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Harvest and Post-Harvest Performance of Autumn-Winter Butterhead Lettuce as Affected by Nitrogen and Azoxystrobin Application

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Abstract: An autumn-winter trial was carried out in Southern Italy in open-field conditions on butterhead lettuce to investigate the effect of the nitrogen (N) fertilizer rate (0, 50, and 100 kg ha^{-1}, N0, N50, N100) and the application of the azoxystrobin, sprayed twice in an earlier vs. a later application scheme, specifically at 65/85 or 65/100 days after transplantation. An untreated control was also included. The evaluation of the product quality was conducted on fresh and stored shredded leaves. The N50 was a suitable rate for autumn-winter butterhead lettuce, but it does not guarantee the color appearance of the fresh leaves (lowest h°, highest L*). Concerning post-harvest changes, the N50- and N100-product were less suitable for storage, accounting for higher decay of visual quality (h°) and physiological senescence (EL) indices. Irrespective of N rate and application time, azoxystrobin improved growth and yield (+16%), visual (lower L*, higher h°, and chlorophylls), and nutritional (higher carotenoids and antioxidant capacity) quality of the fresh leaves. The application of azoxystrobin improved the shelf-life of butterhead lettuce leaves, by keeping higher turgidity (RWC), lower color decay (CHLs, h°), and higher nutritional value (carotenoids), and by limiting the browning spreading in shredded leaves.

Keywords: strobilurin; *Lactuca sativa* L. var. *capitata*; PPOs; shelf-life; fresh-cut

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1. Introduction

Lettuce is an important leafy vegetable, available all over the world for 12 months per year, and despite its high perishability, is one of the major fresh-cut vegetables.

The distinctive characteristics of fresh-cut lettuce quality can be described by several attributes (color and appearance, consistency, off-flavors, nutritional and antinutritional traits, etc.) [1]. The main problem with shredded lettuce is the browning of cut surfaces, which significantly affects the visual quality and limits the shelf-life of the product [2].

Pre-harvest factors including cultural practices, genotype, and climatic conditions could successfully improve the quality of lettuce as a fresh and stored product [3–5]. Among cultural practices, N fertilization is crucial for optimizing productivity as well as visual (color and consistency) and nutritional quality (low concentration of nitrates) [5].

In recent decades, strobilurins have been investigated for their fungicidal activity and even for their 'biostimulant' effects, on crops not infected or threatened by pathogens, especially cereals [6,7]. Strobilurins are natural compounds that were isolated for the first time in 1977 from basidiomycetes [8]. During the early 1980s, some commercial chemical groups identified stable synthetic strobilurin and, due to the successive improvement of their physical characteristics, strobilurins such as azoxystrobin, picoxystrobin, pyraclostrobin, and trifloxystrobin have been brought to the commercial fungicidal market.

Among the synthetic strobilurins, azoxystrobin (methyl(E)-2-{2-[6-(2-cyanophenoxy) pyrimidin-4-yloxy]phenyl}-3-methoxy acrylate), has been more explored for its 'biostimulant' effects on herbaceous species such as durum wheat [9], and also on fruit vegetables as

tomato [10–12] or leafy ones such as butterhead lettuce [13], baby-leaf spinach [14], and baby-leaf rocket [15–17]. Additionally, the pre-harvest application of azoxystrobin has also been found to affect the shelf-life of cold-stored leafy species such as shredded [13] or baby-leaf type [14,16].

A few of these researchers showed contrasting results concerning N fertilization rates and azoxystrobin application, as well as their interaction.

In wild rocket, the pre-harvest application of azoxystrobin promoted yield and the quality of fresh leaves (higher chlorophylls and carotenoids, lower nitrate content) [16,17], and increased the product shelf-life in terms of less chlorophyll decay (yellowing) and off-odour presence [16]. These authors did not find any interaction with N fertilization rate either in the first (24/84 kg ha^{-1} of N) or second (32/112 kg ha^{-1} N) autumn-winter trials. In spring butterhead lettuce, irrespective of the N level (0, 50, 100 kg ha^{-1}), azoxystrobin application has been proven effective in improving yield and reducing the nitrate content of fresh leaves. In a spring-summer cycle, azoxystrobin in combination with moderate N fertilization (50 kg ha^{-1}) improved leaf turgidity of butterhead lettuce and delayed senescence by reducing membrane alteration, chlorophyll degradation, and tissue browning in 12-day-stored shredded leaves [13]. Conversa et al. [14] reported that azoxystrobin improved the physiological quality of baby-leaf spinach (higher chlorophylls and lower electrolyte leakage), harvested both in winter (0, 80, and 120 kg ha^{-1} of N) and in spring (N rate 80 kg ha^{-1}). However, a positive effect of azoxystrobin on the nutritional (increasing ascorbic acid and phenols) and safety quality (lowering nitrate) was only observed for spring fresh leaves. In winter baby-leaf spinach, azoxystrobin application was effective in limiting the post-harvest losses of the weight observed in the fertilized product [14].

Considering that the response of lettuce to N and azoxystrobin was evaluated only in spring/spring-summer cycles [13], we hypothesize that this species could behave differently to N and azoxystrobin treatment under different climate conditions (lower temperature and radiation level). Since lettuce is cultivated throughout the year, the present work aims to widen the knowledge on the effects of azoxystrobin, with increasing N level, on harvest and post-harvest butterhead lettuce grown in an autumn-winter cycle. Moreover, since the efficiency of strobilurins as a fungicide/biostimulant could be affected by the application time [18,19], two different scheduling schemes of azoxystrobin applications were also evaluated.

2. Materials and Methods

2.1. Field Experimental Site and Climatic Conditions

Lettuce plants of butterhead morpho-type (*Lactuca sativa* L., var. *capitata*) (cultivar Faustina, ISEA s.r.l., San Severino Marche, MC, Italy) were open field cultivated in autumn-winter 2012–2013 on a commercial farm, located in Foggia province (Puglia Region, Southern Italy, latitude 41°46′ N, longitude 15°55′ E, 74 m above sea level). The experimental farm is representative of soil and climate conditions in the area. The soil characteristics are 24% clay, 34% loam, 42% sand, 7.52 pH (soil:water 1:2.5), 1.8% organic matter, 1.25‰ total N, 382 ppm NH4OAc-extractable K, 24 ppm Olsen P, and 7% active carbonate.

2.2. Field Management of Crop, Experimental Design, and Treatments

Plantlets (at 4–5th true-leaf stage) were transplanted 30 cm apart in 40 cm spaced rows (7.4 plants per m^2) on 17 October 2012.

Two experimental treatments included: (A) nitrogen fertilization rates: 0, 50, and 100 kg ha^{-1} (N0, N50, N100); (B) azoxystrobin application (Ortiva®-Syngenta Crop Protection, Fulbourn, Cambridge, UK), which included: (i) no azoxystrobin, only water application (Azox-) (control); (ii) azoxystrobin application at 65 and 85 days after transplant (DAT) (Azox+$_{65/85}$); (iii) azoxystrobin application at 65 and 100 DAT (Azox+$_{65/100}$). At each application time, the azoxystrobin was sprayed at the dose of 416.7 mg L^{-1} active ingredient in a volume of 600 L ha^{-1} of water). In detail, the first application was at 65 DAT

(17th true-leaf stage, approx. 50 g f.w.), while the second application was twenty (85 DAT) or five (100 DAT) days before harvest (as outlined in Figure 1A).

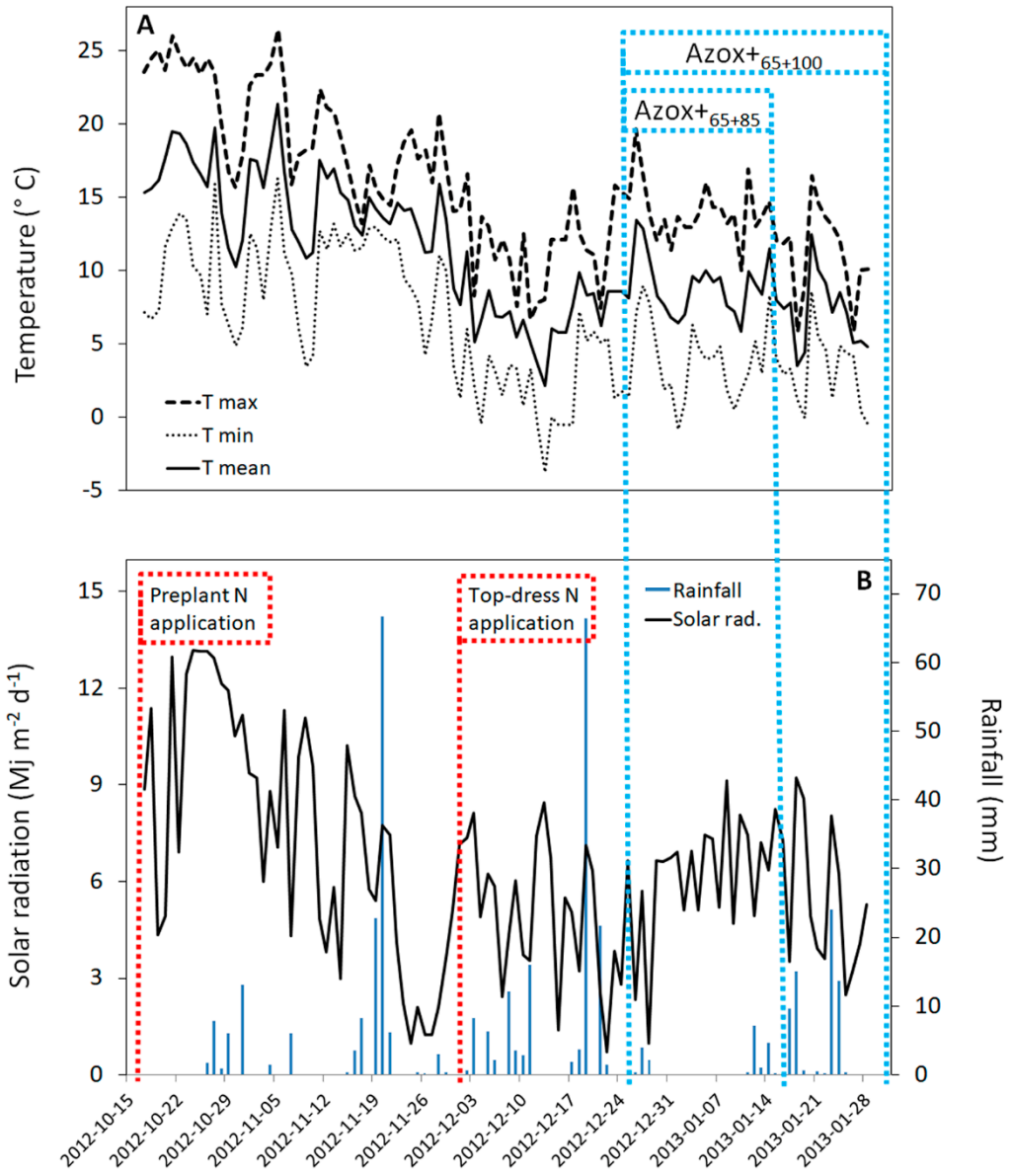

Figure 1. (**A**) The daily temperatures (the minimum, maximum, and average) during the lettuce crop cycle. The blue dotted lines indicate the dates of azoxystrobin application: Azox+$_{65/85}$ and Azox+$_{65/100}$, respectively, application at 65 and 85 or 65 and 100 days after transplant (DAT). (**B**) The daily solar radiation and rain events during the lettuce crop cycle. The red dotted lines indicate the dates of the nitrogen application.

Nitrogen fertilizer was applied as NH_4NO_3 (34-0-0, N:P:K) (Yara International ASA, Oslo, Norway). Forty per cent of the N rate was applied in surface strips and dished before planting (15 October 2012) and the remaining part was side-dressed at the fifteenth true-leaf stage (29 November 2012) (as outlined in Figure 1B). The level of PK fertilization was adjusted to the plants' nutritional requirements. A sprinkler irrigation system was used to satisfy the water requirements of the crops.

A strip-plot design with azoxystrobin application as the vertical factor and N rate as the horizontal factor was applied with four replications. The experimental unit (1.8 m wide, 5.8 m long, and 10.4 m^2 surface) was represented by four rows with a total of 77 plants.

2.3. Sampling and Measurements

The harvest of heads was performed at the optimal stage for fresh consumption and took place on 28 January 2013 (105 DAT). The plants for measurements were sampled in the central area of the plots.

2.3.1. Effect of N Levels and Azoxystrobin Application on Plant Material

After the harvest, the fresh plant material was directly transported to the laboratory at the Department of Agriculture, Food, Natural Resources and Engineering (DAFNE) ($\sim$10 km away) and processed within 1 h after harvest. Physical (yield, dry matter concentration, specific leaf area), morphological (leaf number, leaf height, leaf area), appearance (chlorophyll concentration, main color indices), physiological (relative water content, osmotic potential, and electrolytic leakage, and PPO enzyme activity), nutritional (ascorbic acid and phenolic concentration, the total antioxidant capacity and its components, lipophilic and hydrophilic capacity) and anti-nutritional (nitrate) traits were evaluated.

Physical and Morphological Traits

Fresh yield (FY) was calculated on 20 lettuce heads randomly selected from each experimental unit.

To determine the dry weight (DW) of leaves, the fresh weight (FW) of plant material was dried in a thermo-ventilated oven at 70 °C until it reached a constant mass. The dry matter concentration (DM) was calculated as (DW/FW) $\times$ 100.

Leaf area (A) was measured using LI-COR 3100 (LI-COR Inc., Lincoln, NE, USA). The specific leaf area (SLA) was calculated as ratio DW/A.

Head height was measured from the cutting point to the top of the head.

Visual Quality

Color index measurements based on the CIE L*a*b* scale 1976-L* (Lightness index), a* (green to red), and b* (yellow to blue) were performed on the images of fresh leaves. The hue angle (h°) and the saturation index (Chroma−C*) were calculated as derived parameters.

Physiological Traits

The relative water content (RWC) was determined on leaf blade discs. The sample was first weighed to determine the fresh weight (FW) and, afterwards, it was hydrated to full turgidity for 24 h, under normal room light and temperature conditions, on deionized water in a closed Petri dish. Then the sample was taken out of the water and well dried off with filter paper and immediately weighed to obtain a fully turgid weight (TW). The sample was then oven-dried at 70 °C and weighed to determine the dry weight (DW). RWC = (FW − DW)/(TW − DW). The osmotic potential (OP) was determined using an automatic cryoscopic osmometer (Micro-Osmometer Automatic 13/13DR; Roebling, Berlin, Germany) on cell sap extracted from fresh leaves. The percentage of electrolyte leakage was calculated as EL (%) = (EC1/EC2) $\times$ 100. A portion of fresh leaf material (3 g) was weighed into a glass beaker containing twice-distilled water. The electrical conductivity of the solution (EC1) was measured using a hand-held conductivity meter (Hanna Instruments Italia s.r.l., Villafranca, PD, Italy). After boiling the sample for 2 min, the electrical conductivity of the solution cooled to room temperature was re-measured (EC2).

Lyophilized samples were mixed with 1.5 mL, 80% ethanol (ethanol:water = 80:20), containing 0.1% hydrochloric acid in a 10 mL tube with a screw cap. The mixture was treated with ultrasonic power using a DU-32 digital ultrasonic cleaner (Argo Lab s.r.l., Carpi, MO, Italy) at room temperature for 30 min. After sonication, the samples were centrifuged in a refrigerated Coulter Allegra TM 25 centrifuge (Beckman Coulter Inc., Fullerton, CA, USA) and then the supernatant was collected. The above steps were repeated twice and supernatants were combined. Finally, the extract was filtered by using 0.22 μm, reinforced nylon membrane filters. The extract solution was measured with an Evolution 201 UV-Visible spectrophotometer (Thermo Fisher Scientific Inc., Waltham,

MA, USA) at 664, 649. Pigment concentration was estimated using the following equation: Total chlorophylls = 17.32 (A649) + 7.18 (A664); Chl a = 13.36 (A664) − 5.19 (A649); Chl b = 27.43 (A649) − 8.12 (A664).

A mass of 0.75 g of lettuce was homogenized in 1.5 mL of 0.05 M potassium dihydrogen phosphate buffer, containing 0.11 g of PVPP. The slurry was centrifuged at 15,000× g for 15 min. The supernatant was used as the crude enzyme extract. The activity of polyphenol oxidases (PPOs) was measured using the method described by Altunkaya and Gökmen [20] with some modifications. A volume of 25 µL of enzyme extract was added to 1.3 mL of 0.08 M catechol solution in 0.05 M phosphate buffer to initiate the reaction (the final volume was 1.3 mL). The initial rate of quinone formation was monitored as an increase in the absorbance at 420 nm using a UV-VIS spectrophotometer (Shimadzu UV-2101 PC, Shimadzu Corp, Kyoto, Japan) with a 1-cm path length cuvette). One unit of PPO activity was defined as an increase in absorbance of 0.1 per minute per gram of fresh weight.

Nutritional and Anti-Nutritional Traits

The concentrations of total phenols, carotenoids, and anions of lettuce leaves were determined, from frozen plant material successively lyophilized and then ground into fine particles.

For total phenol (TP) content, the lyophilized samples were previously double extracted in water/methanol (20:80, v/v) solution and centrifuged. TP content successively was determined by mixing the methanolic extracts with the Folin-Ciocalteu reagent and the absorbance was read at 750 nm. The results are expressed as gallic acid equivalents (g.a.e.) using a calibration curve.

Total carotenoids were determined as followed: $MgCO_3$ (0.05 g) was added to a 0.1 g of sample to neutralize cytosolic acids; 0.01 g of celite was used for better tissue disruption. The extraction was with 10 mL of ethanol:hexane (4:3 by volume); 1 mL of pyrogallol solution (5%) was added as an antioxidant. The mixture was placed in a mechanical shaker for 15 min and then centrifuged at 6700 rpm for 10 min, and the supernatant was collected. The residue was re-extracted; the two extracts were combined and decanted into a 50-mL tube. The supernatant hexane phase was transferred into another tube, and the lower aqueous phase was discarded. To overcome the problem of carotenoid overestimation by the presence of chlorophylls, a saponification step was included during extraction. In brief, an equal volume of 10% methanolic KOH was added to the recovered hexane phase, the mixture was shaken vigorously for 1 min and placed on ice for 15 min. After centrifugation at 6700 rpm for 10 min, the supernatant (hexane phase) was collected and washed two times with 15 mL of NaCl 10% solution and two times with 15 mL of water. The aqueous phase was discarded. The total carotenoids in the extract were measured at 450 nm using a UV-visible spectrophotometer and estimated according to the "Method of Mean" reported by Biehler et al. [21].

For nitrate, the lyophilized samples (0.5 g) were extracted with 50 mL of eluent solution (3.5 mM sodium-carbonate and 1.0 mM sodium bicarbonate) in a shaking water bath at room temperature for 30 min. The mixture was filtered through the Whatman n. 2 paper. The filtrates were filtered again through a 0.22 µm Millipore filter, before injection into the ion chromatography system. The ion chromatography system was equipped with: An isocratic pump, conductivity detector; a model AS-DV auto-sampler; a self-generating ERS-500 suppressor (4 mm), an Ion-Pac AS23 analytical column (4 × 250 mm, particle size 6 µm), and an eluent solution (3.5 mM sodium carbonate and 1.0 mM sodium-bicarbonate) at a flow rate of 1 mL min^{-1} (Dionex—Thermo Fisher Scientific, Waltham, MA, USA).

The antioxidant capacity (AC) was assessed as TEAC (Trolox equivalent antioxidant capacity). The hydrophilic fraction was extracted twice from samples (30 mg) with 1 mL of 70% methanol in a shaking water bath (100 rpm, 30 °C) for 15 min and by centrifugation (13,000 rpm for 10 min). The supernatants were combined. The lipophilic components were extracted twice with 1 mL of hexane, using the above conditions

2.3.2. Effect of N Levels and Azoxystrobin Application on Stored Plant Material

The fresh leaves were used for post-harvest evaluation. After being directly transported to the laboratory, the soiled and decayed external leaves were removed, and the internal leaves were washed in tap water to remove residuals, and dried in a manual salad spinner. Leaves were manually cut with a sharp knife into pieces of about 2–3 cm.

The storage of shredded lettuce was performed by packaging 150 g of fresh cut leaves in OPP bags (20 µm) (Metalvuoto spa, Roncello, MI, Italy) (29.5 × 24.3 cm), kept in the dark for 7 days in a refrigerator (5 °C).

The DM, visual quality, chlorophyll concentration, nutritional and anti-nutritional traits as well antioxidant capacity of lettuce leaves were determined on stored plant material as reported for fresh leaves. The post-harvest weight losses were calculated as reported below.

At each final storage period (T7), fresh weight (FW) was measured on un-packaged (FWup) (when the bag was opened after storage, and after drying of leaves) and on packaged samples (FWp) (entire closed bag). Weight losses (WL) were calculated as:

— total, as: WLTot = (FWup(T7) − FWup(T0))/FWup(T0) × 100;
— by respiration, as: WLResp = (FWp(T7) − FWp(T0))/FWup(T0) × 100;
— by transpiration, as: WLTrans = WLTot − WLResp.

2.4. Statistical Analysis

Data were analyzed using the GLM procedure of SAS software (SAS, Cary, NC, USA). The least significant difference (LSD) test (p = 0.05) was used to establish differences between means.

3. Results

3.1. Weather Conditions

Figure 1 reports the weather conditions during the lettuce growing season. In detail, the mean maximum and mean minimum temperatures were 15.7 and 6.1 °C, respectively (Figure 1A); total rainfall was 380 mm, and 6.2 MJ m^{-2} d^{-1} was the mean solar radiation (Figure 1B) which accounted for a total of 667 MJ m^{-2} on the whole cycle.

3.2. Productive, Morpho-Biometric, Visual Quality and Bio-Physiological Attributes of Butterhead Lettuce at Harvest

The supply of N50 boosted FY (+3%), DY (+8%), and specific leaf area (SLA) (+10%) in comparison with the N0 and N100 levels (Table 1). N50-fed plants showed leaves with the highest lightness (L*) and the lowest hue angle (h°), while the product from N50 and N100 levels showed the highest saturation (C*) index (Table 1).

Irrespective of the application time, azoxystrobin increased FY and DY by 16% (Table 1). The earlier application (Azox+$_{65/85}$), the higher the SLA and dry matter concentrations (Table 1). The Azox+ $_{65/85}$ and the Azox- gave, respectively, the highest and the lowest head length and leaf number, while the later application (Azox+$_{65/100}$) showed an intermediate value (Table 1). The Azox+ $_{65/100}$ and the Azox- showed, respectively, the highest and the lowest value of the head weight and leaf area, while the Azox+$_{65/85}$ showed an intermediate value (Table 1). The head height was not affected by either pre-harvest treatment (21.2 cm, on average) (Table 1).

Irrespective of application time, azoxystrobin treatments increased the h° and C* color indices, while decreasing the L* one (Table 1).

Azoxystrobin treatments did not affect the physiological indices of leaves, including the osmotic potential, the relative water content, and the electrolyte leakage (respectively −6.0 bar, 96%, and 17.9%, on average) (Table 2). When applied earlier, azoxystrobin determined a higher chlorophyll concentration (CHL *a*, CHL *b*, and CHL *total*) compared with Azox- (Table 2).

Table 1. Effect of N rate supply and azoxystrobin application on productive, morpho-biometric and visual quality traits of butterhead lettuce at harvest.

Treatments	Fresh Yield	Dry Yield	Head Weight	Head Height	Head Length	Leaf Number	Leaf Area	Dry Matter	Specific Leaf Area	L*	h°	C*
	(t ha^{-1})	(t ha^{-1})	(g)	(cm)	(cm)	(no.)	(cm^2)	(g kg^{-1} f.w.)	(mg cm^{-2})	(-)	(-)	(-)
N rate (N)												
N0	23.7b [2]	1.3b	321.0a	12.8a	21.8a	45.0a	3.686a	56.4a	4.9b	56.9b	105.9a	40.1b
N50	24.2a	1.4a	327.4a	12.2a	21.1a	43.4a	3.475a	57.2a	5.4a	59.6a	104.9b	41.8a
N100	23.3b	1.3b	315.1a	12.3a	20.7a	44.6a	3.635a	56.2a	4.89b	57.0b	105.8a	41.4a
Azoxystrobin (Azox)												
Azox-	21.5b	1.2b	290.0b	12.3a	19.7b	40.6b	3.349b	55.8b	4.8b	58.8a	105.0b	42.2a
Azox+$_{65/85}$	24.2a	1.4a	327.8ab	12.6a	22.9a	47.2a	3.527ab	58.6a	5.5a	57.2b	105.6a	40.4b
Azox+$_{65/100}$	25.6a	1.4a	345.7a	12.4a	21.1ab	45.3ab	3.923a	55.6b	4.9b	57.4b	105.9a	40.5b
Significance [1]												
N	*	*	ns	ns	ns	ns	ns	ns	*	***	***	**
Azox	*	*	*	ns	**	*	*	**	**	*	***	***
N x Azox	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

[1] n.s., *, ** and *** not significant or significant at $p \leq 0.05$, 0.01 and 0.001, respectively. [2] Different letters within the column indicate significant differences at $p = 0.05$.

Table 2. Effect of N rate supply and azoxystrobin application on bio-physiological traits of butterhead lettuce at harvest.

Treatments	Activity of Polyphenol Oxidases	Osmotic Potential	Relative Water Content	Electrolyte Leakage	Chlorophylls		
					a	*b*	Total
	(Unit Activity g^{-1} fw)	(bar)	(%)	(%)	(μg cm^{-2})		
N rate (N)							
N0	283.0a [2]	−6.2a	95.0a	18.2a	9.0a	2.7a	11.8a
N50	214.4a	−5.8a	96.3a	18.1a	8.2a	2.4a	10.7a
N100	205.4a	−5.9a	96.6a	17.4a	8.3a	2.4a	10.8a
Azoxystrobin (Azox)							
Azox-	375.6a	−5.8a	95.9a	17.0a	6.7c	2.0c	8.9c
Azox+$_{65/85}$	128.0b	−6.1a	97.0a	18.7a	10.1a	3.0a	13.2a
Azox+$_{65/100}$	179.3b	−6.0a	95.1a	18.0a	8.6b	2.5b	11.2b
Significance [1]							
N	ns	ns	ns	ns	ns	ns	ns
Azox	**	ns	ns	ns	***	***	***
N x Azox	ns	ns	ns	ns	ns	ns	ns

[1] ns., ** and *** not significant or significant at $p \leq 0.01$ and 0.001, respectively. [2] Different letters within the column indicate significant differences at $p = 0.05$.

Irrespective of the application time, azoxystrobin-treated leaves had lower activity of polyphenol oxidases (PPOs) than the untreated ones (−60%) (Table 2).

3.3. Nutritional and Anti-Nutritional Attributes of Butterhead Lettuce at Harvest

The chloride, phosphate and sulfate concentrations were not affected by any pre-harvest treatment, being on average 23.8, 8.4, and 2.5 g kg^{-1} d.w., respectively (Table 3).

The nitrate concentration of leaves, expressed both on a dry and fresh weight basis, increased with the increasing N level, but it did not change in response to the azoxystrobin treatment (Table 3).

Irrespective of application time, the azoxystrobin-treated leaves had a higher total and hydrophilic antioxidant capacity (AC) than untreated ones (+83%), while the early-treated plants had the lowest lipophilic component of AC (Table 3).

Carotenoids and phenols were higher in N0 plants, while Azoxy+ improved carotenoid concentration (Table 3).

Table 3. Effect of N rate supply and *Azoxystrobin* application on nutritional and anti-nutritional traits of butterhead lettuce at harvest.

Treatments	Carotenoids	Phenols	Antioxidant Capacity			Anions				
			Lipophilic	Hydrophilic	Total	Nitrate	Nitrate	Chloride	Phosphate	Sulfate
	(mg 100 g^{-1} f.w.)	(mg a.g.e. 100 g^{-1} f.w.)	(µmol T.E. kg^{-1} f.w)			(mg kg^{-1} f.w.)	(g kg^{-1} d.w.)			
N rate (N)										
N0	10.5a [(2)]	34.0a	102a	1383a	1484a	1001b	17.4b	3.6a	7.9a	2.6a
N50	9.0b	30.0ab	108a	1234a	1342a	1430ab	25.0ab	3.3a	8.7a	2.6a
N100	9.7ab	25.3b	107a	1054a	1162a	1668a	29.1a	2.9a	8.5a	2.3a
Azoxystrobin (Azox)										
Azox-	8.3c	28.3a	118a	736b	855b	1031a	18.5a	4.5a	8.7a	2.1a
Azox+$_{65/85}$	11.1a	31.8a	90b	1367a	1458a	1632a	27.4a	2.7a	7.9a	2.7a
Azox+$_{65/100}$	9.8b	29.3a	109a	1567a	1676a	1433a	25.6a	2.6a	8.7a	2.6a
Significance [(1)]										
N	*	**	ns	ns	ns	*	*	ns	ns	ns
Azox	***	ns	**	***	***	ns	ns	ns	ns	ns
N x Azox	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

[(1)] ns, *, ** and *** not significant or significant at $p \leq 0.05$, 0.01, and 0.001, respectively. [(2)] Different letters within the column indicate significant differences at $p = 0.05$.

3.4. Biometrical, Physiological and Nutritional Changes during Post-Harvest Storage

N supply and azoxystrobin application did not affect weight losses of butterhead leaves after 7 days of storage, being 1.0 g 100 g^{-1} f.w. (Table S1). The weight loss due to respiration (0.4 g 100 g^{-1} f.w.) was similar to that due to transpiration (0.5 g 100 g^{-1} f.w.) (Table S1).

The dry matter concentration and leaf color saturation index (C*) were not affected by storage time or by the interaction of storage with pre-harvest treatments (Table 4).

The lightness index generally decreased (−1.7%) after 7 days of storage (Table 4). The variation during storage of the hue angle (h°) occurred only in the N50 and N100 treatments, which showed a lower value of h° than the N0 treatment at the end of this period (Figure 2A). The change of h° during the storage was different according to the azoxystrobin treatments, and at the end of storage, the later-treated leaves showed a higher value of h° than the untreated and the early-treated ones (Figure 3A).

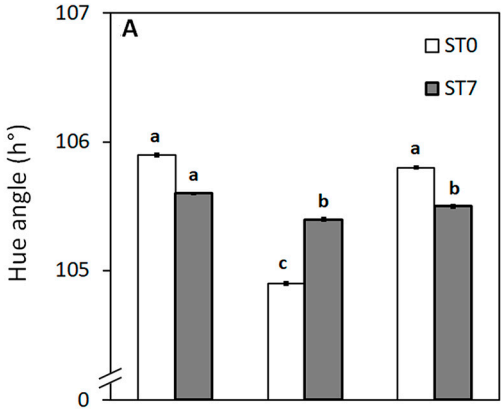

Figure 2. *Cont.*

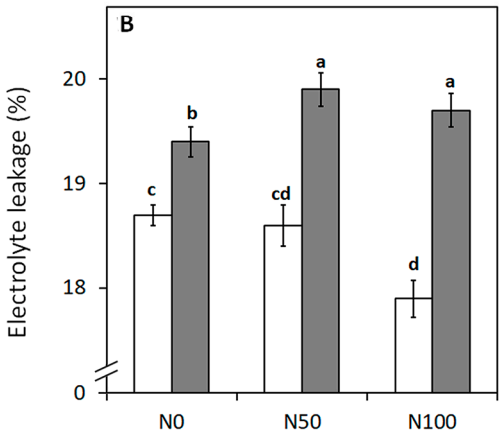

Figure 2. Effect of N rate on hue angle (**A**) and electrolyte leakage (**B**) in fresh and stored butterhead lettuce. Vertical bars (standard error) (n = 24) with different letters are significantly different according to the LSD test (p = 0.05).

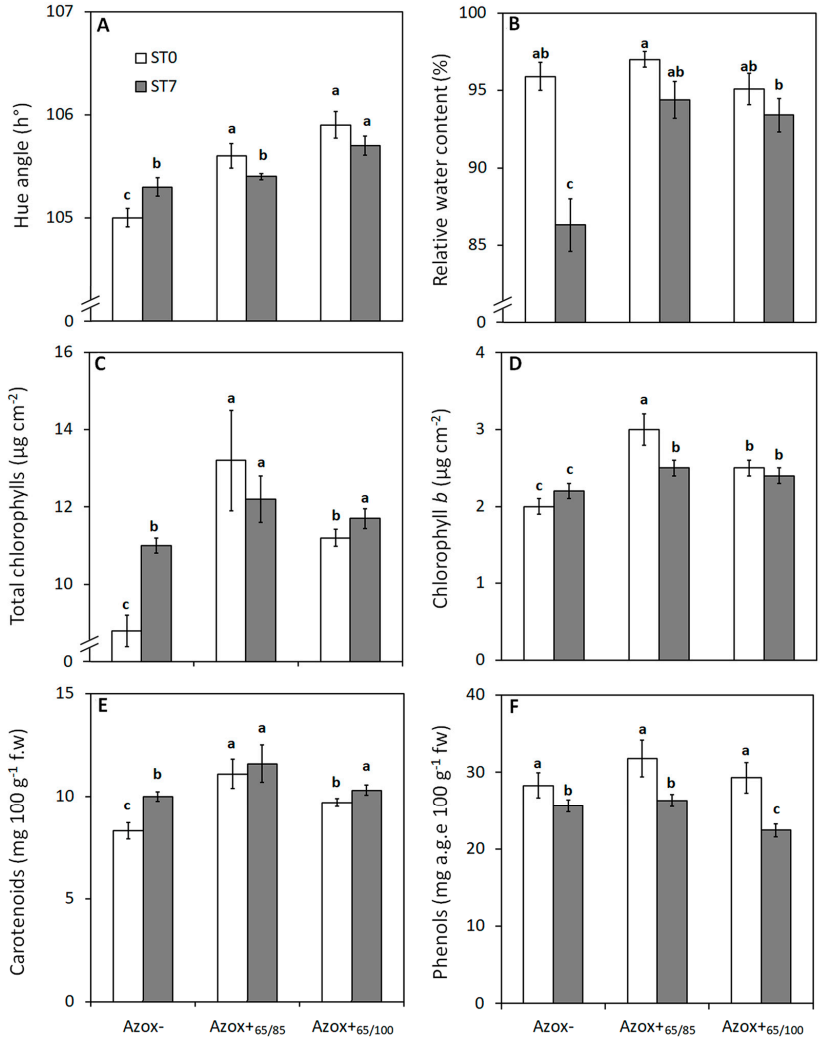

Figure 3. Effect of azoxystrobin application time on hue angle (**A**), relative water content (**B**), concentration of total chlorophylls (**C**), chlorophyll b (**D**), carotenoids (**E**), and phenols (**F**) in fresh and stored butterhead lettuce. Vertical bars (standard error) (n = 24) with different letters are significantly different according to the LSD test (p = 0.05).

Table 4. The significance of F test of ANOVA for the effects of storage time (ST), N rate (N), azoxystrobin application time (Azox) and their interactions on biometrical, physiological, and nutritional traits in butterhead lettuce leaves.

	Dry Matter (g kg^{-1} f.w.)	Lightness Index (L*)	Hue Angle (h°)	Saturation Index (C*)	Osmotic Potential (bar)	Relative Water Content (%)	Electrolyte Leakage (%)	Chlorophyll *a* (µg cm^{-2})	Chlorophyll *b* (µg cm^{-2})	Total Chlorophylls (µg cm^{-2})	Carotenoids (mg 100 g^{-1} f.w.)	Phenols (mg a.g.e. 100 g^{-1} f.w.)	Lipophilic Antioxidant Capacity (µmol T.E. kg^{-1} f.w)	Hydrophilic Antioxidant Capacity (µmol T.E. kg^{-1} f.w)	Total Antioxidant Capacity (µmol T.E. kg^{-1} f.w)	Nitrate (mg kg^{-1} f.w.)	Nitrate (g kg^{-1} d.w.)	Chloride (g kg^{-1} d.w.)	Phosphate (g kg^{-1} d.w.)	Sulphate g kg^{-1} d.w.)
Storage time (ST)																				
Time 0	56.6a [2]	57.8a	105.5a	41.1a	−5.9a	96.0a	17.9b	8.5a	2.5a	11.1a	9.7b	29.8a	106a	1224b	1329b	1366a	23.8a	3.3b	8.4b	2.5a
Time 7	58.1a	56.8b	105.5a	41.6a	−5.6b	91.4b	19.1a	9.2a	2.4a	11.7a	10.6a	24.8b	57b	1816a	1872a	951b	16.5b	7.2a	11.0a	1.9b
Significance [1]																				
ST	ns	*	ns	ns	*	***	*	ns	ns	ns	*	**	***	***	***	**	***	***	**	**
ST*N	ns	ns	***	ns	ns	ns	*	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
ST*Azox	ns	ns	**	ns	ns	***	ns	***	***	***	***	**	ns	ns	ns	ns	ns	ns	ns	ns
ST*N*Azox	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

[1] n.s., *, ** and *** not significant or significant at $p \leq 0.05$, 0.01 and 0.001, respectively. [2] Different letters within the column indicate significant differences at $p = 0.05$.

The osmotic potential of leaves became more negative after 7 days of storage (Table 4). The electrolyte leakage of leaf tissues increased differently during the storage period according to the N level: +10% in N100-, +7% in N50-, and +4% in N0-fed plants (Figure 2B).

During storage, the relative water content (RWC) only sharply decreased in leaves from Azox-, and at the end of the 7 storage days, the product from azoxystrobin treatment showed a higher RWC than the untreated plants (Figure 3B).

Several variations occurred in total chlorophylls-CHLs, CHL *a* (Figure 3C), CHL *b* (Figure 3D), and total carotenoids (Figure 3E) concentration during storage, with the leaves from azoxystrobin-treated plants showing higher concentrations of these pigments in comparison with the untreated ones at end of the storage period.

During storage, the antioxidant capacity (AC) increased in the total values (+41%) and the hydrophilic component (+48%), while the lipophilic component decreased (−46%) (Table 4).

The concentration of phenols decreased during the storage differently according to azoxystrobin application: −10% in untreated plants, −17% in the earlier application, −23% in the later application, and the stored leaves from the later application showed the lowest phenolic concentration in comparison to the Azox- and the earlier application of azoxystrobin (Figure 3F).

After 7 days of storage, the leaf concentration of nitrate, expressed both on a dry and fresh basis decreased (−31%), as well as that of sulfate (−24%), while that of chloride and phosphate increased (+118% and +31%, respectively) (Table 4).

4. Discussion

4.1. Effects of N and Azoxystrobin Application on Fresh Leaves of Butterhead Lettuce

4.1.1. Productive, Morpho-Biometric, and Bio-Physiological Attributes

Nitrogen (N) fertilization supplied during the autumn-winter cycle affected the vegetative growth (DY) and crop productivity (FY) of butterhead lettuce. Specifically, the N50 treatment can be considered as an optimal N level, because it gave the highest DY (consistently with the highest specific leaf area-SLA) and also the highest FY. The larger nitrate accumulation in the N100-fed plants indicates an N luxury consumption status, thus N100 may be considered an excessive N level for autumn-winter butterhead lettuce. An N-nutrition higher than the optimal could have provoked the speeding up of plant/leaf senescence and could have caused the reduction in dry and fresh biomass [22]. Concerning visual quality, the N50-product revealed a lower greenness of fresh leaves, as indicated by the highest lightness index-L* and the lowest hue angle-h°, although no difference in chlorophylls occurred.

Several studies on some herbaceous crops such as wheat, corn, and soybean [23–25] and vegetables such as tomato, lettuce, spinach, and wild rocket [10–17] have reported that strobilurin-based fungicides, applied in the absence of foliar disease, are associated with a yield improvement. The present study confirms that the azoxystrobin application significantly boosted the growth and production of butterhead lettuce, and the effect was irrespective of the N rate.

The yield-boosting effect could be imputable to the ability of several strobilurins to activate the enzyme catalyzing the first stage of N/nitrate assimilation in plants (nitrate reductase-NR), as previously demonstrated on leaf tissues of spinach by strobilurin kresoxim-methyl (KROM) [26], on hydroponically-grown *T. aestivum* wheat by strobilurin F 500® [27], and on cucumber leaves in recent studies [28].

In the present study, no specific investigation was performed; however, the potential effect of azoxystrobin in reducing nitrate concentration did not show up.

The effect of azoxystrobin on FY could be associated with the leaf morphological characteristics (leaf number, area, head length), related to an evident increase in leaf expansion. The increase in chlorophyll concentration (CHL) due to azoxystrobin application, at all N doses, could have induced the stimulation of light interception as well as photosynthetic

efficiency [17,29–31], thus leading to better plant performance in terms of boosting DY and FY.

According to a study on strobilurin KROM [6], it is possible to suppose that the general increase in CHL pigments under strobilurin application is imputable to its capacity to favor chloroplast development and to increase the cytokinins involved in CHL biosynthesis. The increase in photosynthetic pigments (measured as CHL concentration or SPAD readings) after strobilurin application had been confirmed in previous studies on herbaceous species (by KROM in *T. aestivum* [6,7,32]; by azoxystrobin in *T. aestivum* [33]; and durum wheat [9]) and in recent studies on ginseng leaves [34], leafy vegetables such as wild rocket by azoxystrobin [16,17], in spring and spring-summer butterhead lettuce by azoxystrobin [13], in spring and in winter spinach by azoxystrobin [14].

Concerning the application time, with the earlier timing (Azox+ $_{65/85}$), despite pushing up chlorophylls, SLA and DM, no significant boosting of DY or FY occurred in comparison with the later time (Azox+ $_{65/100}$). Thus, no clear differences in effectiveness between the two application times could be highlighted.

Irrespective of the application time, Azox+ determined a better visual quality of the product, showing leaves with more vivid (lower chrome index-C*) and more green (lower lightness-L*, higher hue angle-h°) color in comparison with the Azox-. These color measurements were also supported by chlorophyll values, thus the general positive effect of strobilurins on the visual quality of leafy species can be strictly linked with the improving effect on chlorophylls.

Since enzymatic discoloration (browning) is the process in which phenolic compounds are oxidized by the enzyme polyphenol oxidases (PPOs) to quinones [35] and then transformed into dark pigments [36], the oxidoreductase PPOs are crucially involved in the browning process pathway of leafy species. By considering the reduction in the activity of PPOs in azoxystrobin-treated fresh leaves and the positive correlation found between the activity levels of PPOs and the development of browning [37], it could be hypothesized that the azoxystrobin treatment makes the fresh plant material less prone to browning, thus supposing a higher shelf-life of the stored leaf product.

4.1.2. Nutritional and Anti-Nutritional Attributes

Azoxystrobin, at any application time, was not effective in reducing the nitrate content in autumn-winter lettuce leaves, as also observed in winter spinach, grown in the same soil and climatic conditions [14]. Conversely, the azoxystrobin application in spring and spring-summer butterhead lettuce (same cultivar 'Faustina') [13] and spring-grown spinach [14] is useful to reduce nitrate accumulation. This points out that the conditions favoring nitrate accumulation [38], such as the radiative and temperature conditions that occurred in the autumn-winter cycle of the present study (Figure 1) could have masked the reduction of nitrate, which was expected due to a higher NR-activation state induced by strobilurin [26,28].

Irrespective of applied treatments, the mean level of nitrate in the winter-grown lettuce (1366 mg kg^{-1} f.w.) was lower than the maximum nitrate limits for winter open-field lettuce imposed by the European Commission (4000 mg kg^{-1} f.w.; EU Regulation N. 1258/2011), thus this product is not hazardous.

Leafy vegetables such as lettuce are considered good sources of antioxidant molecules such as phenolic compounds and carotenoids [39]. The antioxidant capacity (AC) in total, measuring the efficiency of all antioxidant compounds in scavenging free radicals [40], accounts for the hydrophilic component (associated with hydrophilic phenols and others such as vitamin C), and the lipophilic one, more negligible (associated with carotenoids, and others such as tocopherols and lipophilic phenols). The application of azoxystrobin, irrespective of application time, induced a product with the highest total and hydrophilic AC (which was 94% of the total AC), as also confirmed by Bonasia et al. in lettuce [13] as well as by Schiattone et al. [16] and Candido et al. [15] in wild rocket salad. These results could be related to the general capacity of strobilurins to alter the antioxidant

metabolism in plant tissues via effects on the mitochondrial electron transport chain [41]. The application of strobilurins seems to lead to the increase in some enzymes involved in oxidative stress—by pyraclostrobin in tomato (Boari et al. [42]) and sugarcane (Lopes et al. [43]); by azoxystrobin in ginseng (Liang et al. [34]) and wild rocket (Candido et al. [17]).

Azoxystrobin increased antioxidant carotenoids in lettuce, especially when applied at an earlier time, as also seen in baby-leaf rocket salad [16]. The response of azoxystrobin on carotenoids was strictly in line with the increase in other pigments such as the chlorophylls, accounting for a linear correlation between the chlorophylls and the carotenoids, as demonstrated in lettuce [44] or other vegetable species such as *Zea mays* plantlets [45].

In N0-leaves, the production of antioxidant phenols and carotenoids was elicited probably as a response to stressing sub-optimal N-nutrition conditions, which prompt an overproduction of antioxidant molecules for scavenging reactive oxygen species [46]. The results concerning phenols are consistent with those reported by other authors in lettuce [47] and other leafy species [48]. However, there are quite contrary result data in the literature concerning carotenoids, for spinach in Xu and Mou [49], for lamb's lettuce in Hernández et al. [50], and red-pigmented lettuce in Zhou et al. [51]. In general terms the response of azoxystrobin or N on phenols (hydrophilic compounds) and carotenoids (lipophilic compounds) was not properly in line with the response on the AC and its components, thus different analytical methods in determining antioxidant AC would probably have been more appropriate.

4.2. Effects of N and Azoxystrobin Application on Stored Leaves of Butterhead Lettuce

Fresh and stored leaves of lettuce are susceptible to deterioration between harvest and consumption, depending on several conditions such as the pre-harvest factors [3].

The weight loss (WL) after 7 days of storage observed in the autumn-winter grown lettuce was in line with that of lettuce grown in a spring-summer cycle (0.98 g 100 g^{-1} fw, 7 days of storage, spring cycle; 0.90 g 100 g^{-1} fw; 12 days of storage spring-summer cycle) [13]. On the whole, the WL of leaves after storage was very low (<1%), since according to Paull [52], leafy greens begin seriously losing the marketable texture with a weight loss greater than 3%.

Leaf color and its post-harvest change is an important index for assessing the visual quality of vegetables and their consumer acceptance. The retention of the original color in shredded lettuce is the most important factor for marketability. The storage conditions usually provoke a significant visual quality decay of lettuce leaves, usually estimated on a colorimetric basis (L*, h°, C*) and/or analytical chlorophyll (CHL) data.

In our case, the alteration of the appearance of lettuce leaves was imputable to storage time and conditions in interaction with the pre-harvest treatments, specifically with N or azoxystrobin application. According to the variation of the colorimetric data observed during storage (lower h°), both pre-harvest N fertilization rates determined a stored leaf product with a higher loss of green color. The more N-nourished plants are more susceptible to post-harvest storage alteration, as seen in Silva et al. [53], and N100-fed spring-summer lettuce [14].

The stored leaves treated with Azoxy+ showed a better visual quality compared with the Azoxy-, as indicated by analytical data (higher CHLs). Specifically, the later application time contributed to maintaining greater post-harvest protection from loss of the green color of leaves, as revealed by the colorimetric data (higher h°). The positive effect of azoxystrobin on keeping the 'green' has been previously highlighted both in the stored product as shredded leaves of lettuce [13] and intact leaves of rocket salad [16] and spinach [14].

Azoxystrobin, irrespective of application time, was efficient in containing the enzymatic discoloration (browning), as seen by a naked-eye evaluation (Figure S1), and as expected according to the relation between color and browning indices [54]. Thus, the post-harvest performance of azoxystrobin during the storage was in line with that predicted by the lower activity of PPOs in the azoxystrobin-treated fresh plant material.

The storage affected the physiological status of the product. The processing and the storage conditions (cutting, post-harvest handling, temperature) determined a more negative value of the osmotic potential (OP), which is a component of leaf water potential [55], thus providing information on a general increase in water stress of plant tissue under storage conditions. The relative water content (RWC), corresponding to the absolute amount of water that the leaf requires to reach artificial full saturation [56], highlighted a generally lower hydration degree of the product. According to the value of another physiological index namely the electrolyte leakage (EL), also identified by the membrane permeability [57], the storage conditions of leaf lettuce induce a generally high degradation of cellular structures.

However, at the end of the 7 days, it emerged that the stored leaves from fertilized plants reached a higher destabilization of the selectivity of the cell membrane in comparison to N0-stored material (EL). Thus, the more nourished plants had tissues more susceptible to cell membrane structural alteration, as also found in spinach [58] and in spring butterhead lettuce [13]. After storage, the Azox+ product showed a greater turgidity than the Azox- leaves (RWC). Thus, azoxystrobin confirmed its ability to retain a better level of tissue hydration, as also observed in lettuce by Bonasia et al. [13].

According to the most sensitive indices for cellular stability (EL) and water (RWC) status in plant tissue, the sub-optimal N condition or azoxystrobin contributed to greater preservation of leaf consistency and senescence in butterhead lettuce.

The total and the hydrophilic antioxidant capacity (AC) of lettuce undergo a significant increase during storage, although a general decrease in phenols occurred during storage. On the whole, the increase in AC is supported by the basic hypothesis that many antioxidant compounds, apart from the phenols, are produced as secondary metabolites in response to cold (storage) stress on plants to compensate for its effects, such as membrane damage [59]. Therefore, in this research, some other hydrophilic compounds, such as vitamin C, not determined in the present research, could have contributed to the general increase in AC.

From a nutritional point of view, azoxystrobin, irrespective of application time, is also effective in maintaining the high nutraceutical value of lettuce after the storage period, as also seen in Schiattone et al. [16], since Azox+ product accumulated higher levels of carotenoids.

Concerning the anti-nutritional profile after the cold and dark storage period, the product showed a lower nitrate concentration. The increase in nitrites following the reduction of nitrates due to storage conditions could be supposed, but this trend is a still controversial issue in the literature regarding fresh plant material [60]. Silalahi et al. [61] found an increase in nitrates along with nitrites in lettuce during storage both at room and at refrigerated temperature. According to other research, no changes in nitrate or nitrite concentration occurred after 7 days at 5 °C in several leafy species such as spinach, crown daisy, Chinese spinach, and Chinese cabbage [62].

5. Conclusions

Moderate nitrogen fertilization (50 kg ha^{-1}) was confirmed as an effective N rate for improving the yield of autumn-winter butterhead lettuce. However, N50 did not guarantee the color appearance of fresh plant material and, along with N100, was less prone to containing green discoloration and membrane damage of the stored leaves.

Irrespective of N rate and azoxystrobin application time, the foliar application of azoxystrobin boosted the growth and productive response of autumn-winter butterhead lettuce. Azoxystrobin also improved the quality of fresh and stored leaves, by maintaining, in this latter case, a high turgidity and limiting visual and nutritional decay. The application of azoxystrobin could be also considered a valuable strategy for extending the shelf-life of shredded lettuce leaves by preventing browning during storage.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13010222/s1, Table S1: Weight loss. Figure S1: Stored shredded leaves.

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