

Organic onion biofortification using microalgae and humic acid

Biofortificação de cebolas orgânicas por microalgas e ácido húmico

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Received: 10/04/2021 | Reviewed: 10/11/2021 | Accept: 10/12/2021 | Published: 10/14/2021

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Abstract

Biofortification can be understood as a strategy to raise nutrients levels in vegetables edible structures, which can positively interfere in human diet. Biofertilizers are an interesting option in alternative production systems, as they can increase food nutritional quality and contribute to plant development, while aiding environment sustainability as natural products. The use of microalgae and humic acid as biofertilizers points to improvements in nutrients and biomolecules content in plants, however, their combined application is still unexplored. In that scenario, it was carried a study with onions about the influence of applications via root immersion in microalgae *Scenedesmus subspicatus* (Sc) and humic acid (HA) solutions, analyzing possible alterations of macro and micronutrients, total sugars, reducing sugar, free total amino acids, , total soluble solids, soluble proteins and antioxidant capacity in the bulbs. Treatments consisted of one minute seedlings roots immersion from two onion cultivars in solutions containing microalgae and humic acid, and then transplanted to organic system field. There were used three concentrations: control, 0.3 g L⁻¹ Sc + 0.3 g L⁻¹ HA (3SH) and 0.6 g L⁻¹ Sc + 0.6 g L⁻¹ HA (6SH). Results show that the treatments with microalgae with humic acid association were able to increase the content of N, carbohydrates and soluble proteins, also elevating antioxidant activity in onion bulbs.

Keywords: *Allium cepa* L.; Humic substances; Organic agriculture; *Scenedesmus subspicatus*.

Resumo

A biofortificação pode ser entendida como uma estratégia para elevar os níveis de nutrientes nas estruturas comestíveis dos vegetais, o que pode interferir positivamente na dieta humana. Os biofertilizantes são uma opção interessante em sistemas alternativos de produção, pois podem aumentar a qualidade nutricional dos alimentos e contribuir para o desenvolvimento das plantas, ao mesmo tempo que auxiliam na sustentabilidade do meio ambiente como produtos naturais. O uso de microalgas e ácido húmico como biofertilizante aponta para melhorias no teor de nutrientes e biomoléculas nas plantas, porém sua aplicação conjunta ainda é inexplorada. Nesse cenário, foi realizado um estudo com cebolas sobre a influência das aplicações via imersão radicular em soluções de microalgas *Scenedesmus subspicatus* (Sc) e ácido húmico (HA), analisando possíveis alterações de macro e micronutrientes, açúcares totais, açúcares redutores, sólidos solúveis totais, aminoácidos livres totais, proteínas solúveis e capacidade antioxidante nos bulbos. Os tratamentos consistiram na imersão das raízes das mudas de duas cultivares de cebola em soluções contendo microalgas e ácido húmico por um minuto, e posteriormente transplantadas para o campo do sistema orgânico. Foram utilizadas três concentrações: controle, 0,3 g L⁻¹ Sc + 0,3 g L⁻¹ HA (3SH) e 0,6 g L⁻¹ Sc + 0,6 g L⁻¹ HA (6SH). Os resultados mostram que os tratamentos com microalgas com associação de ácidos húmicos foram capazes de aumentar o teor de N, carboidratos e proteínas solúveis, elevando também a atividade antioxidante em bulbos de cebola.

Palavras-chave: *Allium cepa* L.; Substâncias húmicas; Agricultura orgânica; *Scenedesmus subspicatus*.

Resumen

La biofortificación puede entenderse como una estrategia para aumentar los niveles de nutrientes en las estructuras comestibles de los vegetales, lo que puede interferir positivamente en la dieta humana. Los biofertilizantes son una opción interesante en los sistemas de producción alternativos, ya que pueden incrementar la calidad nutricional de los alimentos y contribuir al desarrollo de las plantas, al mismo tiempo que ayudan a mantener el medio ambiente como productos naturales. El uso de microalgas y ácido húmico como biofertilizantes apunta a mejoras en el contenido de nutrientes y biomoléculas en las plantas, pero su aplicación conjunta aún no se ha explorado. En este escenario, se realizó un estudio con cebollas sobre la influencia de aplicaciones vía inmersión radicular en soluciones de microalgas *Scenedesmus subspicatus* (Sc) y ácido húmico (HA), analizando posibles cambios en macro y micronutrientes, azúcares totales, azúcares reductores, sólidos solubles totales, aminoácidos libres totales, proteínas solubles y capacidad antioxidante en bulbos. Los tratamientos consistieron en sumergir las raíces de plántulas de dos cultivares de cebolla en soluciones que contenían microalgas y ácido húmico durante un minuto, y luego trasplantarlas al campo del sistema orgánico. Se utilizaron tres concentraciones: control, 0,3 g L⁻¹ Sc + 0,3 g L⁻¹ HA (3SH) y 0,6 g L⁻¹ Sc + 0,6 g L⁻¹ HA (6SH). Los resultados muestran que los tratamientos con microalgas con asociación de ácidos húmicos fueron capaces de incrementar el contenido de N, carbohidratos y proteínas solubles, aumentando también la actividad antioxidante en bulbos de cebolla.

Palabras clave: *Allium cepa* L.; Sustancias húmicas; Agricultura orgánica; *Scenedesmus subspicatus*.

1. Introduction

Originated from the Asian continent, onion (*Allium cepa* L.) is a horticultural specie with great importance. The world production of this bulb grew by 22% between 2010 and 2017, led by China, and Brazil ranking among the first ten (FAO, 2020). Given onion importance in Brazil, its production is only surpassed by potatoes and tomatoes (Kurtz, et al., 2013).

The organic vegetable production in recent years has grown globally (Madail, et al., 2015), driven by a greater consumer demand for foods with higher nutritional value and free from chemical contamination, when compared to the conventional system (Barański, et al., 2017). In addition, Ren, et al., (2017) observed that bulbs obtained from organic systems have a higher concentration of flavonoids and antioxidant activity.

In that matter, onion bulbs have relevant nutritional properties, being rich in minerals, carbohydrates, proteins, ascorbic acid (Vethamoni & Gomathi, 2018), and polyphenols that can significantly contribute to the human diet (Hossain, et al., 2018). Additionally, bulbs also contain molecules that confer a high antioxidant capacity, such as quercetin, kaempferol, miricetin and catechin flavonoids (Karadeniz, et al., 2005).

O'Hare (2015) defines plant Biofortification as a practice that promotes nutrients levels during growth and development of vegetable edible parts. In this sense, technologies that can be used at organic production system that promote fresh food biofortification become relevant not only for human health, but also the environment.

In that scenario, as microalgae use in agriculture is a growing subject, it's noted that biofertilizers from its source are still little explored (Ronga, et al., 2019). Meanwhile, researches already show the potential of microalgae *Scenedesmus* sp. application as a plant growth promoter by increasing proteins and the activity of primary metabolism enzymes (Puglisi, et al., 2020).

In similarity, humic acid is another promising biofertilizer source due to its concrete results in agriculture (Canellas, et al., 2015). Studies with onions and tomatoes subjected to the application of humic substances, show productivity gains as well as greater accumulations of amino acids, sugars and vitamin C (Bettoni, et al., 2016; Hussein, et al., 2015)

Furthermore, humic acids and algae extracts applications in plants can be considered agronomic biofortification techniques, as they have potential to increase nutrient content and positively alter the concentration of total sugars, amino acids, proteins and phenolic compounds (Billard, et al., 2014; Conselvan, et al., 2017; Dineshkumar, et al., 2018). Nevertheless, the association of microalgae with humic substances is still untapped. In this sense, this works objective was to explore microalgae *Scenedesmus subspicatus* (Sc) and humic acid (HA) applications as biofortification substances in organic onion.

2. Methodology

The biomass from *Scenedesmus subspicatus* (Sc) Chodat microalgae (synonym: *Desmodesmus subspicatus*), deposited in the “Elizabeth Aidar” Microalgae Collection at the Fluminense Federal University, Niterói, Rio de Janeiro - Brazil, was obtained through an autotrophic cultivation system using WC culture medium in constant temperature (20-22°C) and light (5500 lux), at the Plant Science and Crop Protection Department of Federal University of Paraná (UFPR), Curitiba, Paraná - Brazil. After 25 days, the biomass was centrifuged and lyophilized, to be ready for use. The Humic acid (HA) used came from Leonardite minerals which contains 33% organic carbon (Powhumus® Humintech GmbH - Germany).

The onion cultivars chosen were BR-29 ('B') and Perfecta F1 ('P') from Topseed®. Seedlings were produced in a tunnel-type nursery covered with polyethylene in beds. After 45 days after sowing (DAS), seedlings were collected for planting in a bed measuring 1.20 x 24 m, and distributed in plots of 1.20 x 1.0 m, using 10 cm spacing between plants and 30 cm between lines, resulting in a population of 230 thousand plants per hectare.

Treatments consisted of aqueous solutions containing microalgae (Sc) and humic acid (HA) were applied via immersion in onion seedling roots for one minute. The solutions concentrations were: 0.3 g L⁻¹ Sc + 0.3 g L⁻¹ HA (3SH), 0.6 g L⁻¹ Sc + 0.6 g L⁻¹ HA (6SH) and distilled water (control). At 150 days after transplanting (DAT), about 80% of the plants had their aerial part dropped close to the ground (top collapse), to help bulb harvest. Thus, after harvesting, bulbs from each repetition and cultivar were sampled for the analysis of nutrient, biochemical and antioxidant content.

Therefore, this experiment was conducted in a completely randomized design in a factorial scheme, with factor A composed of cultivars ('B' and 'P') and factor B of treatments (control, 3SH and 6SH) with four repetitions each. This study was performed at the Organic Vegetable Production Research Area, under organic system since 2006, at the Federal University of Paraná, metropolitan region of Curitiba (latitude 25° 25' south, longitude 49° 06' west and 920 m altitude), in June 2019. The region climate, according to Köppen-Geiger classification is Cfb, which characterizes this region as Temperate. The soil chemical analysis at a depth of 0-20 cm, identified the following levels: 6.30 pH (H₂O), 33.30 g dm⁻³ organic matter; 133.10 mg dm⁻³ P; 1.44 cmol cdm⁻³ K; 9.30 cmol cdm⁻³ Ca; 4.30 cmol cdm⁻³ Mg; 0 cmol cdm⁻³ Al; 3.7 cmol cdm⁻³ Al + H; 18.34 cmol cdm⁻³ CTC and 80% base saturation.

Further, for the chemical analysis, 2 bulbs were collected per repetition to quantify macro and micronutrient contents (N, K, P, Ca, Mg, Cu, Mn, Fe, Zn and B). After that, in order to obtain the dry mass from each sample, a greenhouse with temperature (60°C±5°C) and forced ventilation were used until the samples presented constant weight. Samples with 0.3 g of dry bulb mass were extracted by acid digestion as described by Martins & Reissmann, 2007. After this procedure, nutrient contents were determined using the induction plasma optical emission spectroscopy methods with a Perkin Elmer Optima 4300 (Perkin Elmer, USA) in triplicate samples. The quantification of nitrogen (n-total) was performed by combustion in a CHONS analyzer (Vario EL III model).

In order to quantify total sugars, reducing sugar, total free amino acids and soluble proteins, for biochemical analysis, two bulbs were collected per repetition from each cultivar. To quantify the total soluble solids (Brix °) a refractometer was used in each treatment. For the quantification of total and reducing sugars, bulbs samples were prepared in microtubes plus distilled water, and subsequently taken to the vortex. After this process, the microtubes were placed in a centrifuge for 10 minutes (10,000 rpm). For total sugars extraction, the homogenized sample was added in test tubes with 1 ml of HCl for acid hydrolysis in water bath for 10 minutes. Then 1 ml of NaOH solution was added, leaving it to rest for 5 minutes. After this procedure, the DNS reagent was added to the total and reducing sugar samples, then samples temperature were cooled by ice bath, allowing spectrophotometer readings (540 nm). The standard curve for reducing and total sugars was established with glucose at 1mg/mL (5.5 mM) with values between 50 to 800 µg/mL (Maldonade et al., 2013).

Following Winters, et al. (2002) methodology the extraction of total free amino acids were carried, and the

colorimetric reaction was according to Magné & Larher (1992), using glutamine for the standard curve. Test tubes with vegetable samples and distilled water were used to extract amino acids. Then they were taken to a water bath at 100°C for 15 minutes, and left to cool, allowing the sample to decant after 20 minutes in an ice bath. The supernatant was removed from the samples into new microtubes and centrifuged at 3000 rpm for 10 minutes. For the colorimetric reaction it was added 1 ml of the sample, 0.5 ml of citrate buffer, 1 ml of ninhydrin and used vortex for 2 seconds. Subsequently, a water bath was applied for 15 minutes at 100°C, allowing it to cool, before adding 60% alcohol. The readings were carried out on a spectrophotometer at 570 nm.

The methodology chosen for soluble proteins extraction was described by Bradford (1976), using BSA reagent for the standard curve construction. It was added 0.5 g of plant sample plus 1.5 ml of phosphate buffer as described by Du, et al. (2010). After this procedure, samples were centrifuged for 15 minutes at 10,000 rpm. Then, 70 µL of the supernatant was added to the homogenized sample, plus 2 ml of the Bradford reagent, letting it rest for 15 minutes, thus making it possible to the sample readings on a spectrophotometer (595 nm).

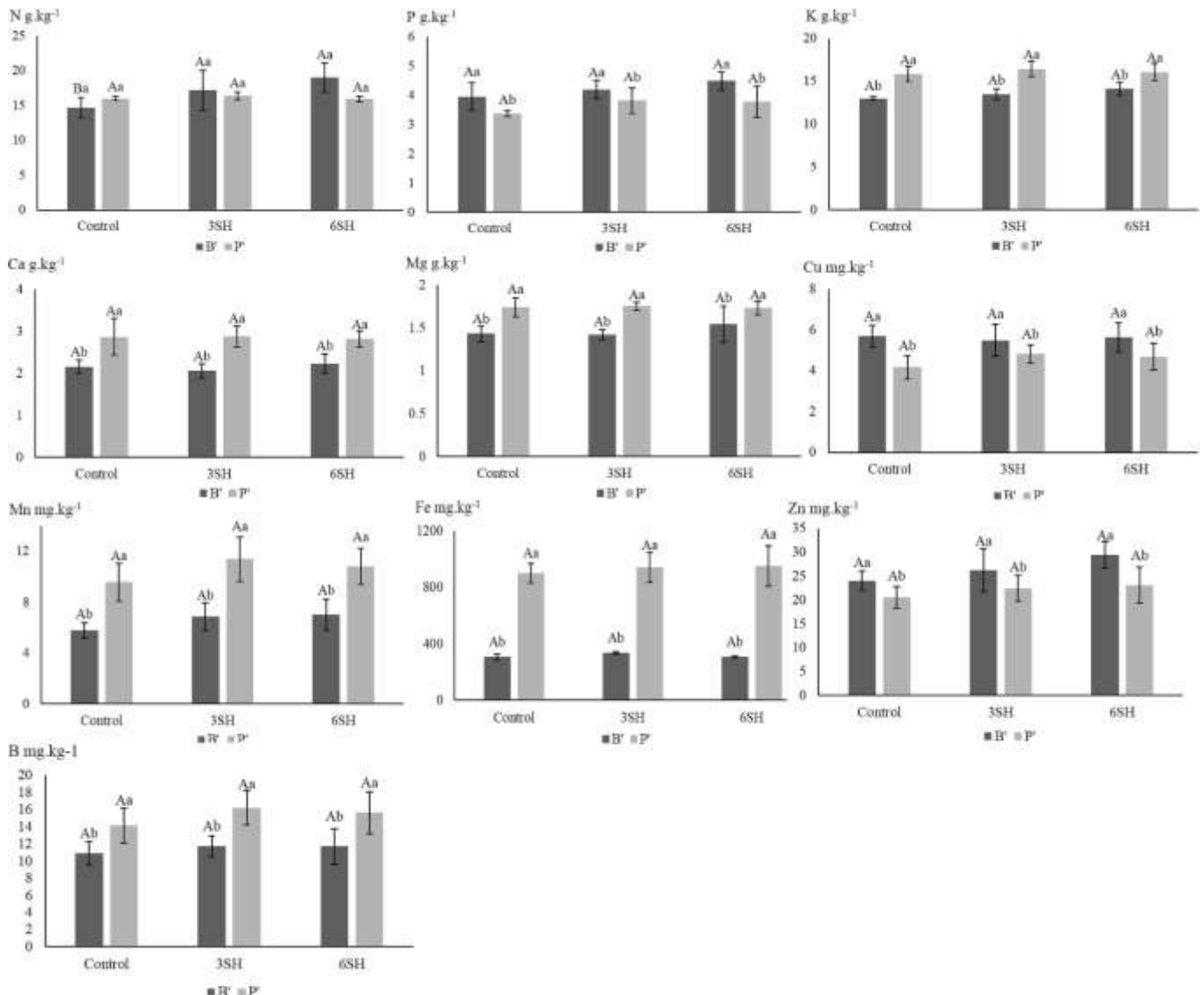
The antioxidant action was estimated using standard 1,1-diphenyl-2-picryl-hydrazil (DPPH) using Brand-Williams et al. (1995) methodology. Samples of 0.5 g from onion bulbs were used, subsequently diluted into distilled water and centrifuged for five minutes. After this procedure, 0.1 ml of the supernatant was removed from the samples and transferred to test tubes that contained 4.9 ml of the DPPH reagent. Then the samples were left to rest for 40 minutes for measurement in a spectrophotometer (517 nm).

Collected data were first verified for its variance homogeneity by Bartlett's test. When significant, the results were subjected to analysis of variance (ANOVA), with averages compared by Scott-Knott's test ($p < 0.05$; $p < 0.01$), using ASSISTAT 7.7 beta version statistical analysis software (Silva & Azevedo, 2016).

3. Results

In 'P' bulbs (Figure 1), treatments containing microalgae associated with humic acid did not have influence in the increase of nutrient content. However, 3SH and 6SH treatments via immersion increased N levels by 17 and 29% in 'B' bulbs, respectively, when compared to the control, showing no statistic difference for other nutrients. In comparison between cultivars, 'P' bulbs presented, on average, higher K (18.9%), Ca (33%), Mg (19%), Mn (66.2%), Fe (296%) and B (33.8%). In contrast, 'B' bulbs showed a higher concentration of P (15.3%), Cu (23%) and Zn (20.4%). The concentration of the mineral nitrogen was on average statistically equal for both cultivars.

Figure 1. Nutrients content in organically grown onion bulbs (dry mass), whose seedlings were immersed for one minute in solutions of the biomass of microalgae *Scenedesmus subspicatus* (Sc) in association with humic acid (HA).



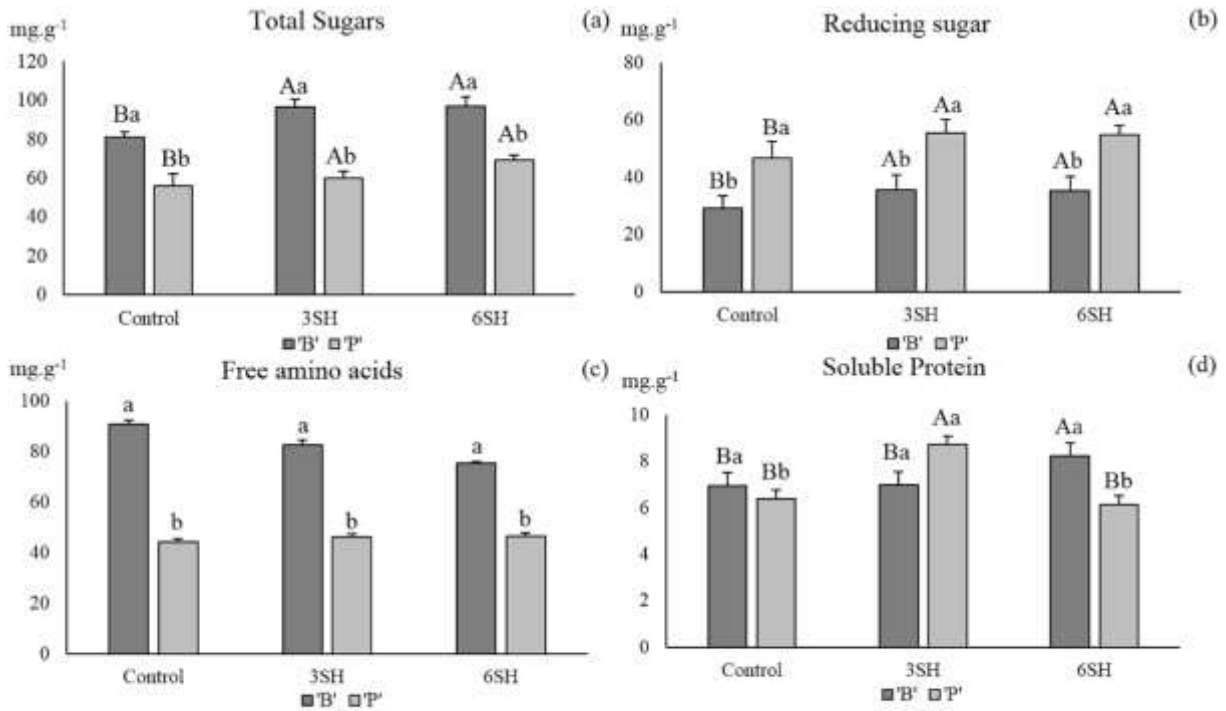
Onion cultivars ('B' = BR-29; 'P' = Perfecta F1). Immersion treatments: control, 3SH = (0.30 g L⁻¹ Sc + 0.30 g L⁻¹ HA), 6SH = (0.60 g L⁻¹ Sc + 0.60 g L⁻¹ HA). Capital letters = treatments. Lower case letters = cultivars. Bars represent standard deviation. Means followed by the same letter do not differ statistically in Skott-Knott test (p ≤ 0.05).

Source: Authors.

The treatments contributed to the increase in total and reducing sugars concentration in bulbs when compared to the control. In 'B' bulbs, the concentration of total sugars (Figure 2AB) showed an increase of 48% when compared to 'P' bulbs. Meanwhile, the content of reducing sugar in 'P' bulbs was significantly higher (62%) when compared to 'B' bulbs.

The content of total free amino acids (Figure 2C) in 'B' bulbs was higher (82%) when compared to 'P', however, without effect between treatments. The content of soluble proteins (Figure 2D), showed interaction factor between cultivars and treatments. Applications of 6SH promoted a significant increase in 'B' bulbs (18%). While in 'P', the application of 3SH significantly increased soluble proteins (37%) when compared to the control.

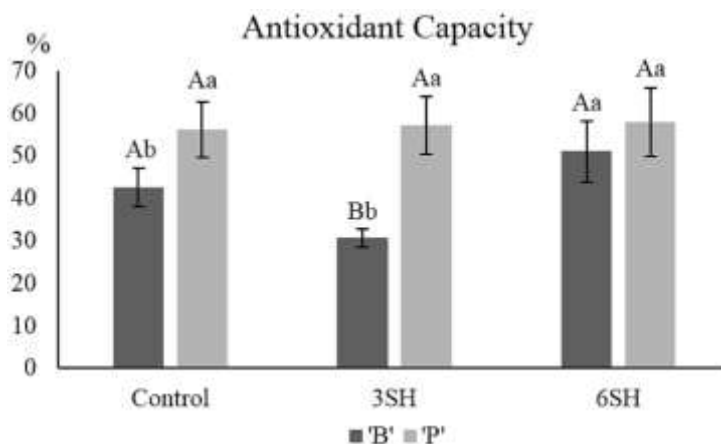
Figure 2. Total sugars content (A), reducing sugars (B), total free amino acids (C) and soluble proteins (D) in organically grown onion bulbs whose seedlings were immersed for one minute in solutions of the biomass of microalgae *Scenedesmus subspicatus* (Sc) in association with humic acid (HA).



Onion cultivars: 'B' = BR-29; 'P' = Perfecta F1. Immersion treatments: control, 3SH = (0.30 g L⁻¹ Sc + 0.30 g L⁻¹ HA), 6SH = (0.60 g L⁻¹ Sc + 0.60 g L⁻¹ HA). Columns with the same letter do not differ statistically by Scott-Knott's test ($p < 0.05$) ($n = 4$). Capital letters = treatments. Lower case letters = cultivars. Bars represent standard deviation.
 Source: Authors.

Additionally, there was a significant difference in antioxidant activity between cultivars and treatments (Figure 3). The 'P' bulbs showed greater capacity (37%) to scavenge free radicals (DPPH) when compared to the 'B' bulbs. There was no statistical difference between treatments in the cultivar 'P'. However, in cultivar 'B' higher antioxidant capacity was observed in the control and 6SH treatments when compared to the 3SH treatment. In addition, the 6SH treatment in 'B', on average, showed antioxidant capacity statistically equal to that found in 'P' bulbs, which also showed greater antioxidant capacity.

Figure 3. Antioxidant capacity in organically grown onion bulbs, whose seedlings were immersed for one minute in solutions of the biomass of microalgae *Scenedesmus subspicatus* (Sc) in association with humic acid (HA).



Onion cultivars: 'B' = BR-29; 'P' = Perfecta F1. Immersion treatments: control, 3SH = (0.30 g L⁻¹ Sc + 0.30 g L⁻¹ HA), 6SH = (0.60 g L⁻¹ Sc + 0.60 g L⁻¹ HA). Columns with the same letter do not differ statistically by Scott-Knott's test (p<0.01) (n = 4). Capital letters = treatments. Lower case letters = cultivars. Bars represent standard deviation.

Source: Authors.

4. Discussion

Studies point to the potential benefits to human health in ingesting fresh onions, as this bulb is considerate to have high nutritional value, which may vary in nutrient composition between different cultivars (Kumar, et al., 2010). In particular, nitrogen is an essential nutrient for human and plant metabolism in stimulating protein synthesis (Al-Fraihat, 2009; Vianna, et al., 2010). In this sense, Bettoni, et al. (2016) observed that about 2.8%, on average, of different onion cultivars mass, is composed of nitrogen. An even superior result to 'B' (1.69% of N) and 'P' (1.60% of N) bulb cultivars used in this work.

An increase in the N content in the 'B' bulbs was observed in the treatments with 3SH and 6SH provided, which may be related to humic acid effect, which when applied to plants promotes involved enzymes activation in the reduction and assimilation of inorganic nitrogen (Nitrate reductase, Nitrite reductase, GS and GOGAT) (Vaccaro, et al., 2015). In addition, microalgae biomass may contain phytohormones, vitamins and enzymes that favor greater assimilation of nutrients in drains of higher priority (Shaaban, et al., 2001).

Moreover, Dias (2012) points out that plants of Liliaceas family, such as onion and garlic, are rich in potassium, calcium and manganese, making possible to supply 10% of human daily needs with the consumption of these vegetables. In 1 g of fresh onion bulb mass, the concentration of potassium and calcium is 1.46 mg and 0.23 mg of calcium respectively (USDA, 2019), which are higher than those observed in the 'B' and 'P' bulbs (Figure 1), as the highest content of these nutrients were quantified in hybrid cultivar bulbs. Phosphorus was the third most accumulated nutrient in bulbs, with magnesium being the nutrient with the lowest content in bulbs, a result similar to that found by Bettoni, et al. (2016).

In addition to iron supplementation in diets to combat anemia, the consumption of foods rich in this micronutrient can help decrease the population risk of iron deficiency (Leão, et al., 2018). A research shown average values of Fe content in onion bulbs of 744 µg g⁻¹ (Vidigal, et al., 2010). Result that may vary from each cultivar, as observed in bulbs of 'P' (930 µg g⁻¹), displaying greater Fe accumulation capacity when compared to 'B' (314 µg g⁻¹), showing that the hybrid cultivar has greater potential in preventing nutritional disorders like anemia.

Additionally, within the same plant species there is variation in chemical elements concentration, as observed in the onion genotypes studied when comparing the micronutrients Mn, Zn, B and Cu (White & Broadley, 2005).

In dry matter fraction of onion bulbs, approximately 80% is composed of non-structural carbohydrates, which are

reducing sugars such as glucose and fructose, as well as sucrose non-reducing sugar and fruits-oligosaccharides (Zhang, et al., 2016). On this wise, soluble solids are related to the sweet taste of onion, being composed of fructans, fructose, glucose and sucrose (Mallor, et al., 2011).

Furthermore, studies with humic acid application in onion plants show an increase in the concentration of reducing sugars and starch in the bulbs (Bettoni, et al., 2016), as well as the microalgae *Nannochloropsi* ssp. and *Scenedesmus subspicatus*, which when applied to tomato and onion plants respectively, promoted an increase in carbohydrates (Coppens, et al., 2016). It was verified that in two onion cultivars bulbs the °Brix values were the same between treatments ($p>0.05$). However, cultivar 'P' showed higher values of °Brix (44%) when compared to 'B'. The average values of °Brix in the 'B' and 'P' bulbs are respectively: control = 5.17 and 8.0; 3SH = 6.25 and 8.05; 6SH = 5.57 and 8.45. The contribution of humic acid and microalgae use as a tool in increasing the carbohydrate content in the bulbs is relevant, since this technology can help farmers to obtain a sweeter onion.

It is known that biofertilizers are capable of promoting the increase of soluble proteins in plants, improving N assimilation and stimulating amino acids metabolism (Nardi, et al., 2016). Although, it was not clear the response to the treatments containing microalgae associated with humic acid, as it promoted 'B' and 'P' greater accumulations of soluble proteins treated with 6SH and 3SH respectively, but it had different results in the concentration of total free amino acids and N content in 'P' bulbs when treated with the biofertilizer.

Among vegetables, onion has a high antioxidant capacity because it contains anthocyanins and flavonoids in its composition, with quercetin being the flavonoid with the highest concentration in its bulbs (Suleria, et al., 2015). In such matter, it was observed a greater antioxidant activity and higher flavonoids concentrations in onions conducted at organic system when compared to conventional, a significant result for alternative production systems and for the consumer (Ren, et al., 2017).

Researches with humic acid application in plants demonstrate the potential to activate secondary metabolism. Schiavon, et al. (2010) shows that humic substances applications increased the expression of phenylalanine amonialisias enzyme, responsible for the first step in phenolic compounds biosynthesis. In addition to having a growth-promoting effect on vegetables, microalgae act as elicitors in the plant's defensive system, increasing the concentration of peroxidases, polyphenol oxidase and phenylalanine amonialiase, enzymes that act as antioxidants in plants (Renuka, et al., 2018). It was observed that the antioxidant capacity varies with the onion genotype, this result was also observed by Lu, et al, (2011). In addition, the biofertilizer application in 6SH concentration stimulates 'B' bulbs to achieve an antioxidant capacity statistically equal to that found in 'P' bulbs, a genotype that showed greater antioxidant capacity.

5. Conclusion

Biofertilizer use containing *Scenedesmus subspicatus* microalgae in association with humic acid promoted an increase in the N content in the open pollinated cultivar. In addition, the biofertilizer provided greater accumulation of carbohydrates and soluble proteins in both cultivars bulbs, and the treatment containing $0.6 \text{ g L}^{-1} \text{ Sc} + 0.6 \text{ g L}^{-1} \text{ HA}$ increased antioxidant capacity in the BR-29 cultivar.

Acknowledgments

The author thanks National Research Council of Brazil (CNPq) for his Doctoral scholarship (grant 142393/2016-9).

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