

EXPOSURE OF ANANAS COMOSUS TO ZINC OXIDE NANO-POWDER *IN VITRO* : PHYSIOLOGY AND BROMELAIN PRODUCTION

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Abstract

The use of zinc oxide nanoparticles to enhance the production of plant metabolites has gained considerable attention as a novel nanotechnology application in plant biotechnology and agriculture. This study aimed to investigate the effect of different concentrations (0, 0.1, 1, 10, 100, and 1,000 mg/L) of biosynthesised zinc oxide (BZnO) on growth parameters, bromelain production and antioxidant activities of the shoot culture of pineapple (*Ananas comosus*). The results showed that the highest amount of shoot (7.2 shoots/explant), fresh weight (FW) (5.390 g), leaf number (68.8 leaves/plantlet) and chlorophyll content (1,161.58 µg/g FW) were achieved at 1 mg/L of BZnO. A significant increase in bromelain production was also observed in the shoot culture of pineapple at 1 mg/L BZnO. The shoot of pineapple grown on plant tissue culture medium supplemented with 1 mg/L BZnO demonstrated the highest total phenolic content (20.11 µg gallic acid equivalent/g FW), reducing power (4.05 µg FeSO₄/g FW) and 2,2-diphenyl-1-picrylhydrazyl-scavenging activity (60.75%). The results revealed that physiological parameters, secondary metabolite production and antioxidant activity increased when the concentration of BZnO was elevated up to 1 mg/L. However, a sudden decrease was observed when the concentration of BZnO was over 1 mg/L BZnO, and it reached to the minimum amount at 1000 mg/L BZnO.

Keywords: Biogenic zinc oxide, shoot culture, bromelain, pineapple, antioxidant activity, nanoparticles

Introduction

Pineapple (*Ananas comosus* L.) is considered as one of the most economically important tropical fruit, with the production reaching approximately 3.2 million tonnes in 2019 (FAO 2020). It is a medicinal plant because of its valuable phytochemical contents, including phenolic compounds, flavonoids and

ascorbic acid. In addition, pineapple has nutritional benefits because it contains sugars, organic acids, essential minerals, fibres and vitamins (Sun *et al.*, 2016). Bromelain is one of the interesting biological substances in pineapple; it is a generic name for proteolytic enzymes in the

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cysteine protease family. This compound is found in plant species belonging to family Bromeliaceae. In pineapple, bromelain is found in all its parts (stem, leaves, fruit and peel), with different amounts and properties observed depending on the part and variety (Ketnawa *et al.*, 2012). The stem and fruit of pineapple are favorable parts for commercial bromelain production (EC3.4.22.32 and EC3.4.22.33, respectively) because of their yield high of this compound (Muntari *et al.*, 2012). Bromelain has been widely used in diverse applications, such as food industry for meat tenderisation, production of protein hydrolysates, tanning for leather production, alcohol production, textile industries and cosmetics. Furthermore, it has been utilised in clinical and pharmaceutical sectors due to several biological activities, including anti-inflammatory, antirheumatic, anticancer, antimicrobial activity, wound healing, cardioprotective and antidiabetic activities (Xie *et al.*, 2005; de la Barrera-Nunez *et al.*, 2014; Saxena and Panjwani, 2014; Kargutkar and Brijesh, 2016; Hammadia and Faisal, 2017. Prakoso *et al.*, 2018; Debnath *et al.*, 2019). Given its diverse applications and high value with the price up to 2400 USD kg⁻¹, numerous studies have attempted to optimise the extraction, isolation and purification processes of bromelain production from pineapple plant using various techniques (Ramli *et al.*, 2017). However, a limited number of reports have investigated bromelain production from pineapples grown in in vitro condition (Perez *et al.*, 2004; Ngampanya and Phongtongpasuk, 2006; Leite *et al.*, 2012; Neta *et al.*, 2012).

Plant tissue culture technique refers to the aseptic culture of plant cells, tissues, and organs under controlled in vitro physical and chemical conditions. This technique is an important biotechnological approach for the production of high value secondary metabolites, which is bromelain in this context. Given their totipotency property, plant cells can produce a range of chemical compounds present in the parent plant. The use of this technique to produce pineapple plant for bromelain production instead of conventional agricultural method has several advantages as follows: independence from geographical and seasonal variations, uniform quality and quantity, production of targeted compounds in high amount within a short period, easy scale up and continuous operation for commercial production (Firoozabady and Gutterson, 2003).

Elicitation, in which chemical or physical stresses are introduced to plant cell cultures, is one of the most successful strategies used to increase and accumulate the produced secondary

metabolites. In this process, stresses trigger the biosynthesis of secondary metabolite through the activation of plant defence mechanism (Ashraf *et al.*, 2018). Different kinds of biotic and abiotic elicitors have been used for the elicitation of plant secondary metabolites. To date, the use of nanoparticles (NPs) as elicitors for the production of secondary metabolites has emerged and drawn substantial attention as a novel nanotechnology application in plant biotechnology and agriculture (Nair *et al.*, 2010). Among NPs, zinc oxide (ZnO) can be used as a phytonutrient and plays a crucial role as signalling molecule to regulate several mechanisms involved in the recognition and activation of abiotic stresses in plants (Sheikh *et al.*, 2009; Dimkpa and Bindraban, 2017). Very few studies focused on the effect of zinc oxide NPs (ZnONPs) as elicitors on the production of secondary metabolites under in vitro conditions. For instance, Javed *et al.* studied the effects of ZnONPs on the physiology and steviol glycoside production of *Stevia rebaudiana*. The 1 mg/L concentration of ZnONPs resulted in the highest amount of steviol glycosides and promoted the proliferation of shoots (Javed *et al.*, 2017). Asl *et al.* showed the use of ZnONPs as elicitors to enhance the production of tropane alkaloids from the hairy root culture of *Hyoscyamus reticulatus* L. The treatment with 100 mg/L ZnONPs showed the highest amount of alkaloid production (hyoscyamine and scopolamine). In addition, the *h6h* gene, which is responsible for the biosynthetic route of hyoscyamine and scopolamine in the hairy root of *H. reticulatus*, increased dramatically and reached the maximum expression level under the ZnONP treatment at a concentration of 100 mg/L (Asl *et al.*, 2019).

ZnONP, produced via a biosynthesis route using agriculture waste, has drawn much attention. This method has advantages over the chemical synthetic process because it is eco-friendly, biocompatible, safe, inexpensive and green (Agarwal *et al.*, 2017). To the best of our knowledge, the use of biosynthesised ZnONPs as elicitors for bromelain production in pineapple under in vitro condition is currently unprecedented. Therefore, this study aimed to investigate the effect of ZnONPs on the growth and production of bromelain from the shoot culture of pineapple.

Materials and Methods

Chemicals

Analytical-grade chemicals were purchased from Sigma Aldrich.

Synthesis of ZnO

ZnO synthesis was carried out via a biogenic route with some modifications (Yuvakkumar *et al.*, 2014). Firstly, 60 g dried rambutan peel was boiled in 600 ml distilled water. After 15 min, the extract was obtained by filtration. The extract was left to cool down. Later, 300 ml extract was mixed with 1500 ml 0.1 M $\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ solution. NaOH solution was dropwise added to the mixture until the solution pH reached to 10. The mixture was further stirred for 2 h at 80°C. The brownish solution was filtered and washed with distilled water. The precipitation was dried in the oven at 60°C for 2 days. Afterward, the powders were calcined at 400°C for 2 h. The calcined powders were called biosynthesised zinc oxide 'BZnO'.

Characterisation of BZnO

X-ray diffraction (XRD) analysis of the prepared powder was conducted on a diffractometer using $\text{CuK}\alpha$ radiation ($\lambda = 1.5406 \text{ \AA}$). Scanning was carried out in the range of 10°-70° in 2 θ angle.

A total of 1 mg BZnO was placed in an agate mortar, mixed and ground thoroughly with 99 mg potassium bromide using a pestle. Infrared spectrum was recorded using a Thermo Scientific model Nicolet 6700 at wavenumbers between 4,000-400 cm^{-1} .

The ultraviolet-visible (UV-Vis) absorption spectrum of BZnO was obtained on a Biochrom Libra S22 UV-Vis spectrophotometer in the 200-800 nm region.

The size, morphology and chemical composition were imaged with a field-emission scanning electron microscope (FE-SEM; JEOL JSM-7610F, Oxford X-Max 20) with an energy-dispersive X-ray spectroscopy (EDX) detector and a transmission electron microscope (TEM; FEI TECNAI G2 20).

Decontamination Method for Explants

The crown-tip meristem of pineapple (*Ananas comosus* var. Pattavia) was used as an explant source. Briefly, the crown leaves were removed and discarded. The crown was washed in detergent using tap water, immersed in 70% ethanol for 3 min, followed by 15% Clorox (5.25% w/v sodium hypochlorite) for 15 min with intermittent shaking and thoroughly washed thrice with sterile distilled water. The crown-tip meristem was cultured in solidified Murashige and Skoog (MS) medium (pH 5.8) with 0.8% w/v agar (Murashige and Skoog, 1962). The culture was incubated at 25°C with a 16 h photoperiod provided by a cool-white fluorescence for 4 weeks.

Shoot culture on medium containing BZnO

The emerged shoot was transferred to the liquid MS medium supplemented with 2 mg/L 6-benzylaminopurine and various concentrations of BZnO (0, 0.1, 1, 10, 100, and 1,000 mg/L). The cultures were placed in a shaking incubator with constant rotating rate of 120 rpm and placed in the same condition as previously mentioned for 3 months. The experiment was carried out in triplicate. Twelve explants were cultured per treatment. Subcultures were conducted every two weeks.

Growth Analysis of *in vitro* Plantlets

To determine the growth of plantlets, we harvested shoot clusters and recorded several physiological parameters, including fresh weight (FW) of induced shoots, the number of shoot formation and the number of leaves. The conductivity of the used liquid MS medium was measured using a conductivity meter (Metler Toledo model seven compact 230) and compared with that of fresh liquid MS medium. Chlorophyll extraction was carried out in accordance with the work of Chen *et al.* (Chen *et al.*, 2018).

Extraction and determination of bromelain

Crude bromelain extraction was performed with a slight modification (Ketnawa *et al.*, 2012). Three-month-old shoots were homogenised with a cold 0.05 M phosphate buffer (pH 7.0) consisting of 0.015 M cysteine and 0.002 M ethylenediaminetetraacetic acid at the ratio of 1:1 (w/v) and centrifuged at 12,000 g at 4°C for 10 min. The supernatant as crude bromelain was collected for bromelain determination. The protease activity was examined using the azocasein method as previously mentioned with some modifications (Bresolin *et al.*, 2013). The reaction mixture containing 0.8 ml 0.5% azocasein (w/v) and 0.2 mL crude bromelain was incubated at 37°C for 30 min. Then, 0.5 mL 5% trichloroacetic acid (TCA) was added to suppress the reaction. The solution was centrifuged at 10,000 g for 10 min, and the precipitate was discarded. A total of 0.8 mL supernatant containing TCA-soluble azo-coupled peptides was collected and mixed with 0.2 mL 2 M NaOH. The absorbance of the supernatant was read at 440 nm using a microplate reader against a blank, which was the solution without the enzyme. One unit of protease activity was defined as the amount of enzyme used to produce an absorbance change of 1.0 in a 1.0 cm cuvette under assay conditions. The results were presented in terms of the percentage of relative activity.

Sample Preparation for the Determination of Antioxidant Activity

Briefly, 0.5 g pineapple shoot was ground using a mortar and a pestle in the presence of liquid nitrogen. Then, 5 mL 0.05 M phosphate buffer (pH 7.0) was added. The mixture was centrifuged at 10,000 g at 4°C for 20 min. The supernatant was collected and kept at 4°C for further experiment.

Determination of total phenolic content

An aliquot of 0.3 mL sample was thoroughly mixed with 1.5 mL Folin Ciocalteu reagent and 1.2 mL 7.5% w/v Na₂CO₃. The mixture was incubated in the dark for 30 min. The absorbance of the mixture was measured at the wavelength of 760 nm. Gallic acid was used as the standard solution. The total phenolic content was expressed as µg gallic acid equivalent per g FW (µg GAE/g FW).

2, 2-diphenyl-1-picryl hydrazyl (DPPH) radical scavenging activity

The in vitro free radical scavenging activity of the samples was carried out by measuring the disappearance of DPPH, in accordance with work of Apea-Bah *et al.* with some modifications. The reaction mixture consisted of 50 µL extract and 150 µL 20 mM DPPH dissolved in methanol and incubated in the dark at room temperature for 30 min in a 96-well plate (Apea-Bah *et al.*, 2014). The absorbance was monitored at 515 nm using a microplate reader. Ascorbic acid was employed as the standard. The percentage of DPPH inhibition was calculated from $((A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}) \times 100$, where A_{control} is the absorbance of DPPH and solvent, and A_{sample} indicates the absorbance of DPPH with the extract.

Ferric reducing antioxidant power (FRAP) assay

FRAP assay was performed referring to a previous study with a slight modification (Wern *et al.*, 2016). The FRAP reagent consisted of 300 mM acetate buffer, 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) in 40 mM HCl and 20 mM FeCl₃·6H₂O. The working solution was freshly prepared by mixing acetate buffer, TPTZ solution and FeCl₃·6H₂O at the ratio of 10:1:1 and incubated at 37°C before the analysis. An aliquot of 20 µL extract was mixed with 180 µL FRAP reagent and incubated in the dark for 15 min at room temperature. The absorbance of the blue solution was measured at a wavelength of 593 nm using a microplate reader. The results were shown in µg FeSO₄·7H₂O/g FW.

Statistical analysis

The data were represented as mean ± standard deviation (SD) of three replicates. Statistical

differences among the treatments were evaluated using one-way analysis of variance followed by Duncan's test at a significance level of 0.05.

Results and Discussions

Characterisation of BZnO

XRD analysis was employed to elucidate the crystal structure of BZnO. All obtained diffraction peaks were assigned to the (100), (002), (101), (102), (110), (103), (200), (112) and (201) planes. All characteristic peaks can be indexed to the hexagonal wurtzite structure of ZnO (JCDPS Card No.36-1451) (Figure 1). The unidentified peak from other phases was not observed. This finding indicates that BZnO has a high purity with a single crystalline phase. The average crystalline size of BZnO was calculated using the Debye-Scherrer equation (Cullity, 1978) from $D = K\lambda/\beta\cos\theta$ where K is the Scherrer constant ($K = 0.89$), λ is the incident X-ray wavelength ($\lambda = 0.154056$ nm), β is the peak width at half maximum (radians), and θ is the Bragg diffraction angle (radians). The average crystallite size was 24 nm.

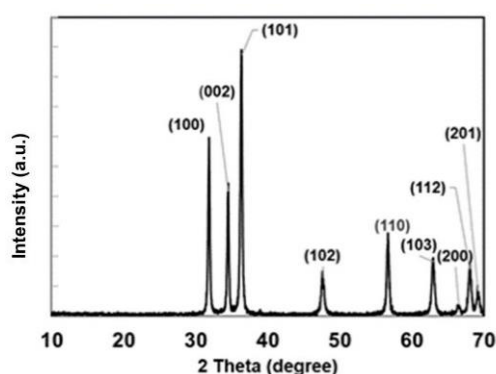


Figure 1. XRD pattern of BZnO

The absorption UV-Vis spectrum was recorded to monitor the formation of BZnO. Figure 2 illustrates the absorption spectrum of BZnO with a characteristic absorption peak at 378 nm, which was related to the electronic transition from the valence band to the conduction band. The band gap energy of BZnO was computed from its UV absorption spectrum as follows: $E_g = 1240/\lambda_g$, where E_g is the band gap energy (eV), and λ_g is wavelength of absorption edge (nm). The band gap energy of BZnO was 3.28 eV, which is lower than that of pure ZnO (3.30 eV) (Tri *et al.*, 2019). This result was likely due to the presence of plant phytochemicals in the lattice of synthesised ZnO

(Fu and Fu, 2015). The energy band gap of ZnONP correlated with the cytotoxicity of the HT1080 cell line. The decrease in energy band gap elevated the oxidative stress in cells, resulting in damaged nuclear material and eventual cell death (Arakha *et al.*, 2017).

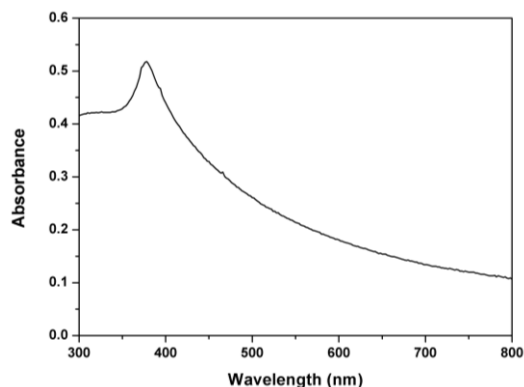


Figure 2. UV-Vis absorption spectra of BZnO

Fourier transform infrared spectroscopy (FTIR) analysis was performed to identify the phytochemicals in the plant extracts assisting in the formation and stabilisation of BZnO. Figure 3 demonstrates the FTIR spectra of BZnO and the extract. The peak at 491 cm^{-1} was assigned to Zn-O stretching vibration, confirming the presence of ZnO (Bhuyan *et al.*, 2015). The peak at 1190 cm^{-1} can be attributed to the C-O stretching vibration of ether. Peaks in the range of $1400\text{--}1700\text{ cm}^{-1}$ were due to the C=C stretching of aromatic ring. The peak at 1790 cm^{-1} is due to the C=O stretching vibration of carbonyl group. The broad band at 3411 cm^{-1} corresponded to the O-H stretching vibration of alcohol and phenolic groups.

For the FTIR absorption spectra of the extract, peaks corresponding to phytochemical compounds in the extract were observed. However, the peaks shifted in comparison with the spectra of BZnO, resulting from the formation of ZnO with bioactive compound surrounded on the surface of the particles.

The surface morphology of BZnO were examined by FE-SEM. Figure 4a demonstrates the irregularly spherical shape of particles. The particles exhibited variable sizes. Figure 4b shows the EDX spectra of BZnO. The strong signals from zinc and oxygen indicated the existence of their atoms in BZnO. The atomic and weight (in brackets) percentages of zinc and oxygen atoms were 39.70 (72.90%) and 60.30 (27.10%), respectively. No impurity signal was detected.

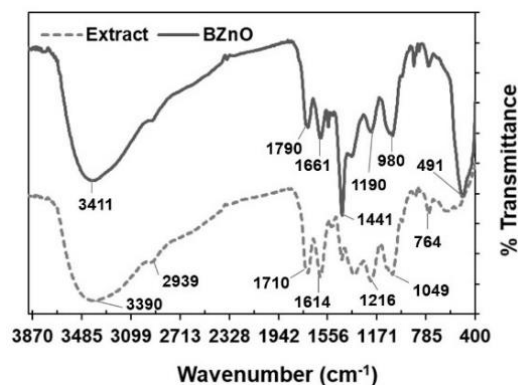


Figure 3. FTIR absorption spectra of BZnO

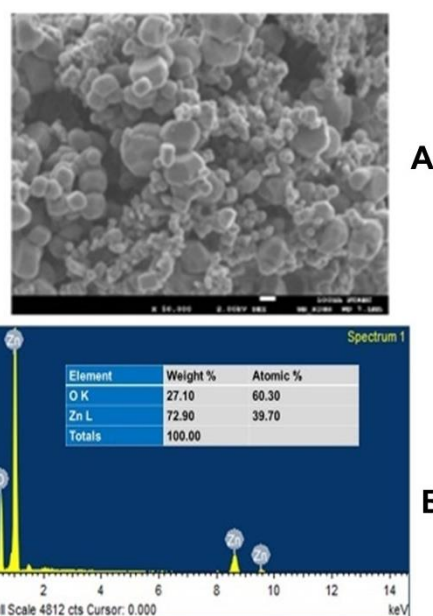


Figure 4. FE-SEM image (A) and EDX spectra (B) of BZnO

The size and shape of BZnO were investigated by TEM. Figure 5 displays the TEM image. The prepared BZnO was irregularly spherical with a broad size distribution, with the diameter varying from 60 nm to 219 nm. The average size of BZnO was 98.8 nm. The size and shape of ZnONPs in this study were different from those of ZnONPs synthesised from rambutan peel extract in previous studies (Yuvakkumar *et al.*, 2014; Karnan and Selvakumar, 2016). This difference was likely due to the phytochemical compositions of the extract used as precursor in the biosynthesis of different extraction solvents (Kobylnska *et al.*, 2020).

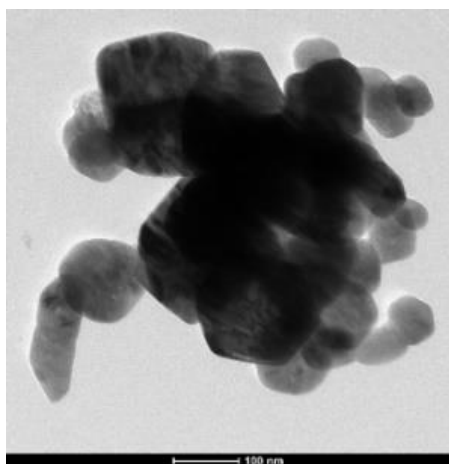


Figure 5. TEM image of BZnO calcined at 400°C

Effect of BZnO on the Shoot Culture of Pineapple

The growth of pineapple shoot was evaluated by measuring physiological parameters, including FW, shoot formation and the number of leaves. Electrical conductivity was also used to examine the plant growth. It represents the electrolyte and salt concentration of the medium available to plants. When the nutrient in the media was uptaken for plant growth, a reduction in the electrical conductivity was observed. In addition, chlorophyll

content was determined for the evaluation of plant growth because it is a key pigment for photosynthesis, producing energy for plant growth. Table 1 demonstrates that the BZnO in the MS liquid medium positively affected the growth of pineapple shoots in comparison with the control. The results showed that the growth of pineapple shoot improved when BZnO was added to the medium in comparison with the control. Among shoot tissues exposed with different concentrations of BZnO (0.1 mg/L to 1,000 mg/L), the highest FW (5.39 g), shoot formation (7.2 shoots), number of leaves (68.8 leaves), percentage reduction of conductivity (23.23%) and chlorophyll content (1,161.58 µg/g FW) of shoot culture were observed at 1 mg/L BZnO-treated plants. The increase in concentration to over 1 mg/L resulted in the decline in pineapple shoot growth. The lowest growth of pineapple shoot was observed under the 1,000 mg/L BZnO treatment.

Figure 6 illustrates the shoot formation of pineapple treated with BZnO at concentrations ranging between 0 and 1,000 mg/L. The finding suggested that raising the concentration of BZnO to 1 mg/L resulted in the maximum shoot growth. However, further increase in BZnO concentration caused toxicity. Meanwhile, Zn is a micronutrient element essential for plant; a high amount of Zn^{2+} dissolved from BZnO can cause phytotoxicity (Chen *et al.*, 2018).

Table 1. Comparison of plant growth indicators of pineapple shoots in liquid MS medium supplemented with various concentrations of BZnO

BZnO concentration (mg/L)	Fresh weight (g)	Shoot formation (shoots)	Number of leaves	% Reduction of conductivity	Chlorophyll content (µg/g FW)
0	1.416±0.099 ^d	1.5±0.5 ^c	12.0±1.4 ^e	15.61	895.32±3.89 ^c
0.1	1.723±0.047 ^c	2.5±0.5 ^b	15.6±1.7 ^d	16.67	959.72±6.33 ^b
1	5.390±0.113 ^a	7.2±0.4 ^a	68.8±2.3 ^a	23.23	1,161.58±3.12 ^a
10	2.115±0.096 ^b	3.2±0.4 ^b	28.8±1.3 ^b	19.46	968.52±11.57 ^b
100	2.052±0.078 ^b	2.8±0.4 ^b	24.8±1.1 ^c	17.07	875.48±10.65 ^c
1,000	1.299±0.173 ^e	1.4±0.5 ^c	10.0±1.2 ^e	12.59	801.93±6.92 ^d

Values represent the means ± SD of each analysis in triplicate. The means with the same lower-case letter in the same column are not significantly different at 95% confidence interval according to Duncan's multiple-range test.



Figure 6. Photograph illustrating the comparative morphologies of pineapple shoots in response to exposure to BZnO at different concentrations (0.1, 1, 10, 100, and 1,000 mg/L)

Bromelain Production

Bromelain production from pineapple shoot significantly increased in the presence of BZnO at concentrations up to 1 mg/L. However, the production of bromelain decreased with the further increase in the concentration of BZnO (Figure 7). The relative activity of bromelain increased from 40% in the control to 67% under 0.1 mg/L BZnO treatment. The highest relative activity of bromelain was found in shoots grown in the medium containing 1 mg/L BZnO. At 10 mg/L BZnO, the relative bromelain activity showed a dramatic decline of 50%. Moreover, the further decrease in bromelain relative activity (47%) was observed at 100 mg/L BZnO. The lowest amount of bromelain relative activity was observed at 1,000 mg/L BZnO.

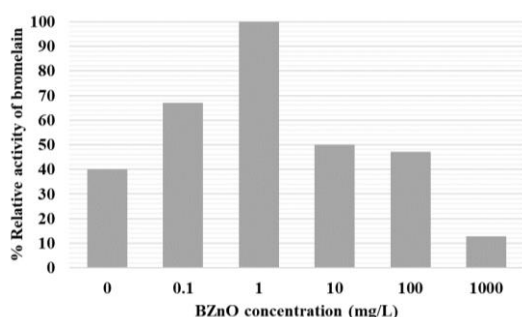


Figure 7. Effect of various concentrations of BZnO on bromelain production

Antioxidant activities

Table 2 reveals that more phenolic content, ferric reducing power and DPPH radical scavenging activity were produced in the presence of BZnO. When the concentration of BZnO in the medium was over 1 mg/L, the phenolic content and antioxidant activities gradually decreased and dropped to the minimum at 1,000 mg/L. The maximum phenolic content, ferric reducing power and DPPH radical scavenging activity were 20.11

µg GAE/g FW, 4.05 µg FeSO₄/g FW and 60.75%, respectively, which were detected at 1 mg/L BZnO treatment. Conversely, the lowest phenolic content (10.72 µg GAE/g FW), antioxidant activity accessed by ferric reducing power (2.66 µg FeSO₄/g FW) and DPPH-scavenging activity (43.45%) were observed in the treatment supplemented with 1,000 mg/L BZnO.

The results demonstrated that antioxidant activities in pineapple shoot were strongly affected by different concentrations of BZnO to various extents when abiotic stress was applied to the pineapple shoot via the addition of BZnO to the MS medium. The enhancement of BZnO concentration up to 1 mg/L positively affected the growth, bromelain production, phenolic content and antioxidant activities of pineapple shoot. The positive impact possibly resulted from the dissolved Zn²⁺ acting as an abiotic elicitor to promote bromelain production. Moreover, zinc is an essential micronutrient for plant growth and assists plant health by stimulating enzymes that produce defence metabolites such as phenolic compounds. Although zinc plays a key role in plant immune responses, the specific mechanism remains unknown. However, the continual application of BZnO to over 1 mg/L caused the decrease in growth, bromelain production, phenolic content and antioxidant activities. The balance of antioxidant mechanisms to eliminate reactive oxygen species (ROS) was possibly disturbed, and the quantity of ROS was enhanced. Consequently, cellular structures, including membranes, proteins and nucleic acids in the plant, were damaged (Laspina *et al.*, 2005). The addition of overdosed BZnO induced oxidative stress by releasing either the excessive quantity of Zn²⁺ or free radicals, including hydrogen peroxide, hydroxyl, and superoxide radical from sequential redox reactions occurring on the particle surface to the medium (Dimkpa *et al.*, 2012; Zudyte and Luksiene, 2021). Usually, plant cells can naturally tackle an oxidative stress by efficient antioxidant mechanisms using phytochemicals to scavenge

Table 2. Phytochemical and antioxidant activities of pineapple shoots grown on the MS liquid medium supplemented with various concentrations of BZnO

BZnO concentration (mg/L)	Phenolic content (µg GAE/g FW)	Ferric reducing power (µg FeSO ₄ /g FW)	DPPH radical scavenging activity (% inhibition)
0	13.84±0.26 ^c	2.85±0.020 ^c	50.28±0.47 ^e
0.1	17.53±0.09 ^b	2.90±0.03 ^c	55.70±0.08 ^b
1	20.11±0.12 ^a	4.05±0.04 ^a	60.75±0.33 ^a
10	14.62±0.11 ^c	3.42±0.04 ^b	53.14±0.14 ^c
100	12.18±0.19 ^d	2.76±0.02 ^d	51.90±0.46 ^d
1,000	10.72±0.09 ^e	2.66±0.03 ^e	43.45±0.24 ^f

Values represent the means ± SD of each analysis in triplicate. The means with the same lower-case letter in the same column are not significantly different at 95% confidence interval according to Duncan's multiple-range test.

free radicals and antioxidant enzymes, such as superoxide dismutase, catalase or other detoxification-related enzymes including acid phosphatase and alkaline phosphatase, to prevent the damage to plant cells (Ma *et al.*, 2020). However, the imbalance of antioxidant activity and oxidative stress occurred at 1000 mg/L BZnO. Therefore, the growth, bromelain production and antioxidant activity were reduced to the lowest values because of the oxidative damage induced by BZnO at 1,000 mg/L. In addition, previous studies have reported the phytotoxicity of ZnONP synthesised from conventional chemical synthesis at 1000 mg/L (Zhao *et al.*, 2013; Watson *et al.*, 2015; Javed *et al.*, 2017). However, the addition of ZnONPs at 1,000 mg/L offered a positive effect on the seed germination of peanuts. This finding is not consistent with the results of this research (Prasad *et al.*, 2012).

Conclusions

In the present study, the physiological, bromelain production and antioxidant responses of *Ananas comosus* exposed to biosynthesised ZnONPs under in vitro condition were elucidated. The growth, amount of bromelain, phenolic content and antioxidant activities dramatically increased when BZnO was introduced to the MS medium up to 1 mg/L concentration. However, the use of BZnO concentration at 10-1,000 mg/L resulted in adverse effects from oxidative damage due to the role of antioxidant activities.

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