Biofertilizer enhances the expression of the *CaWRKY6* gene in water-stressed plants of *Capsicum annuum* L.

Biofertilizante mejora la expresión del gen *CaWRKY6* en plantas de *Capsicum annuum* L. sometidas a estrés hídrico

Santiago Amariles Barrera[1*], Diego Mauricio Martínez Rivillas[1], and Alejandra Vargas Ruiz[1]

ABSTRACT

Colombia has agroecological conditions for bell pepper (*Capsicum annuum* L.) cultivation. However, traditional management practices have depended mainly on agrochemicals. Organic production could enhance the value of products and facilitate their entry into international markets. Research indicates that biofertilizers and biostimulants can activate the gene expression that helps plants tolerate both biotic and abiotic stress. This study assessed the activation of the *CaWRKY6* gene in response to water stress tolerance in *C. annuum*, after applying a biofertilizer to greenhouse-grown young plants to mitigate the impacts of drought. Foliar samples were taken 48 h after the application of five treatments: biofertilizer at two concentrations (5 ml L^{-1} and 10 ml L^{-1}), salicylic acid (5 mg L^{-1}), and two controls (water), followed by RNA extraction and an RT-qPCR test to determine the relative expression of the gene *CaWRKY6*. To compare the adjuvant effect of biofertilizer and salicylic acid, treated young plants were exposed to water depletion for 21 d. The ANOVA indicated differences between treatments with 5 ml L^{-1} and 10 ml L^{-1} of biofertilizer, and they had the highest *CaWRKY6* gene expression, as well as higher growth and less wilting against water stress.

Keywords: organic agriculture, organic fertilizers, abiotic stress resistance, drought protection.

RESUMEN

Colombia presenta condiciones agroecológicas para la producción de pimentón (*Capsicum annuum* L.). Sin embargo, su manejo tradicional ha sido principalmente por medio del uso de agroquímicos. La producción orgánica podría dar valor agregado a los productos, y facilitar su entrada a los mercados internacionales. Se ha evidenciado que los biofertilizantes y bioestimulantes pueden activar la expresión de genes que ayudan a las plantas a tolerar estrés biótico y abiótico. En este estudio se evaluó la activación del gen *CaWRKY6*, uno de los más destacados para *C. annuum*, tras la aplicación de un biofertilizante sobre plántulas de pimentón cultivadas bajo invernadero. Se analizaron muestras foliares 48 h después de aplicar cinco tratamientos: biofertilizante a dos concentraciones (5 ml L^{-1} y 10 ml L^{-1}), ácido salicílico (5 mg L^{-1}) y dos controles (agua). Después se extrajo ARN y mediante una prueba RT-qPCR se determinó a la expresión relativa del gen *CaWRKY6*. Para comparar el efecto adyuvante del biofertilizante y el ácido salicílico, las plantas jóvenes tratadas se expusieron a la falta de agua durante 21 d. El ANOVA indicó diferencias entre los tratamientos con 5 ml L^{-1} y 10 ml L^{-1} de biofertilizante; además, estos tuvieron la mayor expresión del gen *CaWRKY6*, mayor crecimiento y menor marchitez por el estrés hídrico.

Palabras clave: agricultura orgánica, fertilizantes orgánicos, resistencia a estrés abiótico, protección contra sequía.

Introduction

Biotic and abiotic stresses are factors that can impact the productivity and quality of food crops worldwide, posing a threat to global food security (Abdou Zayan, 2020). Specifically, water deficit is the main abiotic factor in soils that limits plant growth and yields worldwide (Campos *et al*., 2014; Yaseen *et al*., 2024).

One of the leading causes of reduced soil water availability is climate change, which brings about increased temperatures and alterations in rainfall patterns. These changes can generate deficiencies in soil moisture, causing loss of biological functions in plants (Abdou Zayan, 2020). Consequently, the stress generated by these conditions may increase susceptibility in plants to disease and pest attack (Sinha *et al*., 2019).

Research conducted on pepper (*Capsicum annuum* L.) plants has shown that water and heat stress lead to decreased growth rate, decreased fruit yield and quality, and increased susceptibility to attack by pathogenic microorganisms (Jang *et al*., 2019; Lee *et al*., 2018; Ntanasi *et al*.,

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[1] Universidad CES, Medellín, Antioquia (Colombia).

[*] Corresponding author: amariles.santiago@uces.edu.co

2025; Yaseen *et al.*, 2024; Zhang Ma *et al.*, 2024). This is why climatic conditions that promote water stress can actively contribute to the deterioration of crop phytosanitary conditions.

Colombia, a country with a strong agricultural tradition, has the potential to provide organic products with minimal reliance on chemical synthetic inputs. However, in bell pepper production, both fertilization and pest and disease management have historically depended on synthetic fertilizers and insecticides (Casilimas *et al.*, 2012; Jaramillo Noreña *et al.*, 2014). The use of these chemicals in agroecosystems poses a risk of toxicity for non-target organisms, leading to alterations in aquatic and terrestrial ecosystems (Campos-Soriano *et al.*, 2020; Mandal *et al.*, 2020) and adverse effects on human health (Elahi *et al.*, 2019). For this reason, export markets are increasingly demanding standards aimed at reducing or eliminating the use of agrochemicals (Martínez Bernal *et al.*, 2012). Countries such as the USA have a high demand for bell peppers, representing a significant export opportunity for emerging economies like Colombia. In international markets, value-added attributes related to the production system, particularly organic certification, play a key role in influencing sales prices and consumer preference (Agronegocios, 2015; MINCIT, 2019; Zhang *et al.*, 2024). However, to access international organic markets, Colombian farmers must be certified in environmentally sustainable and agrochemical-free practices, and thus be able to give added value to their products (MADR, 2020; Martínez Bernal *et al.*, 2012). To achieve this goal, farmers must fertilize their crops through the application of environmentally friendly methods that protect human health, among which are biofertilizers, effective microorganisms, and plant extracts (MADR, 2020).

The biofertilizers, besides containing nutrients of high bioavailability for plants (Goñi *et al.*, 2018; Kour *et al.*, 2020), include some biostimulant substances such as indole-acetic acid (Etesami *et al.*, 2015), humic and fulvic acids, phosphites (Han *et al.*, 2021), and free amino acids (Ahammed *et al.*, 2020; Vijayakumar *et al.*, 2019); these can activate genes and metabolic pathways that help plants to tolerate and/or to resist adverse environmental factors and pathogen attack that may alter their development (Malo *et al.*, 2015; Orozco-Mosqueda *et al.*, 2020; Xu *et al.*, 2019; Yaseen *et al.*, 2024). Accordingly, *CaWRKY* genes are one of the most representative gene families in *C. annuum* for protecting plants against biotic and abiotic stresses (Bulle *et al.*, 2025; Hussain *et al.*, 2019; Jingyuan *et al.*, 2011; Zheng *et al.*, 2019).

An extract of *Equisetum arvense* can act as a biostimulant in tomato (*Lycopersicum esculentum*) plants by activating action on *SlWRKY* genes (Malo *et al.*, 2015). This species shares phylogenetic similarities with *C. annuum* (Park *et al.*, 2011) and has comparable growing conditions and management practices (Roselló I Oltra & Porcuna, 2012). It can serve as a reference to evaluate the activation of *CaWRKY* genes in *C. annuum* and elucidate its relationship with protective activity against biotic and abiotic stresses.

It is necessary to find data that verify the effectiveness and supports the application of biofertilizers and/or biostimulant substances for the nutrition of *C. annuum* plants. Such applications will increase *CaWRKY6* gene expression, enhance drought tolerance, and improve growth under greenhouse conditions. This research aimed to evaluate the effect of the application of biofertilizers on young plants of *C. annuum* in order to investigate the activation of the *CaWRKY6* gene associated with water stress, as a strategy to face adverse environmental growing conditions.

Materials and methods

Plant material

Young plants of *Capsicum annuum* 'California Wonder' were obtained from a commercial supplier and established in the greenhouse facilities of CES University, Medellín, Colombia. The study was conducted in a greenhouse maintained under semi-controlled conditions. Relative air humidity ranged between 60% and 85%, with an average temperature of 24°C, reaching a maximum of 32°C and a minimum of 17°C. The greenhouse was equipped with a forced ventilation system consisting of an air injector and extractor to ensure adequate air exchange. The structure was covered with anti-thrips mesh to prevent pest entry. Plants were placed on welded wire mesh benches that allowed proper drainage and air circulation around the pots. The greenhouse had a concrete floor, which improved sanitization and weed control.

To ensure the quality of the potting soil substrate and verify that the aluminum content was within acceptable limits for planting, a physicochemical analysis of the substrate was performed. Additionally, a water retention curve was conducted to determine the field capacity of the substrate. Once the young plants reached an average height of 11 cm, they were subjected to the different treatments considered in this study.

Young plants of *Capsicum annuum* of approximately 30 d were used; at this stage they are more susceptible to changes in irrigation and water stress, because their root system is less developed than in adult plants (Bykova *et al*., 2019). This made it possible to detect changes in their growth more accurately when the young plants were subjected to different treatments.

The biofertilizer used for this research is a filtered extract derived from a fermentation process. The composition of the biofertilizer is as follows: oxidizable organic carbon 18.7 g L^{-1}, total nitrogen 1.10 g L^{-1}, phosphorus 1.16 g L^{-1}, potassium 5.75 g L^{-1}, calcium 3.14 g L^{-1}, magnesium 4.41 g L^{-1}, zinc 7.91 g L^{-1}, copper 0.32 g L^{-1}, iron 0.55 g L^{-1}, manganese 1.07 g L^{-1}, sulfur 8.70 g L^{-1}, boron 1.02 g L^{-1}. The potting soil used as substrate for planting had the following characteristics: pH 5.7, sandy loam texture, 17.24% organic matter, 7.63% organic carbon, and aluminum contents 1.2 cmol kg^{-1}.

Application of biofertilizer and salicylic acid

Thirty plants grown in the greenhouse were randomly selected and distributed using five treatments:

TABLE 1. Description of the five treatments applied to the plants.

Nomenclature	Treatment description
Bio 5	Liquid biofertilizer dissolved in water at a concentration of 5 ml L^{-1}
Bio 10	Liquid biofertilizer dissolved in water at a concentration of 10 ml L^{-1}
SA	Salicylic acid (SA) dissolved in water at a concentration of 5 mg L^{-1}, similar values have been proposed by other authors (Jingyuan *et al*., 2011)
CLI	Control - limited irrigation (CLI), a group of young plants to which water was applied and subsequently exposed to water depletion
CTI	Control - total irrigation (CTI), a group of young plants that had constant irrigation throughout the experiment to exclude the possibility that the environmental conditions of the greenhouse, the type of substrate in which they were planted, and the irrigation water did not have an undesirable effect on the plants

Only one application of the biofertilizer (5 ml L^{-1}, 10 ml L^{-1}) and salicylic acid was made at the beginning of the experiment. The application was sprayed on the leaves and added to the irrigation water, guaranteeing the same volume of prepared solution for all the plants.

RNA extraction and quantification

For the analysis of gene expression, the methodologies proposed by Han *et al*. (2021) and Verly *et al*. (2020) were considered; these suggest that the time required to show gene activation after the application of biostimulant substances and to proceed to take samples to extract RNA was between 48 and 72 h. Foliar samples were taken 48 h after the application of the treatments. RNA was extracted following the instructions of the Thermo Scientific™ GeneJET RNA Purification Kit. Subsequently, the RNA of each sample was quantified using the NanoDrop™ 2000 equipment.

Detection of the *CaWRKY6* gene expression

To determine the transcription of the *CaWRKY6* gene and its control, *CaActin*, the methodology proposed by Cai *et al*. (2015) was followed by performing RT-qPCR using the primers:

CaWRKY6 – Forward
5′GGTAGCTAGACAATTATGCTGC 3′

CaWRKY6 – Reverse
5′CAAAAAAAAATCTTATCAACTTG 3′

CaActin – Forward
5’AGGGATGGGTCAAAAGGATGC 3’

CaActin – Reverse
5’GAGACAACACCGCCTGAATAGC 3’

To detect *CaWRKY6* gene expression, the Verso 1-Step RT-PCR Kit ReddyMix from Thermo Fisher was used; the program suggested by Dang *et al*. (2013) was followed: 95°C for 30 s; 40 cycles of 95°C for 5 s; 60°C for 34 s; 95°C for 15 s; 60°C for 1 min; 95°C for 15 s; 60°C for 15 s; and 95°C for 15 s in an Eppendorf Mastercycler® ep realplex kit. Each reaction consisted of 1 μl of sample (100 ng μL^{-1}), 10 μl of enzyme, 1.6 μl of primers, and 7.4 μl of water, for a total volume of 20 μl.

For relative expression analysis, five replicates of each experiment were performed; the data were analyzed using the “Livak” $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001). This quantifies the normalized relative expression level of the *CaWRKY6* gene of interest, comparing it to the actin reference gene *CaActin* that was previously reported in bell pepper (Cai *et al*., 2015; Dang *et al*., 2013).

Induction of water stress, plant height, and proportion of wilted leaves

Three days after the application of the Bio 5, Bio 10, SA, and CLI treatments the plants were subjected to water depletion, suppressing irrigation for 21 d (Ahammed *et al*. 2020; Goñi *et al*. 2018). The effect of water stress was evaluated by monitoring height growth and the proportion of wilted leaves during a 21-d water depletion period, with data collected on days 0, 3, 5, 8, 11, 14, 16, 18, and 21.

We assessed plant height using a tape measure; and the length in centimeters was determined by recording the distance from the plant base to the apical meristem of each plant. To quantify leaf wilting, we calculated the ratio of wilted leaves to the total number of leaves per seedling. Criteria for identifying wilted leaves included curled leaf margins and downward curvature below the horizontal plane (epinasty). We determined the presence or absence of wilting when one or both characteristics were observed.

Statistical analysis

For the analysis of relative *CaWRKY6* gene expression, plant height, and wilting, normality parameters were evaluated. An analysis of variance (ANOVA) was performed to identify differences between treatments, and Tukey's multiple range test was performed (P<0.05). The studies were conducted with R statistical software, R Studio 3.6.2 version.

Results and discussion

Relative expression of the *CaWRKY6* gene

Forty-eight hours after the application of the substances there were no significant differences in relative expression between biofertilizer treatments (P=0.797). Although the SA treatment had gene induction, it was significantly lower than the Bio 5 treatment (P=0.0001). Additionally, the expression of the SA treatment was different from that of the controls. There were no significant differences between the controls (P = 0.9986) (Fig. 1).

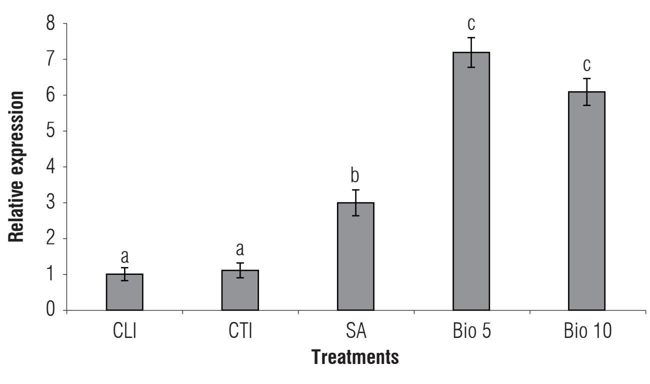

FIGURE 1. Relative expression of the *CaWRKY6* gene ("Livak" $2^{-\Delta\Delta CT}$) 48 h after application of the five treatments (Tab. 1). Different letters indicate significant differences between treatment means according to the Tukey's test ($P\leq$0.05); bars represent the standard error, n=6.

Plant height

From the beginning of the experiment until day 21, the increment in height of the biofertilizer treatments showed a nearly equal trend; there were no significant differences between them. The SA and CLI treatments had a similar growth pattern. However, from day 16 there was a slight increase in the growth of the plants of the SA treatment with no differences between them (Fig. 2).

On day 8 the treatments with biofertilizer had average heights of 15 cm with no significant differences between them (P=0.855). It is important to note that both treatments resulted in slightly greater heights than the CLI treatment; only the Bio 10 treatment showed significant differences with CLI (P=0.035). By day 11, the biofertilizer treatments and the CLI treatment showed an average height of 16 cm and showed no significant differences.

On day 14, the biofertilizer treatments showed an average height of 17 cm. This was lower than the 18.7 cm of the CTI treatment and was significantly different from the other treatments. From day 16 after water depletion, only the plants under the CTI treatment continued to grow. In contrast, those in the other treatments ceased their growth.

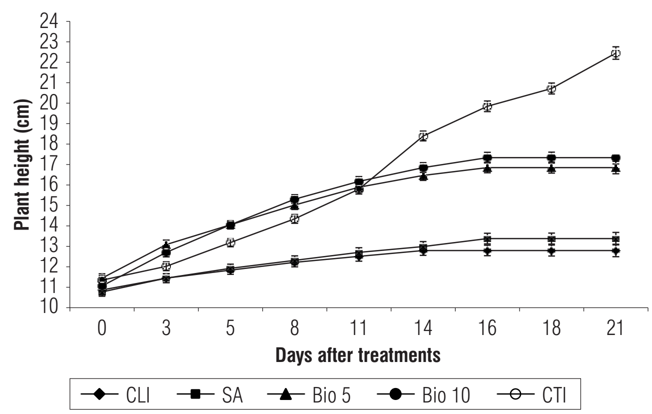

FIGURE 2. Average height (cm) of pepper plants for the five treatments (Tab. 1) during the 21 d after the onset of water depletion. Bars represent the standard error, n=6.

Proportion of wilted leaves

The five treatments showed no signs of wilt during the first 5 d. From day 5 to day 18, the percentage of biofertilizer treatments showed a similar trend and remained relatively constant during the 21 d there were no significant differences among them. A similar situation was observed between the SA and CLI treatments. It is important to note that the CTI treatment was constantly irrigated throughout the experiment; this may explain the absence of signs of wilting (Fig. 3).

On day 8 it is essential to note that there were no significant differences between the treatments with biofertilizer and CTI (P=0.99). However, the SA and CLI treatments showed differences (P=0.03); the SA treatment had a lower percentage of wilt of 15.2%. On day 11, the biofertilizer treatments showed values of 8%; in contrast, the SA and CLI treatments showed a wilting rate of approximately 55%.

By day 14, the treatments with biofertilizer showed an average wilt of 19%, while the SA and CLI treatments had values close to 100%. On day 18, all treatments showed values close to 100% wilt; at 21 d all plants appeared dry, except in the CTI control treatment with constant irrigation.

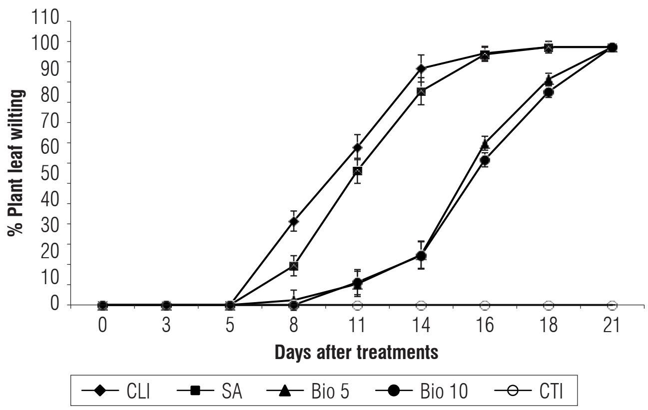

FIGURE 3. Average leaf wilting (± SE) (n = 6) recorded in pepper plants for the five treatments (Tab. 1) during the 21 d after the onset of water depletion.

Results indicated that the plants with a higher expression of *CaWRKY6* gene were those treated with biofertilizer, presenting greater height and fewer signs of wilting compared to the SA and CLI that had higher tolerances to water stress (Figs. 1 and 4). These results are consistent with the findings of other authors (Cai *et al*., 2015; Hussain *et al*., 2019; Zheng *et al*., 2019), who describe *CaWRKY6* as a gene involved in several responses of tolerance and resistance to both biotic and abiotic stresses in *C. annuum*. Hussain *et al*. (2019) propose that the *CaWRKY6* gene plays a significant role in signaling pathways mediated by jasmonates, ethylene, and abscisic acid, substances that are not only important in the defensive response of plants but also in the signaling network related to biotic and abiotic stresses. Therefore, the jasmonate, ethylene, and abscisic acid-mediated pathways constitute vital components of *CaWRKY6*-mediated induction of tolerance to heat stress, drought, and immune responses in peppers.

Additionally, Cai *et al*. (2015) found that, in *C. annuum*, the *CaWRKY6* gene partially induces the *CaWRKY40* gene by binding to its promoter and activating it, like how *CaWRKY40* participates in resistance to biotic stress conditions caused by pathogen attacks and tolerance to high temperatures. The preceding studies suggest that the

FIGURE 4. Pepper plants at day 14 after the onset of water depletion. Treatments from left to right: A) CLI, B) SA, C) Bio 5, D) Bio 10, and E) CTI (Tab. 1).

activation of *CaWRKY6* detected in our study is a possible mechanism that allowed biofertilizer-treated plants to tolerate water stress better than the SA and CLI treatments.

A possible correlation between the activation of *CaWRKY6* in *C. annuum* and enhanced height growth could be associated with the fact that the *CaWRKY6* gene is expressed in the root, stem, leaves, flower buds, and flowers, in addition to participating in the development of leaves and fruits (Zheng *et al*., 2019). Additionally, it supports the fact that the gene was expressed in the control treatments, as it is present in vegetative development.

About the application of the SA treatment, the relative expression of the *CaWRKY6* gene induced by salicylic acid had a value of 3.0 (Fig. 1). On day 8 of this experiment, comparing the proportion of wilted leaves of treatment SA 15.2% with CLI 32.5% (Fig. 3) a significant difference could be seen (P = 0.032). On days 11 and 14 although there was no significant difference between treatments SA and CLI (day 11: P = 0. 68, day 14: P = 0.77), the wilting percentage of the SA treatment (day 11: 52.1%, day 14: 82.9%) was slightly lower than CLI (day 11: 61.2%, day 14: 91.7%). This shows that salicylic acid activated *CaWRKY6*, and with it there was a decrease in wilting signs during the experiment. However, activation by Bio 5 and Bio 10 treatments had a higher relative expression (Bio 5: 7.2, Bio 10: 6.1) and a higher protective effect against withering.

Salicylic acid has been considered as an inducer of response to biotic and abiotic stresses through the modulation of metabolic processes, acting as an elicitor of the systemic acquired resistance in plants (Estaji & Niknam, 2020; Thakur *et al*., 2019). In *C. annuum*, salicylic acid is shown to activate genes of the *CaWRKY* complex, which are related to protection against biotic and abiotic stresses. Jingyuan *et al*. (2011) report that two cultivars of *C. annuum* treated with salicylic acid had a rapid induction of transcripts and activation of the *CaWRKY30* gene, reflected in plant protection against pathogens such as *Meloidogyne incognita*, *Phytophthora capsici*, tobacco mosaic virus, *Ralstonia solanacearum*, and low-temperature stress. In addition, Dang *et al*. (2013) report that *CaWRKY40* transcripts are induced by signaling mechanisms mediated by salicylic acid, jasmonic acid, and ethylene, which have protective effects against heat stress, high relative humidity, and attack by *R. solanacearum* bacteria.

The results of the relative *CaWRKY6* expression show that, although the Bio 10 treatment had a higher concentration of biofertilizer, the Bio 5 treatment showed a higher expression of *CaWRKY6* (Fig. 1). This may be due to a possible saturation point in the plant by the mineral nutrients contained in the biofertilizer. Research by Campos-Soriano *et al*. (2020) and Melnikova *et al*. (2015) show that variation in nutrient concentration can affect the expression of genes related to protein and enzyme metabolism in plants.

The activation of the *CaWRKY6* gene, after the application of the biofertilizer, and its potential protective activity when activated before exposing the plants to 100% water stress, could be supported by the behavior of the plants that were treated with the biofertilizer and exposed to 11 d of drought, as they had similar growth to the plants with constant irrigation but were not treated with the biofertilizer. The description of the increase in height of the different treatments was noted on days 8, 11, and 14, considering that during this range of time the treatments had the following behavior: on day 8, Bio 5, and Bio 10 with values of 15.2 cm and 15.5 cm surpassed the height of 14.5 cm of the CTI treatment. Later on day 11, Bio 5 and Bio 10 had values of 16.1 cm and 16.4 cm, which were close to the 16 cm of the CTI treatment; and on day 14, the heights of the Bio 5 and Bio 10 treatments with values of 16.7 cm and 17.1 cm were surpassed by the 18.7 cm of the CTI treatment, which continued to increase until the end of the experiment (Fig. 2).

Day 5 was chosen to start describing the proportion of wilted leaves, because after this day, the five treatments began to show signs of wilting. By day 8, the plants treated with the biofertilizer showed tolerance to dehydration, such that Bio 10 had a total absence (0%) of wilted leaves and Bio 5 had a slight presence of wilting of 1.85% (Fig. 3). One of the possible factors that may be involved here, is the presence of indole-acetic acid detected in the composition of the biofertilizer; this growth regulator is reported in *C. annuum* plants as a promoter of water accumulation in leaf tissues under water stress conditions (Pérez-Jiménez *et al*., 2016).

In the plants of the CLI treatment, the growth of 12.9 cm up to day 14 and the absence of 0% wilting in the first 5 d after water depletion can be explained by the presence of water remaining in the substrate where the plants were planted, which helped to keep the roots hydrated, promoting their growth and resistance to dehydration. However, there was a marked difference in the treatments with biofertilizer, which supports its protective action against dehydration.

Conclusions

This study demonstrated that foliar application of biofertilizer, at 5 ml L^{-1} and 10 ml L^{-1}, significantly enhanced the expression of the *CaWRKY6* gene in *C. annuum* plants, conferring improved drought tolerance under greenhouse conditions. While salicylic acid, at 5 ml L^{-1}, also induced *CaWRKY6* activation, its protective effect against water stress was lower than that of the biofertilizer. These findings highlight the potential of biofertilizers as effective substances to mitigate water stress in bell pepper crops, offering a sustainable alternative to conventional agrochemical practices.

These results contribute to understanding the role of *CaWRKY* transcription factors in plant water stress responses and increase the agronomic value of biofertilizers to promote tolerance to climate-induced abiotic stress, such as drought in bell pepper crops.

Further research is needed to clarify the molecular mechanisms involved in the action of biofertilizers, as well as their potential synergies with other biostimulants. In addition, field trials are essential to validate their efficacy under diverse agroecological conditions and varying levels of abiotic stress. Moreover, incorporating studies focused on stress responses associated with pathogen pressure would contribute to a more comprehensive understanding of the range of conditions under which biofertilizers are effective. Taken together, these considerations could open new opportunities for the effective integration of biofertilizers into agricultural systems, particularly in regions vulnerable to drought and climate change.

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Conflict of interest statement

The authors declare that there is no conflict of interests regarding the publication of this article.

Author's contributions

SAB and DMMR designed and developed the methodology. SAB performed field and laboratory experiments and data collection; AVR oversaw and contributed to the laboratory experiments. SAB conducted the data analysis. SAB and DMMR contributed to the result description. SAB wrote the draft. All authors reviewed the final version of the manuscript.

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