Biofortification and antioxidant improvement of onion bulbs using calcareous algae and storage[1]

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ABSTRACT

Along the line that health-beneficial foods must also be accompanied by sustainable agricultural practices, the red alga *Lithothamnium* sp. (Rhodophyta), frequently used for animal and human nutrition, it was shown that it could be a biofertilizer, regarding their bioactive humic acid content, released by micronization. Also, onions present well-known benefits to health and are among the main vegetable crops grown worldwide. Thus, the objective this work was to evaluate the effects of foliar sprays with micronized *Lithothamniun* sp. on yield, mineral nutrients, flavonoids, phenolic content, and antioxidant activity before and after storage of bulbs of two organically grown onion cultivars. The yield, mineral content, antioxidant activity, and phenolic and flavonoid content in onion bulbs were improved through sprays, highlighting the dose of 1.5 g L^{-1} of *Lithothamnium* sp. in solution. Genotype interactions and storage effects were observed. The benefits with the use of *Lithothamnium* sp. as biofertilizer were towards the biofortification of organically grown onions by improving mineral nutrient acquisition as it was followed by upgrading antioxidant capacity.

Keywords: *Lithothamnium* sp.; *Allium cepa*; biofertilizer; biostimulant; nutrients; phenolics.

INTRODUCTION

Onions (*Allium cepa*) are among the main vegetable crops grown worldwide. It isa bulb with a typical flavor sold and consumed in different ways, such as seasoning, cooked, fried, and dehydrated, with many beneficial effects on health (Marrelli *et al.*, 2019). It has been present in human life since 3500 BC (Arshad *et al.*, 2017). In addition to its unmistakable aroma, the onion is also well known for its nutritional attributes, as a source of calcium, phosphorus, and other essential nutrients. (Corzomartinez *et al.*, 2007).

Onion have been used for their medicinal effects since ancient times. In addition, its antibacterial, anti-inflammatory, and antioxidant activities have been widely investigated (Zamri & Hamid, 2019). Therefore, onions are part of our diet, not only for their flavor but for their functionality in the human body (Marrelli *et al.*, 2019).

In this sense, healthy food production gains relevance health-beneficial foods should be accompanied by sustainable agricultural practices. Following this nature-friendly approach, the effect of algae extracts on the improvement of onion growth and yield has already been reported, regarding their biostimulant/ biofertilizer action (Sharma *et al.*, 2014; Szczepanek *et al.*, 2017; Mógor *et al.*, 2021).

Recently, the red alga *Lithothamnium* sp. (Rhodophyta), frequently used in animal and human diets (Zenk *et*

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al., 2018), was reported as a natural plant biostimulant, promoting better growth related to the release of humic acid through a micronization process (Amatussi *et al.*, 2020; Mógor *et al.*, 2021).

The *Lithothamnium* sp. is a calcium-based alga, composed mainly of calcium and magnesium carbonate. It can absorb minerals from the aquatic environment and transform them into compounds that can be absorbed by plants (Melo & Moura, 2009). The depositions of calcareous algae at the bottom of the ocean could follow organic matter transformation, similar to that in the soil, where the algae organic fraction is being transformed into bioactive humic substances, promoting plant growth and yield (Amatussi *et al.*, 2020).

Considering the importance of onion in the human diet, combined with the need to present natural alternatives to grow onions, the objective of this work was to evaluate the effect of micronized *Lithothamniun* sp. on yield, mineral nutrients, flavonoid, phenolic content, and antioxidant activity of organically grown onions. Also, to compare antioxidant profile in bulbs, before and after storage, considering that the onion storage by family farmers is customary to achieve better market prices despite causingbiochemical changes in the bulbs (Marrelli *et al.*, 2019).

MATERIAL AND METHODS

Growing conditions and plant material

Onion plants were grown in the Organic Vegetables Research Area, in an organic system since 2006, at the Federal University of Paraná, Paraná State, Curitiba, Brazil., under the geographical coordinates 25˚25'S and 49˚06'W and altitude of 920 m. The climate, according to the Köppen's classification, is subtropical of the Cfb type. The soil chemical analysis indicated the following values: pH ($CaCl_2$) = 5.60; pH SMP = 5.80; $Al^{+3} = 0$; $H^{+} + Al^{+3} = 5.80$ cmol·dm^{-3}; $Ca^{2+} = 7.0$ cmol·dm^{-3}; $Mg^{2+} = 3.90$ cmol·dm^{-3}; $K^{+} = 1.64$ cmol·dm^{-3}; P = 78.40 mg·dm^{-3}; C = 40.8 g·dm^{-3}; soil base saturation = 68% and CEC = 18.34 cmoldm^{-3}, Cu = 16.80 mg·kg^{-1}, Mn = 154.20 mg·kg^{-1}, Fe = 98.40 mg·kg^{-1}, Zn = 9.40 mg·kg^{-1}. It was prepared according to the Brazilian regulation for organic agriculture, with the incorporation of 12 t ha^{-1} organic compost with the following values: C = 30.3 g kg^{-1}; N = 30.3 g kg^{-1}; P = 8.5 g kg^{-1}; K = 6.6 g kg^{-1}; Ca = 8.1 g kg^{-1}; Mg = 4.1 g kg^{-1}. The moisture was kept at 80% through irrigation and tensiometer.

After the dispersion of the compost, beds were made with a dimension of 1.20 x 24 m. Four rows were spaced by 25 cm between them and 10 cm between plants, equivalent to a plant population of 250.000 per hectare. Two onion cultivars often used by organic growers in southern Brazil were chosen: i) Br-29, an open pollination cultivar, producing white, rounded, with dark yellow skin and ii) a hybrid cultivar "Perfecta F1", a white, rounded, with golden yellow skin, both short-day type from TopSeed®, Agristar®. Seedlings transplantation was performed 40 days after sowing in seedbeds, with seedlings showing five leaves.

Lithothamnium sp. *sample*

The *Lithothamnium* sp. sample was obtained from legal collection extraction located off Espírito Santo State coast, Brazil (20°19′ 10″ S–40° 20′ 16″ W). The micronization (mechanical breakage, caused by the friction between the particles until sizes among 1 to 10 μm) was done, and the sample provided by Valeagro Comércio Importação & Exportação/ NaturVita® Bioagroindustria – (Petrolina City, Pernambuco State, Brazil). The sample (white grayish color dry powder) presented: Calcium 23.3 % (w/w); Magnesium 1.3% (w/w) and solubility (water 20ºC) 3.0 g L^{-1}. The sample humic acid concentration was determined through UV-VIS spectrophotometry and the ratio E4/E6 was calculated through readings at 465 nm and 665 nm in triplicates (Javanshah & Saidi, 2016), achieving a humic acid concentration of 31.36 μg.g^{-1} and E4/E6 of 1.35, indicating high humification degree (Saab & Martin-Neto, 2007). The sample (micronized powder) was suspended in deionized water at two concentrations: 1.5 g L^{-1} and 3.0 g L^{-1} (maximum soluble concentration) just before they were sprayed on the leaves of the onion plants.

Treatments

Ten foliar sprays of *Lithothamnium* sp. suspensions (*Lit*) were performed, starting 15 days after seedlings were transplanted and repeated weekly, using a pressurized sprayer at constant pressure (40 psi). The control plants were sprayed with deionized water. The plots with 1.20 × 1.0-m were distributed in a completely randomized design with four replications (n = 4). The plants were managed according to Brazilian regulations for organic agriculture.

Harvest and storage

Bulbs were harvested at 120 days after sowing, clas-

sified according to the mass and diameter following the Brazilian market classification. The yield was determined with bulbs in commercially viable classes: IV (70 mm to 90 mm) and class III (50 mm to 70 mm). Bulbs were randomly selected and used for biochemical and nutritional analysis. The samples were separated, and the bulbs were randomly characterized as "at harvest" (*Har*) and "after storage" (*Sto*). The *Har* samples were immediately submitted to determinations of minerals, antioxidants, total phenolic, and total flavonoid contents. The *Sto* samples went on to the storage shed, where they remained for 60 days at room temperature (25 °C +/- 2 °C). After, they were submitted to antioxidant, total phenolic content, and total flavonoid content analyses.

Mineral analysis

For nitrogen content analysis (N-total), bulbs were weighed into 15 mg samples. They were packed in tin capsules for posterior dry combustion in a CHONS analyzer. For determination of phosphorus (P), potassium (K), magnesium (Mg), calcium (Ca), manganese (Mn), iron (Fe), zinc (Zn) and copper (Cu) contents, samples with 0,3 g of dry bulb mass were used. These samples were diluted in HNO_3 and dissolved in H_2O_2. Then, the nutrient contents were determined using induction induced plasma optical emission spectroscopy Perkin Elmer Optima 4300 (ICP-OES) (Perkin Elmer, USA), in triplicate.

Antioxidant activity

The measurement of the sequential activity of the DPPH radical (1,1-diphenyl-2-picrilidrazil) was performed according to the methodology applied by Brand-Williams *et al.* (1995). To assess the antioxidant activity, measures of 0.05 g were used from the bulb samples (*Cur* and *Har)* diluted in 2 mL of distilled water and centrifuged for 5 minutes, then the supernatant was collected. After collecting 0.1 mL of each sample, and transferring to test tubes, 4.9 mL of the DPPH solution (0.1 mL) were added, then they sat in a dark room for 40 minutes for the reaction. The same procedure was carried out in a test tube without an onion aliquot to read the blank. The radical DPPH has an absorption characteristic at 517 nm, which disappears after reduction by hydrogen organized by an antioxidant compound. The reduction of the DPPH radical was measured by reading the absorbance at 517 nm in 40 minutes of reaction. The antioxidant activity was expressed according to the methodology equation, including: $\%\ AA = 100 - \{[(Aa - Ab) \times 100] / Ac\}$ where Aa = absorbance of the sample; Ab = blank absorbance; Ac = absorbance of the control. The antioxidant activity was expressed according to the methodology equation, registered as potential antioxidant percentage.

Total phenolic compounds

The content of total phenolic compounds was determined by the Folin-Ciocalteu spectrophotometric method using gallic acid as a reference standard (Folin & Ciocalteu, 1927). Folin Ciocalteu's reagent is a solution of complex polymeric ions formed from phosphomolybdic and phosphotungstic heteropoly acids. This reagent oxidizes the phenolates, reducing the acids to a blue Mo-W complex, in an alkaline medium. A measure of 0.5 g of each sample (no-cure and post-cure separately) was taken for extraction in 20 mL of methanol: water (40:60) solution, processed in a centrifuge for 20 minutes in two 10-minutes steps. The supernatant was transferred to a 25 mL volumetric flask and filled with distilled water. This extraction was stored in a refrigerator for later analysis. From this extract, a 0.1 mL aliquot was taken and transferred to a test tube, where 1.5 mL of distilled water were added, and then 0.1 mL of the reagent Folin Ciocalteu. This procedure was repeated in all 24 samples tested at a time, and by the time the last test tube was reached with the Folin-Ciocalteu reagent, enough time had passed to start adding 0.3 mL of sodium carbonate at 20% in the first tubes. The tubes were shaken and placed in a grid to take the water bath at 40 °C for 30 minutes. Then, the absorbance reading was performed at 740 nm in a spectrophotometer.

Flavonoid content

The determination of flavonoid content was done according to the methodology proposed by Woisky & Salatino (1998). The technique is based on the absorbance measurement, at 420 nm, of the complex formed between the flavonoid and the aluminum of the color reagent, forming yellowish compounds. A measurement of approximately 0.05 g was taken from each sample (no-cure and post-cure separately), placed in test tubes and diluted in 2 mL of distilled water. A blank tube was made with only water and reagent. The tubes with samples were centrifuged and 0.5 mL of each were selected, transferred to other clean tubes, in duplicate, where 0.5 mL of 2% aluminum chloride were added to each tube. All samples were incubated for one hour. Then the samples were read at 420 nm on a spectrophotometer.

Statistical calculations

The experiment was conducted in a completely randomized design, and the data collected were subjected to a factorial scheme being: i) cultivars (2) x treatments with *Lit* (2 plus control) for yield and mineral content, and ii) cultivars (2) x treatments with *Lit* (2 plus control) x harvest/storage (2) for antioxidant activity, total phenolic compounds, flavonoid content. The analysis was performed using Assistat statistical assistance software.

RESULTS

Yield

The foliar sprays of *Lithothamnium* sp. suspensions (*Lit*) improved the average yield of the cultivar Perfecta-F1 (PF) (Table 1). Yield increments were 26.7% for *Lit* 1.5 g L^{-1} and 21.7% for 3.0 g L^{-1} over the control.

Minerals

The foliar sprays of *Lit* influenced the content of all nutrients in onion cultivars (Fig. 1). Interactions were found among cultivars and treatments for nitrogen (N), iron (Fe), zinc (Zn), and copper (Cu), and effect of treatments at both cultivars on average for phosphorous (P), potassium (K), calcium (Ca), magnesium (Mg) and manganese (Mn).

Regarding the interactions among cultivars and treatments, the N content (Fig. 2a) in BR-29 (BR) bulbs was incremented by *Lit* at both concentrations (1.5 g L^{-1} and 3.0 g L^{-1}). Perfecta-F1 (PF) presented a higher content in the control-treatment, indicating genotype differences among cultivars. The Fe content (Fig. 2f) was incremented only in BR by *Lit* 1.5 g L^{-1}, while Cu (Fig. 2i) was incremented only in BR by both *Lit* concentrations. On the other hand, Zn (Fig. 2g) content was incremented in PF by *Lit* 1.5 g L^{-1} and in BR by both *Lit* concentrations, yet when comparing cultivars, PF had higher Zn content than BR.

Regarding to both cultivars on average, the treatments showed similar effects for the contents of P (Fig. 2b) and K (Fig. 2c), being incremented by *Lit* 1.5 g L^{-1} over the control. Whereas, both *Lit*concentrations did not differing each other for P and K, and also *Lit* 3.0 g L^{-1} not differing from control, indicating that doubling *Lit* concentration did not increment P and K uptake by onion. The Ca content (Fig. 2d) was incremented only by *Lit* 1.5 g L^{-1}, while Mg and Mn were incremented by both *Lit* concentrations, showing differences in each other, with *Lit* 1.5 g L^{-1} promoting higher Mg and Mn accumulation in the onion bulbs than 3.0 g L^{-1}.

In general terms, the foliar sprays of *Lithothamnium* sp. micronized suspensions at 1.5 g L^{-1} were efficient to improve the mineral content in onion bulbs, in which genotype interactions are taken into account.

Antioxidants

Data in Table 2 indicate that the total content of flavonoids (*Flav*) was affected by the three factors (*Lit* x 'Cult' x Har/Sto). At *Har*, both *Lit* treatments have increased *Flav* over the control in PF, not affecting BR. After *Sto*, both *Lit* increased *Flav* over the control in BR, while *Lm* 1.5 g L^{-1} improved *Flav* content in PF by 26.7% over the control.

The phenolic content (*Phe*) in bulbs was affected by *Lit*, 'Cult' and *Sto*; however, without interaction between them. Comparing *Har*/*Sto*, the *Phe* was higher at *Har* in comparison to after *Sto*. The comparison between 'Cult'

Table 1: Yield of two organically grown onion cultivars (PF = Perfecta-F1, BR = BR-29) submitted to foliar sprays with solutions containing Lithothamnium sp. micronized (1.5 g L^{-1} and 3.0 g L^{-1})

	Control	**1.5 g L^{-1}**	**3.0 g L^{-1}**	$\bar{X}$
(a) Average yield (ton ha $^{-1}$)				
P	24.87 aB ±1.52	31.51 aA ± 1.98	30.28 aA ± 0.58	131.29 a
B	26.57 aA ± 2.19	27.98 bA ± 1.62	26.58 bA ± 2.25	122.94 b
$\bar{X}$	25.72 b ± 1.97	29.75 a ± 2.52	28.42 a ± 2.49	
C	*			
T	**			
C x T	*			

Onions cultivars: P = Perfecta F1; B = BR 29. Means followed by the same letter do not differ statistically at the 5% probability level by the Tukey test (n = 4) ± SD. Upper case letters at lines, lowercase letters at columns. ANOVA: ns = not significant, * and ** significant at p 0.05 and 0.01 respectively. C = cultivars. T = treatments and C x T = interactions.

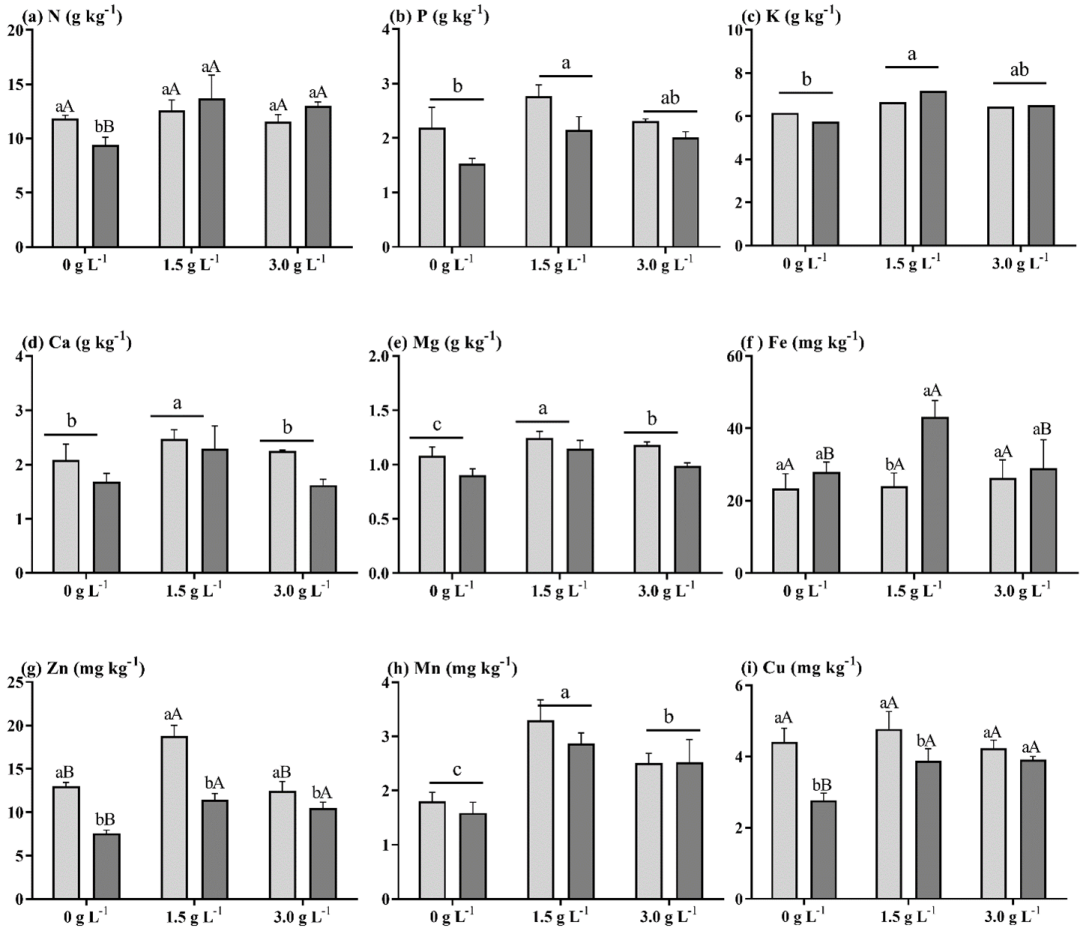

Figure 1: Contents of nutrients in bulbs of two organically grown onion cultivars submitted to foliar sprays with solutions containing Lithothamnium sp. micronized (1.5 g L-1 and 3.0 g L-1). For N, Fe, Zn and Cu, upper case letters = cultivars, lowercase letters = treatments. For P, K, Ca, Mg and Mn, the ANOVA did not found interaction among cultivars and treatments, being lowercase letters = treatments. PF = Perfecta-F1 light gray column, BR = BR-29 dark gray column. Means followed by the same letter do not differ statistically at the 5% probability by the Tukey test. Bars represent standard error.

showed that *Phe* was higher in PF bulbs than BR and the comparison between*Lit*, showed that *Phe* was improved at *Lit* 1.5 g L-¹ by 23.5 % over the control, but not differing from 3.0 g L^{-1} which in turn did not differ from control.

The antioxidant activity (*Aox*) was affected by *Har/Sto* and *Lit* treatments. Comparing *Har/Sto*, the 60-day storage at room temperature improved *Aox* by 17.4%. The *Lit* at 1.5 g L-¹ improved *Aox* by 23% over the control, but not differing from 3.0 g L^{-1}, which in turn, did not differ from control.

In general terms, *Lit* 1.5 g L^{-1} increased *Flav*, *Phe* and *Aox* in bulbs. The *Sto* also increased *Flav* and *Aox* but reduced *Phe* content in bulbs. It could be seen in 'Cult' that PF was richer in *Phe* than BR, although PF had responded mainly to *Lit* before. On the other hand, BR showed responses after *Sto*.

DISCUSSION

The effect of humic substances (HS) is widely discussed in literature in regards to their auxin-hormone like action on plant metabolism, promoting increases in the H + ATPase enzyme activity with consequences in the cell expansion and biomass accumulation (Canellas *et al.*, 2015).

The effect on onion yield through the use of HS was previously reported, showing improvements on bulbs biomass and caliber (Bettoni *et al.*, 2016), increasing marketable yield of bulbs, as well as enhancing the average weight of bulbs through foliar application (Kandil *et al.*, 2013), which are effects also presented by *Lit* applications (Table 1).

Table 2: Values of antioxidants (flavonoid, phenolics and antioxidant activity) in onion bulbs of two organically grown cultivars submitted to foliar sprays with solutions containing *Lithothamnium* sp. micronized (1.5 g L^{-1} and 3.0 g L^{-1})

Antioxidants	**Control**	**1.5 g L^{-1}**	**3.0 g L^{-1}**	**$\bar{X}$**
Flavonoids (mg g)				
Har/Sto x 'Cult' x *Lm*	*			
PF *Har*	306.10 bB ± 3.61	384.97 bA ± 3.84	384.87 aA ± 3.84	
BR	321.67 bA ± 3.21	373.36 bA ± 3.73	326.38 bA ± 3.26	
PF *Sto*	381.29 aB ± 3.81	438.17 aA ± 4.38	377.51 aB ± 3.77	
BR	312.99 bB ± 3.12	384.97 bA ± 3.84	369.21 aA ± 3.69	
Phenolics (mg g)				
Har x *Sto*	**			
Har	31.54	42.05	39.29	37.63 a ± 8.27
Sto	19.02	20.38	20.46	19.95 b ± 4.38
'Cult'	**			
PF	31.86	40,42	35.09	35.79 a ± 7.87
BR	18.70	22.01	24.66	21.79 b ± 4.79
Lm	*			
$\bar{X}$	25.28 b ± 5.56	31.22 a ± 6.86	29.88 ab ± 6.57	
Antioxidant activity (%)				
Har x Sto	**			
Har	0.542	0.693	0.593	0.609 b ± 0.10
Sto	0.639	0.761	0.743	0.715 a ± 0.11
Lm	**			
$\bar{X}$	0.591 b ± 0.09	0.727 a ± 0.12	0.668 ab ± 0.11	

Analysis done at harvest (*Har*) and after storage (*Sto*). Onion cultivars – 'Cult' (PF = Perfecta-F1, BR = BR-29). *Lithothamnium* sp. micronized - *Lm* (1.5 g L^{-1} and 3.0 g L^{-1}). For flavonoids, ANOVA found triple interaction (*Har*/*Sto* x 'Cult' x *Lm*) = upper case letters at lines, lowercase letters at columns. There were no interactions for phelonics, with significances (*Har/Sto,* 'Cult', *Lm*) on averages $\bar{X}$. There were no interactions for antioxidant activity, with significances (*Har/Sto*, *Lm*) on averages $\bar{X}$. Means followed by the same letter do not differ statistically at the Tukey test = * ($p \geq 0.05$) and **($p \geq 0.01$). (n = 4) +/−SD.

The experiment with HS is, in general, was conducted using the mineral leonardite obtained from mining, a source used in biostimulants to promote plant growth (Du Jardin, 2015). The novelty in this work is that HS is released from calcareous red algae through micronization, showing improvements on onion yield by 26.7% for *Lit* 1.5 g L^{-1} and 21.7% for 3.0 g L^{-1} over the control (Table 1), an achievement that is in line with the sustainable agricultural practices.

The effect of HS on improving root growth can also improve the mineral uptake by onion plants (Gemin *et al.*, 2019). Considering that the diets of over two-thirds of the world's population lack one or more essential mineral elements, agronomic practices to increase the concentration of minerals on food (biofortification) are strategic for an adequate human nutrition (White & Broadley, 2009). Thus, *Lit* treatments improved the nutritional quality of onion bulbs (Fig. 1).

The N content in food supply is determinant in terms of dietary protein adequacy (Shaheen *et al.*, 2016), in this sense, *Lit* has contributed by improving N content in bulbs on average up to 23.7% through foliar sprays with 1.5 g L^{-1}. Just as well, *Lit* has showed effects over major mineral nutrients in bulbs, those that play key roles in our body

for the necessary functions for a healthy and lengthy life (Gharibzahedi & Jafari, 2017), improving P, K, Ca and Mg, respectively in 32.2%, 16.4%, 26,5%, 21,2% by *Lit* 1.5 g L^{-1} (Fig. 1).

Micronutrient malnutrition in humans is derived from deficiencies of these elements in soils and foods (Yang *et al.*, 2007). The *Lit* 1.5 g L^{-1} sprays improved remarkably the micronutrient content in bulbs, with 30.7% (Fe), 46.6% (Zn), 82.2% (Mn) and 20.3% (Cu). Therefore, *Lit* contributed to onion biofortification.

The significant increases in mineral concentrations in bulbs promoted by *Lit* could be related to the achieved biochemical changes (Table 2) as the nutrients may participate in secondary metabolites pathways in plants, such as calcium, magnesium, iron and manganese, linked to the biosynthesis of *Phe* and *Aox* (Lattanzio *et al.*, 2009).

Phenolics are known to be the largest group of secondary metabolites in plants, varying from single aromatic rings to more complex ones. Phenols are divided into several groups such as phenolic acids and *Flav* (Sharma *et al.*, 2019). Onions are a recognized source of *Flav*, strongly linked to the *Aox*, with beneficial roles in human health as often discussed (Rodrigues *et al.*, 2017).

As a consequence of *Lit* sprays, the *Aox* of bulbs were improved, as well the *Phe* and *Flav* content, effect which was more pronounced at 1.5 g L^{-1}.

The *Aox* and *Phe* often increase during storage (Rodrigues *et al.*, 2017). However, a decrease was observed in *Phe* after *Sto* in comparison to 'Cult'. Such decrease could be related to metabolic changes to other phenolics in BR over the *Sto* period, such as phenolic acids, and other, not detected by the analytical method. These same acids may have been degraded or transformed during *Sto*, as increases in *Aox* and *Flav* concentration were observed after *Sto*. The greatest *Aox* potentials of onions are in its *Flav*, compared to its other varied *Phe* (Kefeli *et al.*, 2003).

The increase in *Aox* compounds in onion bulbs can bring many benefits for their consumers (Rodrigues *et al.*, 2017), enforcing the advantages of *Lit* sprays to grow onions beyond the biofortification, with the improved mineral nutrient acquisition, which is followed by a better antioxidant capacity.

Foliar sprays with micronized *Lithothamnium* sp. solution, under the evaluated conditions can improve yield and mineral content in onion bulbs, antioxidant activity in bulbs with their phenolic and flavonoid contents.

CONCLUSIONS

Foliar sprays with micronized *Lithothamnium* sp. solution can improve yield and mineral content in onion bulbs also the antioxidant activity in bulbs improving their phenolic and flavonoid contents.

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The authors declare that they have no conflict of interest.

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