

Multielemental Microanalysis in Root and Leaves, Growth and Biomass in Tomato Seedling in Response to Ca Levels and Induction of Stress: Non-Destructive Spectroscopy

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How to cite this paper: Pérez-Dueñas, O.A., Hernández-Mendoza, F., García-Gaytán, V. and García-Morales, S. (2021) Multielemental Microanalysis in Root and Leaves, Growth and Biomass in Tomato Seedling in Response to Ca Levels and Induction of Stress: Non-Destructive Spectroscopy. *Open Journal of Applied Sciences*, 11, 672-683.

<https://doi.org/10.4236/ojapps.2021.116049>

Received: May 21, 2021

Accepted: June 21, 2021

Published: June 24, 2021

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Abstract

Tomatoes are one of the main vegetables in the food industry that are consumed fresh and processed. A multi-element microanalysis was performed with the use of scanning electron microscopy (SEM) in conjunction with energy dispersion spectroscopy (EDS), to see changes in nutrient concentration in roots and leaves, as well as fresh and dry biomass in tomato seedlings. Exposed in nutrient solution (NS) with different levels of Ca (control, 25, 50 and 100 mM). The root was put in also to H₂O₂ for 8 hours to observe the changes that occurred. It was observed that NS with 100 mM Ca in fresh stem biomass and fresh leaf biomass increased by 25% and 38% compared to the control. It was observed that the NS with 100 and 50 mM of Ca, the content of Ca and Cl in foliar tissue increased by 34.50% and 13.15% compared to the control. Treatment with H₂O₂ and with 25 and 100 mM of Ca at NS increased the height of the seedling and fresh leaf bio-mass by 19.75% and 60.80% compared to the control. The treatment with H₂O₂ and with the different levels of Ca in the NS increased the content of C, P, K, Ca and Cl in the leaf tissue. Only S was significant for the control. Treatment with H₂O₂ and 100 mM Ca in the NS increased Ca and Mg by 83.48% and 40.47% for the root compared to the control. The K and Cl in the root was higher in the control treatment by 47.61% and 55% respect to the highest level of Ca. It is concluded that multielemental microanalysis is a powerful, non-destructive, fast and accurate tool for the determination of plant nutrients. Also, it is applica-

ble for the areas like horticulture, physiology and agronomy.

Keywords

Microanalysis, Nutrient Solution, Calcium, Root, Leaves, Stem

1. Introduction

Hydroponic systems have been used as one of the standard methods for plant biology research and for the commercial production of vegetables such as tomatoes. In addition, it has served to study the responses of plants to biotic and abiotic stress [1]. Tomato seedlings with a good root system will allow a good anchorage to the substrate. Abiotic stress factors, such as salinity, affect the uniformity and nutrition of the tomato [2] [3]. Root pretreatment with hydrogen sulfide (H₂S) protects strawberry plants against salt stress (NaCl) [4]. The exposure of *Populus x capeskins* hydroponically to Cd and H₂O₂ showed that the first agent inhibited the antioxidant enzymes. The peroxide caused the accumulation of GSH and the loss of ascorbate [5]. In another study, the immersion of the roots of strawberries (*Fragaria x ananassa* Dusch.) in H₂O₂ increased plant growth, the concentration of photosynthetic pigments, the relative water content in leaves and antioxidant activity [6]. For the previous cases, the exposure of H₂O₂ in roots increased the resistance of the plants to salinity stress. In another study, the addition of 10 mM H₂O₂ induced nitric oxide production in Mung Bean (*Phaseolus aureus*) [7]. However, previous investigations did not determine the nutritional profile. Plant nutrition studies are carried out using methods that require chemical extraction to determine an element [8], such as total N [9] [10] and organic C [11] [12]. Equipment such as plasma spectrometry (ICP-MS), or atomic absorption spectroscopy, several elements are analyzed at the same time in plant nutrition [13] [14] [15] [16]. Experiments are required to demonstrate the accuracy of the data on the nutritional content of plants. We suggest that multielemental microanalysis by SEM/EDS is the adequate, fast, and precise tool for the determination of nutrients in plant tissue.

Scanning electron microscopy with energy dispersive X-ray spectrometry (SEM/EDS) is an elemental microanalysis technique widely applied across a broad range of the physical and biological sciences, engineering, technology, and forensic investigations. Electron-excited characteristic X-ray peaks provide identification and quantification for all elements of the periodic table, with the exceptions of H, He, and Li [17] [18]. The previous technique has been used in plant tissue for nutritional diagnosis, to confirm deficiency symptoms or optimal nutritional ranges, in addition, to identify the accumulation of metals in different organs of the plant [19] [20] [21].

In order to evaluate the precision and reliability of the SEM/EDS in the relative content of nutrients. It was experimentally simulated the development of the

seedlings under two conditions.

Semi-indeterminate growth tomato seedlings Pony Express were exposed hydroponically to different levels of Ca, to see changes in the concentration of nutrients, growth and biomass. As well as the immersion of tomato roots in H₂O₂ for 8 hours and find out if it affected the characteristics of the roots and elemental content, root, and leaves. And evaluate the differences in growth, biomass and elemental analysis in each organ of the seedlings using SEM coupled to EDS.

2. Materials and Methods

2.1. Biological Material and Nutrient Solution

Pony Express HM[®] cv tomato seedlings of semi-indeterminate growth, from Yurecuaro, Michoacan were used. The roots of the seedling were removed from peat moss using tap water. The experimental units consisted of an airtight container with a capacity of 3.5 L⁻¹, and 2.5 L⁻¹ distilled water was added to each container. Each container contained 20 plants, and five replicates were used. Tomato plants were grown for three weeks in modified Hoagland nutrient solution containing 1.2 mM KNO₃, 0.8 mM Ca(NO₃)₂, 0.2 mM KH₂PO₄, 0.2 mM MgSO₄, 50 mM CaCl₂, 12.5 μM H₃BO₃, 1 μM MnSO₄, 1 μM ZnSO₄, 0.5 μM CuSO₄, 0.1 μM (NH₄)₆Mo₇O₂₄, 10 μM Fe-EDTA, and 0.1 μM NiCl [21] [23].

Hoagland solution was supplemented with four concentrations of Ca (25, 50, and 100 mM), and Hoagland solution served as the control. To avoid root hypoxia, oxygen was supplied by a pump (Elite[®] 802). The roots were pretreated with 10 mM H₂O₂ for 8 hours. Plants were conditioned again in the nutrient solution containing the corresponding treatment for one week. The average CO₂ level in the experiment was 502 ppm. The relative humidity was 37%, and the temperature was 23 °C (WatchDog model A160 temp/HR/CO₂, Spectrum Technologies Inc.).

2.2. Experiment I

2.2.1. Stem Diameter, SPAD, Root Length and Length Stem

The growth variable was evaluated 15 days after incubating at different concentrations of Ca(NO₃)₂. For the stem diameter (DT) it was measured with a digital vernier (mm) and was the average of three measurements on the stem (low, medium and upper). The value of the SPAD readings was the average of three readings per plant; it was performed using a SPAD-502 meter (Konica-Minolta, Japan). Plant length (LP) was measured with a graduated ruler (cm) from the base of the stem to the apex. Root length (LR) was measured with a graduated ruler (cm) from the base of the stem to the most distal part of the root.

2.2.2. Fresh and Dried Root, Stem and Leaf Biomass

After evaluating the growth variables, the roots, stem and leaves of the seedlings were separated, subsequently labeled and weighed. The root, stem and leaf were placed in an oven (Felissa[®], model Fe-292 AD) at 60 °C for 72 hours. Fresh and dried biomass was weighed on a RADWAG[®] AS 310/X precision balance.

2.3. Experiment II

2.3.1. Root Immersion in H₂O₂

Once the first experiment is evaluated, and before preparing and adding the Hoagland solution with the different concentrations of Ca. To each tray, containing distilled water was added 10 mM hydrogen peroxide (H₂O₂) and temporarily left for 8 hours with the oxygenation system with tomato seedlings. Subsequently, the Hoagland solution was renewed with calcium concentrations. Seven days later, the growth variables were measured: stem diameter, SPAD value, root length and seedling length. Fresh and dried root, stem and leaf biomass.

2.3.2. Mineral Profile in Root and Leaves Tomato Seedlings

Once the samples of the treatments with Ca levels and peroxide treatments have been quantified. The processing of the samples was developed according to those described by García-Gaytán *et al.* [19] and Bautista *et al.* [20]: the total fresh biomass was quantified, then it was introduced in an oven (Felissa®, model Fe-292 AD) at 60 °C for 72 hours. The resulting dry biomass weight was milled in an Osterizer blender. The samples, once ground, were put into capsules for a micro-grinding process. The micro-grinding was carried out in a mill (Retsch, MM400) for 9.0 minutes. To obtain a pellet of the particles of the micro-grinding, the sample was subjected to a hydraulic press (Retsch, PP25) at 20 tons of pressure. The resulting tablet was placed in an aluminum mold. The relative content was determined in a scanning electron microscope (Scanning Electron Microscope, Model 7582, England), equipped with energy-dispersive spectroscopy. The value of the elementary composition corresponds to the average of three replicates between the treatments.

2.4. Statistic Analysis

Data were analyzed using SAS software ver. 9.3. An analysis of variance (Proc ANOVA) was performed. The comparison of means was made using the Tukey test with a significance value of 95% ($P \leq 0.05$) in order to determine the significant differences between the variables.

3. Results and Discussion

Figure 1 shows the characteristics of roots, stem, and leaves of tomato seedlings in response to Hoagland's nutrient solution, with the addition of different levels of calcium (mM). While **Figure 2** shows a higher biomass of leaves and stems in the seedlings with the immersion of roots in H₂O₂. Although there is a greater abundance of root hairs, necrosis is also evident (**Figure 2**).

3.1. Growth, Fresh and Dry Biomass without Peroxide Immersion

The statistical analysis showed that root length, seedling height, stem diameter, chlorophyll content and fresh root biomass did not present statistically significant differences ($P \leq 0.001$) with the NS or with the additions of the levels of Ca (**Table 1**). There were statistically significant differences in fresh stem and leaf



Figure 1. Tomato seedlings with different calcium levels without peroxide immersion. (A) Control; (B) 25 mM; (C) 50 mM; (D) 100 mM. Bar 30 cm.



Figure 2. Tomato seedlings with different levels of calcium and immersion in peroxide for 8 hours. (A) Control; (B) 25 mM; (C) 50 mM; (D) 100 mM. Bar = 30 cm.

Table 1. Nutritive solution and different levels of calcium on growth, fresh biomass and dry biomass in tomato seedlings.

Growth variable	Nutrient solution				C.V	P-value
	Control	25	50 (mM)	100		
Length root (cm)	26.98 a	29.74 a	24.98 a	26.64 a	17.61	0.86
Plant length (cm)	19.72 a	20.08 a	16.12 b	17.62 ab	7.61	8.81
Stem diameter (mm)	4.12 a	4.13 a	3.37 a	4.69 a	13.43	2.22
Chlorophyll (SPAD index)	34.66 a	39.12 a	31.20 a	38.40 a	13.35	2.93
Fresh biomass of root (g)	0.82 a	0.99 a	1.36 a	0.93 a	51.13	0.95
Fresh biomass of stem (g)	2.17 ab	2.48 ab	1.68 b	2.89 a	22.44***	4.34
Fresh biomass of leaves (g)	1.41 b	1.66 ab	1.47 ab	2.31 a	27.60***	3.79
Dry biomass of leaves (g)	0.15 a	0.22 a	0.13 a	0.23 a	52.62	1.16
Dry biomass of stem (g)	0.09 a	0.11 a	0.15 a	0.13 a	79.86	0.38
Dry biomass of root (g)	0.02 a	0.03 a	0.03 a	0.03 a	49.74	0.20

Means followed by different letters in each column indicate significant differences among treatments (*** $P \leq 0.001$; ** $P \leq 0.01$). C.V coefficient of variation.

biomass when 100 mM Ca was added to the SN, the increase was 25% and 38% compared to the control (Table 1). There were no significant statistical differences ($P \leq 0.001$) between treatments with the variables dry biomass of leaves, stem and root (Table 1).

3.2. Multielemental Microanalysis in Leaves, without Peroxide Immersion

In the multi-elemental microanalysis for the leaves, only nine nutrients (macro- and micronutrients) were detected among the treatments (Table 2). Statistical analysis showed that there were no statistically significant differences for C, O, N, P, K, Mg, and S with NS and the different levels of Ca (Table 2). However, it was observed that as the level of Ca (100 mM) in the NS increased, the Ca content in the leaf increased by 34.50% compared to the control treatment (Table 2). There were statistically significant differences ($P \leq 0.001$) for Cl, since with the addition of 50 mM Ca in the NS, it increased by 13.15% compared to the control.

3.3. Growth, Fresh and Dry Biomass, with Peroxide Immersion

The statistical analysis showed that the immersion of roots in peroxide in a period of 8 hours had no effect on the variable root length, stem diameter, chlorophyll content, fresh root biomass and fresh stem biomass (Table 3). There were statistically significant differences in seedling height, with the 25 mM Ca treatment, the increase was 19.75% compared to the control. There were statistically significant differences for the fresh biomass of leaves with 100 mM Ca, the increase was 60.80% compared to the control (Table 3). There were no statistically significant differences for the variables of dry biomass of leaf, stem, and root. A

Table 2. Mineral nutrients in leaves due to the effect of different levels of calcium.

Leaf mineral nutrients	Nutrient solution					C.V	P-value
	Control	25	50 (mM)	100			
C (%)	49.66 a	49.96 a	49.38 a	48.71 a	1.79	1.07	
O (%)	41.09 a	41.13 a	39.78 a	41.43 a	1.71	3.56	
N (%)	3.00 a	3.05 a	3.56 a	3.69 a	15.58	1.39	
P (%)	0.71 a	0.56 b	0.75 a	0.41 c	8.20	29.08	
K (%)	2.51 a	1.98 b	2.78 a	1.91 b	5.49	32.90	
Ca (%)	1.86 c	2.33 b	2.52 ab	2.84 a	5.90***	25.46	
Mg (%)	0.36 a	0.35 a	0.40 a	0.29 a	15.13	2.19	
S (%)	0.26 a	0.26 a	0.31 a	0.25 a	17.11	0.86	
Cl (%)	0.33 ab	0.26 bc	0.38 a	0.21 c	14.07***	9.10	

Means followed by different letters in each column indicate significant differences among treatments (** $P \leq 0.001$; ** $P \leq 0.01$). C.V coefficient of variation.

Table 3. Nutritive solution and different levels of calcium on growth, fresh biomass and dry biomass in tomato seedlings with peroxide immersion.

Growth variable	Nutrient Solution					C.V	P-value
	Control	25	50 (mM)	100			
Length root (cm)	31.14 a	33.80 a	21.42 a	26.88 a	24.98	2.31	
Plant length (cm)	19.50 ab	24.30 a	16.80 b	22.08 ab	12.54***	6.34	
Stem diameter (mm)	4.15 a	4.66 a	4.18 a	5.02 a	18.61	1.99	
Chlorophyll (SPAD index)	39.92 a	43.44 a	36.27 a	50.38 a	18.26	1.07	
Fresh biomass of root (g)	2.44 a	3.73 a	1.39 a	5.64 a	3.42	57.69	
Fresh biomass of stem (g)	3.14 ab	5.09 a	2.70 b	7.21 a	34.11	6.69	
Fresh biomass of leaves (g)	1.41 b	3.06 ab	1.71 b	4.52 a	33.76***	7.11	
Dry biomass of leaves (g)	0.28 a	0.46 a	0.21 a	0.60 a	45.64	3.73	
Dry biomass of stem (g)	0.26 a	0.40 a	0.15 a	0.96 a	110.36	2.0	
Dry biomass of root (g)	0.12 a	0.20 a	0.06 a	0.28 a	58.29	3.70	

Means followed by different letters in each column indicate significant differences among treatments (** $P \leq 0.001$; ** $P \leq 0.01$). C.V coefficient of variation.

study by Souri and Dehnavard [24] on 17-week-old tomato plants found that Ca nitrate significantly affected plant height, leaf area, and number of lateral roots compared to urea, and sulfate of ammonium.

3.4. Multielemental Microanalysis in Leaves and Root, with Peroxide Immersion

When the roots are exposed to peroxide and then restored in nutrient solution, it was shown that those with 25 mM Ca, carbon in leaves increased by 1.46% compared to the control and by 7.33% compared to the treatment with 50 mM Ca (Table 4). There were no statistically significant differences for O and N. The

Table 4. Multielemental microanalysis in leaves and roots in tomato seedlings with roots immersion in peroxide.

Leaf mineral nutrients	Nutrient solution					<i>P-value</i>
	Control	25	50 (mM)	100	C.V	
C (%)	50.40 ab	51.15 a	47.40 b	48.56 ab	1.75***	7.41
O (%)	41.03 a	40.67 a	40.93 a	41.91 a	1.08	3.52
N (%)	2.89 a	2.20 a	3.18 a	3.03 a	18.09	3.04
P (%)	0.38 c	0.43 bc	0.68 a	0.33 b	11.37***	16.83
K (%)	1.48 c	1.53 c	2.11 a	1.20 d	4.50***	54.60
Ca (%)	2.36 c	2.85 bc	3.44 ab	4.02 a	8.21***	17.80
Mg (%)	0.39 a	0.30 a	0.32 a	0.34 a	15.81	1.30
S (%)	0.57 a	0.32 b	0.23 b	0.25 b	8.33***	62.91
Cl (%)	0.32 abc	0.26 bc	0.49 a	0.16 c	20.18***	10.68
Fe (%)	0.12 a	0.10 c	0.11 b	0.07 d	0	infin

Root mineral nutrients	Nutrient solution					<i>P-value</i>
	Control	25	50 (mM)	100	C.V	
C (%)	50.59 a	50.34 a	50.11 ab	49.22 ab	1.19	4.80
O (%)	42.83 a	42.64 a	42.88 a	43.60 a	0.88	3.12
N (%)	2.34 a	2.90 a	3.22 a	2.50 a	19.20	2.0
P (%)	0.29 ab	0.40 a	0.38 a	0.25 b	11.06	9.40
K (%)	2.52 a	2.11 bc	1.98 c	1.32 d	4.84***	63.79
Ca (%)	0.34 b	0.82 b	0.69 bc	2.06 a	9.80***	130.86
Mg (%)	0.25 b	0.32 ab	0.29 ab	0.42 a	16.11***	5.41
S (%)	0.18 a	0.19 a	0.21 a	0.15 a	16.67	1.51
Cl (%)	0.20 a	0.14 ab	0.11 b	0.09 b	17.37***	8.12
Fe (%)	0.05 b	0.00 c	0.00 c	0.13 a	0	infin

Means followed by different letters in each column indicate significant differences among treatments (*** $P \leq 0.001$; ** $P \leq 0.01$). C.V coefficient of variation.

statistical analysis showed that with 50 mM of Ca in the NS, the concentration of P and K in leaf tissue increased by 44.11% and 29.85% compared to the control (Table 4). The Ca concentration in the leaf increased with the 100 mM Ca treatment by 41.29% compared to the control (Table 4), and higher by 29.35% compared to the Ca concentration in the treatment without peroxide immersion (Table 2). There were no significant differences for Mg and Cl between treatments. The S and Fe in leaf tissue was significantly higher in the control treatment by 56.14% and 8.33% compared to the treatment with 100 mM Ca (Table 4).

The values in tomato roots once exposed to peroxide and its reconditioning to NS are shown in Table 4. The multielemental microanalysis detected 10 nutrients in the root (macro- and micronutrients). The statistical analysis showed that there were no significant statistical differences for C, O, N, P, and S. The root control treatment presented significant statistical differences for K and Cl,

as it was higher by 47.61% and 55% with respect to the treatment with 100 mM Ca (**Table 4**). The statistical analysis showed that with 100 mM of Ca in the NS, the Ca and Mg in the root increased by 83.48% and 40.47% (**Table 4**).

Although, in the experiment without root immersion to peroxide, it presented statistically significant differences for fresh stem biomass and fresh leaf biomass (2.89 g and 2.31 g) (**Table 1**). However, the values in the growth parameters and dry weights were lower than the results observed in the experiment with root immersion (**Table 3**), for example, H₂O₂ induced an increase in root length and seedling height in a 12.01% and 17.36% (**Table 3** and **Figure 2**), with respect to the treatment without immersion (**Table 1** and **Figure 1**). While H₂O₂ had a positive effect on growth, however, when tomato plants are subjected to salinity stress (NaCl), it is accompanied by significant reductions in shoot weight, plant height and number of sheets [25].

Plants uptake and translocate nutrients from the root to the conducting vessels (xylem and phloem) [20]. Nutrients are redistributed to the entire system, and in the case of the tomato, the greatest demand for nutrients is given to developing organs such as leaves, flowers, and fruits [20].

The O and N values did not differ between the experiments with and without H₂O₂ in the leaf tissue (**Table 2** and **Table 4**). For the case of P, K, and Mg (0.75, 2.51, 0.40%) the concentration in foliar tissue was higher (**Table 2**), with respect to plants with root immersion for the same macronutrients P, K, and Mg (0.68, 2.11, and 0.34%, respectively) (**Table 4**). H₂O₂ induced the concentration of C, Ca, S, Cl and Fe (51.15%, 4.02%, 0.57%, 0.49% and 0.12%) in the leaves (**Table 4**) being higher than the same nutrients C, Ca, S and Cl (49.96, 2.84, 0.31 and 0.38%) reported in the experiment without immersion in peroxide (**Table 2**).

The majority accumulation of nutrients in the root due to the effect of immersion in H₂O₂ were C, O, N, K, Ca, Mg, P, S, Cl, and Fe (50.59%, 43.60%, 3.22%, 2.52%, 2.06%, 0.42%, 0.40%, 0.21%, 0.20%, and 0.13%, respectively). However, in this case K, Ca, Mg, S, Cl, and Fe presented statistically significant differences (**Table 4**). Bautista *et al.* [20] performed a multi-element analysis on roots, stem/branches and leaves in cherry tomato plants in fruiting stage. They found that the concentration of mineral nutrients in roots such as C, O, N, P, K, and Ca, were below the values observed in roots with immersion in H₂O₂ (**Table 4**).

Some nutrients were below the optimal range reported, such as, P, K, and Cl (**Table 2**). The N, Ca, Mg, and S in leaves without root immersion were within the optimal range reported (**Table 2**). Nutrient levels in tomato leaves during the five-leaf phenological stage were 3.0% N, 1.0% Ca, 0.3% Mg, and 0.3% S [26]. In the case of seedlings with root immersion in H₂O₂, K was below the optimal range reported (**Table 4**).

The N, P, Ca, Mg, and S were within the optimal range reported (**Table 4**), according to the values described by Hochmuth *et al.* [26]. For their part, Bautista *et al.* [20] in the multielemental profile in tomato leaves, found in their values that Mg, S, Cl and Fe were higher than the values reported in our experiment with and without the addition of H₂O₂ (**Table 2** and **Table 4**). While C, O, N

and P the highest values were in our experiment, possibly due to the phenological stage (seedling). According to Hartz *et al.* [27] the optimal ranges for the main macronutrients in tomato leaf tissue are 3.89% N, 0.32% P, 2.24% K, 3.04% Ca, 1.25% Mg, and 0.97% S. On the other hand, Campbell [28] reports that the optimal ranges for tomato are 3.5% N, 0.30% P, 3.5% K, 3.0% Ca, 0.35% Mg, and 0.2% S respectively.

4. Conclusions

From the results obtained we conclude the following:

- 1) The experiment with NS and with 100 mM of Ca, increased the fresh biomass of stem and leaf by 25% and 38% compared to the control. The addition of 100 and 50 mM Ca to the NS significantly increased the Ca and Cl content in the leaf by 34.50% and 13.15% compared to the control.
- 2) The experiment with H₂O₂ and with 25 and 100 mM of Ca in the NS increased the seedling height and biomass of fresh leaves. With the different levels of Ca in the NS increased the content of C, P, K, Ca and Cl in the leaf tissue. With the highest Ca level in the NS, Ca and Mg increased at the root by 83.48% and 40.47%. K and Cl increased at the root without any Ca level. With this experiment we demonstrate that multielemental microanalysis is a powerful, non-destructive, fast and accurate tool for the determination of plant nutrients.

Acknowledgements

Engineer Esteban Sánchez-Rodríguez, analyst at LADiPA.

Conflicts of Interest

The authors declare no conflict of interest regarding the publication.

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