

ZNO AND TiO₂ NANOPARTICLES STIMULATE CALLUS INDUCTION AND SECONDARY METABOLITE PRODUCTION IN *Indica* RICE

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Abstract

Metal nanoparticles are widely used in various fields of industry and their impact in the agricultural field is increasing. This research was carried out to investigate the effects of ZnO and TiO₂ nanoparticles on callus induction and plant metabolism in *indica* rice. *Indica* rice was assessed in plant tissue culture condition to estimate the influence of metal nanoparticles on morphological and physiological factors over 4 weeks. The seeds of *Oryza sativa* L. var. KDML105 were exposed to 1-3 mg L⁻¹ of 2,4-dichlorophenoxyacetic acid (2,4-D) in the callus induction medium. The induced-calli were cultured on the callus induction medium supplemented with 2 mg L⁻¹ of 2,4-D and showed a high increase in size and dry weight contents under the light condition when compared with the control and other treatments. Moreover, this research also investigated the potential impacts of ZnO and TiO₂ nanoparticles at 200, 400 and 800 mg L⁻¹ on morphological and metabolic responses of the induced-calli cultured on the callus induction medium supplemented with 2,4-D and each type of metal nanoparticles. At 4 weeks after treatment, the induced-calli were analyzed for plant growth and assays on aspects of their metabolism were undertaken. The induced-calli exposed to 200 mg L⁻¹ of ZnO or TiO₂ nanoparticles were found to have significant increase of biomass (size and dry weight of callus), total phenolic compounds and total flavonoid contents in KDML105 variety. The results demonstrated that utilization of ZnO and TiO₂ nanoparticles at appropriate concentration could positively induce the production of beneficial secondary metabolites in the induced-calli of *indica* rice and are worthy of further study for applications in other plant species.

Keywords: Callus induction, Flavonoids, *Indica* rice, Phenolic compounds, TiO₂ nanoparticles, ZnO nanoparticles

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Introduction

Rice (*Oryza sativa* L.) is the most important crop for domestic consumption and export in many countries and especially in Thailand and is a model monocot plant for research throughout in the world (Bakhshandeh *et al.*, 2015; Ghabooli *et al.*, 2015). Currently, there is a need to increase the value of rice for increased income of the agriculturist. Previous researchers have found that rice callus produced many types of metabolites such as flavonoids, ascorbic acid, tocopherol, linoleic acid, linolenic acid and proline (Rico *et al.*, 2013; Wu *et al.*, 2017). Numerous conditions and elicitors are available to induce an advantageous metabolite in rice callus such as biotic and abiotic stresses (Gill and Tuteja, 2010; Frank *et al.*, 2012; Scalabrin *et al.*, 2016). Plant cells have to suitable elicitor responses against effects of abiotic stress as well as nanomaterials by stimulation of metabolic processes. Under abiotic stress condition, defense systems of plant cells responded by accumulation and production in some compounds or metabolites (Ramakrishna and Ravishankar, 2011). Plant metabolites are significant source of compounds with various benefits as well as having pharmaceutical properties (Chatterjee, 2015). These metabolites also play a role in plant adaptations and have applications in protection against many diseases of both human and other organisms as well as application as a supplementary food (Balbuena *et al.*, 2009; Bicer *et al.*, 2017).

Metal nanoparticles are one of the plentiful engineering materials available in the world and are widely applied in many industrial products such as fertilizers, electronics, cosmetics, medicals, agriculture and various consumer products (Klaine *et al.*, 2008; Gardea-Torresdey *et al.*, 2014). The positive and negative effects of metal nanoparticles on organism response and metabolism depends on the type, size, dose, exposure time and condition of metal nanoparticles (Kim *et al.*, 2012; Conway *et al.*, 2015). The applications of metal nanoparticles for the benefits of organisms are very wide ranging with considerable future potential.

Therefore, the major aim of this research is to investigate the morphological and physiological responses of rice callus to various concentrations of ZnO and TiO₂ nanoparticles exposure. The callus samples were measured for the biomass accumulation (size and dry weight) and contents of the secondary metabolite (total phenolic compounds and flavonoids) of *indica* rice callus cv. KDML105. The results of this research contributes to the future application and potential function of ZnO and TiO₂ nanoparticles in the induction of essential metabolites from *indica* rice callus.

Materials and Methods

Plant Material and Effect of Plant Growth Regulator Concentrations on Callus Induction Efficiency

Seeds of *Oryza sativa* L. cv. Khao Dawk Mali 105 (KDML105) were washed thoroughly with 70% (v/v) of ethanol for 2-3 min. The seeds were sterilized with 5% (v/v) commercial bleach (6% sodium hypochlorite) for 30 min followed by 30% (v/v) commercial bleach (6% sodium hypochlorite) for 30 min and rinsing (5-6 times) in sterile distilled water. The sterilized-seeds were placed on sterilized-filter paper (Whatman™ No. 1) and then transferred to NB (Li *et al.*, 1993) medium containing 500 mg L⁻¹ proline, 300 mg L⁻¹ casein hydrolysate, 30 g L⁻¹ sucrose, 8 g L⁻¹ agar and different concentrations (1-3 mg L⁻¹) of 2,4-dichlorophenoxyacetic acid (2,4-D) for callus induction. The pH of the medium was adjusted to 5.6-5.8 with 1 M KOH or 1 M HCl prior to sterilization. The cultures were incubated at 25±2°C under dark and light conditions (1000 lux intensity by fluorescence tubes and 16 h light: 8 h dark photoperiod). The size and weight of callus produced were recorded after 4 weeks of culture.

Effect of Metal Nanoparticle Concentrations on Callus Induction Efficiency

The appropriate concentrations of 2,4-D from the previous section were used. The

sterilized-seeds were placed on sterilized-filter paper (WhatmanTM No. 1) and then transferred to NB medium containing 500 mg L⁻¹ proline, 300 mg L⁻¹ casein hydrolysate, 30 g L⁻¹ sucrose, 8 g L⁻¹ agar, the appropriate concentrations of 2,4-D and different concentrations (0-800 mg L⁻¹) of ZnO (average size 35.65±4.15 nm; Global Chemical Co., Ltd., Thailand) and TiO₂ (average size 28.16±2.25 nm; Evonik Degussa GmbH, Germany) nanoparticles for callus induction. The pH of the medium was adjusted to 5.6-5.8 with 1 M KOH or 1 M HCl prior to sterilization. The cultures were incubated at 25±2°C under the appropriate conditions from the previous section. The size and weight of callus were recorded after 4 weeks of culture.

Effect of Metal Nanoparticle Concentrations on Plant Metabolites

Total phenolic compound contents were analyzed using Folin Ciocalteu's assay (Allothman *et al.*, 2009). The mixtures were centrifuged at 8000 rpm for 15 min and the supernatants were collected. The extracts were mixed with 1.8 mL of diluted Folin–Ciocalteu reagent (1:10 v/v water) and kept in the dark at room temperature for 5 min. Then, 7.5% sodium carbonate (Na₂CO₃) was added to the reaction and kept in the dark at room temperature for 60 min. The absorbance was measured using a UV–Vis spectrophotometer (TG92+, PG Instruments, United Kingdom) at 765 nm and the results were expressed as milligrams of Gallic acid equivalents per 1 g sample on a fresh weight (mg Gallic acid g⁻¹ FW).

For total flavonoid determinations, the callus of each treatment was extracted in acidic-methanol (99:1 methanol:HCl) for 2 h at room temperature in the dark condition. After 2 h, the samples were centrifuged at 8000 rpm for 15 min. The extracts were measured using a UV–Vis spectrophotometer (TG92+, PG Instruments, United Kingdom) at 374 nm. The results of total flavonoids were expressed as mg per g fresh weight (Pedro *et al.*, 2016).

Statistical Analysis

All experiments were repeated 5 times (n=5). The data were subjected to Completely Randomized Design (CRD) and analyzed by

one-way analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT) at a significant level of $P \leq 0.05$.

Results and Discussion

Plant species, plant explants, cultural condition and medium compositions had an important impact on callus formation and plant regeneration. Callus induction has been achieved and produced in different plant species on media containing plant growth regulators such as 2,4-D, kinetin and NAA (Shahsavari *et al.*, 2010; Lee and Huang, 2014). The preliminary research has studied the effects of various concentrations of 2,4-D on callus induction efficiency from mature seeds of KDML105 variety. After 2 weeks, callus formation had been initiated from mature seeds. Callus formation was induced on at all concentrations of 2,4-D and cultural condition (light and dark conditions). The morphological responses (size and dry weight) of callus formation varied with the concentration of 2,4-D (1-3 mg L⁻¹) and cultural condition after 4 weeks of culture (Table 1). The size and dry weight of callus formation were significantly higher under light condition (0.56-0.88 cm and 30.08-33.78 mg, respectively) more than dark condition (0.26-0.54 cm and 13.56-22.24 mg, respectively) in all concentrations of 2,4-D. The size and dry weight of callus formation *in vitro* varied significantly between the concentration of 2,4-D treatments (Table 1). The highest size (0.88±0.08 cm) and dry weight (33.78±0.74 cm) of callus formation were obtained in the medium containing 2 mg L⁻¹ 2,4-D. In this research, the appropriation of 2,4-D concentration produced callus formation on mature seeds in KDML105 variety, indicating that 2,4-D played a role as a callus inducer in *indica* rice. The results of 2,4-D activity in this research corresponded with the results obtained from previous researches in other plant species. These investigations have also revealed the positive effects of 2,4-D on callus induction and formation in various rice varieties using mature seeds as explant experiments (Verma *et al.*, 2011; Zhao *et al.*, 2011).

Table 1. Comparative changes in the callus formation (size and dry weight) in *indica* rice cv. KDML105 exposed to different concentrations of 2,4-D after 4 weeks

2,4-D Concentrations (mg L ⁻¹)	Size (cm)		Dry Weight (mg)	
	Light	Dark	Light	Dark
1.0	0.56 ± 0.05e	0.26 ± 0.05d	30.14 ± 1.07c	13.56 ± 1.02d
1.5	0.70 ± 0.07c	0.36 ± 0.06b	32.06 ± 1.02b	16.52 ± 1.04c
2.0	0.88 ± 0.08a	0.54 ± 0.05a	33.78 ± 0.74a	22.24 ± 1.05a
2.5	0.76 ± 0.04b	0.40 ± 0.07b	31.24 ± 0.078b	17.66 ± 0.75b
3.0	0.66 ± 0.06d	0.30 ± 0.07c	30.08 ± 0.079c	16.70 ± 1.04c

Values within a column followed by the different letters are significantly different at $P < 0.05$ analyzed by Duncan's Multiple Range Test (DMRT). The values are the average of five replicates ± standard deviation (SD).

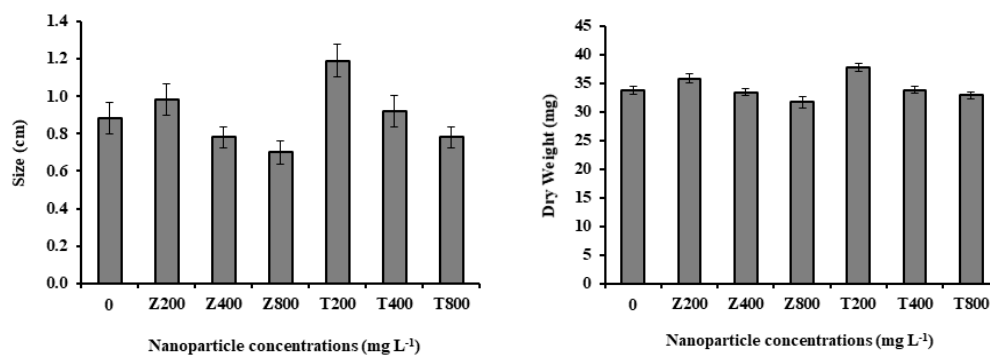


Figure 1. Comparative changes in the callus formation (A; size and B; dry weight) in *indica* rice cv. KDML105 exposed to different concentrations of metal nanoparticles [200 (Z200), 400 (Z400), 800 (Z800) mg L⁻¹ ZnO or 200 (T200), 400 (T400), 800 (T800) mg L⁻¹ TiO₂ nanoparticles] after 4 weeks. Values above a bar followed by the different letters are significantly different at $P < 0.05$ analyzed by Duncan's Multiple Range Test (DMRT). The values are the average of five replicates ± standard deviation (SD)

Callus induction medium supplemented with 2 mg L⁻¹ 2,4-D was used for the effect of metal nanoparticle concentrations on callus induction efficiency. Metal nanoparticles have been shown to be effective or ineffective for plant growth, plant metabolism and callus formation in various plant species (Helaly *et al.*, 2014; Mousavi Kouhi *et al.*, 2014; Adhikari *et al.*, 2015; Chutipaijit and Sutjaritvorakul, 2018). Callus formation of KDML105 variety was also affected by the type and concentration of metal nanoparticles. Mature seeds which developed callus formation were cultured on callus induction medium containing 2 mg L⁻¹ 2,4-D and different concentrations (0-800 mg L⁻¹) of ZnO and TiO₂ nanoparticles (Figure 1). Callus induction medium supplemented with 2 mg L⁻¹ 2,4-D and 200 mg L⁻¹ of ZnO or TiO₂

nanoparticles was found to be the most efficient medium to produce the size and dry weight of callus formation more than other media. Interestingly, at the same concentration (200 mg L⁻¹) of metal nanoparticles, the size and dry weight of callus formation were induced in mature seeds cultured on TiO₂ nanoparticles-containing medium (1.19 ± 0.08 cm and 37.76 ± 0.78 mg, respectively) higher than ZnO nanoparticles-containing medium (0.98 ± 0.08 cm and 35.86 ± 0.82 mg, respectively). However, the size and dry weight of callus formation on metal nanoparticles-containing medium at high concentrations (400 and 800 mg L⁻¹) were lower than those on control medium (0 mg L⁻¹ ZnO or TiO₂ nanoparticles). Metal nanoparticles have been confirmed to be a highly effective in the application of

morphological and physiological responses in various plant species (Qi *et al.*, 2013; Chutipaijit, 2015; Wang *et al.*, 2016; Samart *et al.*, 2018). ZnO and TiO₂ nanoparticles were promoted in plant cell growth by supporting water accumulation and enhanced oxygen uptake (Khot *et al.*, 2012). In previous researches TiO₂ nanoparticles have been shown to stimulate nitrate reductase activity in plant cells and promoted organic nitrogen accumulation (Feizi *et al.*, 2012; Servin *et al.*, 2012).

The impact of metal nanoparticles is often assessed through the physiological and metabolic responses in plant cells (Ghorbanpour and Hadian, 2015; Oloumi *et al.*, 2015; Chutipaijit and Sutjaritvorakul, 2017). Metal nanoparticles-containing medium treatments had effects on total flavonoid contents in rice callus. As compared to control (0 mg L⁻¹ metal nanoparticles), a significant increase of total phenolic compounds and flavonoid contents were occurred in ZnO nanoparticles-containing medium at 200 mg L⁻¹ (185% and 118%, respectively) and 400 mg L⁻¹ (152% and 106%, respectively). In addition, total phenolic compound and flavonoid contents were significantly increased under 200 mg L⁻¹ (272% and 134%, respectively) and 400 mg L⁻¹ (176% and 113%, respectively) TiO₂ nanoparticle treatments (Figures 2 and 3). As compared to control treatment, there were significant decreases in total phenolic compounds and flavonoid contents under 800 mg L⁻¹ ZnO nanoparticle treatment (84% and 93%, respectively) as well as 800 mg L⁻¹ TiO₂ nanoparticle treatment (89% and 96%, respectively) was observed in KDML105 callus at 4 weeks. Enhanced contents of phenolic compounds and nonenzymatic antioxidants such as flavonoid under metal nanoparticle treatments is an indication of the induction in plant defense mechanism to combat reactive oxidant species (ROS) and oxidative stress damage (Song *et al.*, 2013; Mirzajani *et al.*, 2014). Moreover, phenolic compounds and flavonoid have been reported as serving a variety of useful functions for humans. Flavonoids are shown to have antibacterial, antioxidants, hepatoprotective,

anti-inflammatory, antiviral activity, anticancer properties and are implicated in heart disease prevention (Ghorbanpour, 2015; Zhao *et al.*, 2016). Therefore, the induction of pharmaceutical metabolite productions in plant cells is considerable potential and especially the application of metal nanoparticles as elicitors. The metabolites of plant cells have biocompatibility and are less toxic to organisms than the synthetic chemicals.

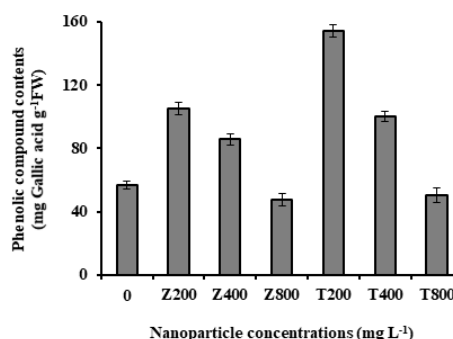


Figure 2. Comparative changes in the total phenolic compound contents in callus of *indica* rice cv. KDML105 exposed to different concentrations of metal nanoparticles [200 (Z200), 400 (Z400), 800 (Z800) mg L⁻¹ ZnO or 200 (T200), 400 (T400), 800 (T800) mg L⁻¹ TiO₂ nanoparticles] after 4 weeks. Values above a bar followed by the different letters are significantly different at $P < 0.05$ analyzed by Duncan's Multiple Range Test (DMRT). The values are the average of five replicates $\pm$ standard deviation (SD)

Conclusions

In this research, the size and dry weight of callus formation increased and the total flavonoid contents were elevated under exposure to low and moderate doses of metal nanoparticles. The results demonstrated the effect of ZnO and TiO₂ nanoparticles on callus formation and metabolism of *indica* rice cv. KDML105 in callus induction medium. At 200 mg L⁻¹ metal nanoparticles, ZnO and TiO₂

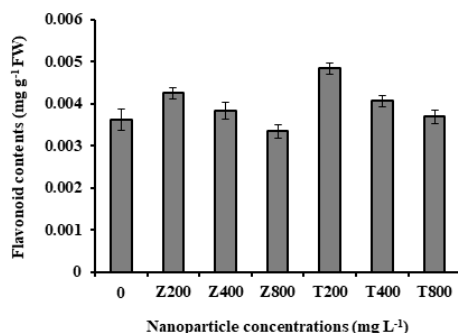


Figure 3. Comparative changes in the total flavonoid contents in callus of *indica* rice cv. KDML105 exposed to different concentrations of metal nanoparticles [200 (Z200), 400 (Z400), 800 (Z800) mg L⁻¹ ZnO or 200 (T200), 400 (T400), 800 (T800) mg L⁻¹ TiO₂ nanoparticles] after 4 weeks. Values above a bar followed by the different letters are significantly different at $P < 0.05$ analyzed by Duncan's Multiple Range Test (DMRT). The values are the average of five replicates $\pm$ standard deviation (SD)

nanoparticles-induced callus led to an increase in biomass and total flavonoid contents; however, ZnO and TiO₂ nanoparticles at the high exposure concentrations (800 mg L⁻¹) used mitigated the effects of metal nanoparticles through reduced in biomass and total flavonoid contents. The differential responses of metabolite contents (total flavonoids) to metal nanoparticles in callus may be attributed to appropriated doses of ZnO and TiO₂ nanoparticles. These results suggested that the appropriated concentration of ZnO and TiO₂ nanoparticles may act as a suitable elicitor for flavonoid compound production and application in pharmaceutically compounds in callus of *indica* rice cv. KDML105 and further research is intended. The increases of total flavonoid contents and the resulting improvement of nutritional composition of *indica* rice resulted in improved added value.

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