclusions

Our experiment demonstrated that crop systems with low humidity levels (plants cultivated at greenhouse without an extra supply of humidity) favored the occurrence of tipburn in endives. In that condition we observed 'Natacha' plants had greater susceptibility to this disorder in comparison to 'Cuartana'. These results suggest that tipburn incidence in endives depends on the interaction of genetic and climatic factors that affect the transpiration and root pressure. Related to the nitrate accumulation in leaves, few differences were found between cultivars that could indicate the possibility of reducing the nitrate content in endives with the selection of a cultivar. Leaf nitrate content was favored by high air temperature conditions, possibly because the temperatures reached in the experiment caused a decrease in the chlorophyll content.

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# References

- 1. AFHORFRESH (Asociación Española de Frutas y Hortalizas Lavadas Listas para su Empleo). 2015. Available online: www.afhorla.com (accessed on 16 October 2019).
- 2. CEBAS-CSIC (Centre for Applied Soil Science and Biology of the Segura—Spanish National Research Council) Cienciaceba's blog. Fresh-cut products. 2015. Available online: www.cienciacebas.wordpress.com (accessed on 20 October 2019).
- 3. Konstantopoulou, E.; Kapotis, G.; Salachas, G.; Petropouls, S.A.; Karapanos, I.C.; Passam, S.A. Nutritional quality of greenhouse lettuce at harvest and after storage in relation to N application and cultivation season. *Sci. Hortic.* **2010**, *125*, 93e.1–93e.5. [CrossRef]
- 4. Mensinga, T.T.; Speijers, J.A.G.; Meulenbelt, J. Health Implications of Exposure to Environmental Nitrogenous Compounds. *Toxicol. Rev.* **2003**, *22*, 41–51. [CrossRef] [PubMed]
- 5. Anjana, S.U.; Iqbal, M. Nitrate accumulation in plants, factors affecting the process, and human health implications. A review. *Agron. Sustain. Dev.* **2007**, *27*, 45–57. [CrossRef]
- OJEU (Official Journal of the European Union). Commission Regulation (EU) No 1258/2011 of December 2011 amending Regulation (EC) No 1881/2006 as regards maximum levels for nitrates in foodstuffs. Official Journal of the European Union. L. 320/15-17. 2011. Available online: http://eur-lex.europa.eu (accessed on 20 October 2019).
- Pavlou, G.C.; Ehaliotis, C.D.; Kavvadias, V.A. Effect of organic and inorganic fertilizers applied during successive crop seasons on growth and nitrate accumulation in lettuce. *Sci. Hortic.* 2007, 111, 319–325. [CrossRef]
- 8. Dapoigny, L.; Tourdonnet, S.; Roger-Estrade, J.; Jeuffroy, M.H.; Fleury, A. Effect of nitrogen nutrition on growth and nitrate accumulation in lettuce (*Lactuca sativa* L.), under various conditions of radiation and temperature. *Agronomie* **2000**, *20*, 843–855. [CrossRef]
- 9. Lillo, C. Light regulation of nitrate reductase in green leaves of higher plants. *Physiol. Plant.* **1994**, *90*, 616–620. [CrossRef]
- 10. Maldonado, J.M. Asimilación del nitrógeno y del azufre. In *Fundamentos de fisiología vegetal*, 2nd ed.; Azcón Bieto, J., Talón, M., Eds.; Mc Graw Hill: Madrid, Spain, 2008; pp. 287–303.
- 11. Reinink, K.; Groenwold, R.; Bootsma, A. Genotypical differences in nitrate content in *Lactuca sativa* L. and related species and correlation with dry matter content. *Euphytica* **1987**, *36*, 11–18. [CrossRef]
- 12. Behr, U.; Wiebe, H.J. Relation between photosynthesis and nitrate content of lettuce cultivars. *Sci. Hortic.* **1992**, *49*, 175–179. [CrossRef]
- Santamaria, P.; Gonella, M.; Elia, A.; Parente, A. Ways of reducing rocket salad nitrate content. *Acta Hortic.* 2001, 548, 529–537. [CrossRef]

- 14. Collier, G.F.; Wurr, D.C.E. The relationship of tipburn incidence in head lettuce to evaporative water loss and leaf dimensions. *J. Hort. Sci.* **1981**, *56*, 9–13. [CrossRef]
- 15. Maroto, J.V. *Fisiopatías. En La lechuga y la escarole;* Maroto Borrego, J.V., Miguel Gómez, A., Baixauli Soria, C., Eds.; Mundi-Prensa: Madrid, Spain, 2000; pp. 216–229.
- 16. Pazkill, D.A.; Tibbits, T.W.; Williams, P.H. Enhancement of Calcium transport to Inner Leaves of Cabbage for Prevention Tipburn. *J. Am. Soc. Hort. Sci.* **1976**, *101*, 645–648.
- 17. Pazkill, D.A.; Tibbit, T.W. Evidence that Root Pressure Flow Is Required for Calcium Transport to Head Leaves of Cabbage. *Plant Physiol.* **1977**, *60*, 854–856. [CrossRef] [PubMed]
- 18. Collier, G.F.; Tibbits, T.W. Tipburn of Lettuce. Hortic. Rev. 1982, 4, 49-65.
- 19. Collier, G.F.; Tibbits, T.W. Effects on Relative Humidity and Root Temperature on Calcium Concentration and Tipburn Development in Lettuce. *J. Am. Soc. Hort. Sci.* **1984**, *109*, 128–131.
- 20. Rosen, C.J. Leaf Tipburn in Cauliflower as Affected by Cultivar, Calcium Sprays, and nitrogen Nutrition. *HortScience* **1990**, *25*, 660–663. [CrossRef]
- 21. Frantz, J.M.; Ritchue, G.; Cometti, N.N.; Robienson, J.; Bugbee, B. Exploring the Limits of Crop Productivity: Beyond the Limits of Tipburn in Lettuce. *J. Am. Soc. Hort. Sci.* **2004**, *129*, 331–338. [CrossRef]
- 22. Konno, H.; Yamaya, T.; Yamakasi, Y.; Matsumoto, H. Pectic Polysaccharide Breakdown of Cell Walls in Cucumber Rootss Grown with Calcium Starvation. *Plant Physiol.* **1984**, *76*, 633–637. [CrossRef]
- 23. Barta, J.D.; Tibbits, T.W. Calcium localization in lettuce with and withouth Tipburn: Comparison of controlled environment and field-gorwn plants. *J. Am. Soc. Hort. Sci.* **1991**, *116*, 870–875. [CrossRef]
- 24. Guardiola Bárcena, J.L.; García Luis, A. *Fisiología vegetal I: Nutrición y transporte*, 1st ed.; Síntesis: Madrid, Spain, 1990; p. 440.
- 25. Wurr, D.C.E.; Fellows, J.R. The influence of solar radiation and temperature on the head weight of crips lettuce. *J Hortic. Sci.* **1991**, *66*, 183–190. [CrossRef]
- 26. Wheelet, T.R.; Hadley, P.; Morison, J.I.L.; Ellis, R.H. Effects of temperature on the growth of lettuce (*Lactuca sativa* L.) and the implications for assessing the impacts of potential climate change. *Eur. J. Agron.* **1993**, *2*, 305–311. [CrossRef]
- 27. San Bautista, A.; López-Galarza, S.; Pascual, B.; Alagarda, J.; Fresquet, J.L.; Bono, M.S.; Palau, V.; Laza, P.; Torres, J.F.; Maroto, J.V. Influencia de distintas dosis de riego y soluciones nutritivas en el rendimiento comercial y la incidencia de Tipburn en cultivo protegido de dos *cvs* de lechuga *Iceberg*. *Cuadernos de Fitopatología* 2003, *78*, 112–121.
- 28. Bres, W.; Weston, A. Nutrient accumulation and Tipburn in NFT-grown Lettuce at Several Potassium and pH Levels. *HortScience* **1992**, *27*, 790–792. [CrossRef]
- 29. Drake, B.G.; Raschke, K.; Salisbury, F.B. Temperatures and Transpiration Resistances of Xanthium Leaveas as Affected by Air Temperature, Humidity, and Wind Speed. *Plant Physiol.* **1970**, *46*, 324–330. [CrossRef]
- 30. Stratton, M.L.; Nagata, R.T. Preliminary determination of parameters to develop and objective procedure for assessing tipburn in lettuce. *Proc. Fla. State Hort. Soc.* **1993**, *106*, 157–159.
- 31. Mabrouk, A.E.; Cock, J.H. Stomatal response to air humidity and its relation to stomatal density in a wide range of warm climate species. *Photosynth. Res.* **1985**, *7*, 137–149.
- 32. Schulze, E. Carbon dioxide and water vapor Exchange in response to drought in the atmosphere and in the soil. *Annu. Rev. Plant Physiol.* **1986**, *37*, 247–274. [CrossRef]
- 33. Leonardia, C.; Guichard, S.; Bertinb, N. High vapour pressure déficit infuences growth transpiration and quality of tomato fruit. *Sci. Hortic.* **2000**, *84*, 285–296. [CrossRef]
- 34. Maroto, J.V. *Etiología y descripción de las principales fisiopatías de la horticultura Mediterránea;* Ediciones y promociones L.A.V.: Valencia, Spain, 1997.
- 35. Everaarts, A.P.; Blom-Zandstra, M. Internal tipburn of cabbage (*Brassica oleracea var. capitae*). J. Hort. Sci. Biotechnol. 2001, 76, 515–521.
- Kuronuma, T.; Wotanabe, Y.; Ando, M.; Watanabe, H. Tipburn Severity and Calcium Distribution in Lisianthus (*Eustoma Grandiflorum* (Raf.) Shim) Cultivars Under Different Relative Air Humidity Conditions. *Agronomy* 2018, *8*, 218. [CrossRef]
- Brumm, I. Einflub des Stickstoffangebots auf das Aufreten von Ca-Mangel bei Kopfsalat (*Lactuca sativa* L.). Ph.D. Thesis, Universität Hannover, Hannover, Germany, 1992.
- 38. Eckerson, S.H. Protein synthesis by plants I. Nitrate reduction. Bot. Gaz. 1924, 77, 377–390. [CrossRef]

- 39. Skok, J. Effect of the form of the available nitrogen on the calcium deficiency symptoms in the bean plant. *Plant Physiol.* **1941**, *16*, 145–157. [CrossRef] [PubMed]
- 40. Gromaz, A. Escarola (*Cichorium endivia* L.): Sistemas de manejo y ciclos como factores de la incidencia de fisiopatias y acumulación de nitratos. Ph.D. Thesis, Universitat Politècnica de València, València, Spain, November 2016.
- 41. Stagnari, F.; Di Bitteto, V.; Pisante, M. Effects of N fertilizers and rates on yield, safety and nutrients in processing spinach genotypes. *Sci. Hortic.* **2007**, *114*, 225–233. [CrossRef]
- Govedarica-Lucik, V.; Mojevic, M.; Percovic, G.; Govedarica, B. Yield and nutritional quality of greenhouse lettuce (Lactuca sativa L.) as affected by genotype and production methods. *Genetika* 2014, 46, 1027–1036. [CrossRef]
- 43. Nicholas, J.C.; Harper, J.E.; Hageman, R.H. Nitrate Reductase Activity in Soybeans (*Glycine max* [L.] Merr.). *Plant Physiol.* **1976**, *58*, 731–735. [CrossRef]
- 44. Blom-Zandstra, M.; Lampe, J.E.M. The Role of Nitrate in the Osmoregulation of Lettuce (*Lactuca sativa* L.) Grown at Different Light Intensities. *J. Exp. Bot.* **1985**, *36*, 1043–1052.
- 45. Fallovo, C.; Rouphael, Y.; Cardelli, M.; Rea, E.; Battistelli, A.; Colla, G. Yield and quality of leafy lettuce in response to nutrient solution and growing season. *J. Food Agric. Environ.* **2009**, *7*, 456–462.
- 46. Parks, S.E.; Huett, D.O.; Campbell, L.C.; Spohr, L.J. Nitrate and nitrite in Australian leafy vegetables. *Aust. J. Agric. Res.* **2008**, *59*, 632–638. [CrossRef]
- 47. Fallovo, C.; Rouphael, Y.; Rea, E.; Battistelli, A.; Colla, G. Nutrient solution concentration and growing season affect yield and quality of *Lactuca sativa* L. var. *acephala in floating raft culture*. *J. Sci. Food Agric.* **2009**, *89*, 1682–1689. [CrossRef]
- 48. Torres, J.F.; Ndamekele, J.B.; San Bautista, A.; Pascual-España, B.; Bono, M.S.; López Galarza, S.; Pascual-Seva, N.; Alagarda, J.; Maroto, J.V. Influencia de ciclos, fertilización nitrogenada y modalidades de cultivo en la incidencia de diversas fisiopatías y la acumulación de nitratos en cuatro cultivares de minilechuga (I). *Phytoma* 2013, 245, 16–21.
- 49. Lorenz, H.P.; Wiebe, H.J. Effect of temperature on photosynthesis of lettuce adapted to different light and temperature conditions. *Sci. Hortic.* **1980**, *13*, 115–123. [CrossRef]
- Rincón Sanchez, L.; Pérez Crespo, A.; Pellicer Botía, C.; Sáez Sironi, J.; Abadía Sanchez, A. Influencia de la fertilización nitrogenada en la absorción de nitrógeno y acumulación de nitratos en la lechuga iceberg. *Invest. Agric. Prod. Prot. Veg.* 2002, *17*, 303–318.



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