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Plant Biostimulant Effects of Baker's Yeast Vinasse and Selenium on Tomatoes through Foliar Fertilization

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Abstract: The application of selenium (Se) to tomatoes enhances accumulation of bioactive compounds. The physiological window of Se is very narrow, and Se overdose reduces the yield. Glycine betaine was shown to reduce Se's negative effects on plants and to potentiate its beneficial effects. In this study, baker's yeast vinasse (BYV), as an affordable source of glycine betaine, was tested for its interaction with Se in an optimized foliar fertilizer. The application dose was selected after a laboratory experiment, wherein assays on plant height, leaves surfaces, stomatal conductance, and chlorophyll fluorescence were done. The Se and BYV supplemented foliar fertilizers were tested for their effects on accumulation of bioactives in drip-irrigated tomatoes cultivated in a greenhouse. Under laboratory conditions, assays demonstrated Se and BYV induced effects on tomatoes plants. Both the stomatal conductance and photosynthesis efficiency increased compared to a water treated control. The greenhouse experiment demonstrated that BYV and Se addition increases the number of tomato fruits in the "extra" marketable class and enhances the accumulation of ascorbic acid, carotenes, polyphenols, and flavonoids. The effects depend on the composition of the foliar fertilizer, the most significant effects being recorded for the foliar applied product with the highest BYV and nitrogen content.

Keywords: tomato plant growth; crop quality; marketable yield; tomato bioactive compounds; chlorophyll fluorescence; stomatal conductance

1. Introduction

Selenium (Se) was selected by biological systems during the course of their evolution to exert several physiological important functions, the best known being the role as redox and interface modulator [1,2]. Se has been proven to be essential to most of the prokaryotes and to several eukaryotic kingdoms, including algae. In plants and fungi its essentiality seems to be lost, most probably due to lower Se bioavailability in initial terrestrial habitats [3]. However, Se application on plants, as soil or

foliar treatments, produces beneficial effects, such as growth promotion [4]; modulation of nutrient up-take and use [5,6]; increased resistance to biotic [7,8] and abiotic stress [9,10], including drought [11]; and enhanced accumulation of bioactive compounds [12–14].

Regarding tomatoes, the selenium effects on the accumulation of bioactives depend on dose and application route. Foliar treatments with Se increase the accumulation of flavonoids in edible fruits and decrease the levels of phenolic acids [15]. Application to leaves of 1 mg L^{-1} selenium delays the tomato ripening, increases the level of antioxidants, decreases the reactive oxygen species (ROS) formation [15], and determines higher accumulation of flavonoids, vitamin C, vitamin E, soluble sugars, and free amino acids [16].

Schiavon et al. [17] showed that radicular application of low doses of Se, up to 10 µM, stimulates the metabolism of both polyphenols and flavonoids. Higher doses of radicular-applied Se promote glutathione accumulation. The application of 1 mg L^{-1} Se nutrient solution on hydroponic grown cherry tomatoes, for five weeks prior to harvest, determined an increase of fruit firmness and enhanced the accumulation of bioactives, such as vitamin C and antimicrobial compounds [18].

The accumulation of bioactives in tomatoes after Se treatment is important because two important targets related to human health are reached: (i) safe food chain biofortification with selenium and (ii) enhanced accumulation of health-promoting phytochemicals [17]. Traditionally, the bioactivity of tomatoes was related to carotenoids and vitamin C [19]. However, in the last few decades, polyphenols and flavonoids were demonstrated to be other important health-promoting phytochemicals from tomatoes [19,20]. These lipophilic (carotenoids), amphiphilic (polyphenols and flavonoids), and hydrophilic (vitamin C, glutathione) antioxidants from tomatoes are a complex phytochemical system, important for preventing cancers [21] and cardiovascular diseases [22]. Such antioxidants were demonstrated to have a complementary action to that of selenium [23].

Vinasse is a by-product of industrial cultivation of yeast on molasses or on raw sugar juices; it results from anaerobic alcoholic fermentation [24] for (bio)ethanol [25] or for alcoholic distilled beverage [26] production, or from aerobic biosynthesis of bakery yeast [27] or of amino/organic acids [28]. Despite variability, all types of vinasse have a specific characteristic—a large content of glycine betaine, usually between 15% and 20%, accumulated during sugar refining process from the initial plant material [29]. Glycine betaine is an osmoprotectant/compatible solute, i.e., a substance highly soluble in water, which protects the proteins from denaturation and enhances plant tolerance to abiotic stress [30,31]. Exogenous glycine-betaine, applied as foliar treatment to tomatoes, enhances plant response to various types of abiotic stress—drought [32,33], salinity [32,34], chilling [35], high-temperatures [36], and water logging [37].

Se application on tomatoes determines negative effects as well. Both foliar and radicular applications were proven to reduce nitrogen accumulation [5]. The ripening delay determined by Se application is associated with a reduced accumulation of β-carotene in tomato fruits [38]. Se has a very low physiological window [9], lower than an order of magnitude. For hydroponically grown tomatoes, the toxicity-threshold value was calculated to be 1.27 mg L^{-1} [39]. The response to Se treatment depends on tomatoes cultivars [40]; thus, the risk of overdosing is high. On sensitive cultivars, Se could induce distorted protein structure and function, and oxidative stress [41]. Therefore, a solution to reduce the negative effects and the over-dosing risks of selenium application is necessary.

Previous studies showed that foliar application of glycine betaine together with selenium reduces the drawbacks of selenium treatments and amplifies the positive effects on plants, enhancing Se protection against abiotic stress and the health-related biofortification [42–44]. One of our objectives for the present study was to determine the influence of baker's yeast vinasse, as a source of affordable glycine betaine, applied together with selenium, on the accumulation of bioactives in tomato fruits.

Vinasse was used mainly as organic soil fertilizer [45]. However, soil fertilization does not benefit from betaine's osmoprotectant effects like foliar fertilization does. Very few attempts were made to use vinasse as a foliar fertilizer and to benefit from its betaine content. A formulation with both selenium

and baker's yeast vinasse (BYV) glycine betaine should benefit from their synergic effects on protecting plants against water stress too [42,43].

The objectives of our present study were: (i) to develop an organo-mineral foliar fertilizer enriched with baker's yeast vinasse and selenium; (ii) to optimize the dose and number of treatments under laboratory conditions, by assaying plant heights, leaves surfaces, stomatal conductance, and chlorophyll fluorescence; and (iii) the effects of treatment with such foliar fertilizers, containing Se and BYV (plant biostimulants), on accumulation of bioactive compounds in tomatoes grown under protected and well-watered conditions—drip irrigated greenhouse.

2. Materials and Methods

2.1. Plant Material and Baker's Yeast Vinasse

Two cultivars of tomato (*Solanum lycopersicum*) were used: Micro-Tom and Prekos F1. The Micro-Tom cultivar was grown under controlled conditions in a climatic chamber (Economic Lux Chamber with Imago 500 controller, model ECD01E, Snijders, Tilburg, The Netherlands), and in a greenhouse, on a vegetable growing substrate (Canna Terra Professional Plus, Canna, Oosterhout, The Netherlands). Hybrid Prekos F1 was cultivated in greenhouse, on a cerno-cambic hortic anthrosol. The concentrated baker's yeast vinasse used in this study, which is produced by Rompak (Pașcani, Romania), contains 3.0% ± 0.2% total nitrogen, 0.5% ± 0.1% total phosphorus, 7.0% ± 0.3% total potassium, and is certified as fertilizer for the organic farming systems [45].

2.2. Assays for Quantification of Baker's Yeast Vinasse Composition

The following ingredients of baker's yeast vinasse were assayed in 5 different batches: mineral nutrients, betaine, proteins/peptides, and polyamines. These were considered to influence plant nutrition, either directly (i.e., the mineral nutrients), or either indirectly—betaine, proteins/peptides, and polyamines. The latter could act as plant biostimulants, enhancing nutrient uptake and nutrient use efficiency [46].

2.2.1. Mineral Nutrients Analysis

Total Kjedahl nitrogen (TKN) was determined according to the EN 15478:2009 method. The analytic system consisted of an infrared rapid digestion equipment (Behrotest® InKjel P, Behr Labor-Technik, Dusseldorf, Germany), a process suction automatic scrubber (Behrosog S4, Behr Labor-Technik) for neutralization of H_2SO_4 vapors, fully automatic steam distillation equipment (Behrotest S4® WD 40, Behr Labor-Technik), and an orbital shaker (Rotamax 120, Heidolph, Schwabach, Germany). Briefly, a 2 mL sample was mineralized in an 800 mL Kjeldahl flask with 20 mL H_2SO_4 98%, one Kjeldahl tablet (5.0 g K_2SO_4 + 0.5 $CuSO_4$), and 10 mL distilled water. Ammonia distillation was performed by adding 40 mL NaOH 30% to the digestion vessel. In the absorption vessel of the Behrotest® S4 distillation system, 50 mL H_2SO_4 0.05 mol L^{-1} were added, with 0.5 mL mixed indicator. The excess of acid was titrated with standard 0.1 mol L^{-1} NaOH solution in the presence of the mixed indicator.

Phosphorus (P) was determined after extraction, by gravimetric determination according to ISO 6598:1996 method. An aliquot of 2 mL of the sample was digested with a mixture of HNO_3 65% and H_2SO_4 98%, for 45 min. To the acid digested solution, precipitating reagent (ammonium quinoline molybdate) was added by dripping for 15 min. After filtration through a G4 filter crucible, the precipitate was dried in a controlled oven (250 ± 5 °C) until constant mass, and then it was cooled and weighed.

Potassium (K) was determined according to EN 15477:2009 method. Briefly, a 2 mL sample with 50 mL distilled water was heated and boiled for 30 min and then quantitatively transferred into a volumetric flask of 100 mL with distilled water. To an aliquot of 25 mL, taken from the volumetric flask, 10 mL EDTA, a few drops of phenolphthalein solution, and 1 mL 30% NaOH solution were added, the latter being added dropwise. For precipitation, 10 mL sodium tetraphenylborate solution was added

dropwise with continuous stirring. The precipitate was filtered through the filter crucible and dried in a controlled oven (120° ± 10 °C), till a constant mass.

The determination of the concentrations of microelements Cu, Mn, Mg, Fe, Zn, and Mo, was performed by inductive plasma optical emission spectrometry (ICP-OES) method, after the sample was extracted in water, using a plasma inductive coupling spectrometer Optima 2100 DV (Perkin Elmer, Waltham, MA, USA). The quantification of the microelements was done by using calibration curves and standard reference material (Certipur® ICP, Merck Group, Darmstad, Germany) and 100 mg L^{-1} stock solution of Quality Control Standard (Perkin Elmer).

The determination of Se was done with Inductively coupled plasma-optical emission spectrometry (ICP-OES) using an Optima 2100 DV (Perkin Elmer, Waltham, MA, USA) instrument equipped with the Agilent Technologies Multimode Sample Introduction System (MSIS) (Santa Clara, CA, USA) [47]. The concentrated hydrochloric acid (37%) used for standard and sample preparation was from Merck Group (Darmstadt, Germany). The reductant used to generate the hydride vapor was 1.5% (*w*/*v*) sodium borohydride ($NaBH_4$) in 1.0% (*w*/*v*) sodium hydroxide (NaOH) from Scharlau (Barcelona, Spain). All the reagents used for selenium determination were of analytical grade.

2.2.2. Glycine Betaine Assays

The determinations were performed on a liquid chromatography Agilent 1260 Infinity system coupled with a mass spectrometer and time-of-flight detector 6224 TOF LC/MS (Agilent Technologies, Santa Clara, CA, USA). The system was equipped with a Zorbax Extend C18 column, 1.8 µm, 10 × 2.1 mm (Agilent Technologies). A mixture of 1% mobile phase A: 99% mobile phase B (*v*/*v*) was used. Mobile phase A was prepared from 0.1% formic acid in 5% methanol and 1 mM ammonium formate, and mobile phase B consisted of 0.1% formic acid in 98% methanol and 1 mM ammonium formate. The reference stock solution consisted of a 0.5 mg mL^{-1} concentrated solution of 99.3% betaine standard (TraceCERT® certified reference material, Sigma-Aldrich, Merck Group, Darmstadt, Germany) dissolved in MilliQ water.

2.2.3. Protein Determination

The protein concentration in vinasse was determined with Bradford assay (using Bradford reagent, Sigma-Aldrich, Merck Group). A calibration curve with bovine serum albumin (BSA, chromatographically purified, Sigma-Aldrich, Merck Group) was done. The absorption was read at 595 nm, in 96 well Nunc plates with flat bottoms, using a microplate reader (CLARIOstar, BMG Labtech, Ortenberg, Germany), with number of flashes per well 22 and settling time 0.5 s. The initial concentration of protein in vinasse was expressed as mg/mL, by interpolating the absorbance in the calibration curve and multiplying the measured concentration with the dilution factor. The UV–Vis–NIR spectrum of centrifuged vinasse was recorded between 200 and 1000 nm, integration time 4000 µs, 3 spectra averaged, with an UV–Vis–NIR spectrophotometer with optical fibers (Ocean Optics, Largo, FL, USA).

2.2.4. Polyamine Assay

The polyamines from the concentrated baker's yeast vinasse were determined after separation of their dansyl chloride, 5-(dimethylamino) naphthalene-1-sulfonyl chloride derivatives, by high pressure liquid chromatography with fluorescent detection [48]. The system was an Agilent 1200 HPLC (Agilent Technologies) with fluorescent detector (FLD). The chromatographic separation was done on a Kromasil SB-C18 column (46 mm × 150 mm, 5 µm). The analytical standards of polyamines: cadaverine, putrescine, histidine, spermine, spermidine, and 1,7-diaminoheptan, used as internal standard [48], were purchased from Sigma-Aldrich, Merck Group. The diluted samples and the reference analytical standards were treated with dansyl chloride (HPLC derivatization grade, ≥98%, Sigma-Aldrich, Merck Group), and the hydrophobic fluorescent derivatives were extracted with diethyl ether (for HPLC, ≥99%, Sigma-Aldrich, Merck Group) [49] and evaporated to dryness. The remaining residue was

re-dissolved in acetonitrile, filtered through a 0.45 µm syringe filter into an injection vial, and injected on the chromatographic column.

2.3. Preparation of Foliar Fertilizer with BYV and Se

The concentrated baker's yeast vinasse (BYV) was combined with mineral solutions, including Se salts, to form different types of foliar fertilizers. Depending on the BYV concentration and the order of the mineral solutions addition, stable and unstable formulations resulted. For a stable formulation, no precipitate was formed during 12 months of storage at room temperature. For an unstable formulation, fine precipitate was formed. From the unstable formulation, the precipitate was separated by centrifugation, at 10,125× *g*, 5 °C, for 15 min (5810 Eppendorf centrifuge, swing bucket rotor) and analyzed by X-ray diffraction (XRD). XRD data were obtained using a Rigaku SmartLab diffractometer (Rigaku, Tokyo, Japan), emitting $CuK_{\alpha 1}$ (λ = 1.54059 Å) radiation, to identify the nature of precipitate and to optimize the order of mineral addition.

Two foliar fertilizer formulations which did not present vinasse components precipitation were selected. The first one, coded LegoFert 1, was a 1:1:1 type NPK fertilizer, equivalent to an ideal $N:P_2O_5:K_2O$ = 60:60:60, and included the micro-elements B, Fe, Cu, Mn, Mg, Zn, Mo, and Se. Within this type of fertilizer formulation, the final concentration of baker's yeast vinasse was 12%, the maximum concentration at which no precipitation was observed. The second formulation, coded LegoFert 2, was a 3:1:1 type NPK fertilizer, equivalent to an ideal $N:P_2O_5:K_2O$ = 105:35:35 and contained the same micro-elements as LegoFert 1. This type of fertilizer supported higher concentrations of vinasse without precipitation, and a final concentration of 16% vinasse was used. A reference mineral fertilizer, called LegoFert, with a mineral composition as LegoFert 1, but without vinasse, was also produced as control. The analysis of the main components of the foliar fertilizers, mineral nutrients and betaines, was done with the methods presented above (Sections 2.2.1 and 2.2.2).

2.4. Tests of Foliar Fertilizers with BYV and Se under Controlled Conditions

Seeds were sterilized by 5 repeated washes with ethanol and bleaching solutions. The sterilized seeds were germinated in aseptic conditions, in sterile Petri dishes ∅ 9 cm, which contained a sterile cotton wool bed, moistened with sterile pure water. The germinated seeds were transferred to small pots containing growing substrate (Canna Terra Professional, Canna), sterilized by ^{60}Co γ-radiation. The plants were maintained for 30 days in the climatic chamber. The light intensity was set at 240 µmoles $m^{-2}s^{-1}$. The light/dark period was set at 16/8 h, with 25 ± 1 °C temperature during the light period and 18 ± 1 °C during the dark period. After 30 days the plants were transferred to larger pots (with the same growing substrate) and to greenhouse. Three different foliar spraying solutions were prepared using appropriate dilutions (1%, 2%, and 3%, *v*/*v*) of the foliar fertilizers coded LF, LF1, and LF2. A control, without foliar application of fertilizer, was sprayed only with water, and a reference product, Cropmax® organo-mineral fertilizer (Holland Farming, Groenekan, The Netherlands), with 0.2% N, 0.4% P_2O_5, 0.02% K_2O, pH 4.5, which contains micronutrients (Fe, Zn, Mn, B, Ca, Mo, and Co), plant amino acids, and plant extracts, applied at 0.2% concentration; treatments, were also included in the experiments. All treatments were performed in triplicate, with 25 tomato plants per each replicate. On tomato plants were applied 1, 2, or 3 treatments according to the following schedule: 1st application at 22 days from germination; 2nd application at 39 days from germination; 3rd application at 73 days from germination. The final measurements were performed at 95 days from germination.

The following features were evaluated in order to select the dose and the number of treatments: plant heights, average leaf surface per plant, stomatal conductance, and leaf fluorescence. The plant height was measured with a digital caliper. For the determination of the leaf surface by image analysis, a morphometric method based on Quick Photo Micro 2.3 software (Promicra, Prague, Czech Republic) was used [50]. Briefly, leaves images were taken by a high resolution camera (Canon EOS 5D Mark IV DSLR 30.4MP CMOS, Canon, Tokyo, Japan), using a macro-objective (Canon EF 100 mm f/2.8 L Macro IS USM Lens, Canon), under constant LED illumination (Kast Led Copy Stand, Kathay Technology

Industrial, Hongkong, China). Within the leaves images, the leaves were selected and measured. The stomatal conductance (nmol·m^{-2}s^{-1}) of tomato leaves was measured with a Delta T AP4 porometer (Delta-T Devices, Burwell, UK). The measurements of chlorophyll fluorescence of tomato leaves were done with a PAM fluorometer (Walz PAM 2500, Effertlich, Germany). The determinations were made on several representative, healthy plant upper leaves from each repetition (10 leaves randomly chosen). Before starting the determination, the leaves were kept in the dark for 30 min, by using a brown paper bag, then the saturation light pulses were applied. The photosystem II (PSII) efficiency was determined as the ratio between F_v, variable fluorescence, and F_m, the maximum fluorescent yield in the dark-adapted state.

2.5. Test under Greenhouse Conditions

The tests under greenhouse conditions were carried-out in Solarium 1, Vegetation Department, V. Adamachi Horticulture Farm, Faculty of Horticulture, Agricultural Sciences and Veterinary Medicine University, Iași, NE of Romania. The soil from the experimental plot was a cerno-cambic-hortic anthrosol. The main properties of this soil are presented in Table 1.

Table 1. The main properties of the cerno-cambic hortic anthrosol from the experimental site, Solarium 1, Vegetation Department, V. Adamachi Horticulture Farm, Faculty of Horticulture, Agricultural Sciences and Veterinary Medicine University, Iași.

The Main Physical, Chemical and Biological Properties of the Soil	Genetic Horizons/Depth (cm)			
	Amho (0–20 cm)	**Amho (20–40 cm)**	**AB (40–60 cm)**	**Bv (60–100 cm)**
Soil texture (% colloidal clay)	36.0	38.1	40.3	44.8
Dry summer consistency of the soil	Moderate cohesive	Very hard	Very hard	Very hard
Soil reaction (pH H_2O)	6.25	6.66	7.02	7.45
Humus content (%)	3.01	2.22	1.08	0.61
Total nitrogen content (Nt %)	0.20	0.18	0.11	0.06
Mobile phosphorus content (ppm)	51	44	32	45
Mobile potassium content (ppm)	225	201	147	153
The degree of saturation in bases (V %)	88	89	91	93
Dehydrogenase (mg formazan)	18.56	10.11	3.52	2.21

The monofactorial experiment was organized in a completely randomized block design with 4 treatments: (1) control; (2) Lego–Fert formulation, 1:1:1 type NPK fertilizer, with micro-elements B, Fe, Cu, Mn, Mg, Zn, Mo, and Se, and without BYV; (3) LegoFert 1, 1:1:1 type NPK fertilizer, with micro-elements B, Fe, Cu, Mn, Mg, Zn, and Mo, 12% BYV, and Se; (4) LegoFert 2, 3:1:1 type NPK fertilizer, with micro-elements B, Fe, Cu, Mn, Mg, Zn, and Mo, 16% BYV, and Se. Each treatment was applied in three replications. The tomato plants were obtained from six-week-old seedlings, and transplanted at 0.35 m between plants in a row. The distance between two rows was 0.8 m, according to the recommended cultivation technology for Prekos F1 hybrid. Each plot consisted of 35 m^2, and included 5 rows, with 20 plants per rows; i.e., 100 plants per repetition. Buffer zones consisting of 2 m wide bare soil were maintained between plots by two weeks of repeated hoeing.

A dripping irrigation system for tomato cultivation was installed into this experimental plot. During the experimentation period the cultivated tomatoes were irrigated according to an automated schedule. The automated schedule was controlled by an electronic tension switch (400C, Tensio-Technik, Geisenheim, Germany), connected to a tensiometer (LT1 28 cm, Tensio-Technik), with the porous cup placed at 25 cm depth. The irrigation was started when a specific soil water potential of −400 hPa was recorded and stopped when the soil water potential reached the set-point value of −100 hPa [51].

The foliar fertilizers, LF, LF1, and LF2, were applied with a dose of 3 L/ha, equivalent to 300 L of 1% foliar fertilizer (*v/v*) normalized spraying norm per ha. The treatments were applied using a backpack sprayer SG20 (Stihl AG, Waiblingen, Germany), with a flat jet and low drift nozzle (TeeJett® flat-fan TT11002 model, Spraying Systems, Wheaton, IL, USA). The foliar fertilizer was applied three

times, in the following tomato growing stages: first treatment—two weeks after the transplanting of tomato seedlings; second treatment—during the flowering growth stage; third treatment—during the growth and development of the fruits. The control was sprayed with the water used to prepare the foliar fertilizer solution.

The tomatoes were grown according to the recommended technology for the Prekos F1 hybrid in a greenhouse, starting from 6 May 2019 until 12 August 2019. The weed control was done by hoeing repeatedly every two weeks, on rows and intervals. The plant pathogens and pest were controlled by using recommended pesticides, applied according to an integrated pest management schedule.

The fruits from each fruiting wave were harvest and the fruits were weighted, and the total weights of tomatoes per treatment were summarized. The fruits were analyzed for their quality class as fresh tomatoes supplied to consumers, according to Regulation (EU) 543/2011. The fruits from "extra" class were defined as the firm fruits, with a color specific to the beginning of the ripening stage for Prekos F1 cultivar, without circular or radial cracks in the skin, without greenbacks, symptoms of insects or diseases damage, or other defects. The fruits from the class one were defined as the fruits with minor (less than 1 cm) healed skin cracks, with no excessive protuberances, free of greenbacks, and with slight defects. The class two fruits were considered those reasonably firm, with healed cracks (especially radial) longer than 1 cm, with skin defects or bruises, and with defects in shape and coloring.

2.6. Determination of the Influence of the Foliar Fertilization on Bioactives Accumulation

The content of the each of following compounds was determined in the tomatoes: vitamin C (ascorbic acid), β-carotene, total polyphenols, and total flavonoids. Ascorbic acid was determined after extraction of a fruit homogenate with 5% metaphosphoric solution. The extraction ratio of tomato fruit homogenate to 5% metaphosphoric acid was 1:2.5 (*v*/*v*). The not extracted vegetable material was separated by centrifugation at 23,897× *g*, at 15 °C, for 25 min (5810 Eppendorf centrifuge, with fixed angle rotor F-45-30-11). The assay of the ascorbic acid was done by a HPLC-UV method, using 10 mM potassium dihydrogen phosphate buffer adjusted to pH 3.5 and acetonitrile as the mobile phase [52]. As instrumentation, a Zorbax NH2 column and the equipment Agilent 1260 Infinity with diode array and multiple wavelength detector (Agilent Technologies) were used.

β-carotene was determined in tomato fruits according to the method described by Giuntini et al. [53], but slightly modified. Briefly, the tomato fruits were cut into pieces and homogenized, using an Ultra-Turrax homogenizer (Ultra-Turrax®, IKA, Staufen, Germany). From a homogenized sample, the carotenoids were extracted with tetrahydrofuran (THF), concentrated, and re-extracted in dichloromethane and a saturated solution of NaCl. The aqueous phase was washed three times with dichloromethane into a separation funnel. The organic phases were reunited and evaporated under vacuum. A fraction of this final concentrated solution was filtered through a 0.45 µm syringe filter into an injection vial and analyzed by HPLC-UV-DAD. Agilent ZORBAX RRHD EclipsePlus C18, 2.1 × 50 mm, 1.8 µm column, and equipment Agilent 1260 Infinity with UV-DAD were used.

Screening for the total phenols changes after applied foliar fertilization treatments was done by Folin–Ciocâlteu method [54], using known concentrations of gallic acid for the calibration curve. Screening the content of flavonoids was done by the aluminum chloride colorimetric method [55], using quercetin (Q) as the standard for the calibration curve. Samples were extracted under sonication, at room temperature, in an 80% ethanol solution. The modification of the profile of flavonoids following treatments with foliar fertilizer was evaluated by using a HPLC-DAD method [56]. Briefly, 100 mg of freeze-dried tomato powder was repeatedly extracted with 3 portions of 5 mL ethanol, 80%. The reunited extracts were concentrated by nitrogen, blown down, normalized to 2 mL in a volumetric flask, and filtered through a 0.45 µm syringe filter. The analysis was done by a Luna C_{18} column (50 × 2.0 mm i.d., 5 µm; Phenomenex, Torrance, CA, USA) and an Agilent 1260 Infinity with UV-DAD. The flavonoids compounds were identified and quantified using standard retention time and spectral characteristics. The fixed wavelengths were 280 nm (naringenin) and 360 nm (quercetin and rutin). The analysis was performed in triplicates per each samples.

2.7. Statistical Analysis

The data from laboratory and greenhouse experiments on tomatoes were statistically analyzed by analysis of variance (ANOVA), using the SPSS 21 software package (IBM, Armonk, NY, USA). The least significant difference (LSD) test was determined to separate treatment means within each measured parameter, at a significance level of $p \leq 0.05$.

3. Results

3.1. Baker's Yeast Vinasse Analysis and Preparation of the Foliar Fertilizer with BYV and Se

Analysis of five different batches of bakery's yeast vinasse showed that it contained: 150.052 ± 14.73 g·kg^{-1} glycine betaine; 10.12 ± 1.23 mg·kg^{-1} total polyamine, from which putrescine was at 8.68 ± 0.73 mg·kg^{-1}; 9.22 ± 0.79 g·kg^{-1} total proteins; 32.43% ± 2.57% g·kg^{-1} total nitrogen; 5.71 ± 1.14 g·kg^{-1} total phosphorus; and 74.42 ± 3.18 g·kg^{-1} total potassium. Glycine betaine was confirmed to be the main ingredient of bakery's yeast vinasse, accumulated during sugar refining process from the initial plant material [27] and not used by yeast during its development on molasses. Based on this analysis, three batches of fertilizer LF1 and LF2 were prepared. The average compositions of these foliar fertilizers containing plant biostimulants (i.e., selenium and glycine betaine from vinasse) are presented in Table 2.

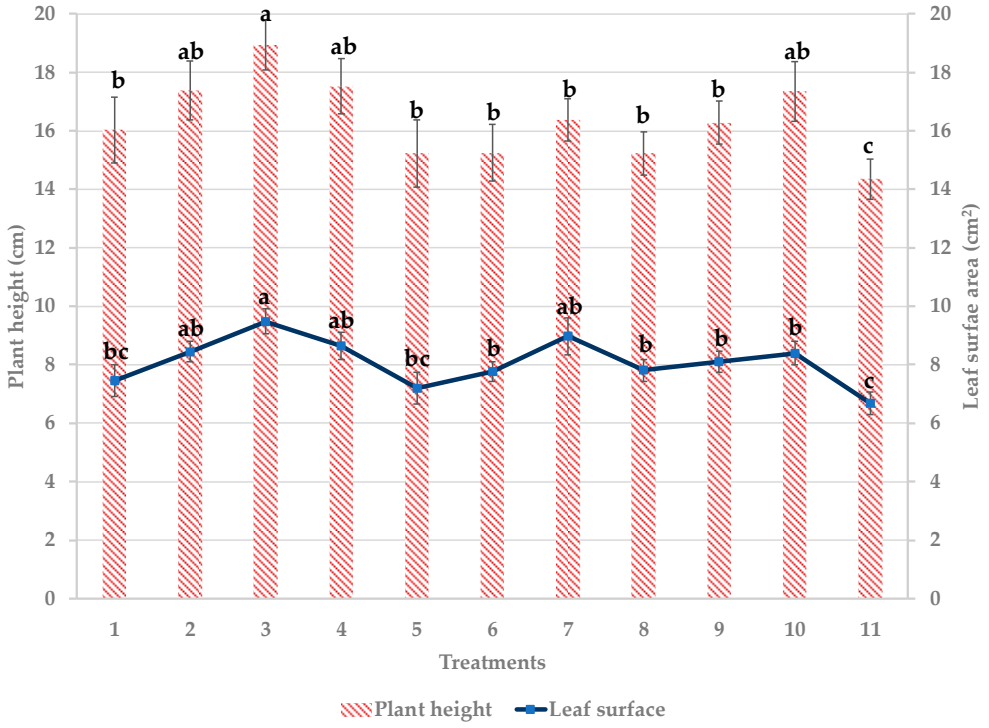
Table 2. Composition of the prepared foliar fertilizers, LF1 and LF2, with bakery's yeast vinasse and selenium, and of the reference fertilizer, LF, without plant biostimulants *.

Analyte	Units	Values *		
		LF	LF1	LF2
N (total)	g L^{-1}	65.7 ± 7.83	65.4 ± 8.02	109 ± 12.3
P (total)	g L^{-1}, as P_2O_5	62.3 ± 1.47	61.5 ± 1.69	36.0 ± 1.17
K soluble water	g L^{-1}, as K_2O	59.3 ± 3.14	58.8 ± 2.86	37.5 ± 0.59
Glycine-betaine (from vinasse)	g L^{-1}	-	1.05	1.60
Fe (λ = 238.204 nm)	g L^{-1}	0.60 ± 0.07	0.62 ± 0.08	0.65 ± 0.05
Mn (λ = 257.610 nm)	g L^{-1}	0.37 ± 0.03	0.38 ± 0.02	0.38 ± 0.02
Cu (λ = 327.393 nm)	g L^{-1}	0.23 ± 0.01	0.23 ± 0.01	0.23 ± 0.005
B (λ = 249.677 nm)	g L^{-1}	0.11 ± 0.01	0.12 ± 0.001	0.11 ± 0.01
Mg (λ = 280.271 nm)	g L^{-1}	0.24 ± 0.03	0.24 ± 0.04	0.25 ± 0.03
Zn (λ = 213.857 nm)	g L^{-1}	0.062 ± 0.014	0.061 ± 0.017	0.063 ± 0.015
Mo (λ = 202.031 nm)	g L^{-1}	0.028 ± 0.003	0.028 ± 0.004	0.028 ± 0.001
Se (λ = 196.026 nm)	g L^{-1}	-	0.073 ± 0.001	0.034 ± 0.003
pH	unit pH	6.55	6.58	6.73
Density	kg L^{-1}	1.175	1.181	1.187

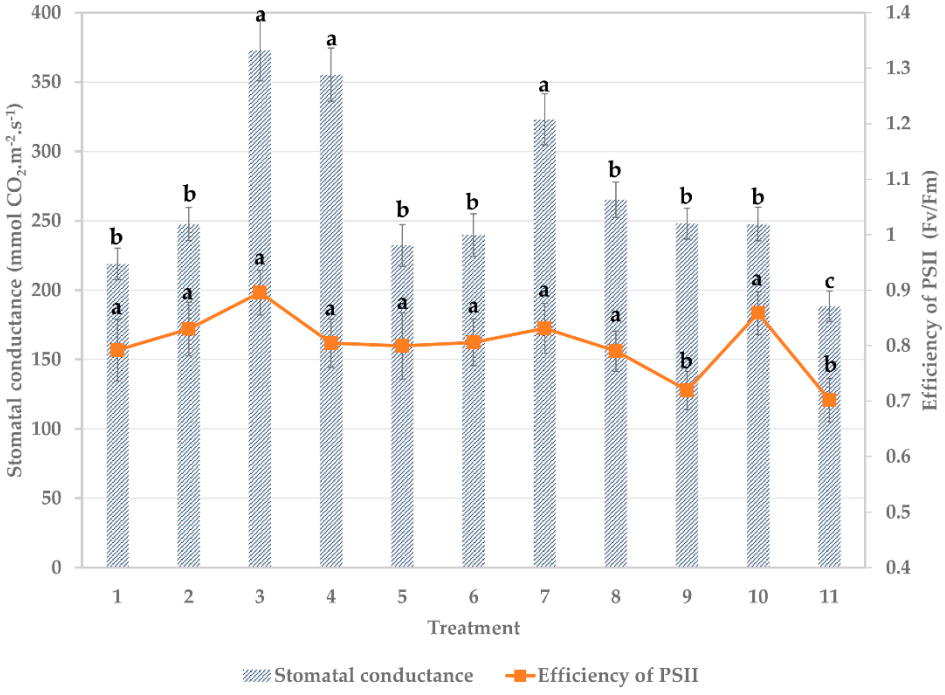
* Results are expressed as mean values ± standard errors (n = 3).

3.2. The Effects of Foliar Fertilizers with BYV and Se on Tomatoes Grown under Controlled Conditions

The foliar fertilizer coded Lego Fert 1 (LF1), containing BYV and Se, was tested on tomatoes (cv. Micro-Tom) grown under controlled conditions. The results related to the morphological (plant height and leaf surface) and physiological (stomatal conductance and efficiency of the photosystem II) characteristics are presented in Figure 1a,b.



(**a**)



(**b**)

Figure 1. The effects of foliar fertilizer Lego Fert 1 on (**a**) morphological (leaf surface, plant height) and (**b**) physiological (stomatal conductance, efficiency of PSII) characteristics of tomatoes (cv. Micro-Tom). Treatments: 1—solution 1%, one treatment; 2—solution 1%, two treatments; 3—solution 1%, three treatments; 4—solution 2%, one treatment; 5—solution 2%, two treatments; 6—solution 2%, three treatments; 7—solution 3%, one treatment; 8—solution 3%, two treatments; 9—solution 3%, three treatments; 10—reference product, 0.2%, two treatments; 11—control, sprayed with water. The values presented represent means ± standard errors (n = 25 plants). Columns labeled with same letter within each parameter do not differ significantly for $p < 0.05$.

Compared to the control, almost all the fertilizer treatments had a significant effect. However, the effects of the foliar fertilizer, including BYV and Se (LF1, LF2), were not in a concentration/dose dependent manner. For 1% LF1, the tomato plants benefit from application of repeated treatments. However, for higher tested concentrations repeated treatment did not have enhanced effects on tomato plants compared to single treatment. Consequently, for larger surface test we chose the concentration of 1%, applied three times.

3.3. The Effects of Foliar Fertilizers with BYV and Se on Production of Tomatoes Grown under Greenhouse Conditions

The effects of the foliar fertilizers with BYV and Se (LF1 and LF2) on the yield of tomatoes (cv. Prekos F1), grown under greenhouse conditions and drip irrigated, are presented in Table 3.

Table 3. The Effects of the prepared foliar fertilizers, LF1 and LF2, and of the reference fertilizer, LF, on the yield of tomatoes (cv. Prekos) grown in greenhouse and drip irrigated *.

Treatment	Average Yield kg m^{-2}	Average Yield Difference kg m^{-2}
Control (sprayed with water)	3.125 ± 0.212 b	-
LF, 3 treatments	4.164 ± 0.244 a	1.039 a
LF1, 3 treatments	4.081 ± 0.306 a	0.955 a
LF2, 3 treatments	4.139 ± 0.187 a	1.013 a

* Values followed by the same letter do not differ significantly for $p < 0.05$.

There are no significant differences regarding the tomatoes yield between the foliar fertilizers with or without BYV and Se, all fertilizers increasing with almost 30% the yield compared to the control. However, the marketable yield of the fruits was more significantly influenced by the treatment with LF1 and LF2 than with LF—Table 4.

Table 4. Effects of prepared foliar fertilizers, LF1 and LF2, and of the reference fertilizer, LF, on the quality of fresh tomatoes (cv. Prekos F1), grown under greenhouse conditions and drip irrigated *.

Treatment	Fruit Quality Class		
	Extra (%)	1st Class Quality (%)	2nd Class Quality (%)
Control (sprayed with water)	10 ± 2 c	28 ± 4 c	62 ± 5 a
LF, 3 treatment	15 ± 2 b	35 ± 3 b	50 ± 5 b
LF1, 3 treatment	18 ± 3 ab	40 ± 4 a	42 ± 6 bc
LF2, 3 treatment	22 ± 3 a	43 ± 4 a	35 ± 3 c

* Values followed by the same letter do not differ significantly for $p < 0.05$.

As was already mentioned, the quality of the fresh tomato fruits was analyzed according to Regulation (EU) 543/2011, for 200 fruits randomly selected from the experimental plants. The application of foliar fertilizers, including BYV and Se, determined an increased number of the fruits from the "extra" class. This increase was higher than two fold compared to control for Lego Fert 2, the product with the highest BYV and nitrogen content. During the classification operation for fruit quality, it was noted that the tomato fruits treated with BYV and Se were firmer fruits, with lower frequency of circular or radial cracks in the skin and with a color specific to the beginning of the ripening stage for Prekos F1 cultivar. Application of the foliar fertilizer without BYV and Se (LF) determined the apparition of longer cracks slight defects compared to control. In the control, the defects on shape and coloring have a higher frequency.

3.4. The Effects of Foliar Fertilizers with BYV and Se on the Accumulation of Bioactive Compounds in Tomatoes Grown under Greenhouse Conditions

The effect of foliar fertilizer with BYV and Se (LF1 and LF2) on the accumulation of bioactive compounds in tomatoes grown under greenhouse conditions is presented in Table 5.

Table 5. The Effects of the prepared foliar fertilizers, LF1 and LF2, and of the reference fertilizer, LF, on accumulation of bioactive compounds in tomatoes (cv. Prekos F1) grown under greenhouse conditions and drip irrigated *.

Bioactive Compounds	Control (Sprayed with Water)	LF, 3 Treatment	LF1, 3 Treatment	LF2, 3 Treatment × 3
Lycopene (mg 100 g^{-1} FW) [1]	9.06 ± 0.59 b	11.93 ± 0.36 a	9.74 ± 0.28 b	12.24 ± 0.47 a
β-carotene (mg 100 g^{-1} FW) [1]	0.94 ± 0.18 b	0.89 ± 0.12 b	1.33 ± 0.09 a	1.23 ± 0.12 a
Phytoene (mg 100 g^{-1} FW) [1]	0.72 ± 0.09 b	1.08 ± 0.08 a	0.84 ± 0.16 b	1.12 ± 0.14 a
Phytofluene (mg 100 g^{-1} FW) [1]	0.41 ± 0.03 b	0.37 ± 0.06 b	0.64 ± 0.07 b	0.75 ± 0.08 a
Total carotenoids (mg 100 g^{-1} FW) [1]	11.09 ± 0.89 b	14.27 ± 0.62 a	12.55 ± 0.60 b	15.32 ± 0.81 a
Ascorbic acid (mg 100 g^{-1} FW) [1]	117.8 ± 8.74 b	112 ± 12.68 b	134.5 ± 11.21 a	147.2 ± 9.73 a
Total polyphenols (mg gallic acid eq. 100 g^{-1} DW) [2]	12.62 ± 1.04 b	12.84 ± 0.69 b	15.35 ± 0.87 a	14.72 ± 0.79 a
Rutin (mg. 100 g^{-1} DW) [2]	4.46 ± 0.22 c	4.29 ± 0.17 c	7.19 ± 0.52 a	5.94 ± 0.37 b
Quercitin (mg 100 g^{-1} DW) [2]	1.17 ± 0.08 b	1.24 ± 0.11 b	1.34 ± 0.14 b	1.70 ± 0.16 a
Naringenin (mg 100 g^{-1} DW) [2]	0.38 ± 0.03 b	0.43 ± 0.04 b	0.65 ± 0.07 a	0.45 ± 0.05 b
Total flavonoids [3] (mg quercetin eq. 100 g^{-1} DW)	9.48 ± 0.87 b	10.62 ± 0.72 b	12.47 ± 0.35 a	12.12 ± 0.42 a

* Values followed by the same letter within each parameter do not differ significantly for $p < 0.05$. [1] HPLC-DAD; [2] Folin–Ciocâlteu colorimetric method; [3] Aluminum chloride colorimetric method.

The pattern is quite similar to that of the effects on the quality of fresh tomato fruits. The foliar fertilizer containing the highest BYV and nitrogen content (LF2) determines an enhancement of the accumulation of bioactive compounds into tomato fruits, both hydrophobic (i.e., carotenoids) and hydrophilic (ascorbic acids, total polyphenols, total flavonoids). The fertilizer with the highest level of Se (LF1) determines the highest significant accumulation of hydrophilic bioactive compounds—except for quercitin, where the LF2 proved to be more efficient. However, the effects on the hydrophobic bioactive compounds are less significant—being even without effect on lycopene accumulation, under our experimental conditions.

4. Discussion

Generally, Se agricultural inputs (used initially for biofortification of the food chains) are presented as fertilizer [4], despite the fact that Se itself is not considered a plant nutrient [57]. Se does not fulfil the "essentiality" criteria; i.e., "When a plant is unable to complete its lifecycle due to the element's absence" [57]. The well-known function of Se in eukaryotes is linked to selenocysteine as the active site of redox modulating selenoproteins [58]. SECIS element (i.e., selenocysteine insertion sequence) was considered to be absent in plant genome, because it was not identified in the nuclear genome [59]. SECIS element was recently identified in the mitochondrial genome of American cranberry and selenoprotein O was identified in *Arabidopsis thaliana* chloroplasts. Therefore, the effects of Se could be linked to the modulation of the level of reactive oxygen species, which these two energy harvesting/producing organites generate inside plant cells. Such physiological effects are rather linked to protection against stress [10] and not to completion of the plant lifecycle.

The effects of Se and BYV presented in our study are similar to those of plant biostimulants. BYV, due most probably to its glycine betaine content, seems to potentiate the plant biostimulant-like

effects of selenium. Under controlled laboratory stress conditions (low intensity light), the plant biostimulant effects were significant on plant height, leaf surfaces, stomatal conductance, and chlorophyll fluorescence. Compared to the control, treated with water, almost all the compositions applied through foliar fertilization have a significant effect. However, the effects of the foliar fertilizer, including BYV and Se (LF1, LF2), are not in a concentration/dose dependent manner. Only for 1% LF1, tomato plants benefit from application of repeated treatments. In the case of the tomatoes grown in low stress conditions, greenhouse and drip irrigated, the foliar application of BYV and Se increases the number of tomato fruits from "extra" marketable class.

We believed that Se should be included among plant biostimulant category of agricultural inputs, because its beneficial effects on plants are similar to those specific for plant biostimulants: it enhances/benefits nutrients uptake, increases plant tolerance to stress, and improves crop yield quality [46]. Several pieces of evidence related to such Se effects were presented already in the beginning of the introduction. The interconnected biochemical mechanisms involved in such effects are related to epigenetic effects [60], including gene activation and enzyme induction [61], regulation of reactive oxygen (and nitrogen) species [62], and recovery of the cell and organelle membrane integrity [10].

The impact of Se can be compared to the activity of silicon, which is also an element which is involved in protection of plants against stress [63], rather than completion of the plant life cycle. Silicon was considered both plant nutrient [57] and plant biostimulant [64]. The mechanisms behind silicon's actions on plants are understood only at physiological level [65–67] without a biochemical basis—the exact biochemical reactions in which silicon is involved are not yet known [67]. Despite the lack of biochemical/metabolic evidences, silicon is still considered a plant nutrient—a non-essential one [68].

Differences of approach between silicon and Se are also related to the toxic nature of Se. We mentioned already that Se has a very low physiological window [9]. Its effects is influenced not only by the applied dose, but also by the selenium forms [69]. In the environment, Se presents five oxidation states: +4, +6, 0, −1, and −2, known under the following forms: selenite (Se^{+4}, SeO_3^{2-}, $HSeO_3^{-}$), selenate (Se^{+6}, SeO_4^{2-}), elemental (zerovalent) selenium (Se^0), selenide (Se^{2-}), and organic selenium (usually with Se^{2-}, in selenomethionine (SeMet), selenocysteine (SeCys), and methylselenocysteine (MeSeCys)) [70–72]. Due to various oxidation states, Se inorganic forms could determine in plant tissue, a pro-oxidant effect in plant tissues, with increased nitro-oxidative stress and hormonal disturbance [73]. Existence of Se-accumulators plants (tolerant to high Se levels) and non-accumulator plants, wherein toxic effects of Se are produced at lower doses, further complicates our understanding of Se effects.

Tomato plants are Se non-accumulator plants, being sensitive to a rather low concentration of selenium. The physiological effects of selenium are very diverse and still not completely understood—besides involvement in the catalytic center of the redox enzymes, selenium seems to be involved into cellular signaling, epigenetic effects, and protein folding [74]. We propose vinasse as an affordable source of glycine betaine, to be used together with Se salts, not only because of similarity between the effects on plants—e.g., increased tolerance to water stress, well known for both Se [11] and glycine betaine [30], but mainly to reduce the potential hazard of selenium. Moreover, glycine betaine could be used also as methyl donor, to support Se metabolism and its physiological effects [74]. Recent studies highlight the role of glycine-betaine on the metabolic processes, including epigenetic effects, protein folding, and association and regulation of enzymes activity [75]. Se and glycine betaine metabolism are interconnected. Betaine homocysteine methyltransferase (BMHT), the enzyme involved in S-Adenosyl methionine restoration and one metabolic pathway of carbon, needs an optimal level of Se for optimal activity [74,76]. The assimilation of Se needs proper function of S-adenosyl methionine cycle [77]. Methylation reduces the toxicity of selenium (compounds) [78]. This inter-connected metabolic cycle between Se and glycine betaine could be involved in the combined effects of Se and glycine betaine.

Our data show that a higher dose of both selenium and glycine betaine (as BYV) does not necessarily lead to a better development of Micro-Tom plants, and may even have a possible inhibitory

effect due to nitrogen and/or selenium excess [39,79]. Such a response of physiological parameters (i.e., stomatal conductance) of tomato plants, not in direct relationship to the applied dose, has been already reported for glycine-betaine [80]. For selenium, considered an "essential poison" [4], the U shape response is specific to its narrow physiological windows [74]. The biostimulant effects of selenium and glycine betaine (from bakery's yeast vinasse) were significant, especially on tomatoes grown in climatic chamber, under stress conditions (low light).

The increased cultivated plant tolerance to abiotic stress induced by plant biostimulants, as one of their main uses is related to enhanced horticultural crop tolerance to stress [81,82]. Both Se and betaine were demonstrated to enhance plant tolerance to abiotic stress—and the evidence for such effects was already presented. Another specific effect of plant biostimulants is the improvement of the crop quality [46,82,83]. Se was demonstrated to improve the quality of treated plants, including tomatoes [16]. Glycine betaine was recently reported to increase the quality of the tomato fruits from plant submitted to waterlogging stress [37]—and therefore, for plants grown under stress. However, to the best of our knowledge, our study is the first-one regarding the effects of the combination between BYV/glycine betaine with Se on tomatoes fruit quality.

In the tomato plants grown under greenhouse conditions and drip irrigated, the stress is reduced to a minimum. Therefore, the effect on plant yield was not significant, because plant biostimulants increase the yield for cultivated plants submitted to stress. The significant effect of the applied plant biostimulants for tomatoes grown under optimal conditions (protected in the greenhouse and drip irrigated) was related mainly to the quality—increase of the marketable yield, especially for extra class, and higher accumulation of bioactives. Such effects have been also presented for other types of plant biostimulants, legume-derived protein hydrolysates, and plant and seaweed extracts, applied on tomatoes cultivated under greenhouse conditions [84]. Even in such optimal conditions, the application of plant biostimulants is still profitable.

The level of accumulated bioactives is modified in the tomatoes produced by plants treated with the Se and BYV composition applied through foliar fertilization. The formulation with a higher level of selenium and lower level of nitrogen and BYV significantly increase the level of flavonoids, especially rutin. Such an effect is less significant for the other formulations with higher nitrogen content. Probably, this is related to the higher level of nitrate in LF2 comparied to LF1—the higher level of nitrate was reported to slightly reduce the level of flavonoids and polyphenols in tomatoes [85]. Higher level of accumulation of polyphenols and specific flavonoids (i.e., rutin) seems to be a result of combination of Se and BYV. The foliar treatment of tomatoes only with Se was reported to increase the level of quercetin and naringenin chalcone [17]. The tomatoes cultivar we used, Prekos F1, was reported to respond to application of other plant biostimulants (humic acid and galactomannan) through a foliar fertilization by increasing the accumulation of rutin in the fruits [86].

5. Conclusions

Enhanced crop quality resulted from application of Se and BYs through foliar fertilization benefits for the whole tomatoes value chain: growers, traders, and consumers. Growers benefit from the increased percentage of tomatoes from extra and 1st class, which have an increased commercial value. Traders should experience less losses during transportation and storage because hydrophilic antioxidants (mainly polyphenols and flavonoids) increase tomatoes' shelf lives. Tomatoes are climacteric fruits and accumulation of reactive oxygen species (ROS) are characteristics of the ripening stage [87]. Antioxidant/ROS scavenger accumulation into fruit tissues were proven to extend shelf life of tomatoes [88]. It was already reported that the increased the level of antioxidants after Se treatment in tomatoes leads to a decreased reactive oxygen species (ROS) formation and to an extended shelf life of tomato fruits [15]. Also, it was demonstrated that the foliar treatment with selenium activates the plant defense system, the resultant tomato fruits being more resistant to gray mold (*Botrytis cinerea*) post-harvest disease [89,90].

Consumers benefit from increased content of bioactive compounds. Tomatoes do not have a very high content on polyphenols and flavonoids [21]. However, because they are consumed on a regular basis and in quite large quantities, tomato polyphenols and flavonoids contribute significantly to the human dietary antioxidant intake [91]. Tomatoes polyphenols synergistically promote the antioxidation of tomato carotenoids, including lycopene [92]. Consumers would benefit from the increased content of bioactive compounds, as well as from the biofortification with Se, due to accumulation of organo-Se compounds into tomato fruit tissues. The antioxidant activity of bioactives from tomatoes is complementary to that of Se [17].

The results of our study provide evidence for the plant biostimulant-like effects of foliar fertilizers that contain Se and BYV applied on tomatoes. Based on these and previous results, we propose Se to be included in the plant biostimulant category of agricultural inputs. Managing the quality of tomato fruits by using a plant biostimulant composition based on Se and BYV, through foliar fertilization, besides adding value to all players in tomato value chains, provides a new opportunity to valorize the by-product of baker's yeast production on molasses. The baker's yeast industry's profitability relies on the low costs of molasses used as carbon source for the growing media [93]. One disadvantage of molasses-based growing media for baker's yeast is the large quantity of resultant vinasse by-product. In a conventional 100,000 tons per year baker's yeast plant working on molasses, the vinasse amounts 230,000 tons per year, almost 10 times more than the dry substance of the principal product, compressed yeast [27]. The efficient valorization of side streams is essential to maintain the profitability of bakers' yeast industry [93]. Our study demonstrates that it is possible.

6. Patents

The Patent Application RO133307 A2 was filled, related to the composition of foliar fertilizers with glycine betaine (from baker's yeast vinasse) and selenium.

Author Contributions: Conceptualization, F.O.; data curation, D.C.-A.; formal analysis, V.Z.-C.; investigation, S.-O.D., C.N., M.D.-A., M.G., L.C., E.R., R.S., and V.-A.F.; methodology, C.N., M.D.-A., M.G., R.S., and D.C.-A.; supervision, F.O.; writing—original draft, S.-O.D.; writing—review and editing, D.C.-A. and F.O. All authors have read and agreed to the published version of the manuscript.

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