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Effect of seaweed (*Ecklonia maxima*) extract and legume-derived protein hydrolysate biostimulants on baby leaf lettuce grown on optimal doses of nitrogen under greenhouse conditions

Ida Di Mola[1*], Eugenio Cozzolino[2], Lucia Ottaiano[1], Maria Giordano[1], Youssef Rouphael[1], Christophe El-Nakhel[1], Vincenzo Leone[2], Mauro Mori[1]

[1]Department of Agricultural Sciences, University of Naples Federico II, via Università 100, 80055 Portici Napoli Italy
[2]CREA-Research Centre for Cereal and Industrial Crops, via Torrino 2, 81100 Caserta Italy

***Corresponding author: ida.dimola@unina.it**

Abstract

In recent years, the demand for green leafy vegetables is increasing. In order to satisfy this trend, the leafy crops are cultivated under high energetic input, especially high doses of nitrogen (N) fertilization that leads to a nitrate accumulation in leaves, sometimes overcoming the legal threshold set by the European Community for their commercialization. The nitrate in leaves can be dangerous for human health because in the human body it can be converted into nitrite, which can cause methemoglobinemia or create cancer-causing compounds. In order to overcome this problem, a correct N management is needed, especially using technical means which can improve the nitrogen use efficiency. In this study, we evaluated the possible effect of two important plant biostimulants on yield and quality traits (nitrate, antioxidants activity, carotenoids) of baby leafy lettuce, grown in a greenhouse with three levels of nitrogen input. Nitrogen doses were 0, 10 (sub-optimal) and 20 (optimal) kg ha^{-1}, N0, N10 and N20 respectively. The biostimulants were *Ecklonia maxima* seaweed extract (3 ml per liter) (named Bio 1), legume-derived protein hydrolysate (Bio 2) and non-treated control (Control). The treatments were distributed in a randomized complete-block design with three replications (3 N levels x 3 Biostimulant applications x 3 replications). Biostimulant applications of seaweed extracts and legume-derived protein hydrolysate improved yield and LAI: 13.4% and 12.0% increase over non treated plants, respectively. The highest yield was reached at 20 kg N ha^{-1}. Application of foliar biostimulants stimulated the antioxidant systems of plants, improved leaves color and increased chlorophyll and carotenoids content. The nitrate concentration in leaves was increased under higher levels of N fertilization, meeting the EC legal limit at N20 treatment in plants sprayed by *E. maxima* seaweed extract. Therefore, in our growth conditions, it seems possible to reduce nitrogen input at 10 kg N ha^{-1}, by applying additional applications of biostimulants to reduce the yield gap upon application of N20 treatment.

Keywords: Biostimulants, nitrogen dose, *Lactuca sativa* L., leafy greens, nutritional quality, sustainable agriculture, yield.
Abbreviations: LAI_Leaf Area Index; SLW_Specific Leaf Weight; SPAD_Soil Plant Analysis Development; LAA_Lipophilic Antioxidant Activity; HAA_Hydrophilic Antioxidant Activity; TAA_Total Ascorbic Acid.

Introduction

In recent years, the spread of baby leaf vegetables is destined in ready-to-eat salads in response to the market demand. In order to satisfy consumer's request, farmers are forcing the cultivation to reach higher production: crops are cultivated in protected environment (tunnel and greenhouse), where more growing cycles per year are carried out and high energetic input (nitrogen fertilization) are used. Nitrate is usually the main source of nitrogen (N) for higher plants (Marshner, 1995) and it can be highly accumulated in plant tissue, especially in green leafy vegetables. Most plants are capable to reduce nitrate in both roots and shoots (Marshner, 1995), but it is mainly and efficiently reduced in leaves, due to the availability of reductants and energy and carbon skeletons produced by photosynthesis (Oaks, 1994; Solomons and Barber, 1990). The nitrate up-taken by plants can also be stored in the vacuoles until released for reduction in the cytosol (Cardenas-Navarro et al., 1999). Moreover, the nitrate reductase activity and consequently the nitrate accumulation depend on several environmental factors, especially light intensity. Light conditions influences directly nitrate-reductase activity that decreases at low level of radiation, and indirectly because the photosynthesis provides NADH to nitrate-reductase and produces carbohydrates and organic acids, used by cells, as osmolytes in alternative to NO_3^- (Gonnella, 2002; Steingröver et al., 1986a; Blom-Zandstra and Lampe, 1985). Therefore, when the photosynthetic activity is low, also nitrate reductase activity decreases and the plant seems to use nitrates like an osmolyte, accumulating them in the vacuoles. So nitrate accumulation is higher in all low radiation conditions: winter, protected environment (e.g., plastic films reduce the sunlight transmission) and high latitudes (Nord Europe regions). But nitrate accumulation strongly depends also to N availability and; therefore, N fertilization.

Nitrate becomes dangerous in human body, when converted into nitrite after the intake, where this latter can cause methemoglobinemia or cause cancer compounds (nitrosamine and nitrosamide) (Gangolli et al., 1994; Walker, 1990). Therefore, the European Community have fixed the legal threshold for commercialization of major leafy vegetables which is 5000 mg NO_3 kg^{-1} for lettuce grown in protected environment and harvested between 1 October and 31 March (Reg. n° 1258/2011).

Consequently, to overcome this problem, an optimal crop management is necessary and in this perspective, the use of biostimulants to replace or couple to nitrogen fertilization can be a valid alternative. Biostimulants have been recently included in the national fertilizers regulation (Amanda et al., 2009). They are organic compounds, such as humic substances or compounds extracted from seaweeds or obtained by chemical hydrolysis of plant- or animal-derived proteins (Rouphael et al., 2017; Colla et al., 2015; Kauffman et al. 2007; Cavani et al., 2006). Generally, they contain active molecules, such as: free amino acids, polypeptides, vitamins, phytohormones and carbohydrates, which can stimulate plant metabolism, development and productivity, also an improvement in nitrogen use efficiency (Rouphael et al., 2017; Colla et al., 2015; Kunicki et al., 2010; Russo and Berlyn, 1992). In addition, biostimulants seem to limit the effect of biotic and abiotic stress on plant growth and ameliorate quantitative and qualitative traits of production (Ziosi et al., 2013): in fact, biostimulants can defense against these abiotic stresses depend on activation of plants antioxidant defense system or increasing phenolic compounds level (Ertani et al., 2013, 2011). Notwithstanding that the positive action of biostimulants is function of different conditions: species, cultivars, abiotic conditions, dosage, origin and time of application (Lisiecka et al., 2011).

Several studies reported an improvement of leaf color by stimulating the chlorophyll content (Abbas and Akladious, 2013); other researchers found an increase of chlorophyll levels and carotenoids in rocket (*E. sativa*) (Abdalla, 2013). Vernieri et al. (2002) showed that the biostimulant triggered lettuce root growth and caused as well a leaf area expansion. Other experiments illustrated that application of plant biostimulants to the roots or leaves can improve leaf color, photosynthetic efficiency, leaf area and number, shoot and root fresh weight, in addition to fruit number and fruit mean weight especially in abiotic stress circumstances (Lucini et al., 2018; Rouphael et al., 2017; Lucini et al., 2015,). Rouphael et al. (2017) showed a positive effect of biostimulants on antioxidant activities and biologically active compounds in tomato. Similarly, Amanda et al. (2009) also found that biostimulants can caused an increase of chlorophyll, total phenols and anthocyanins in baby leaf lettuce grown in protected environment.

In winter 2018, an experiment was carried out on baby leaf lettuce (Di Mola et al., 2019) in plastic tunnel (semi-protected environment), where we tested four levels of nitrogen (0, 10, 20 and 30 kg ha^{-1}) and three plant biostimulants (seaweed *Ecklonia maxima* extract, legume-derived hydrolysate protein and tropical plant extract). We found an increase in yield, LAI, antioxidant activity, total ascorbic acid, chlorophyll and SPAD index, in plants treated with seaweed extract and legume-derived hydrolysate protein. Instead, the supra-optimal nitrogen dose (30 kg ha^{-1}) showed a plateau of yield (N20 and N30 showed no difference) and an overcoming of legal EU threshold of nitrates in leaves.

Therefore, starting from our previous findings, we decided to carry out another experiment on baby leaf lettuce, testing the two best performing biostimulants (seaweed *Ecklonia maxima* extract and legume-derived hydrolysate protein) combined to three level of nitrogen (0, 10 and 20 kg ha^{-1}, no nitrogen, suboptimal and optimal nitrogen level respectively), in order to verify the effects on yield and some quality traits (especially nitrate content, but also antioxidants activity, ascorbic acid, color, etc.), but in fully protected conditions (plastic greenhouse).

Results and Discussion

Influence of nitrogen-fertilization dose and biostimulants on crop productivity and growth parameters

In Figs 1 and 2, the results relative to marketable yield and leaf area index (LAI) are reported, respectively, while in Table 1 leaf succulence and specific leaf weight (SLW) are reported. For the first two parameters the interaction between the two experimental factors (N fertilization and biostimulants application) was significant (Figs. 1 and 2), while only the main effect of the two factors was significant for succulence and SLW (Tab. 1).

Baby leaf lettuce fresh yield was positively affected by increasing N level, reaching the maximum at 20 kg of N per hectare (Fig. 1). The biostimulants application always improved the marketable yield without differences between seaweed extract (Bio1) and legume-derived PH (Bio2). The increase was 17.0%, 15.1% and 8.0% for N0, N10 and N20, respectively, compared to non-treated plants.

Leaf Area Index (LAI –Fig. 2) had the same trend like fresh yield, where it was increased positively with N fertilization increase. The biostimulants application averagely elicited 12% increase, compared to untreated plants, but no difference among them was found.

Irrespective of biostimulant application, the leaf succulence and SLW were increased from no fertilized to fertilized plants with no significant difference among the two N treatments (Table 1). Averaged over N fertilization levels, applying biostimulants induced significant differentiation only on leaf succulence. In particular, legume-derived PH (Bio2) showed the highest value, while seaweed extract (Bio1) was not different from untreated plants.

The stimulation effect of biostimulants application was observed in the current research is consistent with the findings of our previous research (Di Mola et., 2019) carried out equally on baby leaf lettuce but treated with tropical plant extract, as well as seaweed extract and legume-derived PH (+19% in no fertilized plants and + 10% in N10 and N20 plants). Moreover, these outcomes are congurent with the increase on fresh greenhouse tomato yield (Colla et al., 2017) (+11.7%, 6.6% and 7.0% using plant extract, seaweed extract and protein hydrolysate, respectively). However, it is so different from those recorded on greenhouse spinach (+51.5% in yield of plants treated by seaweed extract - *E. maxima* or *Ascophyllum nodosum*- and protein hydrolysate) by Rouphael et al. (2018). Therefore, the different crop response to biostimulants application warrants further crop-specific research to achieve maximum efficiency of plant biostimulants implementation also in respect to environmental conditions and/or management practice (Colla et al., 2016). The improvement in growth parameters and consequently in yield can be due to the presence of several bioactive molecules in two commercial tested biostimulants (amino acids, soluble peptides (in PH), polysaccharides, phenolic compounds, osmolytes and phyohormones (in SwE) (Colla et al., 2017; Battacharyya et al., 2015), that might have initiated a *signal transduction pathway* throughout elicitation of intrinsic phytohormone synthesis (Rouphael et al., 2018). However, it is possible that the stimulation effect of biostimulants on LAI and marketable fresh yield can be due to another mechanism of action. In fact, they stimulate the development of roots system (length, volume and biomass production) that results in an improvement of nutrient uptake and then translocation and assimilation to the above ground biomass (Szczepanek et al., 2019; Trevisan et al., 2019; Rouphael et al., 2018; Rouphael et al., 2017a).

Influence of nitrogen-fertilization dose and biostimulants on SPAD index and Leaf Colorimetry

Food quality influences consumer's choice, particularly some qualitative characteristics that are immediately detectable, such as color parameters. In the current research, the CIELAB color parameters (brightness-L*, redness-a*, yellowness-b*) and SPAD index showed no interaction between N fertilization level and biostimulants application (Table 2). They were positively affected by both main factors. The leaves of baby leaf lettuce at N20 were lighter (higher L* value) with high green intensity (lower a* value). Averaged on N fertilization doses, the application of biostimulants elicited the same effect (higher L* values and lower a* values) without statistical differences among them. Irrespective from biostimulants application, the SPAD index increased with N fertilization increase. Generally, SPAD values can be attributed to the improvement of nitrogen uptake efficiency. The SPAD index is also an important pointer of chlorophyll biosynthesis and photosynthetic system operation that improves crop growth (Colla et al., 2017a; Ertani et al., 2017). An increase of 7.8% compared to untreated plants was recorded for SPAD index of plants sprayed by seaweed extract and legume-derived PH, without significant differences among them. The increase over control value is consistent with several researches on some horticultural plants treated with different biostimulants (du Jardin, 2015; Ertani et al., 2014; Craigie, 2011). It can be a result to a greater movement of soluble sugars via phloem, an increase in the biogenesis of chloroplast and/or a reduction in chlorophyll degradation (Battacharyya et al., 2015; Ertani et al., 2014; Jannin et al., 2013).

Influence of nitrogen-fertilization dose and biostimulants on nitrate, chlorophyll and carotenoid contents

For green leafy vegetables (lettuce, spinach, rocket) nitrate content is the utmost quality traits. These cultivated plants greatly and easily accumulate nitrates in leaves and this process is worsened by specific environmental conditions, such as low solar radiation (Blom-Zandstra, 1989; Steingröver et al., 1986a, 1986b) and also by some crop management practices, such as nitrogen fertilization. Nitrate accumulation causes problems to human health, when it is converted into nitrite after intake by diet into the human body, where nitrites can cause methemoglobinemia or originate cancer-causing compounds (nitrosamine and nitrosamide) (Gangolli et al., 1994; Walker, 1990).

In our test, the application of biostimulants and N fertilization both influenced nitrate content, but no significant interaction was noted regarding their interaction (Table 3). N fertilization dramatically raised nitrate concentration in baby lettuce leaves. In fact at 20 kg per hectare of nitrogen fertilization, it overcame the legal threshold (5000 mg NO_3 kg^{-1} of lettuce grown in protected environment and harvested between 1° October and 31 March) set by European community with the Reg. n° 1258/2011 (Table 3). Averaged on N fertilization levels, the nitrate concentration of leaves sprayed by seaweed extract equally exceeded the EC upper limit for lettuce marketing (Table 3). Probably these results depend both on the plastic film cover of the greenhouse that considerably reduced the solar radiation transmission, a condition worsened by originally low winter sunlight and high availability of nitrogen (combined effect of fertilization, biostimulant application and soil fertility). However, the legume-derived PH seems to implement a molecular process, like genes positive retro-control that are incorporated in N metabolism (nitrate reductase) with a consequent increasing assimilation of nitrates into amino acids (Ali et al., 2019; Colla et al., 2018).

The total chlorophyll including a and b and carotenoids content showed no F × B interaction. However, only the main effects of biostimulants application significantly affected the chlorophyll a, b and total content (Table 3). For instance, fertilization levels elicited an increment in total chlorophyll and chlorophyll a and b with statistical difference also between the two N treatments, but with no effect on carotenoids content (Tab. 3). Furthermore, the biostimulants application had a positive impact on chlorophyll and carotenoids with an increase of 10.3%, 26.7%, 11.1% and 15.5%, compared to untreated plants for chlorophyll a, chlorophyll b, total chlorophyll and carotenoids respectively. Particular, application of seaweed extract on leaves showed the best performance (Table 3).

This positive effect of seaweed extract and legume-derived PH has been found in several agricultural crops and horticultural commodities such as corn, eggplant, jute (Ali et al., 2019; Carillo et al., 2019; Carillo et al., 2019a; Tadros et al., 2019). The increase in chlorophyll could be due to the higher content of primary amino acids (e.g., alanine, aspartate, asparagine and glutamate) in the plants sprayed with vegetal-based biostimulants, which helped to boosting chlorophyll content and consequently increase the net photosynthetic activity of baby leaf lettuce (Carillo et al., 2019a).

Table 1. Effects of fertilization doses (0, 10 and 20 kg ha^{-1}; N0, N10 and N20, respectively) and biostimulants applications (Control= non-treated control, Bio 1= Seaweed extract; Bio 2= Legume-derived protein hydrolysate) on succulence and specific leaf weight (SLW).

Treatments	Succulence *mg H_2O cm^{-2}*	SLW *mg d.m. cm^{-2}*
Fertilization		
N0	43.24 b	2.13 b
N10	44.16 a	2.20 a
N20	44.11 a	2.20 a
Biostimulants		
Control	43.60 b	2.16
Bio1	43.43 b	2.18
Bio2	44.48 a	2.20
Significance		
Fertilization	**	*
Biostimulants	*	NS
F x B	NS	NS

NS,*,** Non significant or significant at P ≤ 0.05 and 0.01. Different letters within each column indicate significant differences according to Duncan's test (P ≤ 0.05)

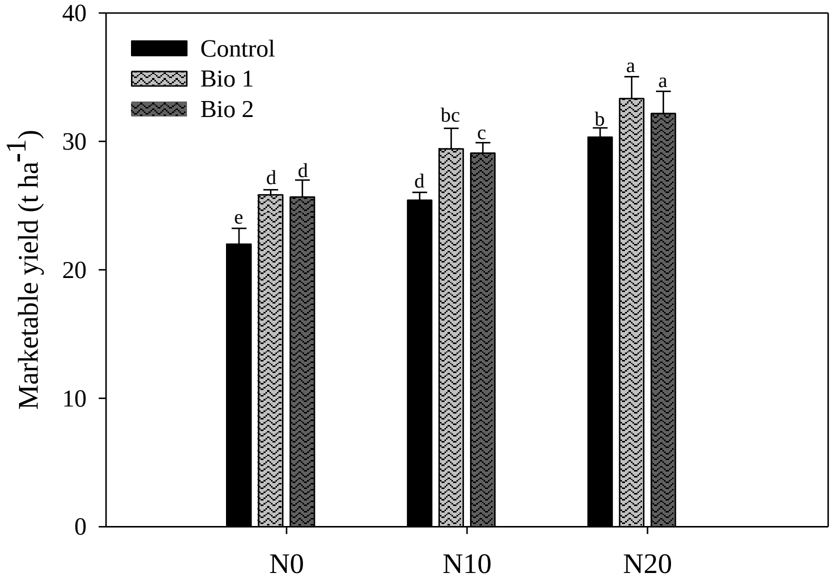

Fig 1. Effect of interaction between nitrogen doses (0, 10 and 20 kg ha^{-1}; N0, N10 and N20, respectively) and biostimulants (Control= non-treated control, Bio 1= Seaweed extract; Bio 2= Legume-derived protein hydrolysate) on marketable yield. Vertical bars indicate ± standard errors. Different letters indicate significant differences according to the Duncan's test (significance level 0.05).

Table 2. Effects of fertilization doses (0, 10 and 20 kg ha^{-1}; N0, N10 and N20, respectively) and biostimulants applications (Control= non-treated control, Bio 1= Seaweed extract; Bio 2= Legume-derived protein hydrolysate) on Soil Plant Analysis Development (SPAD) index and color parameters L* (brightness), a* (-a* = green; +a* = red) and b* (-b* = blue; +b* = yellow).

Treatments	Spad index	L*	a*	b*
Fertilization				
N0	14.7 c	53.60 c	-19.33 c	36.65 c
N10	16.2 b	55.16 b	-20.44 b	38.46 b
N20	17.2 a	57.66 a	-21.35 a	39.22 a
Biostimulants				
Control	15.4 b	53.86 b	-19.77 b	37.54 b
Bio1	16.4 a	56.45 a	-20.56 ab	38.37 a
Bio2	16.8 a	56.79 a	-21.12 a	38.86 a
Significance				
Fertilization	**	**	**	**
Biostimulants	*	*	*	*
F x B	NS	NS	NS	NS

NS,*,** Non significant or significant at P ≤ 0.05 and 0.01. Different letters within each column indicate significant differences according to Duncan's test (P ≤ 0.05)

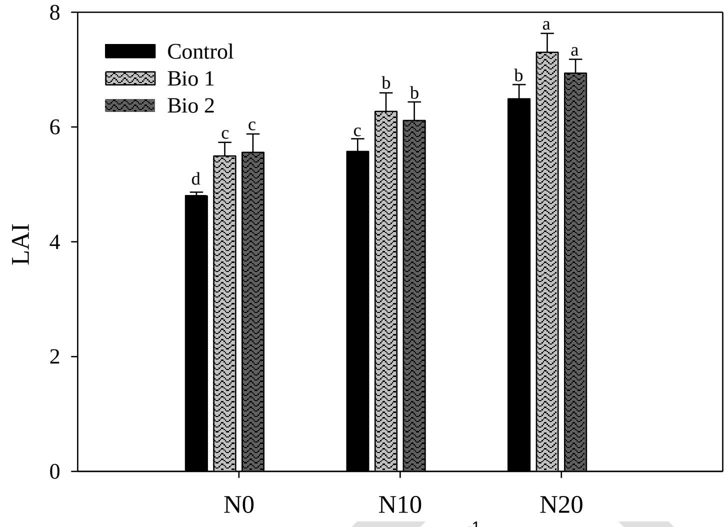

Fig 2. Effect of interaction between nitrogen doses (0, 10 and 20 kg ha^{-1}; N0, N10 and N20, respectively) and biostimulants (Control= non-treated control, Bio 1= Seaweed extract; Bio 2= Legume-derived protein hydrolysate) on leaf area index (LAI). Vertical bars indicate ± standard errors. Different letters indicate significant differences according to the Duncan's test (significance level 0.05).

Table 3. Effect of fertilization doses (0, 10 and 20 kg ha^{-1}; N0, N10 and N20, respectively) and biostimulants applications (Control= non-treated control, Bio 1= Seaweed extract; Bio 2= Legume-derived protein hydrolysate) on nitrate, chlorophyll A, chlorophyll B, total chlorophyll and total carotenoids.

Treatments	Nitrate *mg g^{-1}fw*	Chlorophyll A *mg g^{-1} fw*	Chlorophyll B *mg g^{-1} fw*	Total Chlorophyll *mg g^{-1} fw*	Carotenoids *µg g^{-1} fw*
Fertilization					
N0	2870.0 c	0.401 b	0.253 c	0.654 c	172
N10	4050.7 b	0.418 b	0.281 b	0.699 b	168
N20	8071.0 a	0.455 a	0.285 ab	0.740 a	173
Biostimulants					
Control	4370.1 c	0.398 b	0.232 c	0.630 c	155 b
Bio1	5665.9 a	0.468 a	0.308 a	0.776 a	200 a
Bio2	4955.8 b	0.410 b	0.280 b	0.690 b	158 b
Significance					
Fertilization	**	*	*	*	NS
Biostimulants	*	*	**	*	**
F x B	NS	NS	NS	NS	NS

NS,*,** Non significant or significant at $P \leq 0.05$ and 0.01. Different letters within each column indicate significant differences according to Duncan's test ($P \leq 0.05$)

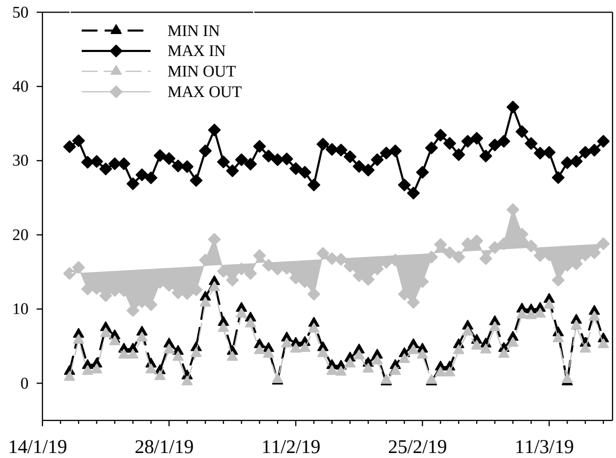

Fig 3. Trend of indoor and outdoor minimum and maximum temperatures.

Table 4. Effect of fertilization doses (0, 10 and 20 kg ha^{-1}; N0, N10 and N20, respectively) and biostimulants applications (Control= non-treated control, Bio 1= Seaweed extract; Bio 2= Legume-derived protein hydrolysate) on lipophilic antioxidant activity (LAA), hydrophilic antioxidant activity (HAA), total phenols and total ascorbic acid (TAA).

Treatments	LAA	HAA	Phenols	TAA
	mmol Trolox eq. 100g^{-1} dw	*mmol ascorbic acid eq.100g^{-1} dw*	*mg gallic acid eq. g^{-1} dw*	*mg g^{-1} fw*
Fertilization				
N0	25.28	6.28 a	2.48	15.45 a
N10	25.98	5.79 b	2.40	12.60 b
N20	25.34	5.94 b	2.52	12.27 b
Biostimulants				
Control	24.86 b	5.77 b	2.36	12.10 b
Bio1	25.84 a	5.87 b	2.67	12.21 b
Bio2	25.90 a	6.37 a	2.37	16.02 a
Significance				
Fertilization	NS	*	NS	**
Biostimulants	*	*	NS	**
F x B	NS	NS	NS	NS

NS,*,** Non significant or significant at P ≤ 0.05 and 0.01. Different letters within each column indicate significant differences according to Duncan's test (P ≤ 0.05)

Table 5. Chemical and physical characteristics of the greenhouse soil.

Soil Properties	Units	Mean Values
Texture		
Coarse sand	%	33.7
Fine sand	%	41.9
Silt	%	16.7
Clay	%	7.7
Chemical properties		
pH	-	6.9
Electrical conductivity	dS m^{-1}	0.60
Organic matter	g kg^{-1}	17.0
Total N (Kjeldahl method)	g kg^{-1}	1.1
P_2O_5 (Olsen method)	mg kg^{-1}	87.0
K_2O (Tetraphenylborate method)	mg kg^{-1}	1811.0

Influence of nitrogen-fertilization dose and biostimulants on functional leaf quality traits

Total ascorbic acid (TAA), hydrophilic (HAA) and lipophilic (LAA) antioxidant activity were influenced by biostimulants application, while N fertilization affected only HAA and TAA, but no F × B interaction was noted (Table 4). The HAA and TAA decreased, when N fertilization levels increased, starting from the sub-optimal nitrogen level (N10).

Our findings are consistent with Di Mola et al. (2019) and Wang et al. (2008) findings, where high N fertilization levels resulted in a decline in quality features of leafy vegetables and fruits, like soluble solids, total sugars and Vitamin C causing a nutritional and commercial decline.

Irrespective of N fertilization levels, the biostimulants application elicited an increase compared to untreated plants: +4.1%, +6.1% and +16.6% for LAA, HAA and TAA, respectively. Interestingly, legume-derived (Bio2) elicited a major increase in HAA and TAA, compared to seaweed extract (Bio1): 8.5% and 31.2%, respectively. The beneficial effect of legume-derived on LAA, HAA and TAA was reported also by Caruso et al. (2019) regarding perennial wall rocket. A possible explanation of this positive effect of biostimulants, particularly legume-derived PH, could be (a) the activity stimulation of key enzymes involved in antioxidant homeostasis in cells and (b) the higher macro and micronutrients assimilation of biostimulant-treated plants which could contribute to the synthesis of amino acids, phenylalanine and tyrosine (Colla et al., 2017a; Colla et al., 2015).

Materials and Methods

Plant material, experimental design and growth conditions

The experiment was carried out on baby leaf lettuce (*Lactuca sativa* L.) cv. "Zarina" (ISI Sementi), in a polyethylene greenhouse during 2019 winter growing season, at the experimental site "Gussone Park" of Department of Agricultural Science in Portici (Naples, Italy N40° 48.870'; E14° 20.821'; 70 m a.s.l.), Southern Italy.

The experiment was projected as a factorial comparison between three increasing levels of N fertilization (0, 10 and 20 kg ha^{-1}, control -N0, sub-optimal dose -N10 and optimal dose -N20, respectively) and two biostimulants of plant origin (seaweed extract –Bio 1 and protein hydrolysate –Bio 2) and a non-treated control. The treatments were distributed in a randomized complete-block design with three replications accounting for 27 experimental units (3 N levels x 3 Biostimulant applications x 3 replications), each one was 1.5 m long and 0.5 m large. The trial soil was a sandy-loam with high content of potassium and phosphorus and good content of total nitrogen and organic matter (Table 5).

Crop management, nitrogen-fertilization doses and biostimulants application

The sowing was hand made in mid-January with 3 grams of seeds per square meter equivalent to 2500 seeds m^{-2}. Nitrogen was added in form of ammonium nitrate (26%) in a single operation in early February (about two weeks after the sowing). The two commercial biostimulants, seaweed extracts *Ecklonia maxima* and protein hydrolysate were Kelpak® and Trainer®, respectively. The first one is produced by Kelpak Products (Pty Ltd., Cape Town, South Africa), which is an extract of brown seaweed *Ecklonia maxima* (Osbeck) containing auxins and cytokinins in 367:1 ratio, but also several vitamins, amino-acids, carbohydrates and nitrogen, phosphorus and potassium in quantity of 3.6, 8.2 and 7.2 g kg^{-1}, respectively (Rouphael et al., 2018, 2017). Trainer® is a protein hydrolysate, produced by Italpollina S.p.A. (Rivoli Veronese, Italy) on the basis of legume-plants. It contains amino acids, peptides, plus nitrogen, phosphorus and potassium equal to 50.0, 0.9 and 4.1 g kg^{-1}, respectively, together with numerous micro-elements. Rouphael et al. (2017a) and Paul et al. (2019, 2019a) reported the detailed aminogram of this biostimulant.
A solution containing 3 ml per liter of biostimulants was sprayed on baby leaf plants, leading to a total of four applications divided in a weekly basis spray starting 25 days after the sowing.
Water losses were calculated by Hargreaves formula and fully restored by irrigation. During the cycle, the air temperature was continuously monitored with probes (Vantage Pro2, Davis Instruments).

Temperatures pattern inside the greenhouse

During the experiment, minimum temperatures in the greenhouse ranged between 13.7°C and 0.2°C (Fig. 3). The differences between indoor and outdoor minimum temperatures were about -0.8°C on average. Instead, the differences between indoor and outdoor maximum temperatures were more marked about 15.2°C on average. In fact, under greenhouse conditions the maximum temperatures ranged between 25.6°C and 37.2°C (Fig. 3).

Sampling, growth analysis, marketable yield and leaf colorimetry

On the first decade of March, the baby leaf lettuce of all experimental plots was hand harvested and the marketable yield was expressed in tons per hectare. A 100 grams representative vegetable sample was used for measuring leaf area by an electronic leaf area meter (Li-Cor 3000, Li-Cor, Lincoln, NE) and subsequently calculating leaf area index (LAI). Then the sample was dried in an oven at 70°C until reaching constant weight for determining leaf dry matter percentage and consequently specific leaf weight (SLW, mg of leaf dry weight per cm^2) and succulence (mg of leaf water content per cm^2).
Moreover, dried leaf samples were used for measuring nitrate content by Foss FIAstar 5000 continuous flow Analyzer. Nitrate was extracted by NH_4Cl-buffer; then the solution was filtered and introduced into a flow injection system. The chemical principle for determination of nitrate is the reduction to nitrite on a cadmium reductor. The described method gives the sum of nitrate and nitrite available in the extract. However, the concentration of nitrite is in most cases negligible in comparison with the nitrate concentration.
Just before harvest, measurements of Soil Plant Analysis Development (SPAD) index were made by a portable chlorophyll meter SPAD-502 (Konica Minolta, Tokyo, Japan). In the laboratory, on ten undamaged leaves per each experimental unit, the color space parameters were measured by a Minolta CR-300 Chroma Meter (Minolta Camera Co. Ltd., Japan); according to Commission international de l'eclairage (CIELAB) the measured parameters were: L***,** a* and b***.**
Finally, a 50 g representative fresh leaves sample per replicate was frozen in liquid nitrogen immediately after harvest, and stored at -80 ºC until being freeze-dried in Christ, Alpha 1-4 (Osterode, Germany) for further analysis.

Chlorophyllous pigments and bioactive molecules analysis

Carotenoids, Chlorophyll a and chlorophyll b, were evaluated on 1 g of fresh sample after extraction in 99% acetone, by a Hach DR 2000 spectrophotometer (Hach Co., Loveland, Colorado, USA) at 470, 662 and 647 nm respectively, as described in details by Lichtenhaler and Wellburn (1983).
Kampfenkel et al. (1995) method was used for measuring total ascorbic acid content (TAA), expressed as mg ascorbic acid (AsA) per 100 g fresh weight. The quantitation of AsA, closely related to reduction of Fe^{3+} to Fe^{2+}, which was measured at 525 nm versus an ascorbate calibration curve.
The total phenol content was determined by Folin-Ciocalteau procedure (Singleton et al., 1999). After extraction with 10 mL of methanol/water solution and combination with Folin-Ciocalteau's reagent (Sigma Aldrich Inc., St. Louis, MO, USA) and sodium carbonate/water solution, the mixture was shaken and absorption was measured after 30 min at 765 nm by a UV-Vis spectrophotometer using an external acid gallic calibration curve (Sigma Aldrich Inc, St Louis, MO, USA).

Antioxidant activity analysis

200 mg extract of freeze-dried leaves sample was used to assess lipophilic (LAA) and the hydrophilic (HAA) antioxidant activity by ABTS (2,20-azinobis 3-ethylbenzothiazoline-6-sulfonic acid) method (Pellegrini, et al., 1999) and N, N-dimethyl-p-phenylenediamine (DMPD) method (Fogliano, et al., 1999), respectively.
The absorbance of solution was measured by UV – Vis spectrophotometry at 505 nm versus an ascorbate calibration curve, and at 734 nm, using Trolox (6-hydroxy-2,5,7,8-tetramethylchro man-2- carboxylic acid) external standard calibration curve, for HAA and LAA, respectively.

Statistical analysis of the experimental dataset

SPSS software package (SPSS version 22, Chicago, Illinois) was used to analyze all data, using a general linear model (2 way ANOVA). Means were separated according to the Duncan Multiple Range Test (DMRT; significance level 0.05).

Conclusions

Biostimulants foliar application, both seaweed extract and protein hydrolysate, improved marketable yield and leaf area index of baby leafy lettuce at all level of N but more under no -fertilized conditions. Moreover, the application of seaweed extract of *E. maxima* and legume derived PH enhanced SPAD index and color parameters, but also chlorophyll and carotenoids content, especially seaweed extract. However, this latter also showed an increase in nitrate content in leaves, which overcame the legal limit of European Community. Interestingly, legume-derived application allowed low nitrate content in leaves, in addition to high LAA, HAA and high content of TAA.

The results of our research highlighted the beneficial effect of biostimulants foliar application, especially legume-derived PH, on increasing yield and quality of baby leaf lettuce, also under sub-optimal nitrogen conditions, probably due to the improvement of nitrogen use efficiency. Therefore, in our growth conditions (greenhouse, winter season and good soil fertility), it seems possible to reduce additional nitrogen input above 10 kg of N per hectare, by increasing the number of biostimulants applications in order to reduce the yield gap with plants fertilized with 20 kg of N per hectare but more interestingly, to increase nutritional and functional quality (high antioxidant activity and low nitrate content). This practice will also allow us to reduce the impact on environmental and human health, by finding large spread among farmers.

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