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Article

Growth and Nutritional Responses of Zucchini Squash to a Novel Consortium of Six *Bacillus* sp. Strains Used as a Biostimulant

Dimitrios Savvas *, Paraskevi Magkana, Dionisios Yfantopoulos, Panagiotis Kalozoumis and Georgia Ntatsi

Laboratory of Vegetable Production, Department of Crop Science, Agricultural University of Athens, Iera Odos 75, 11855 Athens, Greece; evimagkana@gmail.com (P.M.); yfantopoulos@aua.gr (D.Y.); kalozoumis@aua.gr (P.K.); ntatsi@aua.gr (G.N.)
* Correspondence: dsavvas@aua.gr; Tel.: +30-2105294510

Abstract: The use of biostimulants consisting of plant growth-promoting rhizobacteria (PGPR) has been rapidly expanding in horticulture in recent years. In the current study, a novel mix of six *Bacillus* sp. strains (*B. subtilis*, *B. pumilus*, *B. megaterium*, *B. amyloliquefaciens*, *B. velezensis*, *B. licheniformis*) was tested as a PGPR biostimulant in two experiments with zucchini squash (*Cucurbita pepo* L.). The first experiment took place in greenhouse soil in winter, while the second experiment was conducted in an open field during summer. In both experiments, seeds of the local landrace "Kompokolokytho" and the commercial hybrid "ARO-800" were either inoculated or non-inoculated with the PGPR biostimulant. The application of the six *Bacillus* sp. strains increased both the vegetative growth and the yield of zucchini squash, and these effects were associated with significantly higher shoot phosphorus levels in both experiments and both genotypes. Furthermore, at the end of the cultivation, the colony-forming units of *Bacillus* sp. were appreciably higher in plants originating from inoculated compared to non-inoculated seeds, indicating that the tested mix of *Bacillus* sp. can be successfully applied through seed inoculation. "ARO-800" produced more vegetative and fruit biomass than "Kompokolokytho" under greenhouse cropping conditions, while in the open field crop, both genotypes performed equally. Presumably, this response occurred because "ARO-800" did not express its full yield potential in the open field due to stress imposed by the high summer temperatures, while the local landrace, which is traditionally grown in open fields, may be more resilient to stress conditions frequently encountered in open fields.

Keywords: *Cucurbita pepo*; greenhouse; integrated crop management; landrace; PGPR; phosphorus

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1. Introduction

Currently, the deleterious effects of climate change on agricultural systems, coupled with the escalating global population, are anticipated to elevate global hunger by 30% by the year 2050 [1]. Considering this complex scenario, agriculture has to face the dual challenge of fulfilling the escalating demand for food production while concurrently alleviating its environmental impact on natural ecosystems and human health [2]. Currently, a range of both traditional and innovative eco-friendly management practices is being widely implemented to augment crop yields and increase food safety standards [3].

Horticulture, and especially vegetable production, heavily depends on external inputs of mineral nutrients, primarily in the form of synthetic fertilizers. The relatively low nutrient use efficiency exhibited by many vegetable crops leads to an excess application of nutrients beyond what the crops actually require, thereby causing substantial environmental impacts [4,5]. This excess application not only raises concerns about resource sustainability but also contributes to environmental issues such as nutrient runoff, soil degradation and water pollution [6]. Addressing the nutrient use efficiency in vegetable production is crucial for mitigating these environmental challenges and promoting sustainable

agricultural practices. Recognizing these concerns, the European Commission has set an ambitious goal to substitute 30% of synthetic fertilizers with greener alternatives by 2050 [7]. Plant biostimulants, acknowledged as natural products, have emerged as eco-friendly tools for the reduction of synthetic fertilizer use, as they can enhance nutrient use efficiency [8]. Their application not only contributes to reducing reliance on chemical fertilizers but also helps plants resist stress induced by climate change [9,10]. Therefore, plant biostimulants have the potential to foster a more sustainable agriculture, ensuring crop yields under lower inputs, and represent a crucial step towards environmental responsibility [8,11–14].

Non-pathogenic soil microorganisms with beneficial effects on plant growth and crop production can be used as microbial biostimulants in commercial crops [15]. Bacterial strains living in the rhizosphere or even within the root tissues with beneficial effects on plant growth are generally termed "plant growth promoting rhizobacteria" (PGPR). Due to their beneficial effects on crop yield, several PGPR have been licensed for commercial distribution as biostimulants [16]. PGPR are usually applied either by inoculating the seeds before sowing or by soil drenching after planting or plant emergence. *Bacillus* spp. are Gram-positive bacteria which have already been studied extensively regarding plant growth stimulation and growth promotion [17] and are categorized as extracellular plant growth-promoting rhizobacteria (ePGPR) [18]. They can promote plant growth by various means, including production of siderophores and phytohormone precursors, phosphate solubilization, and induction of systemic resistance [19,20].

The treatment of seeds with microbial preparations is based on specific techniques, which may have different effectiveness–success in coating the seeds, depending on the size, weight, and texture of each seed. Biopriming is a seed-presoaking technique along with the inoculation of beneficial microorganisms. It combines both the biological agent (microorganisms) and physiological soaking (seed hydration) phase [21]. The exudates released from the seed may serve as a source of energy and nutrients to the biocontrol agents during biopriming [22], thus facilitating the proliferation and the colonization of these biocontrol agents over the surface of seeds which facilitate the nutrient/water uptake. Film-coated seed treatment is based on seed coating with a thin film, which contains the active substance or microorganisms, and one or two inert materials that function as a carrier to preserve the microorganisms and/or as adhesives. This film is created after treating the seeds with a liquid-dense solution or suspension [23].

Zucchini squash (*Cucurbita pepo* L.), a member of the Cucurbitaceae family, is a popular vegetable worldwide [24] and of great economic importance due to its high nutritional value [25]. Zucchini squash is widely cultivated both in fields and in greenhouses in the Mediterranean region [26] and in arid climatic zones characterized by limited water resources and hot weather conditions as well as in soils with low organic matter content [27].

Taking the above into consideration, an investigation was designed to assess the effectiveness of a novel inoculant containing six *Bacillus* sp. strains (*B. subtilis*, *B. pumilus*, *B. megaterium*, *B. amyloliquefaciens*, *B. velezensis*, *B. licheniformis*) in improving nutrient uptake and concomitantly plant growth and yield when used as a PGPR biostimulant through biopriming of zucchini squash seeds.

2. Materials and Methods

2.1. Plant Material and Experimental Layout

Two consecutive experiments were conducted at the Laboratory of Vegetable Production at the Agricultural University of Athens (latitude 37°98′ N, longitude 23°70′ E, altitude 24 m). The first experiment was conducted in the natural soil of a greenhouse from November 2020 to February 2021, while the second experiment was conducted in an open experimental field in summer 2022. More specifically, in the greenhouse experiment, zucchini seedlings were transplanted to the greenhouse soil on 28 November 2020 and the experiment was terminated on 23 February 2021, while in the open field experiment, planting was performed on 15 June 2022 and the experiment was terminated on 4 August 2022. In both experiments, integrated crop management (ICM) practices [28] were

consistently applied in all treatments. In the greenhouse experiment, temperature was adjusted by active heating and cooling equipment which maintained temperatures between 16 °C and 28 °C. The open field experiment was conducted under Mediterranean summer conditions, with temperatures ranging between 22 °C and 37 °C. Chemical properties of the greenhouse soil and the open field soil before transplanting are presented in Table 1.

Table 1. Soil physical and chemical properties in the greenhouse (GH) and in the open field (OF) as determined in samples obtained just before planting.

Parameter	GH	OF	Parameter	GH	OF
Clay (%)	21.7	21.1	Organic matter (%)	3.71	4.62
Silt (%)	30.9	15.3	NO_3 (mg kg^{-1})	60.81	36.00
Sand (%)	47.4	63.6	NH_4 (mg kg^{-1})	13.95	2.27
pH	7.68	7.74	Available P (mg kg^{-1})	17.42	24.50
EC (dS m^{-1})	0.90	0.81	Exchangeable K (mg kg^{-1})	213.00	154.00

Two zucchini squash (*Cucurbita pepo* L.) genotypes were tested. The first genotype of zucchini was the local landrace "Kompokolokytho" from Agrogen S.A., while the second genotype was the commercial hybrid, "ARO-800" from AROSEED (https://aroseed.gr/, accessed on 18 December 2023). In both experiments, half of the zucchini seeds were inoculated with PGPR (mix of six *Bacillus* sp.) before sowing, while the remaining seeds were not inoculated in both genotypes. Consequently, the resulting treatments were as follows:

1. "Kompokolokytho", +PGPR,
2. "Kompokolokytho", −PGPR,
3. "ARO-800", +PGPR,
4. "ARO-800", −PGPR.

The experiments were designed and analyzed as a two-factorial design (PGPR, genotype) with two levels for each factor (PGPR: inoculation or not; genotype: landrace "Kompokolokytho" or "ARO-800") and the experimental design was randomized complete blocks in both trials. Each of the 2 × 2 = 4 experimental treatments was replicated 4 times in one of the 4 groups formed.

Irrigation was performed daily through drip irrigation systems in both experiments, using tensiometers to control the frequency of water supply. Irrigation was activated when the electronic tensiometers reached −25 kPa [29]. Fertilization was applied through the drip irrigation system at all irrigation events. In both experiments, the concentrations of the applied nutrients (in mmol/L) were K: 3.5, Ca: 2, Mg: 1, NH_4-N: 1, NO_3-N: 7, P: 0.5, and SO_4-S: 1.5, as suggested by the decision support system NUTRISENSE (https://nutrisense.online/ accessed on 18 December 2023), which specializes in fertigation management in horticulture. In the greenhouse experiment, the only plant protection treatment was the application of the organic fungicide "SEPTUM" (extract of *Equisetum* sp., AGRIPRO, www.agripro.eu accessed on 18 December 2023) two times. In the open field experiment, for insect control, the organic insecticide "PYREGARD" (pyrethrins 4%, BIOGARD, Athens, GREECE) was applied two times at 15 and 22 days after transplanting. Weed management was based on hoeing throughout the cultivation period in both experiments.

2.2. Biostimulant Application

2.2.1. Microbial Inoculant

The microbial inoculant used as biostimulant contained the six *Bacillus* species *Bacillus subtilis*, *Bacillus pumilus*, *Bacillus megaterium*, *Bacillus amyloliquefaciens*, *Bacillus velezensis*, and *Bacillus licheniformis* at a concentration of 1×10^{12} CFU (colony-forming unit) mL^{-1} of each one. Every species was, separately, cultivated at 37 °C, aerobically, in a liquid growth medium containing 0.1 g L^{-1} K_2HPO_4, 0.4 g L^{-1} KH_2PO_4, 0.01 g L^{-1} $FeSO_4 \times 7\ H_2O$, 0.20 g L^{-1} $MgSO_4 \times 7\ H_2O$, 0.20 g L^{-1} $MnSO_4$, 0.10 g L^{-1} NaCl, 0.02 g L^{-1} $CaCl_2 \times 2\ H_2O$,

and 2 g L^{-1} glucose. The cultures were stopped 10 h after entering stationary growth phase. Then, the cultures were centrifuged and the biomass from each culture was redissolved with 0.01% PBS (phosphate-buffered saline). The six biomasses were mixed and subsequently diluted in PBS, so that the final microbial inoculant contained bacteria of the *Bacillus* genus at a concentration of 6×10^{12} CFU L^{-1}.

2.2.2. Seed Treatment with the Microbial Inoculant

Zucchini seeds were treated using the biopriming technique. Seeds were placed in a sealed plastic bag, covered with the liquid microbial inoculant and hydrated at 15–20 °C for 1 h. After that, seeds were air-dried for 24 h. At the end of the process, the seeds were checked for both their germination and their microbial load concentration. Each zucchini seed possessed bacteria of the *Bacillus* genus at a concentration of 1×10^5 CFU.

2.3. Determination of Plant Biomass, Total Yield, and Fruit Quality Characteristics

In both experiments, for the determination of the plants' fresh and dry biomass, one zucchini plant per replicate was sampled before commencement of harvest (1st sampling date) and at the end of the experiment (2nd sampling date). When plant samples were collected, shoot and root fresh biomass was recorded. Shoot biomass was the sum of leaves and stems. Then, samples were oven-dried at 65 °C for at least 72 h until constant weight was reached. Dried samples were then used to determine the plants' dry weight and nutrient content. For the estimation of the plants' total yield, zucchini fruits were harvested when they reached marketable size (length over 12 cm) [30]. In the greenhouse experiment, the harvesting started on 3 January 2021 and terminated on 23 February 2021. In the open field experiment, harvesting started on 9 July 2022 and terminated on 4 August 2022. For the determination of fruit quality characteristics, analyses for total soluble solids and fruit firmness were conducted. Total soluble–solid content (TSSC) was determined by squeezing and extracting the zucchini fruits' juice directly onto the refractometer (SCHMIDT + HAENSCH HR32B, Berlin, Germany), and values were expressed in ∘Brix units against a refractive index. Fruit firmness was measured using a table penetrometer (Chatillon DFIS 10, Ametek, Berwyn, PA, USA). One zucchini fruit from each replicate was punctured three times and the mean value was recorded.

2.4. Shoot Mineral Analysis

Nutrient analyses of both experiments were carried out at the Laboratory of Vegetable Production of the Agricultural University of Athens. Dried leaf tissues were ground in a Wiley mill to pass through a 20-mesh screen, and 1 g of the dried tissues was analyzed for the macronutrients N, P, and K. The N concentration of leaf tissues was determined after mineralization with sulfuric acid by the "Regular Kjeldahl method" [31], whereas P and K concentrations were determined by dry-ashing at 550 °C for 8 h. Then, extraction for the measurement of nutrients was carried out with a solution of HCl 1N placed into the capsule. The solution contained in the capsule was filtered with Whatman No. 42 filters into 100 mL volumetric flasks and distilled water was added up to 100 mL. Potassium was measured by placing diluted or undiluted extraction solution in the flame photometer (Sherwood 410, Cambridge, UK), while phosphorus was determined as phosphomolybdate blue complex at 880 nm using a spectrophotometer (Anthos Zenyth 200; Biochrom, Cambridge, UK).

2.5. Estimation of Root Colonization by Bacillus sp.

At the end of the greenhouse experiment, the roots of one randomly selected plant per replicate were placed in a sealed plastic bag and immediately transferred to the laboratory to determine the concentration of *Bacillus* sp. bacteria on the surface and interior of the roots. In the laboratory, the zucchini roots were rinsed with sterile distilled water to remove adhering soil particles. Then, roots were cut into smaller segments (about 1–2 cm in length) and placed in sterile containers, and 10 mL of 0.01 M phosphate buffer solution (PBS) was added to the containers and heated at 80 °C for 10 min to eliminate vegetative

cells. The root homogenate was diluted by transferring known volumes into sterile tubes containing sterile PBS (10^{-1}, 10^{-2}, 10^{-3}, etc.). An amount of 0.1 mL from each dilution was transferred onto separate sterile agar plates with TSA medium, and incubated plates were left in a microbiology incubator, aerobic, at 37 °C for three days to allow colony growth. After incubation, colonies were examined for size, pigmentation, form, margin, and elevation. The moderate to large, raised, cream-white colonies with circular or irregular forms and well-defined merge were selected. Bacteria of these colonies were tested for their morphological characteristics such as Gram's reaction and endospore-forming. Rod-shaped, endospore-forming aerobic or facultatively anaerobic Gram-positive bacteria are tested using biochemical tests such as catalase, citrate, urease, indole, starch hydrolysis, and sugar fermentation, according to standard procedures. Colonies of the catalase, citrate, urease, indole, and starch hydrolysis-positive bacteria were counted on each plate [32–35].

2.6. Gas Exchange Assessment

To search for possible effects of the tested microbial biostimulant on the primary plant metabolism of zucchini squash, the rates of net assimilation (μmol CO_2 m^{-2} s^{-1}) and transpiration (mmol H_2O m^{-2} s^{-1}), and the stomatal conductance were measured in all treatments. The measurements were conducted by using a portable photosynthetic system, LCpro T (ADC BioScientific Ltd., Hoddesdon, UK).

2.7. Statistical Analysis

Two-factor analysis of variance (ANOVA) was applied to evaluate the two selected zucchini genotypes and the pre-inoculation of zucchini seeds with plant growth-promoting rhizobacteria, and the interactions between them. Data were analyzed as mean ± SE (n = 5). A multiple-range (Duncan) test was conducted for all parameters at a $p \leq 0.05$ level of significance. Statistical analysis was performed using the STATISTICA software package for Windows 12.0 (Tulsa, OK, USA).

3. Results

3.1. Greenhouse Zucchini Crop

The seed inoculation with the PGPR used as biostimulant significantly increased the vegetative growth of zucchini squash grown on greenhouse soil. This is indicated by the substantial increase in the fresh and dry shoot weight in both genotypes tested in the current study (Table 2). The fresh and dry shoot mass of the commercial hybrid "ARO-800" was higher than that of the local landrace, both in treated and in non-treated plants with PGPR. The dry matter content in the shoot was not influenced by the treatment of the seed with PGPR but was significantly higher in the commercial hybrid compared with that measured in the local landrace.

The length of the zucchini fruit was not influenced by the inoculation of the seeds with PGPR in the greenhouse crop, while it was similar in both genotypes tested in the current study (Table 3). However, the total fruit yield was significantly enhanced by seed priming with PGPR in both the commercial variety and the local landrace. The commercial hybrid rendered a significantly higher yield than the local landrace, without any interaction between seed priming with PGPR and genotype. The yield increase in the greenhouse zucchini crop was exclusively a result of a higher fruit number per plant, while the mean fruit weight was not influenced by PGPR application in the seeds in both genotypes. Unlike the yield, the fruit quality characteristics determined in the current study (TSS, firmness) were not influenced by PGPR inoculation or by the zucchini genotype.

Table 2. Impact of seed inoculation with a PGPR strain used as biostimulant on shoot fresh weight, shoot dry weight, and shoot dry matter content of two zucchini squash genotypes (landrace "Kompokolokytho" or "ARO-800" F1) grown in the soil in a greenhouse.

Genotype	PGPR	Shoot Fresh Weight (kg/Plant)	Shoot Dry Weight (g/Plant)	Shoot Dry Matter Content (%)
Landrace	−PGPR	1.373	106.4	7.8
	+PGPR	1.699	130.6	7.8
"ARO-800"	−PGPR	1.616	137.8	8.6
	+PGPR	1.983	160.9	8.2
Main Effects				
PGPR				
−PGPR		1.494 b	122.1 b	8.2
+PGPR		1.841 a	145.8 a	8.0
Genotype				
Landrace		1.536 b	118.5 b	7.8 b
"ARO-800"		1.799 a	149.4 a	8.4 a
Significance				
PGPR		**	**	n.s.
Genotype		*	***	*
PGPR × genotype		n.s.	n.s.	n.s.

Mean values (n = 5) followed by different letters are significant according to ANOVA. The symbols *, **, and *** indicate that the differences were significant at $p < 0.05$, $p < 0.01$, $p < 0.001$, respectively, while n.s. denotes non-significant differences.

Table 3. Impact of seed inoculation with a PGPR strain used as biostimulant on mean fruit length, mean fruit weight, and total yield in two zucchini squash genotypes (landrace "Kompokolokytho" or "ARO-800" F1) grown in the soil in a greenhouse.

Genotype	PGPR	Total Yield (kg m^{-2})	Mean Fruit Length (cm)	Mean Fruit Weight (g)
Landrace	−PGPR	1.392	16.9	146.4
	+PGPR	1.765	16.7	149.6
"ARO-800"	−PGPR	1.991	16.6	152.4
	+PGPR	2.355	16.8	156.1
Main Effects				
PGPR				
−PGPR		1.692 b	16.7	149.4
+PGPR		2.060 a	16.8	152.8
Genotype				
Landrace		1.578 b	16.8	147.6 b
"ARO-800"		2.173 a	16.7	154.3 a
Significance				
PGPR		**	n.s.	n.s.
Genotype		***	n.s.	*
PGPR × genotype		n.s.	n.s.	n.s.

Mean values (n = 5) followed by different letters are significant according to ANOVA. The symbols *, **, and *** indicate that the differences were significant at $p < 0.05$, $p < 0.01$, $p < 0.001$, respectively, while n.s. denotes non-significant differences.

The inoculation of the seeds with PGPR in the greenhouse crop of zucchini squash had no impact on the shoot nitrogen and potassium levels but significantly increased the phosphorus concentration at both sampling dates (Table 4). The shoot P concentration was significantly higher in the landrace on the first sampling date compared to the commercial

hybrid, but this difference disappeared on the 2nd sampling date at crop termination. On the other hand, the shoot K concentration was similar at both genotypes on the 1st sampling date, while it was significantly lower in the local landrace at crop termination compared to the commercial hybrid. No interaction between inoculation and genotype was found in the shoot N, P, and K concentrations. Finally, the gas exchange parameters (rates of net photosynthesis and transpiration, stomatal conductance) were similar in both genotypes tested and not influenced by the PGPR application.

Table 4. Impact of seed inoculation with a PGPR strain used as biostimulant on N, P, and K concentrations in shoot samples collected at two sampling dates (1st SD and 2nd SD, respectively) from two zucchini squash genotypes (landrace "Kompokolokytho" or "ARO-800" F1) in the soil in a greenhouse.

Genotype	PGPR	Shoot N (mg g^{-1} d.wt.)		Shoot P (mg g^{-1} d.wt.)		Shoot K (mg g^{-1} d.wt.)	
		1st SD	2nd SD	1st SD	2nd SD	1st SD	2nd SD
Landrace	−PGPR	3.38	3.40	3.29	2.88	35.2	25.0
	+PGPR	3.58	3.36	4.14	3.30	35.4	28.1
"ARO-800"	−PGPR	3.52	3.49	2.73	3.08	35.4	29.3
	+PGPR	3.68	3.51	2.82	3.33	37.2	32.9
Main Effects							
PGPR							
−PGPR		3.45	3.45	3.01 b	2.98 b	35.3	27.1
+PGPR		3.63	3.44	3.48 a	3.32 a	36.3	30.5
Genotype							
Landrace		3.48	3.38	3.71 a	3.09	35.3	26.5 b
"ARO-800"		3.60	3.50	2.77 b	3.20	36.3	31.1 a
Significance							
PGPR		n.s.	n.s.	*	*	n.s.	n.s.
Genotype		n.s.	n.s.	***	n.s.	n.s.	*
PGPR × genotype		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

Mean values (n = 5) followed by different letters are significant according to ANOVA. The symbols * and *** indicate that the differences were significant at $p < 0.05$ and $p < 0.001$, respectively, while n.s. denotes non-significant differences.

3.2. Open Field Zucchini Squash Crop

The inoculation of the seeds with PGPR stimulated the vegetative growth of zucchini squash cultivated conventionally in an open field, as indicated by the increased fresh and dry shoot weight in both inoculated genotypes compared to non-inoculation (Table 5). Similar to the greenhouse crop, in the open field crop the commercial hybrid "ARO-800" produced more shoot biomass compared to that rendered by the local landrace, irrespective of seed inoculation with PGPR or not. The dry matter content in the shoots of zucchini squash grown in the open field was not influenced by the treatment of the seeds with PGPR but was significantly higher in the commercial hybrid compared with that measured in the local landrace.

The total fruit yield of zucchini squash cultivated in an open field was significantly enhanced by seed priming with PGPR in both the commercial variety and the local landrace (Table 6), in agreement with the respective results in the greenhouse crop. However, unlike in the greenhouse crop, in the open field, the commercial hybrid rendered a similar yield to the local landrace. The yield increase imposed by seed inoculation with PGPR in the open field crop was exclusively a result of a higher fruit number per plant in both genotypes, similar to the greenhouse zucchini crop. The mean fruit weight was not influenced by

PGPR application in the seeds (Table 6). Total soluble solids (TSS) and fruit firmness were not influenced by either PGPR inoculation or the two zucchini genotypes tested.

Table 5. Impact of seed inoculation with a PGPR strain used as biostimulant on shoot fresh weight, shoot dry weight, and shoot dry matter content of two zucchini squash genotypes (landrace "Kompokolokytho" or "ARO-800" F1) grown in an open field according to conventional farming practices.

Genotype	PGPR	Shoot Fresh Weight (kg/Plant)	Shoot Dry Weight (g/Plant)	Shoot Dry Matter Content (%)
Landrace	−PGPR	1.373	106.4	7.8
	+PGPR	1.699	130.6	7.8
"ARO-800"	−PGPR	1.616	137.8	8.6
	+PGPR	1.983	160.9	8.2
Main Effects				
PGPR				
−PGPR		1.494 b	122.1 b	8.2
+PGPR		1.841 a	145.8 a	8.0
Genotype				
Landrace		1.536 b	118.5 b	7.8 b
"ARO-800"		1.799 a	149.4 a	8.4 a
Significance				
PGPR		**	**	n.s.
Genotype		*	***	*
PGPR × genotype		n.s.	n.s.	n.s.

Mean values (n = 5) followed by different letters are significant according to ANOVA. The symbols *, **, and *** indicate that the differences were significant at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively, while n.s. denotes non-significant differences.

Table 6. Impact of seed inoculation with a PGPR strain used as biostimulant on mean fruit length, mean fruit weight, and total yield in two zucchini squash genotypes (landrace "Kompokolokytho" or "ARO-800" F1) grown in an open field according to conventional farming practices.

Genotype	PGPR	Total Yield (kg m^{-2})	Fruit Number Per m^2	Mean Fruit Weight (g)
Landrace	−PGPR	1.592	14.00	113.5
	+PGPR	1.883	16.36	115.2
"ARO-800"	−PGPR	1.601	13.67	117.2
	+PGPR	2.014	17.00	118.6
Main Effects				
PGPR				
−PGPR		1.587 b	13.83 b	115.3
+PGPR		1.942 a	16.68 a	116.9
Genotype				
Landrace		1.743	15.18	114.3
"ARO-800"		1.807	15.33	117.4
Significance				
PGPR		**	*	n.s.
Genotype		n.s.	n.s.	n.s.
PGPR × genotype		n.s.	n.s.	n.s.

Mean values (n = 5) followed by different letters are significant according to ANOVA. The symbols * and ** indicate that the differences were significant at $p < 0.05$ and $p < 0.01$, respectively, while n.s. denotes non-significant differences.

The inoculation of the seeds with PGPR in the conventional cultivation of zucchini squash in the open field significantly increased the phosphorus concentration at both sampling dates, while it had no impact on the shoot nitrogen and potassium levels (Table 7). Similar to the greenhouse crop, the shoot P concentration in the open field crop was significantly higher in the landrace on the first sampling date compared to the commercial hybrid but not on the second sampling date. Furthermore, the shoot K concentration was significantly higher in the local landrace than in the commercial hybrid at both sampling dates. Finally, the estimated gas exchange parameters (net assimilation rate, net transpiration rate and stomatal conductance) were not influenced by the application of PGPR or by the genotype.

Table 7. Impact of seed inoculation with a PGPR strain used as biostimulant on N, P, and K concentrations in shoot samples collected at two sampling dates (1st SD and 2nd SD, respectively) from two zucchini squash genotypes (landrace "Kompokolokytho" or "ARO-800" F1) grown in an open field according to conventional farming practices.

Genotype	PGPR	Shoot N (mg g^{-1} d.wt.)		Shoot P (mg g^{-1} d.wt.)		Shoot K (mg g^{-1} d.wt.)	
		1st SD	2nd SD	1st SD	2nd SD	1st SD	2nd SD
Landrace	−PGPR	4.78	3.40	4.90 b	3.98	42.0	34.7
	+PGPR	5.48	3.66	6.45 a	4.14	42.5	34.3
"ARO-800"	−PGPR	3.99	2.97	4.32 bc	3.35	33.5	25.0
	+PGPR	4.08	3.58	3.69 cd	4.49	36.0	29.3
Main Effects							
PGPR							
−PGPR		4.38	3.19	4.61	3.67 b	37.8	29.1
+PGPR		4.78	3.62	5.07	4.32 a	39.3	31.8
Genotype							
Landrace		5.12	3.53	5.67	4.06	42.3 a	34.4 a
"ARO-800"		4.03	3.28	4.00	3.92	34.8 b	27.1 b
Significance							
PGPR		n.s.	n.s.	ns	*	n.s.	n.s.
Genotype		n.s.	n.s.	*	n.s.	*	*
PGPR × genotype		n.s.	n.s.	*	n.s.	n.s.	n.s.

Mean values (n = 5) followed by different letters are significant according to ANOVA. The symbol * indicates that the differences were significant at $p < 0.05$, while n.s. denotes non-significant differences.

3.3. Root Colonization by PGPR

As shown in Figure 1, the number of *Bacillus* sp. bacteria counted at crop termination on the root system of zucchini squash was appreciably higher in plants originating from seeds treated with the PGPR biostimulant than in plants originating from untreated seeds. The differences were highly significant in both genotypes tested in the current study.

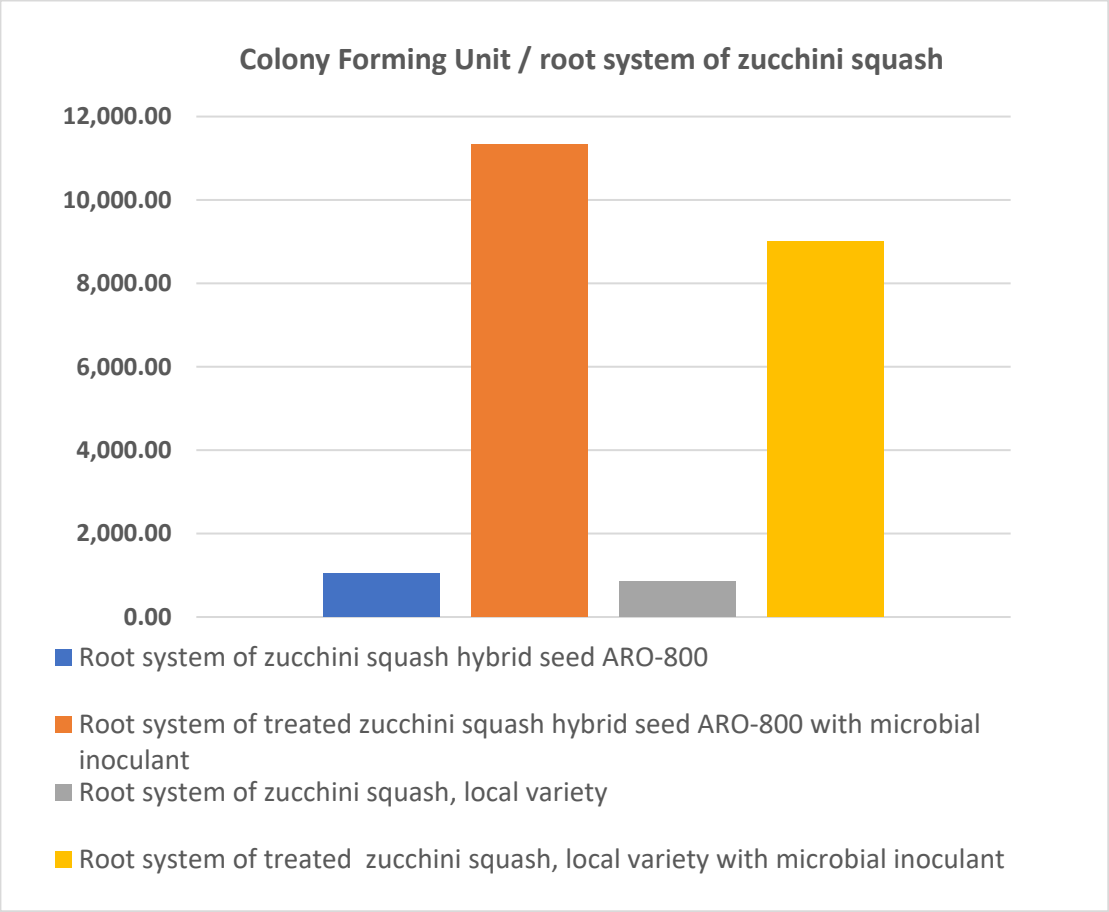

Figure 1. Number of *Bacillus* sp. bacteria counted at crop termination on the root system of two zucchini squash genotypes (landrace "Kompokolokytho" or "ARO-800" F1) originating from seeds either treated or non-treated with the PGPR biostimulant.

4. Discussion

In the current study, the inoculation of zucchini squash seeds with the novel mix of *Bacillus* sp. strains significantly improved both the vegetative growth and the fruit production of the plants. These results clearly show that this mix of *Bacillus* strains includes plant growth-promoting rhizobacteria (PGPR) and thus could be used as a microbial biostimulant. Soil microorganisms have an active role in natural processes that affect soil fertility and soil quality, such as atmospheric nitrogen fixation, organic matter decomposition, and mineral nutrition [36]. These processes are intimately associated not only with the growth and yield of cultivated plants but also with the quality of the obtained products [37]. Several studies have shown that the use of beneficial rhizobacteria (PGPR) as root inoculants in vegetables promotes plant growth, while it can improve soil composition or resistance to pests and diseases. Therefore, many PGPR strains or mixtures of them are used as commercial biostimulants in agriculture and horticulture. According to European Council Regulation 2019/1009, biostimulants are defined as plant substances, mixtures, and products of microorganisms, which "stimulate plant nutrition processes independently of the nutrient content in the product with the sole aim of improving one or more of the following characteristics of the plant or the plant rhizosphere: (i) nutrient use efficiency, (ii) tolerance to abiotic stress, (iii) quality traits, or (iv) availability of confined nutrients in the soil or rhizosphere" [38]. The PGPR can be classified into the following two main categories: extracellular root-forming bacteria (ePGPR) and intracellular root-forming bacteria (iPGPR) [39]. ePGPR live in the rhizosphere or in the spaces between the cells of the root cortex, whereas iPGPR live mainly within specialized nodal structures of the root cells. Also, due to their

ability to bind to soil nutrients, they improve the levels of nutrients involved in plant cellular processes [40]. Furthermore, some PGPR strains promote the development of disease suppressants or produce phytohormones that increase plant tolerance to biotic stress, thereby acting as biostimulants [41].

The tested *Bacillus* sp. strains mix used as a microbial biostimulant in the current study effectively colonized the zucchini roots, as *Bacillus* spp. were present at substantially higher populations in the roots of inoculated plants at the end of the experiment, compared to the non-inoculated plants. Patakioutas et al. [42] has reported a significant reduction in the population density for *Bacillus amyloliquefaciens* when it was used as biostimulant in a soilless tomato crop in a greenhouse. In the current study, the population density was also lower than the initial inoculant, and this result indicates that repeated PGPR applications by drenching during the cropping period could potentially increase their effectiveness in terms of crop productivity.

The tested mix of *Bacillus* sp. strains improved specifically the uptake of P and its transport to the aboveground shoots, as indicated by the higher leaf P levels in plants from seeds inoculated with the tested PGPR mix. Phosphorus is an essential macronutrient for plants but its availability to plants is marginal in most soils [43,44]. Although total P is abundant in the soil [45], its concentration in the soil solution is mostly lower than 0.1 mg L^{-1} (i.e., 0.1 ppm) because of the poor solubility of the phosphoric salts occurring in the soil, which are mostly complexes of calcium phosphates and magnesium phosphates [46]. As a result, the water-soluble P is quickly depleted in the rhizosphere, and this causes a concentration gradient between the rhizosphere and the bulk of the soil. This gradient contributes to a continuous diffusion of P from the bulk of the soil to the root surface. However, due to the low soluble P concentration in the soil solution, the contribution of diffusion to delivery of P to the root hairs can hardly cover the plant needs, thereby constituting a bottleneck in plant nutrient status [47]. More specifically, as has been reported by Olsen and Watanabe [48], the diffusion coefficient for soil P is relatively low, especially when the P concentrations in the soil solution are less than 0.2 mg kg^{-1} [48]. Mass flow also contributes to transport of P from the soil bulk to the surface of the root hairs. However, due to the low P concentration in the soil solution, the transport of this macronutrient to the root surface through mass flow is negligible. Indeed, zucchini plants generally require from 0.6 to 1.6 L of water per day [49]. Thus, even with a P concentration of 1 mg L^{-1} in the soil solution, the P delivery via mass flow cannot exceed 1.6 mg day^{-1}, which is far below the actual plant needs.

Given these limitations in P delivery from the bulk of the soil to the root surface through natural processes, plants have evolved other P transport pathways beyond mass flow and diffusion to fully cover their P needs. These pathways are mostly associated with beneficial effects of soil microorganisms. The most widely known alternative pathway of P transport from the soil bulk to the plant roots is through the hyphae of beneficial fungi, such as the arbuscular mycorrhizal fungi which form symbiotic relationships with the plant roots [30]. Furthermore, several studies have concluded that many beneficial microorganisms, including bacteria [50] and fungi [30] living in the soil, are capable of solubilizing otherwise insoluble soil P [51]. Thus, through natural selection, phosphate-solubilizing bacteria and plants have evolved mutual relationships of reciprocal symbiosis [52].

Bacullus subtilis, *Bacillus pumilus*, and *Bacillus amyloliquefaciens* have already been reported by researchers as bacteria that can solubilize soil organic and inorganic phosphate [53–56]. On the one hand, inorganic phosphate can be solubilized by the production and secretion of low molecular weight organic acids and metal chelates produced by the above strains [57]. Furthermore, phytic acid, a compound which accounts for 20 to 50% of soil organic P [58], can be converted to orthophosphates when hydrolyzed by the enzyme phytase [59]. *Bacillus amyloliquefaciens* and *Bacullus subtilis* are phytase-producing microorganisms and, due to phytase production, they are able to release three molecules of Pi from each molecule of phytic acid [53].

Considering the increased shoot P levels in the plants obtained from seeds inoculated with the mix of the six *Bacillus* sp. strains in the current study, compared to those from non-inoculated seeds, it is reasonable to conclude that this mix acted as a biostimulant contributing to a higher P availability for the roots of zucchini squash. Nevertheless, the current study did not reveal whether the tested mix of microorganisms increased the P availability to the zucchini squash crop through an increased solubility of soil P or through other mechanisms. Hence, further research is needed to unravel the mechanisms deployed by the microorganisms tested in the current study to increase the soil P availability in soil-grown zucchini crops.

Adequate P levels in zucchini shoots range between 4 and 6 mg g^{-1} [60]. Considering this P range, the shoot P levels measured in non-inoculated plants in the current study are considered marginally low, while seed priming with PGPR shifted the shoot P to levels well within the adequacy range. Hence, plants originating from seeds treated with PGPR increased plant growth and fruit production, a result which is in agreement with Batista et al. [61] and Souza et al. [62], who concluded that increased P availability and absorption leads to yield increase in zucchini plants.

Regarding N concentration, several studies have found that PGPR can increase the N levels in tomato and zucchini shoots [10,63,64]. However, in the present study, PGPR application did not increase the N levels in plant shoots in both experiments. The lack of any effect of the tested mix of PGPR on plant N status was presumably a result of the adequate N supply to all treatments, which was ensured by using the DSS NUTRISENSE to calculate a balanced fertigation scheme throughout the experiment. Thus, the PGPR tested in the current study could not provide an additional benefit to the plants from inoculated seeds. Furthermore, the tested mix of PGPR did not increase the potassium levels in the shoot of plants originating from inoculated seeds. In agreement with our results, the potassium status in cultivated vegetable plants is usually not affected by the presence of PGPR in their roots [10,65].

Organically cultivated zucchini produced from 1.2 to 1.7 kg of fruit per m^{-2} [66], while plants in soilless production systems can reach 3.5–6.0 kg m^{-2} [49]. In the current study, the application of PGPR in a conventional zucchini crop resulted in substantially higher yield levels than those reported by Montemurro et al. [66] in organically cultivated zucchini but lower than in the soilless zucchini crop. Nevertheless, the yield performance of fruit vegetables is also dependent on the duration of the harvesting season, which can vary widely depending on the cropping season, the cropping system, and the local cultivation practices.

The comparison of the two genotypes tested in the current study revealed that the local landrace "Kompokolokytho" produced lower shoot fresh and dry biomass and lower total yield compared to the commercial hybrid under greenhouse conditions. However, under open field conditions, the local landrace, and the hybrid "ARO-800" resulted in similar total yields. A likely explanation for the different responses of the two genotypes is the season of the year, which was winter in the greenhouse crop but summer in the open field crop. Presumably, the local landrace has either a lower adaptability than the commercial hybrid under cold conditions or a higher adaptability to hot summer conditions. The latter is more likely, as "Kompokolokytho" is a native landrace of Greece, a country with a hot summer season, and this landrace is traditionally grown in the open field.

5. Conclusions

The current study showed that the tested mix of *Bacillus* sp. strains possesses a biostimulant activity, as its application through seed priming had a positive effect on both the vegetative growth and the yield of zucchini squash grown conventionally under both greenhouse and open field conditions. The increased biomass and fruit production in the plants originating from inoculated seeds compared to those obtained from non-inoculated plants was associated with significantly higher shoot P levels in both the greenhouse and the open field crop and in both tested genotypes, i.e., the local landrace and the commercial

hybrid. Furthermore, at the end of the cultivation, the colony-forming units of *Bacillus* sp. were appreciably higher in the plants originating from inoculated seeds compared to those from non-inoculated seeds. This finding suggests that the roots of zucchini squash can be effectively colonized by the tested mix of *Bacillus* sp. microorganisms when this is applied through seed inoculation. Furthermore, the effective colonization of the zucchini squash roots with the tested PGPR points to causal relationships between the tested *Bacillus* sp. strains and the increased shoot P levels and, in turn, between the higher shoot P levels and the increased vegetative and fruit biomass production. Nevertheless, further research is needed to unravel the mechanisms underlying the increased P availability for the roots of zucchini squash that is achieved after seed priming with the tested PGPR mix.

The commercial zucchini squash hybrid "ARO-800" produced more vegetative and fruit biomass than the local landrace "Kompokolokytho" under greenhouse cropping conditions, while in the open field crop, both genotypes performed equally, presumably because the local landrace is traditionally grown in, and thus adapted to, open field cropping systems.

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