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Influence of 2iP and 2,4-D Concentrations on Accumulation of Biomass, Phenolics, Flavonoids and Radical Scavenging Activity in Date Palm (*Phoenix dactylifera* L.) Cell Suspension Culture

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Abstract: Plant hormones are chemical compounds that serve as crucial signal molecules. The growth and induction of bioactive compounds in plant suspension cultures depend on the exogenous application of auxins and cytokinins at different concentrations. In this study, date palm (*Phoenix dactylifera* L., cv. Shishi) cell suspension cultures initiated in Murashige and Skoog (MS) medium were treated with 2.5 and 5 mg/L 2-isopentenyladenine (2-iP) in combinations with 1, 2.5, 5 and 10 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), and compared to the standard cell multiplication medium as the control, which contained 1.5 mg/L 2iP and 10 mg/L naphthaleneacetic acid (NAA). The optimum biomass accumulation, based on the packed cell volume and dry weight, was found in the cell suspension culture augmented with 1 mg/L 2,4-D + 5 mg/L 2iP followed by 5 mg/L 2,4-D + 2.5 mg/L 2-iP. In addition, the study assessed the total phenolic, flavonoid and radical scavenging activity, and high performance liquid chromatography (HPLC) was also used to determine the content of polyphenols (apigenin, caffeic acid, catechin and kaempferol) quantitatively. Cell suspension cultures containing 5 mg/L 2,4-D and 2.5 mg/L 2iP yielded the maximum accumulation of phenolics, flavonoids and radical scavenging activity (90.65%), and also a significantly higher content of caffeic acid (37.1 µg/g DW). The present finding facilitates the scaling up and commercial production of polyphenols from date palm cell suspension culture.

Keywords: antioxidants; bioactive compounds; biomass; cell suspension culture; date palm; polyphenols; secondary metabolites

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1. Introduction

Date palm, *Phoenix dactylifera* L., an Islamic religions holy plant, comes under the family Arecaceae [1]. It is one of the oldest and most economically important fruit tree species in the Arab world. In Saudi Arabia, 320 varieties represented in 23 million trees are grown, with an annual income of USD 500 million [2,3]. In addition to its food value and socio-economic importance, date palm provides raw materials for shelter, clothing, fiber, furniture and aesthetics [4].

Date fruits are the source of various phytochemicals such as flavonoids, sterols, tannins, anthocyanins, carotenoids and procyanidins and also exhibit a variety of nutraceutically important phenolic compounds of high antioxidant value [5–8]. These phenolic compounds possess anticancer properties and also have the properties to prevent cardiovascular diseases [9,10]. In vitro callus and cell suspension cultures show a great potential to produce commercially useful bioactive compounds with a great antioxidant activity [11–13]. Plant growth regulators (PGR) often serve as key factors in the accumulation of bioactive

compounds. The type and concentration of auxin and cytokinin affects growth and product formation in callus and plant cell suspension culture [14–16]. The medicinal value of these polyphenols is well known, as they have antioxidant properties and are used as raw materials for industries such as food and pharma [9,10]. Limited research has been reported with respect to polyphenols production from date palm in vitro cultures [12,17–19].

The objective of the current study is to assess the influence of adding combinations of 2-isopentenyladenine (2-iP) and 2,4-dichlorophenoxyacetic acid (2,4-D) concentrations to the cell suspension culture of date palm with respect to the accumulation of biomass, total phenolic and flavonoid contents, antioxidant activity and polyphenols production (apigenin, caffeic acid, catechin and kaempferol). As per our knowledge, there has been no previous report that has addressed the influence of the studied hormones on polyphenols accumulation in date palm cell suspension culture. The results from this study may provide a new avenue for pharmaceutical industries.

2. Materials and Methods

2.1. Plant Material

The date palm (*Phoenix dactylifera* L.) cultivar Shishi was selected for this study because of its abundance and popularity in the Eastern Province of Saudi Arabia, where the experiment was performed. The shoot tip region was excised from 3-year-old offshoots. After the removal of surrounding leaves, the shoot tip was isolated, and the surface was sterilized, sectioned into small pieces and inoculated on the callus initiation medium in accordance with the procedures described by Naik and Al-Khayri [20].

2.2. Cell Suspension Culture

To induce the cell suspension culture, 0.5 g of the resultant callus of 12 weeks old culture was macerated aseptically using a scalpel and inoculated in 250-mL conical flasks, each containing 50 mL of liquid Murashige and Skoog (MS) medium [21]. The cell suspension cultures were cultured for 11 weeks under a 16-h photoperiod of 40 μmol/m^2/s light at 25 ± 2 °C and a shaker speed of 150 rpm. The standard culture medium was augmented with 1.5 mg/L 2iP and 10 mg/L naphthaleneacetic acid (NAA) that served as the control for this experiment [12]. The hormone 2iP (2.5 and 5 mg/L) in combination with 2,4-D (0, 1, 2.5, 5 and 10 mg/L) were evaluated in comparison to the standard culture medium.

2.3. Biomass Determination

To determine the packed cell volume (PCV), 15-mL sterile graduated centrifuge tubes were filled with 10 mL of cell suspension and centrifuged for 5 min at 2000 *g*. The packed volume of cell mass is recorded as a percentage. To determine the dry weight (DW), cells were collected by filtering through Whatman No. 1 filter paper and then were oven-dried (60 °C) for 24 h. The dried cell mass was stored at 4 °C for 1 month in a vial, and subsequently extraction and analysis were carried out.

2.4. Extraction of Cell Suspension Culture

Using a pestle and mortar, a fine powder was made from the dried cell mass. A finely powdered 100 mg sample in a centrifuge tube (15 mL) was used to carry out the cell extraction [12]. Aqueous methanol (80%, *v*/*v*, 10 mL) was used as the extraction solvent (for 2 h at 60 °C under a water bath). The extract was centrifuged at 6000 rpm for 20 min, and then a polyvinylidene fluoride (PVDF) membrane filter (Merck Millipore, Cork, Ireland) with a pore size of 0.45 μm was used to filter the supernatant. This filtrate was collected in a round bottom flask (125 mL), and solvent was evaporated under reduced pressure. A total of 1 mL of 80% methanol was used to dissolve dried extracts, and this was filtered through a PVDF 0.45-μm membrane filter, and the filtrate was collected in 2-mL high performance liquid chromatography (HPLC) sample vials.

2.5. Total Phenolic Contents (TPC)

The determination of TPC in date palm cell suspension culture was carried out according to the Modified Folin-Ciocalteu (F-C) method [22]. A total of 20 µL of methanolic extract was mixed with deionized water (1.58 mL), and then 100 µL of F-C reagent was added. These were mixed thoroughly and kept for 8 min, thereafter adding 20% sodium carbonate (300 µL). This mixture solution was shaken and mixed well, and kept at 20 °C for 2 h to develop the color. The absorbance was measured at 765 nm using an Ultraviolet-visible (UV-Vis) spectrophotometer (Yoke, Shanghai, China). Finally, we recorded the TPC of cell extract expressed as mg gallic acid equivalents (GAE) 100/g DW.

2.6. Total Flavonoid Content (TFC)

The quantification of TFC in the date palm cell suspension culture extracts was based on the modified colorimetric method [23]. The mixture of 100 µL extract, 10% aluminum chloride (50 µL), 1 M potassium acetate (50 µL) and 1.8 mL deionized water was thoroughly mixed. The mixture was kept at room temperature (30 min) for incubation, and the absorbance was measured at 415 nm using a UV-Vis spectrophotometer (Yoke, China). The quantity of TFC of the date palm cell extract was expressed as mg quercetin equivalents (QE) 100/g DW.

2.7. Evaluation of Radical Scavenging Activity (RSA)

2,2-Diphenyl-1-picrylhydrazyl (DPPH), the stable free radical, has a hydrogen ion-donating ability, and this property is used to evaluate the antioxidant activities of various compounds/cell extracts [24]. A modified Farag et al. protocol was applied to determine the antioxidant activity [5]. The original sample was suspended in methanol, and a stock solution was made at value 1 mg/mL. Sample solutions were prepared from this stock solution for various concentrations (2–1000 µg/mL). A standard control, butylated hydroxylanisole (BHA), was prepared similarly to the samples. Extracts of 250 µL were taken from the original sample, and the same solvent was used to make up the 1 mL. To each test, 2 mL of DPPH (0.1 mM in methanol) was added. Methanol (1 mL) and DPPH solution (2 mL) was used to prepare the blank. Incubation condition: dark, room temperature for 30 min. The UV-Vis spectrophotometer reading was kept at 517 nm, and for methanol the absorbance was set to zero and the absorbance was recorded for the blank, standard control and samples. The DPPH disappearance was recorded; from the samples and standard control, the percentage of RSA of the DPPH was calculated based on the following equation:

$$\% \text{ DPPH RSA} = [(A_0 - A_1)/A_0] \times 100,$$

where the absorbance of blank is denoted as $A_{0,}$
absorbance of standard control (BHA) or the sample is denoted as A_1.

2.8. Determination of Polyphenols

The determination of polyphenols was achieved by using the HPLC unit (Shimadzu Prominence Liquid chromatography, Japan), as per the experimental protocol conducted by Naik and Al-Khayri [12]. The mobile phases were 0.5% acetic acid in Millipore water (solvent A), methanol (solvent B) and acetonitrile (solvent C). A PVDF membrane filter (0.45 µm) was used to filter the mobile phase, and was then deaerated ultrasonically using a sonicator. The standards, catechin, caffeic acid and kaempferol (Sigma-Aldrich, St. Louis, MO, USA), were dissolved in methanol; apigenin was dissolved in solution containing 1:1 methanol and acetonitrile, and then all the standards were diluted with the mobile phase before injecting in the HPLC system. The concentration ranges of 1, 2, 5, 10, 30 and 40 µg/mL were used to develop a calibration curve. Solutions of standard compounds were chromatographed and served as external standards. When comparing the retention times of the standards, polyphenols of cell extracts were identified (Figure 1a,b). The quantities of each polyphenol present in the samples were expressed as µg/g.

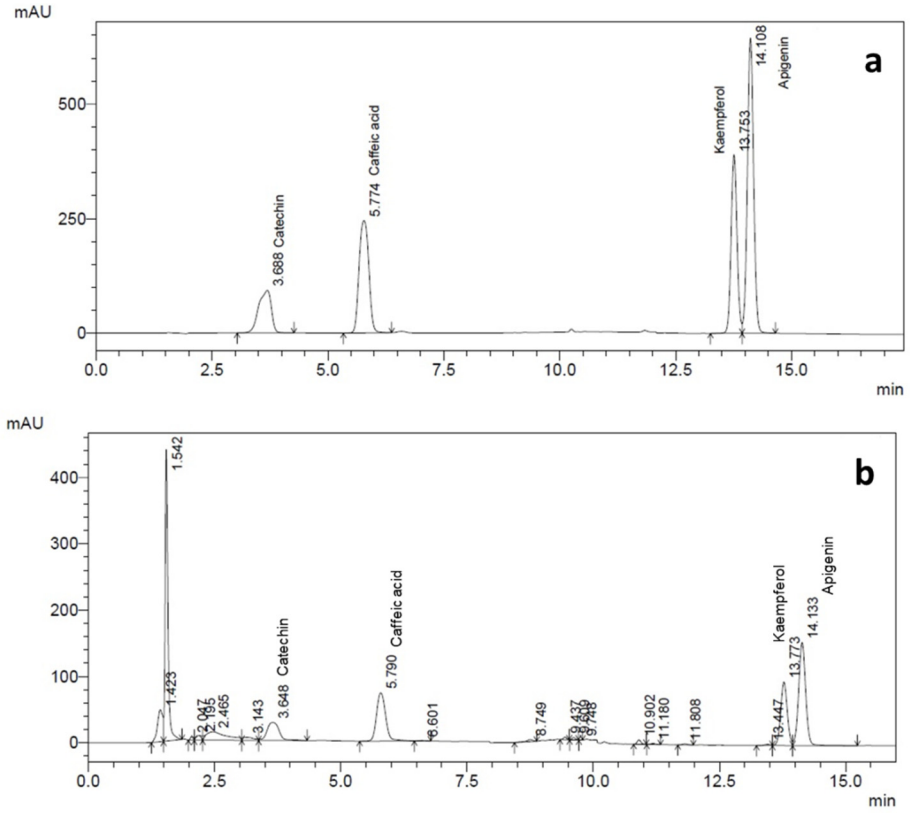

Figure 1. HPLC chromatograms: (**a**) Chromatogram showing the retention time of standard compounds, and (**b**) chromatogram showing the retention time of tested polyphenols.

2.9. Statistical Analysis

The data were collected in three replications per treatment, and an analysis of variance (ANOVA) was applied to the collected data using SPSS (Statistics version 22.0; IBM corp. New York, NY, USA). Based on Duncan's multiple range test (DMRT) at $p \leq 0.05$, the mean values were separated.

3. Results

3.1. Biomass Accumulation

For any cell suspension culture system, the evaluation of biomass accumulation is the most important feature. In the present experiment, the results showed that there was an increase in biomass when the culture was grown in medium treated with 2.5 and 5 mg/L 2iP. However, the 1 mg/L 2,4-D + 5 mg/L 2iP hormone combination in cell suspension culture produced an optimum PCV and DW accumulation followed by 5 mg/L 2,4-D + 2.5 mg/L 2iP, when compared to the control and other hormonal combinations tested (Figures 2 and 3). The hormone combination 10 mg/L 2,4-D + 5 mg/L 2iP culture showed the least accumulation of biomass. The result indicating higher concentrations of the auxin and cytokinin combination resulted in a reduction of the biomass accumulation.

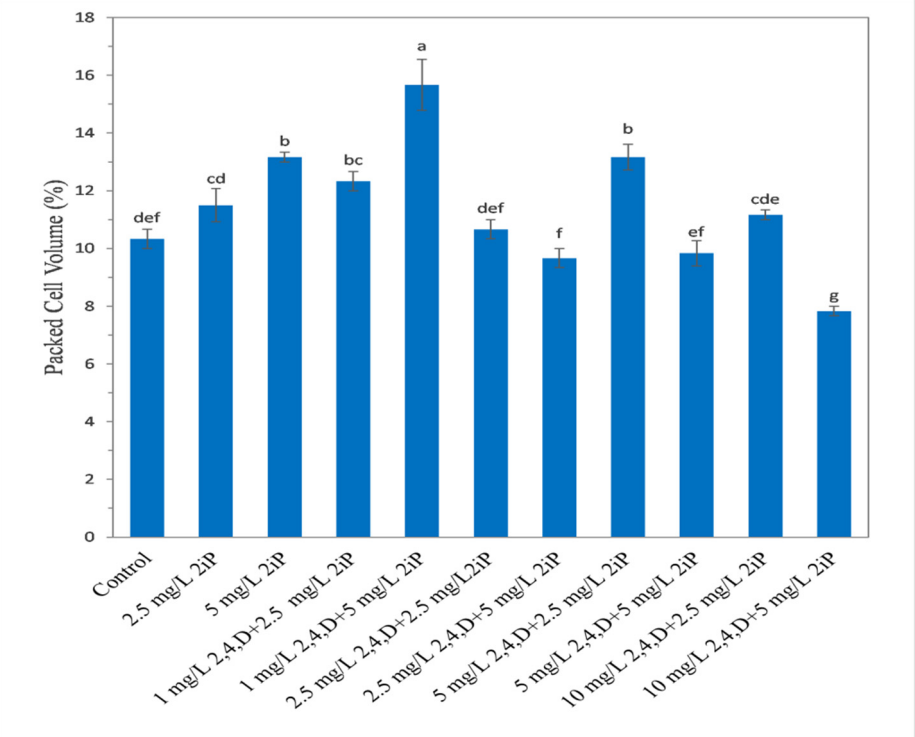

Figure 2. Effect of 2iP and 2,4-D on packed cell volume (%) in date palm cell suspension culture. At the eleventh week of culture, data were recorded. Line bar represents the standard error (SE). The same letters indicate no significant difference according to DMRT at $p \leq 0.05$.

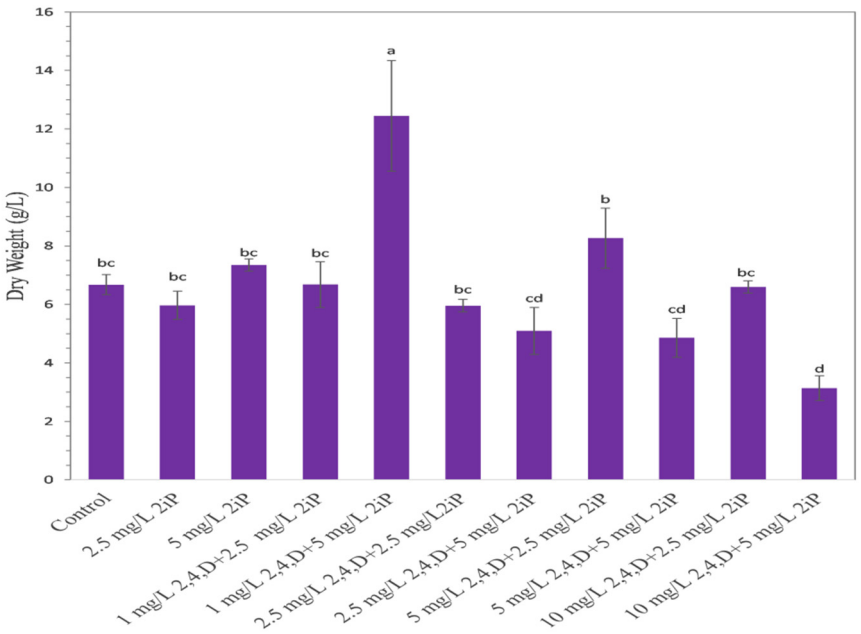

Figure 3. Effect of 2iP and 2,4-D on dry weight (g/L) in date palm cell suspension culture. At the eleventh week of culture, data were recorded. Line bar represents the SE. The same letters indicate no significant difference according to DMRT ($p \leq 0.05$).

3.2. Total Phenolic Content, Flavonoids and Radical Scavenging Activity (RSA)

The cell suspension culture grown in the medium fortified with 2.5 and 5 mg/L 2iP showed that there was an elevation in the content of the total phenolic content, flavonoids and RSA when compared to the control. On the other hand, the hormone 5 mg/L 2,4-D + 2.5 mg/L 2iP supplemented culture yielded the maximum accumulation

of total phenolic content, flavonoids and RSA (90.65 %) over the control experiment, and also over other tested hormones and their combinations (Table 1). In the auxin and cytokinin combination, the culture medium treated with 2.5 mg/L 2,4-D + 2.5 mg/L 2iP induced a significantly lower concentration of total phenolic content, flavonoids and RSA (49.29%) when compared to the control and other hormone concentrations. This indicates that the hormones with low and equal concentrations are not favoring the accumulation of the total phenolic content, flavonoids and RSA. The cell suspension culture medium supplemented with a higher concentration of auxin/cytokinin (10 mg/L 2,4-D + 5 mg/L 2iP) also accumulated the least concentration of the total phenolic content, flavonoids and RSA. The cell suspension cultures with the other hormone combinations are inconsistent with the accumulation of the total phenolic content, flavonoids and RSA value. However, the hormone-treated date palm cell suspension culture showed a strong positive correlation between the total phenolic content, flavonoids and RSA.

Table 1. Effect of 2iP and 2,4-D on total phenolic content (TPC), total flavonoid content (TFC) accumulation and RSA (%) in cell suspension of date palm at the eleventh week of culturing.

Hormones	Total Phenolic Content mg GAE 100/g DW *	Total Flavonoid Content QE 100/g DW *	DPPH Radical Scavenging Activity (%) *
Control	266.75 ± 8.75 d	68.66 ± 12.22 cdef	89.86 ± 1.57 ab
2.5 mg/L 2iP	297.77 ± 56.95 d	45.51 ± 8.03 efg	82.61 ± 0.53 b
5 mg/L 2iP	546.81 ± 71.86 b	94.24 ± 4.37 c	89.32 ± 0.21 ab
1 mg/L 2,4-D + 2.5 mg/L 2iP	363.50 ± 45.90 cd	139.70 ± 22.11 b	88.84 ± 0.66 ab
1 mg/L 2,4-D + 5 mg/L 2iP	310.03 ± 74.85 d	48.57 ± 1.96 defg	88.76 ± 0.91 ab
2.5 mg/L 2,4-D + 2.5 mg/L 2iP	198.90 ± 28.01 d	25.19 ± 1.36 g	49.29 ± 6.51 e
2.5 mg/L 2,4-D + 5 mg/L 2iP	243.07 ± 17.07 d	59.92 ± 5.41 cdefg	72.60 ± 2.66 c
5 mg/L 2,4-D + 2.5 mg/L 2iP	774.36 ± 104.20 a	209.70 ± 31.27 a	90.65 ± 1.04 a
5 mg/L 2,4-D + 5 mg/L 2iP	497.63 ± 84.69 bc	89.61 ± 6.34 cd	88.81 ± 1.97 ab
10 mg/L 2,4-D + 2.5 mg/L 2iP	562.40 ± 37.35 b	79.72 ± 0.20 cde	88.96 ± 0.66 ab
10 mg/L 2,4-D + 5 mg/L 2iP	233.52 ± 6.18 d	31.48 ± 2.40 fg	63.58 ± 2.04 d

* Values presented as mean ± SE. The same letters within a column indicate no significant difference according to DMRT ($p \leq 0.05$).

3.3. Polyphenol Content

The culture medium augmented with 2.5 and 5 mg/L 2iP showed the same level of polyphenol production when compared to the control. From the different hormones and combinations tested, the 5 mg/L 2,4-D + 2.5 mg/L 2iP supplemented cell suspension culture resulted in a significantly higher content of caffeic acid-37.1 µg/g DW production when compared to the control culture (Table 2). It also showed the highest accumulation of catechin (30.6 µg/g DW). With respect to the other polyphenols (kaempferol and apigenin), the control culture performed well compared to the other tested hormones in cultures (Table 2). The cell suspension culture medium fortified with a higher concentration, that is to say the 10 mg/L 2,4-D + 5 mg/L 2iP hormone, accumulated the overall lowest concentration of polyphenols when compared to the control and other hormone concentrations. The culture medium treated with 2.5 mg/L 2,4-D + 2.5 mg/L 2iP also showed a significantly lower synthesis of polyphenols.

Table 2. Effect of 2iP and 2,4-D on polyphenol content accumulation in cell suspension of date palm at the eleventh week of culturing.

Hormones	Catechin µg/g DW *	Caffeic Acid µg/g DW *	Kaempferol µg/g DW *	Apigenin µg/g DW *
Control	21.8 ± 0.5 a	23.2 ± 2.4 cd	13.2 ± 0.7 a	30.4 ± 5.7 a
2.5 mg/L 2iP	22.5 ± 4.4 a	34.0 ± 5.9 ab	8.3 ± 1.7 b	27.7 ± 3.4 a
5 mg/L 2iP	26.6 ± 5.7 a	29.8 ± 1.5 abc	6.8 ± 0.5 b	30.7 ± 0.2 a
1 mg/L 2,4-D + 2.5 mg/L 2iP	23.3 ± 5.1 a	27.0 ± 0.2 bc	0.0 ± 0.0 c	17.9 ± 1.1 b
1 mg/L 2,4-D + 5 mg/L 2iP	22.3 ± 1.1 a	23.1 ± 1.7 cd	0.0 ± 0.0 c	7.8 ± 0.4 c
2.5 mg/L 2,4-D + 2.5 mg/L 2iP	8.8 ± 1.9 b	7.2 ± 1.6 f	0.0 ± 0.0 c	12.9 ± 3.3 bc
2.5 mg/L 2,4-D + 5 mg/L 2iP	10.5 ± 1.9 b	8.9 ± 0.6 f	1.7 ± 0.1 c	7.4 ± 1.6 c
5 mg/L 2,4-D + 2.5 mg/L 2iP	30.6 ± 3.3 a	37.1 ± 4.2 a	6.3 ± 1.7 b	7.5 ± 0.8 c
5 mg/L 2,4-D + 5 mg/L 2iP	32.2 ± 2.0 a	28.6 ± 3.0 abc	7.0 ± 0.5 b	5.4 ± 1.3 c
10 mg/L 2,4-D + 2.5 mg/L 2iP	22.8 ± 3.3 a	17.2 ± 0.1 de	5.5 ± 2.0 b	8.5 ± 1.9 c
10 mg/L 2,4-D + 5 mg/L 2iP	4.0 ± 0.4 b	12.2 ± 1.3 ef	0.0 ± 0.0 c	8.8 ± 0.4 c

* Values presented as mean ± SE. The same letters within a column indicate no significant difference according to DMRT ($p \leq 0.05$).

4. Discussion

Plant growth regulators are the key elements in the suspension/cell culture medium and are essential for cell proliferation and differentiation. The concentration and type of auxin or the ratio of auxin/cytokinins significantly influence both cell growth and the in vitro synthesis of polyphenol compounds [25]. Praveen and Murthy reported an altered cell biomass accumulation in *Withania somnifera* (L.) Dunal when the culture media were augmented with auxins at different concentrations [26]. They recorded the optimum accumulation of biomass in the medium treated with 2 mg/L 2,4-D. However, the maximum biomass accumulation was also noted with the culture medium in combination with 2 mg/L 2,4-D + 0.5 mg/L kinetin (KN). In the *Fagonia indica* (L.) adventitious root culture, NAA resulted in the highest accumulation of biomass [27].

Hormone concentration is the major factor in secondary product accumulation such as phenolics and flavonoids [14]. Corroborating our results with a number of previous research suggested that there was a tight association between the observed antioxidant activities and the phenolic compounds produced by in vitro cultures [18,28]. In the current investigation, RSA levels were found to be at a maximum in the cell suspension culture of date palm, which indicates that the commercial-scale utilization of date palm cell suspension cultures for the production of nutraceuticals is promising. The MS medium augmented with 27.1 µM 2,4-D favored the optimum accumulation of total phenols in the callus culture of *Cnidium officinale* Makino. [29]. Similarly to our findings, the *Garcinia brasiliensis* Mart. callus culture containing 2,4-D and 2iP produced a significantly higher content of total flavonoids [30].

Plant hormones are chemical compounds and a group of key signal molecules that are actively involved in the synthesis of plant secondary metabolites and also in regulating development and plant growth [31]. Plant hormones play a crucial role in plant tissue and cell suspension cultures, not only in cell growth, morphogenesis and plant regeneration but also in metabolite accumulation [29,30]. In the cell suspension culture of *Panax quinquefolium* L., where several growth regulators such as BA, KN, NAA and 2,4-D augmented the MS medium, the optimum accumulation of ginsenosides was observed in 1.0 mg/L 2, 4-D and 0.25 mg/L KN fortified medium [32]. In another study, 4.5 and 6.8 µM 2,4-D concentrations played an important role in the production of optimum levels of phthalide and 3-butylidenephthalide, respectively, in the callus culture of *C. officinale* Makino [30]. The above discussion indicates that different auxin/cytokinin combinations had key effects on various plant species and affected the synthesis of bioactive compounds at different levels.

5. Conclusions

In the cell suspension culture system, auxin/cytokinin with various concentrations and combinations induces biomass and polyphenols at different levels with respect to the

selected plant species. The results obtained from the present investigation indicated that 1 mg/L 2,4-D + 5 mg/L 2iP-supplemented date palm cell suspension culture favored the accumulation of biomass. Cell suspension cultures treated with 5 mg/L 2,4-D and 2.5 mg/L 2iP induced significantly higher levels of phenolics, flavonoids and radical scavenging activity (90.65%) and also produced the highest content of caffeic acid (37.1 µg/g DW). However, a higher concentration of 2,4-D + 2iP in date palm cell suspension culture is not suitable for the production of biomass and polyphenols. The valuable information obtained in this study is encouraging for the large-scale production of nutraceutically important polyphenols, which are the basic materials for various pharma and food industries.

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Data Availability Statement: All the related data are presented in the manuscript.

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