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Green Synthesized ZnO Nanoparticles Improve the Growth and Phytohormones Biosynthesis and Modulate the Expression of Resistance Genes in *Phaseolus vulgaris*

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THE present study was conducted for green synthesis of zinc oxide nanoparticles by zinc acetate and leaf extract of *Chenopodium album* and emphasize its impact on *Phaseolus vulgaris* growth. Evaluation of the synthesized ZnO NPs was done through spectrophotometer, X-ray diffraction, and Zeta-potential. Spherical crystals with an average diameter of 25 nm were captured in the Transmission electron microscopy photographs. *Phaseolus vulgaris* seeds early germinated when was treated with 300mg/L ZnO NPs, as radicles were observed in 40% of the sowed seeds. ZnO NPs supplementation at 500mg /L displayed a good performance in *P. vulgaris* plants, which was reflected in high measurements of root length, plant height, dry biomass and chlorophylls. Endogenous plant hormones elevated in all treatments when compared with that of control or *C. album* extract. Indole-butyric acid (IBA) and indole-acetic acid (IAA) were induced by 300mg /L ZnO NPs. Abscisic acid (ABA) was only recorded in plants treated with 1000mg /L. High level of α-amylase was measured in plants treated with 300 and 500mg/L ZnO NPs. 500mg /L ZnO NPs up-regulated NAC gene (no apical meristem gene) with 1.2 fold as compared to the control using qRT-PCR analysis. However, the same dose had no effect on glucanase gene (GLU) expression. This study supports the possibility of using ZnO NPs as a growth promoter and may improve the plant tolerance toward stresses.

Keywords: *Chenopodium album,* NAC and GLU genes, Nanoparticles green synthesis, *Phaseolus vulgaris*, Seed germination, ZnO NPs.

Introduction

Phaseolus vulgaris L. is one of the most important edible legumes for the entire world and participates in the global economy. Zinc is one of eight micronutrients essential for plant health and reproduction, membrane integrity. Zn takes part as a structural component of a variety of proteins, such as transcription factors and metalloenzymes, and regulates phytohormone activity (Figueiredo et al., 2012; Bharti et al., 2013). Application of nanoparticles (NPs) in agrisciences is the newest approach for yield improvement. Extensive physical and chemical methods were used for metal NPs synthesis (Kolekar et al., 2013). Tons of polluted materials were yielded upon applying these methods. Therefore, biological ways of synthesizing NPs from plants or microorganisms are trustworthy and safer (Abdelkader et al., 2021; Ghosh et al., 2021; Mandal et al., 2022). Many research articles have been disseminated details on the green synthesis of ZnO NPs from different plant materials such as *Zingiber officinale* (Raj & Jayalakshmy, 2015), *Cassia tora* (Manokari & Shekhawat, 2017), *Catharanthus roseus* (Gupta et al., 2018), *Ailanthus altissima* (Awwad et al., 2020). Based on concentration, plant species, developmental stage and the application method, NPs play a positive role in enhancing plant growth and self-protection against stresses (Subbaiah et al., 2016; Venkatachalam et al., 2017; Adhikari et al., 2021). On the contrary, phytotoxic effects of Zn NPs are reported on some plant species, especially at high doses (Salah et al., 2015; Wang et al., 2016). Therefore, more detailed studies are needed to elucidate their potential efficacy or toxicity.

Plant hormones play relevant roles in seed physiology, starting from germination until the dormancy of the produced seeds. Auxins such as indole-acetic acid (IAA) and indole-butyric acid (IBA) appear to contribute to multiple auxin-related developmental processes. For example, the auxin-rich phenotypes contributed to cotyledon enlargement and root hair elongation (Strader et al., 2020). Gibberellins (GAs) are essential for several physiological events in plants, including activation of vegetative growth of the germinated seeds and mobilization of the stored food. GAs promote growth by increasing the plasticity of the cell wall, followed by activation of many hydrolytic enzymes such as α- amylase, lowering the intracellular potential, causing water entry into the cell, cell elongation, and seed germination (Taiz & Zeiger, 2000). Abscisic acid (ABA) regulates vast masterful processes involved in plant development and adaptation to stresses. Plants under stressful conditions produce ABA in multiple organs to initiate protective mechanisms like controlling stomatal opening and inducing genes that promote resistance to environmental stressors (Lim et al., 2015; Taiz & Zeiger, 2000).

No apical meristem (NAC) gene is an important transcription factor (TF) regularly involved in regulation of abiotic stress response (Guo & Gan, 2012; Pascual et al., 2018). Several NAC proteins participate in crop development to enhance the varieties stress tolerance. Salt and drought tolerance in transgenic *Arabidopsis* plants can be improved by expressing ANAC019 (Tran et al., 2004). Transgenic rice plants with the overexpression of OsNAC5 or OsNAC45 enhanced the plant tolerance to drought and salt stresses (Sperotto et al., 2009; Zhang et al., 2020). Abiotic stress responses in wheat plants have been reported as a result of TaNAC29 overexpression (Huang et al., 2015). For maize plant, overexpression of ZmNAC49 could confer the plant's ability to face drought stress (Xiang et al., 2021). Up-regulation of NtNAC028 controlled the high salinity, dehydration, and abscisic acid (ABA) stresses (Wen et al., 2022). Even, the NAC gene acted as a positive regulator of natural leaf senescence in tobacco (Li et al., 2018); a negative regulation toward the abiotic stresses and promotion of leaf senescence via ABA biosynthesis had been reported (Shen et al., 2017).

Plant β-glucanases play a crucial role in regulating the plant–microbe interactions (Perrot et al., 2022). These enzymes have function in plant self-defense systems and can hydrolyze β-glucans. β-glucans are the major constituent in the wall of bacteria, metazoan, viruses, and some fungi (Bachman & McClay, 1996; Ruiz-Herrera & Ortiz-Castellanos, 2019). Therefore, the glucanase gene (GLU) is dominated in gene transformation technique to exceed the fungal plant resistant (Amian et al., 2011; Zhang et al., 2019; Wang et al., 2021).

The goal of this research is to improve the growth of *P. vulgaris* plant using ZnO NPs as a safe and alternative way to boost germination. Therefore, ZnO NPs were synthesized with the contribution of *C. album* leaves, which is a plant weed. The synthesized NPs were characterized using UV- visible spectroscopy, Fourier transform infrared (FTIR), X- ray diffraction (XRD), Transmission electron microscopy (TEM) and zetapotential. The impact of ZnO NPs on *P. vulgaris* seed germination and the endogenous level of some major plant hormones were assessed. In conjunction with the morphological parameters, the biological activity of ZnO NPs was probed for its effect on the expression of NAC and GLU genes. These two genes had been selected as they represent stress resistance genes.

Materials and Methods

Plant materials

Phaseolus vulgaris L. (var. Giza 6) seeds were donated from Agriculture Research Centre, Giza, Egypt. *Chenopodium album* plants wildly grow in the decoration garden at Cairo University. Accumulation of *C.album* plants represents a huge agricultural waste. Using that waste in green synthesis of nanoparticles represents a good solution to reduce agricultural waste on the campus.

Preparation of ZnO NPs in presence of C. album leaf extract

Fifty grams of fresh *C. album* leaves were gathered from the Cairo University garden, washed with distilled water and left in air to dry then cut into small pieces to be used in ZnO NPs preparation according to Abbasi et al. (2020) with some modifications as indicated. The extract was obtained by soaking and macerating these pieces with 1L of boiled distilled water kept at 70°C for 15min., then extract was filtered through Whatman no. 3. To investigate the effect of pH on ZnO NPs formation, the obtained *C. album* leaf

extract was divided into four flasks and 1 mM Zn acetate was added to each extract. The pH of the extract was adjusted at 6, 8, 10 or 12 by adding of 1M NaOH or 0.1 N HCL and continuous stirring for 2 h. All other experimental variables, including temperature and time, were held steady. Absorption spectrum was established for each flask started from 300 to 700nm. The formed precipitates at the highest absorbance at 400nm (pH 10) were collected by centrifugation at 10000rpm for 15min. The precipitate was rinsed with sterilized deionized water, dehydrated and calcined at 600°C for 3h.

Characterization of ZnO NP$_s$
UV- vis spectrum
Absorption spectrum was recorded for each pH from 300 to 700nm using the Perkin-Elmer spectrophotometer instrument.

FTIR spectrum
The powder of ZnO NPs was mixed with 10 mg KBr and then pressed and dried to remove moisture content and pressed into a plate for FTIR characterization (Mishra & Sharma, 2015). Infrared spectrum was recorded in the region of 500-4000 nm using Perkin-Elmer instrument.

X-ray diffraction (XRD)
Analysis was performed on an X-ray diffractometer (Bruker D8) operating at 30 KV and 30mA. The routine protocol at Characterization Lab of Nanomaterials, Faculty of postgraduate of nanotechnology, Cairo University was followed. The size of synthesized ZnO NPs was calculated using Scherer's equation (Holzwarth & Gibson, 2011).

$$D = K \lambda / \beta \cos \varnothing$$

where: D= size of nanoparticle, k= 0.9, λ= 1.5406, β= FWHM and ∅ is the peak position.

Transmission electron microscopy (TEM)
The shape of the synthesized ZnO NPs was photographed by TEM (JEOL- 1011, Japan). Samples were prepared by fixing drops of aqueous particle suspensions onto a carbon-coated copper grid. The solvent was evaporated under vacuum (Nagaonkar & Rai, 2015).

Zeta-potential analysis
The Z-potential defines the colloidal stability and the surface electric charge on the nanoparticle (Modena et al., 2019; Faisal et al., 2021). This analysis was performed using NiCOMP Nano (Z 3000 zls).

Estimation of imbibed water amount by P. vulgaris seeds
In 50mL Falcon test tubes, six sets of five weighed P. vulgaris seeds were immersed in 15mL of distilled water (C) or C. album leaf extract (CE) 50g/L, or in different concentrations of ZnO NPs: 300mg /L (T1), 500mg /L (T2), 700mg /L (T3) and 1000mg /L (T4). The tubes were incubated at 25°C for 12h. All seeds were removed, blotted dry and re-weighed. The amount of the imbibed water per gram of seed was calculated (Saikia & Sarma, 2017).

Germination of P. vulgaris seeds
P. vulgaris seeds of equal size were surface sterilized by shaking at 100rpm in a solution of 3% sodium hypochlorite for 3min and then rinsed with sterile distilled water. Eighteen sterile Petri dishes (15cm) contained double layers of filter paper Whatman no. 3 were grouped as follows: Control (C), C. album leaf water extract (CE), treatment with ZnO NPs: 300mg /L (T1), 500mg /L (T2), 700mg /L (T3) and 1000mg /L (T4). Twenty sterilized seeds and 20mL of corresponding solution/suspension was placed in the petri dishes, and were incubated at 26±1°C for 7 days. The seed germination percentage was calculated by the following equation:

G %= (no. of germinated seeds/ total no. of seeds) X 100

Planting of P. vulgaris seeds
Eighteen sterile plastic pots (15cm in diameter) were filled with a sterile mixture of clay and sand (50:50 w/w). The pots were arranged into six groups as follows: Control (C), C. album leaf water extract (CE), treatment with different concentrations ZnO NPs as used in previous experiments: T1, T2, T3 and T4. Each group consisted of three pots. Five surface sterilized seeds were planted and watered with 200mL of the corresponding solution/suspension every three days, and were placed in the green house at 26±1°C for 30 days. Morphological measurements were studied on the collected plant samples. Samples were kept in a deep freezer for further analyses.

Determination of photosynthetic pigments
From health and fresh leaves, 0.3g was

homogenized in a prechilled mortar with acetone: water (4:1, v/v) mixture. The homogenates were centrifuged at 3000 g for 10min. The debris was rewashed until decolorization of the tissues. The absorbance was determined at 663, 645 and 470 nm by using the Perkin-Elmer instrument (Yang et al., 1998). The concentration of each pigment was calculated from the following formula:

$$Chl\ a = 12.25\ A_{663} - 2.25\ A_{645}$$

$$Chl\ b = 20.31\ A_{645} + 4.91\ A_{663}$$

$$Total\ carotenoids = 1000\ A_{470} - 2.27\ (Chl\ a) - 81.4\ (Chl\ b)/\ 227$$

The results were expressed as μg m/L extract, then calculated as mg /g Fwt.

Determination of endogenous P. vulgaris plant hormones

Endogenous *P. vulgaris* plant hormones were extracted from different samples according to Topçuoğlu & Ünyayar (1995). Agilent 1260 infinity HPLC equipped with Quaternary pump, a Zorbex Eclipse Plus C18 column (100 mm x 4.6mm), operated at 35°C. The injected volume was 20 μL. The elution gradient was mixture of 5 mM ammonium acetate/ 0.05% formic acid in water and acetonitrile. The detector set at 25 nm. The ambient temperature was 20°C. Authentic hormones (ABA, BAP, IBA and IAA) were used as standards.

Determination of α- amylase activity

α- amylase activity was assayed according to Goncalves et al. (2010). One g of 30 days old *P. vulgaris* leaves was homogenized with 10mL of extraction buffer (50mM sodium succinate, 3 mM $CaCl_2$, 3mM β-mercaptoethanol and 20% glycerol). The homogenate was centrifuged

for 20min at 10000. The reaction mixture was prepared by mixing 1mL of the enzyme extract, 1ml starch solution and 1mL phosphate buffer at pH 6.8 and incubated for 30min at 37°C. The reaction was terminated by adding 2mL dinitrosalicylic acid reagent (5g dinitrosalicylic acid in 250mL distilled water, 100mL of 2N NaOH, and 150 g of potassium sodium tartrate-hydrate), and boiling for 15min. The absorption was determined at 540nm. A standard calibration curve was established using 0.1-10mg/L glucose.

The real-time PCR analysis of NAC and GLU genes expression in response to ZnO NPs

Total RNA isolation and cDNA synthesis

Total RNA was extracted from 100 mg of frozen 30 days *P. vulgaris* terminal bud using the RNeasy Plant Mini Kit (Qiagen, Germany). NanoDrop ND-1000- Italy was used to estimate the RNA yield purity and concentration. cDNA was synthesized from the yielded RNA using cDNA synthesis kit (Bioneer, Korea).

Quantitative PCR (qPCR) assays

Table 1 shows the sequence of reverse and forward primers which used for RT-PCR analysis. Primer sequences were designed by using BLAST (NCBI: http://www.ncbi.nlm.nih.gov/BLAST). In a final volume of 25μL: 5μL of cDNA sample, 12.5μL of qPCR Master Mix, and 0.4μM of primers were mixed. Three replicates of qPCR reactions were performed in the Real-Time System (Bio-Rad). The qPCR thermal cycle was: denaturation at 95°C for 10min, followed by 40 cycles at 95°C for 20sec; at 60°C for 45sec. The fold change in the expression level of the NAC and GLU genes was calculated using CFX Manager Software (Bio-Rad). Data were analyzed using the comparative Ct method (Kamranfar et al., 2018).

TABLE 1. Primers sequences used for real time PCR

Gene	Forward primer	Reverse primer
Actin 1 (Accession no. AY305733)	ATCCTCCGTCTTGACCTTG	TGTCCGTCAGGCAACTCAT
NAC	GGTGATCAGCCGTGATCCAA	CGACCGAAAGGGGTTGGTTTTC
GLU	CCCTACGGCTTCGGTCTTTT	CGCGCAAGGTAATGAAGACG

The specificity of each primer was confirmed by using the BLASTN tool to ensure that the primers targets a unique site within the set of predicted *P. vulgaris* sequence. Actin 1 was used as reference gene.

Statistical analysis

All data were presented as the mean (±SD) of three replicates. Data were statistically analyzed by one-way ANOVA using SPSS 18.0 software. A Tukey test was performed with a P-value <0.05 to identify significant differences compared to controls.

Results

Preparation and characterization of ZnO NP$_s$

pH value is considered as the most key factor affecting the amount and size of NPs synthesized by the green method. Four different pHs (6, 8, 10 and 12) was studied (Figs. 1, 2). It was observed that pH 10 was optimum for green synthesizing ZnO NPs from *C. album* leaf extract (Fig. 2). While, the absorbance was lower at other pH values. Hence, *C.album* leaf extract was found to be a typical source for the biosynthesis of ZnO NPs. From the absorbance spectrum, the absorbance peak was between 300 and 420nm. The stabilizing, reducing, and capping agents required to synthesize ZnO NPs are present in the secondary metabolites of the leaf extract.
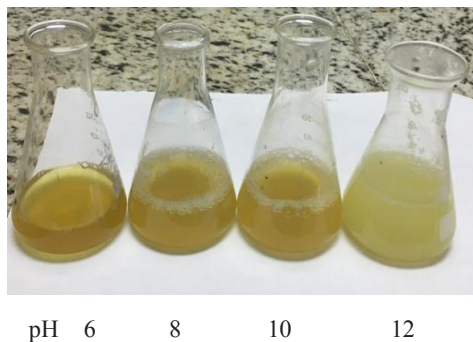

Fig. 1. Developing of ZnO NPs in presence of *C. album* leaf extract at different pHs
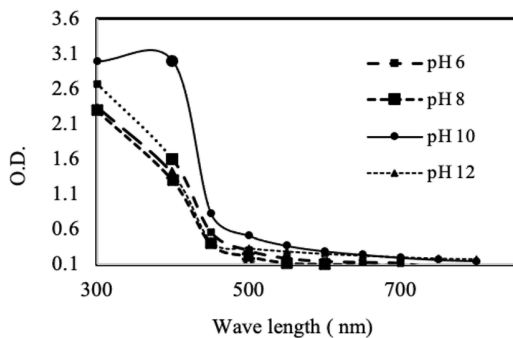

Fig. 2. Absorption spectra for ZnO NPs synthesized in presence of *C. album* leaf extract

The FTIR spectrum of ZnO NPs from *C. album* leaf extract represented in Fig. 3. FTIR peaks have functional groups exposed at 3822-3433, 2211, 1618 and 1063 /cm. The peak between 700 - 800/cm confirmed the formation of ZnO NPs.

The XRD analysis confirmed the crystalline nature of the synthesized ZnO NPs as the obtained peaks observed in Figure (4) are sharp and not broad. The XRD spectra showed distinct direction peaks with 2 ∅value of 20.214, 29.22, 33.83, 35.40, 40.50 and 43.72 with intensity values of 110, 344, 149, 186, 96.7 and 79.4, respectively. The ZnO NPs were determined to have an average size of ~9 nm. The presence of numerous peaks was due to the organic compounds present in the *C. album* leaf crude extract. These compounds play a significant role in the reduction of zinc ions and the stabilization of NPs.

Zeta potential analysis was performed to detect the surface of green synthesized ZnO NPs. In this study, the average zeta potential value was – 6.51mV, with – 0.48 mobility (Fig. 5). This result confirmed that there is a biomolecular cap present on the obtained ZnO NPs.

TEM images provided the morphology and the size of ZnO NPs at direct magnification (Fig. 6). Spherical crystals of ZnO NPs were observed with an average diameter ranging from 25 to 33nm. This range was higher than that got from calculated size by applying Schere's equation on XRD analysis pattern.

Imbibition and growth parameters of P. vulgaris seeds impacted by ZnO NPs

Figure 7 displays the absorbed water volume of *P. vulgaris* seeds after being soaked for 12 hours in distilled water, which acted as the control (C) or *C. album* leaf extract (CE) or different concentrations of ZnO NPs suspension. ZnO NPs application decreased the imbibed water amount. The lowest imbibed water amount was in T4. The amount of water absorbed by seeds in T4 was 40% lower than the control. CE did not influence the amount of the imbibed water. T1 and T2 impacted the seed germination, the radicle length of the seedlings reached 2.5 cm in both treatments. However, radicle length was 2 and 0.3 cm in T3 and T4, respectively. No radicle was observed in C or CE (Fig. 8).
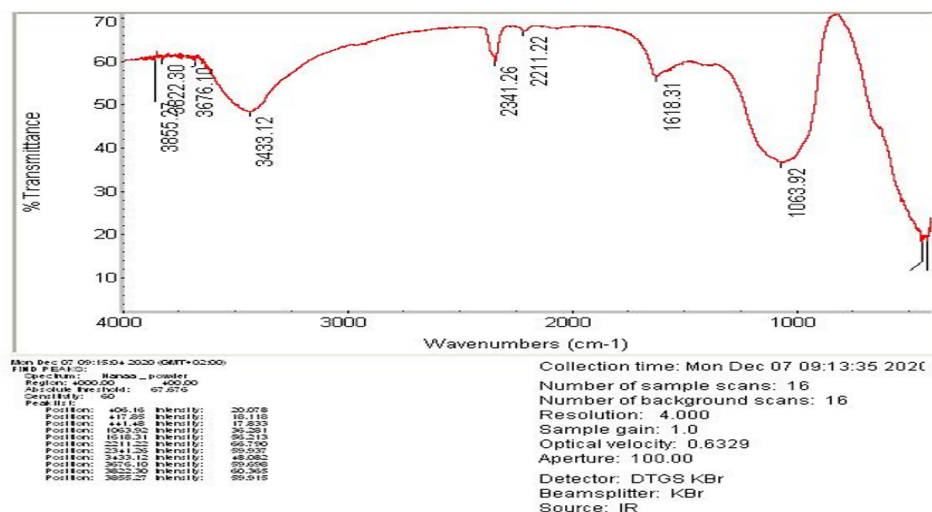
Fig. 3. FTIR spectrum of synthesized ZnO NPs in presence of *C. album* leaf extract measured in % transmittance in frequency range of 4000 – 500/cm
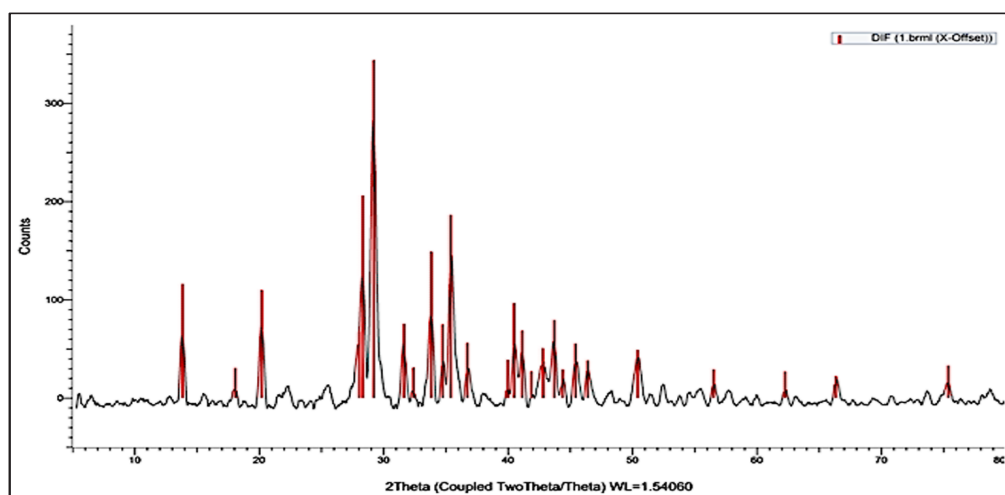

Fig. 4. X- ray diffraction pattern (XRD) of synthesized ZnO NPs in presence of *C. album* leaf extract

Early seed germination was observed in T1, as radicles were observed in 40% of the sowed seeds. Nonetheless, the germination percentage is negatively impacted by the rise in concentration of green synthesized ZnO NPs. In C and CE, the seeds started to germinate after 5 days of sowing (Fig. 9).

Growth parameters and photosynthetic pigments level were assessed in response to CE or ZnO NPs application at four doses and compared with control (C). These results were represented in Table 2. The highest values for all measurements were in T2 except the total carotenoids. The untreated plants (C) and CE contained high carotenoids compared to NPs-treated plants.

The root length of T2 demonstrated a significant

doubling compared to C. ZnO NPs application led to a slight increase in shoot length across all concentrations. Regarding the fresh weights, the highest root fresh weight was in T2, as it reached 150% compared with C. The highest shoot fresh weight was also in T2. The shoot fresh weight of the plants in this treatment increased by 60% relative to control. Despite the constancy of the leaves number, the leaf area increased by 20% in case of T2. ZnO NPs biosynthesized from CE exhibited positive impact on photosynthetic pigments (Table 2). The high Chl a and b contents were measured in T2. The plant leaves of that treatment contained 22% and 44% more Chl a and b than control, respectively. However, carotenoids content in T2 plants was half of its value in control.
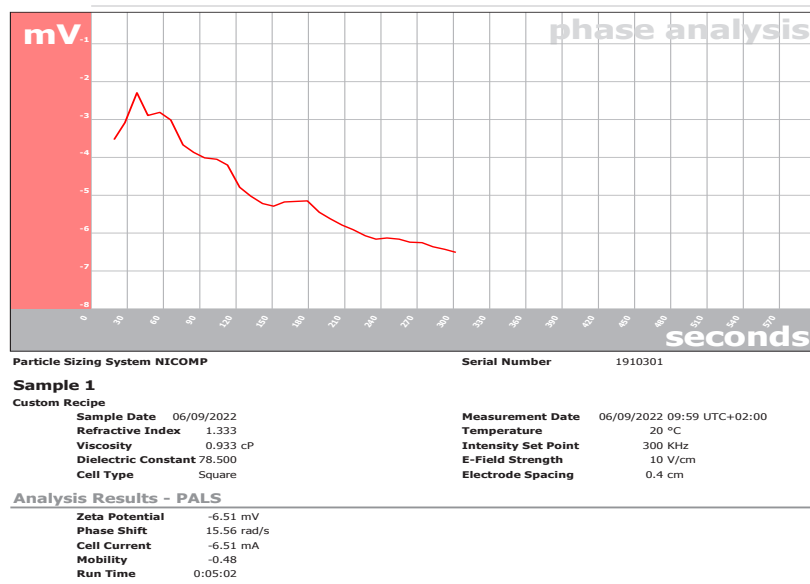
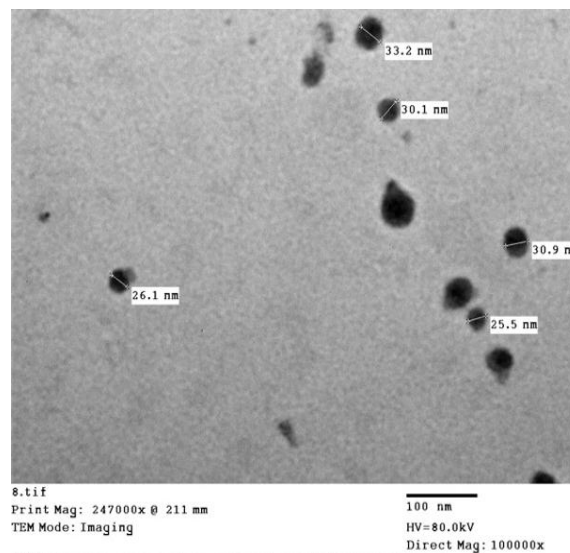**Fig. 5. Zeta-potential analysis of the of synthesized ZnO NPs in presence of *C. album* leaf extract**

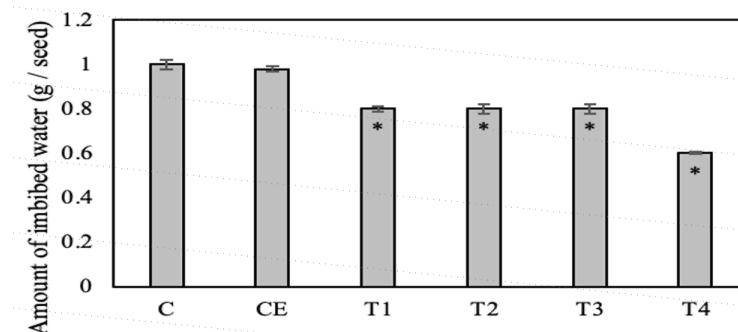**Fig. 6. TEM for the ZnO NPs synthesized in presence of *C. album* leaf extract**

Fig. 7. Amount of imbibed water by *P. vulgaris* seeds after 12 h of immersing in different solutions. C: control; CE: *C. album* leaf extract; T1: 300; T2: 500; T3: 700; T4: 1000mg/L ZnO NPs [Bars indicate SD. (*) indicates significant differences compared to control ($P < 0.05$)]

C   CE   T1   T2   T3   T4

Fig. 8. Germination of *P. vulgaris* seeds in presence of different ZnO NPs concentrations [C: control; CE: *C. album* leaf extract. Legend as the same as described in Fig. 7]
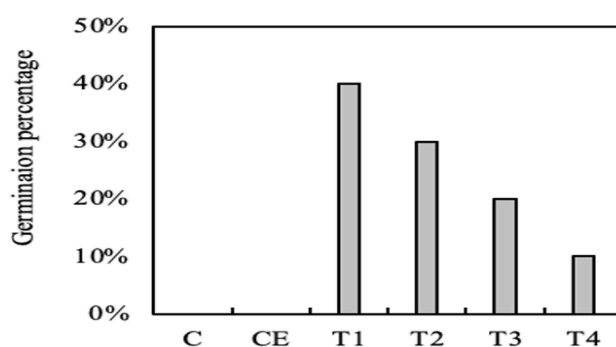

Fig. 9. Germination percentage of *P. vulgaris* seeds after two days of planting [C: control; CE: *C. album* leaf extract; T1: 300; T2: 500; T3: 700; T4: 1000mg/L ZnO NPs]

TABLE 2 Growth parameters and photosynthetic pigments in 30 days *P.vulgaris* plants in response to the application of CE or ZnO NPs mediated by *C.album* leaf extract

Treatments	Root length	Root Fwt	Shoot length	Shoot Fwt	No. of leaves	Leaf area	Chl. a	Chl. b	Total carotenoids
C	9.0 ±0.1 c	0.2 ±0.01 cb	17.0 ±0.3 c	1.5±0.01 b	2 ±0.0 a	19.91±0.11 c	1.57±0.01 c	1.20±0.03 c	0.53 ± 0.03 a
CE	8.9 ±0.15 c	0.3 ±0.01 b	17.5 ±0.1 c	1.3±0.02 cb	2 ±0.0 a	20.09±0.15 c	1.55±0.01 c	1.30±0.01 c	0.57 ±0.01 a
T1	12 ±0.2 b	0.3 ±0.02 b	19.0 ±0.18 ab	2.5±0.01 a	2 ±0.0 a	21.02±0.13 b	1.64±0.04 b	1.56±0.04 b	0.38 ±0.04 b
T2	18 ±0.15 a	0.5 ±0.01 a	19.9 ±0.11 a	2.4±0.02 a	2 ±0.0 a	34.10±0.14 a	1.92±0.01 a	1.73±0.03 a	0.27 ±0.03 c
T3	17.7±0.13 a	0.49±0.01 a	19.7 ±0.12 a	2.3±0.03 a	2 ±0.0 a	35.10±0.15 a	1.89±0.02 a	1.77±0.01 a	0.28 ±0.01 c
T4	17.9±0.11 a	0.5 ±0.02 a	19.5 ±0.15 a	2.1±0.02 a	2 ±0.0 a	33.10±0.11 a	1.99±0.02 a	1.69±0.06 a	0.28 ±0.05 c

C: control; CE: *C. album* extract; T1: 300; T2: 500, T3: 700, T4: 1000 mg /L ZnO NPs. Length (cm); Fwt (g/plant); area (cm^2); Chl a, b and total carotenoids (mg/g Fwt). (±): SD; different letters indicate significant differences at P< 0.05.

Four plant hormones were studied in 30 days *P. vulgaris* plants and represented in Fig.10. Among all treatments, including C and CE, IBA reigned supreme as the dominant plant hormone. The induction of IBA synthesis in T1 was twice that of C. Despite this, the IBA content in the plants decreased with the rise in ZnO NPs concentration. Nevertheless, the IBA content in NPs-treated plants remained superior to that of C or CE. All tested plants exhibited IAA, albeit T1 had a significantly elevated level. The BAP content was the least in T4, with only 34.55% retention compared to C. The induction of ABA synthesis was exclusively observed in the 1000mg/L ZnO NPs treatment, which stands out as the most remarkable result of this experiment.

α-amylase activity has been assessed in 30 days plants either in C, CE, or plants treated with ZnO NPs (300, 500, 700 and 1000mg/L). The results were illustrated in Fig. 11. CE has no effect on α-amylase activity. However, T1, T2 and T3 significantly increased α-amylase activity. Significant inhibitory effect of ZnO NPs in T4 has been shown.

NAC and GLU genes expression in P. vulgaris plants treated with ZnO NPs

qRT-PCR was employed on 30-day-old *P. vulgaris* plants, treated with CE or 500mg/L ZnO NPs (T2), and untreated plants (C) to determine whether ZnO NPs induced the expression of NAC and GLU genes. Data were analyzed using the comparative Ct method. The fold change in the expression level of the NAC and GLU genes

transcripts expression levels were calculated based on the levels in control. Figure 12 shows that, treatment with CE had no effect on NAC gene expression. However, NAC gene was up-regulated with 500mg /L ZnO NPs which have an average diameter of 25nm. The fold of change of NAC gene expression was 1.2 as compared to control. Figure 13 illustrates the alteration in fold change of GLU gene expression in *P. vulgaris* plants after 30 days of treatment with CE or 500mg/L ZnO NPs (T2), compared to untreated plants (C). This figure reveals that neither CE nor ZnO NPs had an effect on expression of this gene.

Discussion

ZnO NPs characters

Plants contain many phytochemicals compounds that act as reducing agents (Abdelrazik et al, 2020; Soliman et al., 2022). Terpenes, alkaloids, flavonoids, and phenolics are among the phyto-compounds that aid in NPs synthesis, leading to cost and environmental hazard reduction which may produce during chemical and physical NPs synthesis methods (Khan et al., 2022). pH value is a key factor affecting the amount and size of NPs by the green synthesis (Iravani, 2011). In this study, pH 10 was the optimum pH for the synthesis of ZnO NPs from *C. album* extract. At this pH, the OH⁻ ions played a role in ZnO conversion and the particle formation. At the alkaline pH, the flocculation rate decreased and so the particle size reduced (Jang et al., 2009). However, at acidic pH, the particles aggregated and produced wider diameter.

Fig. 10. Plant hormones contents in 30 days *P. vulgaris* plants in re-sponse to treatment with different concentrations of ZnO NPs synthesized in presence of *C. album* leaf extract [C: control; CE: *C. album* leaf extract; T1: 300; T2: 500; T3: 700; T4: 1000mg /L ZnO NPs. IAA: indole acetic acid; IBA: indole butaric acid; BAP: Benzylaminopurine; ABA: abscisic acid. (*) indicates significant differences compared to control (P<0.05)]

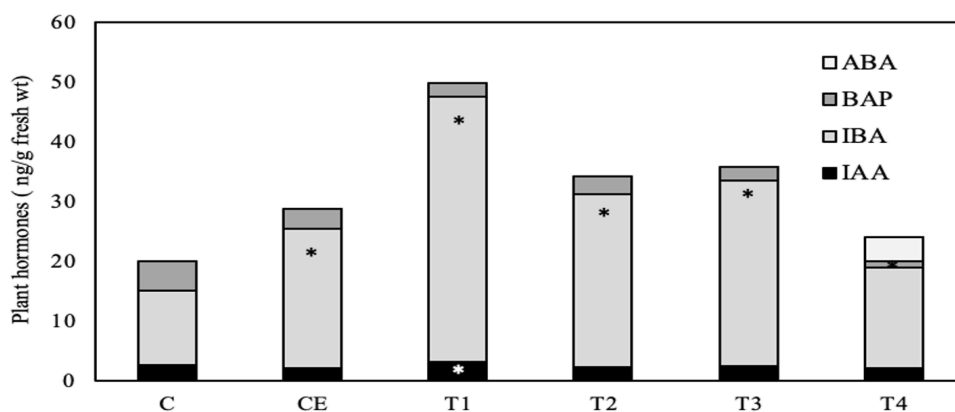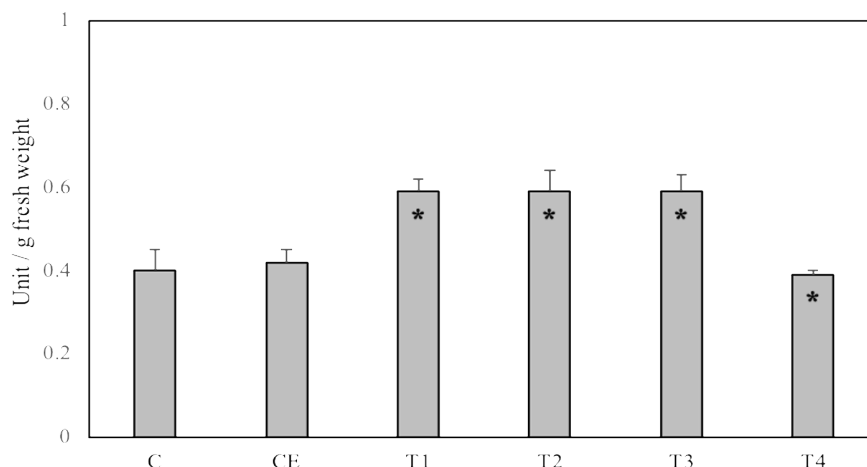**Fig. 11.** α- amylase activity in 30 days *P. vulgaris* plants in response to treatment with different concentrations of ZnO NPs synthesized in presence of C. album leaf extract [C: control; CE: *C.album* extract; T1: 300; T2: 500; T3: 700; T4: 1000 mg /L ZnO NPs. Bars indicate SD. (*) indicates significant differences compared to control (P<0.05)]
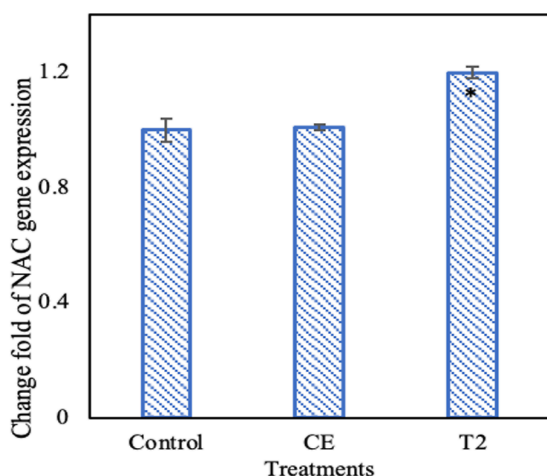

Fig. 12. Change fold of NAC gene expression in 30 days *P. vulgaris* plants treated with *C. album* extract (CE) or 500mg /L ZnO NPs treatment (T2) [Bars indicate mean ±SD. (*) indicates significant as compared to control]
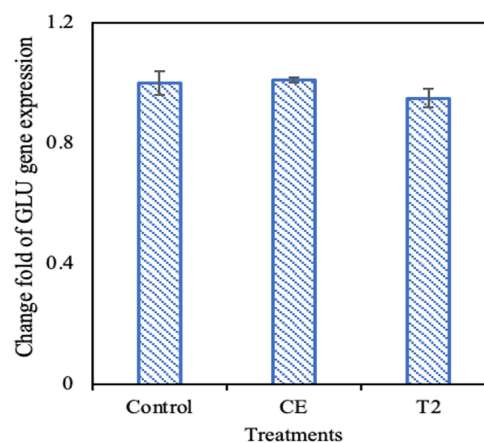

Fig. 13. Change fold of GLU gene expression [Legend as the same as described in Fig. 12]

The UV-vis spectrum revealed that the absorption peak of ZnO NPs was observed between 300 and 420 nm. From the previous literatures, the absorption peak was at 272 nm synthesized from the almond seed coat (Khan et al., 2022), at 366 nm from *Catharanthus roseus* (Gupta et al., 2018), at 380 nm from *Passiflora caerulea* leaf (Santhoshkumar et al., 2017), and at 398 nm from *Geranium wallichianum* (Abbasi et al., 2020).

In this investigation, the FTIR spectrum shows numerous peaks. A high - intensity broad band between 3822 - 3433/cm is due to stretching alkenyl group made of zinc acetate salts and their reduction to ZnO NPs. Peak between 700 - 800/cm confirmed the formation of ZnO NPs, and indicated the dual role of plant extract as a green reducing and stabilizing agents (Khan et al., 2022). According to Aldalbahi et al. (2020), the Zn–O stretching modes resemble the absorption peaks at 700 – 400. The remained peaks in the figure could be elucidated according to Gupta et al. (2018) as follows: at 2211/cm

shifted to C-C bond; at 1618 /cm corresponding to C= O stretching of functional group; at 1063/cm resulted from C-N stretching amino group. From literature, ZnO NPs using the almond seed coat extract for green synthesis prominent band at 527/cm (Khan et al., 2022).

In the present work, the XRD analysis confirmed the crystalline nature of the synthesized ZnO NPs as the obtained peaks were sharp and not broad. The size of synthesized ZnO NPs was calculated using Scherer's equation was ~ 9nm. This was consistent with that reported on ZnO NPs synthesized from *G. wallichianum* (Abbasi et al., 2020); *Olea europaea* (Issam et al., 2021).

Zeta-potential analysis was performed to detect the surface of green synthesized ZnO NPs. In this study, the average zeta potential value was − 6.51mV, with − 0.48 mobility. This value revealed that there was an incipient stability with a fair degree of coagulation and confirmed the presence of biomolecular cap on the ZnO NPs (Barzinjy & Azeez, 2020; Faisal et al., 2021). The detected negative charges on the nanoparticles revealed the electrostatic repulsion between these particles. The value of zeta potential in this study was very coincides with that from pomegranate (*Punica granatum*) fruit peel extract (Yassin et al., 2022). However, it was less than that from *Myristica fragrans* fruit extract (Faisal et al., 2021).

The definite morphology and the size of the synthesized ZnO NPs were provided with TEM. The average diameter of the spherical crystals of ZnO NPs was 25 nm. This range was comparable with that formerly reported (Khan et al., 2022). However, ZnO NPs with high diameter (50-92nm) was reported by Gupta et al. (2018); small diameter (~18 nm) was reported by Abbasi et al. (2020). A wide size range of spherical ZnO NPs (20nm to 60nm) was measured Tymoszuk & Wojnarowicz (2020). It is worth saying, the great efficacy and impact of nanoparticles are parallel with the small size.

Impact of ZnO NPs application on P. vulgaris plant

The growth of *P. vulgaris* is impeded by diverse biotic and abiotic stresses leading to several challenges, decreasing them is a noble goal to prevent universal food shortage (Diaz et al., 2018). The process of seed imbibition is subject to various factors, including temperature, pressure, pH, texture of imbibing medium, and electrolyte presence. Presence of electrolyte represents the cardinal one of the mentioned factors, as the others were fixed in this study. Significant decreased in the imbibed water amount was measured in ZnO NPs- treated seeds. Zinc not only promotes seed viability and root development, but also affects water retention and transport capacity. In addition, it reduces the negative effects of abiotic stresses and plays an active role in gibberellin biosynthesis (Tsonev & Lidon, 2012; Sedghi et al., 2013). Different effects after NP application have been reported in several plant species. An instance would be the triggering or hindering of seed germination and seedling growth through the activation or repression of genes that regulate plant metabolism. As NPs are easily cross cell walls and deliver target genes to specific organelles (Gupta et al., 2018).

In this work, early seed germination was noted in T1. Due to the barrier function of the plant cell wall, any external agent, including nanoparticles, needs to have a smaller diameter than the cell wall's pore diameter to penetrate and reach the plasma membrane (Nair et al., 2010). They may also cross the membrane using embedded transport carrier proteins or through ion channels. NPs have the ability to bind with different cytoplasmic organelles, ultimately impeding metabolic processes in those locations (Nair et al., 2010). Hence, it has been served in the maintenance of cell organelles such as chloroplasts and mitochondria (Ahmad et al., 2018). Several reports addressed the ZnO NPs impact on improving the plant seed germination and vigor such as alfalfa (Bandyopadhyay et al., 2015), *Gossypium hirsutum* (Venkatachalam et al., 2017), *Portulaca oleracea* (Iziy et al., 2019), onion (Raskar & Laware, 2014). ZnO NPs exhibited biphasic effects on *Allium cepa* seed germination, promoting it at low concentrations but hindering it at high concentrations (Tymoszuk & Wojnarowicz, 2020), while it also suppressed the germination of ryegrass seeds (Lin & Xing, 2007). Supplementation of NPs Zn restored the uptake of both micro and macronutrients that are lost in stressful conditions. It can be used as a seed germination booster when applied at controllable dose.

In this study, ZnO NPs increased plant biomass and chlorophylls content. Clearly, there

is a partial correlation between the plant species, NPs dose and size, and their response (Rizwan et al., 2017). From literature, ZnO NPs (32nm) at concentrations of 1, 2, and 4mg /L enhanced barley seed germination, shoot/root elongation (Plaksenkova et al., 2020). Root length of corn seedlings was promoted by 10mg /L ZnO NPs (Zhang et al., 2015). Growth of mung bean and chickpea was increased by ZnO NPs (1~20 ppm) (Mahajan et al., 2011). Zinc is likely stimulating the plant growth through its involvement in enzymes participate in protein synthesis, and plays a verified function in the tryptophan production which is a foundational amino acid in the auxin biosynthesis (Taiz & Zeiger, 2000). However, high dose of ZnO NPs (1000mg/ L) reduced the root length of corn and cucumber (Zhang et al., 2015). Application of ZnO NPs in concentrations up to 500μg m/L decreased the shoot and root length of rapeseed germinated seedlings (Mousavi Kouhi et al., 2014). Onion root growth inhibited by 5, 10, 20mg ZnO NP (Ahmad et al., 2018). Meanwhile, no significant difference in the shoot length of those exposed to ZnO NPs with size over 50nm (Adhikari et al., 2021).

The degree to which a plant can photosynthesize effectively is inextricably linked to its chlorophyll content, which is an essential determinant of its growth. The presence of Zn is indispensable for plant growth as it is an obligatory trace element that facilitates Chl biosynthesis (Cakmak & Braun, 2001). Nevertheless, an excessive amount of Zn can hinder the synthesis of photosynthetic pigments and, consequently, hamper the photosynthetic process (Li et al., 2013). The association of ZnO NPs treatment in photosynthetic pigments, implying a potential improvement in photosynthesis efficiency. The previous literature indicated that maize showed a 19% increase in Chl. a and wheat exhibited a 25% increase in Chl. a upon the administration of 200mg/L ZnO NPs (Srivastav et al., 2021). For Chl. b, maize saw a 30% increase, while wheat experienced a 24% increase under the same conditions (Srivastav et al., 2021).

Recently, lower doses of ZnO NPs than that used in this study (20μg/mL) have 34% more chlorophyll content as compared to control (Mazumder et al., 2020). However, decrease the chlorophyll content in higher plants, such as soybean and kidney bean was reported (Priester et al., 2017; Medina-Velo et al., 2017).

Carotenoids are non-enzymatic antioxidant compounds soluble in plant cells operate to reduce oxidative damage to the plant (Hashimoto et al., 2016). Carotenoids are the precursors for the abscisic acid biosynthesis in the plants, so they can generate plants tolerant to stresses (Taiz & Zeiger, 2000; Sun et al. 2022). Controversy to the result in this investigation, ZnO NPs application enhanced the carotenoids content in green pea, peanut, and cluster bean (Prasad et al., 2012; Mukherjee et al., 2016). Phytoene synthase is an enzyme in carotenoid biosynthesis pathway (Sun et al. 2022). As the content of carotenoids in this study decreased by ZnO NPs supplements either at 300 or 500mg/ L, there is a suggestion that the ZnO nanoparticles have negative feedback on this key enzyme (Oh et al., 2006; Yang et al., 2006).

Zinc serves as a crucial element of an enzyme that has a profound impact on the production of IAA, the primary plant hormone responsible for regulating plant growth. By increasing the level of IAA, zinc gives a positive response in seed germination (Moghaddasi et al., 2017). The improved germination of *P. vulgaris* seeds in this study is attributed to the stimulation of ZnO NPs, which in turn promoted the biosynthesis of auxin. IBA appears to contribute to several auxin-related developmental processes and enhances the cell expansion (Strader & Bartel, 2009).

Exposure to NPs may lead to alteration of transcriptive genes that impact biosynthesis or signal transduction of plant hormones such as auxin repressor, auxin response genes, ABA-biosynthetic genes, or ethylene signaling components (Kaveh et al., 2013; Syu et al., 2014). The augmentation of cytokinin biosynthesis resulting from the application of low levels of ZnO NPs can be inferred by the rise in cytokinin precursors or by the activation of plant defense, which is concomitant with a marked upsurge in stress hormones such as ABA (Vankova et al., 2017).

α -amylase activity enhanced by NPs application up to 700mg/ L. This result was correlated with the induction of ABA, as it was believed that ABA counteracted the action of GA_s that induced α -amylase activity in germinated seeds. Augmenting the ZnO NPs concentration hindered the α -amylase activity, a concurrence with findings reported by Sandip et al. (2008).

Impact of ZnO NPs application on NAC and GLU genes

Few studies have been published to discuss different gene expression after ZnO NPs applications. As the primary focus is on the pivotal function of green synthesized NPs in revamping plant traits, such as seedling germination, growth parameters, and photosynthetic efficiency (Kumari et al., 2022). In this study, the results of qRT-PCR revealed a significant increase in the expression level of NAC gene. The transcription factor (TFs) play an important role of development of the programmer and function of the cell. This role entails the direct control of gene expression through protein interactions to take part in plant growth and physiological operations (Cui et al., 2019). Recent studies implied several TFs such as MYB, bZIP, and NAC, all of them are represented major regulators of the core stress responsive genes (Hong et al., 2016; Chen et al., 2021). NAC genes are expressed in various developmental stages and tissues. Improvement of stress tolerance in maize plants associated with overexpression of ZmNAC33, ZmNAC49 genes was significant in rice (Xiang et al., 2021). Overexpression of OsNAC10 in rice plants by 1.25 fold increased the root diameter therefore enhancing drought tolerance (Jeong et al., 2010). The optimal dose of ZnO NPs can potentially restructure or alter the transcription program, thereby affecting tissue differentiation and bestowing stress resilience in crops (Pejam et al., 2021). According to earlier research, the application of ZnO NPs resulted in the up-regulation of several critical genes, including *MKK2*, *bHLH*, *WRKY1*, *EREB*, *HsfA1a*, and *R2R3MYB* that brought about alterations in the signaling molecules and hormones (Iranbakhsh et al., 2021). In this investigation, ZnO NPs displayed no change in expression of GLU gene. The crucial role played by GLU gene in plants expressing β-1,3-glucanases in plant-microbe interactions can be attributed to the prevalence of β-1,3-glucans in fungi, bacteria, and metazoan, thereby, gaining significant attention. The hydrolysis of pathogen walls is attributed to the direct participation of certain β-1,3-glucanases that gather upon pathogenic assault (Moore & Stone, 1972; Perrot et al., 2022). Therefore, up-regulation of GLU genes may be a noble target to improve the antimicrobial mechanisms in plants (Amian et al., 2011; Chun & Chandrasekaran, 2019).

Conclusions

ZnO NPs green synthesized in presence of *C. album* leaves increased the germination percentage of *P. vulgaris* seeds and cause early germination when applied with concentration from 300 and up to 1000mg/L. Moreover, ZnO NPs diminish the quantity of water necessary for starting germination specially when applied at 1000 mg/L. Plant biomass and chlorophylls increased by ZnO NPs treatments. ZnO NPs triggered the biosynthesis of ABA "antistress hormone" as well as IBA and IAA, while also increased α - amylase activity which helps in reserve food mobilization. So far, there is no report discuss the action of ZnO NPs on NAC or GLU gene expression in *P. vulgaris* plants. NAC gene in *P. vulgaris* was up-regulated through the application of 500 mg /L ZnO NPs. This gene plays a significant role as a regulator of the core antistress responsive genes. Despite that, the GLU gene was not influenced by the same dose. As GLU gene has well documented exhibition with antifungal activity, therefore more attempts need to be exerted to up-regulated it.

Ethics approval: Not applicable.

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جزيئات أكسيد الزنك النانوية المُصنَّعة خضريا تعمل على تحسين النمو والتخليق الحيوي للهرمونات النباتية وتعديل التعبير في جينات المقاومة لنبات الفاصولياء

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أجريت الدراسة الحالية لتحفيز تصنيع جزيئات أكسيد الزنك النانوية بواسطة أسيتات الزنك والمستخلص المائي لأوراق نبات الزربيح الأبيض الغضة. تم تقييم الخصائص المورفولوجية والهيكلية لمركبات أكسيد الزنك النانوية المُصنَّعة باستخدام مقياس الطيف الضوئي، حيود الاشعة السينية وتحليل جهد زيتا. أظهر التصوير الفوتوغرافي TEM بلورات كروية من أكسيد الزنك النانوية بمتوسط قطر يبلغ 25 نانومتر. نبتت بذور نبات الفاصولياء مبكرًا عند معالجتها بـ 300 مجم/ لتر من أكسيد الزنك النانوية، حيث لوحظت الجذور في 40 ٪ من البذور المزروعة. أدى تطبيق أكسيد الزنك النانوية إلى تقليل كمية المياه المشبعة التي تتطلبها البذور الجافة لبدء الإنبات. أظهرت المكملات التي تحتوي على 500 مجم/ لتر من أكسيد الزنك النانوية أداءً جيدًا في نباتات الفاصولياء، والذي انعكس بشكل ملحوظ على طول الجذر وطول النبات والكتلة الحيوية الجافة. علاوة على ذلك، فإن معاملة نباتات الفاصولياء لمدة 30 يومًا باستخدام أكسيد الزنك النانوية أثر إيجابيًا على مستوى أصباغ البناء الضوئي (كلوروفيل أ ، ب). وفي قياسات الهرمونات النباتية الذاتية لنبات الفاصولياء بعمر 30 يوم بعد الزراعة فقد ارتفع مستواها في جميع المعالجات بالمقارنة مع تلك الموجودة في المجموعة الضابطة أو المعالجة بمستخلص أوراق الزربيح الأبيض. حيث سجل تحفيز في انتاج كلا من IAA ، IBA عن طريق المعاملة بـ 300 مجم /لتر أكسيد الزنك النانوية. وقد تم تسجيل هرمون ABA فقط في المعاملة باستخدام 1000 مجم /لتر أكسيد الزنك النانوية. و كان أعلى مستوى من انزيم الفا- اميليز في النباتات المعالجة بـ 300 و 500 مجم /لتر أكسيد الزنك النانوية الكروية. وبالنسبة لتأثير أكسيد الزنك النانوية على جين NAC ، فقد زاد تعبيره بـ 1.2 ضعف مقارنة بالنباتات الضابطة. ومع ذلك، فإن نفس الجرعة ليس لها أي تأثير على التعبير الجيني للجلوكانيز (GLU). فإن هذه الدراسة تدعم فكرة استخدام أكسيد الزنك النانوية كمحفز للإنبات والنمو، حيث يلعب الزنك دورًا مهمًا في تخليق الكلوروفيل وقد يحسن تحمل النبات للإجهادات المختلفة.