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Improvement of Phytopharmaceutical and Alkaloid Production in Periwinkle Plants by Endophyte and Abiotic Elicitors

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Abstract: Periwinkle plant represents a major source of immensely vital terpenoid indole alkaloids and natural antioxidants which are widely used in cancer chemotherapy. A pot experiment was done to evaluate the role of two periwinkle endophytes (*Streptomyces* sp. and *Bacillus* sp.) with or without abiotic elicitors (aluminum chloride, tryptophan, and chitosan) on plant biomass, physio-biochemical attributes, phytopharmaceutical constituents, and alkaloid production. Inoculation with endophyte microbes significantly increased plant growth, nitrogen, phosphorus, potassium, carotenoids, ascorbic acid, and alkaloid yield. It also decreased oxidative biomarkers (hydrogen peroxide and malondialdehyde) and had no significant effects on flavonoids and anthocyanin. In this regard, *Streptomyces* sp. was more effective than *Bacillus* sp. Foliar spraying with chitosan significantly increased plant growth, chlorophyll, ions, antioxidant capacity, phytopharmaceutical constituents (total soluble phenols, flavonoids, and anthocyanin), and alkaloid yield, associated with a decline in oxidative biomarkers. Conversely, aluminum chloride application generally increased oxidative biomarkers, which was associated with a decreasing effect on plant growth, chlorophyll, and ions. Application of either tryptophan or chitosan with endophyte microbes increased plant growth, chlorophyll, ions, antioxidants, and alkaloid; meanwhile, it decreased oxidative biomarkers. On the contrary, aluminum chloride with endophytes evoked oxidative damage that was associated with a reduction in plant growth, chlorophyll, ions, and phytopharmaceutical constituents. The current study provides a proof-of-concept of the use of the endophyte *Streptomyces* sp. with chitosan for enhancing periwinkle plant biomass, phytopharmaceuticals accumulation, and alkaloid production.

Keywords: alkaloid; *Bacillus* sp.; elicitors; endophytes; phytopharmacetical; *Streptomyces* sp.

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1. Introduction

Cancer is one of the greatest widespread destructive diseases, influencing millions of people each year, and it has been anticipated as the second principal causative agent of human death following cardiovascular disease [1]. In 2018, about 18.1 million people worldwide had cancer, and 9.6 million died from the disease, those numbers are projected to nearly double by 2040 [1]. Global annual expenses on anticancer drugs are approximately $100 billion and are predicted to rise to $150 billion by 2020 [2].

Periwinkle (*Catharanthus roseus* L. (G.) Don; Apocynaceae), is a significant source of high natural antioxidants and terpenoid indole alkaloids (approximately 130 TIAs) involving two essential dimeric alkaloids utilized in cancer chemotherapy [3]. The most important periwinkle alkaloids are vincristine (VCR) and vinblastine (VLB) which have been applied as a foremost active constituent of different marketable chemotherapy drugs (ONCOVIN and VELNR respectively) for chronic cancers such as leukemia, breast carcinoma, lung

cancer, and Hodgkin's disease [3,4]. Formerly, they were isolated in trace amounts (0.0003% and 2.56% of dry leaf weight for VCR and VB respectively) from periwinkle leaves [5]. Additionally, it contains numerous imperative bioactive constituents—such as anthocyanins, flavonol glycosides, phenolic acids, saponins, steroids, and terpenoids—that display antidiarrheal, antidiabetic, antihyperglycemic, antimicrobial, wound healing, and antioxidant activities [6,7]. Periwinkle plant water extracts are disbursed for several applications, i.e., diabetes, fever, or rheumatism [8]. Additionally, the plant leaves have been ground to suppress feelings of hunger and fatigue [9]. Phenolics represent the most plentiful and widespread plant natural products, occupying imperative purposes for the plant, i.e., defense against environmental stresses, herbivore restriction, and signaling in plant–microbe interactions. For humans, plant phenolics are a source of numerous plant-derived drugs and they have recently attracted more consideration owing to their implication in protection against cancer, cardiovascular, and neurodegenerative diseases, in relation to their antioxidant action [10,11].

To keep up with the growing requirements for anticancer drugs and the attendant challenge of their production costs (from \$1–3.5 million kg^{-1}), widespread interest over the past 25 years has intensified for increasing their production [2]. Due to intricate structures of the alkaloid (particularly VLB and VCR), their chemical synthesis at a large scale is not efficiently practicable [9]. Therefore, various approaches—i.e., in vitro cultures, metabolic engineering [12,13], and semi-chemical synthesis [14]—are being explored to achieve improved specific indole alkaloid production. Semi-synthesis and derivatization of intricate biochemical construction by chemical methods are also reliant upon TIA precursors that are likewise fed-up from medicinal herbs [14]. The reconstitution of herb metabolic pathways in heterogonous hosts has led to restricted achievement [15]. Distinction of intracellular compartmentalization and enzymes involved in TIA biosynthetic pathways to the production of the end product are still unexplored through system biology approaches [16]. One of the most imperative ways for improving the assimilation of secondary metabolites and phytopharmaceuticals is the utilization of elicitors [17,18]. Elicitors are biotic or abiotic materials that are able to induce an enhancement in the assimilation of secondary metabolites via the stimulation of defensive responses, biochemical modification, and accretion of phytoalexin. There are several abiotic elicitors (metal ions, inorganic compounds, and precursors) and biotic elicitors (endophytes microbes, plant cell wall components) that are normally applied [5,17].

Plant endophytes colonize and spend their entire life cycle or part of their life cycle within the healthy plant tissues devoid of any obvious infection symptoms or noticeable manifestation of diseases, also, they could be a promising resource of novel natural products for medicinal, agricultural, and industrial uses [14,18,19]. The endophyte microbes enter the plant through root hairs or the leaf stomata followed by systemic distribution all over the plant. Endophytes can produce plant growth substances, accelerating nutrient availability and antioxidant capacity that induces plant growth and secondary metabolite accumulation [20–22]; however, there is very little pre-existing research on endophyte utilization for improving the phytopharmaceutical biosynthesizing capacity in periwinkle plants.

Recently, feedings with specific precursors (i.e., tryptophan) have proved to be practical and successful approaches for raising the level of phytopharmaceuticals, including alkaloids [23]. Tryptophan application motivates the growth and biochemical processes of numerous plants by accelerating indole acetic acid biosynthesis, as well as increasing the content of chlorophyll, soluble and insoluble sugars, as well as total alkaloids [24,25]. Chitosan has attracted massive deliberation as a significant biological resource owing to its biological features—i.e., non-toxicity, biodegradability, and eco-friendliness—with different usages in agriculture [26,27]. Recently, a few reports have revealed that chitosan has been established as an efficient abiotic elicitor for improved plant growth, activating antioxidant capacity, as well as enhancing secondary metabolites production in diverse plants [26,28]. Additionally, chitosan application increases the superoxide dismutase activity and reduces oxidative biomarkers [28–30]. Inorganic chemicals, i.e., aluminum chloride, have been

widely applied in several herb species for the production of phytopharmaceuticals via changing plant secondary metabolism processes [13,26]. Aluminum, a noxious soil metal, was formerly recognized as a biogenic elicitor that upregulates genes associated with plant defense strategies under environmental stresses [31].

Previous studies have separately recognized that endophytes or abiotic elicitors, as a cost-effective agent, have multiple biochemical functions in plant development and biochemical pathways as well as secondary metabolite assimilation. Conversely, their integrative application in inducing phytopharmaceutical production in periwinkle plants to our knowledge has not been documented. Therefore, the current study aims to examine whether the application of abiotic elicitors with or without endophytes could be a valuable strategy for improving the biomass and phytopharmaceutical production of the periwinkle herb.

2. Materials and Methods

The current study was done in the research farm and lab of the Agricultural Botany Department, Mansoura University, Egypt (latitude 31°02′40.6″ N, longitude 31°22′40.3″ E, altitude 15 m above sea level), in the 2018 and 2019 seasons, to evaluate the role of two periwinkle endophyte microbes (*Streptomyces* sp. and *Bacillus* sp.) with or without abiotic elicitors (aluminum chloride, tryptophan, and chitosan) on the plant biomass, physio-biochemical attributes, phytopharmaceutical constituents, and alkaloid production.

2.1. Endophyte Micro-Organisms Isolation, Selection, and Identification

Healthy periwinkle plants were collected from the Mansoura University garden, to isolate the endophytic microbes. The plant shoots were washed thoroughly in sterilized distilled water (SDW) and then surface-sterilized for 1 min in 70% ethanol, 2.5% sodium hypochlorite, 70% ethanol respectively, and finally rinsed in SDW three times to ensure that all isolated microorganisms are endophytes and to kill saprophytic or parasitic microbes [32]. To validate the surface sterilization efficiency, we used the protocols described by Coombs and Franco [33].

The surface-sterilized shoot segments (1 cm long from stems and leaves) were divided into three parts, the first part was placed in Petri dishes (9.00 cm) with potato dextrose agar (PDA) with 0.003 mL/l rose bengal and streptomycin (250 mg/L) to isolate the endophytic fungi and then incubated at 26 ± 2 °C until growth was observed. The second part was crushed in SDW for isolation of the endophytic bacteria. An aliquot (1 mL) of sterilized crushed samples was spread onto nutrient agar media (NA) plates and incubated at 30 ± 2 °C [34]. The third part was crushed as mentioned before, spread onto starch nutrient agar (SNA) plates at a dilution of 10^{-6}, and incubated at 28 ± 2 °C to isolate the endophytic actinomycetes. Regular observations for endophyte growth were done from the 2nd to 10st days from inoculation. Individual hyphal tips of the various fungi from internal tissues or colonies from crushed segments were removed and cultured again on PDA, NA, and SNA plates, and subsequently incubated at 28 ± 2 °C for at least 10 days. All endophyte cultures were assessed for purity, transferred to fresh culture slant by hyphal tips, as well as a single spore, and stored at 4 °C for additional observations [35].

Identification of endophyte isolates was done depending on the morpho-taxonomical features and microscopic observations of the mycelia, spore and colony shapes, Gram staining, spore formation, and colony pigmentation via the regular mycological guides [36,37]. For tentative identification, microscopic slides were prepared and checked under a binocular compound microscope for morphological identification. Numerous online databases (MycoBank, Fungal Planet; Index Fungorum, Bibliography of Systemic Mycology) are useful in the identification of fungal isolates. On the other hand, the physio-biochemical features of bacterial isolates were determined following *Bergey's Manual of Determinative Bacteriology* (eighth edition). A total of 15-endophyte microorganisms were recognized. For choosing the most effective endophytic isolates, we prepared an initial experiment in

the greenhouse. This experiment contained 16 groups (each one containing three pots) for 15 endophytes and a non-inoculated treatment.

For bacterial inocula preparation, isolated bacterial strains were grown on liquid NA to maximize bacterial cell numbers for 2 days at 30 ± 2 °C. Subsequently, the bacterial cultures were collected by centrifugation (3000 rpm for 30 min) at lab. temperature. The sediment was re-suspended in 50 mL of 0.8% potassium chloride solution, then the concentration of bacteria was adjusted to 10^{-8} cell mL^{-1} with SDW. Meanwhile, the isolated fungi and actinomycete strains were grown on a solid medium (PDA and SNA respectively) and incubated at 28 ± 2 °C for 5 days. To inoculum preparation, spores were collected from the surface of the medium and suspended in sterile potassium chloride (0.9%) solution. Spore suspension (100 µL from a 10^6 cfu/mL suspension) was transferred to a 50 mL liquid medium and incubated in a rotary shaker incubator at 150 rpm (at 28 ± 2 °C). After 3 days, the culture was centrifuged at 3000 rpm for 10 min. the sediment was re-suspended in a 50 mL sterile solution (containing 0.8% potassium chloride), then the concentration of spores ml^{-1} was adjusted to 1.5 × 10^6 with SDW. The inoculums were mixed well with the experimental soil at 20 mL kg^{-1} soil and the pots were filled. Prepared inoculums were mixed well with the experimental soil at 10 mL kg^{-1} soil and the pots were filled separately. Then the healthy and uniform periwinkle seedlings (5/pot) were transplanted. Ten mL of inoculums were then added to each pot after 10 days following transplanting. At the end of the experiment (30 days following transplanting), the plants were collected for estimation of shoot dry weight and alkaloid content. Depending on the obtained results from this experiment, two endophyte isolates were chosen for the main investigation as indicated below.

The two selected isolates were identified by 16S rRNA gene sequences. The genomic DNA of endophytes was extracted [38], and 16S rDNA was amplified in polymerase chain reaction using the genomic DNA as a template and universal primers, 27F (5′GAGTTTGATCACTGGCTCAG-3′) and 1492R (5′TACGGCTACCTTGTTACGACTT-3′) [39]. To know the identity of isolates, obtained sequences were compared with nucleotides via GenBank [40]. The selected endophytes were *Bacillus* sp.-JN256920 and *Streptomyces* sp.–HE591384.

2.2. Plant Material and Experimental Layout

Outdoor pot experiments were done at the experimental farm of the Agricultural Botany Department, Mansoura University, Egypt, in a completely randomized block design. The experiment is composed of two factors; the first factor includes endophyte microorganisms (no endophytes, *Bacillus* sp., *Streptomyces* sp.). The second factor involved the abiotic elicitors (no-elicitors 'water', aluminum chloride, tryptophan, chitosan). Therefore, the experiment consisted of 12 treatments replicated five times. The appropriate abiotic elicitor concentration in the present research was based on previous studies.

The plastic pots (30 cm inner diameter) were filled with 7 kg clay loam soil (43.54% clay, 34.69% silt; 21.77% sand). The physicochemical properties of the experimental soil were pH (1:2.5 soil suspension, *w*/*v*) 7.43 and 7.46; nitrogen (N) 208 and 209 mg kg^{-1}; phosphorus (P) 5.3 and 5.4 mg kg^{-1}; potassium (K) 179 and 181 mg kg^{-1}; organic matter 1.03% and 1.05%, in the experimental seasons respectively, based on the protocols summarized in Motsara and Roy [41]. Each pot was supplemented with a basal amount of NPK fertilizer (26-13-22 mg kg^{-1} soil) as ammonium sulfate, single superphosphate, and K-sulfate at the time of planting, which was repeated each month.

Seeds of periwinkle plants were sourced from the Horticulture Research Institute, Egypt. Thirty sterilized seeds were sown in each pot on 15 February each year and then irrigated regularly (90–100% of field capacity). After one month, the seedlings in each pot were thinned to leave 10 healthy and uniform seedlings, and subsequently thinned again to 3 plants pot^{-1} 45 days after sowing. At 60 days after sowing, the pots were divided into 12 groups, each one including 5 replicates as indicated in Figure 1 for endophytes and abiotic elicitors' treatments. The *Bacillus* sp. (CFU of 10^{-8} mL^{-1}) and *Streptomyces* sp.

(CFU of 1.5×10^6 mL^{-1}) inoculum (as mentioned previously) were poured into the soil in irrigation water (30 mL pot^{-1}), then mixed with the upper soil surface. Foliar spraying of different elicitors (133 mg L^{-1} aluminum chloride; 150 mg L^{-1} tryptophan; and 500 mg L^{-1} chitosan) with Tween 20 as a wetting agent was done three times in 20 days intervals (60, 80, and 100 days from sowing) until dripping using a hand sprayer. Five plants for every treatment were harvested at 120 days from sowing for plant growth, physiological attribute, and secondary metabolite assessment.

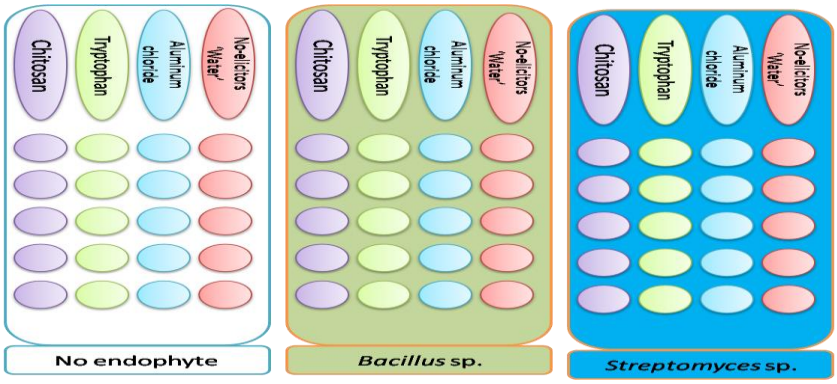

Figure 1. The experiment layout containing two factors (endophytes and abiotic elicitors).

2.3. Growth Parameter

The plant samples were collected carefully, cleaned with SDW, and then shoot fresh (FW) and dry (DW) weights were determined.

2.4. Total Chlorophyll and Carotenoid (mg g^{-1} FW)

The photosynthetic pigment was extracted and estimated following Lichtenthaler and Welburn [42] protocol. Commonly, 200 mg FW from the fourth upper leaves on the main stem were rinsed for 24 h in pre-cooled methanol (96%) supplemented with sodium bicarbonate (0.05%). The absorbance was recorded at 470, 653, and 666 nm spectrophotometrically (T60 UV–Visible spectrophotometer, UK).

2.5. Shoot Ion Percentage

Nitrogen (N), phosphorus (P), and potassium (K) were extracted from the plant shoot and then estimated following the protocol of Motsara and Roy [41]. About 200 mg of shoot dry powder was carefully transferred to a digestion flask containing 5 mL of concentrated H_2SO_4. Digestion was done at 100 °C for 2 h, subsequently; the mixtures were chilled for 15 min at lab. temperature. Then, an aliquot of $H_2SO_4/HClO_3$ mixture was added dropwise. Total N and K were determined with the micro-Kjeldahl method and flame-photometrically respectively. The scheme of Cooper [43] was followed to determine the P in the digested samples against the phosphate standard curve.

2.6. Oxidative Biomarkers

Hydrogen peroxide (H_2O_2; μM g^{-1} FW) in the shoot was determined spectrophotometrically as described by Tariq et al. [44] using titanium reagent. The absorbance was recorded at 415 nm against a blank. The hydrogen peroxide concentration was calculated according to a standard curve of H_2O_2.

Lipid peroxidation (μM malondialdehyde g^{-1} FW) was estimated following the Djanaguiraman et al. [45] method using a thiobarbituric acid reagent. The malondialdehyde concentration was deliberate via an extinction coefficient of 155 mM^{-1}cm^{-1}.

2.7. Antioxidant and Phytopharmaceutical Constitution

For estimation of ascorbic acid (mg g^{-1} FW), homogenization of shoot fresh tissues was performed in oxalic acid and thereafter centrifuged, the supernatant was stored for the assessment of ascorbic acid. A Sadasivam and Manickam [46] method was used to determine the concentration of ascorbic acid in a periwinkle shoot with 2, 6- dichlorophenol indophenol reagent following the equation

$$\text{Ascorbic acid } \left(\text{mg g}-^{1}\right) = \frac{e \times d \times b}{c \times a} \times 100$$

where: a, weight of sample; b, volume made with metaphosphoric acid; c, volume of aliquot taken for estimation; d, dye factor; e, average burette reading for sample

Antioxidant enzymes and soluble protein were extracted from plant tissues with K-phosphate buffer (100 mM, pH 7.0) following the methods of Chrysargyris et al. [47].

Catalase activity (EC 1.11.1.6; unit mg^{-1} protein) was assayed in a 3 mL reaction volume containing 1 mL of 50 mM K-phosphate buffer (pH 7.5), 0.1 mL EDTA, 0.2 mL enzyme extract, and 0.1 mL hydrogen peroxide. The activity was estimated spectrophotometrically at 240 nm [48], 1 unit catalase =1 mM of H_2O_2 decline min^{-1}. The activity of peroxidase activity (EC 1.11.1.7) was estimated spectrophotometrically at 436 nm with guaiacol as a substrate in the existence of hydrogen peroxide using the method of Zhang et al. [49]. The peroxidase activity (unit mg^{-1} protein) was determined using the coefficient of extinction of 2.47 mM cm^{-1} (1 unit =1 µmol of H_2O_2 decay min^{-1}). Soluble protein was estimated in the extract following the technique of Bradford [50].

Total soluble phenolic compounds (mg gallic g^{-1} DW) were assessed following the procedure described in Sadasivam and Manickam [46]. Plant samples (1.0 g DW) were extracted with 10 mL ethanol (80%) with a pre-cooled pestle and mortar. The mixture was centrifuged and then evaporated to dryness. Afterward, Folin–Ciocalteu reagent (0.5 mL) and Na_2CO_3 (20%) were added to every tube. The absorbance was recorded at 650 nm aligned with a blank.

Total flavonoid (mg quercetin g^{-1} DW) was estimated with $AlCl_3$ technique [51]. To 5 mL plant extracts, 0.3 mL of sodium nitrite (5%, w/v) was added and 3 mL of $AlCl_3$ (10%). Subsequently, 2 mL of sodium hydroxide (1 M) was added and mixed well, and then the absorbance at 415 nm was recorded by spectrophotometer.

The total anthocyanin (mg 100 g^{-1} FW) was estimated by Abdel-Aal and Hucl [52] method. One gram of fresh herb tissues was homogenized with 5 mL pre-chilled acidified methanol (1% HCl), then centrifuged. The supernatant absorbance was estimated at 530 nm via spectrophotometrically. The total anthocyanin concentration was calculated as cyaniding-3-glucoside based on Abdel-Aal and Hucl [52] equation.

For total alkaloid determination, a dry powdered shoot was extracted with acetic acid (10%) in ethanol for 4 h, then filtered and concentrated to one-quarter of the original quantity in a water bath. Ammonium hydroxide (AH) was added drop-wise for the entire precipitation and the solution was permitted to stand. The collected precipitates were washed with dilute AH and then filtered, dried, and weighed [53]. Total alkaloid percentage (TAC) was determined by the equation

$$\text{TAC\%} = \frac{\text{Weight of porcelain dish following evaporation (g)} - \text{Weight of empty porcelain dishing (g)}}{\text{Weight of the powder (g)}} \times 100$$

After that, the alkaloid yield (mg $plant^{-1}$) was calculated by the formula

$$\text{Alkaloid yield} = \text{shoot DW} \times \text{TAC\%}.$$

2.8. Statistical Analysis

Homogeneity of error variance for all variables was done before the analysis of variance (ANOVA). The outputs displayed that all data fulfilled the homogeneity to achieve additional ANOVA tests. The data were statistically analyzed by a two-way ANOVA, at a

95% confidence level, using CoHort Software, 2008 statistical package (CoHort software, 2006; release, Cary, NC, USA). The statistical significance was considered as: * $p \leq 0.05$, ** $p \leq 0.01$; *** $p \leq 0.001$, and ns (not significant). The difference between treatment means was assessed by Tukey's HSD Multiple Range Test at $p \leq 0.05$. The values are presented in tables as the means ± standard error (SE).

3. Results and Discussion

3.1. Shoot Biomass

The response of shoot fresh and dry weights to endophyte inoculation, abiotic elicitors, and their interactions are shown in Table 1 during both experimental seasons. Amendment with endophytes significantly increased shoot fresh and dry weights as compared with non-inoculated plants. The greatest shoot fresh weights (28% and 15%) and dry weights (24% and 28%) were obtained by *Streptomyces* application over untreated plants in both seasons. Foliar spraying with tryptophan or chitosan significantly increased shoot fresh and dry weights as compared with untreated or aluminum chloride treated plants (Table 1). Commonly, chitosan was more effective than tryptophan in enhanced shoot fresh and dry weights. The lowest shoot fresh and dry weights were obtained under aluminum chloride relative to water or other treatments. Figure 2 revealed that, in general, the application of chitosan or tryptophan with endophytes gave additive effects on improving plant growth as compared with each one alone. The greatest shoot fresh weight (88% and 65%), shoot dry weight (48% and 123%) was recorded under the application of chitosan with *Streptomyces* sp. relative to the non-inoculated water-spraying herb. Alternatively, the lowest values were obtained with aluminum chloride application without endophytes.

Table 1. Effect of endophytic microorganisms and abiotic elicitors on shoot fresh and dry weight (g); as well as chlorophyll concentration (mg g^{-1} FW) in periwinkle plant shoot in both seasons. Means of five replicates are presented with ±SE.

Treatment	Shoot Fresh Weight (g)		Shoot Dry Weight (g)		Chlorophyll	
	First Year	Second Year	First Year	Second Year	First Year	Second Year
Endophytic microorganisms						
No endophytes	25.5 ± 1.11 ^{c}	24.2 ± 0.95 ^{c}	3.63 ± 0.29 ^{c}	3.18 ± 0.27 ^{c}	1.41 ± 0.03 ^{b}	1.37 ± 0.04 ^{b}
Bacillus	29.1 ± 2.11 ^{b}	25.4 ± 1.29 ^{b}	4.08 ± 0.36 ^{b}	3.66 ± 0.36 ^{b}	1.53 ± 0.07 ^{a}	1.40 ± 0.04 ^{a}
Streptomyces	31.3 ± 2.42 ^{a}	27.9 ± 1.83 ^{a}	4.52 ± 0.37 ^{a}	4.09 ± 0.38 ^{a}	1.62 ± 0.08 ^{a}	1.46 ± 0.05 ^{a}
ANOVA *p* value	***	***	***	***	**	ns
Abiotic elicitors						
Water	25.0 ± 0.65 ^{c}	23.1 ± 0.46 ^{c}	3.57 ± 0.10 ^{c}	2.99 ± 0.13 ^{c}	1.41 ± 0.02 ^{bc}	1.37 ± 0.04 ^{bc}
Aluminum chloride	22.0 ± 0.46 ^{d}	21.0 ± 0.49 ^{c}	2.56 ± 0.08 ^{d}	2.25 ± 0.06 ^{d}	1.32 ± 0.03 ^{c}	1.25 ± 0.03 ^{c}
Tryptophan	29.5 ± 0.74 ^{b}	27.2 ± 0.58 ^{b}	4.61 ± 0.22 ^{b}	4.20 ± 0.15 ^{b}	1.53 ± 0.04 ^{b}	1.47 ± 0.05 ^{ab}
Chitosan	38.0 ± 2.16 ^{a}	32.0 ± 1.51 ^{a}	5.57 ± 0.17 ^{a}	5.13 ± 0.25 ^{a}	1.81 ± 0.08 ^{a}	1.56 ± 0.04 ^{a}
ANOVA *p* value	***	***	***	***	***	***

Levels of significance are represented by ** $p < 0.01$, *** $p < 0.001$ and ns not-significant. For each parameter in the year, different letters within the column show significant differences between the treatments and control according to Tukey's HSD test at $p < 0.05$.

The promoting effect of endophytes or abiotic elicitors recorded in the current investigation is consistent with earlier reports that indicated that inoculation with endophytes [19,20], application of tryptophan [23,54], or chitosan [29,30] possibly will boost the plant growth. Endophytes can enhance plant growth through upregulating and enhancing the synthesis of phytohormones (especially, indole-3-acetic acid) which promotes root elongation and production of root hairs, which accelerates water and nutrients uptake [55,56]; in addition, it suppresses ethylene assimilation through 1-aminocyclopropane-1-carboxylate deaminase activity [57]. Moreover, Zhang et al. [20] advocate that, upon endophytes colonization, the inoculated plant undergoes modification in chloroplasts, associated with increased photosynthesis efficiency. Additionally, endophyte microbes induce

extra-cellular enzyme production that increases ion availability, uptake, and translocation from the soil [58].

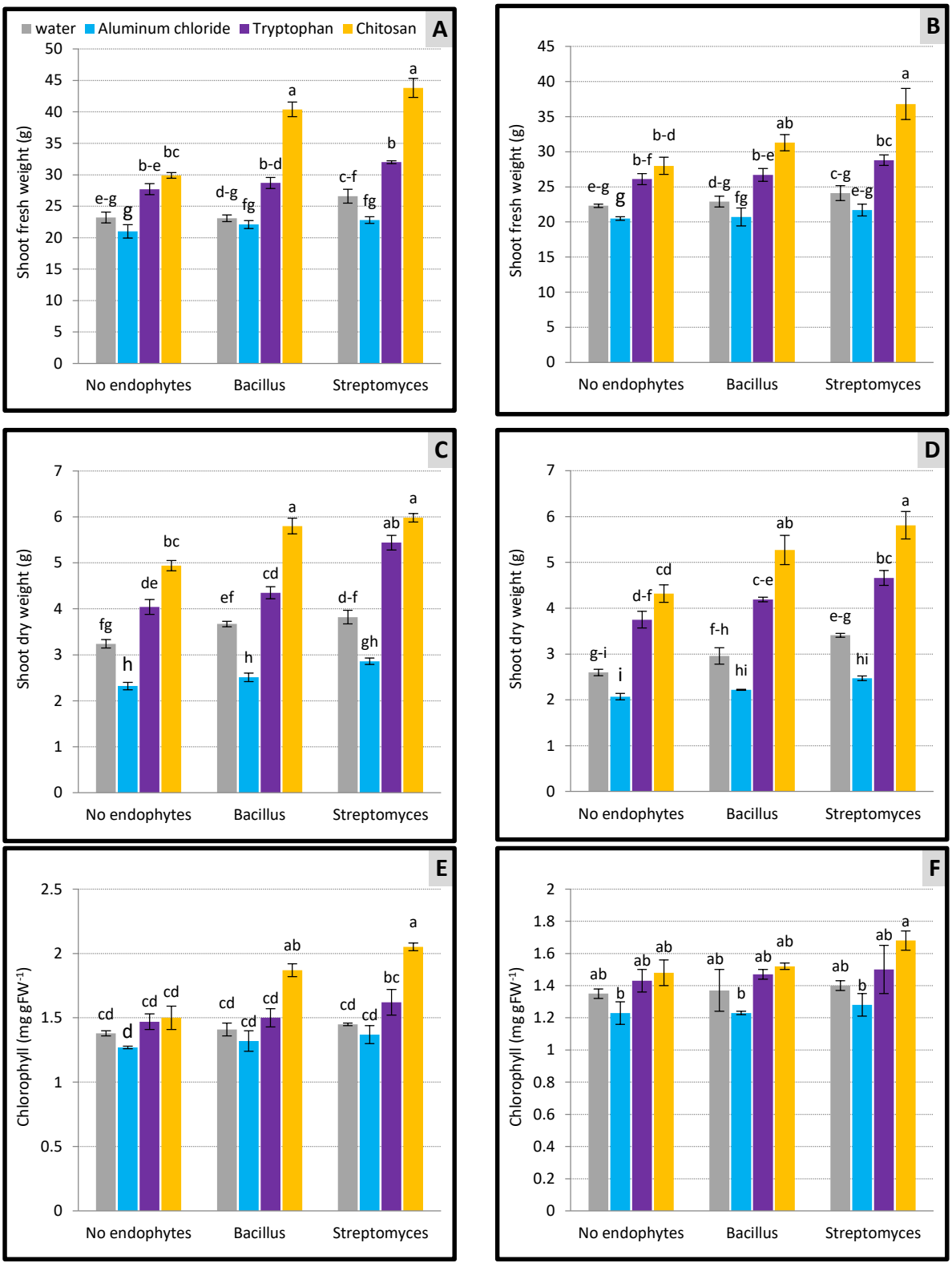

Figure 2. Interactive effect of endophytic microorganisms and abiotic elicitors on (**A**,**B**) shoot fresh weight, (**C**,**D**) shoot dry weight (g); and (**E**,**F**) chlorophyll concentration (mg g^{-1} FW) in periwinkle plant shoot in both seasons. Columns with the same letter in each chart are non-significantly ($p \leq 0.05$) different according to Tukey's test. Means of five replicates are presented with ±SE.

The stimulation effect of either tryptophan or chitosan on plant biomass production may be associated with the elicitors' impact on improving antioxidant capacity and maintaining plant homeostasis as indicated in the present study. Chitosan is a safe and eco-friendly plant growth enhancer [26,27] that increases plant growth and yield of several plants [26,28]. The growth encouraging impact of chitosan is dependent upon a signaling pathway that is linked to gibberellic acid and auxin assimilation [59]. Additionally, the enhancement impact of chitosan on plant growth is possibly ascribed to an improvement in accessibility, as well as water and nutrient uptake [29,60]. Moreover, elicitors can maintain plant water status and photosynthetic activity that could be reflected in enhancing growth. In this concern, Khan et al. [61] revealed that chitosan addition improved net photosynthetic rates, associated with improvement in stomatal conductance and transpiration rate, devoid of several influences on internal CO_2 level. On the other hand, tryptophan application could stimulate the activity of tryptophan decarboxylase (TDS), which increases IAA biosynthesis and induces cell division followed by the increment of biomass production [62]. Ordinarily, elicitor applications may stimulate cell elongation via inducing the expression of genes encoding proteins, such as xyloglucan endotrans glycosylases, which are possibly implicated in cell wall formation [63].

3.2. Chlorophyll Concentration

Periwinkle plants inoculated with either *Bacillus* sp. or *Streptomyces* sp. significantly increased the concentration of total chlorophyll relative to non-inoculated plants. The highest chlorophyll concentration was obtained by *Streptomyces* sp. relative to non-inoculated or *Bacillus* sp. applications in both seasons (Table 1). As for the abiotic elicitor's effect, the greatest chlorophyll concentration was obtained by chitosan application; meanwhile, the lowest concentration was obtained under aluminum chloride supplementation in either the first or second season. As for the interactions (Figure 2), foliar spraying of chitosan with *Streptomyces* sp. inoculation significantly increased total chlorophyll concentration by 48% and 24% in the first and second season as compared with untreated non-inoculated plants. On the other hand, the lowest concentration was recorded following adding aluminum chloride without endophytes.

Assessment of chlorophyll level is one of the major features utilized to consider the rate of plant photosynthesis. Inoculation with endophytes [19,20] and/or foliar application with tryptophan [23] or chitosan [28,30] elevated total chlorophyll concentration relative to untreated plants, conversely, the essential mechanism remains unidentified. Either endophytes and/or abiotic elicitors improved the antioxidant capacity of treated plants that were reflected by nullifying reactive oxygen species (ROS) and maintaining chloroplast functions and chlorophyll stability [64]. Additionally, the current outcomes revealed a considerable increment in N and K that may participate in raising the chloroplast number in each cell, leaf area, and enhanced chlorophyll biosynthesis. Chitosan improves chlorophyll stability and motivates the expression of genes that are implicated in the chlorophyll biosynthetic processes [65]. Additionally, chitosan supplementation induces a decline in the hypothetical chloroplast open reading frame (ycf2) gene expression in the leaves generating extended chloroplasts, which illustrated that one of the target organelles for chitosan achievement is chloroplast and the improvement in its dimension may also contribute to superior photosynthetic efficiency. Moreover, the transcript level of chlorophyllase is withdrawn which is an imperative enzyme in the catabolic pathway of chlorophyll once chitosan treatment [66]. Thus, chitosan could limit chlorophyll degradation and improve the rate of photosynthesis.

3.3. Ion Percentage

Table 2 shows that the inoculation of the periwinkle plant with endophyte in both seasonsm, in most cases, significantly increased ion percentage. Additionally, the application of *Streptomyces* sp. was more effective than *Bacillus* sp. in increasing N by 11% and 12%, P by 11% and 15%, and K by 8% and 12% in the first and second season respectively

over the untreated plants. Table 2 revealed that foliar spraying of either tryptophan or chitosan increased ion accumulation; meanwhile, aluminum chloride decreased it relative to untreated plants. The highest ion percentage was obtained by foliar application of chitosan. The application of abiotic elicitors with endophyte gave an additive effect as compared with each one alone (Figure 3). The greatest percentages of nitrogen (19% and 20%) and potassium (27% and 42%) were recorded under the treatment of chitosan spraying with *Streptomyces* sp., relative to control herbs (Figure 3). Meanwhile, the greatest P was recorded in the first season under chitosan plus *Bacillus* treatment, but in the second season, the highest value was recorded by inoculation with *Streptomyces* sp. plus foliar application with chitosan (Figure 3).

Table 2. Effect of endophytic microorganisms and abiotic elicitors on the percentage of nitrogen, phosphorus, and potassium in periwinkle plant shoot in both seasons. Means of five replicates are presented with ±SE.

Treatment	Nitrogen (%)		Phosphorus (%)		Potassium (%)	
	First Year	Second Year	First Year	Second Year	First year	Second Year
Endophytic microorganisms						
No endophytes	1.93 ± 0.08 ^{b}	1.93 ± 0.09 ^{b}	0.505 ± 0.02 ^{b}	0.457 ± 0.03 ^{b}	2.31 ± 0.08 ^{b}	2.20 ± 0.10 ^{b}
Bacillus	2.06 ± 0.06 ^{a}	1.8 m9 ± 0.09 ^{b}	0.556 ± 0.02 ^{a}	0.478 ± 0.03 ^{b}	2.38 ± 0.09 ^{b}	2.31 ± 0.10 ^{b}
Streptomyces	2.15 ± 0.05 ^{a}	2.04 ± 0.06 ^{a}	0.569 ± 0.02 ^{a}	0.530 ± 0.02 ^{a}	2.52 ± 0.08 ^{a}	2.47 ± 0.10 ^{a}
ANOVA *p* value	***	***	*	**	***	***
Abiotic elicitors						
Water	2.04 ± 0.02 ^{b}	1.96 ± 0.03 ^{b}	0.519 ± 0.01 ^{b}	0.486 ± 0.01 ^{b}	2.33 ± 0.04 ^{c}	2.18 ± 0.05 ^{c}
Aluminum chloride	1.70 ± 0.06 ^{c}	1.46 ± 0.07 ^{c}	0.447 ± 0.01 ^{c}	0.327 ± 0.02 ^{c}	1.97 ± 0.03 ^{d}	1.85 ± 0.03 ^{d}
Tryptophan	2.17 ± 0.02 ^{a}	2.08 ± 0.03 ^{ab}	0.571 ± 0.01 ^{b}	0.548 ± 0.01 ^{a}	2.56 ± 0.04 ^{b}	2.54 ± 0.07 ^{b}
Chitosan	2.28 ± 0.04 ^{a}	2.19 ± 0.02 ^{a}	0.636 ± 0.02 ^{a}	0.593 ± 0.01 ^{a}	2.75 ± 0.04 ^{a}	2.73 ± 0.05 ^{a}
ANOVA *p* value	***	***	***	***	***	***

Levels of significance are represented by * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. For each parameter in the year, different letters within the column show significant differences between the treatments and control according to Tukey's HSD test at $p < 0.05$.

Earlier reports proved that the ion contents were increased with elicitor application, i.e., Akram et al. [55] for endophytes, Wang et al. [30] for chitosan, and Mohamed Manal et al. [54] for tryptophan. This increment could be caused by enhanced nutrient uptake through sustaining plasma membrane function with improving root development [57]. Moreover, Gaudinier et al. [67] established that the transcriptional regulatory network regulates the architecture of plant organs in reaction to alterations in nitrogen availability. Plants supplemented with chitosan accelerate the buildup of different ions such as N, P, K, Mg, Ca, and Si [29,30,68]. Chitosan-mediated growth encouragement might be recognized as the nitrogen content of this polysaccharide that is approximate, 8.7% [69]. It is currently well-known that the application of chitosan may be occupied in sustaining the nutrient status via membrane stabilization and improved antioxidant capacity; therefore, defending plasma membranes from oxidative damage and improving plant cell permeability ultimately improves the ion uptake [70]. Thus, the proliferative role of chitosan on plant growth is owed to the improved nutrient status of various plants.

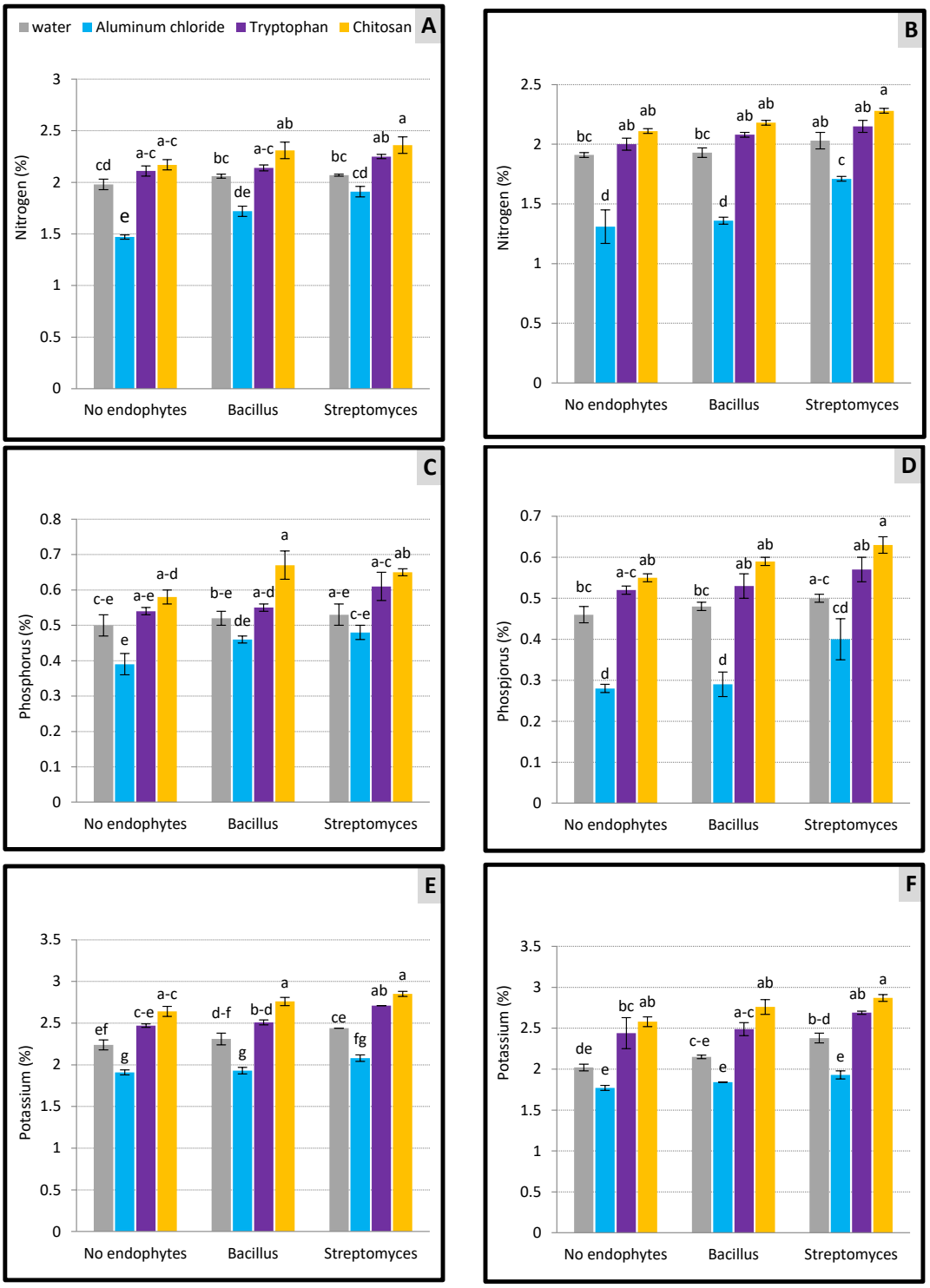

Figure 3. Interactive effect of endophytic microorganisms and abiotic elicitors on the percentage of (**A**,**B**) nitrogen, (**C**,**D**) phosphorus, and (**E**,**F**) potassium in periwinkle plant shoot in both seasons. Columns with the same letter in each chart are non-significantly ($p \leq 0.05$) different according to Tukey's test. Means of five replicates are presented with ±SE.

3.4. Oxidative Biomarkers and Antioxidant Attributes

Endophyte microbe inoculation significantly decreased oxidative biomarkers (hydrogen peroxide and malondialdehyde) through enhancing antioxidant systems represented as carotenoid and ascorbic acid concentration, as well as catalase and peroxidase activity

in both seasons (Tables 3 and 4). Application of *Streptomyces* sp. resulted in the highest antioxidant aptitude and lowest oxidative injuries relative to *Bacillus* sp. or untreated plants (Tables 3 and 4). Table 3 shows that, in both seasons, the application of aluminum chloride significantly increased hydrogen peroxide and malondialdehyde relative to other abiotic elicitors or untreated plants. Alternatively, the antioxidant system was enhanced in both years by foliar spraying of abiotic elicitors. The greatest carotenoid (57% and 63%), ascorbic acid (42% and 48%), catalase (44% and 43%), and peroxidase (54% and 99%) were recorded under chitosan treatment over untreated plants. The data presented in Figures 4 and 5 indicate that foliar spraying with abiotic elicitors with or without endophyte enhances the antioxidant capacity of periwinkle relative to untreated plants. The higher antioxidant capacity was recorded under the *Streptomyces* sp. inoculation with chitosan spraying in both seasons. This enhancement was accompanied by the reduction in hydrogen peroxide accumulation and malondialdehyde production relative to other elicitors or non-treated herbs.

Table 3. Effect of endophytic microorganisms and abiotic elicitors on hydrogen peroxide (H_2O_2, μM g^{-1} FW), malondialdehyde (MDA, μM g^{-1} FW), and carotenoid (mg g^{-1} FW) in periwinkle plant shoot in both seasons (FW, fresh weight). Means of five replicates are presented with ±SE.

Treatment	H_2O_2		MDA		Carotenoid	
	First Year	Second Year	First Year	Second Year	First Year	Second Year
Endophytic microorganisms						
No endophytes	38.2 ± 1.37 ^{a}	36.1 ± 1.50 ^{a}	12.0 ± 0.94 ^{a}	10.6 ± 0.65 ^{a}	0.408 ± 0.02 ^{b}	0.390 ± 0.02 ^{b}
Bacillus	32.0 ± 2.27 ^{b}	27.6 ± 2.02 ^{b}	9.75 ± 0.72 ^{b}	8.85 ± 1.12 ^{ab}	0.460 ± 0.02 ^{a}	0.458 ± 0.03 ^{a}
Streptomyces	26.0 ± 3.04 ^{c}	22.6 ± 2.47 ^{c}	8.02 ± 0.91 ^{b}	7.31 ± 0.77 ^{b}	0.495 ± 0.02 ^{a}	0.492 ± 0.03 ^{a}
ANOVA *p* value	***	***	**	**	***	**
Abiotic elicitors						
Water	30.7 ± 2.34 ^{b}	27.2 ± 2.48 ^{b}	9.64 ± 0.63 ^{b}	8.47 ± 0.91 ^{b}	0.357 ± 0.01 ^{d}	0.342 ± 0.02 ^{c}
Alumium chloride	43.7 ± 0.77 ^{a}	39.4 ± 1.25 ^{a}	13.1 ± 1.21 ^{a}	12.1 ± 1.13 ^{a}	0.415 ± 0.01 ^{c}	0.419 ± 0.02 ^{b}
Tryptophan	27.9 ± 2.43 ^{bc}	25.1 ± 2.51 ^{bc}	8.76 ± 0.86 ^{b}	7.98 ± 0.87 ^{b}	0.482 ± 0.01 ^{b}	0.472 ± 0.02 ^{b}
Chitosan	26.2 ± 2.55 ^{c}	23.5 ± 2.21 ^{c}	8.21 ± 0.75 ^{b}	7.13 ± 0.66 ^{b}	0.563 ± 0.02 ^{a}	0.556 ± 0.03 ^{a}
ANOVA *p* value	***	***	***	***	***	***

Levels of significance are represented by ** $p < 0.01$ and *** $p < 0.001$. For each parameter in the year, different letters within the column show significant differences between the treatments and control according Tukey's HSD test at $p < 0.05$.

Among the different reactive oxygen species (ROS), only hydrogen peroxide is comparatively constant and easily infiltrates the plasma membrane in an unchanged form [71]. In addition to being noxious to chloroplasts and being prevailing inhibitors of the Calvin cycle, hydrogen peroxide is regarded as a signal molecule with a regulatory function in gene expression [71]. The ameliorative role of endophytes on mitigation of the hyper-accumulation of ROS might be due to the upregulation of antioxidant capacity besides ion absorption [20,56]. Generally, amendment with *B. subtilis* improved the redox homeostasis by improving the antioxidant enzyme activities, as well as increasing the concentration of antioxidant solutes [55].

Defensive mechanisms against oxidative injury, including activation of superoxide dismutase, catalase, and peroxidase, can be encouraged by the elicitor's application [55,72]. Numerous studies have proved that abiotic elicitors and endophytes can have valuable impacts on plant development, throughout the eradication of ROS production and maintaining plant water status [28,55]. The lower malondialdehyde concentration in plants treated with chitosan suggests that chitosan protects against oxidative injury. Antioxidant properties of chitosan are largely caused by its abundant energetic hydroxyl and amino groups that react with ROS, forming constant and comparatively harmless macromolecular radicals [73]. Furthermore, the current investigation and others proved that chitosan appli-

cation increased levels of carotenoids, ascorbic acid, and total soluble phenolic compounds, while decreasing the production of ROS [29,30].

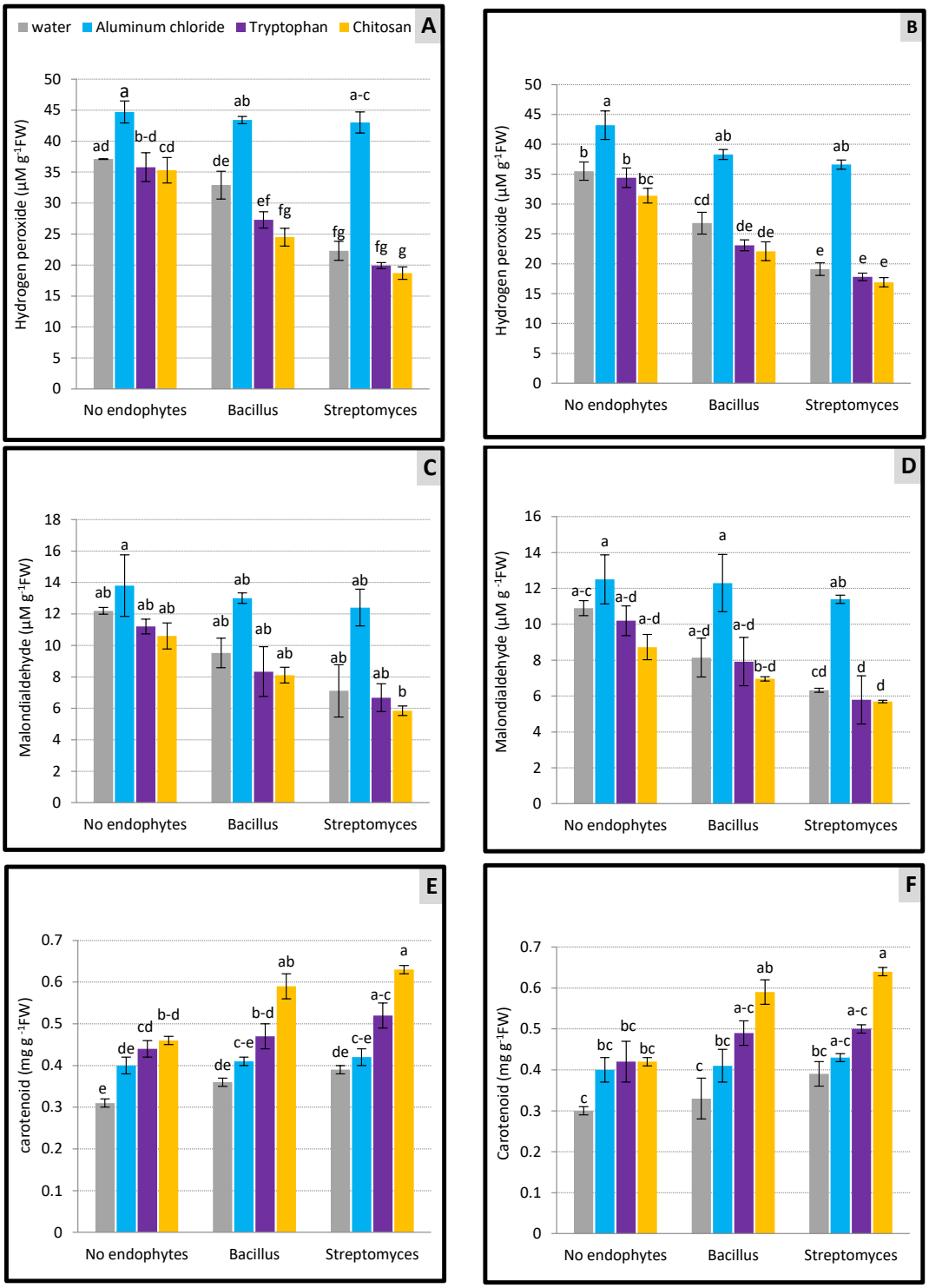

Figure 4. Interactive effect of endophytic microorganisms and abiotic elicitors on (**A**,**B**) hydrogen peroxide (µM g^{-1} FW), (**C**,**D**) malondialdehyde (µM g^{-1} FW), and (**E**,**F**) carotenoid (mg g^{-1} FW) in periwinkle plant shoot in both seasons (FW, fresh weight). Columns w the same letter in each chart are non-significantly ($p \leq 0.05$) different according to Tukey's test. Means of five replicates are presented with ±SE.

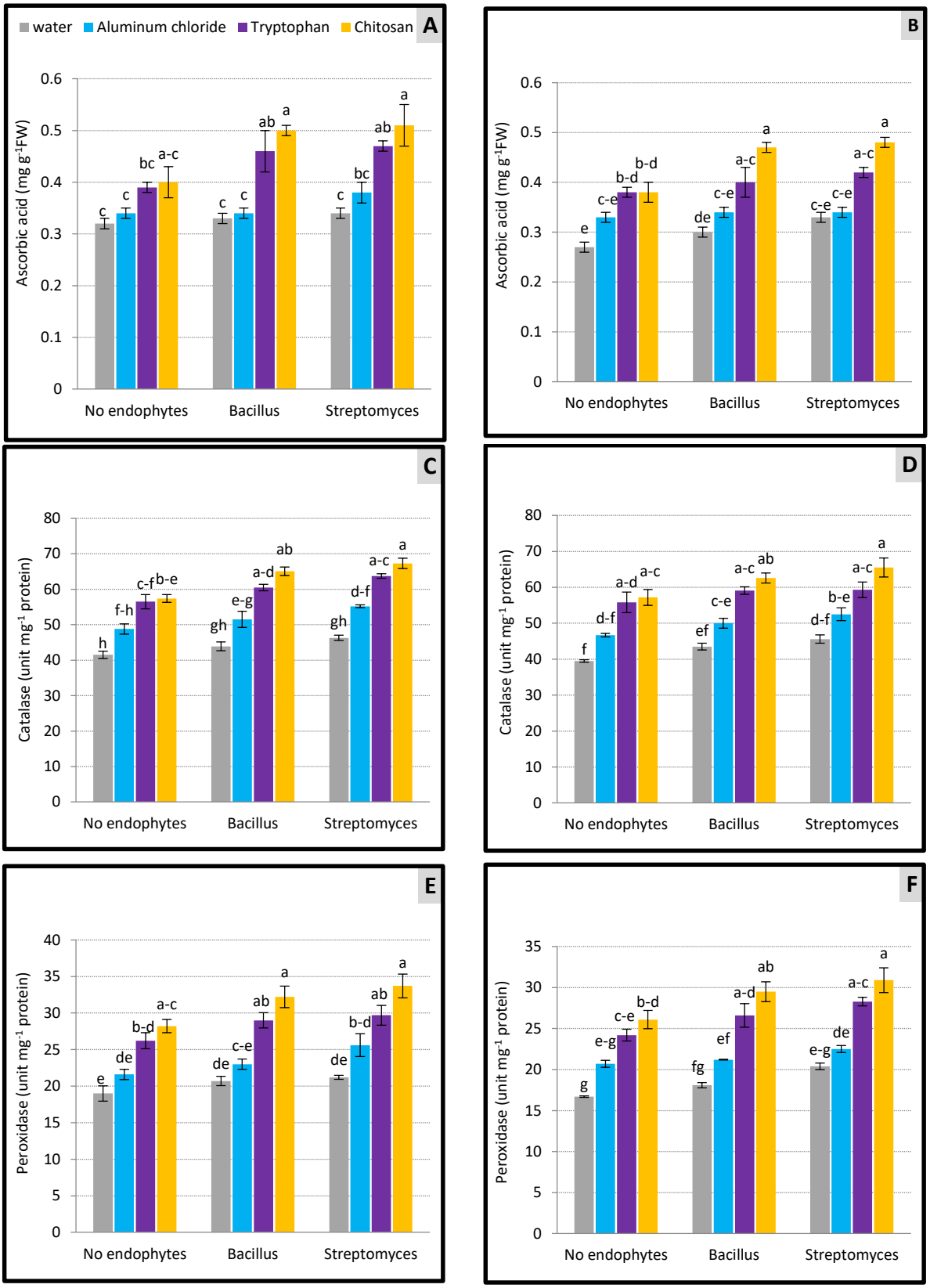

Figure 5. Interactive effect of endophytic microorganisms and abiotic elicitors on (**A**,**B**) ascorbic acid (mg g^{-1} FW), and activity (unit mg^{-1} protein) of (**C**,**D**) catalase and (**E**,**F**) peroxidase enzymes in periwinkle plant shoot in both seasons (FW, fresh weight). Columns with the same letter in each chart are non-significantly ($p \leq 0.05$) different according to Tukey's test. Means of five replicates are presented with ±SE.

Table 4. Effect of endophytic microorganisms and abiotic elicitors on ascorbic acid (AsA; mg g^{-1} FW), and activity (unit m^{-1} protein) of catalase (CAT) and peroxidase (POD) enzymes in periwinkle plant shoot in both seasons (FW, fresh weight). Means of five replicates are presented with ±SE.

Treatment	AsA		CAT		POD	
	First Year	Second Year	First Year	Second Year	First Year	Second Year
Endophytic microorganisms						
No endophytes	0.36 ± 0.01 [b]	0.34 ± 0.01 [b]	51.1 ± 2.10 [c]	49.8 ± 2.30 [b]	23.8 ± 1.17 [b]	21.9 ± 1.12 [c]
Bacillus	0.41 ± 0.02 [a]	0.38 ± 0.02 [a]	55.2 ± 2.59 [b]	53.8 ± 2.32 [a]	26.2 ± 1.45 [a]	23.9 ± 1.40 [b]
Streptomyces	0.42 ± 0.02 [a]	0.39 ± 0.01 [a]	58.1 ± 2.47 [a]	55.5 ± 2.44 [a]	27.5 ± 1.51 [a]	25.5 ± 1.32 [a]
ANOVA *p* value	**	**	***	***	***	***
Abiotic elicitors						
Water	0.33 ± 0.00 [b]	0.30 ± 0.01 [d]	43.9 ± 0.86 [c]	42.9 ± 1.31 [c]	20.3 ± 0.48 [d]	18.4 ± 0.57 [d]
Aluminum chloride	0.36 ± 0.01 [b]	0.34 ± 0.01 [c]	51.8 ± 1.38 [b]	49.7 ± 1.07 [b]	23.4 ± 0.79 [c]	21.5 ± 0.31 [c]
Tryptophan	0.44 ± 0.01 [a]	0.40 ± 0.01 [b]	60.2 ± 1.40 [a]	58.1 ± 1.38 [a]	28.3 ± 0.79 [b]	26.4 ± 0.77 [b]
Chitosan	0.47 ± 0.02 [a]	0.44 ± 0.01 [a]	63.3 ± 1.62 [a]	61.4 ± 1.52 [a]	31.4 ± 1.06 [a]	28.8 ± 0.96 [a]
ANOVA *p* value	***	***	***	***	***	***

Levels of significance are represented by ** $p < 0.01$ and *** $p < 0.001$. For each parameter in the year, different letters within the column show significant differences between the treatments and control according to Tukey's HSD test at $p < 0.05$.

3.5. Phytopharmaceutical and Alkaloid Yield

The application of endophytes drastically increased alkaloid % and alkaloid yield in both seasons relative to non-inoculated plants. The greatest values were obtained by *Streptomyces* sp. relative to *Bacillus* sp. or untreated plants in both seasons (Tables 5 and 6). *Streptomyces* sp. amendment significantly decreased the concentration of total soluble phenols treatment; meanwhile, application of *Bacillus* sp. nonsignificantly decreased it in both seasons relative to non-inoculated. Additionally, application of endophytes in both seasons non-significantly decreased either flavonoids or anthocyanin relative to non-inoculated plants.

Spraying plants with aluminum chloride increased total soluble phenolic compounds, flavonoids, and anthocyanins in both growing seasons. Alkaloid percentage significantly increased with tryptophan application in both seasons, compared with other elicitors or untreated plants. In the meantime, the application of chitosan in both seasons gave the highest alkaloid yield (34.54 and 31.34 mg $plant^{-1}$ respectively) due to its role in increasing shoot dry weight. Figures 6 and 7 introduce the combination effect between endophyte and abiotic elicitors, which indicate the foliar application of aluminum chloride without endophyte gave the highest total soluble phenolic compounds, flavonoids, and anthocyanins above the untreated plants. Conversely, the greatest alkaloid percentage was recorded via tryptophan spraying with *Streptomyces*. Moreover, foliar application of chitosan with *Streptomyces* significantly increased plant alkaloid yield over untreated plants or the other abiotic elicitors.

Natural antioxidants (total soluble phenolic compounds, flavonoids, anthocyanins, ascorbic acid, and carotenoids) are among the most important phytopharmaceuticals molecules that are assimilated by several herbs [74]. The prospective of antioxidant constituents of medicinal herbs for sustaining health and defense against coronary heart disease and cancer is also increasing awareness amongst research groups and food manufacturers as consumers shift to future antioxidants with precise health benefits. Ascorbic acid stands out among the majority of roughly examined non-enzymatic water-soluble antioxidants that play a defensive role against stress-induced ROS, by eliminating or hindering the oxidative chain reactions and decreased risk of cancer and inflammatory diseases [64]. The adequate quantity of ascorbic acid in the leaves of periwinkle is a sign of the capability of the leaves to avoid the development of carcinogens and nullify ROSs that are produced throughout metabolic processes in humans.

Table 5. Effect of endophytic microorganisms and abiotic elicitors on total soluble phenolic compounds (TSPC, mg gallic g^{-1} DW); flavonoids (mg quercetin g^{-1} DW), anthocyanin (mg 100 g^{-1} FW) of periwinkle plant shoot in both seasons. Means of five replicates are presented with ±SE.

Treatment	TSPC		Flavonoids		Anthocyanin	
	First Year	Second Year	First Year	Second Year	First Year	Second Year
Endophytic microorganisms						
No endophytes (N)	18.1 ± 1.44 ^{a}	16.3 ± 3.72 ^{a}	2.28 ± 0.13	2.14 ± 0.15	4.17 ± 0.33	3.75 ± 0.32
Bacillus (B)	17.2 ± 1.36 ^{a}	15.5 ± 1.13 ^{ab}	2.24 ± 0.10	2.06 ± 0.11	3.96 ± 0.32	3.59 ± 0.27
Streptomyces (S)	15.8 ± 1.07 ^{b}	15.0 ± 1.01 ^{b}	2.18 ± 0.11	2.05 ± 0.10	3.81 ± 0.24	3.45 ± 0.25
ANOVA *p*-value	**	*	ns	ns	ns	ns
Abiotic elicitors						
Water (W)	12.0 ± 0.15 ^{d}	11.5 ± 0.20 ^{c}	1.84 ± 0.03 ^{b}	1.65 ± 0.05 ^{c}	2.73 ± 0.16 ^{d}	2.52 ± 0.09 ^{d}
Aluminum chloride (A)	22.5 ± 0.69 ^{a}	20.9 ± 0.50 ^{a}	2.71 ± 0.09 ^{a}	2.60 ± 0.11 ^{a}	5.17 ± 0.16 ^{a}	4.84 ± 0.17 ^{a}
Tryptophan (T)	14.4 ± 0.81 ^{c}	12.6 ± 0.15 ^{c}	1.96 ± 0.01 ^{b}	1.90 ± 0.02 ^{bc}	3.59 ± 0.13 ^{c}	3.09 ± 0.16 ^{c}
Chitosan (C)	19.2 ± 0.56 ^{b}	17.4 ± 0.74 ^{b}	2.42 ± 0.10 ^{a}	2.18 ± 0.07 ^{b}	4.42 ± 0.17 ^{b}	3.93 ± 0.11 ^{b}
ANOVA *p* value	***	***	***	***	***	***

Levels of significance are represented by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns not-significant. For each parameter in the year, different letters within the column show significant differences between the treatments and control according to Tukey's HSD test at $p < 0.05$.

Table 6. Effect of endophytic microorganisms and abiotic elicitors on alkaloid percentage and alkaloid yield (mg $plant^{-1}$ DW) of periwinkle plant shoot in both seasons. Means of five replicates are presented with ±SE.

Treatment	Alkaloid%		Alkaloid Yield	
	First Year	Second Year	First Year	Second Year
Endophytic microorganisms				
No endophytes	0.592 ± 0.01 ^{c}	0.548 ± 0.01 ^{c}	21.5 ± 1.7 ^{c}	17.6 ± 1.7 ^{c}
Bacillus	0.620 ± 0.01 ^{b}	0.603 ± 0.01 ^{b}	25.3 ± 2.3 ^{b}	22.2 ± 2.4 ^{b}
Streptomyces	0.639 ± 0.01 ^{a}	0.620 ± 0.01 ^{a}	29.0 ± 2.6 ^{a}	25.5 ± 2.6 ^{a}
ANOVA *p*-value	***	***	***	***
Abiotic elicitors				
Water	0.558 ± 0.01 ^{c}	0.514 ± 0.02 ^{b}	20.0 ± 0.71 ^{c}	15.5 ± 1.11 ^{c}
Aluminum chloride	0.641 ± 0.01 ^{a}	0.617 ± 0.01 ^{a}	16.4 ± 0.72 ^{d}	13.9 ± 0.58 ^{c}
Tryptophan	0.652 ± 0.01 ^{a}	0.625 ± 0.01 ^{a}	30.2 ± 1.81 ^{b}	26.3 ± 1.26 ^{b}
Chitosan	0.617 ± 0.01 ^{b}	0.606 ± 0.01 ^{b}	34.5 ± 1.50 ^{a}	31.3 ± 2.04 ^{a}
ANOVA *p* value	***	***	***	***

Levels of significance are represented by *** $p < 0.001$. For each parameter in the year, different letters within the column show significant differences between the treatments and control according to Tukey's HSD test at $p < 0.05$.

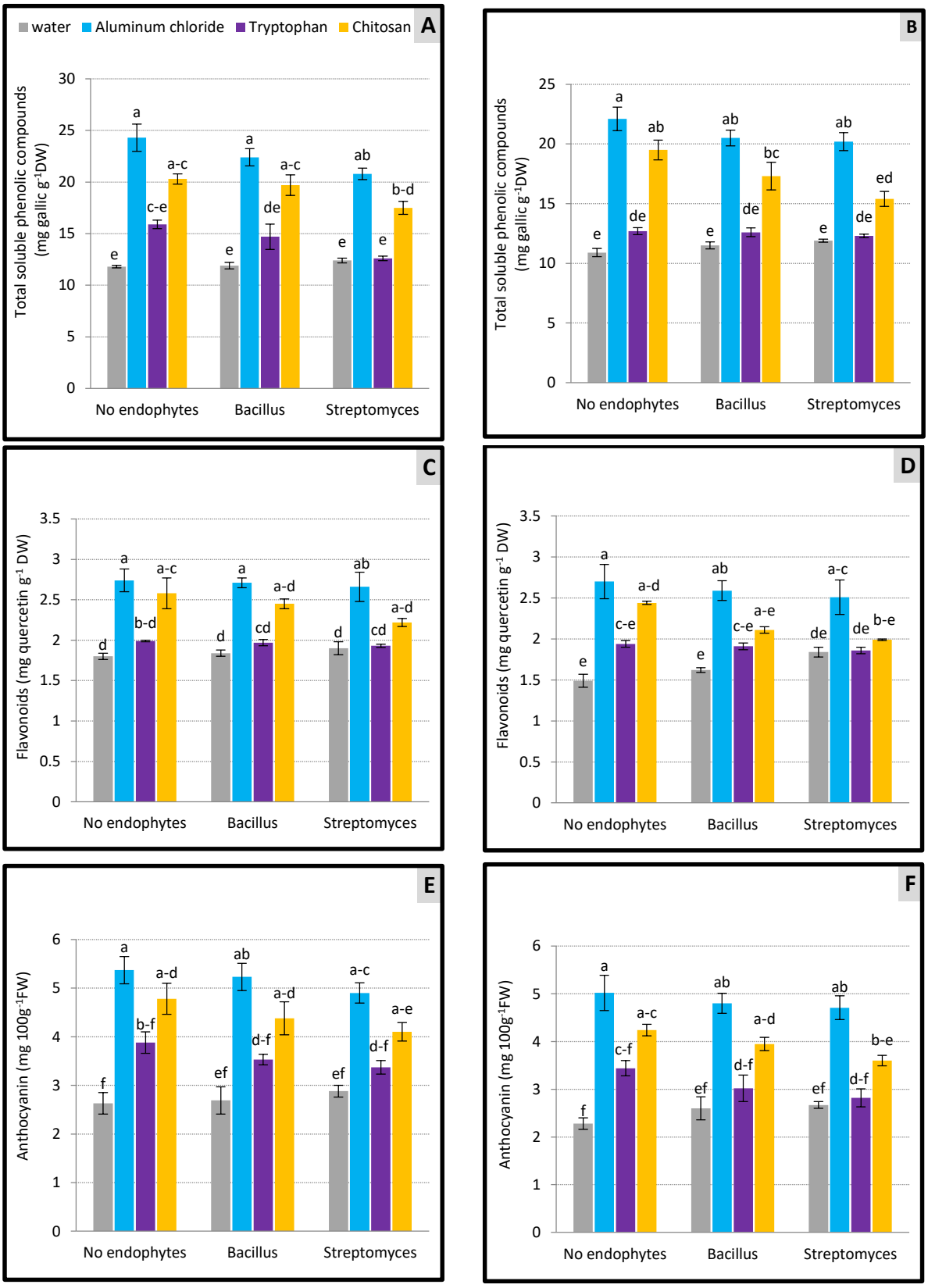

Figure 6. Interactive effect of endophytic microorganisms and abiotic elicitors on (**A**,**B**) total soluble phenolic compounds (TSPC, mg gallic g^{-1} DW), (**C**,**D**) flavonoids (mg quercetin g^{-1} DW), and (**E**,**F**) anthocyanin (mg 100 g^{-1} FW) of periwinkle plant shoot in both seasons. Columns with the same letter in each chart are non-significantly ($p \leq 0.05$) different according to Tukey's test. Means of five replicates are presented with ±SE.

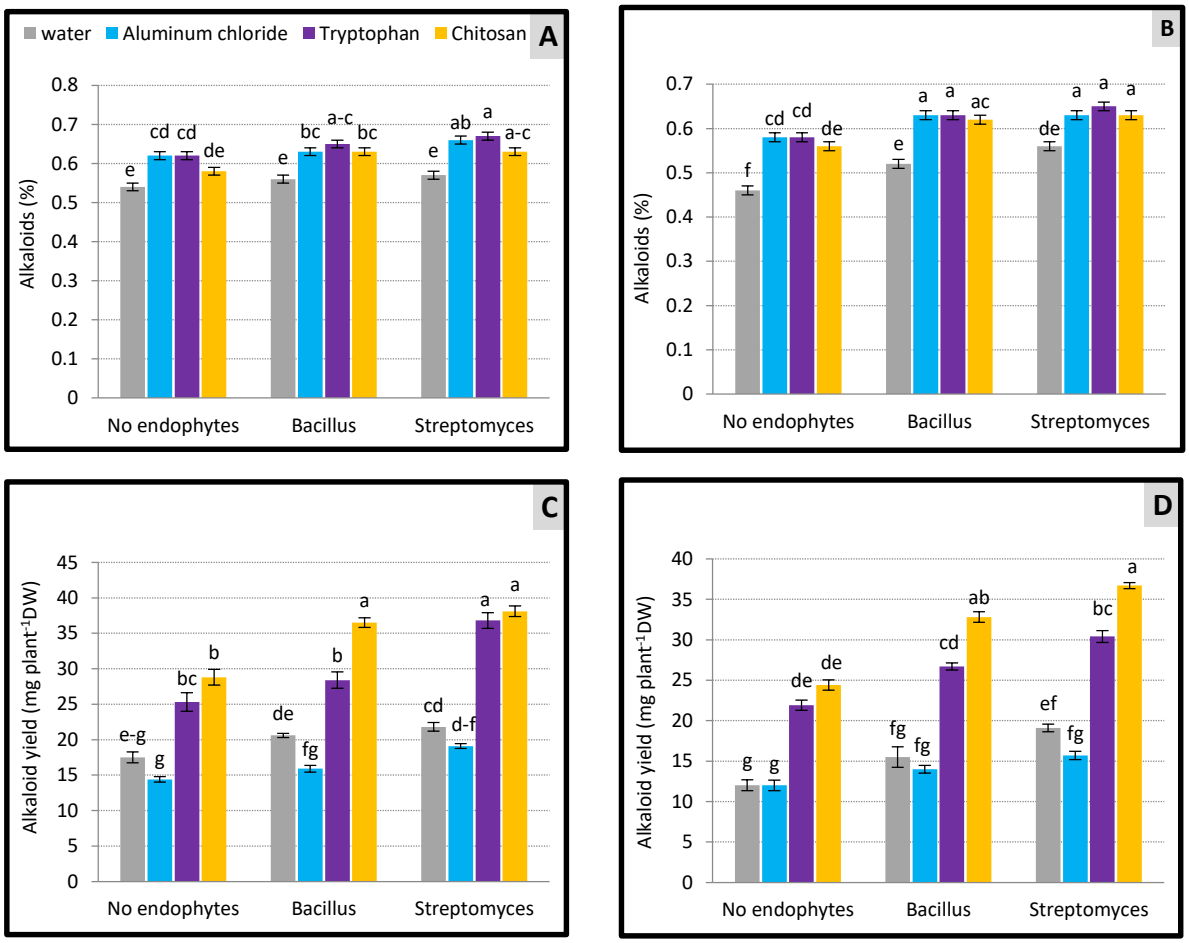

Figure 7. Interactive effect of endophytic microorganisms and abiotic elicitors on (**A**,**B**) alkaloid percentage and (**C**,**D**) alkaloid yield (mg plant^{-1} DW) of periwinkle plant shoot in both seasons. Columns with the same letter in each chart are non-significantly ($p \leq 0.05$) different according to Tukey's test. Means of five replicates are presented with ±SE.

Phenolic compounds (phenolic acids, flavonoids, and anthocyanins) have been considered as a major bioactive substance with a strong antioxidant and have been revealed to be more efficient than ascorbic acid, tocopherol, and carotenoid [21,75], so they have drawn extra consideration in recent years. They possess different biological functions, such as anti-inflammatory, antioxidant, antiviral, cytotoxic, and anticarcinogenic [76]. Observational epidemiology studies have revealed that a considerable nutritional intake of flavonoids and total soluble phenolic compounds are connected with lower rates of occurrence of diverse cancers [75]. The antioxidant capacities of total soluble phenolic compounds are mediated by several strategies [77]: (1) nullify ROS/reactive nitrogen species (RNS); (2) suppress ROS/RNS production by inhibiting several enzymes or chelating trace ions occupied in ROS; (3) upregulate antioxidant system. Many phytopharmaceuticals acquire considerable antioxidant capacities that may be linked with minor incidence and lower death rates of cancer in human populations [78]. Several valuable bioactive constituents' production was recorded to be motivated by elicitor [5,18]. The mechanism of elicitation was, nevertheless, varied in diverse plants, and in the majority, and 'elicitor-receptor' complex was produced and a vast array of physio-biochemical responses were manifested [5,64]. The present outcomes have proved that elicitors induced the excess-accumulation of total soluble phenolic compounds in a periwinkle shoot. This could be due to triggering signal transduction systems and inducing gene expression of secondary metabolic enzyme pathways in special phenyl aminolyase (PAL), thus consequently leading to biosynthesis of phenolic constituents. Chitosan amendment enhanced phenol assimilation in different plants [79,80].

The encouraging effect of chitosan on phenolic compounds may be due to the buildup of an aromatic amino acid (phenyl alanine and tyrosine) and phenylpropanoid compounds [81]. Additionally, chitosan motivates the phenyl propanoid pathway in spinach as it elicits PAL activity besides improved cinnamic acid assimilation [82]. Increased PAL activity with chitosan treatment is coupled with the accumulation of phenolic compounds in different plants [83,84].

In earlier reports, endophytes [85]—abiotic elicitors such as chitosan and aluminum chloride as well as alkaloid precursor [5,13]—could boost the accumulation of phytopharmaceuticals of interest by activating specific secondary metabolic pathways, but the synergistic effect of elicitors is not completely understood. The alkaloid biosynthesis pathway is an incorporation complex and highly regulated, which needs linking of numerous steps, continuous precursor assimilation, transport and translocation to the biosynthesis site, and finally transport to the accumulation position. This sequence of steps depends on the ordinary functioning of related metabolic processes. Elicitors are effective signals that have recognized efficiency in changing metabolic pathways, in numerous medicinal herbs such as periwinkle [25,86]. Additionally, *Streptomyces* inoculation was established to be highly advantageous compared with *Bacillus* for increasing alkaloid production. Presently, it has been revealed that microorganisms' amendment could be applied as a possible method to boost the concentration of main TIAs in periwinkle [87]. Chitosan is known to elicit activation, leading to a variety of defensive responses including accumulation of secondary metabolites [5]. They observed that chitosan at 100 mg L^{-1} improved alkaloid production. To the best of our knowledge, the effect of aluminum chloride on alkaloid assimilation is yet to be studied. Aluminum chloride has been validated as a significant elicitor in improving growth and later motivating enriched levels of phytochemicals in plants [13]. The precise mechanism of aluminum chloride in enhancing alkaloids is not identified so far; however, it possibly upregulates gene encoding enzymes, especially tryptophan decarboxylase which participates in the biosynthetic pathway of alkaloid biosynthesis [88].

Finally, it has been effectively established that the values of plant growth, physiological trials, and phytopharmaceuticals were declined in the second year compared with the first year. This decrease may be related to the exposure of plants to high temperatures in late March. Commonly, growth reduction elicited by extreme temperature is frequently connected with up- and downregulation of the cell cycle, as well as decreased photosynthetic pigments, and this disrupts a plant's water status [89]. Moreover, Alhaithloul et al. [90] proved that heat stress generally decreased *Mentha piperita* and *Catharanthus roseous* growth and its phytopharmaceutical components.

4. Conclusions

The current results proved that the application of *Streptomyces* sp. as a bio-inoculant with chitosan as foliar spraying could enhance periwinkle plant biomass, phytopharmaceutical accumulation, and alkaloid production. Such interactions could provide increased plant biomass coupled with elevated alkaloid production that could, in turn, contribute an essential role in reducing the cost of producing bis-indole alkaloid.

Author Contributions: This work was carried out in collaboration with the authors. Conceptualization, S.F., A.A.A. and S.M.A.E.-G.; Methodology, S.F.; Software, S.F.; Validation, S.F., A.A.A. and S.M.A.E.-G.; Formal analysis, S.F.; Investigation, S.F. and S.M.A.E.-G.; Resources, S.F. and A.A.A.; Data curation, S.F., A.A.A. and S.M.A.E.-G.; Writing—original draft preparation, S.F., A.A.A. and S.M.A.E.-G.; Writing—review and editing, S.F.; Visualization S.F., A.A.A. and S.M.A.E.-G.; Supervision, S.F.; Funding acquisition A.A.A. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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