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Effect of calcium phosphate on growth, pigment and secondary compound accumulation in *Cannabis sativa* L. callus

Tebdoie, C.[1], Thipphaaut, T.[1], Deewatthanawong, R.[2], Kongchinda, P.[3], Singhavorachai, P.[2], Tontiworachai, B.[2], Chanapan, S.[2], Boonchom, B.[4] and Montri, N.[1,5*]

[1]Department of Plant Production Technology, School of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand; [2]Expert Center of Innovative Agriculture, Thailand Institute of Scientific and Technological Research, Pathum Thani, Thailand; [3]Expert Center of Innovative Herbal Products, Thailand Institute of Scientific and Technological Research, Pathum Thani, Thailand; [4]Department of Chemistry, Faculty of Science, King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand; [5]Office of Administrative Interdisciplinary Program on Agricultural Technology, School of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand.

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Abstract The application of calcium phosphate in the form of tricalcium phosphate ($Ca_3(PO_4)_2$) on cannabis (*Cannabis sativa* L.) callus cultures for 7 days significantly increased fresh and dry weights at concentrations ranging from 800 to 2000 mg/L, with no statistical differences between concentrations. However, higher calcium phosphate concentrations led to reductions in pigments and DPPH radical scavenging activity. By contrast, callus cultures treated with 800 and 1600 mg/L showed enhanced total phenolic content and carotenoids, while total flavonoid content increased proportionally. These findings suggested that calcium phosphate concentration influenced the biosynthesis of secondary metabolites in cannabis callus cultures.

Keywords: Cannabis, Callus, Calcium phosphate, Secondary metabolite

Introduction

Cannabis (*Cannabis sativa* L.) is a medicinally valuable plant. The main bioactive compounds are the cannabinoids, Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD), which have analgesic, anti-inflammatory, neuroprotective, and anticonvulsant properties (Devinsky *et al*., 2017). Cannabis also synthesizes various secondary metabolites, including phenolics, flavonoids, carotenoids, and chlorophylls, which contribute synergistically to its therapeutic

* **Corresponding Author:** Montri, N.; **Email:** nattaya.mo@kmitl.ac.th

effects; a phenomenon referred to as the "Entourage effect" (Sommano *et al*., 2020).

The secondary metabolites biosynthesis in plants is frequently induced by various environmental conditions, which activate intricate intracellular signaling networks mediated by phytohormones, proteins, and plant nutrients (Reshi *et al*., 2023). Plant tissue culture offers a controlled and reproducible environment that enables precise manipulation of growth factors and elicitors, resulting in plant growth, optimizes yield, and secondary metabolites production improvement when compared to plant growing in the field condition (Smetanska, 2008). Elicitation strategies have been applied in different species of medicinal plants such as adding with auxins in *Panax ginseng* C.A. Mey adventitious roots increased ginsenoside accumulation (Amin *et al*., 2013). Calcium modulation has also been shown to influence embryogenesis and metabolic outcomes in *Daucus carota* L. cultures (Xu *et al*., 2023). In potato, *Solanum tuberosum* L., callus cultures treated with $CaCl_2$ resulted in enhanced growth (Calabuig-Serna *et al*., 2023). The cytokinin-type regulator thidiazuron (TDZ) in cannabis has been reported to enhance cannabinoid-related traits in callus culture systems (Trajkovska *et al*., 2023). *In vivo*, preharvest foliar salicylic acid (SA) increased total phenolics, total flavonoids, and total chlorophyll in *C. sativa* L. (Tebdoie *et al*., 2024).

The application of abiotic elicitors such as calcium and phosphorus compounds enhances secondary metabolite accumulation in several plants. For example, calcium salts increased lycopene and the total phenolic content in tomato (Mazumder *et al*., 2021) and enhanced total flavonoids and capsaicin accumulation in chili pepper (Montri *et al.*, 2024). Treatment with calcium phosphate nanoparticles (CaP-NPs) at 50 mg/L stimulated antioxidant enzyme activities in rice and increased metabolite levels, while a notable increase in total glutathione was observed at 20 mg/L. Calcium ions (Ca^{2+}) serve as crucial secondary messengers in these pathways, modulating the gene expression and biosynthesis of stress-related compounds via the activation of calcium-dependent protein kinases (CDPKs) (Dell'Aglio *et al*., 2019; Park and Ronald, 2012). Phosphate (PO_4^-) plays a central role in plant growth and metabolism by supporting ATP-dependent energy transfer and regulating gene expression through phosphate/ATP signaling pathways (Wagner, 2024). Application of 50 mg/L of CaP-NPs in callus cultures of carob (*Ceratonia siliqua* L.) increased some secondary compounds such as flavonoids, tannins, terpenoids (Elsherif *et al*., 2025), but Ca/P-based elicitation strategies remain underexplored in cannabis.

This research examined the effect of various calcium phosphate concentrations on biomass, pigments, and some secondary compounds in *C. sativa* L. callus cultures. Our findings contribute to the development of reproducible, eco-friendly methodologies for maximizing bioactive yields, with potential applications in pharmaceutical and biotechnological industries. This method can also be applied to growing cannabis in outside environments.

Materials and methods

Callus induction

Young shoots of *Cannabis sativa* L. 'Hang Kra Rog Phu Phan' were used as the primary explants. The explants were sterilized with 10% sodium hypochlorite added with 2 drops of Tween-20 as a surfactant for 20 min. After surface disinfection, the explants were thoroughly rinsed with sterile distilled water and then cultured on Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962) supplemented with 0.5 mg/L thidiazuron (TDZ) to promote friable callus culture induction. The cultures were incubated in the culture room under control conditions at 25 ± 2°C and a 16/8 h (light/dark) photoperiod, with light intensity ranging from 3000 to 3500 lux. After three weeks, the induced callus tissues were excised and standardized into uniform 2.00 g fragments for subsequent treatments.

Treatments and experimental design

This completely randomized design (CRD) experiment comprised five replicates per treatment, with each replicate consisting of four independent culture vessels. The 2.00 g fresh weight of callus were cultured on MS medium supplemented with 0, 800, 1200, 1600, and 2000 mg/L calcium phosphate. Calcium phosphate is used in the form of tricalcium phosphate ($Ca_3(PO_4)_2$). The cultures were incubated in the culture room.

Growth parameters

After culturing on for 7 days, the callus cultures were weighed to determine the fresh weight. A fresh callus (one replicate per treatment) was dried in a hot air oven at 40°C for 3 days. After drying, the samples were weighed, and the dry weight percentage was calculated using the equation:

$$\text{Dry weight percentage} = (\text{Dry weight} / \text{Fresh weight}) \times 100$$

Analysis of pigments and secondary compounds

Callus extraction

The dried callus samples were finely ground using a grinding machine, and then 0.30 g of the callus powder was extracted with 10 mL of 95% ethanol at 40°C for 30 min using an ultrasonicator (Elmasonic P 120H). This procedure was repeated three times. The ethanolic extracts were then filtered through Whatman No. 1 filter paper and analyzed for secondary metabolite content using a microplate reader (SpectraMax i3x; Molecular Devices, San Jose, CA, USA).

Analysis of pigments

The chlorophyll and carotenoid contents were measured following a modified protocol adapted from Thambavani and Sabitha (2011). Spectrophotometric measurements of the ethanolic extract were conducted at wavelengths of 664, 649, and 470 nm using a microplate reader. Chlorophyll a, chlorophyll b, total chlorophyll, carotenoids, and total pigment were calculated using standard equations based on the absorbance values at the specified wavelengths.

Chlorophyll a (Chl a) = 13.36A664 - 5.19A649

Chlorophyll b (Chl b) = 27.43A649 - 8.12A664

Total chlorophyll = chlorophyll a + chlorophyll b

Carotenoid = [1,000A470 - 2.13(Chl a) - 97.63(Chl b)]/209

Total pigment = Total chlorophyll + carotenoid

Analysis of total phenolic content

The total phenolic content was quantified using an adapted Folin–Ciocalteu colorimetric method, following Velioglu *et al*. (1998). In this procedure, 67 µL of the ethanolic extract was mixed with 500 µL of Folin–Ciocalteu reagent and left at room temperature for 5 min. Then, 500 µL of 6% (w/v) sodium carbonate (Na_2CO_3) solution was added to the mixture and incubated in the dark condition for 90 min, with absorbance measurements recorded at 765 nm using a microplate reader. The results were expressed as mg of gallic acid equivalent (mg GAE) per g of dry sample weight.

Analysis of total flavonoid content

The total flavonoid content was measured using a modified aluminum chloride colorimetric assay following the protocol outlined by Li *et al*. (2013). In brief, 25 µL of the ethanolic extract was diluted with 475 µL of distilled water and left at room temperature for 3 min. Then, 250 µL of a 2% (w/v) aluminum chloride ($AlCl_3$) solution was added, followed by an additional 250 µL of distilled water to complete the reaction volume. The mixture was incubated under dark condition at room temperature for 30 min. Absorbance measurements were performed at a wavelength of 415 nm using a microplate reader. Quercetin was used as the calibration standard, with results expressed as mg of quercetin equivalent (mg QE) per g of dry weight.

Analysis of antioxidant activity by the DPPH assay

The ability of the sample to scavenge free radicals was determined using a modified 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, based on the methodology described by Sadeer *et al.* (2020). In brief, 150 µL of the sample extract was mixed with 650 µL of 0.01% (w/v) DPPH solution. Following thorough homogenization, the mixture was incubated in the dark at room temperature for 30 min to complete the reaction. The absorbance was then measured at 517 nm using a microplate reader. Ascorbic acid was used as a standard reference compound, with results expressed as the percentage of DPPH radical inhibition relative to the control.

Statistical analysis

This study employed a CRD. Data were subjected to analysis of variance (ANOVA), with post hoc comparisons among treatment means conducted using Tukey's Honestly Significant Difference (HSD) test at 99% confidence level ($p \leq 0.01$) and 95% confidence level ($p \leq 0.05$). All the analyses were performed using Statistix 8 software.

Results

Fresh and dry weight

Different concentrations of calcium phosphate application significantly influenced the fresh and dry weights of *C. sativa* L. callus, with the highest fresh weight of 4.362 g observed at 1600 mg/L treatment. However, no significant differences were found among the treatments of 800, 1200, and 2000 mg/L. The lowest fresh weight of 3.462 g was recorded in the control group (0 mg/L) or non-treated callus.

Callus treated with 800 mg/L calcium phosphate resulted in dry weight and percentage of dry weight of 0.094 g and 4.715%. These values were not significantly different from the treatment with 1200 mg/L. By contrast, the lowest dry weight of 0.080 g and dry weight percentage of 3.989% were found in the control group (Table 1).

Table 1. The effects of various calcium phosphate concentrations on fresh weight, dry weight, and dry weight percentage in *Cannabis sativa* L. callus after 7 days of culture

Calcium phosphate (mg/L)	Fresh weight (g)	Dry weight (g)	Dry weight percentage (%)
0	3.462b	0.080b	3.989**c**
800	4.153a	0.094a	4.715a
1200	4.083a	0.092ab	4.59ab
1600	4.362a	0.085ab	4.227bc
2000	4.037a	0.087ab	4.011c
F-test	**	**	**
C.V. (%)	6.59	3.68	3.14

Note: Values followed by the same letter within a column do not differ significantly at $p \leq 0.01$.

Chlorophyll a, chlorophyll b, total chlorophyll, carotenoids and total pigments

The application of varying calcium phosphate concentrations on photosynthetic chlorophyll a, chlorophyll b, total chlorophyll, total pigments, and carotenoid content in cannabis callus under aseptic conditions over a 7-day preharvest period revealed highly significant statistical differences. Non-treated calcium phosphate callus showed the highest concentrations of chlorophyll a (1.119 mg/g DW), chlorophyll b (0.463 mg/g DW), total chlorophyll (1.582 mg/g DW) (Table 2), and total pigment (1.639 mg/g DW) (Table 3). A pronounced and statistically significant decline in chlorophyll a, chlorophyll b, total chlorophyll, total pigments, and carotenoid content was observed with increasing calcium phosphate concentrations. Cannabis callus supplemented with 1600 mg/L treatment had the lowest levels of chlorophyll a, chlorophyll b, total chlorophyll, and total pigment content. However, these values did not differ significantly from the 1200 mg/L or 2000 mg/L treatments.

The cannabis callus culture treated with 800 mg/L calcium phosphate showed elevated carotenoid levels (0.184 mg/g DW), but this increase was not statistically different from callus cultures supplemented with 1200 mg/L and 1600 mg/L. The lowest carotenoid content at 0.058 mg/g DW was found in the control group (Table 3).

Table 2. The effects of various calcium phosphate concentrations on chlorophyll a, chlorophyll b, and total chlorophyll content in *Cannabis sativa* L. callus after 7 days of culture

Calcium phosphate (mg/L)	Chlorophyll a (mg/g DW)	Chlorophyll b (mg/g DW)	Total chlorophyll (mg/g DW)
0	1.119a	0.463a	1.582a
800	0.773b	0.412b	1.185b
1200	0.635bc	0.391bc	1.026bc
1600	0.528c	0.323c	0.850c
2000	0.680bc	0.364d	1.044bc
F-test	**	**	**
C.V. (%)	6.69	2.82	5.36

Note: Values followed by the same letter within a column do not differ significantly at p ≤ 0.01.

Table 3. The effects of various calcium phosphate concentrations on carotenoids and total pigment content in *Cannabis sativa* L. callus after 7 days of culture

Calcium phosphate (mg/L)	Carotenoids (mg/g DW)	Total pigment (mg/g DW)
0	0.058c	1.639a
800	0.184a	1.369b
1200	0.160ab	1.186bc
1600	0.132c	0.982c
2000	0.154b	1.197bc
F-test	**	**
C.V. (%)	8.00	5.65

Note: Values followed by the same letter within a column do not differ significantly at p ≤ 0.01.

Total phenolic content

The total phenolic contents in all the calcium phosphate-treated groups (800-2000 mg/L) significantly improved compared to the control group. The lowest total phenolic content of 12.272 mg GAE/g DW was recorded in the non-treated callus cultures, with the highest at 14.100 mg GAE/g DW observed in callus cultures treated with 800 mg/L of calcium phosphate. However, this value was not significantly different from the 1200, 1600, and 2000 mg/L treatments (Table 4).

Total flavonoid content

A statistically significant difference among calcium phosphate concentrations was observed for the total flavonoid content in *C. sativa* L. callus cultures. The flavonoid content increased with rising calcium phosphate concentrations, reaching a maximum of 12.046 mg QE/g DW at 2000 mg/L, which was significantly higher than the control. However, no significant differences were observed among the 800, 1200, and 1600 mg/L treatments compared to either the 2000 mg/L treatment or the control (Table 4).

Antioxidants

The DPPH radical scavenging activities of the cannabis callus cultures were significantly influenced by the calcium phosphate concentrations. A decreasing trend in DPPH activity was observed with increasing calcium phosphate levels. The highest percentage of DPPH inhibition (41.179%) was obtained from callus cultured in calcium phosphate-free medium. By contrast, the 800 to 2000 mg/L calcium phosphate treatments did not differ significantly, with values ranging between 23.774 and 25.024% (Table 4).

Table 4. The effects of various calcium phosphate concentrations on total phenolics, total flavonoids, and DPPH scavenging capacity in *Cannabis sativa* L. callus after 7 days of culture

Calcium phosphate (mg/L)	Total phenolic content (mg GAE/g DW)	Total flavonoid content (mg QE/g DW)	DPPH scavenging (%)
0	12.272b	11.138b	41.179a
800	14.100a	11.822ab	25.024b
1200	14.045a	11.869ab	24.843bc
1600	13.859a	11.932ab	24.151bc
2000	13.594a	12.046a	23.774c
F-test	**	*	**
C.V. (%)	2.21	2.55	1.12

Note: Values followed by the same letter within a column do not differ significantly at $p \leq 0.01$ (**) and $p \leq 0.05$ (*).

Discussion

Fresh and dry weight

The accumulation of fresh and dry weights of *Cannabis sativa* L. callus cultured on MS medium supplemented with varying concentrations of calcium phosphate showed statistically significant differences after 7 days of culture. Among the tested concentrations, 800 mg/L calcium phosphate resulted in the highest dry weight accumulation at 0.094 g, while 1600 mg/L yielded the optimal fresh weight at 4.362 g. However, no statistically significant differences were observed in fresh weight between the 400 to 2000 mg/L treatment groups.

Phosphorus plays an essential role in plant development as a structural component of adenosine triphosphate (ATP), which functions as the main energy currency in cellular metabolism. ATP plays an important role in photosynthesis, sugar production, and storing carbohydrates. These processes help plants to accumulate dry matter, which is important for their growth (Raghothama, 1999; Schachtman *et al*., 1998). The energy from phosphorus promotes cell elongation and division, thereby expanding tissue capacity for biomass storage and resulting in fresh weight increasing (Poirier and Bucher, 2002). Calcium also improves plant growth by strengthening cell walls and acting as a secondary messenger that mediates auxin and cytokinin hormone signaling, which are essential for plant cell growth and division (Hepler, 2005; Dodd *et al*., 2010). When applied at the optimal amount, calcium phosphate supports the structure of plant cells and the hormonal balance. Baroutkoob *et al*. (2024) found that adding 0.23 g of calcium phosphate in tomato plants (*Solanum lycopersicum* L.) significantly increased fresh and dry root weights.

Chlorophyll a, chlorophyll b, total chlorophyll, total pigments and DPPH radical scavenging activity

Our results showed that supplementation of MS medium with varying concentrations of calcium phosphate significantly affected the levels of chlorophyll a, chlorophyll b, total chlorophyll, total pigments, and DPPH radical scavenging activity in cannabis callus cultures. Callus cultured on MS medium without calcium phosphate supplementation exhibited the highest concentrations of all pigment components and antioxidant activity. By contrast, increasing calcium phosphate concentrations led to a significant reduction in pigment contents and % DPPH activity.

The observed reduction in callus viability was linked to the elevated intracellular levels of calcium (Ca^{2+}) and phosphate (PO_4^{3-}), which may trigger oxidative stress within the callus cells, thereby promoting excessive generation of reactive oxygen species (ROS), and leading to significant cellular damage, particularly affecting the lipid membrane integrity (Apel and Hirt, 2004). ROS-mediated degradation may target essential pigments, such as chlorophyll, thereby impairing photosynthetic efficiency (Gill and Tuteja, 2010).

The observed increase in malondialdehyde (MDA) content in calcium phosphate-treated callus further supports this hypothesis, as MDA is a well-established marker of lipid peroxidation and a direct indicator of ROS-mediated oxidative damage (Jaleel *et al*., 2007). These findings concurred with Tanaka and Tsuji (1980), who suggested that elevated levels of calcium and phosphorus may interfere with the activity of key enzymes involved in pigment biosynthesis, such as chlorophyll synthase, and adversely affect pigment accumulation (Tanaka and Tanaka, 2007).

Carotenoids

Our results indicated that the calcium phosphate concentration significantly affected carotenoid accumulation in *C. sativa* L. callus cultures, with the highest levels observed at 800 mg/L calcium phosphate. Interestingly, this group was not significantly different from the 1200 mg/L treatment, indicating a possible stress response triggered by nutrient imbalance to generate excessive intracellular calcium accumulation.

High calcium concentration may interfere with the uptake of other cationic nutrients like magnesium (Mg^{2+}), iron (Fe^{2+}/Fe^{3+}), and ammonium (NH^{4+}), since these ions are competitively transported through the cell membrane (Marschner, 2012). These elements also play a vital role in chlorophyll biosynthesis, especially magnesium, which constitutes the central atom of the chlorophyll molecule. Diminished Mg and Fe availability leads to decreased chlorophyll biosynthesis efficiency and photosynthesis, thereby stimulating carotenoid production as a protective mechanism against ROS accumulation (Demmig-Adams and Adams, 2002).

Phosphate (PO_4^{3-}), may facilitate calcium influx into plant cells (Raghothama, 1999), leading to ionic imbalance and cellular stress. Phosphates are also stimuli for carotenoid synthesis as major antioxidants during nutrient and oxidative stress (Nisar *et al*., 2015). This phenomenon may be related to increased carotenoid production at moderate calcium phosphate concentrations.

Total phenolic and total flavonoid contents

Our results indicated that different concentrations of calcium phosphate significantly affected the total flavonoid and total phenolic contents in cannabis callus cultures. A calcium phosphate concentration of 2000 mg/L resulted in the highest accumulation of total flavonoids, while 800 mg/L significantly increased total phenolics. No significant differences were observed among treatments within the 800-2000 mg/L range, indicating that calcium phosphate at these concentrations effectively promoted the biosynthesis of secondary metabolites, particularly phenolics and flavonoids.

The observed increase resulted from calcium-triggered signaling cascades that induced the expression of pivotal biosynthetic genes such as PAL (Phenylalanine Ammonia-Lyase) (Calabrese *et al*., 2004) and CHS (Chalcone Synthase) (Dudareva *et al*., 2004), involved in phenolic and flavonoid production, respectively (Hamada *et al*., 2012). Exogenous calcium has also been shown to stimulate the accumulation of stress-related phytohormones, including jasmonic acid (JA), salicylic acid (SA), and abscisic acid (ABA)., which then further stimulate secondary metabolite synthesis (Reddy *et al*., 2011).

In parallel, phosphorus delivered as phosphate plays a complementary role by promoting intracellular phosphorylation reactions that activate stress-response proteins and upregulate secondary metabolite biosynthesis pathways (Park *et al*., 2012). The synergistic effect of calcium and phosphate enhances the metabolic activity, leading to increased production of phenolic compounds and flavonoids, as previously observed by Vallejo *et al*. (2003).

Adding calcium phosphate to the culture media for 7 days had a significant impact on fresh and dry cannabis callus weights, which increased from the 800 to 2000 mg/L treatments. However, adding calcium phosphate had an opposite effect on the contents of chlorophyll a, chlorophyll b, total chlorophyll, total pigment, and DPPH radical scavenging activity, which all significantly declined with increasing levels.

After 7 days, the callus cultures treated with 800 and 1600 mg/L calcium phosphate showed improved total phenolic and carotenoid contents, paralleling the increase in total flavonoid content. The results showed that the application of 800 mg/L calcium phosphate significantly increased both the fresh and dry weight of *C. sativa* L. callus cultures, inducing the optimal accumulation of total phenolics and carotenoids. Conversely, the maximum production of total flavonoids was found at 2000 mg/L, while calcium phosphate treatments did not influence chlorophyll a, chlorophyll b, total chlorophyll, total pigments, and DPPH radical scavenging activity. These findings indicated the potential of calcium phosphate as an elicitor to enhance the biomass and certain secondary metabolites in *C. sativa* L., and for further applications under greenhouse or field conditions.

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Conflict of interest

The authors declare no conflict of interest.

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