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Optimizing Crop Water Productivity in Greenhouse Pepper

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Abstract: Although advanced production systems have been developed in the last 20 years, water scarcity is still a growing problem in agriculture. This study aims to evaluate the effect of different strategies that combine the application of seaweed and microbial biostimulants with regulated deficit irrigation (RDI) strategies on the irrigation water productivity (WP_I), fruit quality parameters and soil enzymatic activity in pepper plants (*Capsicum annum* sp.) under two commercial greenhouse conditions. In each trial, two treatments were applied: (i) irrigation according to Farmer criteria without biostimulant applications and (ii) a combined treatment of RDI and the same biostimulation program, composed of *Bacillus paralicheniformis* and *Ascophillum nodosum* extracts. RDI was applied in different phenological stages in each greenhouse after the establishment until the 1st harvest in trial 1 or during the ripening and harvest period in trial 2. On average, the irrigation was reduced by 600 m^3 ha^{-1} compared to the Farmer irrigation schedule. In both trials, biostimulation promoted an increase in fruit numbers, punctually in trial 1, leading to yield precocity, or generally in trial 2, obtaining a higher yield. Globally, WP_I was increased when RDI was combined with biostimulation. This combined treatment also enhanced the root water absorption and improved the soil enzymatic activity in both greenhouses, suggesting that nutrients in the soil would become more available to plants. Thus, the combined action of biostimulation under different RDI strategies has been proved to be a useful strategy to improve agricultural sustainability.

Keywords: regulated deficit irrigation; soil water content; biostimulants; fruit quality; soil enzymatic activity

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1. Introduction

The world is facing a growing water crisis. Global water scarcity, exacerbated by climate change and population growth, is a pressing issue that threatens our ability to meet the needs of agriculture for feeding the population. Currently, this sector is the major consumer of freshwater resources [1]. In many regions, water scarcity has led to desertification and the loss of soil fertility [2,3], threatening the development of agriculture. Particularly, in arid and semi-arid regions where water resources are naturally limited, water scarcity has not only consequences for the environment, but also for society and other economic sectors that are confronting a crisis marked by declining economic benefits and significant challenges ahead [4].

To achieve properly sustainable agriculture, the first step must be water resource optimization, as sustainable water management helps society adapt to climate change by building resilience against climate change [5].

As it is often pointed out, the Mediterranean areas are highly productive regions that combine favorable growing conditions with limited water resources [6–8]. Jägermeyr et al. [9] quantified a potential increase in crop water productivity, of about 9 to 15%, just by transitioning to improved sprinkler or drip irrigation systems. These more efficient systems allow us to develop other techniques, such as regulated deficit irrigation (RDI), which consists of imposing water deficits at certain developmental stages [10]. This strategy has been broadly studied in woody crops, but is relatively 'unexplored' in horticulture because the water deficit could easily reflect a reduction in the obtained yield [11].

The importance of assessing the benefits of water stress management in crops also has been pointed out by different researchers [11]. A light to moderate deficit irrigation strategy could promote certain benefits, such as improving the soil water extraction [12], flowering [13], or enhancing the synthesis of osmoprotectant compounds [14,15] and secondary metabolites [16]. Furthermore, this water stress could be a signal for the rhizosphere to ameliorate the drought tolerance [17].

However, deficit irrigation management requires a deeper understanding of the sensitivity to water stress in the different phenological stages of a crop, as well as monitoring the soil water status through sensors [18].

Pepper has been classified as a crop susceptible to water deficit; the blossom and germination stages are the most sensitive periods, while the pre-blossom stage is less sensitive [19]. Pepper production in Spain has been increasing since 2012, currently reaching more than 22,000 ha, 73% of which is from greenhouses due to the higher productivity of those systems [20]. Spanish pepper production in 2022 was 1,533,280 t, accounting for 41% of the total European crop [21]. A case worth mentioning is the Almeria region, where 12.294 ha of greenhouse pepper are allocated and 64% of the national crop is grown [20].

Although numerous researchers have conducted studies on the impact that RDI techniques have in pepper water relations [22,23], flowering [24], yield [25–28] or quality [29–31], their findings have demonstrated variations among different varieties and locations.

The term plant biostimulant has become familiar to society in recent years, going through different classifications and definitions in the scientific community [32–34]. Until 2019, biostimulants were included in broad terms in the European regulation (Regulation (EU) 2009/1107) [35], which led to different regulations for these products in each country. With the aim of harmonizing the definition across countries, a new EU rule for fertilizing products was introduced in 2019 (Regulation (EU) 2019/1009) [36], in which plant biostimulants were defined as 'Certain substances, mixtures and micro-organisms that not being an input of nutrients, stimulate plants' natural nutrition processes'. This definition includes the terms substances and microorganisms indiscriminately, as it now takes into account the effect produced in the plant more than its nature, 'Where such products aim solely at improving the plants' nutrient use efficiency, tolerance to abiotic stress, quality traits or increasing the availability of confined nutrients in the soil or rhizosphere, they are by nature more similar to fertilizing products than to most categories of plant protection products. They act in addition to fertilizers, with the aim of optimizing the efficiency of those fertilizers and reducing the nutrient application rates', as industry claimed [37].

Nowadays, both the legislation and papers in the scientific literature report that biostimulants can effectively mitigate abiotic stress [33,38], a problem that needs to be addressed, as it is often faced by crops that grow in semiarid climates, where water resources are scarce and of low quality. Although the quantification of these effects across all crop varieties remains to be accomplished, it would be of great help in minimizing the drawbacks of RDI in crops growing in these areas.

Among biostimulants, some of the nonmicrobial types, such as those derived from seaweeds, have been reported to promote plant growth, acting on the differential regulation of genes implied in nutrient uptake or in the secondary metabolism as phytohormone regulators, or mediating the response to abiotic stress through physiological and biochemical changes, the accumulation of osmolytes, or effects on the rhizosphere [39]. Sometimes, those effects have been cited for their phytohormone-like activity [39,40], but the complex composition of biostimulants (polysaccharides, phenolic compounds, vitamins, osmolytes, etc.) [40,41] involves numerous interactions among their components [32], and as a result, the observed effects are not explained by their individual components, but by holistic view of their combination. On the other hand, microorganisms known as plant growth promoting rhizobacteria (PGPR), have been mainly used to ensure the yield under low-input conditions [42]. As not all bacteria possess the same mechanisms, direct and indirect mechanisms have been reported for the PGPR, related to nutrient uptake and availability (translocation, nutrient transporter activity, production of enzymes), stimulation of the root system (in different ways), improved water relations or antioxidative system, the regulation of plant hormones, the production of volatile compounds, or the excretion of low- and high-molecular weight organic compounds into the rhizosphere [42,43].

The combination of microbial and nonmicrobial biostimulants can have additive or synergistic effects [42] that, in fact, have been effectively used to manage abiotic stresses by modifying physiological, biological and biochemical processes of the crop [44].

RDI is widely known in woody crops as a technique to optimize the water use. Its implementation in horticulture has not been achieved, as it has shown some disadvantages in production.

With the aim of improving the crop water productivity in pepper, in this study, two different techniques were combined: the implementation of deficit irrigation in pepper through the control of soil water status, together with the application of commercial biostimulation strategies combining different products. Our hypothesis is that the biostimulation will promote plant performance when subjected to water stress created by the RDI. Therefore, the aim of this research was to evaluate the combination of a moderate water deficit with the application of microbial and nonmicrobial biostimulants in two commercial greenhouse pepper crops, in relation to their effect on irrigation water productivity, commercial quality parameters and soil enzyme activity.

2. Materials and Methods

2.1. Study Sites and Experimental Conditions

Two trials in pepper (*Capsicum annuum* sp.) were conducted between July 2022 and May 2023 in two commercial greenhouses located in the Almería region, SE Spain. Table 1 details the experimental conditions of each farm during the experimental period. The climate in Almería region is hot semi-arid, belonging to Bsh in Köppen classification; the average temperature is 19 °C, with annual rainfall of around 200 mm in 25 days [45] and basal evapotranspiration of 1285 mm [46].

Table 1. Experimental conditions for each trial location.

	Trial 1	Trial 2
Crop	Pepper Italian	Pepper California
Variety	Sweet Palermo (Rijk Zwaan)	Masami (Syngenta)
Planting date	26 July 2022	3 August 2022
Coordinates	36.762764, −2.88874	36.790612, −2.665999
Location	Balanegra, Almería	Vicar, Almería
Nearest climatic station	Adra, AL-10	La Mojonera, AL-01
Harvesting	8 harvests 132–273 days after transplant	6 harvests 210–286 days after transplant
Soil properties	Clay-loam (38–34–28) OM: 1.23% N-NO_3^-: 48 Ppm pH: 8.23 EC_{Sat}: 3.25 mS cm^{-1}	Clay-loam (42–30–28) OM: 1.33% N-NO_3^-: 33 Ppm pH: 7.23 EC_{Sat}: 2.64 mS cm^{-1}
Irrigation water	pH 7.67 EC: 1.04 mS cm^{-1} NO_3^-: 34.1 mg l^{-1}	pH 7.98 EC: 1.61 mS cm^{-1} NO_3^-: 6.0 mg L^{-1}

OM: organic matter; EC: electrical conductivity; EC_{Sat}: electrical conductivity in saturated extract; SAR: sodium adsorption ratio.

The trials were carried out in two plastic greenhouses, whose structures corresponded to the traditional regional type "raspa y amagado", without heating and with natural rooftop and lateral ventilation. The cultivation system for all greenhouses was sandy soil, a mix of clay, manure and sand arranged on top of the original soil base [47].

The planting frame was double row, the width between rows and between the plants in each row was 2 m and 0.5 m, respectively, with a density of 20,000 plants ha^{-1}.

Two treatments were established: (i) the Farmer treatment, following the conventional irrigation management of farmers, without the application of any biostimulant (F, hereafter); and (ii) the Biostimulated treatment, in which a biostimulation program and irrigation reduction were applied (B, hereafter).

The biostimulation program consisted of Accudo® (*Bacillus paralicheniformis* [48]), applied at 0.5 L ha^{-1} via irrigation at 1 and 15 DAT; Seamac Rhizo® (*Ascophilluym nodosum* extract with amino acids), applied at 2.5 L ha^{-1} via fertigation at 15, 45 and 75 DAT; and Seamac PCT® (*Ascophilluym nodosum* extract), applied via foliar spray at a dose of 2.5 L ha^{-1} at 45, 60 and 75 DAT.

The experimental design was split plot, with four replications per treatment (n = 4). Each field replicate was composed of nearly 800 plants distributed in three adjacent lines; the central plants were monitored, and the rest served as plant border. The total test area amounted to 3200 m^2 for each trial. All treatments followed the same phytosanitary programs, according to the normal practices.

2.2. Irrigation Scheduling

Farmer irrigation was established according to usual practices in the zone, with daily short irrigation events, avoiding water lixiviation below the root system. Outdoor reference evapotranspiration (ET_0) was obtained from the nearest agroclimatic station for each greenhouse, namely, Al-10 and Al-01, belonging to the RIA [46] for trials 1 and 2, respectively. Crop coefficient (K_c) values for the initial, mid season and late season were 0.2, 1.3 and 0.9, respectively, as suggested for plastic greenhouses of the southeastern coast of Spain [7].

In the Biostimulated treatment, irrigation was reduced with respect to Farmer irrigation protocols in different phenological stages for each greenhouse: after establishment to the first harvest in trial 1 or during the ripening and harvest in trial 2. During the remaining period, the Biostimulated treatment was irrigated with the farmer's criteria. The water applied in both treatments was quantified by using volumetric meters.

A conventional fertilization was followed [49,50], corresponding to a $N–P_2O_5–K_2O–Ca$ dose of 241–125–242–177 kg ha^{-1} in trial 1 or 152–97–250–123 kg ha^{-1} in trial 2, respectively. Since the fertilization was carried out through irrigation, the Biostimulated treatment received a lower dose of nutrients.

2.3. Soil Water Status

Soil water matric potential (Ψ_m, kPa) was measured at a 20 cm depth with a thermal compensation capacitive sensor, model TEROS-21 (Decagon Device Inc., Pullman, WA, USA), in four replicates per treatment (n = 4) for each trial.

The water stress integral (WSI, kPa day) was calculated according to the difference between the B treatment Ψ_m values and the Ψ_m at field capacity for each trial (trial 1 $\Psi_{mFC} = -11.5$ kPa, trial 2 $\Psi_{mFC} = -10.0$ kPa). WSI was calculated according to the growth phases considered: vegetative growth (0–56 and 0–63 DAT), reproductive growth (56–98 and 63–147 DAT), maturation (98–133 and 147–210 DAT) and harvest (133–273 and 210–283 DAT), in trials 1 and 2, respectively.

The Ψ_m signal intensity (SI_Ψ) was obtained for F and B treatments through the ratio between the minimum daily Ψ_m and field capacity values. For the water stress period in each trial (28–132 DAT for trial 1 or 150–190 & 230–286 DAT for trial 2), SI_Ψ was averaged.

The soil volumetric water content (θ, m^3 m^{-3}) was measured using an FDR-type probe model Drill & Drop (Sentek Technologies, Stepney, Australia) every 10 cm, between 10 and 60 cm depth in trial 1 due to the soil in trial 2 being inappropriate for this sensor. Four probes per treatment (n = 4) were installed at 10 cm from the dripper in the wetting bulb closest to the plant.

The root water absorption rate was calculated from the daily variation in soil volumetric water content (mm day^{-1}) at the root active depths (20 to 60 cm). Data for the 10 cm depth were discarded, as it was affected by soil evaporation.

The sensor data were acquired every minute and averaged each 10 min and recorded with a datalogger.

2.4. Harvest and Fruit Quality

Yield was determined by hand-harvesting of 160 plants per field replicate (n = 4); all harvests were carried out under commercial criteria, harvesting on different days when the fruits reached the optimum state of maturity required for their sale.

Two categories were established for each variety according to the cooperatives in the area. The 1st quality for California pepper (cv. Masami) included fruits in categories G and GG, between 110 and 70 mm in upper fruit diameter. The 1st-quality fruits for Italian pepper (cv. Sweet Palermo) presented a length of between 20 and 25 cm. In both varieties, the 2nd quality comprised fruits below the 1st-quality references or deformed peppers.

After the last commercial harvest in each greenhouse, the remaining green peppers were cut and classified into the categories previously established.

2.5. Irrigation Water Productivity (WP_I)

WP_I was calculated as the relationship between total harvest, the sum of the harvested yield of all categories (kg ha^{-1}) and the applied irrigation water (m^3 ha^{-1}) accumulated at the end of the trial [51,52].

2.6. Soil Enzymatic Activity

Bulk soil sampling was carried out at full harvest for each of the trials, on 24 February 2023, corresponding to 213 DAT in trial 1, and on 4 May 2023, corresponding to 274 DAT for trial 2. A soil sample per replicate was taken (n = 4 per treatment) from the surface layer (0–20 cm), air-dried and sieved to 0.5 mm. The β-glucosidase (EC 3.2.1.21), alkaline phosphatase (EC 3.1.3.1) and urease (EC 3.5.1.5) enzymatic activities were assessed as follows: Urease activity was determined as the amount of NH_4^+ released after the incubation with urea, according to the procedure described by [53,54]. Alkaline phosphatase and β-glucosidase activities were determined as the amount of *p*-nitrophenol (PNP) released from the hydrolysis of the substrate *p*-nitrophenyl phosphate [55] or *p*-nitrophenyl-β-D-glucopyranoside [56], respectively. All reagents used were of analytical quality.

2.7. Statistical Analysis

To determine the individual effects of the treatments F and B within each trial, a paired Student *t*-test ($p < 0.05$) was performed. Linear mixed-effect models (LMMs) were used to evaluate the effect of the biostimulation program in both trials 1 and 2 on the soil enzymatic activity and productivity parameters. Using the treatments (F, B) as fixed effects, field replicates were included as a nested random effect in each trial (1, 2). All statistical analyses were carried out using the InfoStat software version 2020e [57].

3. Results

3.1. Irrigation Scheduling

The weather conditions did not show significant differences among the trial locations. The average climatic demand (ET_0) outside the greenhouse was very similar between both locations: 3.0 mm day^{-1} and 3.12 mm day^{-1} in trials 1 and 2, respectively. However, during the months of November, December, and January, the climatic demand did differ between locations, being reduced in trial 2 with respect to trial 1 (1.5 vs. 2.0 mm day^{-1}).

Different irrigation schedules were applied in the studied greenhouses. In the trial 1 greenhouse, irrigation water reduction was carried out after establishment of the crop, from 28 DAT until the beginning of the harvest. During this period, weekly irrigation water was reduced between 12% and 42%, resulting in 642 m^3 ha^{-1} saved, which represented 11% of the water applied by the Farmer treatment, i.e., 5945 m^3 ha^{-1} throughout the cycle (Figure 1A). The water reductions in each phenological period, with respect to the F treatment, were 367 m^3 ha^{-1} during vegetative development, 308.9 m^3 in flowering and 56.6 m^3 ha^{-1} at the first stage of maturity.

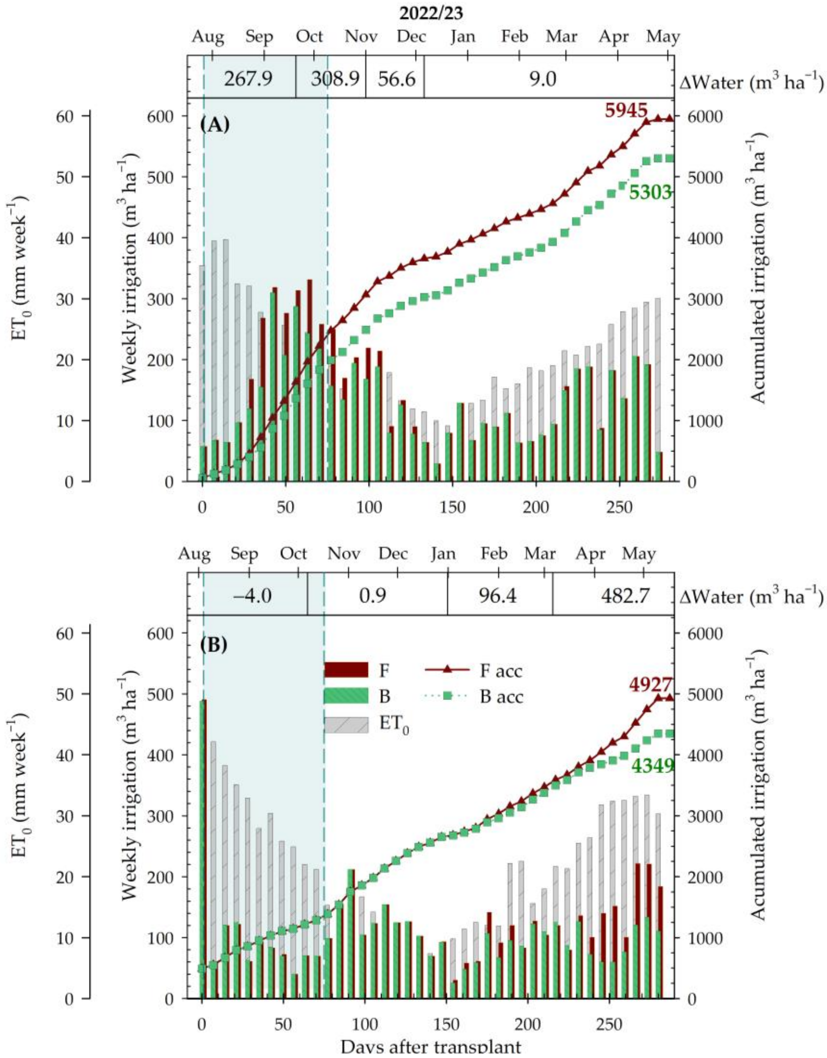

Figure 1. Weekly (bars) and accumulated (lines) irrigation for each irrigation treatment, F and B, in (**A**) trial 1 and (**B**) trial 2, 0 DAT corresponding to (**A**) 26 July 2022 or (**B**) 3 August 2022. Weekly outdoor ET_0 from the nearest climatic station is shown in grey bars. The period for biostimulant application is shaded in blue. Differences (Δ) in applied water between F and B treatments are shown for each period.

For trial 2, the two treatments were irrigated similarly during most of the crop cycle, except for two periods. The first period was from 150 to 195 DAT, in which the water applied in the B treatment was reduced by 15–20% compared to the farmer treatment. The second period comprised 230 DAT until the end of the crop cycle (286 DAT); during this period, as there was a considerable increase in climatic demand, the farmer treatment increased the irrigation water inputs, and the largest reductions in the B treatment were carried out, of up to 60% less in the weekly water supply, which implied 482.7 m^3 ha^{-1} of water savings. Both irrigation reduction periods implied water savings of 579 m^3 ha^{-1}, a 12% reduction of the water applied by the F treatment in the whole cycle (Figure 1B).

Farmer fertilization in each greenhouse was carried out according to commercial criteria and pepper variety. By the end of the crop cycle, N fertilization amounted to 241 kg ha^{-1} for trial 1 and 152 kg ha^{-1} for trial 2 (Table 2). Due to the irrigation reduction applied, the fertilization was also reduced in the Biostimulated treatments. Even though the irrigation reduction percentage was within similar ranges for both greenhouses (around 11.5%), the different fertigation strategies carried out by the two farmers and the phenological stage in which the irrigation reduction occurred resulted in different reduction percentages for the macronutrients supplied, being around 10% in trial 1 or 3% in trial 2.

Table 2. Macronutrients applied through fertigation (kg ha^{-1}) during the entire crop cycle for trial 1 and 2 in Farmer (F) and Biostimulated (B) treatments. The reduction percentage for each macronutrient is shown.

	Trial 1			Trial 2		
kg ha^{-1}	F	B	Δ (%)	F	B	Δ (%)
N	240.98	213.86	11%	152.31	146.57	4%
P_2O_5	124.99	111.32	11%	96.88	95.60	1%
K_2O	242.32	214.28	12%	249.50	240.37	4%
Ca	177.12	158.99	10%	122.50	116.86	5%
Mg	10.94	9.98	9%	0	0	0%

3.2. Soil Water Status

The soil water matric potential (Ψ_m) was monitored throughout the pepper growing cycle in the two trials (Figure 2).

In trial 1, the Ψ_m values at field capacity were around −11.5 kPa (Figure 2A). The Farmer treatment maintained these values throughout the cycle. The exception was between 80 and 100 DAT, when the Farmer treatment decreased the weekly irrigation rate, which reduced Ψ_m values to −30 kPa. The irrigation reduction in the Biostimulated treatment occurred between September and December (40 to 130 DAT), including the vegetative growth stage, after establishment and up to the first harvest, during which the Ψ_m was maintained between −40 and −50 kPa. At the end of the crop cycle, after 250 DAT, despite maintaining the same irrigation levels for both treatments, the Ψ_m in the Biostimulated treatment was reduced to −40 kPa, in alignment with the high values for the root absorption rate (Table 3). Considering the whole cycle, the Biostimulated treatment accumulated a water stress integral (WSI)—calculated from the Ψ_m values—of 3034 kPa day.

Table 3. Average root absorption rate (mm day^{-1}) in each period defined between corresponding days after transplant (DAT) for Farmer (F) and Biostimulated (B) treatments in trial 1.

Period	Differential Irrigation	Harvest	Final
$DAT_{i–f}$	30–131	132–241	242–273
F	−4.27	−3.81	−3.85
B	−3.79	−3.83	−4.46
p-value (t-Student)	<0.0001 ***	0.5268 ^{ns}	<0.0001 ***

***, *p*-value < 0.001, ^{ns} indicates no significant differences were found in *t*-test for the same period.

In trial 2 (Figure 2B), both treatments were irrigated, maintaining the Ψ_m at field capacity level of around −10 kPa, except between 40 and 80 DAT, when the irrigation was reduced to induce flowering, reaching Ψ_m values of −80 kPa. The two irrigation reduction periods applied in the B treatment during the maturation and harvest stages reduced the Ψ_m values to around −28 and −37 kPa, respectively. The WSI calculated for these periods was 109.5 and 236.6 kPa day, respectively; that, together with the water stress applied to promote flowering (863 kPa day), led the B treatment to accumulate 1294 kPa day.

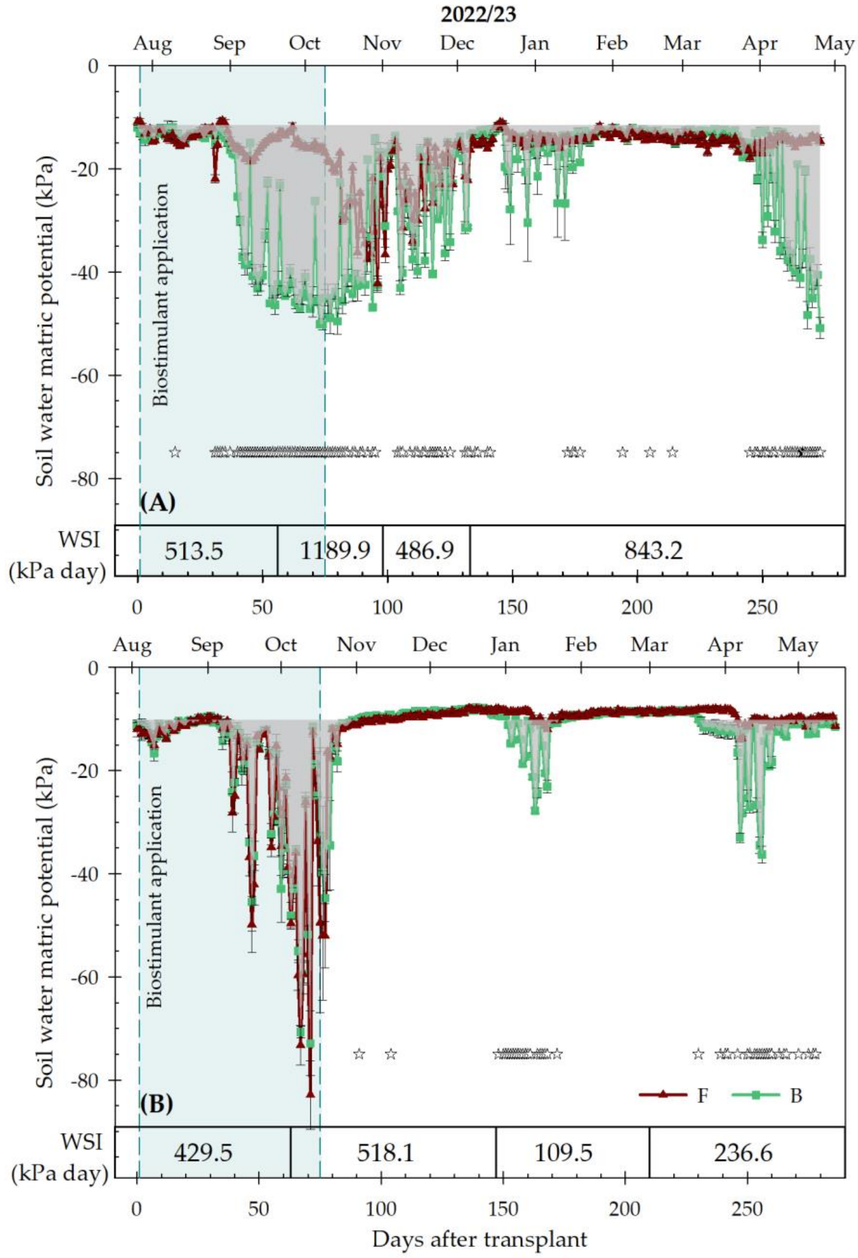

Figure 2. Daily evolution of minimum soil matric potential at 20 cm depth in (**A**) trial 1 and (**B**) trial 2. Stars mean significant differences according to Student *t*-test ($p < 0.05$). Grey areas indicate stress integral between B and F treatment. The period for biostimulant application is shaded in blue. The water stress integral (WSI) values for B treatment in relation to field capacity (Trial $1_{FC} = -11.5$ kPa, Trial $2_{FC} = -10.0$ kPa) are shown for each period.

The average signal intensity, SI_Ψ, calculated through the relativization of each treatment's minimum daily Ψ_m values during the stress period to field capacity in each trial (Trial 1 = −11.5 kPa, Trial 2 = −10 kPa), was 1.70 and 2.77 in trial 1 and 1.07 and 1.48 in trial 2 for the Farmer and Biostimulated treatments, respectively. The Biostimulated treatment SI_Ψ was significantly increased in both greenhouses compared to the Farmer's SI_Ψ (*t*-Student test *p*-value Trial 1 = 0.0002, Trial 2 = 0.0004). The irrigation schedule applied in each greenhouse played a role in the Farmer treatment SI_Ψ values, being higher in trial 1, because the particular irrigation scheduling implemented in trial 2 kept matric potential near field capacity during most of the cycle.

Figure 3 shows the evolution of the soil matric potential (Ψ_m) for water deficit and recovery periods in the two trials. In trial 1, the scheduling for the irrigation reduction in the B treatment was based on a reduction in the daily irrigation time of the F treatment, whereas for trial 2, the irrigation reduction was made through the reduction of irrigation events.

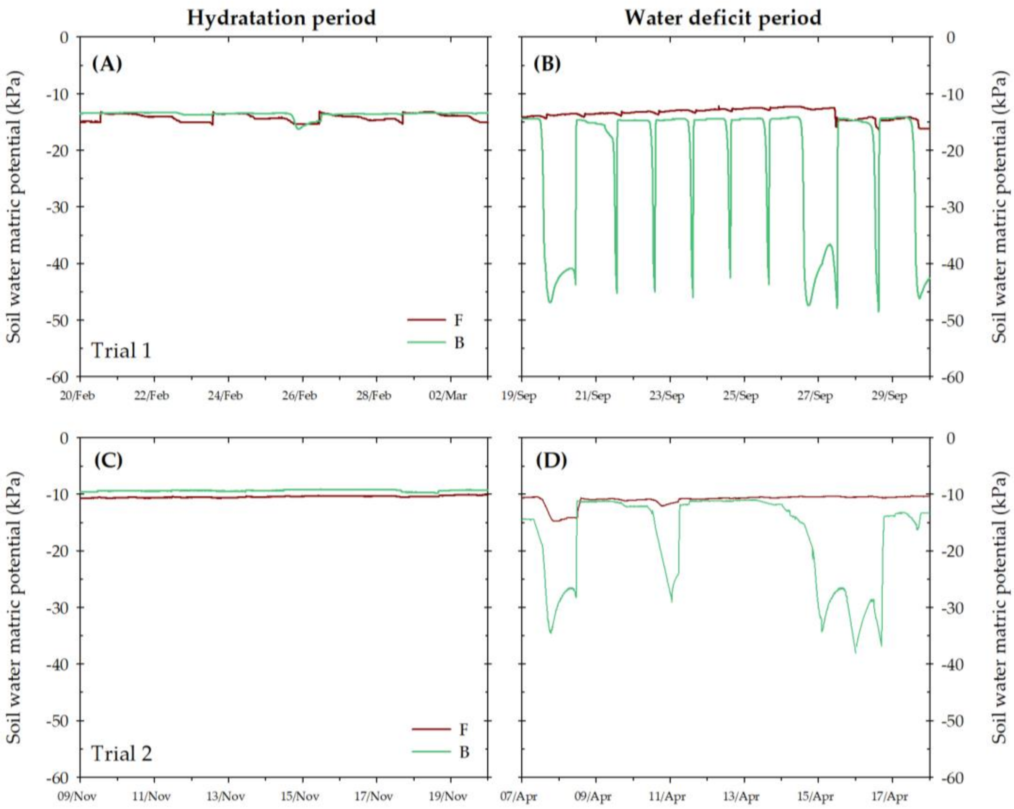

Figure 3. Daily evolution of soil matric potential at 20 cm depth for F and B treatments during a hydration period (**A**,**C**) or water deficit period (**B**,**D**), in trial 1 (**A**,**B**) and trial 2 (**C**,**D**).

3.3. Root Water Absorption

During the beginning of the crop cycle in trial 1, from day 30 to 131 after transplanting, the root water absorption of the F treatment was around −4.27 mm day^{-1}, significantly higher than that of the B treatment. During this stage, the B treatment was submitted to water deficit, with less water available in the soil than in the F treatment. At the beginning of the harvest period, both treatments were under the same irrigation program. During this period, the daily root absorption rate reached similar levels in both treatments, of around 3.82 mm day^{-1}. Further, during the last harvests, even under the same irrigation program, a significant increase in root water absorption was observed in the B treatment, of an average 0.61 mm day^{-1} higher than that in the F treatment (Table 3).

3.4. Soil Enzymatic Activity

Soil bulk sampling was carried out in full harvest for each trial at 20 cm depth (Table 4). β-glucosidase activity oscillated between 0.1 and 0.3 mmol pNP g^{-1} h^{-1} in both greenhouses. The Farmer treatment in trial 2 was found to have the lower activity rate, being this treatment significantly increased in trial 1. The application of biostimulant significantly increased β-glucosidase activity in both trials. Alkaline phosphatase activity was greatly influenced by the location, being higher in trial 1, with values between 0.97 and 1.95 mmol pNP g^{-1} h^{-1}, whereas the range in trial 2 was between 0.39 and 0.97 mmol pNP g^{-1} h^{-1}. Despite the differences among the values found in both trials, the Biostimulated treatment significantly increased phosphatase activity. Urease activity was greatly influenced by the location, showing different trends in each one. Trial 1 showed similar levels of urease activity in both treatments (0.53–0.67 mmol NH_4^+ g^{-1} h^{-1}), while trial 2 showed an increment in this activity in the B treatment, from 0.41 to 1.39 mmol NH_4^+ g^{-1} h^{-1}.

Table 4. Soil enzymatic activities in 0–20 cm depth for β-glucosidase, alkaline phosphatase and urease for Farmer (F) and Biostimulated (B) treatments in the two trials (1, 2).

Trial	TRT	β-Glucosidase (mmol pNP g^{-1} h^{-1})	Alkaline Phosphatase (mmol pNP g^{-1} h^{-1})	Urease (mmol NH_4^+ g^{-1} h^{-1})
1	F	0.190	0.968	0.533
	B	0.293	1.950	0.673
p-value (t-Student)		0.0283 *	0.0027 **	0.2139 ^{ns}
2	F	0.098	0.390	0.408
	B	0.313	0.970	1.393
p-value (t-Student)		0.0010 **	0.0001 ***	0.0037 **
LMM	F	0.144	0.679	0.47
	B	0.300	1.477	1.033
	p-value	0.0001 ***	<0.0001 ***	0.0044 **

* *p*-value < 0.05, **, *p*-value < 0.01, ***, *p*-value < 0.001, ^{ns} indicates no significant differences were found in *t*-test or LMM.

Despite the differences shown in both trials, linear mixed models (LMM, Table 4) indicated global differences in the enzymatic activity analyzed when both treatments and the two locations were considered, being increased by the Biostimulated treatments.

3.5. Harvest, Fruit Quality and Water Productivity

In trial 1, harvesting was carried out according to commercial criteria for eight cuts during the cycle (Figure 4). There was no cumulative effect in the harvest between the treatments F and B, with both showing a yield of around 40 t ha^{-1} and 337,000 fruits ha^{-1} (Table 5). However, the B treatment showed more yield precocity than the F treatment, since the yields obtained in the first and third harvest were significantly higher than those obtained with the F treatment (Figure 4A). The number of fruits harvested was higher in the first cut (Figure 4B), while in the second and third, the average fruit weight was increased in the B treatment (Figure 4C). During the remaining cuts, no significant differences were observed in yield, but fruit weight was significantly increased in the last one.

Table 5. Total productivity parameters for Farmer (F) and Biostimulated (B) treatments in trials 1 and 2.

Trial	TRT	Applied Water (m^3 ha^{-1})	Yield (t ha^{-1})	Fruits (10^3 Fruits ha^{-1})	FW (g)	WP_I (kg m^{-3})
Trial 1	F	5945.08	39.33	341.81	111.64	6.62
	B	5302.74	40.91	333.27	116.58	7.72
t-Student *p*-value			0.3217 ^{ns}	0.3732 ^{ns}	0.2960 ^{ns}	0.0163 *
Trial 2	F	4927.39	46.07	185.52	242.53	9.35
	B	4348.67	54.49	243.97	222.96	12.53
t-Student *p*-value			0.0071 **	0.0039 **	0.0901 ^{ns}	0.0004 **
LMM	F	5436.24	42.70	263.67	175.83	7.98
	B	4825.71	47.70	288.62	170.97	10.12
p-value			0.0061 **	0.0568 ^{ns}	0.3848 ^{ns}	0.0001 ***

* *p*-value < 0.05, **, *p*-value < 0.01, ***, *p*-value < 0.001, ^{ns} indicates no significant differences were found in Student *t*-test or LMM.

Regarding the harvest classification according to quality, first-quality fruits accounted for 83 and 86% of the total harvest, which implied 32.8 and 35.0 t ha^{-1} for the Farmer and Biostimulated treatments, respectively.

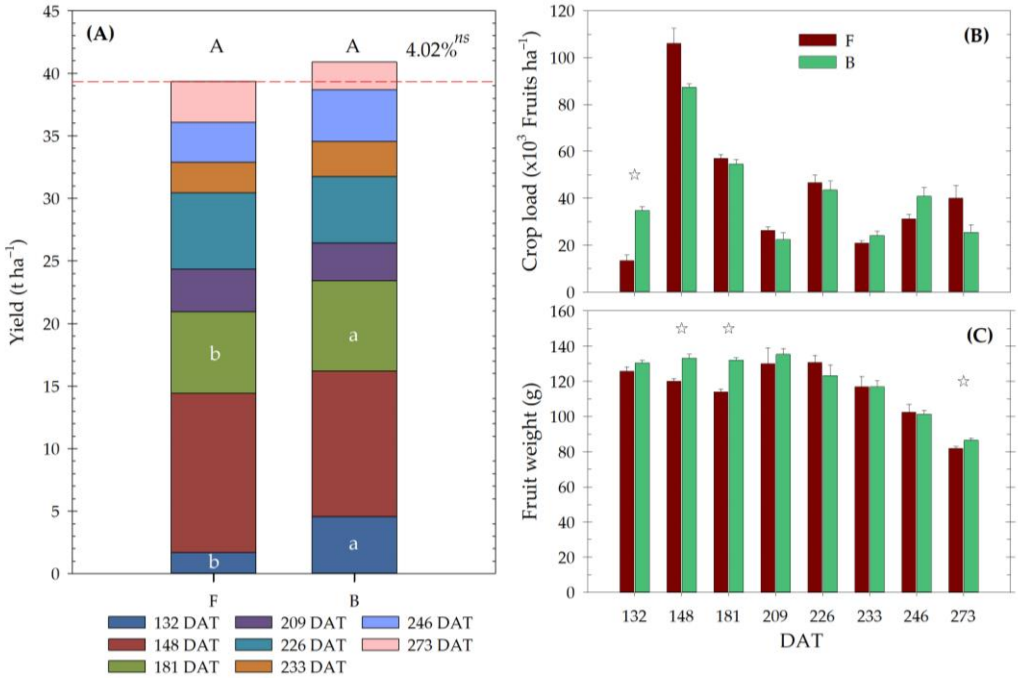

Figure 4. (**A**) Total yield (t ha^{-1}), (**B**) number of fruits and (**C**) average fresh fruit weight of each harvest for Farmer (F) and Biostimulated (B) treatments in trial 1. The stacked bars represent the different harvests in each treatment, and different lowercase letters or stars indicate significant differences between treatments on the same day after transplant (DAT). Capital letters indicate significant differences in the total harvest (^{ns} = non significant differences, *p*-value < 0.05) according to *t*-test.

Similarly to the total harvest, in the first-quality distribution, the Biostimulated treatment obtained a significantly higher yield for the first and third cut. The yield obtained was lower in the last harvest, corresponding to green fruits, due to a smaller number of fruits remaining on the plants (Figure 5A). The average fruit weight in the trial was increased by B treatment in the first three harvests of 1st category, with fruits of 136 g compared to the 125 g obtained in the F treatment harvest. From the 6th cut, the average weight of the fruits began to decrease in both treatments, obtaining smaller fruits of around 105 g (97 g F and 112 g B); these differences were statistically significant in the last harvest carried out in red, with fruits 33 g smaller in the F treatment compared to the B.

Regarding the second-quality yield, in the third cut, the non-biostimulated treatment (F) showed a higher yield, due to a higher number of fruits (16,920 vs. 11,930 fruits ha^{-1}, Figure 5B). However, these fruits presented a lower average weight than the biostimulated ones in the same category (83.4 g vs. 100.8 g).

Trial 2 had a lower number of commercial harvests, although these were of greater volume than those obtained in trial 1, resulting in similar yields obtained between both. The F treatment accumulated 46 t ha^{-1} (Table 5), while the accumulated harvest in the B treatment resulted in an enhanced yield of 8.42 t ha^{-1}, 18.3% higher, due to the increase in the number of fruits per hectare by 58,450, 31.5% more than in the F treatment. Among the individual cuts, the third one, carried out on 241 DAT, 1 April 2023, was the main one, accounting for about 36% of the total harvest (Figure 6A). In this cut, the yield of the biostimulated plants was significantly higher than that obtained by the F treatment in both yield and number of fruits per ha, increasing them by 3.85 t ha^{-1} and 22,150 fruits ha^{-1}, respectively (Figure 6B). During this cut and in the two subsequent ones, the weight of the harvested fruit was affected, resulting in 18 g to 49 g lower weights in the B treatment (Figure 6C).

When classified into categories, the first category harvest comprised 85% of the total yield. The distribution of individual cuts in the first category did not show significant differences in yield between treatments (Figure 7A). Although the increase in the number of fruits was statistically significant in the third harvest (the main one), increasing by 17,950 fruits ha^{-1} (Figure 7A), the average weight of the biostimulated fruit was negatively affected in this harvest and the subsequent ones, although it was higher when the remaining fruits of the last harvest were considered.

Within the second category, differences in yield were found in the last cut, carried out on 286 DAT; in that cut, the B treatment almost doubled the yield obtained for the F treatment, due to an increase in the number of fruits (Figure 7B). This increase in the number of second-category fruits also occurred in the 3rd and 4th harvests, on 241 and 264 DAT, without affecting the average weight of the fruits in any of them (Figure 7B).

Although the total yield obtained in trial 1 was similar for the F and B treatments, as the irrigation water applied was lower in the latter, the water productivity increased significantly in the B treatment, reaching 17% higher than that obtained in the F treatment (Table 5).

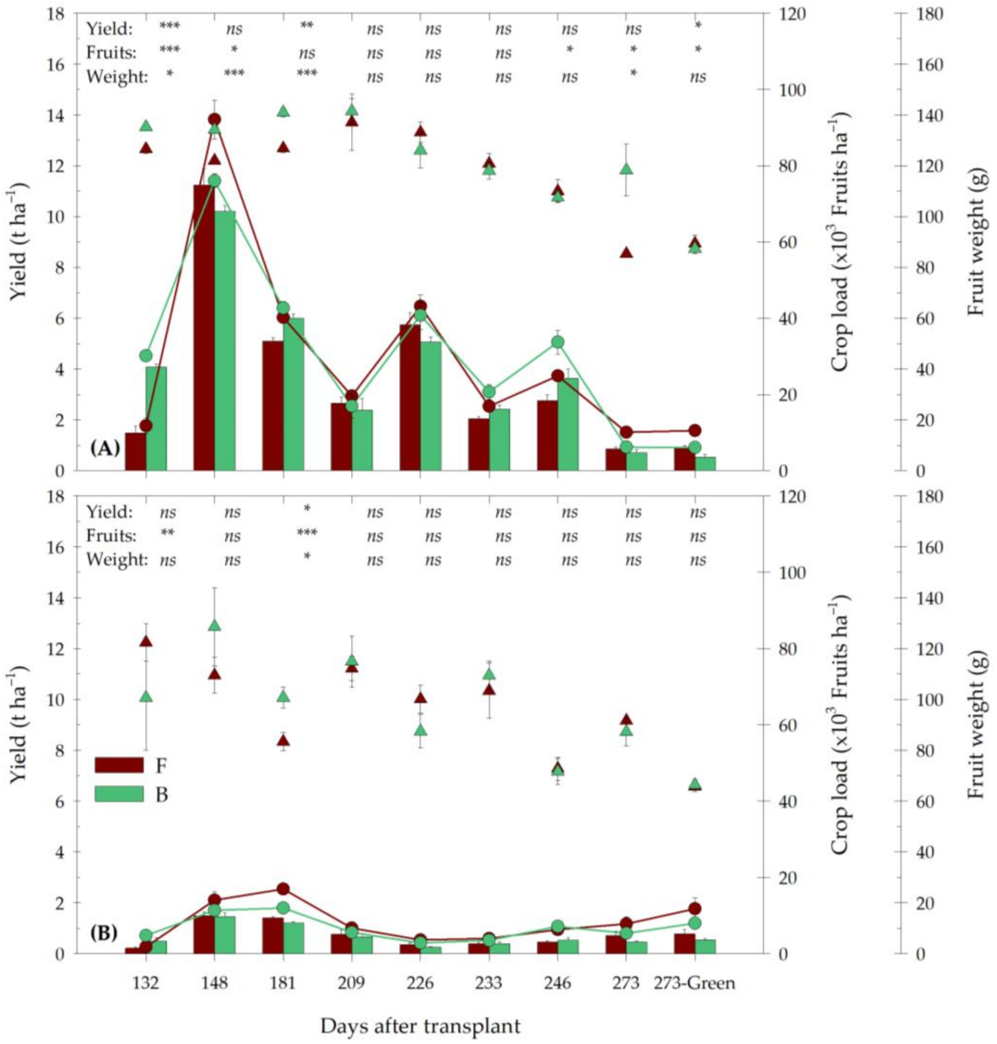

Figure 5. Productive parameters in trial 1 for each individual harvest: yield (bars), fruit per hectare (lines) and fruit fresh weight (triangles), classified according to harvest categories: (**A**) 1st quality, (**B**) 2nd quality. Statistical differences between Farmer (F) and Biostimulated (B) treatments according to *t*-test are shown: * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001, ^{ns} indicates no significant differences.

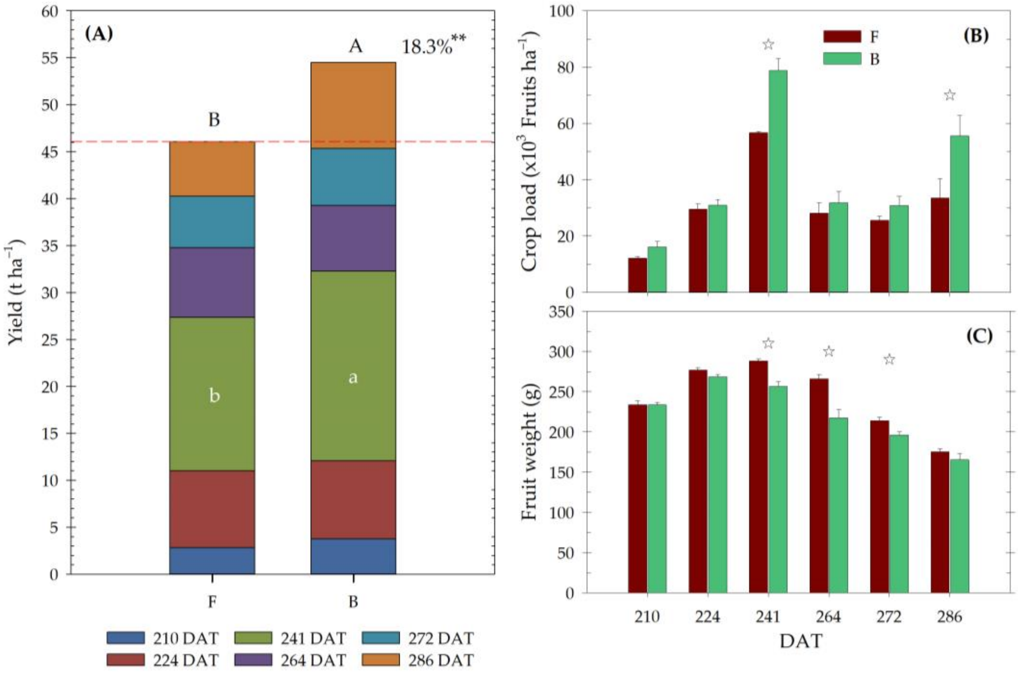

Figure 6. (**A**) Total yield (t ha^{-1}), (**B**) number of fruits and (**C**) average fresh fruit weight of each harvest for Farmer (F) and Biostimulated (B) treatments in trial 2. The stacked bars represent the different harvests in each treatment, and different lowercase letters or stars indicate significant differences (p-value < 0.05) between treatments on the same day after transplant (DAT). Different capital letters indicate significant differences in the total harvest (**, p-value < 0.01) according to t-test.

In trial 2, the yield and crop load significantly increased in the Biostimulated treatment, while the fruit weight was not significantly affected despite averaging 10 g less in the B treatment. As the irrigation was reduced and the yield increased, the B treatment improved its water productivity to 3.18 kg m^{-3}, a 34% gain compared to the Farmer’s WP_I (Table 5).

To analyze the overall effect of biostimulation and irrigation reduction in the different trials, regardless of the phenological phase where it is applied, a linear mixed model (LMM) was applied. The LMM indicated that water productivity would increase due to both the reduction in water applied and the increase in yield obtained.

With respect to the harvest classification into categories, similarly to the total harvest, in trial 1, the cumulative cuts did not result in yield differences for any category. However, the number of small or malformed fruits per hectare (included in the second category) was significantly higher in the Farmer’s treatment (Table 6). Within the categories of trial 2, the cumulative effect of the total cuts appreciated, and the yield and the number of fruits per hectare in both categories were incremented in the B treatment. The average fruit weight, despite having been affected at some cuts, did not show an overall decrease in the cycle (Table 6).

Table 6. Productivity parameters according to pepper quality categories for Farmer (F) and Biostimulated (B) treatments in trials 1 and 2.

Trial	TRT	1st Quality Yield (t ha^{-1})	1st Quality Fruits (10^3 Fruits ha^{-1})	1st Quality FW (g)	2nd Quality Yield (t ha^{-1})	2nd Quality Fruits (10^3 Fruits ha^{-1})	2nd Quality FW (g)
Trial 1	F	32.80	269.12	116.78	6.53	72.68	95.71
	B	35.00	273.13	123.23	5.91	60.14	96.35
t-Student p-value		0.1534 ^{ns}	0.6814 ^{ns}	0.1566 ^{ns}	0.3040 ^{ns}	0.0458 *	0.9087 ^{ns}

Table 6. *Cont.*

Trial	TRT	1st Quality Yield (t ha^{-1})	1st Quality Fruits (10^3 Fruits ha^{-1})	1st Quality FW (g)	2nd Quality Yield (t ha^{-1})	2nd Quality Fruits (10^3 Fruits ha^{-1})	2nd Quality FW (g)
Trial 2	F	40.19	148.56	262.80	5.88	36.97	162.80
	B	45.46	183.35	243.80	9.03	60.62	156.05
t-Student *p*-value		0.0436 *	0.0179 *	0.0751 ^{ns}	0.0004 **	0.0007 **	0.3221 ^{ns}
LMM	F	36.49	208.84	188.50	6.21	54.82	128.85
	B	40.23	228.24	184.76	7.47	60.38	126.54
	p-value	0.0110 *	0.0316 *	0.4745 ^{ns}	0.0634 ^{ns}	0.3583 ^{ns}	0.5994 ^{ns}

* *p*-value < 0.05, **, *p*-value < 0.01, ^{ns} indicates no significant differences were found in Student *t*-test or LMM.

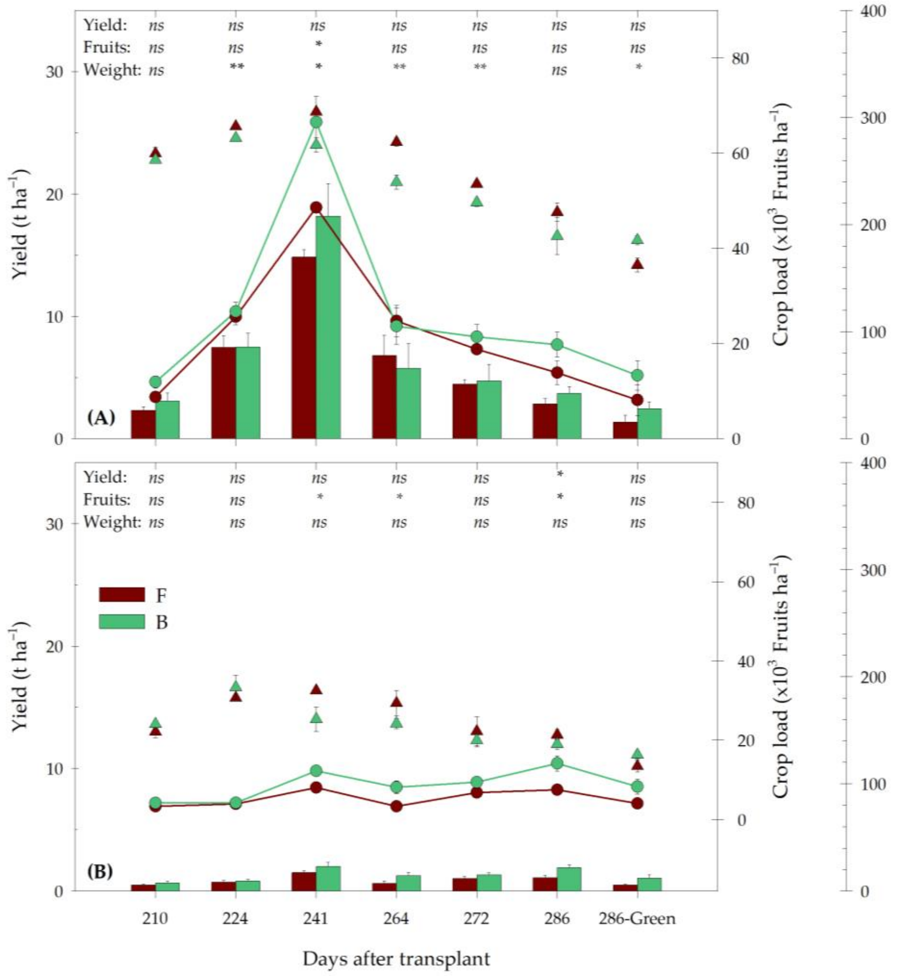

Figure 7. Productive parameters in trial 2 for each individual harvest: yield (bars), fruit per hectare (line) and fruit fresh weight (triangles), classified according to harvest categories: (**A**) 1st quality, (**B**) 2nd quality. Statistical differences between Farmer (F) and Biostimulated (B) treatments according to *t*-test are shown: * *p*-value < 0.05, ** *p*-value < 0.01, ^{ns} indicates no significant differences.

The LMM model indicated that biostimulation could increase the yield and the number of fruits per hectare in the 1st category, without affecting the average fruit weighs, whereas the 2nd quality would not be affected (Table 6). However, these effects would vary depending on the phenological stage when the irrigation reduction was applied (trial 1 or 2).

4. Discussion

The combined effect of regulated deficit irrigation (RDI) and biostimulation (B treatment) increased water productivity (WP_I) by 17 and 34% compared to the Farmer treatment in each of the two trials, due to a reduction in applied irrigation of 642 and 579 m^3 ha^{-1}, respectively (Table 5). In trial 1, yield in the B treatment remained similar to that in the Farmer treatment, although the earliness of production was increased in the first and third cuts, resulting in a greater benefit for the farmer. In trial 2, the yield was significantly improved, due to an increase in the number of fruits harvested (Table 5).

The irrigation reduction strategies were carried out mainly in the phenological stages of vegetative development until the first harvest (trial 1) or ripening and harvest (trial 2), although in the latter trial, both treatments were subjected to a slight water stress promoted by the farmer criteria. The deficit irrigation was applied differently in each trial, while in trial 1, a continuous deficit irrigation was applied, maintaining the soil water potential (Ψ_m) homogeneously between −40 and −50 kPa (Figure 3B). On the other hand, in trial 2, irrigation reduction days were alternated with Farmer irrigation, which resulted in Ψ_m values ranging from −37 kPa to field capacity values for this period (Figure 3D).

As mentioned, the irrigation reduction in trial 1 maintained Ψ_m values at −50 kPa during the water deficit period, (Figure 2), similar to the threshold established for pepper at −58 kPa by Thompson [6]. Instead, this threshold value was exceeded in trial 2 for 4 days, at the end of the vegetative stage (Figure 2). However, other authors have proposed different threshold values, such as −25 kPa [27] for well-watered plants. Dalla Costa and Gianquinto [58] cited critical periods for Ψ_m in pepper, establishing a value above −20 kPa between the beginning of fruit set and first maturing fruits. This threshold was slightly surpassed in trial 2, reaching −28 kPa during the fruit ripening, and completely exceeded in trial 1, reaching up to −40 kPa after the fruit setting until days prior to the harvest (Figure 2).

Although the volume and percentage of water reduction were similar in both greenhouses when compared to the Farmer treatment, the water stress integral (WSI)—according to the soil matric potential values—showed a significant difference between the two trials. Specifically, trial 1 was subjected to a higher WSI than trial 2, oscillating their values for the whole season between 3034.12 and 1299.32 kPa day, respectively (Figure 2). Up to now, the WSI index has been used to quantify the water stress to which a crop has been subjected in a period of water deficit, according to Myers [59]. In this study, we applied this methodology to the Ψ_m values, obtaining a differentiation between the two trials.

The reduction in water supply during the vegetative development of the plant has been previously used to promote yield precocity [26], as occurred in this study (Figures 4 and 6). In this manner, the combination of irrigation reduction with Biostimulation (B treatment) was applied in both trials during this period, accumulating a water stress integral of 1700 and 800 kPa day in trial 1 and 2, respectively. This strategy enabled the yield precocity of the crop in the B treatment of trial 1 (1st and 3rd harvest, Figure 4) and in both treatments of trial 2 (greater yields in a shorter time interval). In this regard, a water deficit applied during the post-veraison period in table grapes promoted an advance in the commercial maturity of the berry, without affecting the total yield [60].

However, Sezen et al. [26] stated that with a continuous reduction in water supply (by about 25 or 50%) applied from the vegetative development until the first picking, an adverse effect on yield could be found. They also indicated that the phenological stages just prior to and during early flowering could be a critical period in pepper, as several researchers have found a reduction in fruit number when irrigation was applied below the crop needs [24,26,29,58,61]. This did not occur in trial 1 (Figure 2), although it should be noted that this treatment was biostimulated with a seaweed extract. In this context, some findings on *Arabidopsis thaliana* reported that treating plants with *Ascophyllum nodosum* extracts reduced the drought effects, leading to a lower concentration of reactive oxygen species or a higher relative water content in leaves [62], allowing us to assume that biostimulation could be the factor that improved the water stress resilience in plants of trial 1. Instead of subjecting the plants to drought conditions, the combination of RDI with biostimulation has been studied in some crops, such as maize, improving both the yield and water productivity [63], similarly to our trial 2, where the mild water stress applied in the biostimulated treatment increased the number of fruits harvested in the first cuts, by approximately 58,500 more fruits than in the farmer treatment (Table 5).

The application of the water stress during the ripening and harvest periods (Figure 2B), which was implemented in trial 2, did not negatively affect the final yield; on the contrary, the yield was significantly increased. However, for this treatment, a greater proportion of fruits were classified as second quality, of 25% compared to the 20% found in the Farmer treatment (Table 6). In fact, the 1st category fruits in trial 2 showed occasional decreases in their average fresh weight, led by this reduction (Figure 7A).

Some authors have implemented RDI strategies to stimulate the spreading of roots at greater depths, achieved by maintaining a threshold for water availability in the soil of around 70% [25,29]. It was also mentioned that this threshold is feasible during the whole crop cycle, but not in harvest, when roots should be provided with at least 90% water availability [25]. In deficit irrigation treatments in pepper plants during this stage, Dorji et al. [31] observed significant decreases in their water status, measured through leaf water potential, due to the competition for water from the reproductive sinks and the demanding temperatures. The yield of the pepper crop was also reduced in various trials when water was reduced during harvest stage, regardless of the strategies used (RDI, partial root irrigation or deficit irrigation) [29,31,64].

The mechanisms by which biostimulants act are not fully understood, given that in the literature, we can find a multitude of references whereby the same raw material, such as the seaweed *Ascophyllum nodosum*, acts at different levels depending on the processing of its formulation [40,65]. Du Jardin [32] remarked that biostimulants should have a development from the field to the laboratory, in order to focus on the mechanisms by which they cause a specific effect, and not the other way around.

In this way, this trial was focused on studying the enzymatic activities of the soil, related to the cycling of the main biologically important nutrients C, N, and P. β-glucosidase catalyzes hydrolytic processes in the organic matter breakdown. Urease and alkaline phosphatase participate in the mineralization of nitrogen and phosphorous compounds. As all of these soil enzymes perform specific biochemical functions, they play an important role in soil fertility [66]. The enzymatic activities of both β-glucosidase and alkaline phosphatase were increased by the Biostimulated treatment in both trials (Table 4). Some researchers [67,68] have observed a decline in β-glucosidase activity in woody crops like citrus or nectarine trees when they are subjected to RDI, due to a reduced nutrient supply. But accordingly with our results, Chen et al. [69] reported that when sugarcane under drought stress is biostimulated with seaweed extract, the activities of different soil enzymes (urease, sucrase, phosphatase, and glucosidase) were enhanced, increasing the availability of nutrients in the soil. Urease activity has been reported to be sensitive to different irrigation regimens under greenhouse conditions, decreasing its activity under full irrigation conditions [70]. In our study, urease activity was enhanced in the biostimulated treatment only in trial 2 (Table 4), due to the soil sampling being carried out in full harvest, coinciding with a water deficit period. Vasconcellos et al. [71] found urease activity to be correlated with a higher microbial biomass, although further studies indicated that soil enzyme activities related to the C, N and P cycles were also correlated with the microbial community structure [67].

Seaweed extracts are reported to promote root water and nutrient absorption, thereby facilitating plant growth [34], and the specific bacterium that was added in this trial has been reported to successfully colonize the root, creating a biofilm and promoting root development [72]. The assessment of root activity is not a common measure, given the difficulty of analyzing the whole root system. In hydroponic trials, it could be inferred by the measure of root biomass [73], although for field trials, the estimation of root water extraction by sensors could be a feasible approach for this parameter [74]. Thus, an increase in water uptake by roots at the end of the growing season was estimated for trial 1 (Table 3). This effect could be related to the increased fineness of pepper roots (roots with greater length and less mass) observed when a lower amount of water is applied through drip irrigation, as reported by Antony and Singandhupe [75]. Similar results have been observed in adult apricot trees subjected to deficit irrigation for four consecutive years, with a significant enhancement in the growth of roots smaller than 1 mm [76]. The enhanced ability of roots to absorb water, as shown in this trial, indicates that root development could happen faster due to the combined application of biostimulants and irrigation reduction.

5. Conclusions

The combined use of regulated deficit irrigation (RDI) and biostimulation strategies significantly increased irrigation water productivity, resulting in water savings of 12% compared to the Farmer treatment. The irrigation reduction strategies were carried out mainly in the phenological stages of vegetative development until the first harvest (trial 1) or ripening and harvest (trial 2) and can be considered as non-critical for the application of RDI strategies with moderate water stress when biostimulation is applied. This combined treatment also improved the soil enzymatic activity in both greenhouses, suggesting that nutrients in the soil will become more readily available to plants. Thus, the combined action of biostimulation with different RDI strategies has been proved to be a useful strategy to improve agricultural sustainability.

This study represents a significant advancement in optimizing water usage in agriculture, combining deficit irrigation strategies and biostimulation. Despite the critical importance of this topic, there has been little research in this area so far, which further highlights the novelty and value of our findings. Our work not only opens new avenues for future research, but also has the potential to transform current agricultural practices, promoting more efficient use of water.

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Conflicts of Interest: P.J.E. and C.M. are employees of the company FMC Agricultural Solutions. The remaining authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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