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Research Article

Using biocontrol agents and sodium nitrophenolate to control powdery mildew and improve the growth and productivity of marigold (*Calendula officinalis* L.)

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Abstract

In vitro and *in vivo* studies were conducted to investigate the potential of four biocontrol agents (BCAs), namely *Bacillus megaterium*, *Pseudomonas fluorescens*, *Trichoderma viride*, and *T. harzianum*, individually and in combination with sodium nitrophenolate (SN) to control marigold powdery mildew. The results showed that all treatments led to a significant inhibition in the conidial germination of *Golovinomyces cichoracearum in vitro*. Maximum inhibition was recorded by *T. harzianum* (1×10^9 CFU mL^{-1}) + SN (1.5%), followed by *T. viride* + SN, and *B. megaterium* + SN at the same concentrations as follows: 83.6, 79.1, and 70.6%, respectively. While the lowest inhibition (20.4%) was recorded by *P. fluorescens* (1×10^5 CFU mL^{-1}). In the greenhouse, all treatments applied significantly reduced the disease severity and the area under the disease progress curve (AUDPC). The combination treatments had a better disease control response than individual treatments. Similar results were achieved in the field, where disease severity reduced to 9.2 and 10.3% in plants treated with *T. harzianum* + SN in the first and second seasons, respectively, compared to 40.2 and 44.1% in control in both seasons. Likewise, AUDPC reduced to 274 and 315 in plants treated with *T. harzianum* + SN in the first and second seasons, respectively, compared to 1207 and 1340 in control in both seasons. The treatments improved growth and productivity characteristics, as well as photosynthetic pigments, total phenolic compounds (TPC), and polyphenol oxidase (PPO) activity, while significantly reducing free proline (FP). In

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conclusion, BCAs applied individually or in combination with SN can be used effectively to suppress powdery mildew of marigold.

Keywords: biocontrol agents; *Calendula officinalis*; morphological/ flowering/ biochemical parameters; powdery mildew; sodium nitrophenolate

Introduction

Marigold (*Calendula officinalis* L.) is one of the medicinal plants that is grown in Egypt, and mainly cultivated for its yellow and orange flowers, which are rich in carotenoids, flavonoids, essential oils, and vitamin A (Escher *et al*., 2019). It is an annual herb belonging to the Asteraceae family, and is native to southern and eastern Europe and the Mediterranean countries (Gazim *et al*., 2008). Marigold is used as raw material in the medical, cosmetic, and food industries, as well as in the landscape (Verma *et al*., 2018). In Egypt, the area planted with marigold reached 711 hectares, producing 26,372 tons of flowers (Anonymous, 2019).

Powdery mildew is one of the most widespread foliar diseases of marigold, which can be very destructive in the greenhouse and field (Mir *et al*., 2012; Abdel-Wahed and Shaker, 2020). This disease is caused by *G. cichoracearum* (DC.) V.P. Heluta (Syn.: *Erysiphe cichoracearum* DC ex Merat.) (Severoglu and Ozyigit, 2012). Small white or gray spots scattered on stems, leaves, and inflorescences are typical symptoms of the disease, which expand under favorable conditions to cover the plant with a white, talcum powder-like mass (Sujatha *et al*., 2018). Affected leaves fall, causing defoliation, decreased photosynthetic capacity, and decreased flower productivity (Garibaldi *et al*., 2008; Kavak, 2011). Powdery mildew control has long relied on the use of two basic practices: resistant varieties and chemical control (Kiss, 2003). Although powdery mildew-resistant commercial varieties are available for many crops, the rapid evolution of pathogen strains hampers this practice (Pérez-García *et al*., 2009). Chemical control is usually achieved through intensive applications of fungicides, the emergence of fungicide-resistant isolates with their harmful residual effects has reduced the efficacy of this practice (Sellitto *et al*., 2021). So, the search for alternative strategies has become indispensable. In this consider, biological control is a promising approach (Ownley *et al*., 2010). It provides a plant protection that is eco-friendly, ecologically viable, and has great potential to promote a sustainable agricultural system.

Biological control of plant diseases is defined as the suppression of pathogen populations to an acceptable level by the organisms (Heimpel and Mills, 2017). In nature, plants and microorganisms live in interactions with each other, which can influence the growth and development of plants and even their defensive responses to different stresses (Nataraja *et al*., 2019). Several reports have documented a variety of beneficial microorganisms that possess antimicrobial activities against plant pathogens (Mmbaga *et al*., 2016; Lahlali *et al*., 2022). These microorganisms, known as biocontrol agents (BCAs), include a number of bacteria such as *Streptomyces* spp., *Pseudomonas* spp., and *Bacillus* spp., and a number of fungi such as *Trichoderma* spp., *Coniothyrium* spp., and arbuscular mycorrhizal fungi (AMFs) (Cordier *et al*., 1998; Xu *et al*., 2019). BCAs play an important role in protecting crops from disease damage through different modes of action. They may induce resistance or prime enhanced resistance in infected tissues without direct interaction with pathogens (Conrath *et al*., 2015). Another indirect interaction with pathogens is competition for nutrients or space (Spadaro and Droby, 2016). BCAs may interact directly with the pathogen via hyperparasitism or antagonism, whereby the parasite invades and kills the mycelium, spores, and resting structures of the fungi, as well as bacterial cells (Ghorbanpour *et al*., 2018). The production of antimicrobial secondary metabolites with inhibitory effects on pathogens is another direct mode of action (Raaijmakers and Mazzola, 2012).

Trichoderma species exhibit a variety of antagonistic mechanisms against plant pathogens, including secretion of lytic enzymes and antifungal secondary metabolites, mycoparasitism, and competition for

nutrients (Stracquadanio *et al*., 2020). They can also penetrate and surround the hyphae of pathogens, thus killing them (Tomah *et al*., 2020). Moreover, they promote root development and plant growth by dissolving phosphate and micronutrients, making them accessible to the plant (Lorito *et al*., 2010). Recently, *Trichoderma* spp. have been successfully used to suppress powdery mildew on celery (Ahmed *et al*., 2021), vineyards (Sawant *et al*., 2017), and sunflower (Esawy *et al*., 2021). Bacteria belonging to the genus *Bacillus* are effective biocontrol agents against phytopathogens (Mahmoud *et al*., 2021). They produce a wide range of antibiotics and lipopeptides, which have shown antimicrobial activities and significant inhibitory capacities (Kim *et al*., 2010). Several studies have demonstrated the ability of *B. subtilis* and *B. megaterium* to reduce powdery mildew on many crops (Hashem *et al*., 2019). Another example of beneficial bacteria is *P. fluorescens*, which has been used in agriculture as biofertilizer, biocontrol agent, and plant growth-promoter (Kiely *et al*., 2006). Strains of these bacteria have the ability to secrete cell wall-degrading enzymes, hydrogen cyanide, and antibiotics to suppress fungal growth, in addition to stimulating plant defense mechanisms (Zian and Aly, 2020).

Crops are exposed to various biotic and abiotic stresses that have negative effects on their growth, productivity and quality (Gull *et al*., 2019). The application of biostimulants has received great attention due to their efficiency in improving plant morphological, physiological and biochemical processes against these stresses (Posmyk and Szafranska, 2016). They play an important role in activating metabolic processes such as photosynthesis, respiration, DNA synthesis, and ion uptake, thus increasing plant tolerance and improving its growth and development (Rafiee *et al*., 2016). Among the plant stimulants that have been successfully used to improve growth and increase productivity of many crops is sodium nitrophenolate (Michalski *et al*., 2008). This compound consists of 3 phenolic compounds: sodium para-nitrophenolate, sodium ortho-nitrophenolate, and sodium 5-nitroguaiacolate. These active ingredients are found naturally in plants and stimulate growth by increasing antioxidant enzyme activities, auxin amount, nutrient uptake, and photosynthetic intensity (Borowski and Michałek, 2009; Przybysz *et al*., 2010). Although sodium nitrophenolate is registered in the European Union as a pesticide for some crops, knowledge about its potential in plant disease management is still limited. This study investigates the effect of four BCAs individually and in combination with SN in controlling marigold powdery mildew and improving growth and productivity. The hypothesis discusses increasing BCAs efficacy to suppress this disease by combining them with a plant biostimulant.

Materials and Methods

Plant and soil materials, treatments, and experimental conditions

The present study was conducted in the laboratory, greenhouse, and experimental farm at the Faculty of Agriculture, Beni-Suef University, Egypt, during the 2021/2022 and 2022/2023 seasons. The trial site is found at latitudes 29° and 3°N and longitudes 31° and 5°E, and at an altitude of 32.4 m above sea level. From October to March in the two study seasons, precipitation averaged 2.5 mm, temperature 20.8 °C, relative humidity 55.2%, and evaporation 145.1 mm. Physicochemical analysis of the experimental soil was performed according to the methods of Page *et al*. (1982) as shown in Table 1. Analysis was done at the Central Fertilizer Analysis Laboratory, Soil, Water, and Environment Research Institute, ARC, Egypt. The purpose of this study was to investigate the efficacy of four biocontrol agents (BCAs), i.e. *B. megaterium*, *P. fluorescens*, *T. viride*, and *T. harzianum*, individually and in combination with SN, in comparison with the fungicide propiconazole 25%, to control powdery mildew of marigold. In combination treatments, BCAs and SN were prepared separately and then sprayed on plants with an interval of 1 to 2 hours between them. Seeds of a local marigold (cv. 'Orange Flower') used in all the experiments were obtained from Sids Agricultural Research Station, ARC, Egypt. The seeds were sown in the nursery on September 1st. Uniform seedlings, 35 days old and 15 cm height, were

transplanted under greenhouse and field conditions. All agricultural approvals related to marigold cultivation have been implemented according to what was stated by the Egyptian Ministry of Agriculture and Land Reclamation.

Table 1. Physicochemical analysis of the experimental soil before sowing marigold during the 2021/2022 and 2022/2023 seasons (average of the two seasons)

Silt %			Coarse sand %			Clay %		Fine sand %			Soil texture		
28.64			4.39			56.35		10.62			Clay		
pH	EC (dS/m)	O. M%	Soluble cations (meq/L)				Soluble anions (meq/L)				NPK (mg kg^{-1} soil)		
			Na^+	K^+	Mg^{++}	Ca^{++}	$CO3^{--}$	$HCO3^{--}$	Cl^{--}	$So4^{--}$	N	P	K
7.90	1.51	1.17	6.87	0.39	2.43	5.25	5.40	2.14	7.58	4.51	38.52	7.69	194

Plant sampling, inoculum preparation, and inoculation

Marigold samples showing powdery mildew symptoms were collected from naturally infected fields. Pathogenicity was performed by shaking infected parts on 60-day-old healthy plants (Yarwood, 1936), which were then kept under polyethylene bags for 24 h at 25 ± 2 °C and sufficient humidity necessary for successful infection (Koike and Saenz, 1997). Plants were checked daily for disease development. To fulfil Koch's postulations, the symptoms and re-isolated fungus must be morphologically identical to those originally observed. To prepare fungal inoculum, profusely growing conidia were carefully collected using a sterile brush and suspended in 100 mL of sterile distilled water mixed with 2 drops of Tween-20. The suspension was centrifuged at 3000 rpm for 5 min, then adjusted to 5×10^5 conidia mL^{-1} (Kitao and Doazan, 1989). Inoculation of 60-day-old healthy plants was carried out by spraying them with the prepared suspension, then the plants were kept under polyethylene bags as mentioned previously. Disease was assessed by estimating disease severity.

Inoculum preparation of biocontrol agents (BCAs)

BCAs isolates were provided by the National Research Center (NRC), Cairo, Egypt. Fungal isolates were cultured on PDA for a week, then the cultures were flooded in 20 mL of sterile distilled water + 0.02% Tween-80. The surface of the colony was gently scraped with a spatula to obtain the spores. The suspension was shaken, filtered, and adjusted to 1×10^5, 1×10^7, and 1×10^9 CFU mL^{-1}. Bacterial isolates were grown in liquid nutrient broth in 250 mL flasks and shaken at 150 rpm for 3-4 days, and then bacterial cells were suspended in tap water and adjusted to 1×10^5, 1×10^7, and 1×10^9 CFU mL^{-1}.

Experimental design and layout

In vitro study

An *in vitro* assay was performed to evaluate the antifungal effect of BCAs individually and in combination with SN on conidial germination of *G. cichoracearum*. Conidia were carefully collected from sporulation lesions on leaves using a sterile brush and placed on sterile glass slides (Nair *et al*., 1999). Three concentrations of each BCAs (1×10^5, 1×10^7, and 1×10^9 CFU mL^{-1}), sodium nitrophenolate (0.5, 1.0, and 1.5%), and fungicide (0.15, 0.20, 0.25 mLL^{-1}) were made. In combination treatments, BCAs and SN were prepared separately and then mixed in a 1:1 (*v/v*) ratio. Two drops of each concentration were applied to the conidia, and the slides were then placed on U-shaped glass rods in sterile Petri dishes (9 cm diameter) containing sterile cotton moistened with water to provide the necessary moisture for germination. Conidia treated with sterile distilled water only were used as a control. Three replicates were used/treatment and three Petri dishes/replicate. Plates were incubated at 25 ± 1 °C for 48 h. Germinated conidia were counted using a hemocytometer, then Germination of conidia (GC) and inhibition were calculated according to the following equations:

$$GC\ \% = [(No.of\ germinated\ conidia/\ Total\ no.of\ conidia) \times 100]$$

$$Inhibition\ \% = [(GC\ in\ control - GC\ in\ treatment/\ GC\ in\ control) \times 100]$$

Greenhouse experiments

During the 2021/2022 season, greenhouse trials were designed to evaluate the efficacy of four BCAs individually and in combination with sodium nitrophenolate (SN) to control marigold powdery mildew. Trials were conducted in a randomized complete block design (RCBD), with 11 treatments, 3 replicates/treatment, and 3 pots/replicate. Marigold seedlings, 45 days old and 15 cm high, were transplanted into 30 cm diameter pots, filled with sterilized sandy loam soil (1:3 w/w) at a rate of one seedling/pot. After 15 days of transplanting, inoculation with a suspension of *G. cichoracearum* spores (5×10^5 CFU mL^{-1}) was performed as mentioned before. One week after inoculation, the plants were sprayed three times at an interval of 15 days with the following treatments: (T1): control (untreated plants); (T2): propiconazole fungicide (0.25 mLL^{-1}); (T3): SN (1.5%); (T4): *B. megaterium* (1×10^9 CFU mL^{-1}); (T5): *T. viride* (1×10^9 CFU mL^{-1}); (T6): *P. fluorescens* (1×10^9 CFU mL^{-1}); (T7): *T. harzianum* (1×10^9 CFU mL^{-1}); (T8): *B. megaterium* (1×10^9 CFU mL^{-1}) + SN (1.5%); (T9): *T. viride* (1×10^9 CFU mL^{-1}) + SN (1.5%); (T10): *P. fluorescens* (1×10^9 CFU mL^{-1}) + SN (1.5%); and (T11): *T. harzianum* (1×10^9 CFU mL^{-1}) + SN (1.5%). Powdery mildew severity was assessed 10, 20, 30, and 40 days after the last application, and then the area under disease progress curve was calculated.

Field experiments

Two successive experiments were carried out in the field during the 2021/2022 and 2022/2023 seasons. The experiments were arranged in RCBD, with three repetitions. Each plot consists of 5 rows, each 3.5 m long and 0.7 m wide. Marigold, 45 days old and 15 cm high, was transplanted at a distance of 25 cm from each other. The plants were left for natural infection with the disease, and as soon as the first symptoms of powdery mildew appeared on the plants, they were sprayed with the previous treatments three times at an interval of 15 days. Powdery mildew severity and AUDPC were calculated as in the greenhouse experiments.

Experiment evaluations

Disease assessment

The severity of powdery mildew on marigold was measured using the scale described by Prakash and Saharan (1999) with minor modifications. The scale ranged from 0 to 5 ratings based on the area of plant covered by powdery mildew (%) as follows: 0 = disease free, 1 = 1 – 10%, 2 = 11 – 25%, 3 = 26 – 50%, 4 = 51 – 75%, and 5 = more than 75%. Disease severity was calculated by the following formula given by Liu et al. (2001);

$$Disease\ severity\ (DS)\% = \frac{\sum n \times v}{5N} \times 100$$

Where n = number of plants in each numerical rating, v = numerical rating, and N = total number of plants examined.

Based on the recorded data of disease severity values, the disease reduction (DR) rate was calculated as in the following equation:

$$DR\ \% = [(DS\ in\ control - DS\ in\ treatment/\ DS\ in\ control) \times 100]$$

The area under disease progress curve (AUDPC) was calculated using the following equation proposed by Pandey et al. (1989):

$$AUDPC = D\ [1/2\ (Y1 + Yk) + Y2 + Y3 + \cdots Yk - 1]$$

Where D = intervals between successive records, Y1 = first disease score, YK = last disease score, and Y2, Y3, YK-1 = intermediate disease scores.

Morphological and flowering parameters

At the end of the growing season (late April), some morphological parameters were recorded as follows: plant height (cm), number of main branches/plant, number of leaves/plant, and leaf area (cm^2). During the flowering stage (from the beginning of December to the end of April), some flowering parameters were measured, such as the number of inflorescences/plant, the diameter of the inflorescences (cm), the number of ray flowers/inflorescence, and the fresh and dry weight of 100 inflorescences (g).

Photosynthetic leaf pigments

Chlorophyll and carotenoids in fresh leaves were evaluated using the method described by Lichtenthaler (1987). 0.2 g of sample was homogenized in 10 mL of 80% acetone. The mixture was filtered, then supplemented to 15 mL by adding acetone. The absorbance of the supernatant was measured at 663.2, 646.8, and 470 nm. Pigments were evaluated using the next equations:

Chlorophyll-a (mg/mL) = (12.5 A663.2) – (2.79 A653)

Chlorophyll-b (mg/mL) = (21.51 A646.8) – (5.1 A663.2)

Carotenoids (mg/mL) = (1000 A470) – (1.8 Chl. a) – (85.02 Chl. b)

Photosynthetic Pigment (mg/g FW) = (C – V/1000 – W)

Where C = conc. of pigment, V = volume of acetone (mL), and W = sample weight (g)

Biochemical parameters

Total phenolic compounds (TPC)

Quantification of total phenolic compounds was done colorimetrically using the Folin-Ciocalteu's reagent, following the method described by Georgé *et al*. (2005). Samples (stems and leaves) were collected, dried at 65 °C for 48 h, and stored at 25 °C in the dark before extraction. 1.0 g of dried material was extracted in 10 mL of 70% ethanol. The extract was shaken at 120 rpm for 2 h on 30 °C and then centrifuged at 1013 × g for 10 min. About 0.2 mL of the extract was mixed with 1.0 mL of Folin-Ciocalteu's reagent 1:10 and 0.8 mL of 7.5% $NaCO_3$. After 30 min of rest in the dark, the absorbance was read at 760 nm, using gallic acid as a standard. Results were expressed as mg of gallic acid equivalent per 100 g of dry weight.

Polyphenol oxidase (PPO) activity

PPO activity in fresh leaves was assessed using the method of Mayer et al. (1965). The sample (1.0 g) was homogenized in 2 mL of 0.1 M sodium phosphate buffer (pH 6.5), and the mixture was then centrifuged at 16,000× g for 15 min at 4 °C. The reaction mixture involved 0.2 mL of supernatant and 1.5 mL of 0.1 M sodium phosphate buffer (pH 6.5). To begin the reaction, 0.2 mL of 0.1 M catechol was added to the mixture. The change in absorbance was recorded at 30 s intervals for up to 3 min at 495 nm. Results were reported as PPO mg $protein^{-1}$ min^{-1}.

Free proline (FP) content

Free proline was quantified by the method of Bates et al. (1973). A known amount of dry matter (about 100 mg) was homogenized in 3% (w/v) 5-sulfosalicylic acid. The extract was reacted with glacial acetic acid and acidic ninhydrin reagent at 100 °C for 1h. Toluene was used to extract the reaction mixture, and the absorbance was read at 520 nm. Results were reported as FP μ mole g^{-1} of dry weight.

Statistical analyses

Data obtained were statistically analysed through ANOVA, by Web Agriculture Stat Package software (WASP 2.0, Central Coastal Agricultural Research Institute, Goa, India). The values given are the means of all recorded measurements. A combined analysis of data noticed over the two study seasons and Duncan's range test were applied to compare significant differences between treatments at $p \leq 0.05$ (Gomez and Gomez, 1984).

To test the relationships between all measured variable parameters and treatments, a hierarchical cluster analysis was performed using Statgraphics XVII software version 17.20.

Results

Activity of biocontrol agents individually and in combination with sodium nitrophenolate against conidial germination of G. cichoracearum in vitro

Data presented in Table 2 reveal that all combination or individual treatments significantly inhibited the germination of *G. cichoracearum* conidia compared to control. The highest inhibition was recorded by *T. harzianum* + SN at 1000 ppm, followed by *T. viride* + SN, *B. megaterium* + SN, *P. fluorescens* + SN, and *T. harzianum* at the same concentration. The corresponding inhibition values were 83.6, 79.1, 70.6, 64.5, and 61.6%, respectively. While the lowest inhibition was recorded by *P. fluorescens* at 250 ppm (20.4%). Complete inhibition of conidia was achieved by propiconazole fungicide at all tested concentrations.

Table 2. Effect of BCAs individually and in combination with SN on conidial germination of *G. cichoracearum*, after 48 h of incubation at 25 ± 1 °C

Treatments	Conidial germination %			* Inhibition %		
	Concentration applied (ppm)			Concentration applied (ppm)		
	A	B	C	A	B	C
Control	24.5 ± 0.7 [a]	24.5 ± 0.7 [a]	24.5 ± 0.7 [a]	–	–	–
Propiconazole	0.0 ± 0.0 [h]	0.0 ± 0.0 [i]	0.0 ± 0.0 [k]	100	100	100
SN	15.9 ± 1.8 [c]	12.3 ± 0.4 [e]	10.7 ± 0.3 [e]	35.1	49.7	56.3
B. megaterium	17.1 ± 1.2 [c]	14.3 ± 0.3 [c]	13.5 ± 0.3 [c]	30.2	41.6	44.8
T. viride	16.2 ± 1.1 [c]	13.5 ± 0.3 [d]	11.4 ± 0.5 [d]	33.8	44.8	53.5
P. fluorescens	19.5 ± 0.7 [b]	17.4 ± 0.7 [b]	15.3 ± 0.2 [b]	20.4	28.9	37.5
T. harzianum	14.0 ± 0.6 [d]	11.2 ± 0.4 [f]	9.4 ± 0.2 [f]	42.8	54.2	61.6
B. megaterium + SN	11.5 ± 0.6 [e]	9.1 ± 0.3 [g]	7.2 ± 0.5 [h]	53.1	62.8	70.6
T. viride + SN	10.1 ± 0.3 [f]	7.5 ± 0.2 [h]	5.1 ± 0.3 [i]	58.7	69.3	79.1
P. fluorescens + SN	13.0 ± 0.3 [d]	11.2 ± 0.4 [f]	8.7 ± 0.3 [g]	46.9	54.2	64.5
T. harzianum + SN	7.9 ± 0.3 [g]	7.1 ± 0.2 [h]	4.0 ± 0.3 [j]	67.7	71.0	83.6

Data represent the mean of three replicates ± standard deviation (SD). Means with different letters represent a significant difference at the statistical level $p \leq 0.05$ between the treatments according to Duncan's multiple range test. A = 1×10^5 CFU mL^{-1}, B = 1×10^7 CFU mL^{-1}, C = 1×10^9 CFU mL^{-1}, and SN = sodium nitrophenolate. * Inhibition values were calculated based on control value.

Potential of biocontrol agents individually and in combination with sodium nitrophenolate in reducing powdery mildew severity and AUDPC in the greenhouse

Data presented in Table 3 show that all treatments applied in the greenhouse significantly reduced the severity of powdery mildew 10, 20, 30, and 40 days after the last application, and also significantly reduced the area under disease progress curve (AUDPC) compared to the control. Moreover, combination treatments had a better disease control response than individual treatments. The highest reduction in disease was recorded by *T. harzianum* + SN, followed by propiconazole, *T. viride* + SN, *B. megaterium* + SN, and *P. fluorescens* + SN as follows: 83.7, 82.7, 75, 71.7, and 70.2%, respectively. In addition, intermediate values of 61.8, 59.4, 57.1, and 53.1% were recorded by *T. harzianum*, SN, *T. viride*, and *B. megaterium*, respectively. While *P. fluorescens* ranked lowest (45.9%).

Table 3. Effect of BCAs individually and in combination with SN on the severity of marigold powdery mildew and AUDPC during the 2021/2022 season in the greenhouse

Treatments	Disease severity %				AUDPC	* Disease Reduction %
	Period after last application (Days)					
	10 Days	20 Days	30 Days	40 Days		
Control	12.3 ± 0.7 [a]	34 ± 3.6 [a]	48.2 ± 3.4 [a]	62.1 ± 1.5 [a]	1194 ± 37.3 [a]	–
Propiconazole	3 ± 0.4 [g]	5.7 ± 0.3 [f]	8.1 ± 0.4 [g]	10.3 ± 0.4 [i]	204.5 ± 22.3 [h]	82.7
SN	9.3 ± 0.8 [c]	14 ± 0.6 [cd]	18.3 ± 0.6 [d]	22 ± 1.1 [de]	479 ± 26.5 [de]	59.4
B. megaterium	10.3 ± 0.3 [b]	15.7 ± 0.7 [c]	21.1 ± 0.8 [c]	26.3 ± 1.5 [c]	551 ± 16.6 [c]	53.1
T. viride	9.7 ± 0.3 [bc]	15 ± 1.0 [cd]	19.3 ± 0.6 [cd]	23.1 ± 1.2 [d]	507 ± 9.4 [d]	57.1
P. fluorescens	10 ± 0.3 [b]	18 ± 0.5 [b]	25 ± 0.9 [b]	31.7 ± 1.7 [b]	638.5 ± 7.0 [b]	45.9
T. harzianum	8.3 ± 0.3 [d]	13.1 ± 0.4 [d]	17.3 ± 0.5 [d]	21 ± 1.0 [e]	450.5 ± 12.7 [e]	61.8
B. megaterium + SN	7 ± 0.3 [e]	10.3 ± 0.3 [e]	11.7 ± 0.5 [ef]	15.3 ± 0.8 [g]	331.5 ± 12.0 [f]	71.7
T. viride + SN	5.3 ± 0.3 [f]	9.7 ± 0.3 [e]	11 ± 0.4 [f]	13.1 ± 0.5 [h]	299 ± 11.5 [g]	75.0
P. fluorescens + SN	7.3 ± 0.2 [e]	9.1 ± 0.3 [e]	13.3 ± 0.6 [e]	17 ± 0.6 [f]	345.5 ± 5.4 [f]	70.2
T. harzianum + SN	3 ± 0.4 [g]	5.2 ± 0.3 [f]	7.3 ± 0.2 [g]	10 ± 0.4 [i]	190 ± 9.1 [h]	83.7

Data represent the mean of three replicates ± standard deviation (SD). Means with different letters represent a significant difference at the statistical level $p \leq 0.05$ between the treatments according to Duncan's multiple range test. SN = sodium nitrophenolate and AUDPC = the area under disease progress curve. * Disease reduction values were calculated based on control value.

Potential of biocontrol agents individually and in combination with sodium nitrophenolate in reducing powdery mildew severity and AUDPC in the field

Data provided in Table 4 demonstrate that after applying the treatments in the field, the severity of powdery mildew decreased significantly from 40.2% in untreated plants to 9.2% in plants treated with *T. harzianum* + SN, in the first season, and from 44.1% in untreated plants to 10.3% in plants treated with *T. harzianum* + SN, in the second season. Likewise, AUDPC decreased from 1207 in untreated plants to 274 in plants treated with *T. harzianum* + SN, in the first season, and from 1340 in untreated plants to 315 in plants treated with *T. harzianum* + SN, in the second season. In general, the highest reduction in disease was recorded by *T. harzianum* + SN, followed by propiconazole, *T. viride* + SN, and *B. megaterium* + SN. The corresponding reduction values were 79.3, 74.6, 73.8, and 71.4%, respectively, in the first season, and 78.9, 74.3, 72.5, and 69.1%, respectively, in the second season. While *P. fluorescens* ranked lowest, recording 44.3 and 43.1% in the first and second seasons, respectively.

Table 4. Effect of BCAs individually and in combination with SN on the severity of marigold powdery mildew and AUDPC during the 2021/2022 and 2022/2023 seasons

Treatments	1st Season (2021/2022)			2nd Season (2022/2023)		
	Disease Severity %	AUDPC	Disease Reduction %	Disease Severity %	AUDPC	Disease Reduction %
Control	40.2 ± 1.0 [a]	1207 ± 11.2 [a]	–	44.1 ± 1.8 [a]	1340 ± 26.8 [a]	–
Propiconazole	9.3 ± 0.4 [h]	276 ± 6.5 [i]	74.6	10.7 ± 0.4 [hi]	318 ± 6.4 [j]	74.3
SN	17.2 ± 0.7 [d]	519.5 ± 3.5 [d]	57.2	19.2 ± 0.7 [d]	579.5 ± 9.9 [e]	56.4
B. megaterium	19.5 ± 1.2 [c]	575 ± 7.8 [c]	51.5	22.2 ± 2.0 [c]	656 ± 7.4 [c]	49.6
T. viride	17.1 ± 0.4 [d]	510.5 ± 5.3 [d]	57.4	20.3 ± 1.9 [d]	603 ± 11.9 [d]	54.0
P. fluorescens	22.4 ± 1.4 [b]	665 ± 7.2 [b]	44.3	25.1 ± 1.0 [b]	749 ± 6.9 [b]	43.1
T. harzianum	15.1 ± 0.9 [e]	455 ± 9.7 [e]	62.4	17.1 ± 0.5 [e]	513.5 ± 7.1 [f]	61.2
B. megaterium + SN	11.5 ± 0.4 [g]	342 ± 9.6 [g]	71.4	13.6 ± 0.8 [g]	404 ± 8.9 [h]	69.1
T. viride + SN	10.5 ± 0.3 [g]	317 ± 4.8 [h]	73.8	12.1 ± 0.4 [h]	367 ± 9.9 [i]	72.5
P. fluorescens + SN	13.4 ± 0.7 [f]	393 ± 8.9 [f]	66.7	15.4 ± 0.7 [f]	453 ± 11.8 [g]	65.0
T. harzianum + SN	9.2 ± 0.3 [h]	274 ± 8.8 [i]	79.3	10.3 ± 0.6 [i]	315 ± 9.7 [j]	78.9

Data represent the mean of three replicates ± standard deviation (SD). Means with different letters represent a significant difference at the statistical level $p \leq 0.05$ between the treatments according to Duncan's multiple range test. SN = sodium nitrophenolate and AUDPC = the area under disease progress curve. * Disease reduction values were calculated based on control value.

Marigold morphological parameters

Data in Figure 1 indicate that all treatments significantly improved the morphological traits of marigold. Combination treatments had a better effect than individual treatments. In the first season, all plants treated with *T. harzianum* + SN and SN recorded the highest values of plant height (90.3 cm in each), followed by those treated with propiconazole (87.3 cm), *B. megaterium* + SN (83.7 cm), *P. fluorescens* + SN (82.3 cm), and *T. viride* + SN (81 cm). While plants treated with *B. megaterium* recorded the lowest value (69.7 cm) (Figure 1A). In addition, the highest number of main branches/plant was recorded by *T. harzianum* + SN, followed by propiconazole, SN, and *T. viride* + SN as follows: 13.7, 13.3, 10.7, and 10.3, respectively, in contrast to *P. fluorescens* had the lowest value (6.7) (Figure 1B). Also, *T. harzianum* + SN, propiconazole, and *T. viride* + SN recorded the best results for number of leaves/plant as follows: 168.3, 160.3, and 155.7, respectively. While *P. fluorescens* recorded the lowest value (107.3) (Figure 1C). Similarly, the highest leaf area was found in plants treated with propiconazole, followed by *T. harzianum* + SN, SN, *T. viride* + SN, *B. megaterium* + SN, and *P. fluorescens* + SN, recording 41.3, 41.2, 39.7, 39.5, 37, and 36.1 cm^2, respectively. While *P. fluorescens* ranks lowest (28.7 cm^2) (Figure 1D). In the second season, the treatments: SN, *T. harzianum* + SN, propiconazole, and *B. megaterium* + SN achieved the highest values for plant height as follows: 87.3, 87, 85, and 81.7 cm, respectively. While *B. megaterium* recorded the lowest value (63.7 cm). Likewise, the best results for number of main branches/plant were obtained by the treatments: *T. harzianum* + SN, propiconazole, SN, and *T. viride* + SN, recording 12.3, 12.3, 10, and 9.7, respectively. While the lowest value was recorded by *P. fluorescens* (6.7). In addition, plants treated with propiconazole, *T. harzianum* + SN, SN, and *T. viride* + SN recorded the highest number of leaves/plant as follows: 157, 154, 149.3, and 140.3, respectively. While *P. fluorescens* recorded the lowest value (97.3). The highest leaf area values were achieved by *T. harzianum* + SN, propiconazole, *T. viride* + SN, *B. megaterium* + SN, and SN as follows: 41.5, 40.1, 38.5, 38.1, and 37.5 cm^2, respectively. While *P. fluorescens* recorded the lowest value (26.5 cm^2).

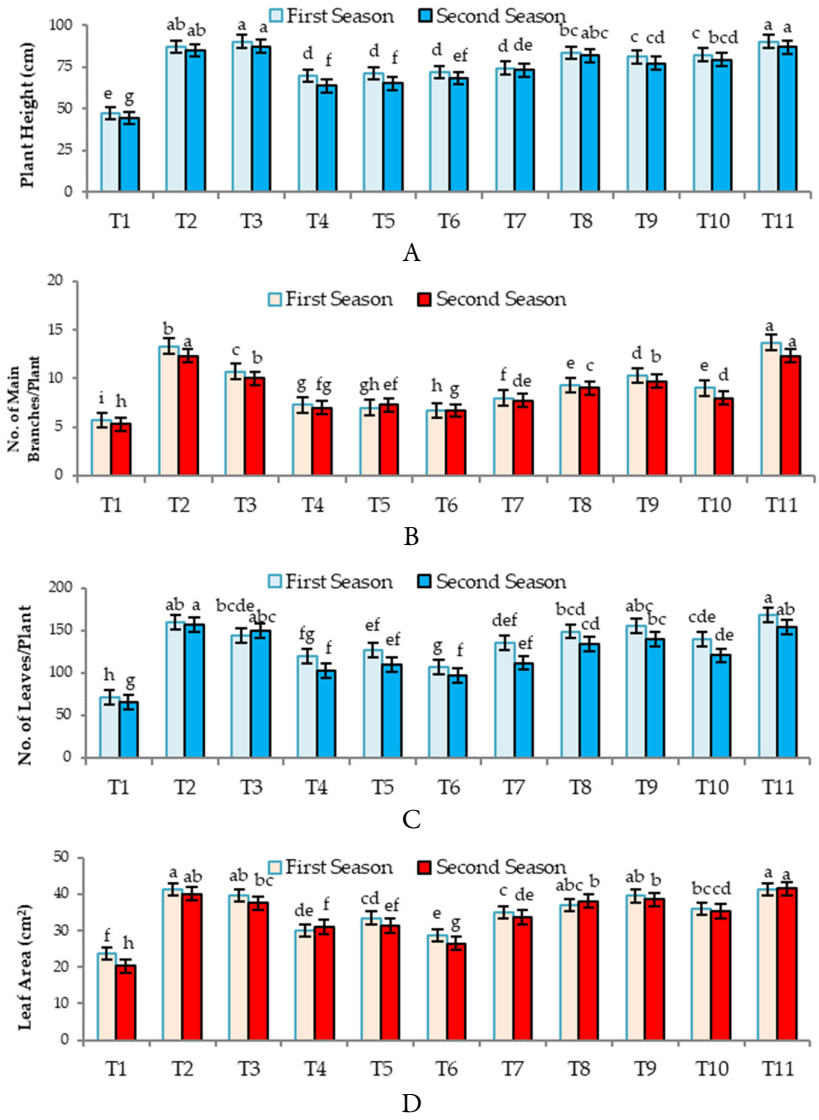


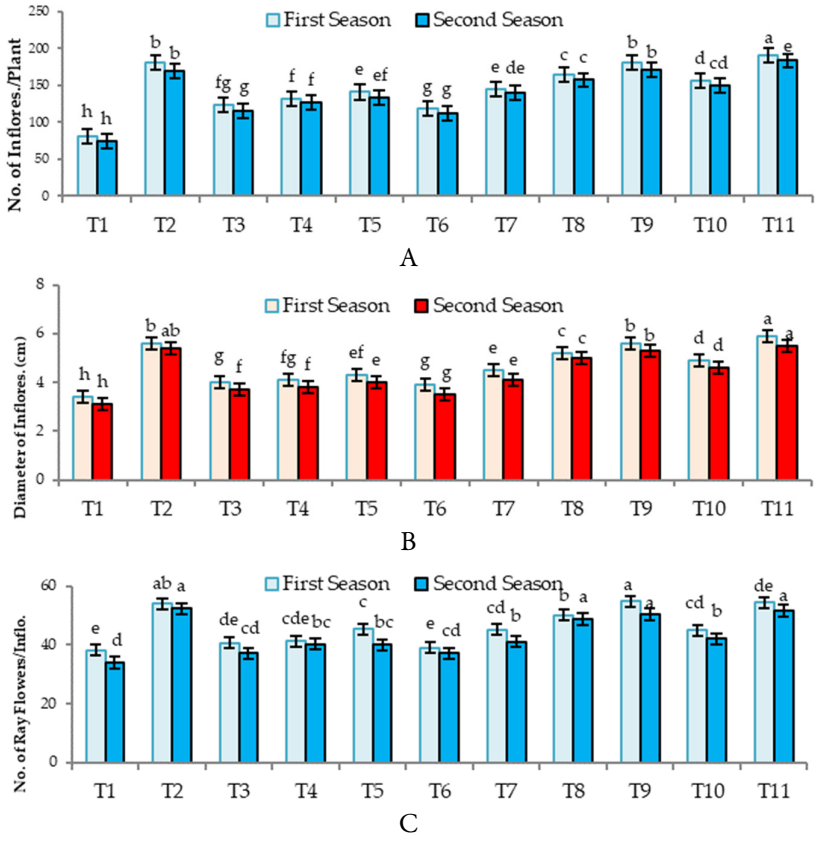
Figure 1. Effect of treatments on the morphological parameters of marigold: (A) plant height; (B) no. of main branches/plant; (C) no. of leaves/plant; and (D) leaf area, during the 2021/2022 and 2022/2023 seasons

Vertical bars represent the mean of three replicates ± standard deviation (SD). Means with different letters represent a significant difference at the statistical level $p \leq 0.05$ between the treatments according to Duncan's multiple range test.

Marigold flowering parameters

As shown in Figure 2, all treatments led to a significant improvement in flowering traits of marigold. Combination treatments had a better effect than individual treatments. In the first season, the highest number of inflorescences/plant was recorded in plants treated with *T. harzianum* + SN, followed by *T. viride* + SN, propiconazole, and *B. megaterium* + SN as follows: 191, 180.7, 180.3, and 165.1, respectively. While *P. fluorescens* ranked lowest (119) (Figure 2A). Also, the highest inflorescence diameters were recorded by *T. harzianum* + SN, *T. viride* + SN, propiconazole, and *B. megaterium* + SN as follows: 5.9, 5.6, 5.6, and 5.2 cm, respectively. While the lowest value was recorded by *P. fluorescens* (3.9 cm) (Figure 2B). In addition, the best

results for number of ray flowers/inflorescence were achieved by *T. viride* + SN, *T. harzianum* + SN, propiconazole, and *B. megaterium* + SN, recording 54.7, 54.3, 54, and 50.1, respectively. While *P. fluorescens* recorded the lowest value (39) (Figure 2C). The results also show that the highest fresh weights of 100 inflorescences were recorded by *T. harzianum* + SN, propiconazole, *T. viride* + SN, and *B. megaterium* + SN as follows: 370.3, 362.1, 359.3, and 340.1 g, respectively. While *P. fluorescens* recorded the lowest value (249.7 g) (Figure 2D). Likewise, the highest dry weights of 100 inflorescences were recorded by *T. harzianum* + SN, *T. viride* + SN, propiconazole, and *B. megaterium* + SN as follows: 80.1, 78.4, 78.1, and 74.5 g, respectively. While *P. fluorescens* was found in the lowest ranking (54 g) (Figure 2E). In the second season, plants treated with *T. harzianum* + SN, *T. viride* + SN, propiconazole, and *B. megaterium* + SN achieved the best results for the number of inflorescences/plant as follows: 183.3, 171, 169, and 157.3, respectively. While *P. fluorescens* was found in the lowest ranking (112.3). In addition, the highest values of inflorescence diameter were achieved by *T. harzianum* + SN, propiconazole, *T. viride* + SN, and *B. megaterium* + SN, recording 5.5, 5.4, 5.3, and 5 cm, respectively. While *P. fluorescens* recorded the lowest value (3.5 cm). Similarly, plants treated with propiconazole, *T. harzianum* + SN, *T. viride* + SN, and *B. megaterium* + SN recorded the highest values for the number of ray flowers/inflorescence as follows: 52.3, 51.7, 50.3, and 48.7, respectively. While the lowest values were recorded by *P. fluorescens* and SN (37.1 in each). As for the fresh and dry weight of 100 inflorescences, the best results were recorded by *T. harzianum* + SN, propiconazole, *T. viride* + SN, and *B. megaterium* + SN as follows: 361, 355.2, 348.5, and 336 g, respectively in FW, and 78.4, 77.3, 75.3, and 73 g, respectively in DW. While the lowest values (241.7 and 52 g) were recorded by *P. fluorescens* in FW and DW, respectively.



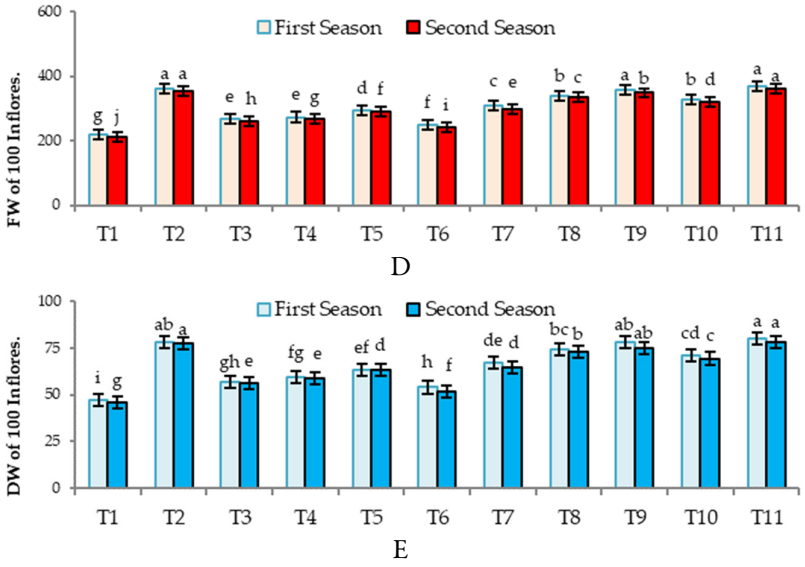


Figure 2. Effect of treatments on the flowering parameters of marigold: (A) no. of inflor./plant; (B) diameter of inflor.; (C) no. of ray flowers/inflor.; (D) 100 inflor. FW; and (E) 100 inflor. DW, during the 2021/2022 and 2022/2023 seasons

Vertical bars represent the mean of three replicates ± standard deviation (SD). Means with different letters represent a significant difference at $p \leq 0.05$ between the treatments according to Duncan's multiple range test.

Chlorophyll a, chlorophyll b, and carotenoids

Data in Figure 3 show that all treatments resulted in a significant increase in the content of chlorophyll a, chlorophyll b, and carotenoids. In the first season, the highest values of these pigments were found in plants treated with *T. harzianum* + SN, *T. viride* + SN, SN, *B. megaterium* + SN, and propiconazole as follows: 9.15, 8.63, 8.14, 7.52, and 7.43 mg g^{-1} leaf FW, respectively in chl. a (Figure 3A), 4.37, 4.21, 4.07, 3.98, and 3.77 mg g^{-1} leaf FW, respectively in chl. b (Figure 3B), and 0.98, 0.91, 0.84, 0.79, and 0.75 mg g^{-1} leaf FW, respectively in carotenoids (Figure 3C). On the contrary, plants treated with *P. fluorescens* recorded the lowest values of 5.22, 2.5, and 0.42 mg g^{-1} leaf FW for chl. a, chl. b, and carotenoids, respectively. In the second season, plants treated with *T. harzianum* + SN, *T. viride* + SN, SN, *B. megaterium* + SN, and propiconazole achieved the highest chl. a (8.72, 8.55, 7.33, 7.24, and 7.10 mg g^{-1} leaf FW, respectively), chl. b (4.20, 4.02, 3.89, 3.80, and 3.80 mg g^{-1} leaf FW, respectively), and carotenoids (0.93, 0.90, 0.80, 0.77, and 0.72 mg g^{-1} leaf FW, respectively). Conversely, plants treated with *P. fluorescens* had the lowest values for chl. a, chl. b, and carotenoids as follows: 5.03, 2.47, and 0.40 mg g^{-1} leaf FW, respectively.

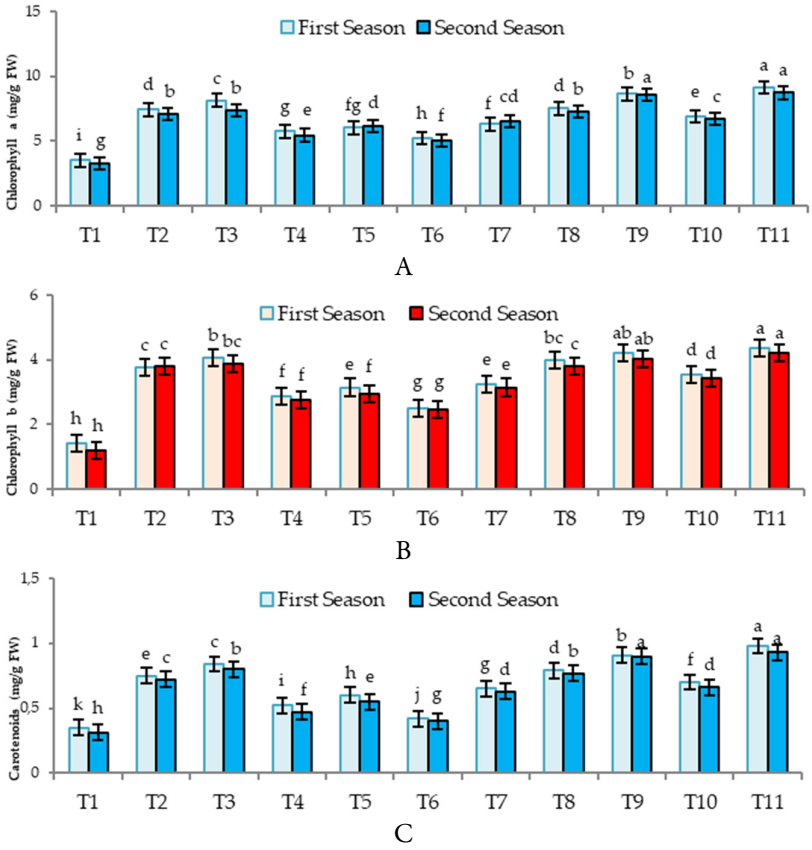


Figure 3. Effect of treatments on (A) chlorophyll-a; (B) chlorophyll-b; and (C) carotenoids of marigold, during the 2021/2022 and 2022/2023 seasons

Vertical bars represent the mean of three replicates ± standard deviation (SD). Means with different letters represent a significant difference at $p \leq 0.05$ between the treatments according to Duncan's multiple range test.

Marigold biochemical components

As shown in Figure 4, all treatments significantly changed the biochemical components of marigold. In the first season, the highest contents of total phenolic compounds were recorded in plants treated with *T. harzianum* + SN, *T. viride* + SN, and propiconazole as follows: 365, 350, and 340 mg 100 g^{-1} DW, respectively. While the lowest content was recorded by *P. fluorescens* (254 mg 100 g^{-1} DW) (Figure 4A). Regarding polyphenol oxidase, the highest activity was recorded by *T. harzianum* + SN, followed by *T. viride* + SN, *B. megaterium* + SN, and *P. fluorescens* + SN as follows: 0.84-, 0.80-, 0.76-, and 0.72-unit mg protein^{-1} min^{-1}, respectively. While *P. fluorescens* recorded the lowest activity (0.55-unit mg protein^{-1} min^{-1}) (Figure 4B). In addition, the lowest amount of free proline was recorded in plants treated with *T. harzianum* + SN, followed by propiconazole, *T. viride* + SN, and *B. megaterium* + SN. The corresponding free proline values were 1.93, 2.10, 2.30, and 2.45 µ mole g^{-1} DW, respectively. While the highest amounts of 4.79 and 3.86 µ mole g^{-1} DW were recorded by control and *P. fluorescens*, respectively (Figure 4C). In the second season, treatments of *T. harzianum* + SN, *T. viride* + SN, *B. megaterium* + SN, and propiconazole recorded the highest content of total phenolic compounds as follows: 354, 339, 328, and 326 mg 100 g^{-1} DW, respectively. While *P. fluorescens* recorded the lowest content (259 mg 100 g^{-1} DW). Concerning the activity of polyphenol oxidase, the maximum level was recorded by *T. harzianum* + SN, followed by *T. viride* + SN, *B. megaterium* + SN, *P. fluorescens* + SN, and propiconazole, recording 0.78, 0.73-, 0.70-, 0.67-, and 0.66-unit mg protein^{-1} min^{-1}, respectively. While *P. fluorescens* ranked lowest (0.48-unit mg protein^{-1} min^{-1}). In addition, treatments of *T.*

harzianum + SN, propiconazole, *T. viride* + SN, and *B. megaterium* + SN recorded the lowest amounts of free proline as follows: 2.44, 2.59, 2.80, and 2.96 µ mole g^{-1} DW, respectively. Conversely, the highest amounts of 5.62 and 4.10 µ mole g^{-1} DW were recorded by control and *P. fluorescens*, respectively.

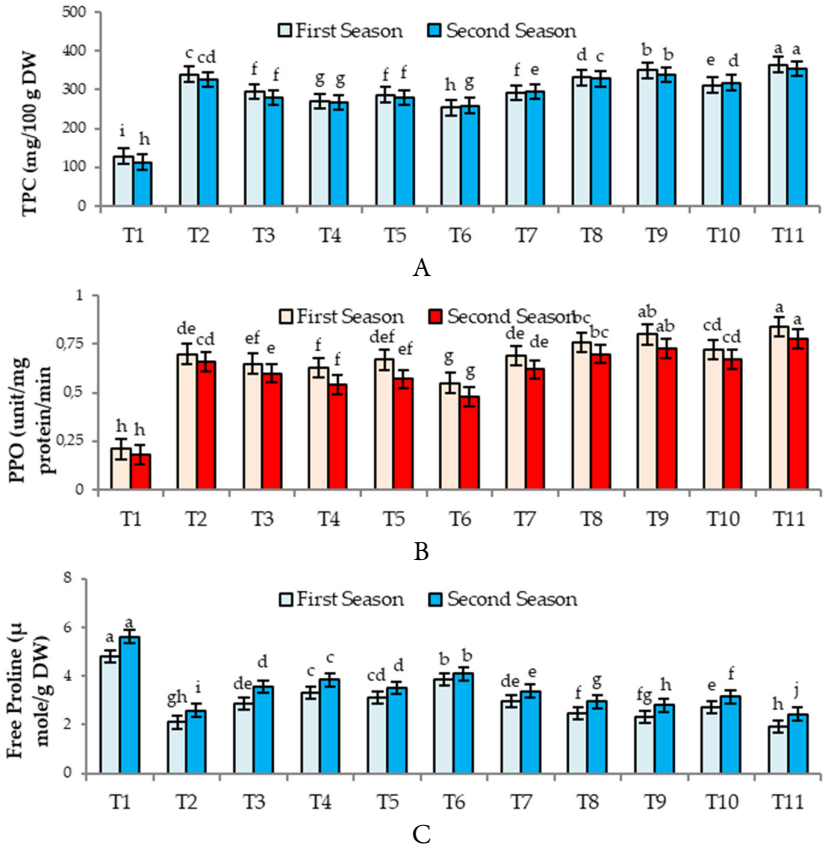


Figure 4. Effect of treatments on the biochemical components of marigold: (A) total phenolic compounds (TPC); (B) polyphenol oxidase (PPO) activity; and (C) free proline (FP), during the 2021/2022 and 2022/2023 seasons

Vertical bars represent the mean of three replicates ± standard deviation (SD). Means with different letters represent a significant difference at the statistical level $p \leq 0.05$ between the treatments according to Duncan's multiple range test.

Correlations

Data in Figure 5 show that the hierarchical analysis divided the study treatments into 3 main groups. The first group (T8, T9, T2, and T11) showed higher performance compared to the second group (T7, T5, T10, T3, T6, and T4). While T1 was clustered in the third group. The first group was clustered into two subgroups (T11 and T2) and (T8 and T9), while the second main group was clustered into two subgroups (T7, T5, T10, and T3) and (T6 and T4). Therefore, the use of biocontrol agents, especially combined with sodium nitrophenolate, significantly reduced the severity of powdery mildew and improved all growth, productivity, and biochemical parameters of marigold compared to untreated plants under *in vivo* conditions.

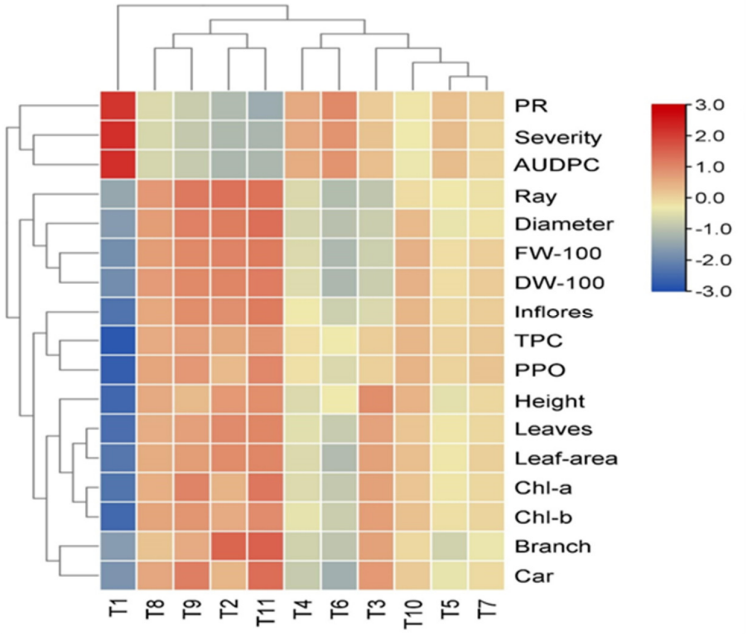


Figure 5. Heat map correlation graph showing the hierarchical clustering analysis between the studied parameters and treatments as an average of two seasons

The colors represent variations in the data. Severity = disease severity, AUDPC = the area under disease progress curve, Height = plant height, Branches = no. of main branches/plant, Leaves = no. of leaves/plant, Leaf area = leaf area, Inflores = no. of inflorescences/plant, Diameter = diameter of inflo., Ray = no. of ray flowers/inflo., FW.100 = fresh weight of 100 inflo., DW.100 = dry weight of 100 inflo., Chl-b = chlorophyll a, Chl-b = chlorophyll b, Car = carotenoids, TPC = total phenolic compounds, PR = proline, and PPO = polyphenol oxidase activity.

Discussion

Powdery mildew caused by *G. cichoracearum* (DC.) V.P. Heluta, is one of the most destructive diseases of marigold in greenhouse and field (Mir *et al*., 2012; Abdel-Wahed and Shaker, 2020). Losses due to this disease range from 37 to 51% in flower productivity (Abdel-Wahed and Shaker, 2020). Although fungicides provide easy management of plant diseases, the negative effects associated with their frequent use have led to reduced reliance on them. This paper discusses the use of four BCAs individually or in combination with SN to control this disease. Our results revealed that all treatments significantly inhibited conidial germination of *G. cichoracearum* compared to control. These results are in harmony with those of Sarhan *et al*. (2020), who found that *Podosphaera xanthii* conidia treated with culture filters of *T. harzianum, T. viride,* and *B. subtilis,* showed a significant reduction in conidial germination. Likewise, *E. heraclei* conidia treated with *B. subtilis, B. pumilus,* and *B. megaterium* showed a significant decrease in germination rate and germ tube length (Ahmed *et al*., 2021). In addition, Hafez *et al*. (2018) reported that treatment of *P. xanthii* conidia with *B. megaterium*, *B. pumilus*, and *Paenibacillus polymyxa* inhibited germination by 98.5, 95.2, and 90.7%, respectively. The activity of BCAs against conidia germination can be attributed to their ability to synthesize of antibiotics and/or other antimicrobial substances, such as hydrolytic enzymes, hydrogen cyanide, and siderophores. These substances degrade conidia cell walls or inhibit enzymes needed to complete the germination (Prasannath, 2017; Rais *et al*., 2017).

Our results showed that all treatments applied *in vivo* significantly reduced powdery mildew severity and AUDPC compared to the control. In this regard, the most effective treatments were *T. harzianum* + SN, propiconazole, *T. viride* + SN, *B. megaterium* + SN, and *P. fluorescens* + SN. These results are consistent with

those of Ahmed *et al*. (2021), who found that foliar application of *B. subtilis,* and *B. megaterium* significantly reduced the severity of celery powdery mildew and AUDPC. Similarly, Esawy *et al*. (2021) reported that among five BCAs, *T. koningii*, *T. harzianum*, and *B. subtilis* were the most efficient in reducing the severity of sunflower powdery mildew and AUDPC. In addition, Hafez *et al*. (2018) found that application of *B. subtilis, B. megaterium, T. harzianum,* and *T. viridi* significantly reduced the severity of squash powdery mildew and AUDPC. Many reports have demonstrated the potential of *Trichoderma* spp. as effective biocontrol agents against powdery mildew of celery, vineyards, and sunflower (Ahmed *et al*., 2021; Sawant *et al*., 2017; Esawy *et al*., 2021). More than 250 Trichoderma-based biofungicides are currently commercially available worldwide (Mukherjee *et al*., 2014). Biological control mechanisms for these fungi include mycoparasitism, competition for nutrients and space, and induction of systemic and local plant resistance (Brunner *et al*., 2005). They also have the ability to produce antibiotics (i.e. viridin, gliotoxin, gliovirin, pyrones, and peptaibols) and cell wall-hydrolytic enzymes (i.e. chitobiosidase, endochitinase, glucan 1,3-β-glucosidase, and n-acetyl-β-glucosaminidase) (Vey *et al*., 2001). Similarly, the antifungal activity of *Bacillus* spp. against powdery mildew fungi has been demonstrated (Ahmed *et al*., 2021; Hashem *et al*., 2019; Hafez *et al*., 2018). This activity is attributed to their ability to synthesize a variety of antibiotics such as iturin, surfactin, and fengicin, which exhibit growth inhibitory effects on many plant pathogens (Kim *et al*., 2010). Some species have the ability to synthesize cyclic peptides, total proteins, and cell wall-degrading enzymes (Schreiber *et al*., 1988). In addition to its ability to synthesize siderophores, plant hormones, volatile compounds, and lipopeptides, that stimulate growth and immune responses in plants (Franco-Sierra *et al*., 2020). Our results also showed that treatments of BCAs combined with sodium nitrophenolate (SN) had a better disease control response than those without SN. This may be due to the multiple positive effects of SN on stimulating growth, increasing productivity, and improving quality (Michalski *et al*., 2008). In addition to its protective role against various abiotic stresses (Borowski and Michałek, 2009). Although sodium nitrophenolate is registered in the European Union as a pesticide for some crops, knowledge about its potential in plant disease management is still limited. It was found that the use of SN (0.1%) achieved positive results in reducing some plant diseases, such as powdery mildew in roses, chrysanthemum rust, rose black spot, and willow rust (Wojdyla, 2004; Oluoch, 2022).

In our study, all treatments significantly improved the growth and productivity parameters of marigold. These results are consistent with those of Sarhan *et al*. (2020), who found that cucumber treated with BCAs showed a significant increase in plant height, weight and number of fruits. Also, Ahmed *et al*. (2021) found that foliar application of BCAs significantly improved celery plant height, number of leaves and umbels, and weight of herb and fruits. Likewise, Ahmed *et al*. (2023) found that roselle treated with a mixture of *B. subtilis, Gliocladium catenulatum,* and *T. asperellum* showed significant improvement in plant height, number of branches and fruits, and weight of sepals and seeds. Many reports have shown the potential of *Bacillus* spp. and *Pseudomonas* spp. to improve the growth and productivity of many crops. This ability may be attributed to their ability to synthesize phytohormones and siderophores, produce amino acids, vitamins and antioxidants, and form biofilms (Khalil *et al*., 2017). They also stimulate the plant to produce ACC-deaminase enzymes that reduce ethylene synthesis in the roots (Gupta and Pandey, 2019). These bacteria mainly contribute to nitrogen fixation and phosphorus availability to plants, and produce plant growth regulators, such as cytokinin, gibberellic acid, and indole-3-acetic acid (Wang et al., 2017; Singh *et al*., 2018). They also promote plant growth indirectly by eliminating biotic stresses on plants caused by pathogens through the production of a wide range of antibiotics, cell wall-degrading enzymes, phenolics, and signaling compounds (Prasannath, 2017). In a similar vein, extensive studies have indicated the role of plant growth-promoting fungi (PGPFs) such as *Trichoderma* spp. in enhancing the growth and productivity of crops, and indicated that they produce hormones that aid in the interaction between soil and plant, thus decompose organic matters and dissolve minerals (Altomare *et al*., 1999). They also play an active role in improving plant growth and vitality indirectly as effective biocontrol agents against a wide range of pathogens (Kumar, 2013). They also directly stimulate

plant growth by enhancing stress tolerance, active uptake of nutrients, supplying plants with many secondary metabolites, enzymes, and PR proteins, in addition to producing plant growth stimulants such as gibberellic acid (Al-Askar *et al*., 2016; Prasannath, 2017). Our results also showed that BCAs combined with SN had a better effect in improving the growth and productivity parameters of marigold than those without SN. Many studies have shown similar results for improving the growth of cotton and maize (Djanaguiraman *et al*., 2005; Batool *et al*., 2022). This potential of SN as a biostimulant may be due to one/or more of the following modes of action: (1) increased activities of antioxidant enzymes such as catalase, peroxidase, and superoxide dismutase. These enzymes search for harmful reactive oxygen species (ROS), which oxidize polysaccharides, proteins and nucleic acids (Matysik *et al*., 2002); (2) increased auxin concentration and inhibition of IAA oxidase activity, ensuring higher activity of naturally synthesized auxins (Przybysz *et al*., 2010); (3) enhancing the uptake of some nutrients such as K, Ca, and Mg (Guo and Oosterhuis, 1995); (4) increased photosynthesis intensity by up to 24.3%, reduced membrane leakage by 34.5%, and increased transpiration rate, but without a decrease in relative water content (Borowski and Michałek, 2009); and (5) increased activity of nitrate reductase, an essential enzyme in nitrogen metabolism (Sharma *et al*., 1984).

Our study showed that all treatments led to a significant increase in the content of chlorophyll a, chlorophyll b, and carotenoids. These findings are consistent with those of Ahmed *et al*. (2021), who found that celery treated with BCAs showed a significant increase in chlorophyll and carotenoid pigments. Gaafar *et al*. (2021) also found that roselle treated with the mixture of *P. fluorescens*, *B. subtilis,* and *Pleurotus ostreatus* showed a significant increase in the content of photosynthetic pigments compared to the untreated ones. In fact, the activity of BCAs in increasing photosynthetic pigments can be attributed to their ability to stimulate the increase of hormones and the uptake of some minerals such as Fe and Mn needed for chlorophyll synthesis (Ben Maachia *et al*., 2013). In addition to its ability to stimulate the formation of pyridoxal enzymes, which play a vital role in the biosynthesis of the beta-aminolevulinic compound necessary for the synthesis of chlorophyll (Adil *et al*., 2017). Our results also showed a significant increase in total phenolic compounds in response to the effects of the treatments. These results are consistent with those of Yousef (2021), who found that the highest content of total phenols was recorded in sunflower treated with the mixture of *T. harzianum* and salicylic acid. Similarly, Sarhan *et al*. (2020) reported that cucumber treated with BCAs showed a significant increase in total phenols content compared to untreated plants. In general, the synthesis of phenolic compounds is a defensive response to pathogen attack, and may be formed before infection (Goodman *et al*., 1986). Phenols exert protection against pathogens through several mechanisms, such as affecting the physiology, morphology, and ultrastructure of pathogen, or by enhancing systemic plant resistance (Mohamed *et al*., 2016). They also activate both resistance genes in plants and pathogen toxicity modulators (Nicholson and Hammerschmidt, 1992). Phenols are participate in the lignification of cell walls, thus impeding the spread of the pathogen in healthy tissues and reducing the transfer of nutrients from host cells to the pathogen (Zaynab *et al*., 2018). In our study, all treatments significantly increased polyphenol oxidase activity. This result is similar to that of Yousef (2021), who found that the highest activity of peroxidase, catalase and polyphenol oxidase was recorded in sunflower treated with mixture of *T. harzianum* and salicylic acid. Also, Sarhan *et al*. (2020) reported that cucumber treated with BCAs showed a significant increase in peroxidase and polyphenol oxidase activity compared to untreated plants. In general, defense-related enzymes play an important role in protecting plants from biotic stresses caused by fungal, bacterial and viral infections (Safdarpour and Khodakaramain, 2018). They act as antioxidants, reducing excess ROS, which are a source of oxidative stress caused by pathogens (Rais *et al*., 2017). Several studies have reported that PPO oxidizes phenolic compounds to quinines that are toxic to microbes (DallAgnol *et al*., 2015). It contributes to the lignification of cell walls, which act as defensive barriers against the penetration of pathogens (Morishita *et al*., 2003). On the other hand, our results showed that all treatments significantly reduced free proline compared to untreated plants. As stated by Gull *et al*. (2019), proline level increases in plants in response to abiotic stresses such as drought, high/low temperatures,

salinity, heavy metal toxicity, etc., or biotic stresses such as infection with plant pathogens, which have adverse effects on growth, productivity and quality. However, proline accumulation is not just a stress signal, but improves the plant's ability to withstand various stresses by increasing photosynthesis, stimulating enzymatic and non-enzymatic antioxidant activities, regulating osmolyte levels, and K and Na balance (Hosseinifard *et al*., 2022). Our results also showed that BCAs combined with SN had a better effect in improving the biochemical traits of marigold compared to those without SN. These results are consistent with those of Przybysz *et al*. (2014), who found that foliar spray of SN improved maize physiological traits, such as relative leaf water (10%), chlorophylls (14%), and carotenoids (15%) under water-deficient conditions. Also, Heikal (2017) found that application of SN with different sources of potassium enhanced chlorophyll, total carbohydrate, and mineral contents of *Salvia farinacea*. It has also been found that SN helps reduce the level of oxidative stress by increasing the activities of antioxidant enzymes and total antioxidant capacity to a greater extent than the increase in the level of anion radicals, in addition to affecting the production of proline and polyols, which are involved in anti-stress mechanisms (Djanaguiraman *et al*., 2005).

Conclusions

Our study concluded that application of BCAs individually or in combination with SN can be successfully used to suppress marigold powdery mildew, as they significantly inhibited conidial germination of *G. cichoracearum in vitro*, as well as disease severity and AUDPC *in vivo*. Combination treatments had a better disease control response than individual treatments. The most effective treatments were *T. harzianum* + SN, *T. viride* + SN, *B. megaterium* + SN, and *P. fluorescens* + SN. The treatments improved growth and productivity traits, as well as photosynthetic pigments, total phenolic compounds, and polyphenol oxidase activity, while significantly reducing free proline.

Authors' Contributions

Conceptualization, H.F.A.A., M.F.S.; methodology, H.F.A.A., G.A.A.; software, R.S.T., M.M.M.; formal analysis, H.F.A.A., M.F.S., N.K., M.M.M.; investigation, H.F.A.A., M.F.S., M.M.M.; resources, H.F.A.A., M.F.S.; data curation, M.F.S., R.S.T..; writing—original draft preparation, H.F.A.A., M.F.S., A.M.M; writing—review and editing, G.A.A., R.S.T., M.M.M., N.K., A.M.M. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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