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Developmental, Phytochemical and Enzymatic Changes in Pot Marigold (*Calendula officinalis* L.) cvs. Hybrid and French with Salicylic Acid (SA) and Polyamine Spermidine (SP) Foliar Spray

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Abstract: Marigolds (*Calendula officinalis* L.) are valuable in ornamentation, human food, and other uses; to enhance productivity, plant growth regulators produce stimulatory effects, including salicylic acid (SA) and spermidine (SP), but there is a lack of scientific evidence about such effects in marigolds. The study assessed, under greenhouse conditions, changes in physico-chemical parameters, enzymatic activity, and bioactive compounds of marigold cvs. Hybrid and French marigolds were sprayed of SA (1 and 2 mM) and SP (2 and 3 mM) and compared to control (pure water). The SA at 2 mM improved leaf length (8.20 cm), flower height and diameter (5.32, 8.28 cm), flower fresh and dry weight (14.30, 1.5 g), and the maximum number of flower petals (55) in 'Hybrid'. Similarly, 2 mM SA gave the maximum number of leaves (40.71) and stem thickness (5.76 mm) in 'French', but 3 mM SP promoted the maximum plant height in 'Hybrid'. Superoxide dismutase, peroxidase, and catalase activities increased in 'Hybrid' with 2 mM SA; with this SA dose, 'Hybrid' had higher contents of total phenolic compounds (68.34 mg GAE g^{-1}), antioxidants (77%), carotenoids (110 mg 100 g^{-1}), and flavonoids (67.5 mg RE g^{-1}) than the control. The best dose for improving growth in both marigold varieties was 2 mM SA.

Keywords: ornamentals; biostimulants; plant growth regulators; secondary metabolites

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1. Introduction

Calendula officinalis L. is an important medicinal and ornamental plant belonging to the family *Asteraceae*, known as pot marigold or calendula [1]. Its flowers are usually big with globular shapes and contain an extensive quantity of healthful elements used to treat different diseases [2]. Cultivation of marigold plants is easy due to its wide adaptability and flexibility to various soils and climatic conditions [3]. It has been utilized in modern medicine, nylons, and dyes industries in raw as well as in processed forms [2].

With the increased interest in and demand for medical products in various industries, creative and eco-friendly methods of increasing the quantity and quality of medicinal plants are being adopted [4]. Poor flower quality of marigolds is always a serious problem in the production of these plants. Its flowers produce and store active ingredients, such as water-soluble carotenoids, flavonoids, essential oil, and mucilaginous compounds [5]. In order to improve production and yield, the world has focused on plant growth regulators (PGRs) and better nutrient management programs that farmers and growers should adopt practically in floriculture to improve their quality and yield components. PGRs have received widespread recognition for optimizing plant production by regulating growth and development in different stressing environments [6]. Previous studies indicate that many experiments had been conducted to encourage ornamental plant growers by treating the plants with environmentally safe substances, such as gibberellic acid, oxalic acid, and salicylic acid [7]. PGRs play a crucial role in flower production by promoting, inhibiting, or quantitatively modifying growth and development [6]. Commercially, PGRs are used to reduce apical dominance, slow vegetative development, induce lateral buds, and produce a large number of flowers in various crops, resulting in a higher flower yield and ease of cultivation [8,9].

Several examples of plant growth hormones are used to regulate flowering in aromatic plants, which quantitatively alters growth and development. For instance, salicylic acid (SA, 2-hydroxybenzoic acid) is a phenolic compound of hormonal nature produced by plants and plays an important role in responses to several abiotic stresses and pathogen attacks [10]. SA has also been studied for its effect on various physiological processes related to vegetative growth and development of plants under normal conditions (no stress) [11]. However, published literature reported that SA is an effective endogenous regulator of flowering plants [12]. Exogenous application of SA enhances various physiological processes, such as seed germination, photosynthesis, plant growth, and development [13]. A genetic study reported that spraying SA on plants during flowering time acts as a regulator that works with both photoperiod-dependent and capable pathways [14]. SA also has an additional effect on medicinal plants; it promotes the synthesis of secondary metabolites, which increases the medicinal and commercial value of the plant [15]. The application of SA in various medicinal plants significantly increased the synthesis of flavonoids in reactions, such as *Matricaria chamomilla* [16]. SA also affects some essential antioxidants, such as peroxidase (POX), catalase (CAT), and superoxide dismutase (SOD) [13].

Spermidine [SP, N-(-3-aminopropyl)-1.4-diaminobutane] is a plant polyamine that promotes membrane protection under stress conditions. It also coexists with ethylene and a common precursor known as S-adenosylmethionine, through which they both compete with ethylene synthesis and are known as anti-aging and anti-stress molecules [17]. Polyamines can help in seed germination, tuber dormancy breakdown, rooting regulation, flower growth and initiation, aging delay, organogenesis, and the response to biotic and abiotic stresses [18]. Polyamines also function as regulatory molecules in basic biological processes, such as cell division, differentiation, gene expression, and DNA and RNA synthesis [19]. In addition, SP produced by bacteria may have a main indirect role in the defense of plants from pathogen attacks [20].

There have been many studies related to the effects of elicitors on various physiological processes related to the vegetative growth and development of plants. However, there has been insufficient scientific evidence about the effects of plant growth regulators, especially polyamines, on biochemical changes in marigolds. Further, it is not clear whether the exogenous application of SA and SP can significantly ameliorate the growth and physiology of marigold plants. With the growing interest in and demand for medicinal products in various industries, a high-throughput approach to increase the quantity and quality of medicinal plants such as marigold is considered necessary. Therefore, in the current study, the effects of phytohormone (i.e., Salicylic acid) and polyamine (i.e., Spermidine) on growth, flowering, and synthesis of secondary metabolites, enzymatic, and antioxidant activity of *Calendula officinalis* plants were evaluated.

2. Materials and Methods

2.1. Experimental Design and Treatments Preparation

The present experiment was conducted under greenhouse conditions at the horticulture nursery, The University of Haripur, Pakistan (72° 54′ 45.7 °E, 33° 58′ 41.4° N, and 610 m above sea level) from 7 October 2017 to 14 January 2018. The seeds of 'Hybrid' and 'French' marigold were purchased from a commercial nursery of Haripur, Pakistan. Nursery of both 'French' and 'Hybrid' marigold cultivars occurred by sowing 5–6 seeds, and when seedlings were 5–7 cm tall with 3–4 leaves, marigold plantlets were transplanted to the main pots (5 L), containing a medium made of sand and clay with farmyard manure (FYM), and maintained at the field water capacity. The experiment was conducted in triplicate, following a completely randomized design (CRD) in a two-factorial arrangement [exogenous application treatment (T), cultivar (C), and interactions (TxC)] with a set of 30 pots. There were five application treatments: CK (Control) (Spray water), SA1 (foliar application of SA 1 mM), SA2 (foliar application of SA 2 mM), SP2 (foliar application of SP 2 mM), SP3 (foliar application of SP 3 mM). The pots were placed in a greenhouse and irrigated daily to maintain the substrate at field capacity throughout the experiment. During the experiment, complete fertilizer of NPK at a ratio of 120:90:60 kg ha^{-1} was applied to all pots. SA and SP were applied by spraying the aerial part of the plants at the four-leaf stage after each 10 days of interval. A total of 100 mL solution was sprayed to each plant, each time.

2.2. Vegetative Growth and Floral Examination

A number of 3 plants per treatment of both 'Hybrid' and 'French' were randomly collected to assess vegetative and floral parameters for each treatment, such as plant height (cm), leaf length (cm), number of leaves per plant, stem thickness (mm), full bloom flowers per plant, flowers height (cm), flowers diameter (cm), number of petals per flower, number of flowers per bud. The flowers were cut at the full flowering stage, separated, cleaned, and air-dried in the shade for two weeks. The air-dried flowers were crushed into fine powder and stored in polyethylene pouches until usage in the extraction of marigold constituents.

2.3. Antioxidant Activity

Total antioxidant activity of the marigold flower heads was measured against 2,2-diphenyl-1-picryl-hydrazul (DPPH) [21]. About 0.4 g of dried inflorescence sample was extracted with 4 mL methanol 99% at room temperature. An amount of 100 µg of the methanolic extract were rapidly mixed with 4 mL methanolic solution of DPPH (40 mgL^{-1}, Sigma-Aldrich, St. Louis, MO, USA). After 50 min incubation in darkness at room temperature, the absorbance of incubated solutions and DPPH was read at 517 nm using the microplate ELISA reader (Epoch, Biotek industries, Highland Park Winoosly, VT, USA). The decline in radical concentration indicated the radical scavenging activity of the sample. The following equation was used to calculate the inhibition percent of DPPH:

$$\% \text{ inhibition of DPPH} = 100 \times [(Abs_{517} \text{ in blank}) - (Abs_{517} \text{ in sample})] \div (Abs_{517} \text{ in blank})$$

2.4. Total Phenolic Compound

The Folin–Ciocalteu (FC) reagent method was used to calculate the total phenolic compound contents (TPC) as stated by Ainsworth et al. [22]. Each dried inflorescence sample was dissolved with 100 µL of FC-reagent mL (10% in distilled water) and vortex carefully; then Na_2CO_3 (700 µL) reagent was dissolved and kept at 25 °C for 1 h. The absorbance of the solution (100 µL) was read at 765 nm. The total phenol content of the samples was calculated according to standard curves of gallic acid and reported in mg of gallic acid equivalent (GAE) per g fresh weight (FW).

2.5. Total Carotenoids Content

Total carotenoids (TC) contents were evaluated in flower heads extract (5 mL), taken out with 1 mL of pure acetone, then the mixture was standardized for 1 min and kept warm at 4 °C in darkness until the cap changed white. The uniform structure was centrifuged at 16,000× *g* for 15 min, and 200 µL of supernatant from each tube were sited in 96-well plates. The reading of absorbance was at 470 nm in a microplate reader (PowerWave HT, Biotek, Biotek Instruments, VT, USA) [23].

2.6. Total Flavonoids Content

For total flavonoids (TF), flower heads were treated with 2 mL of acetone and thereafter the solution was homogenized for 2 min and placed at 4 °C under shadow. The uniform structure was centrifuged at 16,000× *g* for 16 min, and 200 µL of supernatant from each tube was taken. Standard curve was drawn using Rutin as standard. Different concentrations of Rutin were prepared and the absorbance was read at 510 nm. Total flavonoids content of samples was calculated according to standard curves of Rutin and reported as mg of Rutin equivalence (RE) per g fresh weight.

2.7. Qualitative Analysis of the Phytochemicals

The qualitative phytochemical screening of the extracts was performed following the procedures outlined by Edeoga et al. [24], Orech et al. [25], Sofowora [26], Makkar et al. [27] to identify the main groups of chemical constituents present in the extracts using the color reactions.

Test for alkaloids

Mayer's Test: after filtering, 1 mL extract was treated with 1 mL of Mayer's chemical (iodine in potassium iodide), which the formation of red and reddish acceleration indicates the presence of alkaloids.

Test for saponins

Froth Test: The extracts were diluted with 20 mL of distilled water and shaken in a graduated cylinder for 15 min. A foam layer of about 10 mm was formed, indicating the presence of saponin.

Test for phytosterols

Salkowski's Test: Extract (5 mL) was mixed with chloroform (2 mL), and concentrated sulphuric acid (3 mL) was carefully added to form a layer. A reddish-brown coloration of the inter face was formed to show positive results for the presence of triterpenes.

Test for tannins

Gelatin Test: A total of 5% of gelatin solution to 1 mL of the extract containing the sodium chloride was added. The formation of white sign indicates the presence of tannins.

Test for flavonoids

Alkaline reagent Test: The development of intense yellow color was detected on addition of a few drops of sodium hydroxide solution to the test solution, which turns colorless after adding a few drops of dilute acid, indicating the presence of flavonoids.

Test for proteins and amino acids

Xanthoproteic Test: The extract was treated with a few drops of concentrated nitric acid. The formation of yellow color indicates the presence of proteins.

Test for diterpenes

Copper acetate Test: Extracts were dissolved in water and treated with 3-4 drops of copper acetate solution. The formation of emerald green color indicates the presence of diterpenes.

2.8. Antioxidant Enzyme Activities

The petal samples were stored in liquid nitrogen at −30 °C. In a mortar, 1 g of frozen petals was homogenized with 5 mL of 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA, 1 mM dithiothreitol, and 2% polyvinyl pyrrolidone (PVP). The homogenate was centrifuged for 25 min at 15,000× *g*, and the supernatant was used for SOD, POD, and CAT assays.

Superoxide dismutase

The superoxide dismutase activity (SOD) was determined according to Fernandez et al. [28] and was taken on the generation of superoxide radicals by a microbial NADH-diaphorase, recognized by oxidation of hydroxylamine, soft nitrite, which is measured calorimetrically with sulphadiazine and naphthyl ethylenediamine. The simultaneous oxidation of superoxide radicals by SOD prevents the oxidation of hydroxylamine avoiding the absorption of these radicals. The SOD reaction was carried out by exposing the mixture to white light for 15 min at room temperature. After 15 min incubation, absorbance of the solution was measured at 560 nm for both blank and control. SOD activity was expressed as unit mg^{-1} dry weight (DW).

Peroxidase

The extraction of peroxidase (POD) was carried out as described by Berger [29]. The reaction mixture contained 3 mL 0.1 M phosphate buffer (pH 7.0), 0.05 mL 20 mM guaiacol, 0.03 mL H_2O_2, and 0.5 mL enzyme. Enzyme action was noticed by an increase in the absorbance at 436 nm in an electronic 20 spectrophotometer (ultraviolet-2600, Shimadzu, Japan). POD activity was expressed as a unit mg protein per min.

Catalase

The activity of catalase (CAT) was assayed according to the method of Clairbone [30]. The method was used directly to measure the reduction in absorbance at 240 nm (ultraviolet-2600, Shimadzu, Japan) due to H_2O_2 intake by CAT. To improve the assessing conditions, relatively low H_2O_2 concentrations were used, which were accepted in 50 mM K-phosphate buffer, pH 7.0, with 10 mM H_2O_2 at 25 °C. The CAT exact activity was expressed as absorbance in mg protein per min.

2.9. Statistical Analysis

Results were statistically analyzed by considering cultivar (C), treatment application (T), and their interaction (C×T) as sources of variation, using two-way analysis of variance (ANOVA). Statistical differences with P-values under $\alpha = 0.05$ were considered significant and comparison of mean values was carried out by applying LSD method using Statistic 8.1 software.

3. Results

3.1. Vegetative Parameters

3.1.1. Plant Height

Statistical analysis of data revealed that exogenous application of treatments at various concentrations significantly affected the plant height (cm) of marigold cultivars. Mean data for plant height among marigold cultivars showed that taller plants (21.5 cm) were recorded for cv. Hybrid, while shorter plants (17 cm) were observed for cv. French (Figure 1A). Effect of different treatments on plant height of marigold indicated that taller plants (22.5 cm) were recorded under exogenous application of 3 mM of spermidine (SP3), followed by treatments with 2 mM of salicylic acid (SA2) (21.8 cm), while the lowest plant height (15.5 cm) was observed in control treatment (Figure 1A). The interactive effects of treatments by cultivars (T × C) significantly affected the plant height of marigold (Table S1). The highest plant height (24.11 cm) was recorded in 'Hybrid' plants under exogenous

application of SP3, while the control plants of 'French' marigold gave the lowest plant height (13.5 cm), followed by 'French' cultivar treated with 2 mM spermidine (14.5 cm).

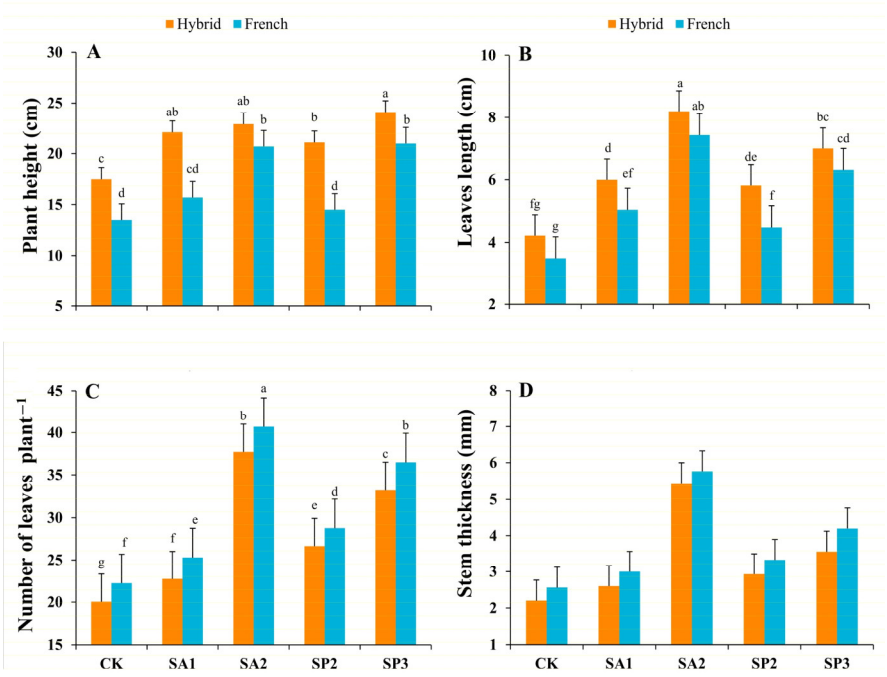

Figure 1. Effects of foliar application of salicylic acid and spermidine on the vegetative parameters of two cultivars (Hybrid and French) of marigold plant; (**A**) plant height, (**B**) leaves length, (**C**) number of leaves plant^{-1}, and (**D**) stem thickness, respectively. CK, control (only water); SA1, salicylic acid @ 1 mM; SA2, salicylic acid @ 2 mM; SP2, spermidine @ 2 mM; SP3, spermidine @ 3 mM. Capped bars represent the standard error (n = 3). Letters above the error bars represent the significant variations among treatments at $p \leq 0.01$.

3.1.2. Leaf Length

Results revealed that the exogenous application treatments and cultivar effects on leaf length were non-significant. However, significant variations for leaf length were found due to their interaction (T × C) (Table S1). The highest leaf length (8.20 cm) was recorded in 'Hybrid' plants under exogenous application of SA2, while the control plants of 'French' marigold gave the lowest leaf length (3.49 cm), followed by 'French' cultivar treated with 2 mM spermidine (4.48 cm) (Figure 1B).

3.1.3. Number of Leaves per Plant

Analysis of the results showed that the number of leaves per plant was not significantly influenced by the various treatments and cultivars. However, significant variations for number of leaves per plant were found due to their interaction (T × C) (Table S1). The highest number of leaves per plant (40.71) was recorded in 'French' plants under exogenous application of SA2, while the control plants of 'Hybrid' marigold gave the lowest value (20.04), followed by 'Hybrid' cultivar treated with SA1 (22.73) (Figure 1C).

3.1.4. Stem Thickness

The multivariate analyses showed significant differences in stem thickness for both sources of variation (cultivars and treatments). However, the interactions treatments by cultivars (T × C) were found non-significant (Table S1). Data indicated that exogenously applied salicylic acid and spermidine caused significant changes in stem thickness of marigold plants. Maximum stem thickness was recorded (5.76 mm) under SA2 treatment (Figure 1D), while the lowest stems thickness (2.22 mm) was found in 'Hybrid' marigolds that had only been sprayed with water (control).

3.2. Reproductive Parameters

3.2.1. Flower Height

Significant variations were found among exogenous application treatments and between the cultivars on flower height, while interactions between treatments by cultivars (T × C) had non-significant effect (Table S2). The highest flower height (5.32 cm) was recorded in 'Hybrid' marigold, while the lowest flower height (4.80 cm) was recorded in 'French' marigold. Greatest flower height (8.01 cm) was obtained under salicylic SA2 treatment, whereas minimum flower height (3.11 cm) was noted in the control treatment where no growth regulators was applied, not statistically different from SA1 treatment (Figure 2A).

3.2.2. Bloom Flowers per Plant

Significant variations were detected in treatment and cultivars, while interaction between (T × C) had a non-significant effect on bloom flowers (Table S2). The maximum number of blooms flowers plant^{-1} (3.70) was recorded in 'Hybrid' marigold compared to 'French' marigold (3.19). Results showed that more bloom flowers plant^{-1} (6.4) was recorded from SP3 treatments, while the control treatment had the minimum bloom flowers plant^{-1} (1.95) (Figure 2B).

3.2.3. Flower Diameter

Results revealed that the various treatments and marigold cultivars significantly influenced the flower diameter, while a non-significant effect was shown in treatments by cultivars interactions (Table S2). The exogenous application of salicylic acid at 2 mM produced the highest flower diameter (8.28 cm), followed by foliar sprayed with SP3 (5.71 cm), whereas the lowest value was recorded in the control plant (3.01 cm) (Figure 2C). 'Hybrid' marigold recorded the highest values of flower diameter with a mean value of 5.23 cm.

3.2.4. Nodes of Flower per Plant

Significant variations were found between treatments and among the marigold cultivars on nodes of flower per plant, while interactions between treatments by cultivars (T × C) had a non-significant effect (Table S2). The exogenous application of salicylic acid at 2 mM showed the highest results of nodes of flower per plant (11.83), followed by marigold treated by SP3 (9.80), whereas the lowest value (6.65) was recorded in control plants (Figure 2D). 'Hybrid' marigold recorded the highest values of nodes of flower per plant with a mean of 10.56, while 'French' marigold recorded only a mean of 7.12 flower per plant.

3.2.5. Fresh Weight of Flowers

The multivariate analyses showed significant differences in flower fresh weight for both sources of variation and their interaction (treatment, cultivar, and T × C) (Table S2). Mean data showed the highest values (7.59 g) for cv. Hybrid, while the lowest values (5.6 g) were observed for cv. French. Effect of different treatments on flower fresh weight indicated that higher values (11.7 g) were recorded under exogenous application of SA2, followed by treatments with SP3 (7.4 g), while the lowest values (3.9 g) were observed in

control treatment (Figure 2E). The interaction effects of treatments by cultivars revealed that 'Hybrid' marigold under exogenous application of SA2 produced the highest flower fresh weight (14.30 g), while the control plants of 'French' marigold gave the lowest fresh weight (3.4 g), although not statistically different from SA1 and SP2 and 'Hybrid' under control treatment.

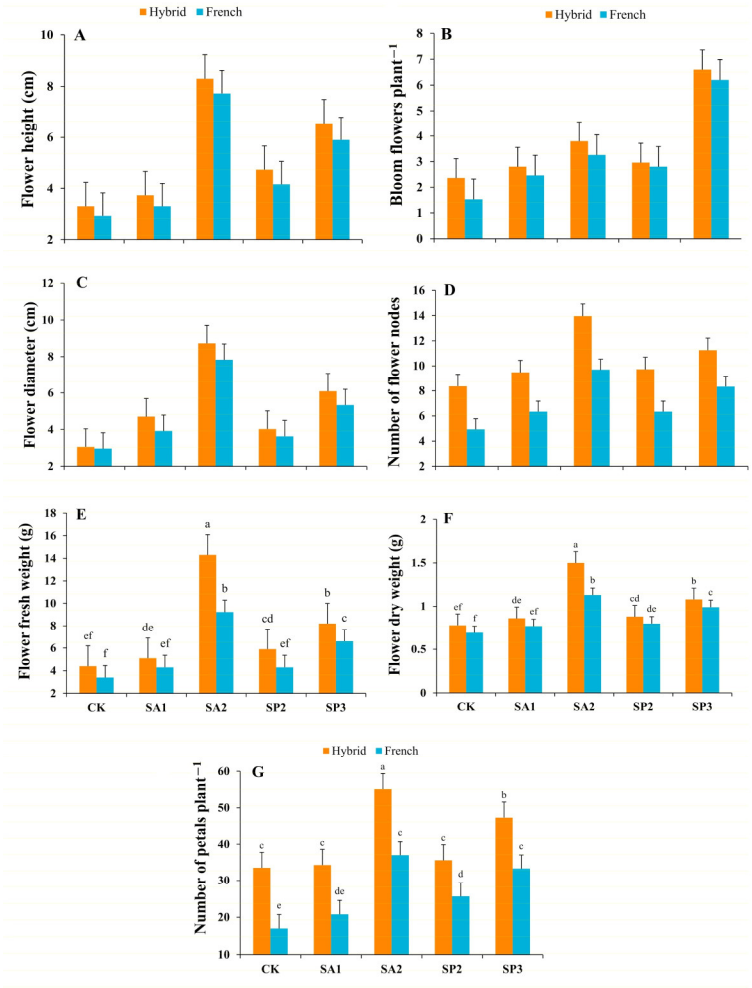

Figure 2. Effects of foliar application of salicylic acid and spermidine on the floral parameters of two cultivars (Hybrid and French) of marigold plant: (**A**) flower height, (**B**) bloom flower plant^{-1}, (**C**) flower diameter, (**D**) number of flower nodes, (**E**) fresh weight of flower, (**F**) dry weight of flower, and (**G**) number of petals. CK, control (only water); SA1, salicylic acid @ 1 mM; SA2, salicylic acid @ 2 mM; SP2, spermidine @ 2 mM; SP3, spermidine @ 3 mM. Capped bars represent the standard error (n = 3). Letters above the error bars represent the significant variations among treatments at $p \leq 0.01$.

3.2.6. Dry Weight of Flower

The ANOVA showed significant differences in flower dry weight for both sources of variation and their interaction (treatment, cultivar, and T × C) (Table S2). The highest value (1.02 g) was recorded for cv. Hybrid, while the lowest value (0.87 gm) was observed for cv. French. For the treatments, the highest value (1.31 g) was recorded under exogenous application of SA2, followed by treatments with SP3 (1.0 g), while the lowest value (0.73 g) was observed in the control treatment. The interaction effects of treatments by cultivars revealed that 'Hybrid' marigold under exogenous application of SA2 produced the highest flower dry weight (1.5 g), while the control plants of 'French' marigold gave the lowest dry weight (0.69 g), not statistically different from 'French' treated with SA1 (0.77 g) or the 'Hybrid' control (0.78 g) (Figure 2F).

3.2.7. Number of Petals per Flower

Significance variations were found between cultivars, treatments, and their interaction (T × C) for the number of petals per flower (Table S2). Mean data for number of petals per flower among marigold cultivars showed that the highest values (41.1) were recorded for cv. Hybrid, while the lowest number of petals per flower (26.7) was observed for cv. French. For the treatments, the highest value (46) was recorded under exogenous application of SA2, followed by treatments with SP3 (40.3) while the lowest values (25.2) were observed in the control treatment. The interaction effects of treatments by cultivars revealed that 'Hybrid' marigold under exogenous application of SA2 produced the highest number of petals per flower (55.1), while the lowest number of petals (17) was recorded in the control plants of 'French' marigold, not statistically different from 'French' marigold (20) treated by 1 mM of salicylic acid (Figure 2G).

3.3. Antioxidant Activity

A significant variation was found between treatments, cultivars, and their interaction (T × C) for the antioxidant activity, as shown in Figure 3. The highest value of antioxidants activity (77%) was detected in the flower extract of the 'Hybrid' marigold treated with 2 mM of salicylic acid followed by (61%) treated by 2 mM of salicylic acid in 'French' marigold. The lowest value was found in 'French' marigold flower extract under control treatment (32%) followed by 'Hybrid' marigold under control treatment (37%).

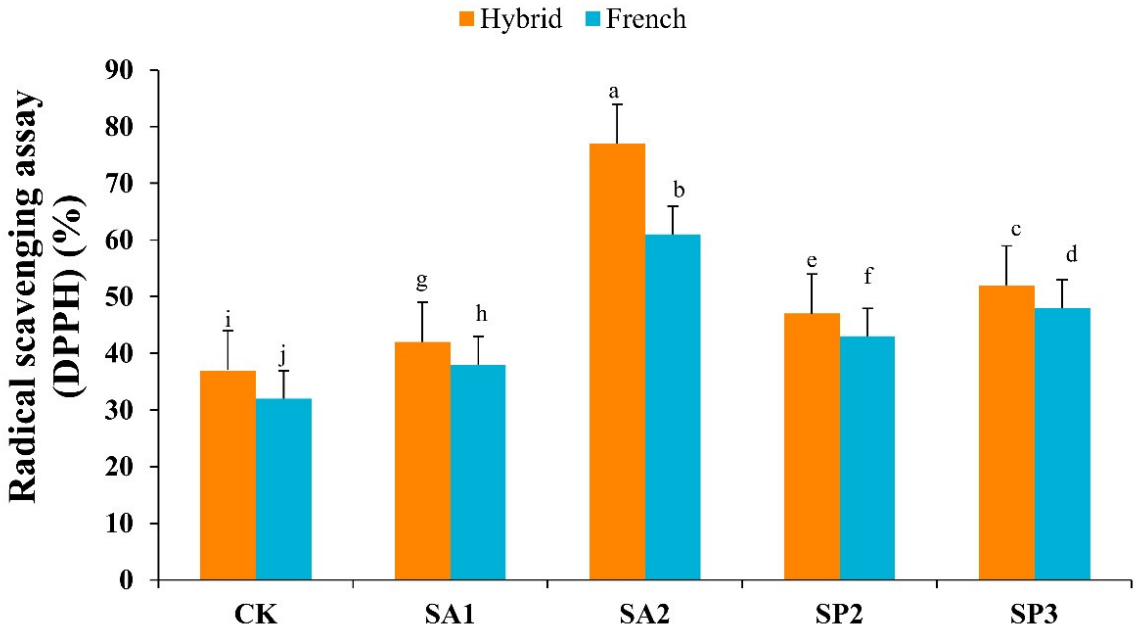

Figure 3. Effects of foliar application of salicylic acid and spermidine on the antioxidant activity of two cultivars (Hybrid and French) of marigold plant. CK, control (only water); SA1, salicylic acid @ 1 mM; SA2, salicylic acid @ 2 mM; SP2, spermidine @ 2 mM; SP3, spermidine @ 3 mM. Capped bars represent the standard error (n = 3). Letters above the error bars represent the significant variations among treatments at $p \leq 0.01$.

3.4. Total Phenolic Compounds

A significant variation was found between treatment, cultivar, and their interaction (T × C) (Table S3). The higher value of total phenolic compounds (68.34 mg GAE g^{-1}) was detected in the flower extract of the 'Hybrid' marigold treated with 2 mM of salicylic acid followed by 'Hybrid' treated with 3 mM of spermidine (63.2 mg GAE g^{-1}). The lowest value was found in the control of 'French' marigold flowers extract (30.2 mg GAE g^{-1})

followed by 'Hybrid' marigold control (33.3 mg GAE g^{-1}), not statistically different from 'French' treated with SA1 (Figure 4).

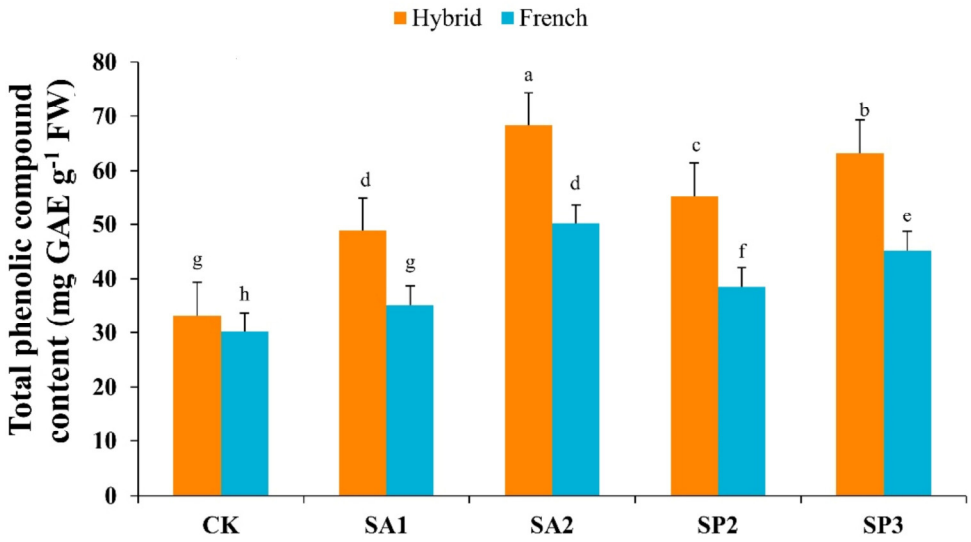

Figure 4. Effects of foliar application of salicylic acid and spermidine on the total phenolic compound contents in the flower extraction of marigold (cvs. Hybrid and French). CK, control (only water); SA1, salicylic acid @ 1 mM; SA2, salicylic acid @ 2 mM; SP2, spermidine @ 2 mM; SP3, spermidine @ 3 mM. Capped bars represent the standard error (n = 3). Letters above the error bars represent the significant variations among treatments at $p \leq 0.01$.

3.5. Total Carotenoids

A significant variation was found between treatments, cultivars, and their interaction (T×C) as shown in Figure 5 and Table S3. The highest value of total carotenoids (110.0 mg 100 g^{-1}) was detected in the flower extract of the 'Hybrid' marigold treated with 2 mM of salicylic acid followed by that of 'French' marigold (89 mg 100 g^{-1}), treated by 2 mM of salicylic acid. The lowest values were found in the control of both cultivars (48 and 53 mg 100 g^{-1}), not statistically different from the values found in 'Hybrid' at SA1 (51 mg 100 g^{-1}) and 'French' at SP2 (58 mg 100 g^{-1}) (Figure 5).

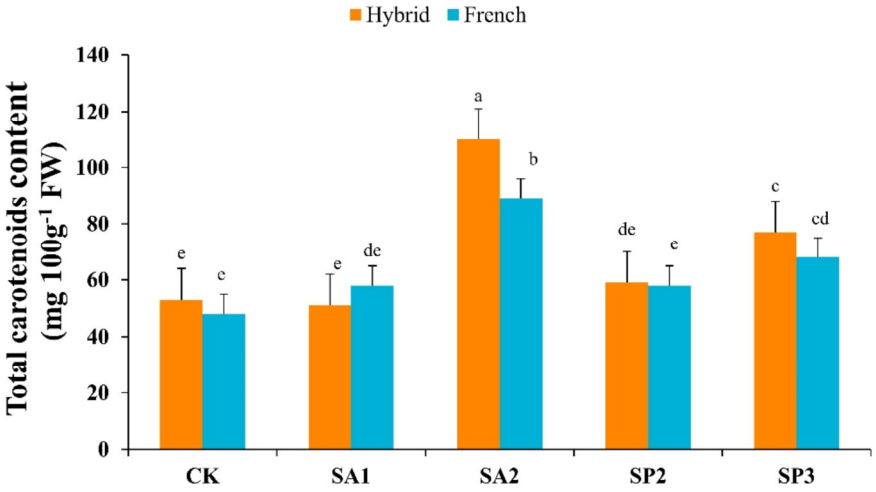

Figure 5. Effects of foliar application of salicylic acid and spermidine on the total carotenoids content in the flower extraction of marigold (cvs. Hybrid and French). CK, control (only water); SA1, salicylic acid @ 1 mM; SA2, salicylic acid @ 2 mM; SP2, spermidine @ 2 mM; SP3, spermidine @ 3 mM. Capped bars represent the standard error (n = 3). Letters above the error bars represent the significant variations among treatments at $p \leq 0.01$.

3.6. Total Flavonoids

Significant variations were found between treatments, cultivars, and their interaction (T × C) (Table S3). The highest value of total flavonoids (67.5 mg REg^{-1}) was detected in the flower extract of the 'Hybrid' marigold treated with 2 mM of salicylic acid followed by that of 'Hybrid' (62.5 mg RE g^{-1}) treated with 3 mM of spermidine (Figure 6).

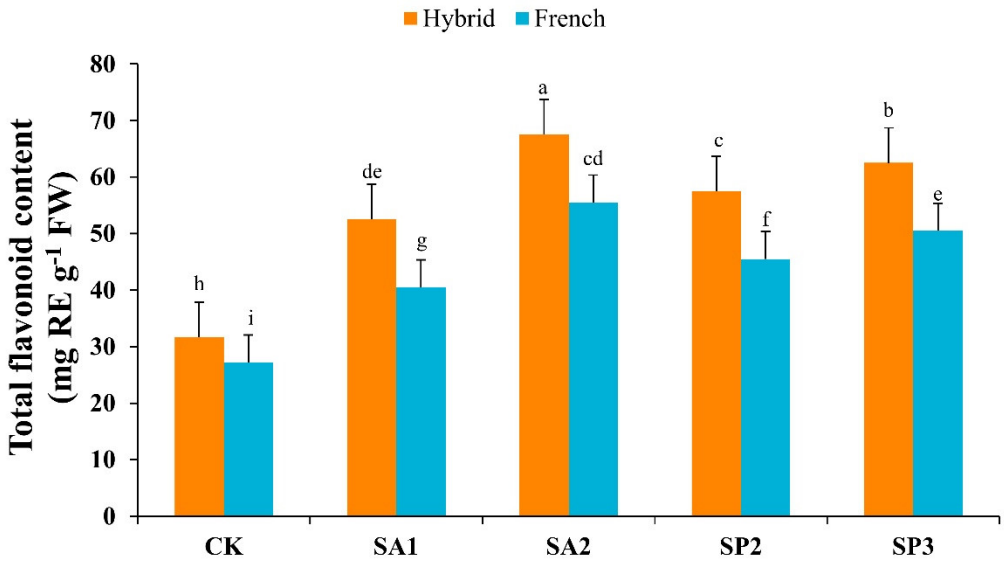

Figure 6. Effects of foliar application of salicylic acid and spermidine on the total flavonoid content in the flower extraction of marigold (cvs. Hybrid and French). CK, control (only water); SA1, salicylic acid @ 1mM; SA2, salicylic acid @ 2 mM; SP2, spermidine @ 2 mM; SP3, spermidine @ 3 mM. Capped bars represent the standard error (n = 3). Letters above the error bars represent the significant variations among treatments at $p \leq 0.01$.

3.7. Qualitative Phytochemical Screening

The extracts were subjected to preliminary qualitative tests to identify the various phytoconstituents present in the flower heads of marigold. The results are presented in Table 1. The qualitative analysis revealed the presence of the phytochemicals, namely tannins, flavonoids, diterpenes, saponins, phytosterols, alkaloids, and proteins. Phytochemical screening results of marigold showed that 'Hybrid' marigold had a significant presence of flavonoids and phytosterols at 2 mM SA application. At the same time, specific deficiency of tannins, diterpenes, saponins, and alkaloids were detected at this treatment; proteins were found at moderate level. On the other hand, in 'French' marigold, tannins and proteins were highly detected at 2 mM SA application. Phytosterols were highly detected with application of 2 mM SP in 'French' marigold (Table 1).

Table 1. Qualitative phytochemical screening of the selected Hybrid and French marigold. CK, control (only water); SA1, salicylic acid @ 1 mM; SA2, salicylic acid @ 2 mM; SP2, spermidine @ 2 mM; SP3, spermidine @ 3 mM.

Treatment	Tannins	Flavonoids	Diterpenes	Saponins	Phytosterols	Alkaloids	Proteins
Hybrid marigold							
CK	−	++	−	−	++	−	++
SA1	++	++	++	++	++	−	+++
SA2	−	+++	−	−	+++	−	++
SP2	++	++	++	++	++	++	+++
SP3	−	++	++	−	−	++	++
French marigold							
CK	−	−	−	++	−	−	−

SA1	++	+++	−	++	++	++	++
SA2	+++	−	−	−	−	−	++
SP2	++	++	++	++	+++	++	++
SP3	++	++	++	++	−	++	++

+++ indicate strong presence, ++ indicates moderate presence, − indicates absence.

3.8. Antioxidant Enzymes

Significant difference was found between treatments, cultivars, and their interaction (T × C) (Table S4), linking metabolic enzyme activities and secondary metabolites at the levels of salicylic acid treatments. The results showed strong positive relationships between superoxide dismutase activities and secondary metabolites content, where the highest value of SOD (12.8 unit mg^{-1} DW) was observed in 'Hybrid' marigold flowers sprayed by 2 mM of salicylic acid (Figure 7A). Spraying marigold flowers with SA at 2 mM yielded the highest value of SOD, with a mean between the two cultivars of 11.4 unit mg^{-1} DW and an increase of nearly 51% over the control.

Peroxidase activity enhanced significantly ($p \leq 0.05$) using growth regulators compared to the control treatment. The results showed highly positive interactions between peroxidase activities and secondary metabolites content in SA at 2 mM foliar treatments. Hybrid marigold recorded the highest value of peroxidase activities (11.9 unit mg^{-1} protein) (Figure 7B). Spraying marigold flowers with SA at 2 mM yielded the highest value of peroxidase activities with a mean between the two cultivars of 10.5 unit mg^{-1} protein and an increase of nearly 47% over the control, whereas the lowest values of peroxidase activities were found in control treatments with a mean between the two cultivars of 55.5 unit mg^{-1} protein (Figure 7B)

Catalase activities enhanced significantly ($p \leq 0.05$) using growth regulators compared to the control treatment. Salicylic acid concentration of 2 mM recorded the highest catalase activities values in 'Hybrid' marigold flowers (12.9 unit mg^{-1} protein) with a mean between the two cultivars of 11.5 unit mg^{-1} protein and an increase of nearly 50% over the control. The lowest value of catalase activities values was recorded in the control treatments (43 unit mg^{-1} protein) (Figure 7C).

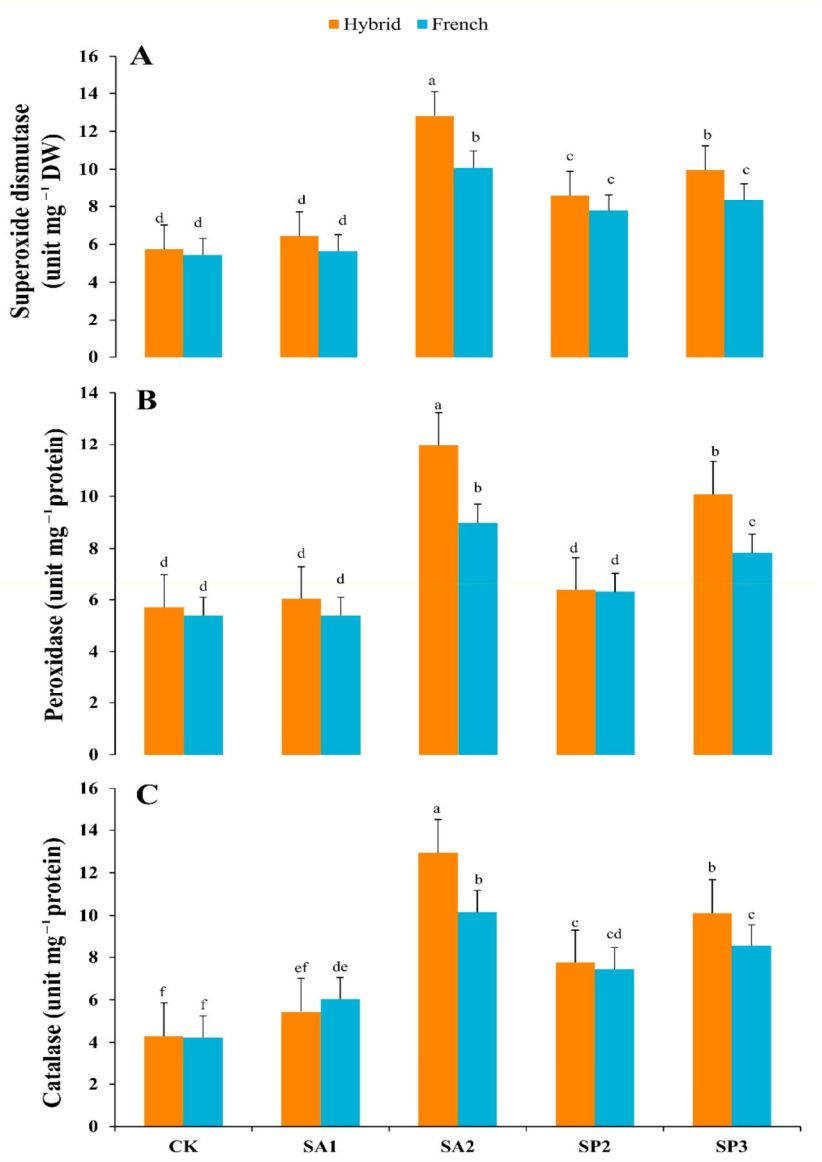

Figure 7. Effects of foliar application of salicylic acid and spermidine on the activities of (**A**) superoxide dismutase, (**B**) peroxidase, and (**C**) catalase. CK, control (only water); SA1, salicylic acid @ 1 mM; SA2, salicylic acid @ 2 mM; SP2, spermidine @ 2 mM; SP3, spermidine @ 3 mM. Capped bars represent the standard error (n = 3). Letters above the error bars represent the significant variations among treatments at $p \leq 0.01$.

4. Discussion

Plant height increased by applying SP (3 mM) compared to control treatments, which were without SA and SP application. This may be due to the high level of SP presence in the youngest vascular tissues, which preceded their change to high cell division and increased nitrogen accumulation in plant shoots. The results are in accordance with the findings of Chen et al. [31], who reported that the nitrogen contents are generally increased with the application of SP, which are necessary for growth and development in most flower crops. Similarly, Tiburcio and Alcazar [20] reported that the foliar application of SP has been known to affect many biochemical and physiological processes and improve vegetative growth due to its effect on cell division, cell elongation, and enzymes activity. In the present work, the highest leaf length was recorded by SA at 2 mM. Interestingly, SA is an important phenolic phytohormone which conspicuously enhances signaling

pathways, improvements in biochemical processes, and biosynthesis of different enzymatic actions [13]. Furthermore, the findings of Gorni et al. [32] also evidenced that SA is an efficient elicitor in the production of secondary metabolites, which enhanced plant aerial and root biomass of *Achillea millefolium*.

The exogenous application of SA (2 mM) prominently increased the number of leaves compared to the control. SA application was most effective on the number of leaves due to the close relationship between leaf and growth regulators. SA is a vital secondary metabolite naturally produced by plants that plays an important role in growth, expected to associate phytohormone, chlorophyll concentration, and photosynthetic rate [13]. It was previously reported that SA increases the number of leaves due to cell division enhanced by plant growth regulators, which increase stem elongation, bud and leaf formation, etc. [5]. Similarly, the work of Youssef et al. [33] confirmed that foliar applications of calcium chloride and SA significantly increased vegetative growth parameters, such as plant length, number of leaves, and chlorophyll content of *Lactuca sativa*.

Foliar application of SA showed a positive effect on stem thickness in French marigold cultivar. Our results are supported by previous work reporting that SA application increased the stem thickness and regulates flowering time and development [14]. The highest bloom flowers were recorded in the Hybrid marigold cultivar treated with 3 mM of SP, significantly higher than the control, possibly due to SP effects on the physiology of flowering and metabolite production; these findings corroborate previously reported results that reported that high levels of foliar application of SP and their conjugates have been created in apical shoots and meristems before flowering [31]. In the present study, maximum flower height was observed in 'Hybrid' marigold treated with 2 mM of SA with a significant difference compared to the control. The findings of Soltani et al. [34] also revealed that application of SA in combination with thiamine increased the number of flowering stems in *Calendula officinalis*. Our results are in line with other findings reporting that SA could affect the exact expression of the factor's protein, which is essential for flowering stimulation [12,13]. Therefore, exogenous application of biologically active compounds, such as SA, can be an alternative approach to improving crop productivity and is also beneficial under stress conditions, particularly heat stress and climate change [35]. Hence, the replacement of synthetic chemicals with natural plant hormones of phenolic nature could be an explicit remedy for reducing plant stress and an excellent option from economic and environmental perspectives.

The highest value of flower diameter was observed in 'Hybrid' marigold treated with 2 mM of SA. The increase in flower diameter in the present study is corroborated by the work of Martínez et al. [14]. They reported that SA increases flower diameter due to signaling pathway activity early in petal development to increase their size. The findings of Bayat et al. [36] also revealed that foliar application of SA significantly increased flower diameter of *Calendula arvensis* as compared to non-treated plants and under salinity stress. In a recent finding, it has been reported that SA prominently increased the flower diameter of two cultivars of African marigold [6].

The maximum value of nodes of flowers per plant was recorded in 'Hybrid' marigold treated with 2 mM of SA. SA acts as signaling molecule of plants under the influence of various biotic and abiotic stresses in marigold, which exerts stimulatory effect on the physiology of plants [3]. Furthermore, foliar application of SA contributes to a rapid translocation of assimilate to sink under the influence of phytohormones, which facilitates instant cell division and increased number of internodes and flowers [13,37].

In this study, maximum fresh weight of marigold flowers was recorded in SA 2 mM treatment; this could be due to SA preventing vascular blockage due to its antimicrobial action [16]. Our results confirm various research studies [38,39] that reported that SA increases water uptake and reduces transpiration level, which increases the fresh weight of cut flowers. The maximum dry weight of flowers was observed in 'French' marigold treated with 2 mM SA. In a similar study, the findings of Basit et al. [3] suggested that exogenous application of SA before flowering stage significantly increased fresh weight

of marigold flowers. Further findings suggest that SA increases vitamin E in plants, a potent antioxidant which increases dry weight of flowers, leaves, and other plant parts [16]. Similarly, it has been reviewed that plant growth regulators, such as SA, increase the dry weight in plants due to the attribute effect of components upon the endogenous phytohormones, especially the plant growth promoters, i.e., auxins, gibberellins, and cytokinin's [40]. Our results also corroborated the work carried out by Saeed et al. [38], as they examined the effects of SA on gladiolus cut flowers and found that the highest levels of SA retained higher fresh weight and increased the number of florets as compared to control. Proper growing substrates are essential for quality flower production as well as the supply of nutrients and water. In our study, the maximum number of petals was recorded in 'French' marigold treated with exogenous application of 2 mM SA. It was recently reported that the positive regulatory effect of SA on stomatal closure improves water retention, which increases petal number and quality [16].

The DPPH assay is a preliminary test used to determine the antioxidant capacity of a sample. It has been widely used to assess the ability of various samples to scavenge free radicals [41]. Plants treated with 2 mM of SA showed the highest total antioxidant activity. Our results are comparable with those of Akshaya et al. [42], that reported that flowers of *Calendula officinalis* had 68 to 82% of DPPH inhibition.

Polyphenols and flavonoids have many physiological functions related to plant survival and are produced naturally during plant growth and development to self-protect plants from biotic and abiotic stresses [43]. Our results are comparable with those of Gong et al. [44], who reported that the total phenolics content of dried marigold residue extracts ranged from 8.50 to 62.36 mg GAE g^{-1} and the total flavonoids content ranged from 19.04 to 97.00 mg RE g^{-1}. Furthermore, it has been reported that flowers of C. *Officinalis* (Marigold) had 45 to 76 mg RE g^{-1} DW of flavonoid content [45]. The exogenous application of either SA or SP plays an important role in the accumulation of secondary metabolites in plants [46], acting on plant defense signal transduction pathways through the expression of defense-related genes, especially those involved in the phenolic compound's biosynthesis, thus increasing their levels in plants [47]. Our results show that the application of SA or SP significantly influenced the biochemical constituents of marigold flower. Such results were in accordance with those stated by Kim et al. [48], who reported a significant increase in total flavonoid contents of marigold flower with the application of SA. Similarly, other works further confirmed the positive effect of SA on total phenolic compounds of plants [10,13]. Our results showed significantly higher values for total phenolic compounds with the application of SA 2 mM treatment in 'Hybrid' marigold than the control; thus, this can be linked to its biosynthesis chemical alternation, which can improve the production of altered groups of secondary metabolites, such as terpenes, alkaloids, flavonoids, and phenolic compounds [2]. Foliar application of salicylic acid has significant effects on plant secondary metabolites and found a positive linear relationship between phenolic content and salicylic acid concentrations [49].

High carotenoid content helps plants to maintain higher rates of photosynthesis and photosystem II activity [13,50]. In another study, marigold genotypes were evaluated and reported a range for carotenoid content from 19 to 525 mg 100 g^{-1} [42]. Apparently, the increase in pigments, particularly chlorophyll and carotenoids, is directly related to the photosynthetic performance of plants and could ultimately contribute to an upsurge in plant growth [51]. Carotenoids also play an important photoprotective role by removing reactive oxygen species (ROS) and suppressing lipid peroxidation [52]. In this study, the foliar application of SA at 2 mM concentration significantly increased carotenoid contents of marigold cultivars. Previous findings evidenced that exogenous application of SA increases carotenoids levels in plants and play an important role in photosynthesis and photoprotection [12].

Superoxide dismutase activity was found to be the highest in 2 mM of SA treatment in 'Hybrid' marigold. These results are supported by the findings of Saeed et al. [38], that

revealed that exogenous application of SA significantly increased the activities of antioxidant enzymes, i.e., SOD, POD, CAT. These antioxidants are very potent in supporting the capacity of scavenging reactive oxygen species (ROS) and improve stress resistance. Moreover, SA induced an increase in POD activities which are helpful for the growth of triterpenoids and flavonoids. In a similar study, Xin et al. [53] reported that antioxidant enzymes POD and SOD might be complex in gathering secondary metabolites, which is widely understood as part of plants defense and stress responses. Our results are similar to those of Janda et al. [12,13] who reported that endogenous and exogenous application of SA induce both gene expression and the enzymatic activity of POD. Catalase activities (CAT) were found to be best in SA 2mM treatment in 'Hybrid' marigold. In a similar study, Mallahi et al. [54] reported that SA significantly boosts the catalase activity in *Tanacetum parthenium* and causes higher tolerance in plants under salinity stress than SA-untreated plant. Apparently, this higher action of CAT in plants plays an important role in cell wall resistance, besides signaling the expression of various plant self-protective genes and one of the essential enzymes that can keep cells from oxidative injury by scavenging responsive oxygen species [14].

Medicinal plants are rich in secondary metabolites that represent different chemical classes and are synthesized through different biochemical pathways [55]. These secondary metabolites accumulate in plant organs through regulatory and biochemical mechanisms, thereby enabling plants to adapt to environmental changes, such as biotic and abiotic stresses [56]. The results of phytochemical screening of marigold leaf extracts showed that marigold leaves contain alkaloids, anthocyanins, beet anthocyanins, cardiac glycosides, coumarins, flavonoids, glycosides, phenols, quinones, saponins, steroids, terpenes, and tannins. Marini et al. [57] showed that marigold leaves contain alkaloids, flavonoids, saponins, and tannins. According to Devika and Koilpillai [58], marigold leaves were found to contain cardiac glycosides, phenols, and coumarins. In addition, marigold leaves contain glycosides and terpenoids [59,60]. The results of qualitative tests showed that 'Hybrid' calendula had significant flavonoids and phytosterols when 2 mM SA was applied. On the other hand, in 'French' marigolds, tannins and proteins were strongly detected by application of 2 mM SA. Various abiotic and biotic elicitors increased the concentration of secondary metabolites. SA also plays an important role in triggering these metabolites [61]. Exogenous application of SA has also been considered as an efficient, cheap, environmentally friendly, and rapid strategy to enhance the synthesis and accumulation of secondary metabolites in plants [61].

5. Conclusions

We achieved the aim of increasing the quantity and quality of medicinal plants like *Calendula officinalis*. In the current study, we evaluated the effects of phytohormones (i.e., salicylic acid) and polyamines (i.e., spermidine) on growth, flowering, and synthesis of secondary metabolites, enzymatic, and antioxidant activity of marigold plants. This study clearly indicated that foliar spray of SA and SP positively affected all the studied vegetative and flowering growth traits of marigold plants. On the other hand, secondary metabolites, antioxidants, and carotenoids content of the plant of the cv. Hybrid of marigold plant increased by application of salicylic acid in the floral extract. Following the treatment of salicylic acid at 2 mM, the enzyme's activity (SOD, CAT, and POD) was also significantly improved. Based on the results of the present study, it seems that the application of salicylic acid constitutes a valuable crop management technique to promote the production of inflorescences and bioactive ingredients in the cultivation of marigold, as well as to enhance the medicinal particularity of marigold.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13010191/s1. Table S1: Analysis of variance calculated for the vegetative parameters of two cultivars (Hybrid and French) of marigold plant. Table S2: Analysis of variance calculated for the floral parameters of two cultivars (Hybrid and French) of marigold plant. Table S3: Analysis of variance calculated for the total phenolic, carotenoids, and flavonoid contents from flower extraction of marigold (cvs. Hybrid and French). Table S4: Analysis of variance calculated for the activities of superoxide dismutase, peroxidase, and catalase.

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