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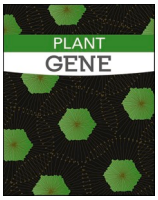

Molecular and ionomic responses of *Solanum lycopersicum* L. (cv. Micro-Tom) plants treated with a novel calcium-based plant biostimulant

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ABSTRACT

In this study, we investigated the leaf treatment effects of a novel trace elements calcium-based fluid mixture with a supposed biostimulant action on *Solanum lycopersicum* L. cv. Micro-Tom. Seedlings were grown on standard peat substrate and treated with two different products: a calcium-based fluid mixture and a common calcium fertilizer, $CaCl_2$. Both treatments were compared to an untreated control. We first investigated the effects of treatments on fruit yield and dry matter production in greenhouse-grown tomato. These effects were then assessed in leaves by gene expression profiling of 60 genes involved in different biological pathways and functional categories, and by ionomic analysis. Leaf treatment on tomato with the calcium-based fluid mixture allowed the highest fruit yield per plant (6.17 fruits plant^{-1}) and above-ground dry matter (13.99 g plant^{-1}) to be obtained. Also, 4 genes related to the nutrient transporter category, *NCX*, *NRAMP3*, *SI BOR2*, and *CHLM*, were upregulated in plants treated with the novel product. *CRK*, a gene related to the calcium-dependent protein kinases (*CDPK*), was upregulated in plants treated with the novel product whereas *SODCC.1*, a gene related to the superoxide dismutase family, was downregulated in the same plants. A substantial reduction of elemental contents was observed for $CaCl_2$-treated plants, while the novel Ca-based mixture increased the leaf mineral content of Zn (+61%) and Mn (+65%). These results highlighted the biostimulant activity of the novel product resulting in changes in fruit yield and dry matter production, gene expression, and ionome profiles of tomato leaves.

1. Introduction

To ensure adequate yields and quality, and provide food security, new sustainable and efficient solutions are needed to meet the requirements of a rapidly growing human world population (Evenson and Gollin, 2003; Godfray et al., 2010; Smith, 2015; Fróna et al., 2019). In this way, the use of bioeffectors, known as biostimulants, helps in various ways to enhance crop yield, exerting a beneficial action at different levels in plants (Alzahrani and Rady, 2019; Semida et al., 2019; Desoky et al., 2021). Plant biostimulants are substances or microorganisms applied to enhance nutrition efficiency, abiotic stress tolerance, and/or crop quality traits, regardless of nutrient content (du Jardin, 2015). The key mechanisms targeted by the biostimulants are strongly related to the nature of the biostimulant itself. Due to the complexity of the chemical composition and the simultaneous action of two or more compounds, a complete mode of action characterization is still lacking (Bulgari et al., 2015; Brown and Saa, 2015; Yakhin et al., 2017; Van Oosten et al., 2017). Tomato is one of the crops that benefit from the use of biostimulants. To study plant response to biostimulants, the variety *Solanum lycopersicum* cv. Micro-Tom is frequently adopted due to its peculiar botanical traits and the availability of a public and completely sequenced genome (Mueller et al., 2005). Recent studies have demonstrated that tomato transcriptome and ionome can be strongly modified by leaf treatment with fertilizers or other substances, such as biostimulants (Caruso et al., 2019; Della Lucia et al., 2022). Particularly, several studies have reported that calcium is a crucial nutritional macroelement for tomato growth and development and it has a still not well-characterized repertoire of metabolic pathways modulated by

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biostimulants in tomato.

Within this context, the aim of this study was to assess the leaf treatment effects of a novel trace elements calcium-based fluid mixture with a supposed biostimulant action on *Solanum lycopersicum* L. (cv. Micro-Tom). We first investigated the effects of treatments on fruit yield and dry matter production in greenhouse-grown tomato. These effects were then assessed in leaves by gene expression profiling of 60 genes coding for microelements transporters, calcium signal pathway, and enzymes involved in abiotic stress tolerance, and by ionomic analysis.

2. Materials and methods

2.1. Plant material and growth conditions

Seeds of tomato (*Solanum lycopersicum* L., cv. Micro-Tom) were provided by Sipcam Oxon S.p.a. and grown in 13 cm diameter pots filled with standard peat substrate. A mineral-based slow-release fertilizer (nitrophoska© type) with nitrogen, phosphorus, and potassium concentration of 12%, 12%, and 17%, respectively; the fertilizer also has an additional 2% of MgO, 24% of sulphur, 0.02% of boron, and 0.10% of zinc and was applied to the substrate, at a rate of about 15–20 g per pot. Each pot was irrigated with around 200–300 mL of water, every two days. Water in excess was resupplied until the complete absorption by the peat substrate. Pots were maintained for 50 days in a climatic chamber at 25/20 °C and a 16/8 light/dark photoperiod. There were two batches, one for the ionome analysis and one for the molecular analysis.

2.2. Treatments application and tissue collection

Plants were divided into three sets: one control and two sets of treated plants. Plants were treated three times. Each treatment was applied as a foliar spray at a volume of 10 mL per plant. This quantity corresponds to the novel Ca-based product volume usually sprayed as treatment in tomato crops. The untreated control was supplied with an equal volume of water. The other two treatments applied were: a calcium-chloride solution with a concentration of 10.050 g L^{-1}, and a novel calcium-based mixture with a concentration of 5 mL L^{-1}. The concentrations of the two products used for the experiments are agronomically effective as reported in the product technical indications by improving tomato agronomic traits. The novel Ca-based product was provided by Sipcam-Oxon S.p.a. and its chemical composition is reported in Table 1. Each solution was diluted in ultra-pure water and the pH was assessed as around 7.

Treatment application occurred at the flowering stage classified by the code BBCH65 in the official BBCH scale classification method, corresponding to the first open flower of the fifth inflorescence. The BBCH scale is defined as a system for uniform coding of phenologically similar growth stages, with a two-digit code that precisely identifies all phenological stages for the majority of plant species. For each code, morphological traits are used and a brief description of each stage is given, from seed germination to harvesting (Meier et al., 2009).

The fruit yield per plant and dry matter (DM) were evaluated to study the growth and product promotion in the treated plants. The sampling for this evaluation occurred 100 days after plant establishment in the growth chamber. For the fruit yield per plant, fruit samples were harvested at BBCH89, when they have the typical fully ripe colour. To measure the above-ground dry matter of plants at harvest time (leaf, stem, and fruit), samples were oven-dried at 50 °C for at least five days. The dried sample was then determined using a digital scale.

Table 1
Composition of the novel Ca-based solution product enriched with an inorganic complexing agent provided by Sipcam Italia S.p.a.

CaO sol. in water (% *w*/w)	5
Mn sol. In water (% w/w)	1.5
Zn sol. In water (% w/w)	0.5
Polysaccharides mixture (% w/w)	20
Electrical conductance (mS cm^{-1})	28.8
pH	4.25
Density (kg L^{-1})	1.407

For the gene expression analysis, three plants per treatment were chosen and sampled 48 h after treatment application. The timing was chosen according to previous experiments in which the best timing after treatment to observe changes in gene expression was assessed. There were three biological replications for each plant and a total of 27 samples were collected. Each sample was formed by three leaf disks collected per single plant. The second, third or fourth unfolded leaf on the main shoot was selected, using all of them alternatively. Samples were immediately stored at −80 °C until RNA extraction. For the ionomic analysis, 6 plants per treatment were selected and sampled before leaf treatment application and after 7 days. Each plant sample consisted of 3 g of fresh tomato leaves, choosing the second, third, or fourth unfolded leaf on the main shoot. Leaves were chosen in according to our previous experiment where it was observed that the optimal accumulation of mineral elements occurs between the second and fourth leaf, for our considered tomato phenological stage. The leaves were stored at −20 °C, ready for the ionomic analysis.

2.3. Selection of candidate genes and PCR primer design

A panel of 60 genes (encoding proteins) involved in different biological pathways and functional categories, such as calcium and other nutrients transport and metabolism, hormonal metabolism, and stress responses were selected and cDNA annotation sequences were provided by the MiBASE database (http://www.pgb.kazusa.or.jp/mibase/) (Aoki et al., 2010). The gene-specific primers were designed using an online PCR Primer Design Tool offered by Eurofins (https://www.eurofinsgenomics.eu/en/ecom/tools/pcr-primer-design).

2.4. RNA isolation and Real-time RT PCR

Total RNA was extracted and purified using an automatized procedure with the BioSprint 96 DNA Plant Kit (Qiagen, Hilden, Germany) in a BioSprint 96 workstation (Qiagen) following the manufacturer's instructions. The molarities of RLT and RPW buffers were increased to achieve higher extracted RNA yields. The quality of the RNA extraction was assessed via quantification, using a Qubit RNA HS Assay Kit in a Qubit fluorometer (Invitrogen, Carlsbad, CA, USA). The total extracted RNA was stored at −80 °C for the next analysis. One-step reverse transcription-quantitative PCR (RT-qPCR) in real-time was run in triplicate on a QuantStudio 12 K Flex Real-Time PCR System (Life Technologies, USA) in a reaction volume of 10 μL containing 9 μL of qPCR BIO Sybrgreen 1-Step Mix (Resnova – PCR Biosystem) and 4 ng of template RNA, using the following thermocycler program: 10 min at 45 °C and 2 min at 95 °C for the holding stages, followed by 40 cycles at 95 °C for 5 s and 25 s at 60 °C. The process also includes a step for amplicons dissociation, recorded by the PCR machine as melting curves graphs. The melting stage was recorded after 40 cycles and starts by heating the amplicons for 15 s at 95 °C, 60 °C for 1 min, and 95 °C for 15 s. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$. Ubiquitin (UBI) and Actin (ACT), two classical tomato housekeeping genes with stable expression levels reported in the literature (Løvdal and Lillo, 2009), were used for the normalization of expression levels in this experiment.

2.5. Ionome analysis

The element concentration in the leaves treated with the two different products and the untreated ones was determined by ionomic analysis. Sampling of the leaves occurred before and seven days after the treatment application Leaf samples were digested with concentrated

HNO_3 in a microwave system. The element concentration was determined by inductively coupled plasma ICP-OES. Elements were quantified using certified multi-element standards. The content of Al, As, B, Ba, Ca, Cd, Cu, Fe, K, Li, Mg, Mn, Mo, Na, Ni, P, S, Si, Sr, Ti, and Zn were evaluated through the ICP-OES optical system. Contents were considered in mg kg^{-1} of dry matter.

2.6. Statistical analysis

The analysis of variance (ANOVA one-way) was performed to determine any statistical differences between treated and untreated samples, for the evaluated variables in the gene expression analysis. A *p*-value <0.05 was considered for each analysis. Treatment means were separated by the Duncan test while variance homogeneity was evaluated using Levene's test. Principal components analysis (PCA) of ionome variation between untreated and treated samples was also performed. Variables were presented in graphs as the mean and standard error of the mean (standard error). All statistical analyses were carried out using Statistica software v. 13.4 (TIBCO Software, USA).

3. Results

3.1. Growth and yield traits

The results indicated that the application of the novel Ca-based mixture had significant effects on tomato plants dry mass and fruit yield. The maximum number of fruits per plant (6.17) was observed with this treatment, which was statistically greater than $CaCl_2$ and the untreated plants (Fig. 1).

Plants treated with the novel Ca-based mixture also produced the significantly highest above-ground dry matter (13.99 g $plant^{-1}$). Instead, no significant difference was recorded for plant DM between the $CaCl_2$ application and untreated plants (Fig. 1).

3.2. Gene expression analysis

Sixty genes reported being involved in several different plant metabolic pathways were selected among all the EST gene sequences reported in the MiBASE – Microtom database. Genes category includes calcium and other nutrients metabolism (Mg, Cu, Zn, B), hormonal metabolism, oxidative stress-triggered metabolism, and water stress-triggered metabolism. All 60 primer pairs were tested for gene expression in leaf samples. Only gene primer pairs with a single peak in the melting curve analysis performed by the machine were selected. A single peak confirms the specificity of PCR amplification and a PCR product of the expected size, as described by Czechowski et al. (2005).

Only twenty primer pairs out of sixty amplified single PCR products with a single peak resulted during the melting curve analysis. The selected primers targeted genes with Thermal Cycle (Ct) ranging from 20 to 29. The twenty genes were divided into 2 groups: one with all the genes related to the nutrient transporter and the other one related to the oxidative and water stress-triggered metabolism (Table 2).

Gene expression was tested by the Duncan posthoc test and only 6 genes out of twenty were selected. These genes showed a statistically significant difference in terms of change in the relative expression as compared to the untreated plants at a *p-value* < 0.05. Genes with no differences between treated and untreated samples were discarded because of the absence of any putative effects from the treatment, meaning that the gene expression of the analyzed genes was not affected. The result of the Duncan test is reported in Table 3. Duncan tests reveal differences in some genes belonging to both categories.

Genes *CRK* and *SODcc.1*, related to the stress signaling metabolism, significantly changed their expression. (Fig. 2). In particular, gene *CRK*, a Ca-dependent protein-kinase of tomato was upregulated eight-fold in samples treated with the Ca-based mixture, as compared to the untreated control. Gene *SODcc.1* instead, was downregulated eightfold in samples treated with the Ca-based mixture, and upregulated in samples treated with $CaCl_2$, but not significantly. Genes *NCX*, *NRAM3*, *SI_BOR2*, and *CHLM*, related to nutrient transporters, were upregulated two-fold in samples treated with the Ca-based mixture, but only *CHLM* and *NRMAP3* showed a difference with the Duncan test (Fig. 3).

3.3. Ionome analysis

The leaf element composition of the treated and untreated leaves was

Table 2
List of genes analyzed, showing statistical significance with ANOVAand $p < 0,05$. All sequences are freely available at http://www.pgb.kazusa.or.jp/mibase/clone_uni_name.htmL

Category	Function	Gene name	EST clone sequence name
Stress related metabolism	CDPK-related protein kinase	*CRK*	*LEFL2013N24*
	SODCC.1 superoxide dismutase [Cu—Zn] 1]	*SODCC.1*	*FC11BA12*
	Probable boron transporter 2	*Si BOR2*	*LEFL1003CF07*
	Generic Fe and Zn vacuolar efflux transporter	*NRAMP3*	*LEFL1039BB08*
Nutrient transporters	Sodium/calcium exchanger NCL	*NCX*	*LEFL1011AD10*
	Magnesium protoporphyrin IX methyltransferase, chloroplastic	*CHLM*	*LEFL1031AD01*

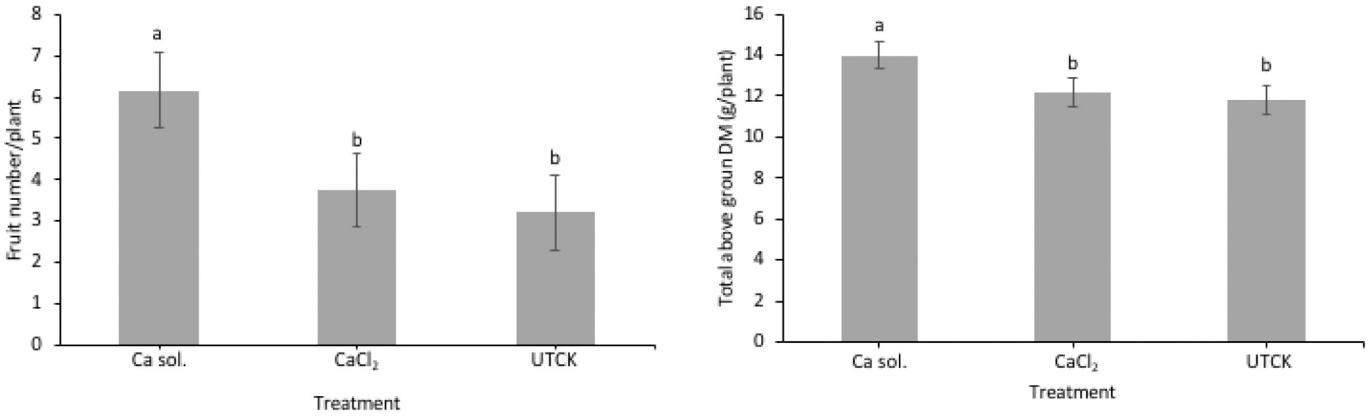

Fig. 1. Fruit number $plant^{-1}$ and total aboveground DM plots for the three treatments. Means and error bars for each treatment are shown. Post-hoc Duncan's test at $p < 0.05$ was performed to discriminate means between treatments.

Table 3
Duncan's Multiple range test ($p < 0,05$) results on relative expression of analyzed genes. Differences are calculated as compared to the untreated control.

	NCX	NRAMP3	SI_Bor2	CHLM	SODcc.1	CRK
Untreated control	–	–	–	–	–	–
Ca solution	*	*	*	*	*n.s.*	**
$CaCl_2$	*	*	*	*n.s.*	*n.s.*	*

analyzed before and seven days after treatment. The comparison of the ionome profile revealed significant differences in the elemental composition, seven days after treatment. ANOVA test results are reported in Table 4. All three main variables, treatment, time, and element, are highly statistically significant. Means of the content profile and increment or decrement for the considered element, before and seven days after the treatment are shown in Tables 5, 6, and 7. Calcium content did not change significantly seven days after the treatment: only in the sample treated with $CaCl_2$, the calcium content showed an increment of 2%, from 31,737.78 mg kg^{-1} to 32,325.79 mg kg^{-1}. This means that neither treatment affected the calcium ionome in leaves. The other plant macro and microelements contents were different. Based on the results of gene expression, some microelements related to the targeted transporter were studied in-depth: B, Mg, Fe, Na, and Zn. Zinc is one of the key elements that showed an increment of 60% after seven days (from 48.99 to 78.93 mg kg^{-1}), in samples treated with the Ca-based mixture, while both untreated control and $CaCl_2$-treated plants decreased their zinc content by about 50% (from 62.66 to 29.40 mg kg^{-1}) and 40% (from 65.81 to 37.23 mg kg^{-1}) respectively. Boron and magnesium content increased by 8% (from 60.61 to 65.84 mg kg^{-1}) and 7% (from 9479.24 to 10,154.01 mg kg^{-1}) respectively in plants treated with the Ca-based mixture, while a decrement of 10% (from 84.03 to 75.44 mg kg^{-1}) was observed for boron and an increment of 13% (from 10,192.13 to 11,588.39 mg kg^{-1}) for magnesium, both in plants treated with $CaCl_2$ For iron, there were no percentage differences in the Ca-based mixture treatment, but a reduction of 24% (from 124.55 to 94.40 mg kg^{-1}) and 20% (from 159.34 to 128.58) mg kg^{-1}) was observed in untreated control and $CaCl_2$ treatment respectively.

A decrease of around 37% in sodium content was observed in all the experimental conditions: from 331.44 to 211.62 mg kg^{-1} for the untreated samples; from 430.50 to 257.55 for the Ca-based mixture treated samples; and from 329.19 to 206.26 mg kg^{-1} for the $CaCl_2$ treated samples. Despite gene expression analysis results not showing changes in the expression of manganese metabolism-related genes, manganese content was 65% higher after seven days in the Ca-based mixture treatment, from 234.94 to 388.15 mg kg^{-1}, but the content decreased by 5% in the other two conditions: from 324.60 to 307.21 mg kg^{-1} for the untreated plants; and from 331.65 to 315.74 mg kg^{-1} for the $CaCl_2$ treatment. Moreover, manganese is the second component of the novel Ca-based mixture, with a concentration of 1.5%.

PCA analysis was performed to evaluate the relationship between treatment and ionome content in treated and untreated samples. PCA analysis revealed separate clustering for the Ca-based mixture treatment, while the others were clustering together (Fig. 4). Factor 1 and factor 2 of the first PCA graph (Fig. 5a) related to data collected before the treatment explained 62.52% and 11.65% of the total variation, respectively. Factor 1 and factor 2 of the second PCA graph (Fig. 5b) related to data collected seven days after the treatment explained 58.30% and 13.90% of the total variation, respectively.

4. Discussion

In this study, we studied the effects of a novel Ca-based product on tomato plants. A $CaCl_2$ mineral fertilizer and an untreated control were included for comparison. The treatment with the Ca-based product showed the highest growth and yield promotion in tomato plants, as compared to the other two treatments. This is in line with other authors who found similar results by applying biostimulants or biostimulant-like substances of different and complex nature with an organic component like the Ca-based mixture (Rouphael et al., 2018; Canellas et al., 2019; Jindo et al., 2020; Canellas et al., 2020). De Hita et al. (2019) reported the effect of the application of humic substances for root and leaf treatments. Root treatments showed more consistent effects compared to leaf treatments, which have a transient effect in increasing plant growth and require several applications during the plant cycle. But despite these findings, our observations on leaf treatment highlight an important effect that resulted in improved plant growth and yield, with only one treatment during the plant life cycle. The effect of treatments on root and shoot development could be explained by the changes we observed in gene expression and the ionome of tomato leaves. Interestingly, the gene related to the calcium CDPK-related protein kinase was notably upregulated in plants treated with the calcium-based mixture. *CDPKs* are a large and partially characterized family of proteins in plants. They are calcium sensor proteins and play an important role as a mediator of responses to endogenous and environmental stimuli (Cheng et al., 2002). The protein structure is also conserved in tomato *CDPKs*, with four characterized domains in the *N*-terminal domain, Ser/Thr kinase domain, autoinhibitory junction domain, and calmodulin-like domain. The *CDPKs* convert the variation of cytosolic [Ca^{2+}] into biochemical and genetic products through a phosphorylation process, like membrane solute transporters (including the Ca^{2+}-ATPase, AtACA2), ion and water channels, *NADPH* oxidases, enzymes involved in carbon and nitrogen metabolism, cytoskeletal proteins, proteases, and DNA-binding proteins, control of the cell cycle,

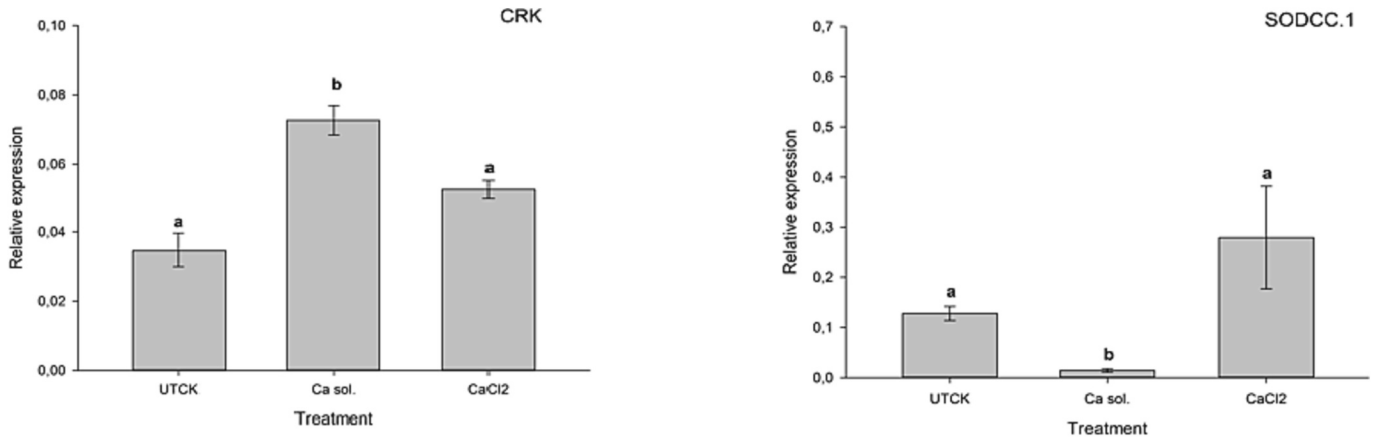

Fig. 2. Relative expression of genes encoding for stress-related proteins. Means and error bars for each treatment are shown. Post-hoc Duncan's test at $p < 0.05$ was performed to discriminate means between treatments.

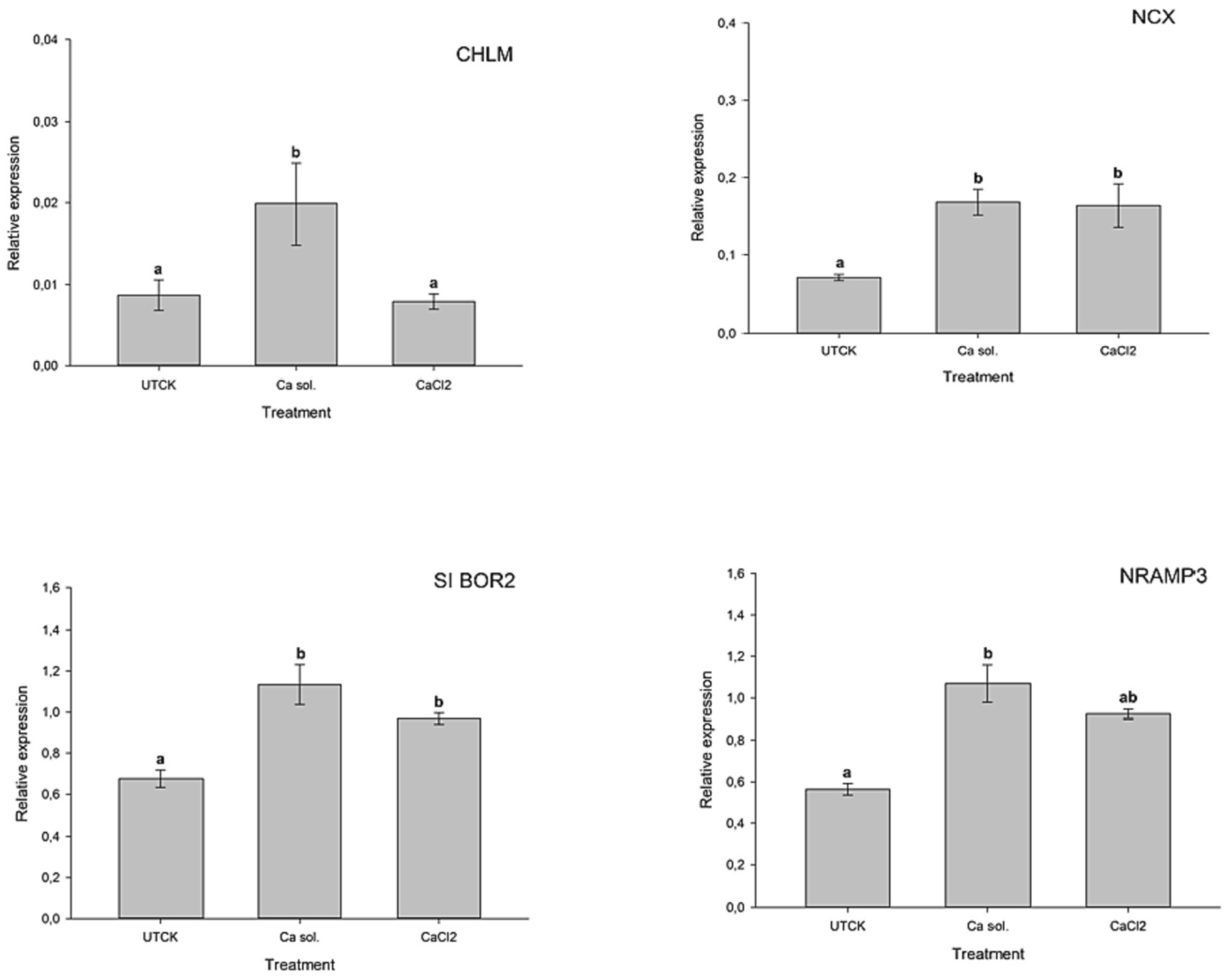

Fig. 3. Relative expression of genes encoding for nutrient transporter. Means and error bars for each treatment are shown. Post-hoc Duncan's test at $p < 0.05$ was performed to discriminate means between treatments.

Table 4
Analysis of Variance for $p < 0,05$, for the ionome content. Means are mg kg^{-1} dm. The statistically significant factors and interactions are highlighted.

	SS	*D.o.f.*	*MS*	*F*	*p*	
Intercept	3.231048E+10	1	3.231048E+10	3632.543	0.000000	
Treatment	1.546550E+08	2	7.732749E+07	8.694	0.000202	**
Time	1.459378E+08	1	1.459378E+08	16.407	0.000062	**
Element	7.422705E+10	12	6.185588E+09	695.422	0.000000	**
Treatment*Time	3.841534E+07	2	1.920767E+07	2.159	0.116767	*n.s.*
Treatment*Element	3.833803E+08	24	1.597418E+07	1.796	0.012841	*
Time*Element	8.046658E+08	12	6.705549E+07	7.539	0.000000	**
Treatment*Time*Element	1.836289E+08	24	7.651205E+06	0.860	0.657598	*n.s.*
Error	3.47E+09	390	8.89E+06			

phytohormone signal transduction, light-regulated gene expression, gravitropism, thigmotropism, nodulation, cold acclimation, salinity tolerance, drought tolerance, and responses to pathogens (White and Broadley, 2003). Neither the treatments with the Ca-based mixture nor the one with $CaCl_2$ significantly affected the concentration of calcium in the leaves, with no significant differences between treated and untreated plants. This is in line with several authors who underline the role of calcium as an elicitor and also the mediator of the signals to activate all the correlated downstream transcriptional processes, like the active regulation of different ion and micronutrient membrane transporters, with a *CDPKs* complex activation, resulting in the overexpression of the related genes (Sanders et al., 2002; White and Broadley, 2003; Dodd et al., 2010). The presence of a polysaccharides mixture in the novel Ca-based mixture could be also a possible explanation for the observed cross-talk between the different metabolic pathways that regulate the growth and development of plants. Polysaccharides are also known as elicitors and this could influence the expression of signaling-related genes, as it is already been reported that carbohydrates and sugars-related compounds can activate PAMP/DAMP responses in plants, which also involves genes related to the CDPKs family, like the one we studied (Bolouri Moghaddam and Van den Ende, 2012). Moreover, oligosaccharides are reported to be effective in enhancing plant tolerance to biotic and abiotic stresses (Ibrahim and Abdellatif, 2016; Davidsson et al., 2017; Chaliha et al., 2018; Zang et al., 2019; Narula et al., 2020).

Table 5
Means (mg kg^{-1} dm) and percentage of variations for the analyzed elements in UTCK samples. Percentages are the increment or decrement after seven days for the considered elements.

	UTCK - 0 h	*UTCK - 7 days*	*% variation*
B	84.552	85.957	1.66
Ba	2.112	1.591	−24.67
Ca	33,046.202	31,378.811	−5.05
Fe	124.550	94.399	−24.21
K	45,467.204	33,323.328	−26.71
Mg	10,564.873	10,674.459	1.04
Mn	324.603	307.209	−5.36
Mo	29.140	31.238	7.20
Na	331.439	211.620	−36.15
P	8820.634	7067.112	−19.88
S	26,684.005	21,727.695	−18.57
Sr	32.933	28.161	−14.49
Zn	62.665	29.396	−53.09

Table 6
Means (mg kg^{-1} dm) and percentages of variations for the analyzed elements in Ca-based solution product treated samples. Percentages are the increment or decrement after seven days for the considered elements.

	Ca solution - 0 h	*Ca solution - 7 days*	*% variation*
B	60.612	65.839	8.62
Ba	1.768	0.947	−46.43
Ca	28,673.219	28,646.059	−0.09
Fe	107.566	108.290	0.67
K	35,425.961	32,008.752	−9.65
Mg	9479.239	10,154.013	7.12
Mn	234.933	388.144	65.21
Mo	17.915	20.351	13.60
Na	430.497	257.547	−40.17
P	6234.708	5880.697	−5.68
S	19,003.177	18,079.315	−4.86
Sr	29.282	28.766	−1.76
Zn	48.992	78.926	61.10

Table 7
Means (mg kg^{-1} dm) and percentages of variations for the analyzed elements in $CaCl_2$ treated samples. Percentages are the increment or decrement after seven days for the considered elements.

	$CaCl_2$–0 h	*$CaCl_2$–7 days*	*% variation*
B	84.036	75.447	−10.22
Ba	2.051	1.751	−14.62
Ca	31,737.784	32,325.792	1.85
Fe	159.340	128.585	−19.30
K	44,437.169	32,294.276	−27.33
Mg	10,192.131	11,588.387	13.70
Mn	331.648	315.745	−4.80
Mo	19.748	17.714	−10.30
Na	329.187	206.264	−37.34
P	8073.711	6403.117	−20.69
S	25,043.394	18,170.229	−27.45
Sr	30.618	29.478	−3.72
Zn	65.807	37.233	−43.42

The *SOD* gene, a Cu/Zn superoxide dismutase (Cu/Zn SOD) was downregulated in plants treated with the calcium-based mixture. Superoxide dismutases instead are a large category of metal-containing enzymes with the important function of catalyzing the dismutation of reduced oxygen species (ROS), which are toxic to plants. Because of this, they play a fundamental role during abiotic and biotic stress events. *SODs* are highly regulated in the plant by gene expression and their activities are also strongly influenced by environmental stimuli (Bowler et al., 1994). In the case of tomatoes, genomic *SODs* gene family classification was studied by Feng et al. (2016). In their study, they revealed the presence of nine genes related to different metal ion-dependent *SODs*, with many *cis-elements in* the promoter sequences, that respond to different stresses. This correlates *SODs* tomato genes to stress responses to drought, low-temperature, defense stresses, anaerobic induction, fungal elicitors, SA, MeJA, GA, IAA, and ethylene. Interestingly, *SlSOD1*, mentioned in the study by Feng et al. (2016), is the same gene sequence used in the current experiment and is overexpressed during salinity stress. Other studies revealed that the *SODs* enzymes are activated under stress events also in other crops, and they work more as stress fighters than stress indicators. Despite the large literature about Ca^{2+} signaling related stress defensive responses, our novel Ca-based mixture product instead acts in an intriguingly different way, enhancing general plant signaling and improving plant nutrients acquisition instead of the plant defense system. We noticed that gene *NRAMP3*, zinc transporter, and CHLM, magnesium transporter, are upregulated in the treatment. In Rai et al. (2021) the authors reviewed many studies in which it was reported that the NRAMPs genes family are directly involved in the iron homeostasis in plants through a complex cross-talk between other micronutrients, such as Zn. Nonetheless, we found an increased level of zinc content in tomatoes treated with the novel Ca-based mixture and no interesting variances in the iron content after seven days, but it was notably decreased in the other two treatments. This supports our hypothesis of a putative biostimulant action as confirmed by previous results showing the ability of biostimulants to improve nutrient uptake with an overexpression of the related nutrient transporters genes. In particular, this improvement includes modifications of the root absorption area and modulation of the plant cell membrane activities related to nutrient acquisition (White and Broadley, 2003; Zandonadi et al., 2016; Zanin et al., 2019; Nardi et al., 2021; González-Morales et al., 2021).

5. Conclusion

In this study, the comparison between treatment with a novel Ca-based mixture with supposed biostimulant activity and calcium-chloride solution showed interesting results with a gene expression/ionome approach. While the calcium-chloride solution did not exert a great impact at the genome level in the leaf and seems to be slightly detrimental to the general leaf ionome, the treatment with the novel substance importantly influenced these two components. These results confirm the initial hypothesis of a supposed biostimulant activity of the novel substance. We have demonstrated, according to the definition of biostimulant substance provided by Du Jardin (2015), that: 1) the novel Ca-based mixture can enhance nutrient use efficiency by increasing the expression of genes related to the nutrient transport metabolism; 2) the novel Ca-based product is a complex mixture of macro and microelements fundamental for plant nutrition without acting like a chemical fertilizer; 3) The novel Ca-based mixture enhances general plant signaling. Given the first two main characteristics extrapolated from our results, the novel product is confirmed as a substance with biostimulant activity, and the way it was characterized is appropriate for understanding more about the mode of action.

Credit author statement

G.B., M.C.D.L, A.B., F.M., M.C., P.S., and S.N. made the conception and design of the study. G.B., M.C.D.L, A.B., F.M., M.C., P.S., and S.N. contributed to the methodology of the study. G.B., M.C.D.L, M.C., and A. B. performed the experiments. G.B. and P.S. conducted statistical analyses and wrote the paper. G.B., M.C.D.L, A.B., F.M., C.C., S.N., G.C., P. S., and S.N. contributed to critical writing and reviewing of the manuscript. All authors reviewed the manuscript and gave final approval for publication.

Declaration of Competing Interest

The authors declare that they have no known competing financial

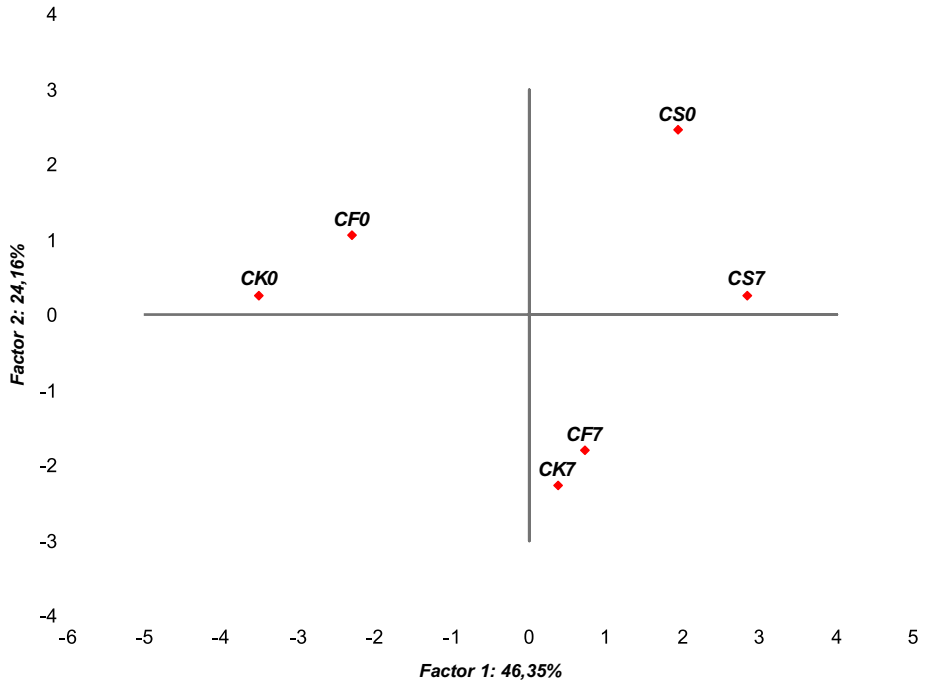

Fig. 4. Classification of the clusters based on leaf ionome content as a function of the treatment. "CK" = Untreated control; "CF" = $CaCl_2$ treatment; "CS" = Ca-based solution product treatment. "0" = before treatment; "7" = seven days after treatment.

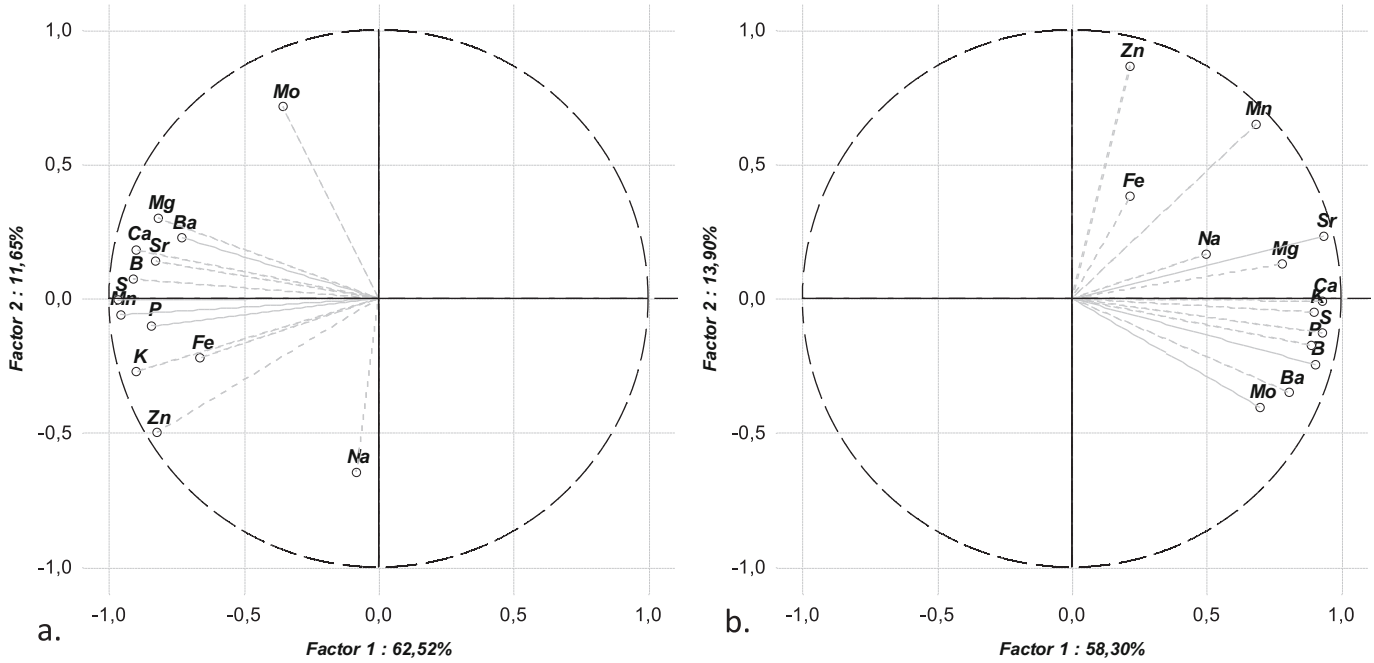

Fig. 5. PCA analysis before the treatment (A) and seven days after (B). These graphs show the correlation between variables, and closeness in the graphs indicates correlation strength.

interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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