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PHYSIOLOGICAL CHANGES AND GROWTH PARAMETERS OF PHASEOLUS VULGARIS L. CALLI CULTURES BY CHITOSAN AND CALCIUM PANTOTHENATE UNDER HEAT STRESS

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Abstract

The current study was carried out with the aim of determining the improvement in physiological parameters as a result of treatment with Chitosan (Chit.) and Calcium Pantothenate (CP) compounds and its impact on the growth parameters of Calli cultures of *Phaseolus vulgaris* L. growing in Murashige and Skoog (MS) media under normal conditions and heat stress conditions. Compounds were used at different concentrations of 0, 50, and 100 µM of Chit. and 0, 2.0, and 4.0 mg L^{-1} of CP. The increase in temperature at 35°C reduced all physiological indicators and had a negative impact on growth indicators. Treatment with Chit and CP contributed to the improvement of physiological study indicators, and its positive effect was reflected in growth indicators, as calli culture growing under natural conditions and treated with concentrations of 100 µM of Chit provided and 4.0 mg L^{-1} of CP. The highest mean content of chlorophyll a and total chlorophyll reached 1.471 and 2.261 mg g^{-1} FW, respectively. The cultures growing under natural conditions were recorded in the media consisting of 0 µM of Chit. and 4.0 mg L^{-1} of CP, the highest mean of chlorophyll b reached 0.750 mg g^{-1} FW. In contrast, the concentration of 50 µM of Chit was recorded. and 4.0 mg L^{-1} of CP had the highest mean relative water content (RWC) of 96.85% under the same conditions.

The positive effect of the chemical compounds (Chit and CP) on the physiological study indicators was reflected in the improvement of growth indicators, and tissue cultures growing under 25°C in a Chit-free media were achieved. The fresh media contained CP at a concentration of 2.0 mg L^{-1}, amounting to 2.808 g. The increase in temperature at 35 °C for cultures growing in a media containing chemical compounds (CP+Chit.) at concentrations of 100 µM + 4.0 mg L^{-1} and 50 µM + 0 mg L^{-1}, respectively, resulted in achieving the highest mean dry weight and calli growth of 131.3 mg and 0.541 respectively.

Keywords: Beans, In vitro, Heat stress, Chitosan, Calcium Pantothenat.

التغيرات الفسيولوجية ومعايير النمو لمزارع كالس *Phaseolus vulgaris* L. باستخدام Chitosan و Calcium Pantothenate تحت الإجهاد الحراري

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الخلاصة

نفذت الدراسة الحالية بهدف تحديد مدى التحسن في الصفات الفسلجية من جراء المعاملة بمركبي Chitosan (Chit.) و Calcium Pantothenate (CP) وانعكاسها على صفات النمو لمزارع كالس نبات *Phasealus vulgaris* L. النامية في وسط Murashige و Skoog (MS) تحت الظروف الطبيعية وظروف الاجهاد الحراري. استعملت تراكيز مختلفة من كلا المركبين بلغت 0 و50 و100 مايكرومول لمركب Chit. و0 و2.0 و4.0 ملغم لتر$^{-1}$ لمركب CP. سببت الزيادة في درجة الحرارة عند 35م في خفض جميع المؤشرات الفسلجية وانعكس تأثيرها السلبي على مؤشرات النمو. ساهمت المعاملة بمركبي Chit. و CP في تحسن مؤشرات الدراسة الفسلجية وانعكس تأثيرها الإيجابي على مؤشرات النمو، إذ حققت مزارع الكالس النامية تحت الظروف الطبيعية والمعاملة بالتراكيز 100 مايكرومول من Chit. و4.0 ملغم لتر$^{-1}$ من CP أعلى متوسط للمحتوى من الكلوروفيل a والكلوروفيل الكلي بلغ 1.471 و2.261 ملغم غم$^{-1}$ وزن طري بالتتابع. بينما سجلت المزارع النامية تحت الظروف الطبيعية في الوسط الغذائي المتكون من 0 مايكرومول من Chit. و4.0 ملغم لتر$^{-1}$ من CP أعلى متوسط من الكلوروفيل b بلغ 0.750 ملغم غم$^{-1}$ وزن طري. بينما سجلت التركيز 50 ميكرومول من

.Chit و4.0 ملغم لتر من CP أعلى متوسط للمحتوى المائي النسبي (RWC) بلغ 96.85% تحت نفس الظروف. انعكس التأثير الإيجابي للمركبات الكيميائية (.Chit وCP) في مؤشرات الدراسة الفسلجية في تحسن مؤشرات النمو وحققت مزارع الانسجة النامية تحت 25 ᵒم في وسط خالٍ من .Chit وحاوٍ على CP بالتركيز 2.0 ملغم لتر أعلى وزن طري بلغ 2.808 غم. في حين سببت الزيادة في درجة الحرارة عند 35 ᵒم للمزارع النامية في وسط غذائي حاوٍ على المركبات الكيميائية (.CP+Chit) بالتراكيز 100 مايكرومول+4.0 ملغم لتر$^{-1}$ و50 مايكرومول+0 ملغم لتر$^{-1}$ بالتتابع في تحقيق اعلى متوسط للوزن الجاف ومعدل نمو الكالس بلغ 131.3 ملغم و0.541 بالتتابع.

كلمات مفتاحية: الفاصولياء، خارج الجسم الحي، الإجهاد الحراري، Chitosan، Calcium Pantothenat.

Introduction

During recent decades, most countries in the world, including Iraq, have suffered from many problems in the field of plant production due to climate change, which has been reflected in the level of food production and thus threatens food security (6). *Phaseolus vulgaris* L. is one of the most important field crops and belongs to the Fabaceae Leguminosae family (20). Beans are an essential food source eaten daily by about 300 million people worldwide. Nutrition experts describe them as an essential source of vegetable protein. It is characterized by its high content of carbohydrates, vitamins, proteins, dietary fiber, and mineral elements such as phosphorus, potassium, magnesium, calcium, iron, and zinc (1 and 4). It is characterized by a high content of phenolic compounds, through which it reflects many therapeutic properties, including controlling blood sugar levels, preventing cardiovascular diseases, obesity, diabetes, cancer, and some kidney diseases, treating gout, rheumatism, and joint pain, and treating cases of malnutrition (10). The continuous development in biotechnology and its applications has contributed to finding more accurate procedures for evaluating development indicators for several vital activities in the plant cell, which contributes to shortening time and effort in breeding and improvement programs and is economical. They represent high-precision tools that track the impact of crop management factors at the cellular and molecular levels (7). Therefore, plant tissue culture technology has been widely utilized to improve plant production because this technology is of great importance in overcoming the dangers facing crop production. The most important of these dangers is heat stress. This will facilitate having plants that are tolerant to this factor while addressing the tolerance mechanisms and the accompanying physiological and biochemical changes (13). Abiotic stress produces excessive reactive oxygen species (ROS), and the plant cell loses its balance, leading to increased free radical production. This causes extensive damage at the cellular level, including the decomposition of cell membranes, proteins, and nucleic acids, leading to oxidative stress and, thus, cellular death (15, 21 and 22). Using chemical compounds, including chitosan, is one strategy to curb free radicals. It is a non-toxic biopolymer that plays an essential role in reducing the harmful effects of heat stress in plants, as it can effectively enhance heat tolerance and improve plant growth by regulating the various mechanisms responsible for

producing antioxidants within the plant cell (2 and 17). It stimulates plant defense enzymes and the synthesis of secondary compounds such as multiple phenols, flavonoids, and lignin (8 and 28).

Vitamins are among the most critical factors that must be available in media prepared for plant development in different environments, as they are added according to the level of the plant's need and participate with other chemical components in stimulating various metabolic processes. It has been shown to affect the calli culture growth, somatic embryo growth, branch formation, rooting, and embryonic development. Calcium Pantothenate, known as Vitamin B5, is a classified vitamin that is important in plant cell development. It is produced as calcium salt or pantothenate alcohol commercially for use in manufactured media (12). Given the importance that calli tissue has in scientific experiments related to programs emerging from tissue culture, the current study aimed to test the effectiveness of the compounds Chitosan (Chit.) and Calcium Pantothenate (CP) in influencing the vital processes of plant tissue and the reflection of that effect on physiological processes, which can enhance the tolerance of plant tissue to the harmful effects of heat stress and thus improves growth.

Materials and Methods

The experiment was conducted in the Plant Tissue Culture Laboratory, Center for Desert Studies/University of Anbar. The Sun Ray variety was used. The work included the following stages:

Preparing media cultures: The nutrient media MS was prepared, Murashige and Skoog (21). The nutrient media was prepared by adding 4.43 g L^{-1} to distilled water; after that, 30 g of sucrose was added. The pH was adjusted to 5.7 ± 0.1 by used NaOH and HCl, and then 7.0 g L^{-1} of agar was added to reach a complete boil under a hot plate stirrer. The prepared media was distributed in Vials in 10 ml. The glass bottles and their content of nutrient media were sterilized in the autoclave.

Sterilization of tools: Sterilization of glass and metal work supplies was accomplished by autoclaving distilled water and the prepared media for 15 min at 121 °C and 1.04 kg cm^{-2}. The cultivation room is sterilized with 70% ethyl alcohol (Ethanol) where the cultivation process carried out place.

Seed Sterilization: The seeds were sterilized by washing them several times, leaving them under running water for 30 minutes, and then brought into the cultivation cabin. The sterilization process was done using 4.0% NaOCl, adding two drops of Tween, with continuous stirring, for 10 min (24). The seeds were then washed with sterile distilled water five times in a row to remove traces of the sterile substance. The seeds were then planted in cultivation tubes containing the nutrient media, with one seed per (vial). The planted bottles were placed inside the growth chamber at 25 °C±1 with a lighting intensity of 1000 lux for 16 h. of light and 8 hrs of darkness to obtain pollution-free seedlings (Figure 1).

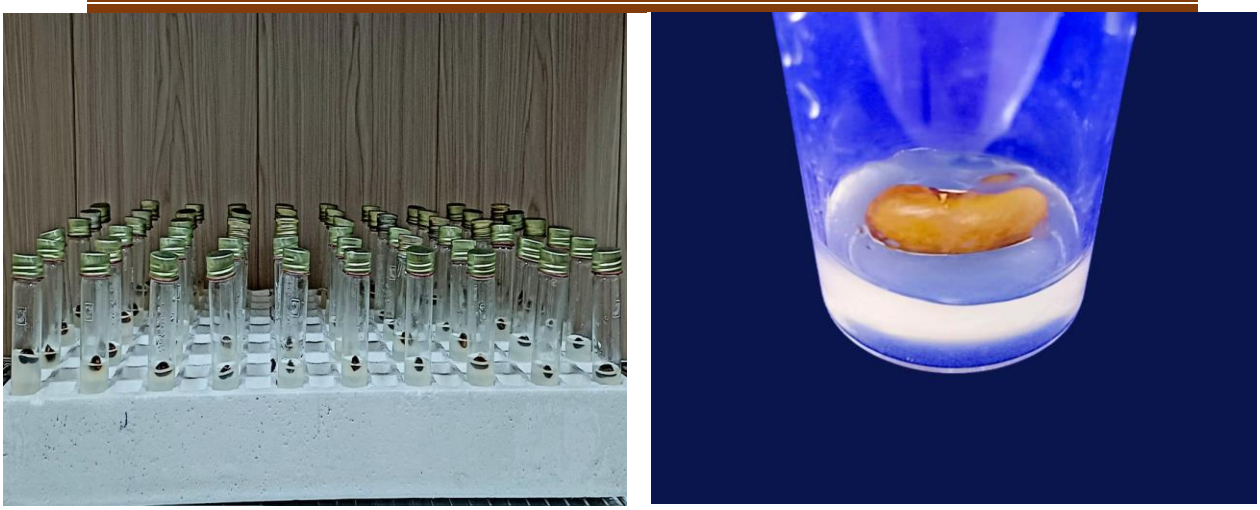


Figure 1: Germinating *P. vulgaris* L. seeds in sterile conditions.

Heat Stress Tolerance Treatment: The effectiveness of the two Chit and CP compounds to the tolerance of cultured calli to heat stress was determined. The calli cultures propagated at four weeks were transferred to a heat-tolerant media consisting of MS nutrient media, sucrose 30 g L^{-1}, and agar 7.0 g L^{-1}. The nutrient media prepared with 0, 50, and 100 µmol of Chit was also included. At concentrations of 0, 2.0, and 4.0 mg L^{-1} of CP, the crops were incubated in the growth chamber at 25°C and 35°C. The crops were left in the growth room at 25 °C±1 and illumination of 1000 lux for 8/16 days, represented by alternating light and darkness. The results for the indicators under study were recorded 21 days after planting.

Determination of Chlorophyll Content: The content of chlorophyll a, b, and total chlorophyll in samples of cultured calli was estimated according to Lichenthaler (18) by using acetone at a concentration of 80% by taking 250 mg of calli tissue, then adding acetone to it and leaving it in the centrifuge at 2500 revolutions for 15 min. After filtering, the estimation by using a spectrophotometer at wavelengths of 646.8 and 663.2 nm and calculation according to the following equations:

Chlorophyll a= (12.24 A663.2–2.79 A646.8)

Chlorophyll b= (21.21 A646.8–5.1 A663.2)

Chlorophyll total= (Chl.a + Chl.b)

Relative water content: The relative water content of calli cultures was measured according to Karimi et al. (14), the details of which are shown in the following equation:

Since:

FW = fresh weight of calli

DW = dry weight of calli

SW = standard calli weight

Fresh and Dry Weight Calculation: The fresh weight was calculated using a sensitive balance after removing the remaining nutrient media that stuck to it. The calli was then dried in an electric oven at 60 °C until the weight was stable so that the dry weight of the calli was recorded.

The growth level was calculated based on the initial fresh weight of the transplanted calli tissue (FWi) and the final fresh weight of the same calli (FWf) according to the following equation:

$$CG = \frac{FW_f - FWi}{FWi} \quad (3)$$

Experimental Design and Statistical Analysis: A factorial experiment was carried out using a completely randomized design. The obtained data were analyzed, and the experimental error was calculated for three replicates using the statistical analysis program Genestat, version 12.

Results and Discussion

Physiological Indicators: The results indicated that there was a significant effect of the study factors on all the physiological indicators under study; the increase in temperature caused a decrease in the relative water content of tissue cultures of P. vulgaris L., while treatment with Chit and CP contributed to reducing this gap and reducing depletion and prevent humidity reduction under the influence of increased temperature. However, both agents (Chit and CP) have a significant effect, whether under normal or abnormal temperature. The combination of 50 µmol of Chit with 4.0 mg L^{-1} of CP achieved the highest relative water content under 25 ^{o}C which was 96.85%. At the same time, the temperature of 35 °C negatively affected the moisture content, reaching 74.08% at the combination of 0 µmol of Chit. with 2.0 mg L^{-1}, sequentially (Figure 3, a1 and a2).

As for the chlorophyll-a content of the culture, the results showed significant differences between the study factors in the indicator. Despite the negative effect of the temperature of 35 °C on the chlorophyll-a pigment, the compounds Chit and CP contributed positively to enhancing the pigment content in the culture of tissues. The combination of 100 micromol of Chit and 4.0 mg L^{-1} under the optimum temperature achieved the highest dye content, which was 1.471 mg g^{-1} of fresh weight, thus superior to all treatments, including the two comparison treatments for both compounds under 35 °C, which showed the lowest dye content which was 0.627 mg g^{-1} FW (Figure 3, b1 and b2).

There was a significant difference between the data obtained as a result of the study factors in the calli tissue content of chlorophyll-b, as the treatment of tissue cultures with two concentrations of 0 + 4.0 mg L^{-1} for the study agents Chit and CP, respectively, under 25 °C providing the highest indicator average, which was 0.750 mg. gm^{-1} of fresh weight; tissue cultures grown in media free of Chit and CP compounds under 35°C showed the lowest indicator average, 0.296 mg gm^{-1} of fresh weight (Figure 3, c1 and c2).

The results indicated a significant difference in the total chlorophyll content of the calli tissue due to treatment with Chit and PC compounds under different temperatures. The temperature at 25°C recorded the highest average, 2.261 mg g^{-1} of fresh weight of the crops whose nutrient media contained high concentrations of both compounds (Chit+ CP). Calli cultures growing under 35 °C, in which the nutrient media was included at 0 µmol Chit + 4.0 mg L-1 CP, showed a fresh weight of 0.970 mg g^{-1} (Figure 3, d1 and d2). The biological activity of Chitosan is mainly related to

its ability to activate the plant's natural defense mechanisms to confront abiotic stress (27). The compound derived this ability from physiological and biochemical changes such as free radical scavenging and activation of secondary metabolism, enzymes, and growth inhibitors. These effects reflect physiological changes at the cellular level, such as inhibition of ATPase activity and protection of cell membranes due to various stress factors (5 and 19). The negative effects of heat stress, primarily the decrease in water content of plant tissue cells, have a significant role in cellular expansion, and it is known that the failure of the cell expansion process negatively affects cellular growth. The rate of cell elongation is susceptible to stress due to the dependence of cell growth on the extent of their ability to retain water, which is directly affected by the lack of access to water, including mineral salts, to the developing plant tissues, thus leading to severe negative repercussions in all biological and physiological processes (27).

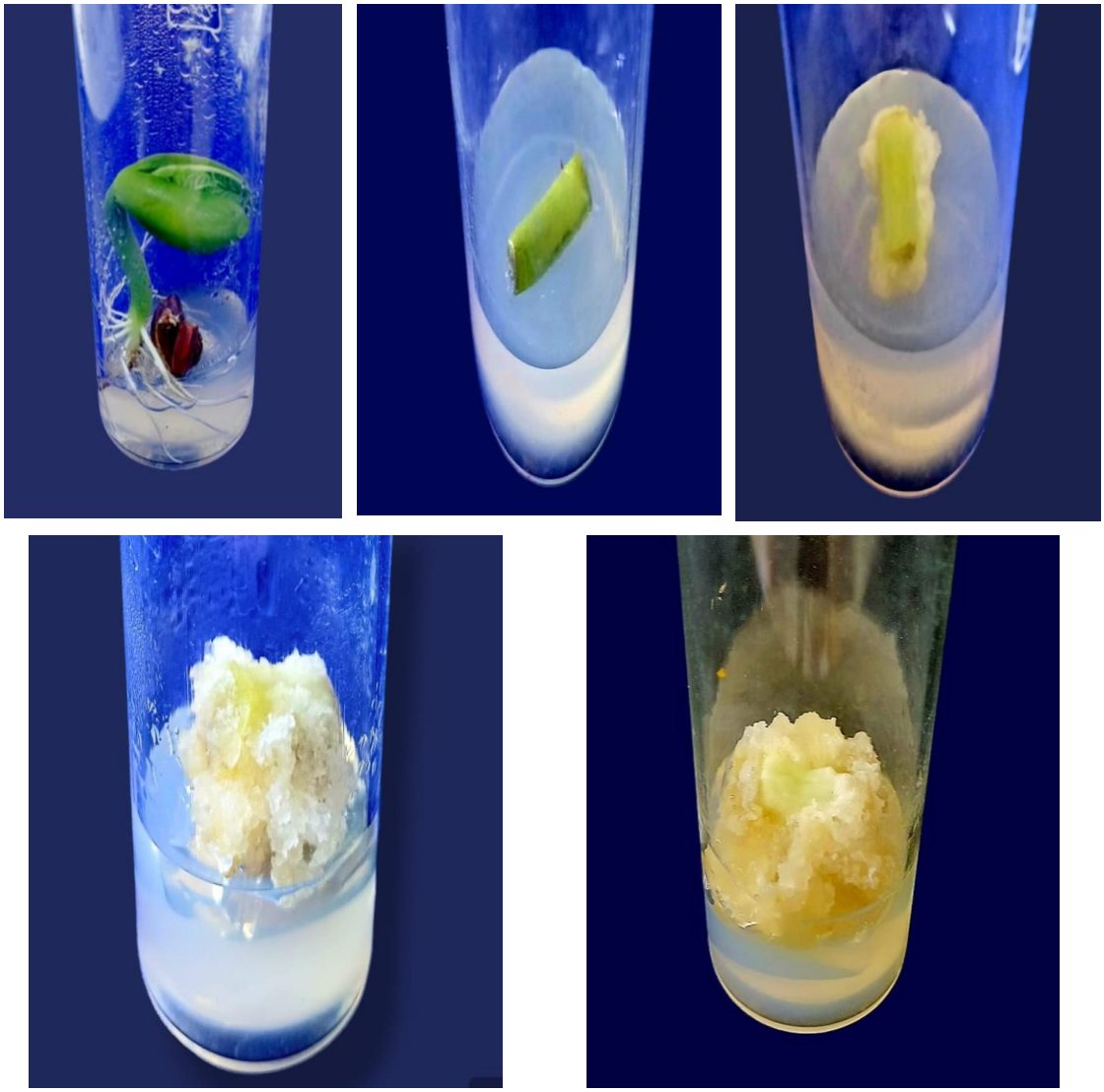
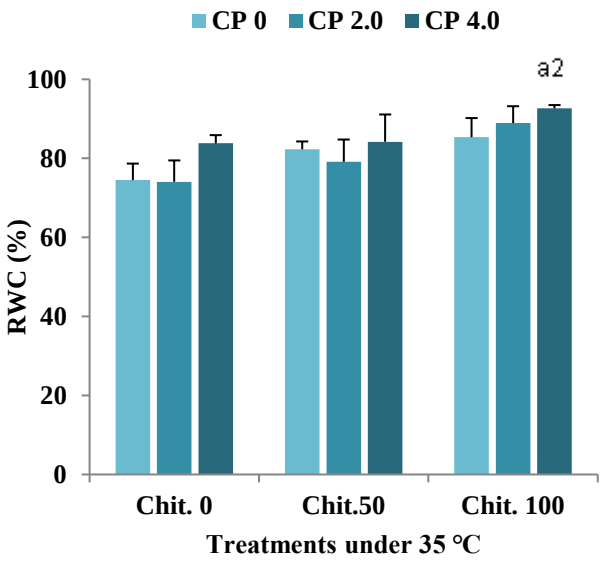
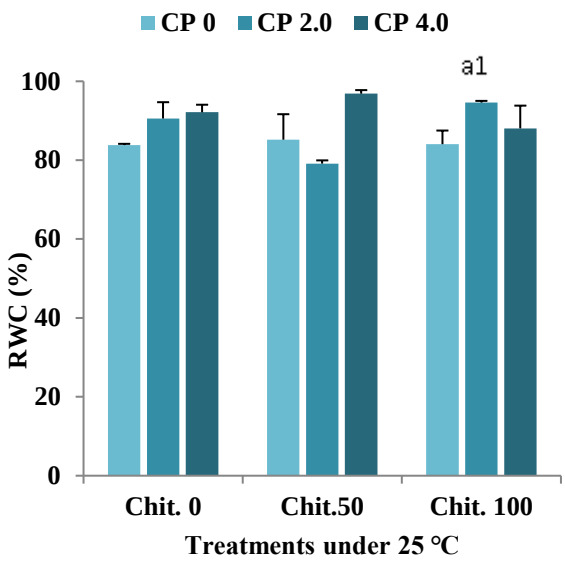
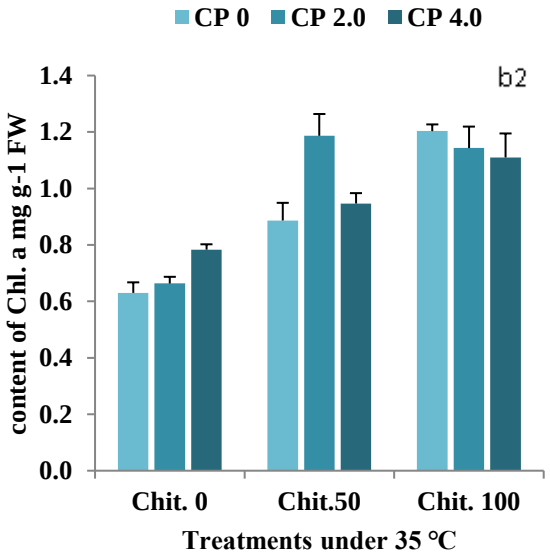
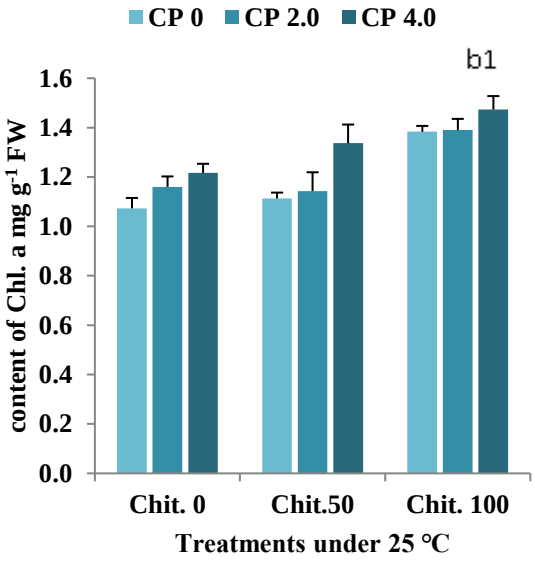


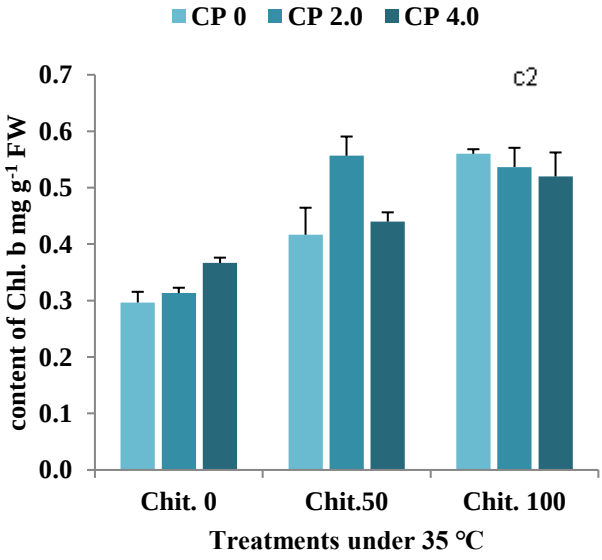
Figure 2: Induction of calli cultures derived from hypocotyl excised from contamination-free seedlings.

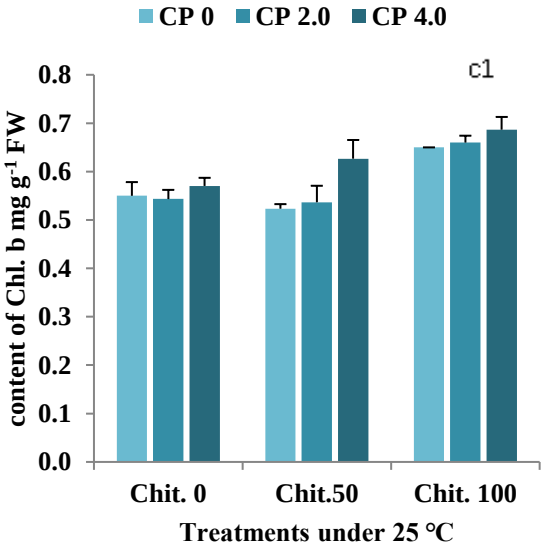

CP 0
CP 2.0
CP 4.0
a2
RWC (%)
100
80
60
40
20
0
Chit. 0
Chit.50
Chit. 100
Treatments under 35 ℃


CP 0
CP 2.0
CP 4.0
a1
RWC (%)
100
80
60
40
20
0
Chit. 0
Chit.50
Chit. 100
Treatments under 25 ℃


CP 0
CP 2.0
CP 4.0
b2
content of Chl. a mg g-1 FW
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Chit. 0
Chit.50
Chit. 100
Treatments under 35 ℃


CP 0
CP 2.0
CP 4.0
b1
content of Chl. a mg g-1 FW
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Chit. 0
Chit.50
Chit. 100
Treatments under 25 ℃


CP 0
CP 2.0
CP 4.0
c2
content of Chl. b mg g-1 FW
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Chit. 0
Chit.50
Chit. 100
Treatments under 35 ℃


CP 0
CP 2.0
CP 4.0
c1
content of Chl. b mg g-1 FW
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Chit. 0
Chit.50
Chit. 100
Treatments under 25 ℃

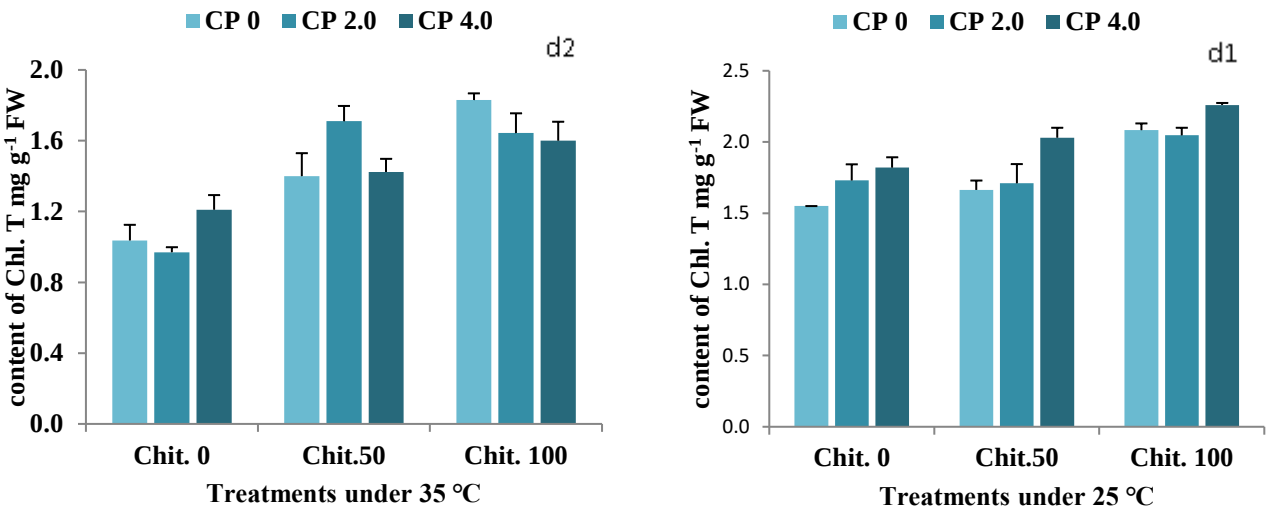


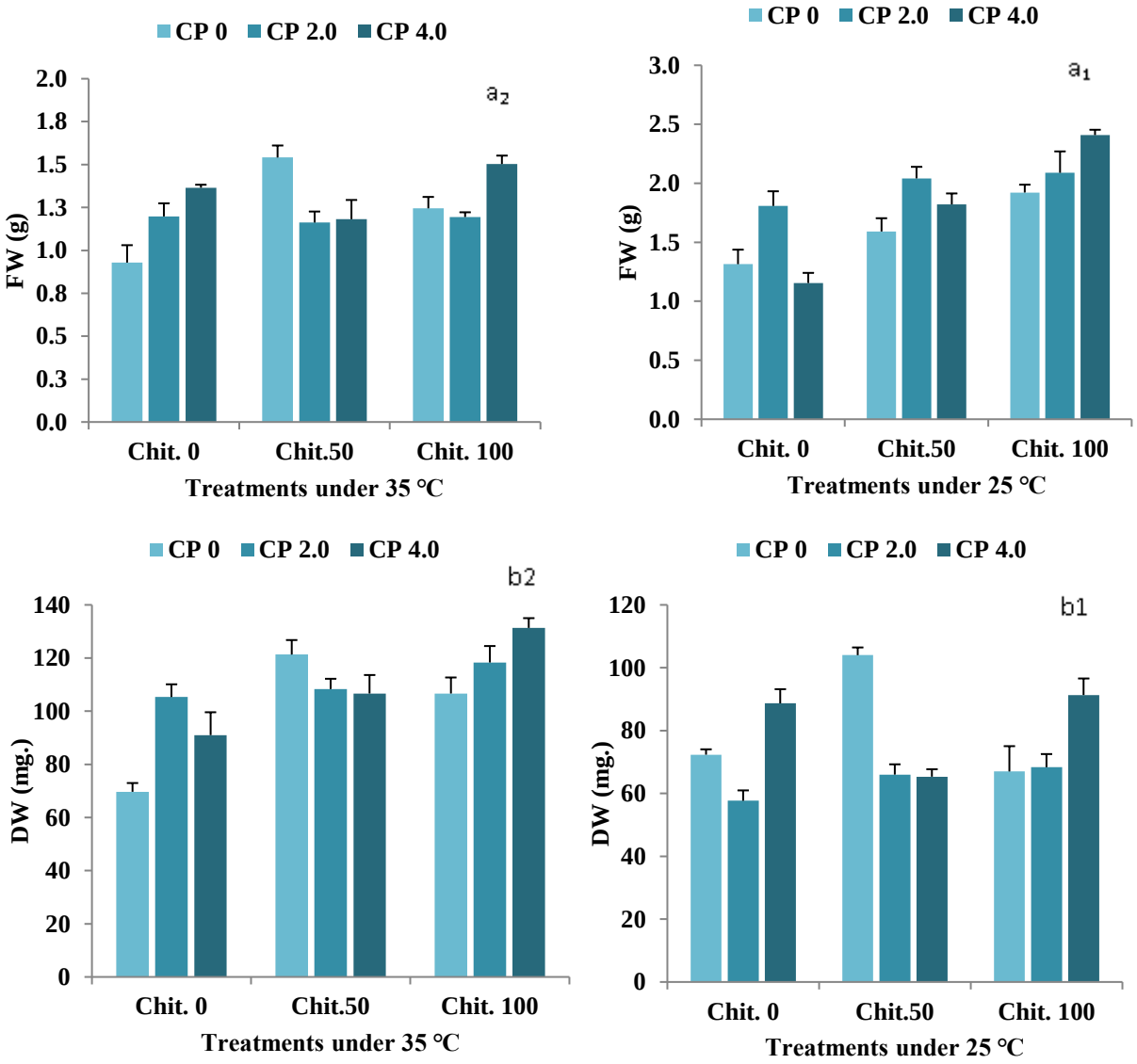
Figure 3: Effect of temperature, Chit, and CP on some growth indicators: (a_1 and a_2) relative water content, (b_1 and b_2) chlorophyll a content, (c_1 and c_2) chlorophyll b content, (d_1 and d_2) total chlorophyll content of calli cultures *P. vulgaris* L. explanted from hypocotyl after 21 days of cultivation on MS media.

Growth Parameters: The results indicated that there was a significant effect of the study factors on some growth indicators of tissue cultures, as the increase in temperature caused a decrease in the fresh weight of the P. vulgaris L. plant, while treatment with Chit and CP contributed to a decrease the fresh weight under the influence of the increased temperature. Both factors (Chit and CP) significantly affect, whether under normal or stressful temperatures. The combination of 0 µmol of Chit with 2.0 mg L^{-1} of CP achieved the highest fresh weight under 25℃ which was 2.808 g, while the 35℃ negatively affected the moisture content, 0.929 g, at the comparison treatments for both Chit and CP (Figure 4 a1 and a2).

The results showed significant differences between the study factors in the indicator. Despite the negative effect of the temperature, 35°C, on the chlorophyll-a pigment, Chit, and CP compounds contributed positively to enhancing the culture pigment content. The combination of 100 µmol of Chit and 4.0 mg L^{-1} achieved the highest dry weight, reaching 131.3 mg, outperforming all treatments, including the comparison treatment of the Chit compound with a concentration of 2.0 mg L^{-1} under 25 °C, which showed the lowest dye content of 57.7 mg (Figure 4, b1 and b2). The results showed that there was a significant difference between the data obtained as a result of the study factors on the growth rate of calli tissue, as the treatment of tissue cultures with the two concentrations 100 µmol + 4.0 mg L^{-1} for the study treatments Chit. and CP, respectively; at 25 °C it showed the highest average of the indicator 1.409, while Tissue cultures growing in media free of Chit. and CP under 35 °C showed a decrease in the growth of the cultures compared to the initial weight, with the lowest average index - 0.071 (Figure 4, c1 and c2).

Chitosan leads to an increase in photosynthesis, which reflects positively on growth indicators, as well as its effect in increasing plant growth regulators such as gibberellins. This is confirmed by GA3 (11 and 25), who showed that the effect of foliar spraying with the compound chitosan stimulated the photosynthesis process through the mechanism that controls the opening and closing of stomata. Also,

spraying plants with chitosan increases the activity of the main enzymes of the photosynthesis process and causes an increase in nitrogen transfer in the leaves, which enhances plant growth as a final result (16). It also affects plant growth through its effect on providing and increasing the absorption of water and necessary nutrients by adjusting cellular osmotic pressure and increasing enzyme activity (9). It increases chlorophyll content, photosynthesis, and synthesis of phenols, lignin, and flavonoid compounds (26) and increases cytosol and calcium ions (30).



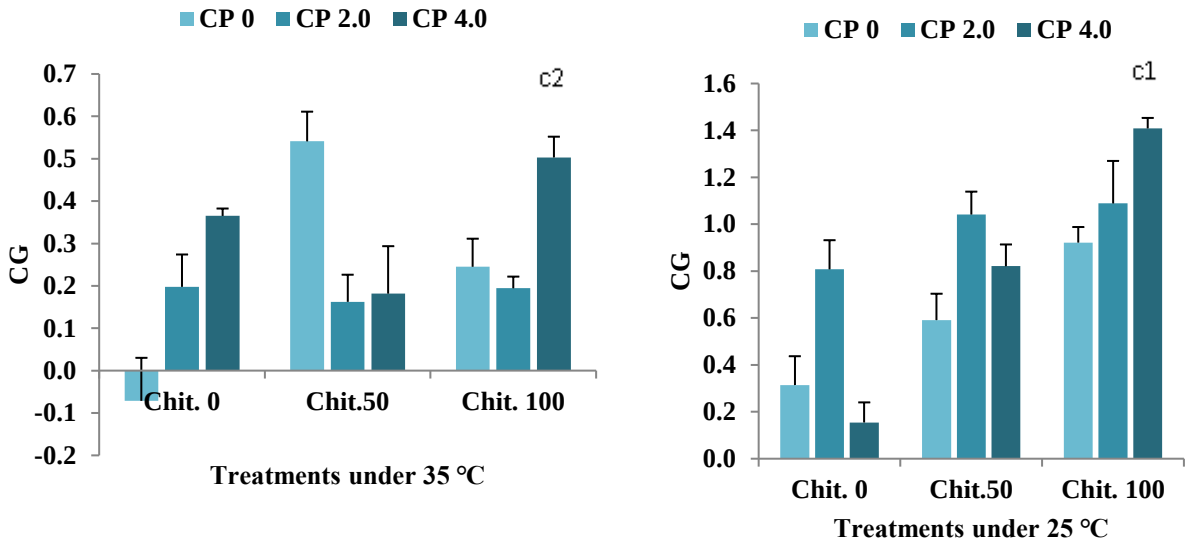


Figure 4: Effect of temperature, Chit, and CP on some growth indicators: (a_1 and a_2) fresh weight, (b_1 and b_2) dry weight, (c_1 and c_2) calli growth rate of *P. vulgaris* L. cultures extracted from the hypocotyl after 21 days of cultivation on MS media.

Principal Components Analysis: Principal component analysis was performed on the primary outcome and indicators to determine the effect of multiple parameters on some essential indicators of *P. vulgaris* L. calli cultures. Components 1 and 2 revealed the maximum contribution among all components, representing 97.3% of the total variance. In the data set, the contribution of component 1 was about 76.7%, while the contribution of component 2 was about 20.6%. The successful distribution of all treatments showed that using Chit and PC treatments positively impacted the physiological and growth indicators of calli plantations *P. vulgaris* L under normal or stressed growth media (Figure 5). Heat stress below 35 °C without Chit or CP compounds treatment showed adverse effects as physiological indicators, which was reflected in growth indicators and caused biomass depletion (fresh and dry weight and calli tissue growth rate), while using those two compounds led to a significant increase in the relative water content and pigment content, thus an increase in fresh and dry calli biomass and growth rate compared to other treatments. Treatment with Chit or PC at high concentrations of both with or without heat stress was more displacing than all other treatments, indicating that their application was more beneficial under heat stress (Figure 5).

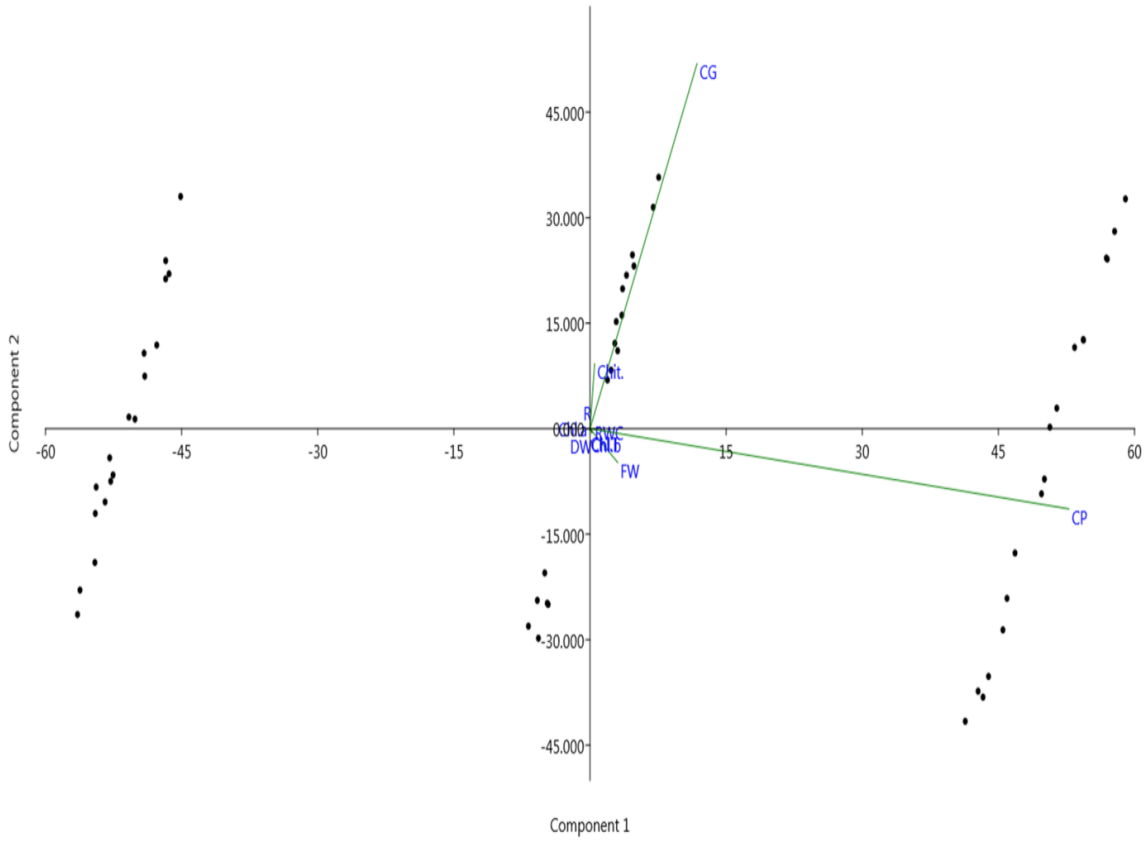


Figure 5: Principal components analysis of study factors and indicators.

Conclusions

Based on the results of the current study, the positive role of Chit treatments is clear to us. And CP on P. vulgaris L. kale culture for the first time. In general, heat stress caused a reduction in parameters within the study. The treatments contributed to the physiological regulation of some vital processes. They thus improved some parameters such as RWC, Chl a, Chl b, and Chl T. This improvement was reflected in its positive effect on basic growth parameters such as FW, DW, and CG. The results of this study help us understand the mechanism that can be implemented to improve the biomass production of calli tissue.

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