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Untargeted metabolites and chemometric approach to elucidate the response of growth and yield attributes on different concentrations of an amino acid based biostimulant in two lettuce cultivars

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ABSTRACT

The study aims to underpin biochemical mechanisms induced by the application of different concentrations of an amino-acid based Phytostim® biostimulant on growth and yield attributes. Different concentrations of Phytostim® biostimulant: 0 (untreated), 1, 3, and 6% were foliar applied on lettuce cultivars ('Lara' and 'Elisa'). After 45 days of transplanting, analysis growth and yield attributes, biochemical analysis and LC-MS untargeted metabolites profile were performed. Application of 3% dose improved both growth and yield parameters in both cultivars in comparison to the 6% dose which inhibited growth. Similar trend was observed for biochemical and antioxidant analysis (phenolic, flavonoids, carotenoids, chloroplyll *a* and *b* and scavenging activity). The PCA and OPLS-DA score-plot clustered the metabolome profile of biostimulant treated vs untreated samples with major heterogeneity distinction observed in the untreated samples. Obtained results validate the use of biostimulants in agriculture while giving information on the effects of the treatments towards changes in the chemical composition within the studied cultivars.

1. Introduction

Application of metabolomics and chemometric analysis has gained interest in research of agriculture. This is based on the principles of the untargeted metabolites profiling which generate holistic profile without being bias to certain metabolites class (Dixon et al., 2006). In addition the targeted metabolites profiling which is likely to include the reference standard offers opportunity to quantify the analyst to observe the impact of the treatment (Dixon et al., 2006). Chemometric analysis including the Principle Component Analysis (PCA) and Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) are the tools used in concomitant to targeted or untargeted metabolites profiling to generate clusters based on the arrangement of the metabolites. The use of metabolites and chemometric approach has been used in different crops in agriculture including tomatoes, amaranth species, lettuce, asparagus and soybean (Garcia et al., 2016; Jiménez-Sánchez et al., 2016; Salem et al., 2020; Teixeira et al., 2017).

In this study, the application of metabolomics and chemometric has been employed to observe the effect of different concentrations of Phytostim® biostimulant and elucidate growth and yield components. Lettuce *(Lactuca sativa* L.) is a common leafy vegetable in South Africa which belongs to Asteraceae family. It is likely to be added in the diets of people who wish to reduce their body weight, due to its predominant contents of the folic acid, potassium and fiber that keeps the good health of digestive system (Kim et al., 2016).

It is loved by consumers for its crispy texture when included as part of leafy green salads. Growth attributes including root length, stem thickness, leaf length and plants height are associated with water and nutrient absorption and contributes to the yield attributes (Caruso et al., 2019). Yield is critical during crop production and it is likely to be affected by the application of biostimulants (Barneix and Causin, 1996; Bulgari et al., 2019; Khan et al., 2019; Ottaiano et al., 2021; Paul et al.,

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2019; Shahrajabian et al., 2021).

Applications of plant origin biostimulants have gained momentum in vegetable production in particular to the amino acid and protein hydrolysed types. They are described as biologically active compounds extracted from plants or animals (Barneix and Causin, 1996; Bulgari et al., 2019; Khan et al., 2019; Ottaiano et al., 2021; Paul et al., 2019; Shahrajabian et al., 2021. An amino acid based biostimulant can be applied either through foliar or drench to be absorbed by the leaf or root systems respectively. They can also be applied as seed priming agent for improved germination (Sorrentino et al., 2021). In fact, these amino acids based biostimulants has been associated with enhanced crop yield, plant quality and can act as elicitors for mitigation of adaptation mechanisms during abiotic stress such as salinity or water stress (Abdelgawad et al., 2018; Cozzolino et al., 2020; Hidalgo-Santiago et al., 2021; Ottaiano et al., 2021; Paul et al., 2019).

Protein hydrolytes (equipped with free amino acids) improved tolerance to the salinity conditions in lettuce by altering the root development, synthesis of chlorophyll and the accumulation of proline metabolite (Rouphael et al., 2017). Furthermore, the application of protein hydrolytes in tomato production had showed to improve the total phenols, ascorbic acid, lycopene and cytokinins or salicylic acids hormones which acts as radical scavengers (Paul et al., 2019). Evidence on the application of amino acid based biostimulants in lettuce have been reported with regards to a single amino acid compound. Indeed, the application of L-methionine (0.2 mg/L) resulted on increased plant and root growth in comparison to the L-tryptophan and L-glycine which resulted in reduced root growth (Khan et al., 2019). However, Khan et al. (2019) reported that the efficiency of the biostimulant is based on the application concentration. Besides the facts about the potential of these amino acids based biostimulant, in particular to its eco-friendly nurture and the ability to improve crop's yield, quality attributes and induce bioactive compounds, information about the effect of different concentrations of the Phytostim® amino acid based biostimulant on growth, yield and biochemical mechanisms induced by the biostimulant is still lacking for lettuce production. This information is necessary to improve sustainable lettuce productivity. Therefore the objective of this study was to evaluate the effect of a commercially available Phytostim®, an amino acid based biostimulant on the growth, yield and to underpin the biochemical mechanisms induced by this biostimulant application.

2. Materials and methods

2.1. Properties of a Phytstim biostimulant

Phytostim® biostimulant is a commercially available product and it was purchased from an agro-chemicals supplier (NTK, Polokwane, South Africa). It is a plant derived biostimulant extracted through enzymatic hydrolysis of proteins in *Moringa oleifera* Lam. crude extract. The enzymatic hydrolysis procedure was also performed to separate the insoluble residues of amino acids compounds following similar procedures to those described by Paul et al. (2019). The final product of Phytostim® biostimulant contained 22 aminogram made-up of valine (323.8 mg/L), isoleucine (246.6 mg/L), leucine (437.4 mg/L), phenylalanine (259.2 mg/L),glutamic acid (507.6 mg/L), aspartic acid (315.0 mg/L), glycine (244.6 mg/L), serine (269.2 mg/L), threonine (249.9 mg/L), alanine (365.2 mg/L) and proline (222.1 mg/L) which constitutes 70% of the active ingredients.

2.2. Study location, research design, and crop establishment procedures

The experiment was conducted during winter season (July-August 2021) under the greenhouse environment at the Green Biotechnologies Research Center of Excellence at the University of Limpopo, South Africa at a 23°53′10″S longitude, 29°44′15″E latitude and 1200 altitude above the sea level.

Day/night temperatures of the greenhouse ranged between 28 and 21 °C, with maximum temperatures controlled using thermostatic activated fans and wet walls. Relative humidity was between 40 and 45% during the study period. Seeds of lettuce cultivars 'Lara' and 'Elisa' were obtained from the Agricultural Research Council, Vegetable, Industrial and Medicinal Plants (ARC-VIMP) seedbank. The seed were sown and germinated in a 200 cavity polystyrene trays filled with a commercial growth medium Hygromix (Hygrotech seed company, South Africa). After four weeks of seed emergence, seedlings were transplanted into a 20 cm plastic pot filled with a mixture of Hygromix growth medium and pasteurized (300 °C) loam and sandy soil at a ratio of 1:1:1. Treatments were four different concentrations of Phytostim® biostimulant including: 1% (T2), 3% (T3), 6% (T4) and untreated (T1). These treatments were laid in a complete randomized block design with 18 replicates per treatment. The blocking in this design was to reduce variation that may be caused by light and fans air. Different concentrations of Phytostim® biostimulant were applied on the leaf (foliar) one time in 14 days. The application rate was linear to the number of leaves such that 250 mL of a single Phytostim® biostimulant concentration covered 18 plants. As the number of leaves increased from 4, 6, 8 to 10, the 250 mL volume covered 18, 14, 10 and 6 plants, respectively. Therefore, this implies those plants at 4, 6, 8 and 10 leaves stage, received 13.89 mL, 17.85 mL, 31.25 mL and 41.67 mL per Phytostim® biostimulant concentration as shown in the Fig. 1. Irrigation with 250 mL of non-chlorinated tap water was performed whenever there was a status of 'dryness' from the moisture probes (T10 Bodentester, South Africa). Phytostim® biostimulant was used as a bio-fertiliser and therefore, no other fertilisers were included during the growth of lettuce in this study.

2.3. Growth and yield attributes in response to Phytostim® biostimilant application in lettuce cultivars

Growth and yield components data collection was performed after six weeks (45 days) of transplanting at the termination day of the trial. Plant height was measured from the base of the stem elongation up to the tip of the plant; the leaf (from petiole to the lengthened tip) and root length (undistracted taproot) were measured using a metered measuring tape. Number of leaves per plant was counted and the stem thickness (10 mm above the soil surface) was measured by placing a digital caliper in a horizontal stature tight around the stem. For yield components, lettuce plants were divided into two separate parts: aerial and roots. Aerial part consisted of the stem and leaves whilst the remaining parts of the plant were considered the root part. After uprooting lettuce from the plastic pot, root were washed with tap water and dried with a paper towel. Then both fresh aerial and root parts were measured for the weight using a balance (Model:AS/60/C/2, Poland). Dried weight was obtained by recording the weight of oven-drying the samples at 40 °C for seven days when there was no further change in weight.

2.4. Sample preparation and chemicals for the untargeted and targeted metabolites analysis in lettuce cultivars exposed to different concentration of Phytostim® biostimulant

A pull of replicates were performed to reduce the number of replicate to six for the analysis of metabolites. Samples were oven dried at 40 °C prior metabolites extraction. For polar metabolites (untargeted metabolites profiling, total phenols, total flavonoids, and scavenging activity), a portion of 2000 mg were homogenized with 2 mL acidified methanol: HCL: distilled water (80:0.5:19.5 v/v/v) in a thermostatic shaking water bath at 70 °C for 15 min following the method described by Mpai and Sivakumar (2018) with slight modifications. Prior to the biochemical analysis, the extract were centrifuged for 15 min dried under the N_2 gas and re-suspended and filtered as described by Mpai and Sivakumar (2020).

2.4.1. Untargeted metabolites analysis

The untargeted metabolites profiling of lettuce cultivars were carried

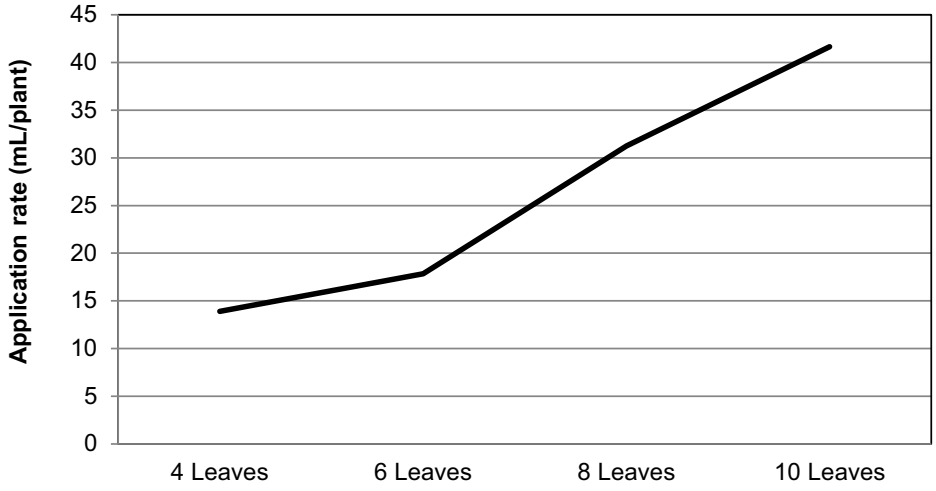

Fig. 1. Phytostim® biostimulant application rate in lettuce cultivar 'Lara' and 'Elisa'.

out using Sciex Exion LC-MS system connected to Sciex X500R QTOF system equipped with electron spray ionization (ESI) probe. The separation was achieved using a Phenomenex Luna C_{18} column (100 × 2 mm, 2.5 μm particle size). Mobile phases A and B consisted of water and Methanol with 20 mM ammonium acetate respectively.

Gradient elution was executed as follows: 0 min, 95% A and 5% B: 1 min, 95% A and 5% B: 22 min, 5% A and 95% B: 27 min, 5% A and 95% B: 27.10 min 95% A and 5% B: 30 min, 95% A and 5% B. The flow rate used was 0.4 mL/min at 40 °C and injection volume was 10 μL. The mass spectrometry was operating in a negative ion electrospray mode and nitrogen (N_2) was used as the desolvation gas. Ion source gas 1 and 2 at 50 psi and 70 psi respectively, curtain gas at 30 psi, CAD gas at 7, Ion source temperature 500 °C, spray voltage −4500, declustering potential at −80 V. Analyst software was used for data acquisition and processing. Due to unavailability of chemical standards to authenticate the identification of compounds, data on MS assigned compounds from the LC-MS–QToF were compared with accurate mass in relation to lettuce and other leafy vegetables reported in literature and in existing online public database including Knapsack (http://www.knapsackfamily.com/knapsack_core/top.php).

2.4.2. Biochemical and antioxidant analysis

Total phenol content was determined following the Folin-Ciocalteu method as the described by Mpai et al. (2018).The results were calculated and reported as mg/100 g using Gallic acid standard curve.

Total flavonoids were carried out following an aluminum chloride method (Makkar et al., 2007). The results were calculated using a catechin standard curve.

Scavenging activity was carried out following the 2, 2-diphenylpicrylhydrazyl (DPPH) method as described by Mpai et al. (2018) and the results were expressed as the concentration of antioxidants required to decrease the initial DPPH absorbance by 50% (IC_{50}).

Non-polar metabolites (total carotenoid, and total chlorophyll *a* and *b)* were extracted following the methods described by Nagata and Yamashita (1992) with slight modification, whereby 2000 mg of powdered sample was homogenized with 2 mL acetone-hexane mixture (40:60 v/v). Equations for calculations of β-carotene, total chlorophyll *a* and *b* was similar with those reported by Mpai et al. (2018), and Managa et al. (2020).

2.4.3. Statistical analysis and LC-MS untargeted metabolites profiling data management

The study adopted a single factorial design consisting different concentrations of Phytostim® biostimulant on individual studied cultivars. Data for growth, yield, biochemical and antioxidant analysis were subjected to one way analysis of variance (ANOVA) with *post-hoc* least significant difference (LSD) test (p-value< 0.05) performed using Genstat® version 20.0 (VSN International Ltd., Hemel Hempstead, UK) statistical package. Chemometric data analysis were performed using SIMCA ver 13.0 (Umetrics, Malmo, Swedan) software to create an unsupervised PCA and supervised OPLS-DA models. The 'explorative' PCA model was carried out following the 'Euclidean' and 'Wards' linkage rule (Paul et al., 2019). To observe clear clustering between different concentrations of Phytostim® biostimulant within the two studied cultivars, supervision was set to demonstrate similarities of metabolites in T1, vs T2, vs T3 and vs T4 at 95–99% confidence limits. Statistical model prediction (R^2Y and Q^2Y) were produced and permutation test were performed to validate the obtained models.

3. Results

3.1. Effect for the application of Phytostim® biostimulant on growth and yield components

The application of different concentrations of Phytostim® biostimulant significantly ($p<0.05$) affected growth and yield components including root length, stem thickness, plant height, leaf length, number of leaves per plant, and aerial mass, in cv 'Lara' (Table 1). Whilst fresh root mass and dried root mass were unaffected by application of Phytostim® bio stimulant different concentrations in cv 'Lara'. In fact, application of 3% Phytostim® biostimulant enhanced growth in all studied growth attributes in concomitant to improved yield component and outperformed other studied treatments. Whilst, 6% application of the Phytostim® biostimulant inhibited plant growth by reducing the root length, leaf length, aerial mass, and dried aerial mass more than other treatments. However, application of 1% dose resulted in similar traits with those of the control (untreated) with regards to plant height, number of leaves, aerial mass, and demonstrated the smallest stem thickness and leaf length comparable to the control (Table 1).

In cv 'Elisa', the application of 3% Phytostim® biostimulant improved: root length, plant height, stem thickness, aerial mass, and dried aerial mass (Table 1). Furthermore, application of 6% Phytostim® biostimulant improved the leaf length similarly to plants that were exposed to 1% concentration. Whilst, the application of 1% dose improved the root length and root dried mass most than the other treatments (Table 1). However, the application of 1% and 6% doses showed similar impact on the plant height and further similarity were observed with the control (untreated) on dried aerial mass (Table 1).

Table 1
Response for the application of Phytostim® biostimulant on growth and yield component in lettuce cultivar 'Lara' and 'Elisa'.

Treatments	Root length (cm)	Stem thickness (mm)	Plant height (cm)	leaf length (cm)	Number of leaves/plant	Root mass (g)	Aerial mass (g)	Dried aerial mass (g)	Dried root mass (g)
Cv 'Lara'									
1% Biostimulant (T2)	11.8 ± 0.11a	11.7 ± 0.02c	26.6 ± 0.14b	18.3 ± 0.01c	8.0 ± 0.13b	13.0 ± 0.01a	107.4 ± 0.01b	6.3 ± 0.04b	0.5 ± 0.21a
3% Biostimulant (T3)	10.3 ± 0.01a	17.2 ± 0.01a	30.8 ± 0.04a	24.2 ± 0.02a	10.0 ± 0.03a	13.5 ± 0.01a	140.6 ± 0.01a	8.7 ± 0.01a	0.6 ± 0.13a
6% Biostimulant (T4)	8.0 ± 0.01b	15.3 ± 0.01b	24.4 ± 0.04c	19.3 ± 0.01c	8.0 ± 0.02b	12.0 ± 0.01a	71.1 ± 0.01c	4.7 ± 0.01c	0.5 ± 0.01a
Untreated (T1)	9.1 ± 0.00b	14.9 ± 0.01b	25.1 ± 0.01b	21.5 ± 0.01b	8.0 ± 0.01b	13.6 ± 0.03a	100.3 ± 0.01b	9.1 ± 0.03a	0.5 ± 0.13a
F-statistics	1.63	1.47	0.94	2.45	0.36	0.87	1.87	2.03	1.69
Cv Élisa'									
1% Biostimulant (T2)	11.8 ± 0.01a	13.4 ± 0.11c	31.2 ± 0.01a	23.7 ± 0.01b	10.0 ± 0.21a	16.2 ± 0.03a	129.1 ± 0.01c	9.4 ± 0.01b	1.1 ± 0.01a
3% Biostimulant (T3)	12.8 ± 0.01a	16.8 ± 0.01a	32.5 ± 0.01a	23.7 ± 0.01b	11.0 ± 0.02a	16.7 ± 0.01a	183.6 ± 0.11a	13.0 ± 0.01a	0,9 ± 0.31b
6% Biostimulant (T4)	10.3 ± 0.01b	15.3 ± 0.11b	26.5 ± 0.01b	26.3 ± 0.01a	10.0 ± 0.01a	15.3 ± 0.01a	125.2 ± 0.10b	8.9 ± 0.01b	0.8 ± 0.13b
Untreated (T1)	10.2 ± 0.01b	13.6 ± 0.01c	27.0 ± 0.01b	24.3 ± 0.04b	10.0 ± 0.13a	15.2 ± 0.03a	123.1 ± 0.01c	9.7 ± 0.12b	1.1 ± 0.01a
F-statistics	1.14	1.89	2.03	0.78	1.64	2.06	1.87	1.96	1.45

Means values and SE were calculated based on 30 samples per treatment. Those followed by a different alphabet letter were significantly different ($p < 0.05$) according to Fisher's protected least significant test.

3.2. Identification of metabolites potentially influenced through the application of Phytostim biostimulant

To identify metabolites induced through the application of different concentrations of Phytostim® biostimulant in lettuce cultivars, changes of the metabolome profile for samples treated with different concentrations of biostimulant were compared to those of the control (Table 2). The selection of 'metabolites' created a metabolome profile with 450 compounds detected from the studied samples. As a result, different metabolites classes such as organic acids, phenolic acids, flavonoids, amino acids, hormones and nitrogen containing compounds and their derivatives were identified (Table 2).

4. Multivariate analysis of HPLC-MS-QTof for untargeted metabolites profile in lettuce cultivars exposed to different concentrations of the Phytosistim® biostimulant

To summarize information generated from the LC- MS untargeted metabolites profiling, chemometric analysis were performed to explore any variations or similarities in the holistic metabolome profile in the studied lettuce cultivars exposed to different concentrations of the Phytostim® biostimulant. The unsupervised PCA (Fig. 2A) grouped the metabolites profiles in two main clusters separated based on the cultivars and there was no clear clustering in relation to different concentrations of Phytostim® biostimulant application (Fig. 2A).

Clearly the metabolome profile in PC 1 (cv Lara) was distinct from those of PC 2 (cv Elisa). A supervised OPLS-DA model was generated to explore the impact of Phytostim® biostimulant on metabolome profile (Fig. 2B). As a result, two main clustering were observed and it demonstrated clear pattern attributed to different concentrations of the Phytostim® doses irrespective of the cultivar (Fig. 2B).

The results suggest that the holistic metabolite profile of the untreated sample (T1) with possess major heterogeneity in comparison to the other Phytostim® treated samples (T2, T3 and T4) (Fig. 2B). Although untreated samples (T1) and those treated with 6% dose (T4) were both arranged on the horizontal PC 1, they showed great metabolic variation upto 86% (R^2X). Whilst the metabolites profile diversity in T1 (untreated) laid between the T3 (3% dose) and T4 (6% dose) along the vertical PC 1 with model statistic of 56% (R^2X value) (Fig. 2B). Furthermore, the OPLS-DA model was generated to demonstrate biomaker of metabolites responsible for variation among different concentrations of the Phytostim® biostimulant (Fig. 2C). As a result, the application of 1% dose (T2) was associated with the accumulation of metabolites with *mz:* 283.26 tentatively identified as *ent-7beta-Hydroxykaurenoic acid*, while the samples treated with 6% dose (T4) were associated with a reduction of metabolites with *mz:* 408.26 tentatively identified as *2-ethylsulfinylethyl glucosinolate* (Fig. 2C). Accumulation of metabolites with *mz*: 449.29 tentatively identified as *typhasterol* can be associated with the application of 3% doses (Fig. 2C).

4.1. Effect for the application of Phytostim® biostimulant on biochemical and antioxidant analysis

The application of different concentrations of Phytostim® biostimulant had significantly ($p<0.05$) affected the contents biochemical and antioxidant activity in both studied lettuce cultivars (Table 3). Clearly, application of Phytostim® biostimulant at 3% dose enhanced the highest accumulation of total phenols (222.54 mg GA/100 g), total flavonoids (425.79 mg Catechin/100 g), chlorophyll *b (*21.32 mg/kg) and total chlorophyll (22.47 mg/kg) contents in comparison to other studied treatments in cv 'Lara'. Whilst, contents of carotenoids (20.55 mg/kg) and chlorophyll *a* (1.30 mg/kg) were the highest in lettuce cv 'Lara' exposed to 1% dose of Phytostim® biostimulant (Table 3). Total phenols content in cv 'Lara' exposed to 1% (201.78 mg GA/100 g) was similar to samples exposed to 6% (205.78 mg GA/100 g) and higher than the content of the control (198.32 mg GA/100 g) (Table 3). On the other hand, samples treated with 3% of Phytostim® biostimulant exhibited the highest contents of all non-nutritive secondary metabolites studied including: total phenols (170.32 mg GA/100 g), total flavonoids (353.72 mg Catechin/100 g), total carotenoids (9.06 mg/kg), chlorophyll a (0.98 mg/kg), chlorophyll *b* (18.37 mg/kg) and total chlorophyll (19.51 mg/kg) (Table 3). Furthermore, a clear trend-line observed in the accumulation of these metabolites increased from untreated upto 3% dose of Phytostim® bistimulant, and a further increase in the concentration upto 6% declined the contents of these metabolites (Table 3).

The scavenging activity was significantly affected by the application of different concentrations of Phytostim biostimulant in both studied lettuce cultivars ('Lara' and 'Elisa'). In cv Lara, application of 3% dose improved the scavenging activity with an IC_{50} of 65.32, this value was comparable to IC_{50} (74.34) recorded for 1% dose in cv 'Lara'. Samples treated with 6% dose showed higher scavenging activity (IC_{50}: 95.23) as compared to the control (control) (IC_{50}: 140.69) (Fig. 3). Similar trend

Table 2
LC-MS tentative identification of detected metabolites in lettuce cultivars exposed to different concentrations of Phytostim® biostimulant.

Retention time (Min)	Exact Mass (g/mol)	Mass generated ESI (-) TOF MS (g/mol)	Chemical Formula	Tentative structural assignment	cv 'Lara'				cv 'Elisa'			
					T1	T2	T3	T4	T1	T2	T3	T4
Organic acids												
1.2	192.06338812	192.05	$C_7H_{12}O_6$	Quinic acid	ND	X	X	X	ND	X	X	X
8.58	219.11067266	219.13	$C_9H_{17}NO_5$	Pantothenic acid	X	X	X	X	X	X	X	X
16.72	264.13615913	264.22	$C_{15}H_{20}O_4$	(+)-Abscisic acid	X	X	X	X	X	ND	X	X
Phenolic acids												
1.98	180.04225874	180.06	$C_9H_8O_4$	Caffeic acid	X	X	X	X	X	X	X	X
1.15	212.06847349	212.08	$C_9H_8O_6$	3,4,5-Trimethoxybenzoic acid	X	X	X	X	X	X	X	X
1.20	126.03169406	127.03	$C_6H_6O_3$	Pyrogallol	X	X	X	X	X	X	X	X
1.14	170.0215233	170.12	$C_6H_2OH_3$	Gallic acid	X	X	X	X	X	X	X	X
2.74	138.03169406	138.01	$C_7H_6O_3$	3-Hydroxybezoic acid	X	X	X	X	X	X	X	X
3.45	164.04734412	164.07	$C_9H_8O_3$	trans-2-Hydroxycinnamic acid	X	X	X	X	X	ND	X	X
4.41	198.05282343	199.04	$C_9H_{10}O_5$	Syringic acid	X	X	X	X	X	X	X	X
5.18	164.04734412	164.04	C_9HO_3	p-coumaric acid	X	X	X	X	X	X	ND	X
5.37	194.05790881	194.05	$C_{10}H_{10}O_4$	Ferulic acid/ Isoferulic acid	X	X	X	X	X	X	X	X
5.63	290.07903818	290.07	$C_{15}H_{14}O_6$	Epicatechin	X	X	X	X	X	X	X	X
6.07	168.04225874	167.09	$C_8H_8O_3$	Vanillic acid	X	X	ND	X	X	X	X	X
7.39	338.10016755	337.09	$C_{16}H_{17}O_8$	Coumaroyl-quinic acid	X	X	X	X	X	X	X	X
15.38	224.06847349	224.06	$C_{11}H_{12}O_5$	Sinapic acid	X	X	X	X	X	X	X	X
18.15	154.02660868	155.14	$C_7H_6O_4$	Protocatechuic acid	X	X	X	X	X	X	X	X
18.24	312.04813198	312.23	$C_{13}H_{12}O_9$	Caftaric acid	X	X	X	X	X	X	X	X
18.34	354.09508217	354.47	$C_{16}H1_8O_9$	Chlorogenic acid	X	ND	ND	ND	X	ND	ND	ND
Flavonoids												
13.49	595.16629532	594.27	$C_{27}H_{30}O_{15}$	Cyanidin 3-rutinoside	X	X	X	X	X	X	X	X
14.16	474.07982604	474.26	$C_{22}H_{18}O_{12}$	Chicoric acid	ND	X	X	X	ND	X	X	X
16.16	270.05282343	271.23	$C_{15}H_{10}O_5$	Apigenin	ND	X	X	X	ND	X	X	X
17.63	432.10564686	432.31	$C_{21}H_{24}O_9$	Apigenin 7-O-glucoside	X	X	X	X	X	X	X	X
17.48	286.04773805	286.23	$C_{15}H_{10}O_6$	Luteolin	X	X	X	X	X	X	X	X
18.23	712.14869347	712.53	$C_{30}H_{32}O_{20}$	Quercetin 3-O-(6″-malonyl-glucoside) 7-O-glucoside	ND	X	X	X	ND	ND	X	X
18.47	742.19564366	741.52	$C_{32}H_{38}O_{20}$	Quercetin 3-(2G-xylosylrutinoside)	ND	X	X	X	X	X	X	X
	476.09547611	474.36	$C_{23}H_{24}O_{11}$	Kaempferol 7,4′-dimethyl ether 3-glucoside	ND	X	X	X	X	X	X	X
Amino acids												
2.27	299.00722271	299.12	$C7H_{10}N_4O$	4-amino-2-methylpyrimidin-5-yl) methyl	ND	X	X	X	X	X	X	X
6.66	152.03476805	153.08	$H_2NC_6H_3(OH)CO_2H$	3-amino-4-hydroxybenzoate;3-Amino-4-hydroxybenzoic acid	ND	X	ND	X	X	X	X	X
11.46	181.07389323	181.19	$C9H_{11}NO_3$	Tyrosine	ND	X	X	X	X	X	X	X
13.04	147.05315778	147.13	$C5H_9NO_4$	Glutamic acid	X	X	X		X	X	X	X
16.21	131.09462867	131.17	$C_6H_{13}NO_2$	Leucine	X	X	X	X	X	X	X	X
17.25	119.05824316	119.12	$C_4H_9NO_3$	Threonine	X	X	X	X	X	X	X	X
Hormones												
19.25	426.3610141	421.28	$C_{28}H_{46}N_2O$	N-stearoyltryptamine	X	X	X	X	X	X	X	X
20.58	216.12626315	217.36	$C_{13}H_{16}N_2O$	Nb-Acetyl-Nb-methyltryptamine	X	X	X	X	X	X	X	X
24.36	219.11201007	217.36	$C_{10}H_{13}N_5O$	cis-Zeatin	X	X	X	X	X	X	X	X
24.98	318.21949482	313.26	$C_{20}H_{30}O_3$	ent-7beta-Hydroxykaurenoic acid	X	X	X	X	X	X	X	X
25.46	304.24023027	306.47	$C20H_{32}O_2$	ent-15alpha,18-Dihydroxykaur-16-ene	X	X	X	X	X	X	X	X
26.00	278.15180919	277.48	$C_{16}H_{22}O_4$	(+)-Methyl abscisate	X	X	X	X	X	X	X	X

T1 = control (0%); T2 = 1%, T3 = 3% and T4 = 6% Phytostim® biostimulant . ND= Not detected, *X*=detected.

was observed in cv 'Elisa, whereby 3% dose outperformed the other studied treatments (Fig. 3).

5. Discussion

The current state of global warming caused by the synthesis of inorganic fertilizers and hypothesizes on population growth, necessitated research on sustainable strategies to improve food production in relation to lettuce. The effect of different concentrations of Phytostim® biostimulant was clearly observed on growth and yield attributes. Application of 3% dose of Phytostim® biostimulant improved the overall plant growth (root length, stem thickness, leaf length, plant height aerial mass and root mass) in both studied lettuce cultivar 'Lara' and 'Elisa' (Table 1). These results have now authenticated the 3% dose recommendations for leafy vegetables made on the labeling of this biostimulants https://www.moringasouthafrica.com/wp-content/uploads/2021/04/PhytoStim-Label.png. The increase in crop growth and yield attributes are attributed to the enclosed 22 essential and non-essential amino acid compounds which contribute directly to growth and developments in plants. In fact, similar results of amino acid based biostimulant demonstrated their efficacy on promoting growth and yield in leafy vegetables such as lettuce, jute and rocket (Abdelgawad et al., 2018; Carillo et al., 2019; Caruso et al., 2019; Khan et al., 2019; Shahrajabian et al., 2021). The improvement of plant growth lettuce was attributed to the role of L-methionines in crop production which have been associated with the absorption of sulfur and nitrogen compounds (Forde and Roberts, 2014; Khan et al., 2019; Vincill et al., 2012). On the other hand, amino acids based biostimulant were associated with improved plant growth due to their contribution in the plant nitrogen biosynthesis, regulating the uptake and fusion of ammonium, accumulation of nitrate, and biosynthesis of proteins (Barneix and Causin, 1996).

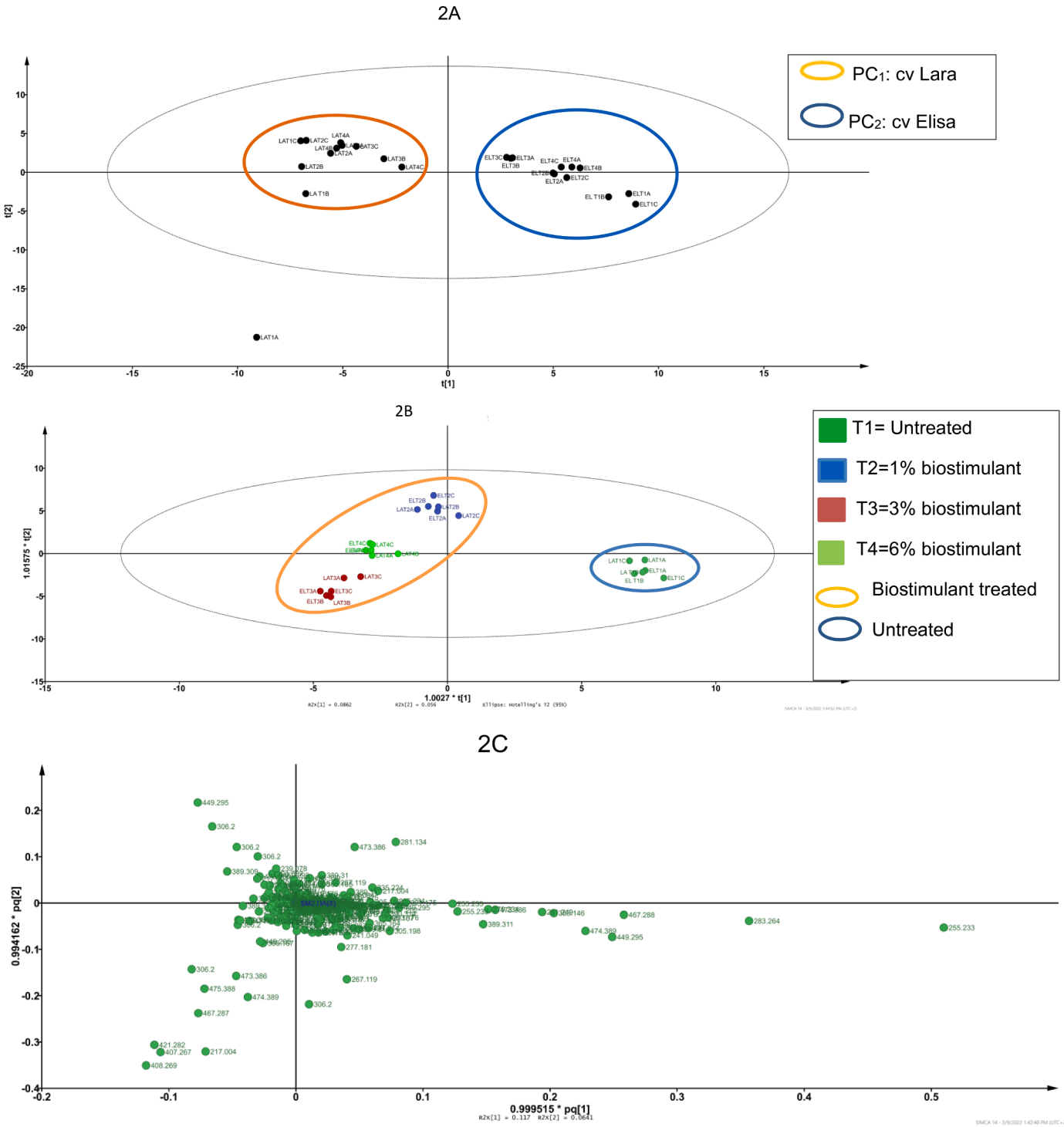

Fig. 2. Chemometric models on unsupervised PCA and supervised OPLSA-DA score plot carried out on LC-MS metabolic profile following the application of different concentrations of Phytostim® biostimulant treatment. (A-PCA, B-OPLS-DA, C—OPLS-DA).The OPLS model was cross-validated using CV-ANOVA ($p< 0.01$ and permutation tested to exclude over fitting.

From our results (Table 1), we postulate that the application of 1% doses is a kickstart, while 3% doses is optimum and 6% dose showed to inhibits plant growth. In this manner, the 22 amino acids profound in the Phytostim® biostimulant acted as transducing signals to induce nutrient uptake acquisition. In fact, Phytostim® biostimulant concentrations of 1% and 3% acted as natural plant growth stimulators to exert auxins or gibbering like action associated with improving the photosynthetic rate and root surface elongation (Forde and Roberts, 2014; Miceli et al., 2019). Whereas, there was an inhibition effect on auxins growth regulators which could have resulted from higher concentrations of the contained amino acid compounds and thus causing poor growth in plant exposed to 6% dose of the biostimulant. Based on the obtained results, we postulate that 6% dose become detrimental to plant growth due to increased plant respiration and the aggravation of mineral nutrients and excess accumulation of auxins causing abiotic stress to the plant (Caruso et al., 2019; Khan et al., 2019). Hence, it has become evident that the concentration level is critical in the efficacy of an amino acids biostimulant (Khan et al., 2019). Khan et al. (2019) reported that

Table 3
The effect of different concentrations of Phytostim® biostimulant on targeted non-secondary metabolites in lettuce cv 'Lara' and 'Elisa'.

	Total phenols (mg gallic acid/100 g)	Total flavonoids mg catechin/100 g)	Total carotenoids (mg/kg)	Chlorophyll *a* (mg/kg)	Chlorophyll *b* (mg/kg)	Total chlorophyll (mg/kg)
Lettuce cv 'Lara'						
1% Biostimulant (T2)	201.78±0.21b	421.75±0.10b	20.55±0.01a	1.30±0.21a	18.19±0.01b	20.49±0.01b
3% Biostimulant (T3)	222.54±0.01a	425.79±0.12a	18.86±0.21b	1.15±0.01b	21.32±0.06a	22.47±0.12a
6% Biostimulant (T4)	205.25±0.01b	415.99±0.14c	16.03±0.02c	0.9 ± 0.04c	17.97±0.61b	18.87±0.12c
Untreated (T1)	198.32±0.01c	411.79±0.14d	13.16±0.04d	1.0 ± 0.46c	17.79±0.03b	18.79±0.14c
F-statistics	1.32	2.36	0.74	1.89	1.98	1.87
Lettuce cv 'Élisa'						
1% Biostimulant (T2)	161.35±0.01b	346.89±0.03b	8.82±0.01b	0.81±0.05b	17.21± 0.04b	17.76±0.12b
3% Biostimulant (T3)	170.32±0.12a	353.72±0.02a	9.06±0.01a	0.98±0.03a	18.37±0.21a	19.51±0.22a
6% Biostimulant (T4)	145.05±0.11c	340.11±0.32c	7.34±0.00c	0.54±0.13d	14.58±0.32c	15.39±0.31c
Untreated (T1)	147.56±0.00c	339.99±0.14c	7.83±0.04c	0.72±0.10c	13.92±0.02c	14.64±0.02d
F-statistics	2.35	4.12	1.63	1.45	1.87	1.65

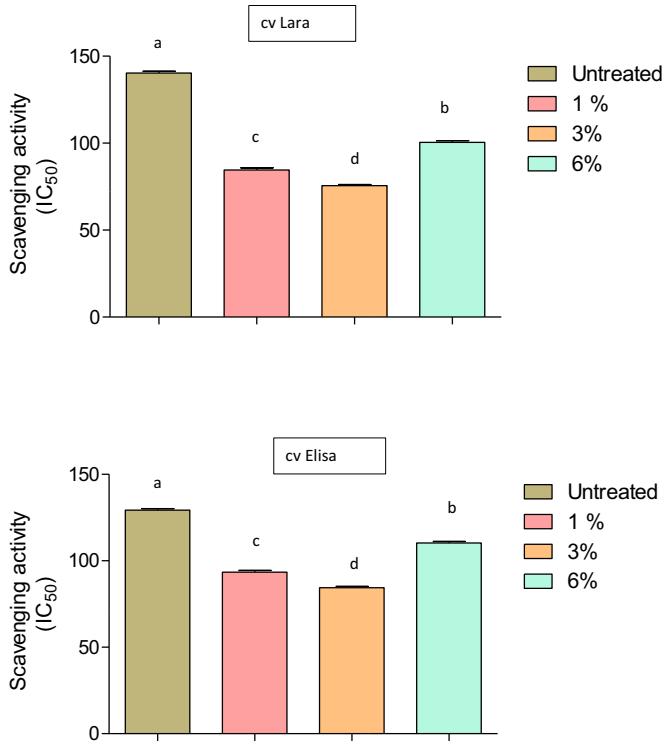

Fig. 3. Effects of different concentrations (1, 3 and 6%) of Phytostim® biostimulant on scavenging activity in lettuce cultivars ('Lara' and 'Elisa'). Means values and SE of each column were calculated based on six samples per biostimulant treatment. Those followed by a different alphabet letter in a column were significantly different (at $p < 0.05$) according to the Fisher's protected least significant test.

the lowest concentrations (0.2 mg/L and 0.02 mg/L) of the L-methionine improved the plant growth more in comparative to higher concentrations that ranged between 2.2 mg/L-2000 mg/L in lettuce samples. From an overall perspective, changes in metabolome profile in response to various concentrations of Phytostim® biostimulant can be associated with different processes aimed at plant adaptation either through cell-wall strengthening and signaling molecules.

Among the tentatively identified untargeted metabolites (Table 2), quinic acid, caffeic acid, ferulic acid and p-coumaric acid which are known as the direct precursor of chlorogenic acid were identified in all treated samples (T2, T3 and T4) in both lettuce cultivars. Considering the role of chlorogenic acid as a metabolic intermediator (Mhlongo et al., 2014; Narukawa et al., 2009; Turner et al., 2016) in the shikimate pathway, its presence in treated samples could elucidate obtained results on growth and yield components in this study. The effect of chlorogenic acid was reported to pose a negative correlation to the rate of growth and biomass, while it positively correlated to the photosynthesis rate (Turner et al., 2016). Although usually related to plant defense and resistance, chlorogenic acid has been reported to play many other regulations in plants, including root hair formation, signaling mediator for deposition of phenolic polymers and cell wall reinforcement (Mhlongo et al., 2014; Narukawa et al., 2009; Turner et al., 2016). A drop in chlorogenic acid was associated with an increase in cell wall bound phenolic polymers, therefore suggesting its action as signaling mediator for deposition of phenolic compounds (including the lignin) during root formation (Narukawa et al., 2009). In fact chlorogenic acid was identified to possess growth inhibition character at higher concentrations (5×10^{-4}) through reduction of stomatal closure (Narukawa et al., 2009). Similar postulation can be associated with detected trend observed in the targeted metabolites including total phenolic acids, total flavonoids, total carotenoids and total chlorophyll content (Table 3). Thus, postulating the impact of different concentrations of Phytostim® biostimulant to regulate total phenolic compounds including chlorogenic acid functionality which resulted in varied response of growth and yield components. Notably, targeted total flavonoids and a pool of flavonoids derivatives (namely quercetin 3-O-(6″-malonyl-glucoside) 7-O-glucoside, Quercetin 3-(2G-xylosylrutinoside), and kaempferol 7, 4′-dimethyl ether 3-glucoside) were additional plant growth regulators induced by the application of biostimulant (Tables 2 and 3). In fact, flavonoids are predominantly detected in leafy vegetables and have been implicated in response against salinity, growth inhibition and retarding biomass (Hofmann and Jahufer, 2011, 2011; Parvin et al., 2019). However, these metabolites are also reported to affect glutathione, signaling pathways and reactive oxygen species (ROS) which could be suggesting high antioxidant activity to maintain oxidative balance (Xu et al., 2019). The concurrent changes in the scavenging activity, total carotenoids, and total chlorophyll contents suggest a fine tuning of the ROS-mediated signaling in studied lettuce cultivars following the application of Phytostim® biostimulant. The impact of ROS were linked with reduction of improving biomass by modulating abiotic and biotic stress (Davaritouchaee et al., 2019).

It is interesting to note that such sustenance to improved biomass in relation to phenolic compounds and this is consistence with our study (Gémes et al., 2017).Even though there was no clear trend to be observed, a wide alteration of the amino acid was observed in this study.

As a result, these alterations could be could be attributed by changes of original mark in different antioxidant compounds including phenolic and flavonoids. Furthermore, it is important to reflect on amino acids role that is aimed at improving plant adaptability (Barneix and Causin, 1996).

6. Conclusion

The use of plant derived hydrolyzed proteins including amino acids has gained interest in agriculture. This is mainly due its proved efficacy in promoting plant growth, yield and modulation of secondary metabolites which exert antioxidant properties for survival in different unfavorable growing conditions. Optimization of biostimulant concentrations is a critical factor that influences the overall growth and alternatively yields components. Application of lower or higher concentrations becomes detrimental to growth and yield components. A novel approach based on the use of untargeted metabolites profiling and chemometric analysis to elucidate the impact of biostimulant on growth and yield creates a platform for introducing possible mechanisms induced in response to the biostimulant application. Phytostim® biostimulant application induced metabolic changes in lettuce by modulating the signaling and cell wall strengthening process that involved phenolic compounds. The coordinated action of plant growth regulators together with antioxidant compounds such as carotenoids and phenolic, might have affected the ROS-mediated signaling pathways. Although further detailed information on specific phenolic acids and carotenoids would strengthen our results, the targeted metabolites pointed out by this approach suggest that application of different concentrations of Phytostim® biotimulant might have reprogrammed the metabolome profile.

CRediT authorship contribution statement

Semakaleng Mpai: Conceptualization. **Lerato M. Mokganya:** . **Lerato Raphoko:** . **Peter Masoko:** Conceptualization. **Ashwell R. Ndhlala:** Funding acquisition, Conceptualization.

Declaration of Competing Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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