



Inoculation of watermelon weeds with *Bacillus* spp. to mitigate water deficit effects

Inoculação de plantas de melancia com *Bacillus* spp. para mitigar os efeitos do déficit hídrico

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Water deficit is one of the main factors limiting watermelon productivity by affecting physiological processes and plant growth. Plant growth-promoting bacteria (PGPB) have emerged as a sustainable alternative to mitigate the effects of water stress. This study evaluated the growth of watermelon plants inoculated with PGPB under different soil moisture levels (SML). The experiment was conducted in a randomized complete block design with five replications using a split-split-plot arrangement over time in a $4 \times 4 \times 5$ factorial design. The treatments consisted of four soil moisture levels (40%, 60%, 80%, and 100% of the soil water-holding capacity), four inoculation treatments (non-inoculated control, *Bacillus velezensis* NB69, *Bacillus subtilis* NB P6.2, and co-inoculation), and five evaluation times (19, 31, 45, 59, and 72 days after sowing). Water deficit from the flowering stage onward significantly reduced watermelon plant growth. Inoculation with *B. subtilis* NB P6.2 mitigated the negative effects of water stress by increasing stem length and diameter, leaf number, and leaf area, regardless of soil moisture level. Co-inoculation improved root development by increasing root length and root volume under soil moisture levels between 66% and 69%, although it did not increase fresh or dry root biomass. These results indicate that specific *Bacillus* strains are a promising and sustainable strategy to reduce the effects of water deficit on watermelon cultivation, particularly in arid and semi-arid regions.

Keywords: rhizobacteria, bacteria, drought.

O estresse hídrico limita a produtividade da melancia ao comprometer processos fisiológicos e o crescimento das plantas. Nesse contexto, bactérias promotoras do crescimento vegetal (BPCV) representam uma alternativa sustentável para mitigar os efeitos do déficit hídrico. Este estudo avaliou o crescimento de plantas de melancia inoculadas com BPCV sob diferentes níveis de umidade do solo (SML). O experimento foi conduzido em delineamento de blocos casualizados, com cinco repetições, em esquema de parcelas sub-subdivididas no tempo, em arranjo fatorial $4 \times 4 \times 5$. Os tratamentos consistiram em quatro níveis de umidade do solo (40%, 60%, 80% e 100% da capacidade de retenção de água), quatro tratamentos de inoculação (controle, *Bacillus velezensis* NB69, *Bacillus subtilis* NB P6.2 e co-inoculação) e cinco épocas de avaliação (19, 31, 45, 59 e 72 dias após a semeadura). O déficit hídrico a partir da fase de floração reduziu significativamente o crescimento das plantas. A inoculação com *B. subtilis* NB P6.2 mitigou os efeitos negativos do estresse hídrico, promovendo maior comprimento e diâmetro do caule, número de folhas e área foliar, independentemente do nível de umidade do solo. A co-inoculação proporcionou melhor desenvolvimento radicular, aumentando o comprimento e o volume das raízes sob níveis de umidade entre 66% e 69%, embora não tenha promovido aumento da biomassa fresca ou seca das raízes. Os resultados indicam que cepas específicas de *Bacillus* constituem uma estratégia promissora para reduzir os impactos do déficit hídrico no cultivo da melancia, especialmente em regiões áridas e semiáridas.

Palavras-chave: rizobactéria, bactéria, seca.

1. INTRODUCTION

Watermelon is a widely cultivated and consumed fruit worldwide, with China being the largest producer and consumer. In Brazil, the crop has economic and social relevance and is cultivated across all regions, including the Northeast, where watermelon is grown under both rainfed and irrigated systems in semi-arid conditions [1]. In this region, irregular rainfall and high evaporative

demand make water deficit a major constraint to plant growth and yield [1]. Watermelon is appreciated for its unique flavor and nutritional value, as it contains sugars, fiber, vitamins, antioxidants such as lycopene and beta-carotene, amino acids such as citrulline and arginine, and minerals [2]. However, watermelon production can be severely affected by several abiotic and biotic stresses during cultivation, including extreme temperatures, drought, flooding, high salinity, heavy metals, insect pests, and pathogenic organisms, which significantly reduce fruit yield and quality [2]. Insufficient water is one of the main limiting factors for agriculture in semiarid regions. Thus, the use of technologies that mitigate its effects is essential for the expansion of agriculture in these regions. Water deficit is a challenging abiotic factor that has been aggravated by climate change, significantly affecting soil quality and crop yields, decreasing the photosynthetic rate, increasing free-radical accumulation, and altering root morphology of plants [3].

As water absorption occurs mainly through the roots, strategies that improve root system development are essential to enhance plant performance under water deficit conditions. In this context, the inoculation of plants with growth-promoting bacteria has emerged as an important approach to modify root architecture and improve water use efficiency. These microorganisms can establish beneficial associations with roots, stimulating root growth, increasing soil exploration, and enhancing plant tolerance to water deficit [4]. Information on plant–microorganism interactions under water deficit conditions can support the development of new technologies, such as inoculants capable of mitigating the negative effects of limited water availability and increasing crop yield. However, selecting plant growth-promoting bacteria requires studies that evaluate their tolerance to water deficit and their beneficial interactions with plants under restricted soil moisture conditions. In addition, understanding how watermelon plants use water in physiological processes can provide a foundation for interventions in their growing environment [5, 6]. In this context, the objective of this study was to evaluate the potential of *Bacillus* strains to promote growth and mitigate the effects of water deficit on watermelon plants at different developmental stages.

2. MATERIAL AND METHODS

The experiment was conducted from September to November 2021 in a greenhouse with a 40% shade screen (Chromatinet®) at the Department of Technology and Social Sciences of Bahia State University (DTCS/UNEB), in Juazeiro, BA, Brazil (09°24'50"S, 40°30'10"W, 368 m altitude). Before initiating the differentiation of moisture levels, inoculated and non-inoculated seeds were placed to germinate in the soil at the end of September, and the pots were irrigated to maintain soil moisture at field capacity (100% moisture level) until the seedlings developed their first true leaf. This period lasted until 17 DAS (days after sowing), at the beginning of October, when the differentiation in irrigation depths was applied. The region's climate is characterized by low mean annual rainfall and high evapotranspiration rates. Mean monthly climatic data were obtained from the DTCS/UNEB automatic meteorological station near the experimental area (Figure 1A), and rainfall data were also measured using a pluviometer (Figure 1B).

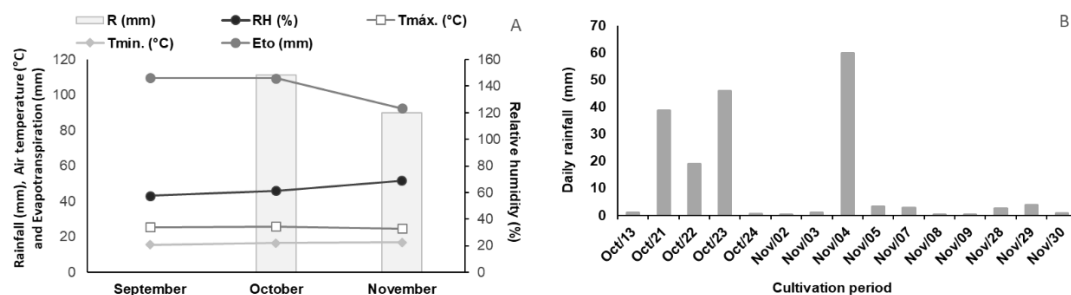


Figure 1. (A) Mean monthly temperature, relative air humidity, and rainfall from September to November; (B) Daily rainfall (mm) during watermelon cultivation 2021. Juazeiro, BA, Brazil. R = Rainfall; Eto = Evapotranspiration; Tmax = maximum air temperature; Tmin = minimum air temperature; RH = relative air humidity. Source: Automatic meteorological station at DTCS/UNEB.

The experiment was conducted in a randomized block design with five replications, in a split-split-plot arrangement and a $4 \times 4 \times 5$ factorial structure. Soil moisture levels, SML, were assigned to the main plots 40%, 60%, 80%, and 100% of soil water retention capacity; inoculation treatments were assigned to the subplots negative control, *Bacillus velezensis* NB69, *Bacillus subtilis* NB P6.2, and co-inoculation with both bacterial strains; and evaluation times were assigned to the sub-subplots 19, 31, 45, 59, and 72 days after sowing, DAS. Evaluations at 19, 31, and 45 DAS represented the vegetative phase, whereas 59 and 72 DAS corresponded to flowering and fruit maturation, respectively. The variables evaluated were leaf area, stem diameter, stem length, and number of leaves. Other morphological variables (relative water content, root length, root volume, and fresh and dry weights of stems, roots, and leaves) were evaluated using a 4×4 factorial arrangement, with plots consisting of four SML (40%, 60%, 80%, and 100% soil water retention capacity) and subplots consisting of four inoculation treatments (negative control; *B. velezensis* NB69; *B. subtilis* NB P6.2; and co-inoculation with both bacteria).

The experimental units consisted of 21-liter black polyethylene pots (38.5 cm height, 30 cm top width, and 22 cm bottom width) spaced 2 m apart between rows and 0.5 m apart within rows. The pots were filled with a layer of crushed stones covered with a fine mesh screen (2.5 kg) followed by a soil layer, totaling 21.5 kg. The pots had holes at the bottom to allow excess water drainage. The soil was classified as a Fluventic Psammaquent, and samples were analyzed at the Soil, Water, and Lime Analysis Laboratory (LASAC/DTCS/UNEB) for chemical characterization (Table 1).

Table 1. Chemical characteristics of the soil used in the study. Juazeiro, BA, Brazil, 2021.

	pH			C.E. (dS m ⁻¹)					M.O. (g kg ⁻¹)
	(CaCl ₂)	KCl	H ₂ O	Ext; Sat;	V (%)	ESP (%)	P (mg dm ⁻³)	(Al ³⁺ / CEC) x 100	
S	5.56	6.13	6.58	0.026	96.83	0.85	5.03	0	9.90
Bulk soil sorption complex (cmol _c dm ⁻³)									
	Ca ⁺²	Mg ⁺²	K ⁺	Na ⁺	SB	Al ⁺³	H ⁺ + Al ⁺³	T	
S	2.18	2.00	0.36	0.04	4.58	0	0.15	4.73	

Ext.Sat. = electrical conductivity of the saturated extract; V = base saturation; ESP = exchangeable sodium percentage (100 Na / CEC); P = phosphorus content; (Al³⁺ / CEC) x 100 = aluminum saturation; M.O. = organic matter; SB = Sum of bases; T = cation exchange capacity; S = sample.

Inoculated and non-inoculated seeds were germinated in the soil, and the pots were irrigated to maintain SML at 100% until the seedlings exhibited the first definitive leaf at 17 days after sowing (DAS). After this period, the water volume applied was defined according to the SML (40%, 60%, 80%, and 100% soil water retention capacity) until the end of the watermelon cycle (72 DAS).

A time domain reflectometry device with fork-shaped probes containing three 10 cm rods was used for irrigation management. The rods were inserted into the pots to measure SML prior to irrigation. Four different probes were used, one for each inoculation treatment, to prevent cross-contamination.

The plants were then manually irrigated using a graduated cylinder to ensure specific and precise water application. The irrigation volume was based on the amount of water consumed, adjusted according to each SML evaluated (40%, 60%, 80%, and 100% soil water retention capacity), maintaining consistency within the established limits of each treatment.

Drought-tolerant plant growth-promoting bacteria from the species *B. velezensis* NB69 and *B. subtilis* NB P6.2 were evaluated using isolates obtained from the Caatinga biome, maintained in the collection of the Semiarid Ecology and Microbial Biotechnology Laboratory at Bahia State University. Previous studies conducted by the laboratory's research group indicated that the strains are effective for maize crops in water-restricted conditions and show potential as plant growth promoting agents in monocotyledons [7, 8].

Bacterial isolates were cultivated for 48 hours at 28 ± 2 °C. The suspension was prepared and adjusted to an optical density of 0.5 at 540 nm, equivalent to 1×10^8 CFU mL⁻¹. The solution was prepared with the addition of 0.1% xanthan gum. Watermelon seeds of the cultivar Crimson Sweet

were immersed for 40 minutes in the suspension of each isolate for seed inoculation. Equal volumes of each suspension were used for co-inoculation with both bacterial strains. A sterile solution with 0.1% xanthan gum was used as the negative control. The seeds were then dried on sterile paper towels at room temperature.

Stem length (cm), stem diameter (mm), leaf area (cm²), and number of leaves were evaluated five times during the watermelon crop cycle, at 19, 31, 45, 59, and 72 DAS. Stem length (main branch) was measured from the stem base to the plant apex using a tape measure. Stem diameter was measured at 1 cm above the soil surface using digital caliper (0.01 mm resolution). The number of leaves was counted, considering only fully expanded leaves. Leaf area was determined as the product of leaf length and width, multiplied by a coefficient (0.70) specific to Cucurbitaceae species [9].

Relative water content, root length, root volume and fresh and dry weights of stems, roots, and leaves were measured only at 72 DAS. Relative water content (RWC; %) was determined following the methodology of Melo et al. (2020) [10], with adaptations, using the equation: $RWC (\%) = [(FW - DW) / (TW - DW)] \times 100$. Leaf discs were weighed on an analytical balance (0.0001 g precision) to obtain the fresh weight (FW), placed in a Petri dish with 30 mL of distilled water for 24 hours for rehydration to obtain the turgid weight (TW), and then dried in an oven at 65 °C for 72 hours to determine the dry weight (DW).

Root length was measured using a graduated ruler. Root volume (mL) was determined by placing the roots in a graduated cylinder with a known water volume, and calculating the difference between the volumes with and without roots.

Plants with roots were collected and cut at the stem base to separate the shoot and root systems, and leaves were detached from the stems. Stems, leaves, and roots were separately weighed on a precision balance (0.0001 g) to obtain their fresh weights and then dried in a forced-air circulation oven at 65–70 °C until constant weight and reweighed to obtain their dry weights.

The data were subjected to analysis of variance (ANOVA) using the F-test at 5% and 1% significance levels. Significant means were compared using regression analysis for SML, and the Scott-Knott test for bacterial treatments, with the aid of Sisvar 4.0 software [11].

3. RESULTS AND DISCUSSION

The analysis of variance revealed that the double interaction (moisture × bacterium) was not significant at a 5% significance level for stem length, stem diameter, number of leaves, and leaf area. However, the triple interaction (moisture × bacterium × evaluation time) was significant for these variables, suggesting that watermelon plant growth was significantly influenced by soil moisture levels (SML) and bacterial inoculation across different phenological stages (Table 2).

Table 2. Chemical characteristics of the soil used in the study. Juazeiro, BA, Brazil, 2021.

Medium square													
FV	GL	CC	DC	NF	AF	TRA	CR	MFC	MFF	MFR	MSC	MSF	MSR
Moisture (M)	3	167760,5**	1,9**	4441,2*	2859,1**	27,2 ^{ns}	3189,7**	55300,8**	496,8*	166,2**	137,4**	11,9*	4,9**
Bacterium (B)	3	19343,4*	14,2**	4948,1**	1408,2 ^{ns}	938,9**	288,9**	13061,1**	2308,4**	360,1**	646,9**	25,2**	0,6**
M X B	9	4633,9 ^{ns}	3,2 ^{ns}	459,3 ^{ns}	894,1 ^{ns}	298,5**	1592,8**	5212,9**	1075,4**	44,5**	248,7**	109,4**	0,8**
Evaluation time (ET)	4	469596,4**	138,3**	77911,9**	34586,5**								
M X ET	12	25268,2**	14,8**	1195,2**	445,1**								
B X ET	12	10282,0**	8,7**	820,0**	194,6**								
M X B X ET	36	5479,4**	21,0**	451,4**	376,8**								
CV(1) (%)		9,60	3,27	23,51	6,46	5,74	9,49	21,0	26,74	11,77	18,58	14,47	13,98
CV(2) (%)		30,26	10,63	46,96	25,23								
CV(3) (%)		5,57	5,66	15,73	4,44								

ns= não significativo; * significativo a 5% de probabilidade; ** significativo a 1% de probabilidade; FV= source of variation; CV= coefficient of variation; GL= degree of freedom; CC= stem length; DC= stem diameter; NF= number of leaves; AF= leaf area; TRA= relative water content; CR= root length; MFC= stem fresh weight; MFF= leaf fresh weight; MSC= stem dry weight;MSF= leaf dry weight; MFR= root fresh weight; MSR= root dry weight.

The double interaction (moisture $\times$ bacterium) was significant for the variables evaluated at the end of the crop cycle (72 DAS): relative water content, root length, stem fresh weight, stem dry weight, leaf fresh weight, leaf dry weight, root fresh weight, and root dry weight. These results indicate that inoculated and non-inoculated plants were significantly influenced by SML at 72 DAS.

The regression analysis for stem length (SL) from the breakdown of the triple interaction (moisture $\times$ bacterium $\times$ evaluation time) is shown in Figure 2. SL of non-inoculated plants in the negative control (NC) increased across evaluation times (Figure 2A). This increase was particularly pronounced with increasing SML. Linear regressions revealed that plants benefited from adequate water availability, as SL reached maximum SL at 100% SML.

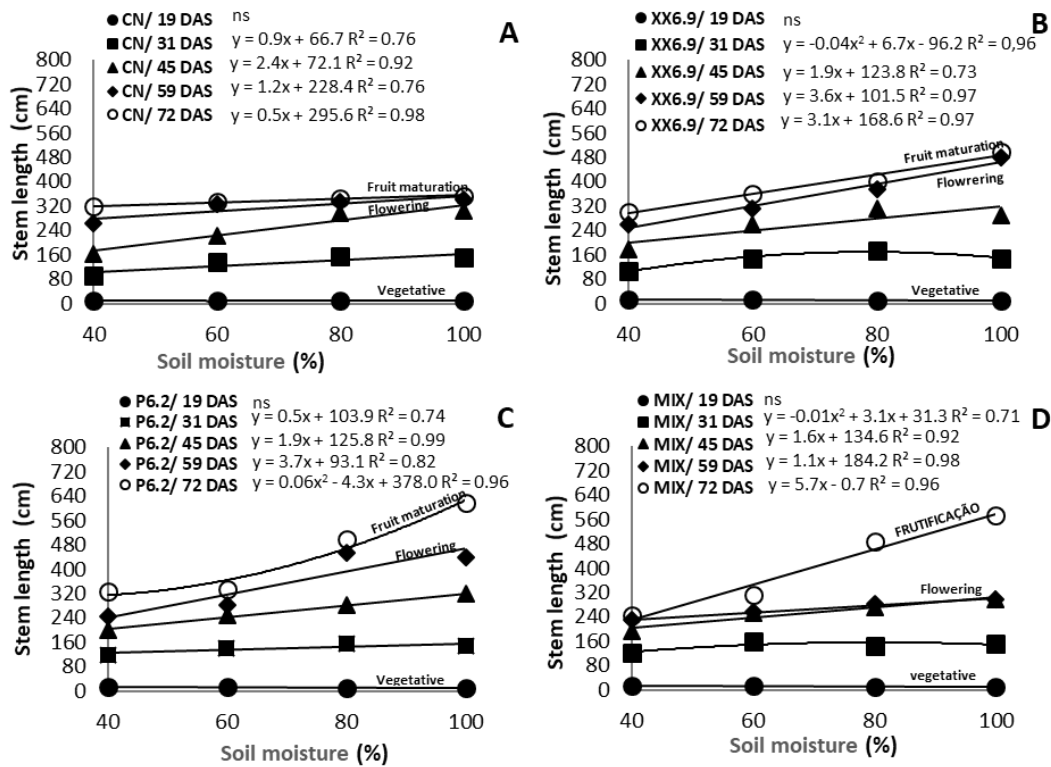


Figure 2. Stem length (cm) of watermelon plants from seeds subjected to inoculation treatments, grown under different soil moisture levels, and evaluated at different times. Inoculation treatments: negative control (NC) (A); inoculated with *Bacillus velezensis* NB69 (BV) (B); inoculated with *Bacillus subtilis* NB P6.2 (BS) (C); and co-inoculated with both bacteria strains (MIX) (D). Soil moisture levels (SML): 40%, 60%, 80%, and 100% soil water retention capacity. Evaluation times: 19, 31, 45, 59, and 72 days after sowing (DAS). ns = not significant at a 5% significance level. Juazeiro, BA, Brazil, 2021.

These results are consistent with those reported by Odhiambo and Aguyoh (2022) [12] for cucumber plants of the cultivar Ashley, which were subjected to different SML (40%, 60%, 80%, and 100% soil water retention capacity) and evaluated between 21 and 77 DAS. They observed greater SL at all evaluation times when the plants were maintained at 100% SML.

SL responses to SML in NC plants were minimal up to 31 DAS (Figure 2A). However, water availability became crucial from 45 DAS onwards, as SL began to decrease significantly under low soil moisture conditions. The mean SL at 100% SML was 305.50 cm, compared with 163.67 cm at 40%, representing a reduction of approximately 46%.

Silva et al. (2015) [13], also observed slow initial growth in watermelon plants, with an increased growth rate after 51 DAS; they attributed this result to low initial water and nutrient absorption and reduced respiration rates. In addition, soil water retention capacity is lower at the initial stage due to the small leaf area of the crop, while soil surface water evaporation drives water loss. Moreover, higher water consumption followed by rapid plant development is observed after the onset of flowering, which explains the plant response at 45 DAS.

Similar to NC, inoculation treatments with *Bacillus velezensis* NB69 (Figure 2B), *Bacillus subtilis* NB P6.2 (Figure 2C), and co-inoculation with both bacteria (Figure 2D) also increased SL over the evaluation period. SL was affected by moderate to severe water stress (40% to 60% SML) from 45 to 72 DAS. However, from 59 to 72 DAS, the single-inoculum treatments, particularly *B. subtilis* NB P6.2, increased SL at 80% and 100% SML levels compared to NC (Figure 2A).

Watermelon plants naturally reach up to 500 cm in length, and some accessions can reach 900 cm [14]. NC reached 353.34 cm under 100% SML at 72 DAS (Figure 2A), while the single-inoculum treatments resulted in 498.67 cm and 616.44 cm, respectively (Figures 2B and 2C). These results are similar to those reported for other Cucurbitaceae species (*Luffa aegyptiaca* 'Alok') inoculated with *B. subtilis*, which exhibited SL between 592 and 632 cm [15].

Various *B. velezensis* and *B. subtilis* strains have been reported in the literature as plant growth-promoting bacteria. These species influence crop development through several mechanisms, including nutrient availability (nitrogen, zinc, phosphorus, and potassium), phytohormone and siderophore production, synthesis of molecules that promote systemic tolerance, and exopolysaccharide production [16, 17]. These bacteria have been reported as growth-promoting agents in maize (*Zea mays* L.), synthesizing indole-3-acetic acid (auxin), producing exopolysaccharides, and modifying plant growth attributes [7, 8].

Soil moisture and evaluation time influenced stem diameter (SD) of watermelon plants (Figure 3). SD increased with increasing evaluation times and SML in the NC (Figure 3A). A thicker stem diameter is expected as plants grow over time due to the synthesis of photoassimilates [18]. The highest SD in the NC (3.35 mm) was observed at 82% SML at 19 DAS. SD decreased linearly at 31, 45, and 59 DAS, with maximum SD (6.09, 6.16, and 6.20 mm) at 40% SML.

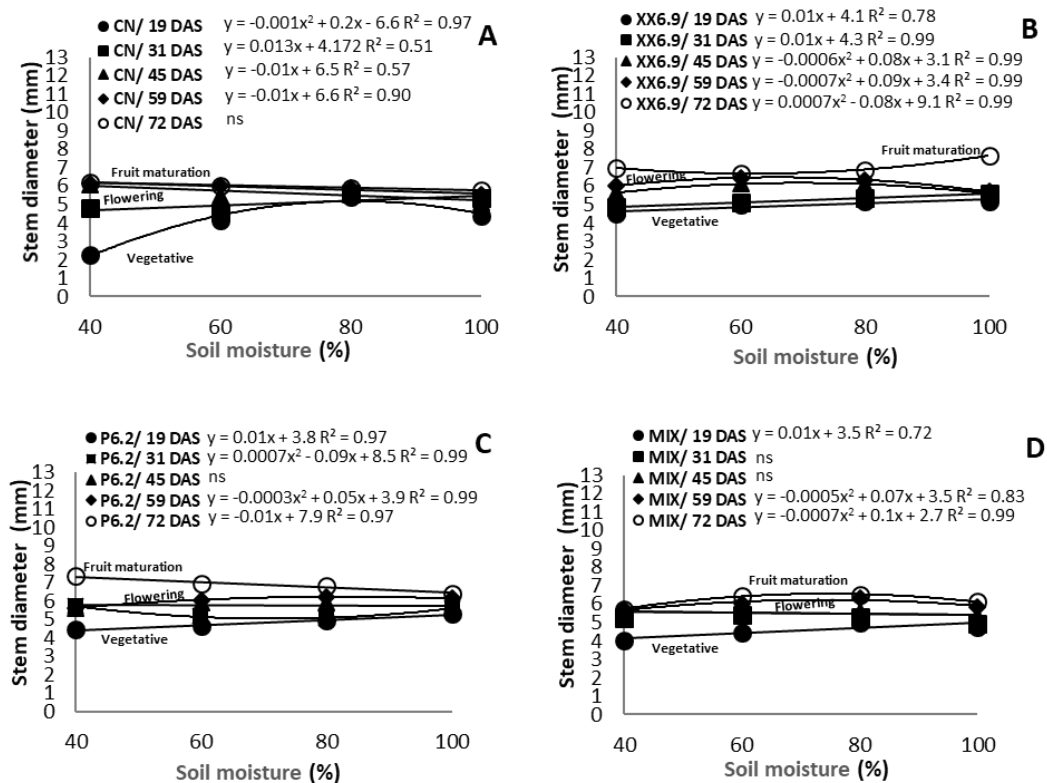


Figure 3. Stem diameter (mm) of watermelon plants from seeds subjected to inoculation treatments, grown under different soil moisture levels, and evaluated at different times. Inoculation treatments: negative control (NC) (A); inoculated with *Bacillus velezensis* NB69 (BV) (B); inoculated with *Bacillus subtilis* NB P6.2 (BS) (C); and co-inoculated with both bacteria strains (MIX) (D). Soil moisture levels (SML): 40%, 60%, 80%, and 100% soil water retention capacity. Evaluation times: 19, 31, 45, 59, and 72 days after sowing (DAS). ns = not significant at a 5% significance level. Juazeiro, BA, Brazil, 2021.

SD exhibited higher sensitivity to water stress at initial stages (Figure 3A), increasing after 19 DAS as water availability increased. Silva et al. (2022) [19] also observed that SD is greatly influenced by water availability at vegetative stages, as water deficits can lead to decreased cell division [20]. Decreased stem diameter is a typical response to water deficit, as plants start to wilt, with stomatal closure reducing water loss by transpiration, impacting photosynthesis [21].

Soil moisture and evaluation time influenced SD of plants treated with *B. velezensis* NB69. An increasing linear regression was observed at 19 and 31 DAS, with maximum SD (4.49 and 4.88 mm) at 100% SML. Maximum SD (6.28 and 6.42 mm) at 45 and 59 DAS were observed at 72% and 65% SML, respectively (Figure 3B). A quadratic regression was observed at 72 DAS, with maximum SD at 100% SML.

SD of plants inoculated with *B. subtilis* NB P6.2 increased linearly at 19 DAS, with a maximum SD (5.33 mm) at 100% SML (Figure 3C). Maximum SD (5.13 and 6.48 mm) at 31 and 59 DAS were observed at 70% and 91% SML. A linear decrease was observed at 72 DAS, with maximum SD (7.35 mm) at 40% SML.

The single-inoculum treatments (Figures 3B and 3C) resulted in higher SD across evaluation times compared to NC up to flowering (Figure 3A). Although NC showed no significant difference at 72 DAS, the treatments with inoculation were superior. The effect of *B. subtilis* NB P6.2 was significant, indicating a high capacity to increase SD under severe water deficit conditions up to the end of the crop cycle. This is associated with the enhanced capacity for photoassimilate translocation in plants, maintaining their metabolic, physiological, and anatomical functions [18].

Soil moisture and evaluation time influenced SD of plants in the co-inoculation treatment (Figure 3D). A linear increase in SD was observed at 19 DAS, with maximum SD (4.77 mm) at 40% SML. Maximum SD (6.14 and 6.46 mm) at 59 and 72 DAS were observed at 70% and 91% SML, respectively. Co-inoculation resulted in the smallest increase in SD over the evaluation period compared to the other treatments (Figures 3A, 3B, 3C).

The treatments with *B. velezensis* NB69, *B. subtilis* NB P6.2, and co-inoculation resulted in higher SD at 19 DAS (Figure 3), particularly at 40% SML, with means of 4.04, 4.47, and 4.49 mm, respectively. Wu et al. (2021) [22] reported SD of 2.6 to 2.8 mm for inoculated watermelon plants under adequate soil moisture at 20 DAS, indicating the superiority of bacterial treatments. Water is essential at the vegetative stage, when potential production is defined, reflecting the plants' capacity to support fruit weight [23]. Thus, an initial boost promoted by growth-promoting bacteria can be advantageous compared to non-inoculated treatments.

Inoculation contributes to seedling germination and vigor, promotes healthy development, and accelerates emergence and the use of available resources to tolerate adverse environments. In addition, direct contact of microorganisms with initial roots allows early interaction between the bacterium and the plant, leading to biostimulant effects and enhancing growth and nutrient absorption by forming a protective zone around the seeds [24].

Soil moisture and evaluation time influenced the number of leaves (NL) of inoculated and non-inoculated plants throughout the evaluation period (Figure 4). NL was not significantly affected by water deficit in inoculated treatments during the vegetative stage (19 and 31 DAS) (Figures 4A, B, C, D). The crop responded positively to the inoculation treatments across the SML at 45, 59, and 72 DAS, corresponding to the vegetative-flowering transition, flowering, and fruit maturation stages, respectively.

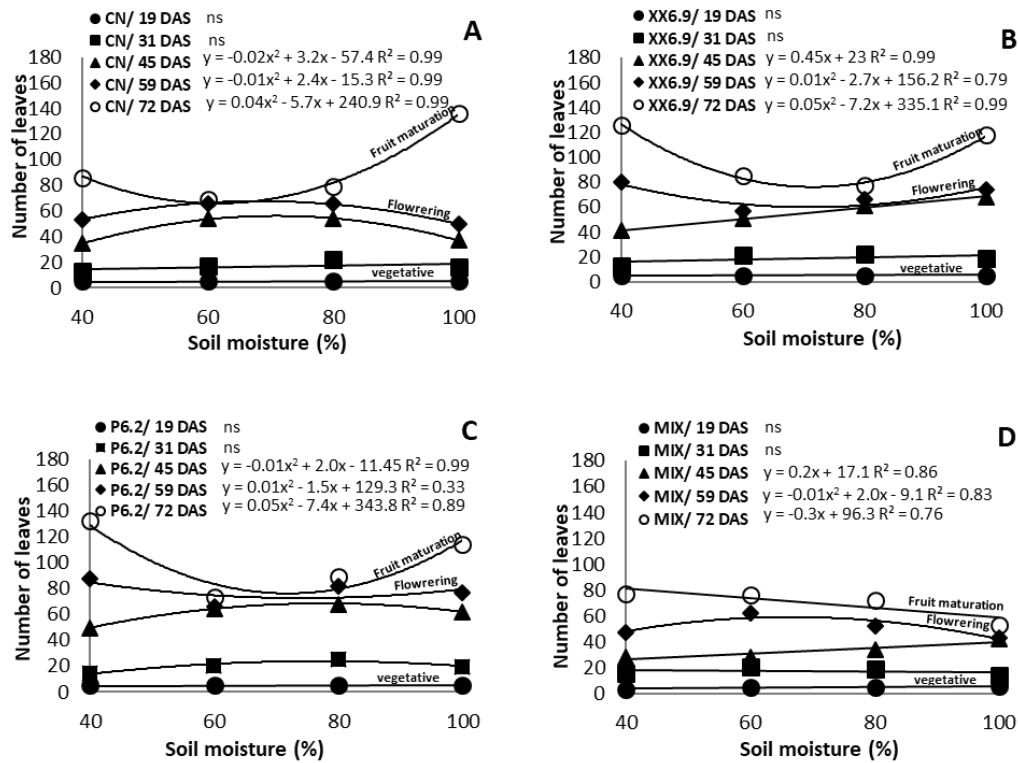


Figure 4. Number of leaves of watermelon plants from seeds subjected to inoculation treatments, grown under different soil moisture levels, and evaluated at different times. Inoculation treatments: negative control (NC) (A); inoculated with *Bacillus velezensis* NB69 (BV) (B); inoculated with *Bacillus subtilis* NB P6.2 (BS) (C); and co-inoculated with both bacteria strains (MLX) (D). Soil moisture levels (SML): 40%, 60%, 80%, and 100% soil water retention capacity. Evaluation times: 19, 31, 45, 59, and 72 days after sowing (DAS). ns = not significant at a 5% significance level. Juazeiro, BA, Brazil, 2021.

NL in the NC treatment increased with evaluation times but varied across SML within each evaluation time, with maximum NL at 45 and 59 DAS (56 and 68 leaves) at 71% and 68% SML, respectively (Figure 4A). Maximum NL at 72 DAS was observed at 100% SML.

Soil moisture and evaluation time influenced NL in the single-inoculum treatments (Figures 4B and 4C), with a linear increase in the treatment with *B. velezensis* NB69 at 45 DAS and a maximum NL (68 leaves) at 100% SML. Maximum NL (69 leaves) in the treatment with *B. subtilis* NB P6.2 was observed at 78% SML. Quadratic responses were observed for NL in single-inoculum treatments at 59 and 72 DAS.

These results indicate that both bacterial strains promoted increases in NL at 45 DAS, although *B. velezensis* NB69 required higher SML (78%) than *B. subtilis* NB P6.2 (71%) for these increases. Additionally, the best responses were observed during flowering and fruiting stages in both treatments, in contrast to NC, particularly at 40% and 60% SML, reinforcing their ability to promote leaf production under water-restricted conditions.

In the co-inoculation treatment, NL increased linearly at 45 DAS, with a maximum NL (42 leaves) at 100% SML (Figure 4D). The maximum NL (59 leaves) at 59 DAS was observed at 67% SML. NL decreased linearly at 72 DAS, with a maximum NL at 40% SML. Increases in NL are attributed to induced resistance to the abiotic stress salinity [25].

An increase in the number of leaves and leaf area enhance net photosynthesis, as shown in a study on *B. subtilis* NB P6.2, which increased NL and photosynthetic rate [26]. NL is a key variable for biomass accumulation in plants, as an increase in shoot production significantly contributes to total plant biomass [27, 28].

The co-inoculation treatment resulted in smaller increase in NL compared to the other treatments (Figure 4 A, B, C), as observed for SL and SD (Figures 2 and 3). According to Nunes et al. (2019) [18], reductions in NL can result from natural senescence and/or prolonged stress exposure. This is a conservation mechanism, as metabolic demand increases after the vegetative

stage, particularly during the fruiting stage, when fruits become strong sinks for photoassimilates [29, 30].

Soil moisture and evaluation time influenced leaf area (LA) of watermelon plants from inoculated and non-inoculated seeds (Figure 5). LA increased over evaluation times in NC, with varying responses depending on SML (Figure 5A). A linear increase was observed at 19 DAS, with a maximum LA at 100% SML. Maximum of LA at 31, 45, and 59 DAS were 124.33, 137.73, and 141.42 cm², observed at 64%, 72%, and 73% SML, respectively. A linear decrease in LA was observed at 72 DAS, with a maximum LA (158.40 cm²) at 40% SML. LA was limited by low water availability at the initial stage (19 DAS) in NC (Figure 5A). However, LA increased from the initial growth to the fruiting stage even in water-restricted environments.

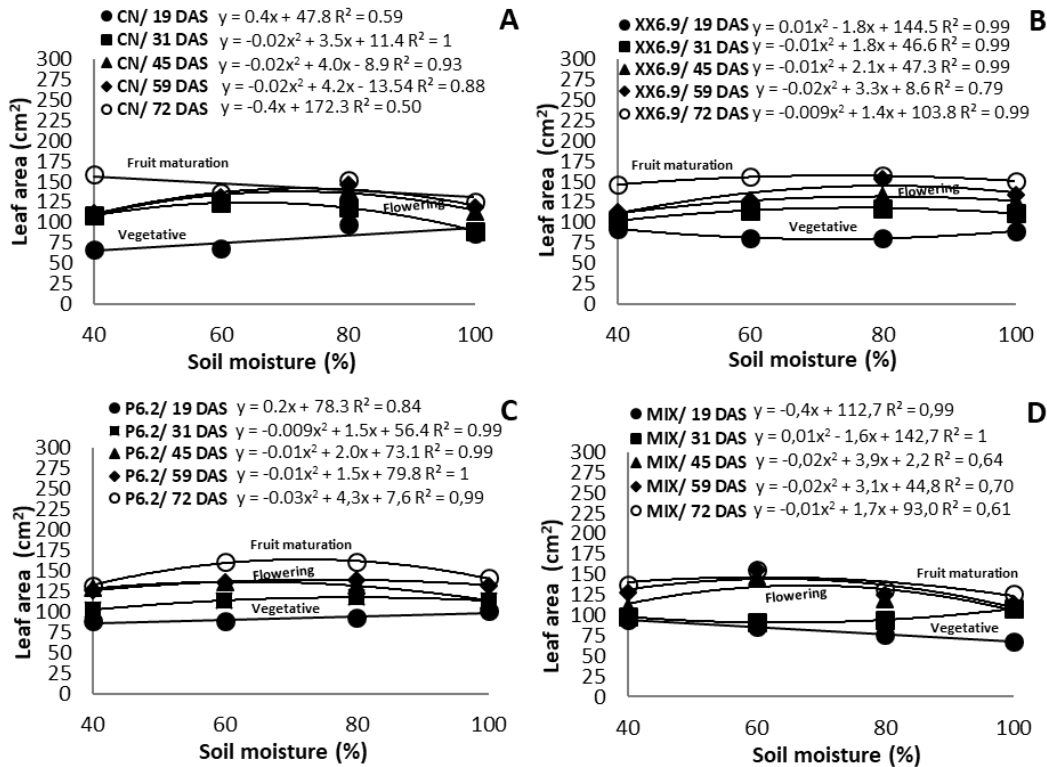


Figure 5. Leaf area (cm²) of watermelon plants from seeds subjected to inoculation treatments, grown under different soil moisture levels, and evaluated at different times. Inoculation treatments: negative control (NC) (A); inoculated with *Bacillus velezensis* NB69 (BV) (B); inoculated with *Bacillus subtilis* NB P6.2 (BS) (C); and co-inoculated with both bacteria strains (MIX) (D). Soil moisture levels (SML): 40%, 60%, 80%, and 100% soil water retention capacity. Evaluation times: 19, 31, 45, 59, and 72 days after sowing (DAS). ns = not significant at a 5% significance level. Juazeiro, BA, Brazil, 2021.

According to Toscano et al. (2018) [31], the flowering and fruiting stages require greater SML for maintaining physiological processes and, during these stages, plants reduce leaf area in advance to improve water use efficiency and minimize water loss through transpiration. In the treatment with *B. velezensis* NB69, quadratic responses were observed for LA at all evaluation times. Maximum LA at 31, 45, 59, and 72 DAS were 118.05, 131.74, 145.05, and 157.64 cm², observed at 76%, 80%, 80%, and 74% SML, respectively.

In the treatment with *B. subtilis* NB P6.2, a linear increase in LA was observed at 19 DAS, with maximum LA at 100% SML (Figure 5C). This treatment led to a 17% increase in LA, with maximum LA at 31, 45, 59, and 72 DAS (117.39, 136.80, 139.17, and 163.63 cm²) observed at 79%, 63%, 75%, and 72% SML, respectively. The increase in LA at the fruiting stage was 31% compared to NC at 100% SML (Figure 5A). Therefore, single-inoculum treatments (*B. velezensis* NB69 and *B. subtilis* NB P6.2) resulted in lower LA compared to NC in most evaluation times under severe to moderate water stress. Reduction of LA is a plant strategy to limit transpiration, retain water in tissues, and protect against potential oxidative damage by decreasing the surface

exposed to light. However, this adaptation also leads to reduced photosynthesis, limiting nutrient uptake, photoassimilate translocation, and decreasing leaf biomass [30, 32].

Unlike the other inoculated treatments, co-inoculation resulted in a linear decrease in LA at 19 DAS, with maximum LA at 40% SML (94.40 cm^2) was higher than those of the other treatments (Figure 5D). Thus, co-inoculation showed the highest LA among inoculated treatments under the lowest SML at the initial stage, and promoted the highest increase in LA at flowering. Leaf area is crucial for photosynthesis and is therefore important to produce carbohydrates, lipids, and proteins. Thus, leaf area index is directly associated with plant growth rate, representing both the photosynthetic agent and the final product [33].

Soil moisture levels significantly influenced relative water content (RWC) in the NC at 72 DAS, with a linear decrease from 65.2% at 40% SML to 47.5% at 100% SML (Figure 6). The reduction in RWC observed at higher soil moisture levels may be associated with hypoxic conditions in the rhizosphere caused by excess water in the soil. In watermelon, waterlogging has been shown to impair root respiration and induce oxidative stress due to oxygen deprivation in the rhizosphere [34]. Under these conditions, root water uptake may be reduced even in the presence of abundant soil water. Conversely, moderate water deficit can trigger adaptive osmotic responses in watermelon roots, helping maintain tissue hydration and relatively high RWC values [35].

This result contrasts with findings by Abdelkhalik et al. (2019) [36], who reported an increase in RWC from 69.9% at 50% of field capacity to 82.3% at 100% of field capacity in watermelon plants of the cultivar Stellar F1. Similarly, Li et al. (2019) [37], observed an increase in RWC from 60% (without irrigation for 8 days) to 90% at 75% of field capacity in watermelon plants of the genotype Y34.

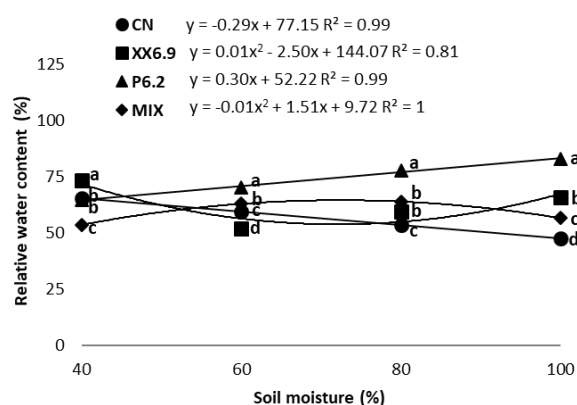


Figure 6. Relative water content (%) of watermelon plants from seeds subjected to inoculation treatments, evaluated at 72 days after sowing under different soil moisture levels (SML). Inoculation treatments: negative control (NC); inoculated with *Bacillus velezensis* NB69 (BV); inoculated with *Bacillus subtilis* NB P6.2 (BS); and co-inoculated with both bacteria strains (MIX). Soil moisture levels (SML): 40%, 60%, 80%, and 100% soil water retention capacity. Juazeiro, BA, Brazil, 2021. Equal letters, comparing the effect of the inoculation treatments within each soil moisture level, are not significantly different by the Scott-Knott test at a 5% significance level.

RWC is correlated with soil water availability; thus, SML directly influences leaf water content and, consequently, plant morphology [38, 39]. Although irrigation levels that ensure adequate SML typically increase RWC, this pattern was not observed in the present study.

In contrast, increasing SML led to higher RWC in the treatment with *B. subtilis* NB P6.2, with results significantly higher than those of the other treatments at 60%, 80%, and 100% SML (Figure 6), reaching a maximum of 82.8%. Similarly, Rajabi-Khamseh et al. (2020) [40], evaluating irrigation levels of 50%, 75%, and 100% of ETc, found that inoculation with *Bacillus amyloliquefaciens* and *Bacillus* sp. increased RWC at 100% ETc, with means ranging from 71% to 73.1%.

Considering the effect of the treatments on RWC within each SML (Figure 6), the highest RWC (73.2%) was observed for *B. velezensis* NB69 at 40% SML, whereas NC showed an RWC

of 65.2%. Both treatments resulted in higher RWC than the 58.7% reported for the Ashley cucumber cultivar at 40% soil water retention capacity [12]. However, the treatment with *B. velezensis* NB69 increased RWC by approximately 12% compared to NC.

Decreases in RWC under water deficit are expected, as low RWC reduces cellular water availability. The resulting loss of cell turgor impairs cell expansion processes, thereby affecting leaf area and disrupting physiological mechanisms [41]. Therefore, the treatment with *B. velezensis* NB69 appears promising, as it may activate defense mechanisms that minimize water loss and assist in maintaining plant water status. Beneficial bacteria can increase abscisic acid (ABA) levels in plants as a strategy used to maintain RWC by inducing stomatal closure. The production of indole-3-acetic acid (IAA), a phytohormone that enhance root development, also contributes to improved water uptake and maintenance of plant turgor [38, 42].

Soil moisture levels significantly influenced root length (RL) (Figure 7A). In NC and co-inoculation treatments, RL increased from 40% to 69% and 66% SML, respectively, with maximum RL of 105.98 cm and 117.01 cm.

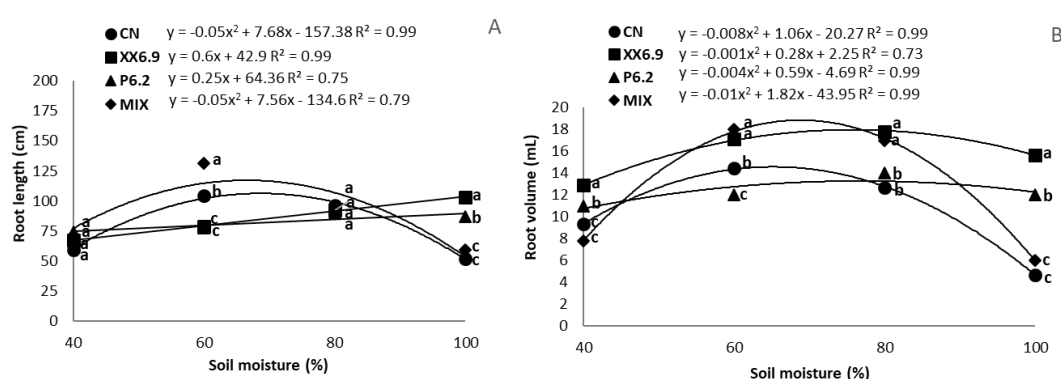


Figure 7. (A) Root length (cm) of watermelon plants from seeds subjected to inoculation treatments, evaluated at 72 days after sowing under different soil moisture levels. (B) Root volume (mL) of watermelon plants from seeds subjected to inoculation treatments, evaluated at 72 days after sowing under different soil moisture levels. Inoculation treatments: negative control (NC); inoculated with *Bacillus velezensis* NB69 (BV); inoculated with *Bacillus subtilis* NB P6.2 (BS); and co-inoculated with both bacteria strains (MIX). Soil moisture levels (SML): 40%, 60%, 80%, and 100% soil water retention capacity. Juazeiro, BA, Brazil, 2021. Equal letters, comparing the effect of the inoculation treatments within each soil moisture level, are not significantly different by the Scott-Knott test at a 5% significance level.

A study reported that NC and co-inoculation treatments resulted in maximum mean estimated RL in watermelon, ranging from 100.85 to 127.30 cm at field capacity [43], highlighting the importance of these treatments in maximizing root development under water stress conditions, particularly co-inoculation. Increased RL improves the plant's ability to penetrate deeper soil layers and enhances water absorption capacity. In addition, it facilitates nutrient absorption, especially potassium and phosphorus, thereby promoting plant growth and increasing crop yield [44].

Inoculation with *Bacillus* spp. can promote root elongation through multiple mechanisms, including the induction of indole-3-acetic acid synthesis. This phytohormone contributes to balancing osmotic stress by increasing root surface area and improving water and nutrient uptake. As a result, water flow from root to shoot is enhanced, helping to prevent reductions in gas exchange in plants [45].

Considering the effect of the treatments on RL within each SML (Figure 7A), the mean RL at 60% SML was 131.5 cm in the co-inoculation treatment and 79 and 77.5 cm in the inoculation treatments with *B. velezensis* NB69 and *B. subtilis* NB P6.2, respectively. Thus, the combination of both bacteria in the co-inoculation nearly doubled the RL observed in the NC at 60% SML, indicating a synergistic effect. Inoculation with two non-antagonistic microorganisms from the same or different species can result in additive or synergistic effects in plant growth. The lack of

a specific activity of one strain may be compensated for by another microorganism that express that trait [46].

Yang et al. (2021) [47], attributed the stronger response of bacterial co-inoculation to the synergy in biofilm formation, as the combined bacteria produced more biofilm than the sum of their individual productions. The main component of this biofilm are water (97%) and polymers (exopolysaccharides) that help to retain water. In addition, biofilm formation improves bacteria survival, competitiveness, and colonization ability [48].

Moreover, Ansari et al. (2023) [49], reported that the combination of *B. subtilis* FAB1 and *Pseudomonas azotoformans* FAP resulted in multiple properties, including production of siderophore, indole-3-acetic acid, ACC-deaminase, and phosphorus solubilization. Additionally, they observed that the co-inoculated bacteria formed denser and more stable biofilms under water stress, which may explain the synergistic effect observed in the present study.

The single-inoculum treatments (*B. velezensis* NB69 and *B. subtilis* NB P6.2) resulted in a linear increase in RL, promoting root growth with increasing water availability (Figure 7A). Considering the effect of the treatments on RL within each SML, the inoculated treatments (*B. velezensis* NB69, *B. subtilis* NB P6.2, and co-inoculation) were superior to the NC at 100% SML, with increases in RL of 98%, 67%, and 13%, respectively.

Soil moisture levels significantly influenced root volume (RV) (Figure 7B). All treatments exhibited a quadratic response, with increases in RV from 40% SML to 65% (NC), 76% (*B. subtilis* NB P6.2), 75% (*B. velezensis* NB69), and 69% (co-inoculation) SML. The maximum estimated RV were 14.55, 13.12, 17.72, and 18.67 mL, respectively, followed by significant decreases up to 100% SML (Figure 7B). The co-inoculation treatment was the most responsive, increasing RV by 28% compared to NC. The soil volume explored and the contact between root surfaces and soil are essential for effective water absorption. Root-soil contact is maximized through root emissions, which increase the surface area and absorption capacity, allowing roots to access deeper soil layers [50, 51].

Co-inoculation resulted in the highest RL at 66% (Figure 7A) and the highest RV at 69% SML (Figure 7B). However, this treatment did not lead to superior performance in shoot-related variables—SL (Figure 2), SD (Figure 3), NL (Figure 4), and LA (Figure 5). Root elongation and reduced shoot growth are typical plant strategies to improve water uptake from deeper soil layers [52]. This likely occurred in the co-inoculation treatment, where the plants reduced the allocation of photoassimilates to the shoot and invested more in root development.

Considering the effect of the treatments on RV within each SML (Figure 7B), treatments with *B. velezensis* NB69 and *B. subtilis* NB P6.2 resulted in higher RV than the NC at 100% SML. The mean RV in the treatment with *B. velezensis* NB69 was 15.7 mL, representing an increase of approximately four times compared to NC. This mean is within the range expected for the crop, 14.7 to 19.6 mL under field capacity [53]. Furthermore, the highest RV at 40% SML was observed in the treatment with *B. velezensis* NB69, representing an increase of nearly 39% compared to NC. This result aligns with findings from a study on nine water deficit-tolerant strains, which reported that *B. velezensis* NB69 increases RV under 40% of ET_c in maize (*Zea mays* L.) [7].

Similar to RL (Figure 7A), RV also benefited from the co-inoculation at 69% SML, but decreased at 100% SML, for which the single-inoculum treatments had the highest RV. This reinforces the synergistic effect of co-inoculation in water-restricted environments and its antagonistic effect under high water availability conditions. The relationship between RL and RV suggests that the treatments influenced not only root extension but also root architecture. In the co-inoculation treatment, the simultaneous increases in RL and RV at intermediate SML indicate a more vigorous and highly exploratory root system, capable of increasing soil exploration while also maintaining greater root biomass and absorptive surface area. This response may enhance root-soil contact and improve water and nutrient uptake efficiency under moderate water restriction.

The success of co-inoculation depends on the type, function, survival, and persistence of each strain in the soil after inoculation, resulting in stimulatory, inhibitory, or neutral interactions. Positive interactions include support networks through cross-feeding, in which one bacterium utilizes metabolic products produced by another, which may have occurred in the co-inoculation treatment at 69% SML. In contrast, negative interactions suppress bacterial activity, disrupting the community structure and function through the production of toxic compounds or competition

for nutrients, water, or space [54], which likely occurred in the co-inoculation treatment at 100% SML.

Watermelon plants have a primary taproot and several lateral roots [55], and they trend to form a shallow root system under optimal resource availability. However, under resource-limited conditions, plants extend their root systems to access low-mobility nutrients such as phosphorus [56], which may explain the highest root length in the NC at 60% SML.

Rhizobacteria tend to stimulate the development of secondary roots, as observed at 80% SML. This stimulation has been attributed to reductions in ethylene levels, since stressed plants synthesize this phytohormone to inhibit root elongation [56].

Soil moisture levels significantly influenced stem, leaf, and root fresh and dry weights across inoculation treatments (Figure 8). The highest stem fresh weights were observed at 100% SML in all treatments (Figure 8A). The treatment with *B. subtilis* NB P6.2 nearly doubled stem fresh weight compared to NC (348 g plant⁻¹). The highest stem dry weight (35.5 g plant⁻¹) was observed at 40% SML, also in the treatment with *B. subtilis* NB P6.2, followed by that with *B. velezensis* NB69 (28.7 g plant⁻¹) (Figure 8B).

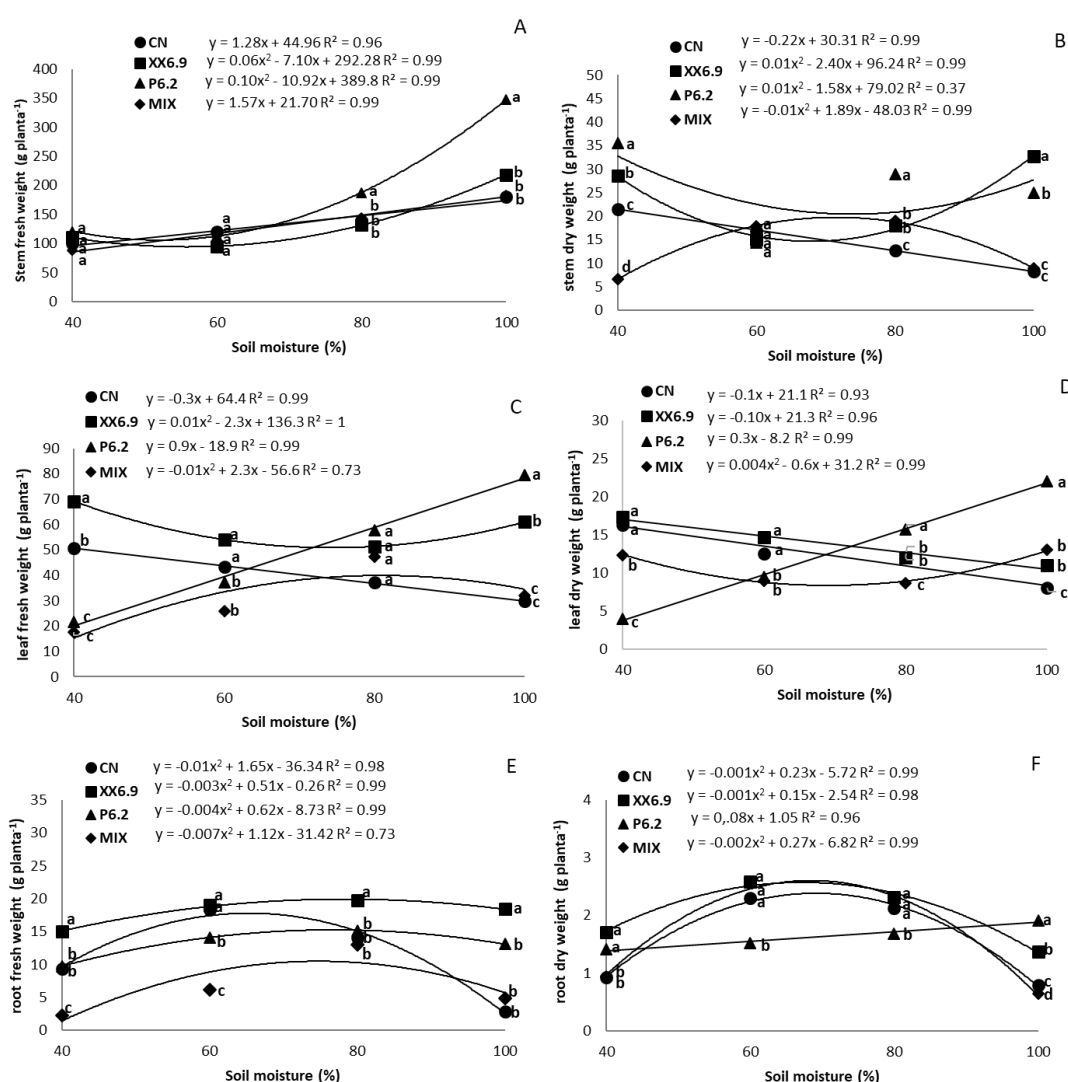


Figure 8. Stem fresh weight (A), stem dry weight (B), leaf fresh weight (C), leaf dry weight (D), root fresh weight (E), and root dry weight (F) of watermelon plants from seeds subjected to inoculation treatments, evaluated at 72 days after sowing under different soil moisture levels. Inoculation treatments: negative control (NC); inoculated with *Bacillus velezensis* NB69 (BV); inoculated with *Bacillus subtilis* NB P6.2 (BS); and co-inoculated with both bacteria strains (MIX). Soil moisture levels (SML): 40%, 60%, 80%, and 100% soil water retention capacity. Juazeiro, BA, Brazil, 2021. Equal letters, comparing the effect of the inoculation treatments within each soil moisture level, are not significantly different by the Scott-Knott test at a 5% significance level.

The increased stem weights resulted from the inoculation of watermelon seeds with bacteria are attributed to the balance of plant hormones, including gibberellins, cytokinins, auxins, and abscisic acid. Higher stem dry weight indicates a more lignified (thicker and heavy) stem, allowing the accumulation of photoassimilates with its elongation [48, 57, 58].

The highest leaf fresh ($79.6 \text{ g plant}^{-1}$) and dry (22 g plant^{-1}) weights at 100% SML were observed in the treatment with *B. subtilis* NB P6.2 (Figures 8C and 8D). These results are consistent with the higher number of leaves (Figure 4C), greater leaf area (Figure 5C), and increased relative water content (Figure 6) found for this treatment. Leaf area and number of leaves are key contributors to photosynthetic capacity and leaf dry matter accumulation [59].

The highest leaf fresh (Figure 8C) and dry (Figure 8D) weights at 40% SML were observed in the treatment with *B. velezensis* NB69. The capacity for exopolysaccharide production, biofilm formation, and osmotic balance maintenance enhances plant tolerance to water deficit, thereby improving shoot dry matter accumulation [60].

The highest root fresh weights (Figure 8E) were 17.68 (NC) , $39.98 \text{ (} B. velezensis \text{ NB69)}$, $15.04 \text{ (} B. subtilis \text{ NB P6.2)}$, and $10.45 \text{ (co-inoculation)}$ g plant^{-1} , observed at 65%, 78%, 76%, and 75% SML, respectively. The highest estimated root dry weights were 2.35 (NC) , $2.73 \text{ (} B. velezensis \text{ NB69)}$, and $2.65 \text{ (co-inoculation)}$ g plant^{-1} , all observed at 69% SML (Figure 8F). The highest root dry weight in the treatment with *B. subtilis* NB P6.2 (1.9 g plant^{-1}) was observed at 100% SML. Thus, the treatment *B. velezensis* NB69 was superior to the other treatments for both root variables, increasing root fresh weight by 126% and root dry weight by 16% compared to NC.

The highest root dry weight observed in the co-inoculation treatment at 69% SML (Figure 8F) is consistent with the highest root volume at 69% SML (Figure 7B) and root length at 66% SML (Figure 7A). However, these variables, along with shoot biomass variables, in the co-inoculation treatment were not superior compared to those in the other inoculated treatments at these SML.

The root-to-stem ratio is used as a criterion to define the drought resistance of plants. According to Souza et al. (2022) [61], a higher assimilate allocation to the roots triggers a competition between shoot and root systems, decreasing aerial biomass and increasing root biomass. This process is explained by the which highlights the importance of an appropriate partition of photoassimilates among plant organs (leaves, stem, and roots) for optimal plant adaptation and performance [62, 63].

According to Silva et al. (2022) [64], a negative photosynthesis-respiration balance reduces dry matter accumulation. Under limited water availability, plants increase respiration rates, leading to energy loss and greater carbohydrate consumption. As new carbohydrates are not produced efficiently, dry weight consequently decreases [65], which may have occurred in the co-inoculation treatment.

Considering the effect of the treatments on stem dry weight, leaf dry weight, and root dry weight (Figures 8B, 8D and 8F) within each SML, the co-inoculation was among the treatments with lower performance at 40% SML. According to Santana et al. (2020) [1], water stress alters carbon allocation patterns. Although plants may maintain high photosynthetic and transpiration rates, the fixed carbon may be redirected to metabolic pathways unrelated to growth, resulting in limited biomass accumulation.

4. CONCLUSION

Bacillus velezensis NB69 and *Bacillus subtilis* NB P6.2 demonstrated the potential to promote watermelon growth under reduced soil moisture conditions, representing a sustainable strategy to mitigate the adverse effects of water limitation in arid and semiarid regions. Among the evaluated treatments, *B. subtilis* NB P6.2 showed the greatest positive effects on plant growth under water-restricted conditions. However, the bacterial consortium promoted the highest root length and root volume values, particularly at intermediate soil moisture levels, indicating a synergistic interaction between the strains and the development of a more vigorous root system. Since this study focused on growth-related traits and relative water content, the observed benefits should be interpreted as improvements in plant performance under water-limited conditions. Further research evaluating physiological, biochemical, and molecular responses, as well as validation

under field conditions, is needed to support the practical application of these strains in agricultural systems.

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