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ORIGINAL ARTICLE

Cattle manure and humic substances stimulate morphophysiological and nutritional processes in pepper plants[1]

Esterco bovino e substâncias húmicas estimulam a morfofisiologia e a nutrição em pimentão

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HIGHLIGHTS:

Humic substances stimulate the growth of pepper roots.
Applying humic substances increases photosynthetic efficiency in pepper plants.
Combining cattle manure with humic substances is a promising alternative to the use of mineral fertilizers.

ABSTRACT: Species of the genus *Capsicum*, especially peppers, are economically important and widely consumed worldwide. The application of animal waste as organic fertilizer is a simple and cost-effective technique for applying nutrients to the soil. Combining cattle manure with humic substances can stimulate plant metabolism, resulting in increased nutrient absorption and assimilation. Therefore, the objective in this study was to evaluate doses of cattle manure combined with humic substances for stimulating plant metabolism and morphophysiological and nutritional processes in pepper plants. A randomized block design with four replicates was used, in a 4×3 factorial arrangement consisting of four cattle manure doses (8, 18, 28, and 38 Mg ha^{-1}) and three humic substance doses (0, 8, and 12 kg ha^{-1}). Humic substances (12 kg ha^{-1}) enhanced gas exchange, increasing net photosynthesis (34.2%), stomatal conductance (12.7%), instantaneous water use efficiency (15.87%), instantaneous carboxylation efficiency (37.37%), and absorbed energy conversion efficiency (23.53%). Cattle manure adequately met the crop's nutritional requirements, while humic substances increased the number and thickness of plant root hairs. The most effective combination to increase photosynthetic activity in pepper plants was 38 Mg ha^{-1} of cattle manure combined with 8 kg ha^{-1} of humic substances. Humic substances stimulated positive morphophysiological responses in organic pepper crops, which resulted in increased production.

Key words: *Capsicum annuum*, biostimulant, organic input, fertilizer

RESUMO: As espécies do gênero *Capsicum*, principalmente pimentão, possuem grande relevância econômica e são amplamente consumidas em todo o mundo. A aplicação de dejetos animais como fertilizante orgânico é uma técnica simples e barata de distribuição de nutrientes ao solo. O esterco bovino em associação com substâncias húmicas pode estimular o metabolismo das plantas, resultando no aumento da absorção e assimilação de nutrientes. Portanto, o objetivo neste estudo foi avaliar doses de esterco bovino associado a substâncias húmicas para estimular o metabolismo vegetal, a morfofisiologia e a nutrição em pimentões. O delineamento utilizado foi em blocos casualizados, distribuídos em arranjo fatorial 4 x 3, referente a quatro doses de esterco bovino (8, 18, 28 e 38 t ha^{-1}) e três doses de substâncias húmicas (0, 8 e 12 kg ha^{-1}), com quatro repetições. Substâncias húmicas (12 kg ha^{-1}) estimularam as trocas gasosas, aumentando a fotossíntese líquida (34,2%), a condutância estomática (12,7%), a eficiência instantânea do uso da água (15,87%), a eficiência instantânea de carboxilação (37,37%) e a eficiência efetiva da absorção conversão de energia (23,53%). O esterco bovino supriu adequadamente as necessidades nutricionais da cultura, enquanto as substâncias húmicas aumentaram o número e a espessura dos pelos radiculares do pimentão. A combinação de manejo mais eficaz para aumentar a atividade fotossintética em pimentões é 38 t ha^{-1} de esterco combinado com 8 kg ha^{-1} de substâncias húmicas. As substâncias húmicas estimularam respostas morfofisiológicas positivas no cultivo de pimentão orgânico, o que resultou no aumento da produção.

Palavras-chave: *Capsicum annuum*, bioestimulante, insumo orgânico, adubação

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Introduction

Species of the genus *Capsicum* have high economic and agricultural importance and are widely produced and consumed worldwide. Global production was 36,972,494 Mg in 2022, in a total area of 2,020,816 hectares (FAO, 2023). Peppers (*Capsicum annuum* L.) are used in cooking, both fresh in salads or as a seasoning. Additionally, they are source of antioxidant compounds, fibers, mineral nutrients, and bioactive compounds such as flavonoids, phenolic acids, carotenoids, capsaicinoids, fatty oils, volatile oils, proteins, and vitamins C, E, and A (Chatterjee et al., 2021).

Pepper crops require proper fertilizer application management and implementation of effective agricultural techniques, as profitability may fall short of production costs. Consequently, there is a pressing need to efficiently utilize organic residues to reduce costs and enhance crop production. In this context, cattle manure stands out for improving nutrient availability, and soil structure, aeration, and water retention capacity (Hijami et al., 2022).

The combined application of humic substances, activating plant metabolism, with cattle manure, providing nutrients for plants, has the potential to enhance photosynthetic efficiency and nutrient absorption and assimilation, thereby reducing fertilization costs and increasing crop production (Nardi et al., 2021). However, the production success by using these products depends on effects emerging from the interaction between the biostimulant, application management, and environmental conditions (Cristofano et al., 2021).

The mechanisms of action and effects of humic substances on plant morphophysiology are not fully elucidated. This is attributed to the high molecular complexity of these substances and the diverse plant responses induced by their application (Franzoni et al., 2022). Therefore, the objective in this study was to evaluate does of cattle manure combined with humic substances for stimulating plant metabolism and morphophysiological and nutritional processes in pepper plants.

Material and Methods

The research was conducted at the agricultural company Canteiro Cheiro Verde, which was undergoing an audit for organic certification by the certifying body IBD – Organis during the research period. The company is in Nova Floresta, in the microregion of Curimataú Paraibano, Paraiba, Brazil (6°27'8"S, 36°12'26"W, and altitude of 660 m). The region's climate was classified as As, tropical with a dry summers and wet winters and autumns, according to the Köppen classification. Climate data are shown in Figure 1 (AGRITEMPO, 2022).

The soil in the experimental area was classified as a Typic Hapludox (USDA, 2014) (Latossolo Amarelo Eutrofico tipico; EMBRAPA, 2018). Soil samples were collected from the 0-20 cm layer, sieved through a 2-mm mesh sieve, and air-dried in the shade for 48 hours. The soil was then chemically characterized at the Laboratório de Matéria Orgânica do Solo (LABMOS) and physically characterized at the Laboratório de Física do Solo (LABFIS) of the Departamento de Solos e Engenharia Rural of the Universidade Federal da Paraíba (DSER – UFPB), as described by Teixeira (2017) (Table 1).

The organic fertilizer source used was cattle manure. Chemical and physical characteristics of the cattle manure used are shown in Table 2.

The bioestimulant used was humic substances derived from leonardite, consisting of 70% humic acids, 15% fulvic acid, 14% potassium (K), and 1% nitrogen (N). It exhibited the following physicochemical characteristics: salinity index = 26%, water solubility = 300 g L^{-1} at 20 °C, cation exchange capacity (CEC) = 200 $cmol_c$ kg^{-1}, and pH = 9.68 in a 1:10 (w v^{-1}) solution.

The experimental area was divided into four sections measuring 20.8 m (length), 0.8 m (width), and 0.2 m (height). Plants were grown in double rows, with spacing of 0.4 m between rows and plants and 0.6 m between beds, resulting in an evaluation area of 0.64 m^2 per plot. A randomized block design with four replicates was used, in a 4 × 3 factorial arrangement consisting of four cattle manure doses (8, 18, 28, and 38 Mg ha^{-1}) and three humic substance doses (0, 8, and 12 kg ha^{-1}). The experimental unit consisted of 48 plants.

The manure was applied as a basal dressing and broadcast on the experimental plots after soil preparation, five days before transplanting. The humic substances were applied to the soil as topdressing, divided into four applications: eight days after

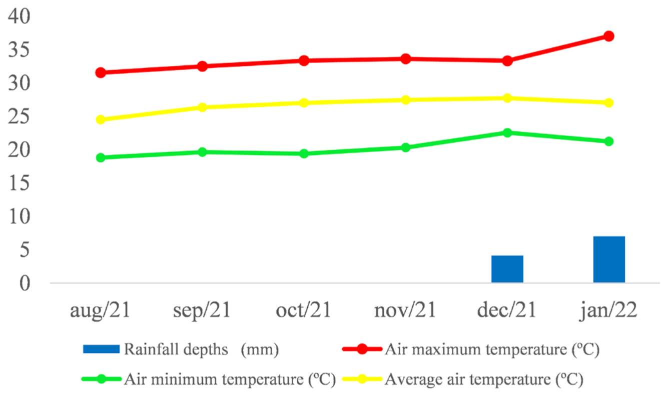


Figure 1. Data of average air temperature and rainfall depths during the experimental period

Table 1. Chemical and physical attributes of the soil prior to experiment implementation

Chemical attributes								
pH	P	K^+	Ca^{2+}	Mg^{2+}	Na^+	Al^{3+}	$H+Al^{3+}$	Organic matter
	(mg kg^{-1})		($cmol_c$ kg^{-1})					(g kg^{-1})
7.4	410.17	0.7	12.24	3.84	2.18	0	0	42

Physical attributes								
Sand	Silt	Clay	Natural clay	Degree of flocculation	Soil density	Particle density	Total porosity	Texture
(g kg^{-1})					(g cm^{3})		(m^3 m^{-3})	
727	149	124	26	790	1.53	2.46	0.38	Sandy Loam

Table 2. Chemical and physical characteristics of the cattle manure

Density (kg m³)	ECES (dS m⁻¹)	pH	C/N	M (%)	N	C	P	K	Ca	Mg
					(g kg⁻¹)					
874.5	5.02	8.98	1.2	9.09	15.8	19	5.2	15.3	12.7	14.4

Micronutrients (mg kg⁻¹)							
Na	S	Si	Cu	Fe	Mn	Zn	B
6.4	3.7	14.8	23.9	11.74	774.2	105.8	7.9

C/N = C to N ratio; ECES = Electrical conductivity of the extracted solution; M = Moisture at 36 °C

transplanting (DAT), during vegetative growth (31 DAT); at the onset of fruiting (58 DAT); and at harvest (87 DAT).

The soil was scarified and turned using a scarifier attached to a mini-tractor, and beds were formed and standardized using an automatic bed shaper. The beds were then covered with plastic mulch. The green pepper hybrid used was Kolima (Top Seed®), which has a 105-day growth cycle, square-shaped fruits (block type) with thick walls, and commercial length and weight of 10 cm and 240 g, respectively.

The seeds were sown in 200-cell trays (0.53 m in length, 0.27 m in width, and 0.42 m in height). The substrate used for seeding contained 60 kg of coconut fiber, 0.02 m^3 of vermicompost, 0.005 m^3 of ashes, and 100 mL of effective microorganisms consisted of unsalted cooked rice and sugarcane molasses, and microorganisms from native forest, applied as topdressing to the substrate. The seedlings were transplanted to the cultivation site 35 days after sowing (DAS), when they reached a height of 0.15 m and exhibited five definitive leaves.

Irrigation was carried out during periods with no rainfall, using a drip irrigation system (drip tape) with a flow rate of 1.5 L h^{-1}. This resulted in an estimated average daily water application depth of 7.5 mm, which was split into two equal applications, in the early morning and late afternoon, applied within 30 min day^{-1}. Water replenishment was monitored using analog tensiometers installed at depths of 0.20 and 0.40 m in the beds. Irrigation was scheduled to maintain soil moisture at 70% of field capacity, following the soil water characteristic curve. Plant staking was not used. No significant incidences of pests and diseases were observed throughout the crop cycle.

Leaf contents of nitrogen (N), phosphorus (P), and potassium (K) were quantified by sampling three leaves from each plant after the first fruiting. Subsequently, the plant material was washed in distilled water, placed in Kraft paper bags, and dried in a forced air circulation oven at 65 °C. The plant material was ground in a Willey mill and sent for chemical analysis at LABMOS-UFPB. N contents were determined using the Kjeldahl method after digestion with sulfuric peroxide. Phosphorus (P) contents were determined by colorimetry, using the molybdenum blue method. Potassium (K) contents were determined by flame photometry (Analyser® model 910).

Physiological analyses were conducted 35 days after transplanting (DAT) between 9:00 a.m. and 11:00 a.m. The third fully expanded leaf from the apex downwards was analyzed, focusing on its middle section.

Gas exchanges were measured using an infrared gas analyzer (LCPro+ Portable Photosynthesis System; ADC BioScientific) under controlled temperature of 25 °C, irradiance of 1200 μmol photons m^{-2} s^{-1}, and an air flow rate of 200 mL min^{-1}. Intercellular CO_2 concentration (Ci - mmol CO_2 mol air^{-1}), stomatal conductance (gs - mol H_2O m^{-2} s^{-1}), transpiration rate (E - mmol H_2O m^{-2} s^{-1}), and net photosynthesis (A - μmol CO_2 m^{-2} m^{-1}) were evaluated. Subsequently, instantaneous water use efficiency (WUE - μmol CO_2 mmol H_2O m^{-2} s^{-1}) and intrinsic carboxylation efficiency (iCE - μmol CO_2 μmol mol air^{-1}) were calculated. Chlorophyll *a*, *b*, and total indices were measured using a portable chlorophyll meter (ClorofiLOG CFL1030, Falker).

Chlorophyll *a* fluorescence was measured using a fluorometer (DS-30p; Opti-Sciences) with closed-chamber clips for 30 minutes for photosystem adaptation. The leaves were then exposed to a weak pulse of far-red light (1-2 μmol m^{-2} s^{-1}) to determine the initial emitted fluorescence (F_0), when all reaction centers of photosystem II (PSII) are in the oxidized state. A saturating light pulse with irradiance of 3000 μmol photons m^{-2} s^{-1} and duration of 5 seconds was then applied to temporarily promote the maximum reduction of the primary electron acceptor of PSII (Qa), determining the maximum fluorescence (F_m). Variable fluorescence (F_v) was calculated by subtracting F_m from F_0. Quantum efficiency of photosystem II (F_v/F_m) and absorbed energy conversion efficiency (F_v/F_0) were calculated based on the obtained data.

Pepper plant roots were excised near the base of the stem. The material was dried in a forced air circulation oven at 65 °C, then, transverse and longitudinal cuts were made in the secondary roots. The samples were then mounted on metal stubs, and images were observed following the protocol of Bozzola & Russel (1999). Photomicrographs of the roots were captured using a scanning electron microscope (A70 ETH TESCAN®).

The obtained data were tested for normality (Shapiro-Wilk) and subjected to analysis of variance ($p \leq 0.05$). Data of manure doses were fitted to polynomial regression models, and the Tukey's test was applied for means of humic substances ($p \leq 0.05$). The analyses were performed using the statistical software R® 4.2.0 (R Core Team, 2022). Figures were created using the software SigmaPlot® 12.5 (Systat Software, San Jose, CA, USA).

Results and Discussion

Leaf phosphorus (P) contents were affected by the interaction between manure and humic substances, and potassium (K) contents were affected only by cattle manure. However, the factor had no effect on nitrogen (N) contents (Table 3).

The application of humic substances did not affect P contents in the lowest (8 Mg ha^{-1}) and highest (38 Mg ha^{-1}) manure doses. The highest P content (2.42 g kg^{-1}) was found for the highest manure rate (38 Mg ha^{-1}) with 12 kg ha^{-1} of

Table 3. Analysis of variance for nutrient contents (N, P, and K) as a function of doses of cattle manure and humic substances

Sources of variation	DF	Mean square		
		N	P	K
Manure	3	8.16^{ns}	7.6*	15.22*
Humic substance	2	12.58^{ns}	3.3^{ns}	13.39^{ns}
Manure × Humic substance	6	8.41^{ns}	3.58**	4.16^{ns}
Blocks	3	4.72^{ns}	0.09^{ns}	7.49^{ns}
Residue	33	186.33	0.05	5.22
CV (%)		27.5	9.1	7.9

*, **, and ns = significant at $p \leq 0.05$, significant at $p \leq 0.01$, and not significant, respectively, by the F-test; DF = degree of freedom; CV = coefficient of variation

humic substances (Figure 2A). Plants fertilized only with manure showed stability among the evaluated doses, with the highest variation in leaf P content of 0.33 g kg^{-1}. Leaf K contents exhibited a linear increase as the manure rate was increased; the highest K content (25.96 g kg^{-1}) was found for the application of 38 Mg ha^{-1} of manure (Figure 2B).

The application of manure to the soil increases the availability of N, P and K, mainly K, and continuous manure application can lead to its accumulation, resulting in high concentrations of this element (Araújo et al., 2007). Potassium is the most demanded nutrient by pepper plants and is important for the translocation and allocation of sugars from plant organs to the fruits, thus improving productivity (Oliveira, 2018). Applying manure causes the chelating activity of humic acid to increase the availability of insoluble phosphorus in the soil, thereby promoting root development and improving absorption of essential nutrients such as N, P, and K (El-Baset & Kasem, 2022).

Phosphorus serves as a structural component of ATPase membranes, and humic acids increase the proton concentration (H^+) in the plasma membrane, activating ATPases; this acidifies the apoplast and reduces the physical resistance of the cell wall, allowing for the elongation of root cells, expanding the root surface area, which is a biological phenomenon known as the acid growth theory (Nardi et al., 2021). Therefore, increases in the electrochemical proton gradient drive ion transport across cell membranes, ultimately improving nutrient absorption (Purwanto et al., 2021).

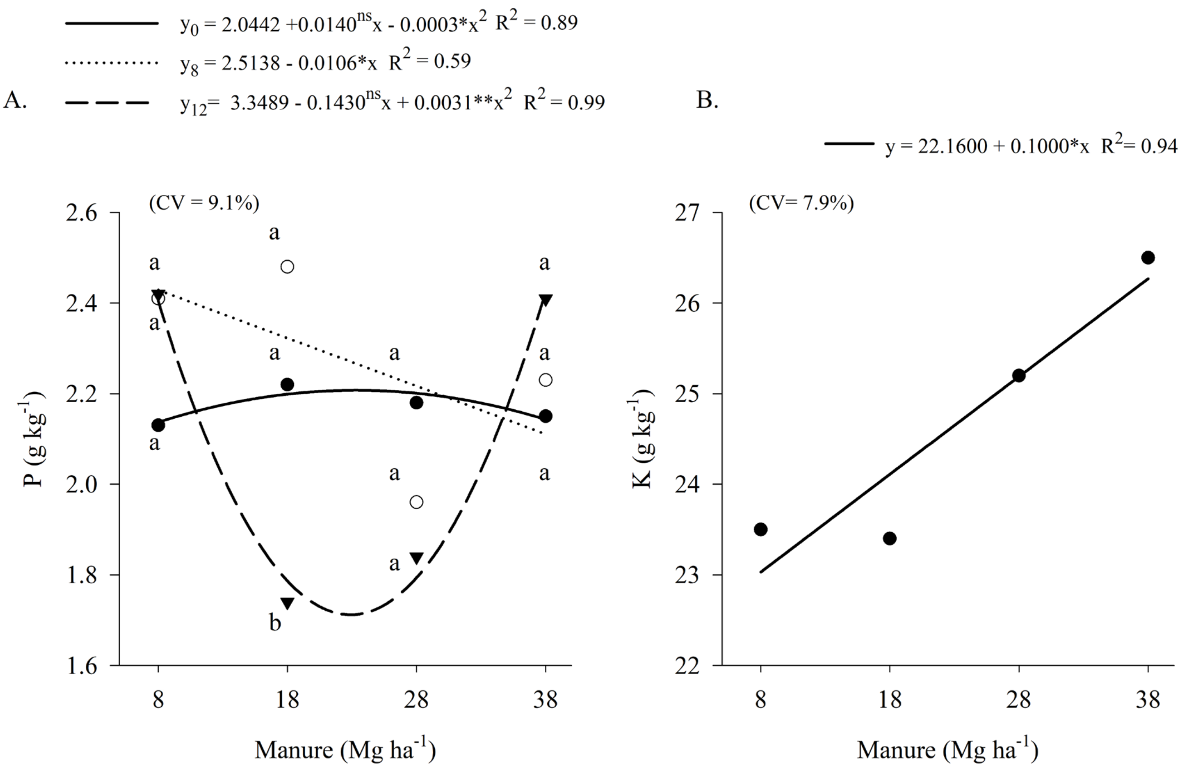
Net photosynthesis (*A*) and stomatal conductance (*gs*) were affected by humic substances, while intercellular CO_2 concentration (*Ci*) was affected by manure; transpiration rate (*E*) was affected by the interaction between manure and humic substances (Table 4).

The application of humic substances increased net photosynthesis from 9.97 (with no humic substance application) to 13.38 μmol CO_2 m^{-2} m^{-1} when applying 12 kg ha^{-1} of humic substances, representing an increase of 34.2% (Figure 3A).

Table 4. Analysis of variance for net photosynthesis (*A*), stomatal conductance (*gs*), intercellular CO_2 concentration (*Ci*), and transpiration rate (*E*) as a function of doses of cattle manure and humic substances

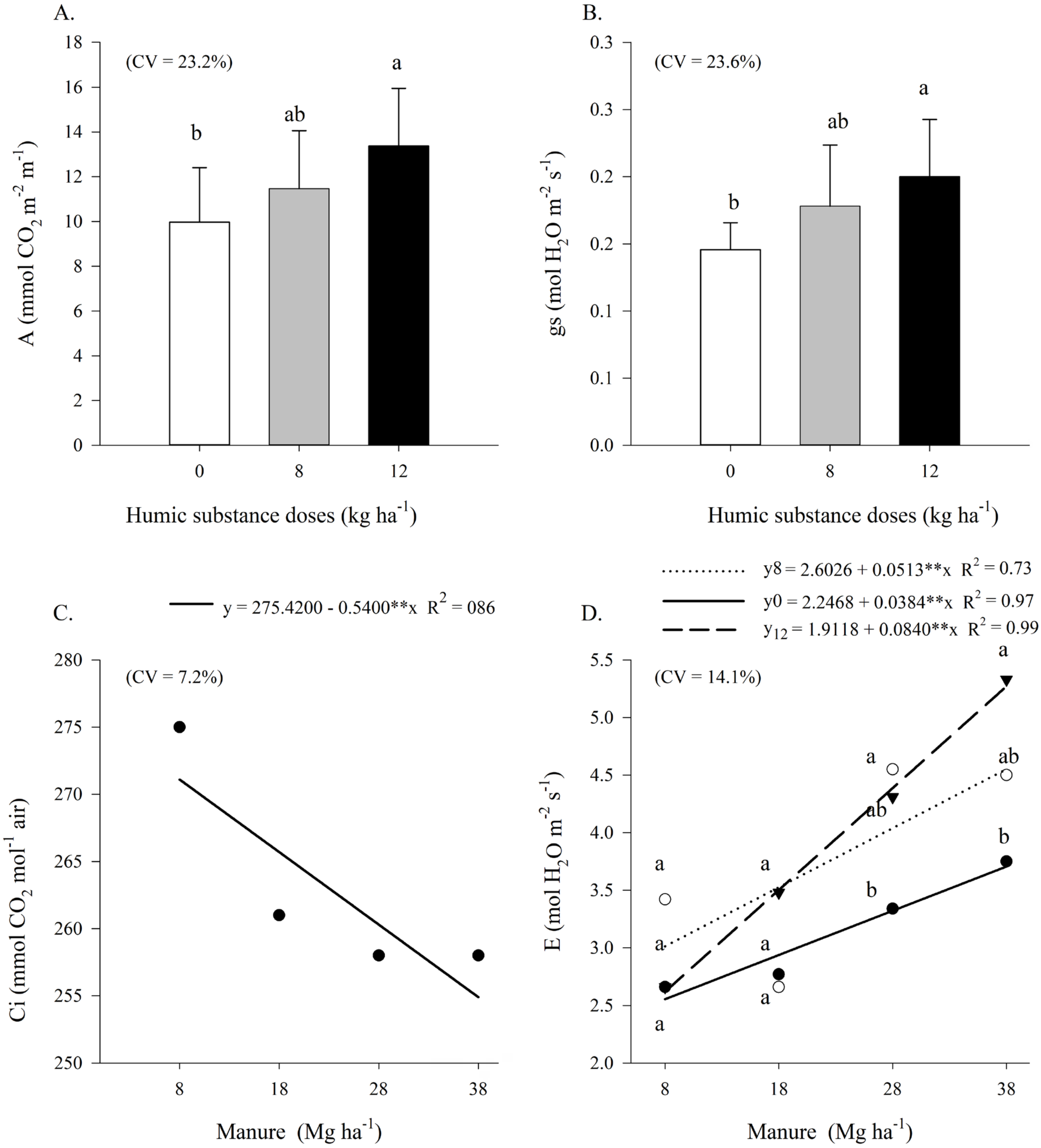
Sources of variation	DF	Mean square			
		A	gs	Ci	E
Manure	3	14.25^{ns}	0.00^{ns}	2740.5**	6.63**
Humic substance	2	46.63*	0.01*	456.46^{ns}	4.07**
Manure × Humic substance	6	11.25^{ns}	0.00^{ns}	1017.82^{ns}	0.52^{ns}
Blocks	3	0.59^{ns}	0.00^{ns}	533.39^{ns}	0.05^{ns}
Residue	33	10.55	0.00	479.94	0.39
CV (%)		23.2	23.6	7.2	14.1

*, **, and ns = significant at $p \leq 0.05$, significant at $p \leq 0.01$, and not significant, respectively, by the F-test; DF = degree of freedom; CV = coefficient of variation



*, **, and ns = significant at $p \leq 0.05$, significant at $p \leq 0.01$, and not significant, respectively, by the F-test; y_0 = 0 kg ha^{-1} of humic substances; y_8 = 8 kg ha^{-1} of humic substances; y_{12} = 12 kg ha^{-1} of humic substances; Means with different letters are not significantly different from each other by the Tukey's test ($p \leq 0.05$)

Figure 2. Phosphorus (A) and potassium (B) contents in pepper leaves as a function of cattle manure doses and three humic substance doses



*, **, and ns = significant at p ≤ 0.05, significant at p ≤ 0.01, and not significant, respectively, by the F-test; y_0 = 0 kg ha^{-1} of humic substances; y_8 = 8 kg ha^{-1} of humic substances; y_{12} = 12 kg ha^{-1} of humic substances; Means with different letters are not significantly different from each other by the Tukey's test (p ≤ 0.05)

Figure 3. Net photosynthesis (*A;* A), stomatal conductance (*gs;* B), intercellular CO_2 concentration (*Ci;* C), and transpiration dose (*E;* D) as a function of cattle manure doses and three humic substance doses

Humic substances improve photosynthetic processes. Ertani et al. (2019) supported this explanation when evaluating the photosynthetic process in maize under application of a biostimulant based on leonardite humate and lignosulfonate, which resulted in increases in Rubisco activation (30%-50%). Furthermore, biostimulants can act as primers for physiological processes, activating specific genes involved in the biosynthesis of phytohormones such as auxins (indole-3-acetic acid) and abscisic acid, thus enhancing photosynthetic activity; this may have resulted in an increase in photosynthesis when applying 12 kg ha^{-1} of humic substances (Figure 3A, Ciriello et al., 2022).

Stomatal conductance increased with the application of humic substances (12 kg ha^{-1}), reaching the highest value of 0.2 mol H_2O m^{-2} s^{-1}, resulting in an increase of 12.7% (Figure 3B). Intercellular CO_2 concentration showed a linear decreasing response to the increasing manure doses, with the highest value (268.1 μmol CO_2 mol air^{-1}) found for the application of 8 Mg ha^{-1} of manure (Figure 3C). Transpiration rate increased linearly with the increasing manure doses (Figure 3D), reaching the highest value of 5.27 mmol H_2O m^{-2} s^{-1} when using the highest manure rate (38 Mg ha^{-1}) combined with humic substances (12 kg ha^{-1}), representing an increase of 42.4% compared to the treatment with application of only manure (Figure 3D).

Increases in stomatal conductance are connected to water use efficiency and production. This physiological effect promoted by biostimulants was reported in a recent study with greenhouse-grown peppers, in which the application of a biostimulant based on algae extract (0.5%) led to a 27% increase in stomatal conductance, resulting in the highest production obtained in the study: 314 kg per 1000 plants (Melo et al., 2020).

Zeist et al. (2018) reported an increase of 18.96% in transpiration rate of pepper plants grown under application of a biostimulant composed of plant phytohormones, which was a result lower than that found in the present study (42.4%). Furthermore, humic substances can affect other metabolic pathways, such as respiration, as they can activate specific genes related to enzymes like glucose-6 phosphate-isomerase and pyruvate kinase in the respiratory metabolic process (Nardi et al., 2021).

Water use efficiency (WUE), instantaneous carboxylation efficiency (iCE), and absorbed energy conversion efficiency (F_v/F_0) were affected by humic substances, although no significant effect of humic substances on quantum efficiency of photosystem II (F_v/F_m) was found. Additionally, chlorophyll indices were affected by the interaction of humic substances and manure, although no significant effects on chlorophyll *b* and total chlorophyll were found (Table 5).

The application of humic substances increased WUE (Figure 4A) from 2.96 (with no humic substance application) to 3.43 μmol CO_2 mmol H_2O m^{-2} s^{-1} when applying 12 kg ha^{-1} of humic substances, representing an increase of 15.87%. Effects of humic substances on WUE are still not clear; one hypothesis is the physical blockage of pores on the cell wall surface of roots, affecting root hydraulic conductivity, leaf growth, transpiration, and tolerance to drought (Lazzarini et al., 2022).

Intrinsic carboxylation efficiency (iCE) increased as a response to humic substances; the highest iCE (0.0563 μmol CO_2 μmol mol air^{-1}) was found when applying 12 kg ha^{-1} of humic substances, representing an increase of 37.37% (Figure 4B). iCE is the rate at which atmospheric CO_2 is converted into organic carbon (Luz et al., 2021); thus, increases in iCE can be explained by an increase in gene expression of the large subunit of Rubisco (rbcL). This chloroplastidic subunit contains a C-terminal loop region essential for CO_2 or O_2 specificity, as this region closes the active binding site for these molecules (Rosgaard et al., 2021). This explanation is supported by Che et al. (2017), who evaluated the effect of fulvic acid application on *Monoraphidium* sp. and analyzed the gene expression of rbcL. They found changes in gene expression of up to 2.5 times, resulting in positive regulation with increased carbohydrates.

Increases in iCE benefit the plant in terms of WUE. When the plant fixes carbon in less time, the stomata remain open for a shorter period, consequently reducing water loss. This is particularly relevant under conditions of low water availability in semiarid regions. Recent studies have linked increases in iCE to the application of biostimulants. For instance, the application of fulvic acid and *Ascophyllum nodosum* to soybean crops under suitable water availability conditions resulted in a maximum iCE of 0.48 μmol CO_2 mol air^{-1} among the tested biostimulant formulations (Rosa et al., 2021). Rice seeds treated with humic substances (150 mL per 100 kg^{-1} seeds) resulted in a maximum iCE in seedlings of 0.025 μmol CO_2 μmol mol air^{-1}, representing an increase of 150% compared to untreated seeds (Luz et al., 2021).

Moreover, another factor that may have contributed to increases in WUE (Figure 4A) and iCE (Figure 4B) was the increase in potassium content in the plants due to the combination of manure with humic substances (Figure 2A). Potassium participates in cellular osmoprotection, enzyme activation, and positive regulation of photosynthesis (Shahid et al., 2023). The application of humic substances increased F_v/F_0, reaching the highest efficiency (3.78) when applying 8 kg ha^{-1} (Figure 4C), representing an increase of 23.53%. F_v/F_0 reflects the efficiency of electron donation to the RCs of PSII and the photosynthetic quantum conversion rate in PSII (Rady et al., 2021). Excess humic substances under these growth conditions appears to reduce the electron transport rate in PSII, affecting the efficiency of ATP and $NADPH^+$ formation, explaining the highest F_v/F_0 found for the application of the intermediate humic substance rate (8 kg ha^{-1}), followed by a reduction at the highest rate (12 kg ha^{-1}).

Chlorophyll *a* index showed a positive response to cattle manure application, exhibiting an increase at the highest manure doses (Figure 4D). The highest chlorophyll *a* index (39.6 FCI) was found when applying 24.74 Mg ha^{-1} of manure; however, higher doses resulted in decreases in chlorophyll contents. The combination of 8 and 12 kg ha^{-1} of humic substances did not yield significantly different results when using the lowest (8 Mg ha^{-1}) or the highest (38 Mg ha^{-1}) doses of manure. The highest chlorophyll *a* index (40.25 FCI) was found when combining 12 kg ha^{-1} of humic substances with 38 Mg ha^{-1} of manure, which resulted in an increase of 3.35%.

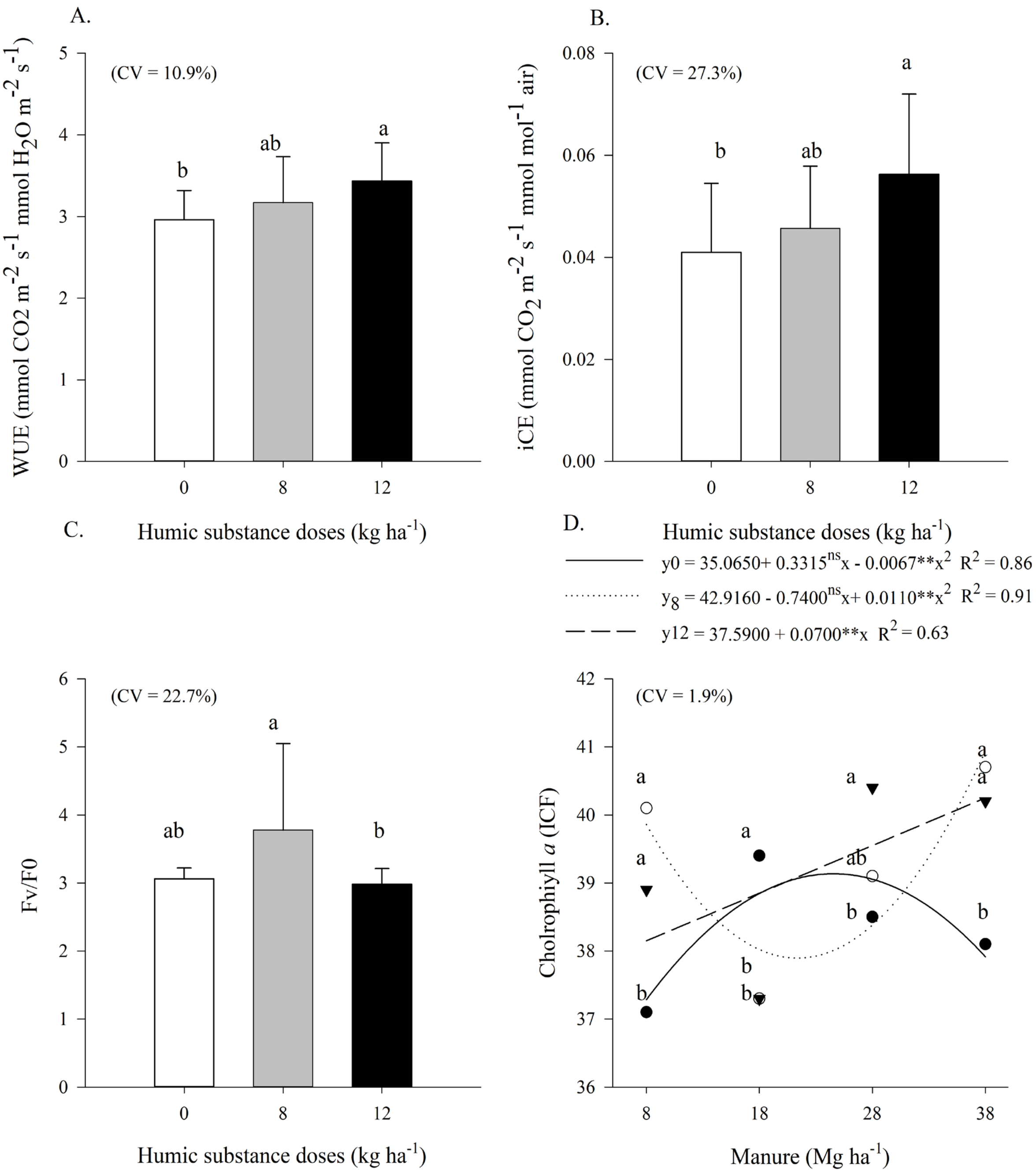
Cattle manure provides essential nutrients, such as nitrogen (N) and magnesium (Mg), for structural development of plants, as well as manganese (Mn) and iron (Fe), which act as enzymatic cofactors in chlorophyll molecule synthesis (Bagale, 2021). Increases in chlorophyll contents due to application of humic substances can be attributed to the activation of chlorophyll synthesis (Alani et al., 2021).

The use of humic substances as plant biostimulants has anatomical, physiological, biochemical, and molecular effects mainly on the root system. In this regard, anatomical changes were found in secondary roots of pepper plants under the

Table 5. Analysis of variance for instantaneous water use efficiency (WUE), intrinsic efficiency of carboxylation (iCE), absorbed energy conversion efficiency (F_v/F_0), efficiency of photosystem II (F_v/F_m), chlorophyll *a* (Chl a), chlorophyll *b* (Chl b), and total chlorophyll (tC) as a function of doses of cattle manure and humic substances

Sources of variation	DF	Mean Square						
		WUE	iCE	F_v/F_0	F_v/F_m	Chl a	Chl b	tC
Manure	3	1.53**	0.00^{ns}	2.31^{ns}	0.73^{ns}	6.28**	35.15*	74.31**
Humic substance	2	0.89*	0.00*	3.06*	0.59^{ns}	5.01**	10.22^{ns}	17.67^{ns}
Manure × Humic substance	6	1.00**	0.00^{ns}	3.14**	1.46*	7.09**	14.10^{ns}	31.86^{ns}
Blocks	3	0.05^{ns}	0.00^{ns}	0.56^{ns}	0.52^{ns}	0.90^{ns}	15.96^{ns}	22.04^{ns}
Residue	33	0.17	0.00	0.8	0.43	0.78	10.18	16.12
CV (%)	-	10.9	27.3	22.7	56.6	1.9	11.8	5.4

*, **, and ns - Significant at p ≤ 0.05, p ≤ 0.01, and not significant, respectively, by F-test; DF - Degree of freedom; CV - Coefficient of variation



*, **, and ns = significant at p ≤ 0.05, significant at p ≤ 0.01, and not significant, respectively, by the F-test; y_0 = 0 kg ha^{-1} of humic substances; y_8 = 8 kg ha^{-1} of humic substances; y_{12} = 12 kg ha^{-1} of humic substances; Means with different letters are not significantly different from each other by the Tukey's test (p ≤ 0.05)

Figure 4. Water use efficiency (WUE - A), intrinsic carboxylation efficiency (iCE - B), absorbed energy conversion efficiency (F_v/F_0 - C), and chlorophyll *a* (D) in pepper leaves as a function of cattle manure doses and three humic substance doses

application of humic substances, increasing the number and thickness of root hairs, as highlighted by the red arrows in Figure 5. Morphological changes in the lateral roots of pepper plants promoted by humic substances (Figures 5C, 5D, 5E, and 5F) can be observed when compared to roots of plants subjected to application of only cattle manure (Figures 5A and 5B).

According to Nardi et al. (2021), morphological modifications in roots at the metabolic level are mediated by auxins and the activation of H^+-ATPase membrane enzymes. They interact with membrane receptors to induce cascade signaling in root cells. Both low and high molecular weight fractions of humic substances stimulate the H^+-ATPase membrane activity, as well as polar auxin flow. The accumulation of auxin and nitric oxide in pericycle cells promotes the emergence and expansion of lateral roots.

These results (Figure 5) shows that humic substances of both low and high molecular weight induce auxin accumulation in the root epidermal cells, leading to an increase in root hair formation and cell elongation (Nardi et al., 2021). Additionally, increased diameter of xylem vessels was found when applying humic substances (Figures 6B and 6C) compared to the treatment without biostimulant application (Figure 6A). The elements of the xylem vessel are highlighted by red circles in Figure 6A.

Increases in photosynthetic efficiency and WUE promoted by the application of humic substances can be explained by the

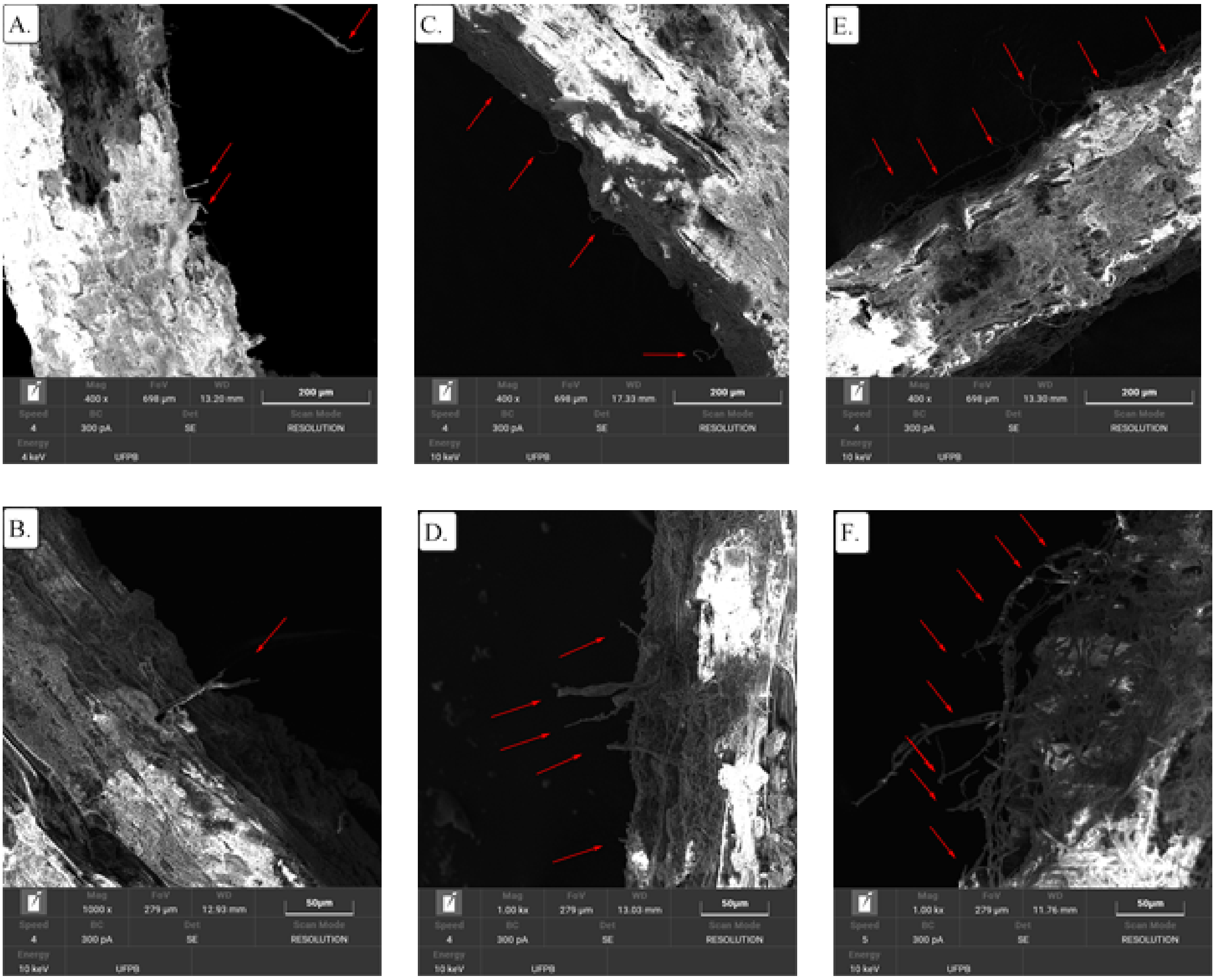


Figure 5. Scanning electron microscopy (SEM) photomicrographs in longitudinal sections highlighting anatomical modifications promoted by humic substances in pepper roots. 0 kg ha^{-1} of humic substances, magnification of 400× (A) and 1000× (B); 8 kg ha-1 of humic substances, magnification of 400× (C) and 1000× (D); 12 kg ha^{-1} of humic substances, magnification of 400× (E) and 1000× (F). Red arrows highlight root hairs

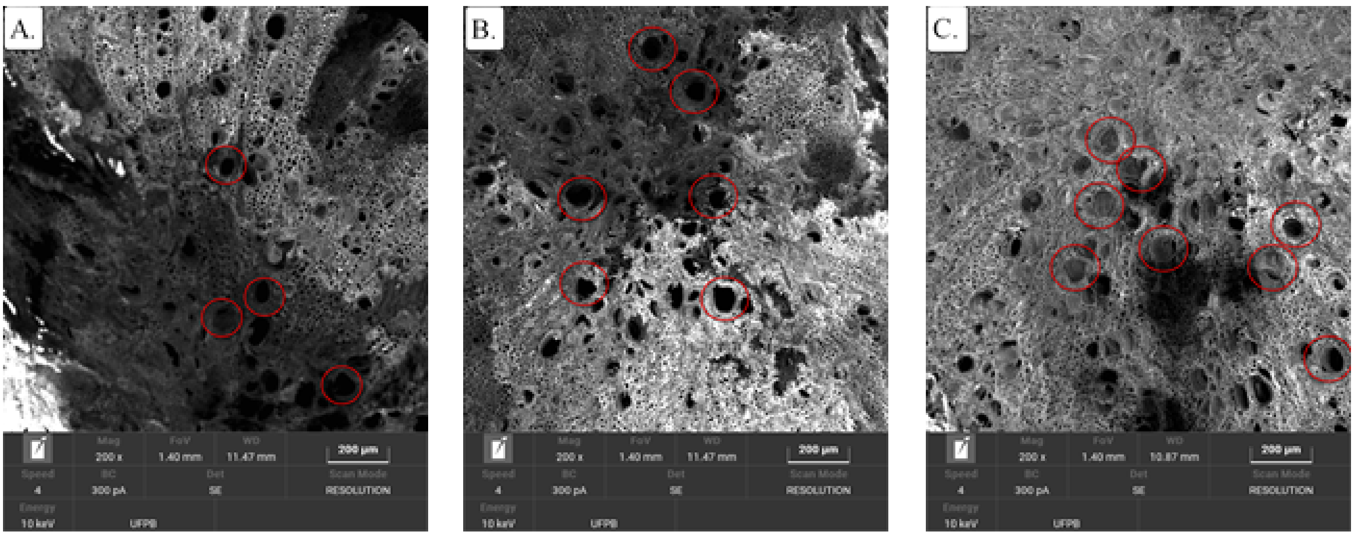


Figure 6. Scanning electron microscopy (SEM) photomicrographs in cross-sections showing anatomical modifications promoted by humic substances in pepper roots. 0 kg ha^{-1} of humic substances (A); 8 kg ha^{-1} of humic substances (B); 12 kg ha^{-1} of humic substances (magnification of 200×, C). Red circles highlight xylem vessel elements

activation of plant metabolism in the aerial part and anatomical changes in the roots of pepper plants. Humic substances can induce different patterns in the central cylinder, positively affecting water conductivity and nutrient flow intensity, as well as in the cortex, resulting in increased root diameter and storage of reserve compounds (Pizzeghello et al., 2020).

Conclusions

1. The best combination to increase the photosynthetic activity (based on intercellular CO_2 concentration and transpiration rate) of pepper plants was the application of 38 Mg ha^{-1} of cattle manure with 8 kg ha^{-1} of humic substances.

2. The application of cattle manure combined with humic substances increased phosphorus contents in pepper plants.

3. The application of manure increased potassium contents in pepper plants.

4. Humic substances stimulated positive morphophysiological responses (number and thickness of root hairs) in organic pepper crops.

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