ENHANCEMENTS OF GROWTH AND METABOLITES OF *INDICA* RICE CALLUS (*ORYZA SATIVA* L. CV. PATHUMTHANI1) USING TiO_2 NANOPARTICLES (NANO-TiO_2)

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In this research, the effects of a various in concentration of TiO_2 nanoparticles (Nano-TiO_2) were applied to study the cell growth, metabolites and antioxidant responses of callus in *indica* rice plants. Dose-dependent changes in growth performances, metabolite accumulations and antioxidant activities of callus were found. Mature seeds of *indica* rice cv. Pathumthani1 were cultured in callus induction medium with different concentrations of Nano-TiO_2 (0-600 mg L^{-1}). After five weeks of cultivation, the callus formations were measured in fresh weights and dry weights, and the contents of phenolic compounds, flavonoids, and the antioxidant activities of callus extracts were assayed. The Nano-TiO_2 induced of fresh weights and dry weights in callus formations when compared with the control treatment (0 mg L^{-1} Nano-TiO_2) were also observed. The flavonoid and phenolic compound contents in rice callus exposed to 400 mg L^{-1} Nano-TiO_2 were 118% and 139%, respectively, higher than rice callus unexposed to Nano-TiO_2. Moreover, the antioxidant activities of the callus metabolites studied shows that the percentages of inhibition were up-regulated (84%) when exposed to 400 mg L^{-1} Nano-TiO_2, as a response to the high-level depletion of the free radicals in metabolites of rice callus. The results obtained from this research showed that the addition of Nano-TiO_2 had impacts on the callus growth and metabolite accumulations in rice callus. The results suggested that addition of Nano-TiO_2 at appropriate levels could role as an elicitor for biosynthesis of valuable metabolites and antioxidant properties for pharmaceutical applications in further research.

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1. Introduction

The potential impacts of nanomaterials have been increasing use in electronics, environment, medicine, pharmaceutical, energy production, consumer products applications, etc. [1]. Amongst the many strategies for sustainable agriculture, the application of nanomaterials for agrochemical technology have significantly affected on agriculture yield and production in present and future periods [2, 3]. The broad applications of nanomaterials in agriculture field have taken advantage and disadvantage of the unique properties, types, duration time, and concentrations as well as organism species [4, 5]. The previous studies have been shown that stimulation of plant growth and accumulation of plant metabolites when exposed to nanomaterials can affected plant responses and proliferation [6]. Adequate knowledge of the types, concentrations and behavior of nanomaterials in growth condition and biological systems for the determination of forecasting and recognizing the plant responses of each species have been still lacking [7].

Rice (*Oryza sativa* L.) is an important staple food crop for more than 50% of people in Asia, especially Southeast Asia region. Moreover, rice is the widespread cultivated food crop in the world [8]. Current situation, biotic and abiotic stresses are the major factors that limits rice growth and productivity worldwide. In the future, rice consumers and feeds will be increase about 26% follow by the expanding population whereas the area of agricultural land will be stable or

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decline [9]. Biotic and abiotic stresses can stimulate plant cells to produce various metabolites for plant survival. Nanomaterials or metals are one of the elicitors in the production of plant metabolites under stress conditions [10, 11]. Some metabolites help plant to communicate with plant response systems and protects plants from stress conditions. These metabolites are significantly essential for growth, proliferation and development in plant cells [12, 13]. Some metabolites, such as phenolic com-pounds, can add value to the plant products and can be applied to agriculture, food and cosmetic goods for value added agricultural products in the further study [14, 15].

In this research, the effects of Nano-TiO_2 on callus formation and plant metabolites, as well as antioxidant activity in callus extract, were investigated in plant tissue culture condition. The comparative studies on the significance of total phenolic compounds and flavonoid contents as well as antioxidant activity in rice callus cv. Pathumthani1 were carried out exposed with and without Nano-TiO_2 condition. The results of the study revealed that the positive impact of Nano-TiO_2 were significantly elicitor for valuable plant metabolites.

2. Experimental

2.1. Rice seed cultivation

Rice seeds (*Oryza sativa* L. cv. Pathumthani1) were obtained from Pathumthani Rice Research Center, Office of grain Rice Department, Ministry of Agriculture and Cooperatives, Thailand. The seeds were dehusked by hand, soaked in 70% ethanol for 3 min, 5% (v/v) commercial bleach (5.25% sodium hypochlorite) for 40 min, and 30% (v/v) commercial bleach for 30 min for surface-sterilization. It was then rinsed 5-6 times with sterilized dis-tilled water and transferred to sterilized tissue paper in a Petri dish.

2.2. Induction of callus formation in rice seeds

The disinfected seeds were transferred to NB medium [16] supplemented with 500 mg L^{-1}, L-proline 500 mg L^{-1}, L-glutamine 300 mg L^{-1}, casein hydrolysate, 30 g L^{-1} sucrose, 8 g L^{-1} agar and different concentrations of 2,4-D (2,4-dichlorophenoxyacetic acid; 0, 1, 1.5, 2, 2.5, 3 mg L^{-1}) with pH 5.6-5.8 for the callus induction medium. The seed cultures were incubated in the light condition with 16/8 h photoperiod (1000 lux) under 25±2 ºC for five weeks. The fresh weight, dry weight and size of callus formation were recorded after five weeks of inoculation.

The appropriate concentrations of 2,4-D from the previous method was used for study the effect of TiO_2 nanoparticles (Nano-TiO_2) on the callus induction and plant metabolites. The disinfected seeds were transferred to the callus induction medium supplemented with appropriate concentrations of 2,4-D from the previous section and different concentrations of Nano-TiO_2 (0, 100, 200, 300, 400, 500 and 600 mg L^{-1}) with pH 5.6-5.8. The average size of the Nano-TiO_2 particles (Evonik Degussa GmbH, Germany) was ranging 25-50 nm and determined by particle analyzer (Delsa™Nano C) with Dynamic light scatter technique. The seed cultures were incubated in the light condition with 16/8 h photoperiod (1000 lux) under 25±2 ºC for five weeks. The fresh weight, dry weight and size of callus formation were recorded after five weeks of inoculation.

2.3. Determination of total phenolic compounds and flavonoid contents in callus extract

To investigate the effect of Nano-TiO_2 on the plant metabolites from rice callus, total phenolic compounds and flavonoid contents of rice callus exposed with and without Nano-TiO_2 was analyzed. The powdered rice callus were extracted with acidic-methanol (methanol:HCl; 99:1) at room temperature for 2 h. After extraction, the samples were centrifuged at 8000 rpm at 4 °C for 15 min. The supernatant solution was used to determine the total phenolic compounds and flavonoid content assays.

The contents of total phenolic compounds in rice callus extracts was determined according to the Folin-Ciocalteu method [17]. The results were expressed as mg of Gallic acid equivalents per g of the fresh weight sample (mg Gallic acid g^{-1} FW). The contents of flavonoids in rice callus

extracts was determined according to Pedro et al. [18]. The results were expressed as mg of flavonoids per g of the fresh weight sample (mg g^{-1} FW).

2.4. Determination of antioxidant activity in callus extract

The percentage of free radical scavenging activity (% Inhibition) was determined according to the ABTS method [19]. The % Inhibition was calculated by using the following equation:

$$\% \text{ Inhibition} = OD_0 - OD_s / OD_0 \times 100$$

where OD_0 was the absorbance of the blank (methanol) and OD_s was the absorbance of the sample. The results were expressed as mg Trolox equivalents per g of the fresh weight sample (mg TE g^{-1} FW).

2.4. Statistical analysis

The means and standard deviations (SD) of five replicates were calculated and analyzed. The level of significance was analyzed from analysis of variance (ANOVA) using SPSS 15.0 software. Comparisons were considered significantly different by Duncan's Multiple Range Test at $P \leq 0.05$.

3. Results and discussions

3.1. The effects of plant growth regulator concentration on callus formation

After five weeks, the callus were induced from mature seeds of *indica* rice cv. Pathumthani1 on the callus induction medium supplemented with different concentrations of 2,4-D and Nano-TiO_2 under light condition. The results were significant differences in the callus formation among different concentration of 2,4-D (Fig. 1). The callus formation was the highest level in callus cultured on the callus induction medium supplemented with 1.5 mg L^{-1} 2,4-D. The results showed that the fresh weight (66.52 g), dry weight (30.66 g) and size (0.60 cm) of callus formation were higher level when rice seeds were cultured on the callus induction medium supplemented with 1.5 mg L^{-1} 2,4-D than the other treatments (Fig. 1A-C).

The results were similar to the research reported by Pawar et al. [20] and Tran and Sanan-Mishra [21] that used 2,4-D for the callus induction from mature seeds of indica rice. The previous researches have been indicated that 2,4-D caused to suitable callus induction in different cultivars of indica rice [22-25].

3.2. The effects of Nano-TiO_2 concentration on callus formation

In this research, callus formation significantly influenced by Nano-TiO_2 concentrations as well as types and concentrations of plant growth regulators. Even though effective callus formation has been succeed in several of plant growth regulator treatments but the combination of 2,4-D with Nano-TiO_2 increased the performances of callus induction. After five weeks of culturing, the color of callus induced from the callus induction medium supplemented with 1.5 mg L^{-1} 2,4-D and Nano-TiO_2 were creamish yellow and the textures were friable (Fig. 2). An optimum level of the fresh weight, dry weight and size in rice callus of Pathumthai1 cultivar was achieved with 1.5 mg L^{-1} 2,4-D and 400 mg L^{-1} Nano-TiO_2. The highest fresh weight (79.74 g), dry weight (39.16 g) and size (1.18 cm) of rice seeds inducing callus formation was observed with the callus induction medium supplemented with 1.5 mg L^{-1} 2,4-D and 400 mg L^{-1} Nano-TiO_2 when compared with the other treat-ments (Fig. 3A-C).

The impacts of nanomaterials on callus induction and plant regeneration from *indica* rice have been examined in the previous researches [26-30]. The Nano-TiO_2 have been shown to affect the biochemical pathway and physiological responses, consequently influencing cell wall formation, cell division, water absorption, the photosynthetic performance etc. [31-33].

3.3. The effects of Nano-TiO_2 concentration on total phenolic compounds and flavonoid contents in callus extract

The physiological responses (total phenolic compounds and flavonoid contents) of the callus extraction under different Nano-TiO_2 concentration conditions were investigated. The contents of total phenolic compounds and flavonoid were detected in this research for study the plant metabolites response to Nano-TiO_2 induced stress. The results showed that the contents of total phenolic compounds and flavonoid were the induction in callus extract among all treatments (Fig. 4).

In the callus extract, the levels of total phenolic compounds and flavonoid contents did vary apparently with the difference of Nano-TiO_2 concentration. The highest contents of total phenolic compounds (108.90 mg Gallic acid g^{-1} FW) and flavonoid (0.005 mg g^{-1} FW) were found when the Nano-TiO_2 supplementation with 400 mg L^{-1} as well as the callus formation performances (Fig. 4A-B).

Nanomaterials or metals have the possible to stimulate the production of plant metabolites in various plant species. The potential to elicit the plant metabolites depend on the plant species, types and concentrations of nanomaterials as well as growth conditions [34, 35]. Bioactive compounds and plant metabolites have been performed when induced under the appropriate nanomaterials types and concentrations for given plant products [36, 37]. Bioactive compounds and plant metabolites performance have been impacted by different types and concentrations of elicitors [38, 39]. The current research indicated that few evidences is available on nanomaterials effects on bioactive compounds and plant metabolites performance in plants. This research reported that nanomaterials cause a significant increase of bioactive compounds and plant metabolites in *indica* rice plants.

3.4. The effects of Nano-TiO_2 concentration on antioxidant activity in callus extract

The level of antioxidant activity in the callus extract exposed with and without Nano-TiO_2 were investigated using ABTS method (Fig. 5). The antioxidant activity of the callus extract was the highest induced by 39.18% and 6.26 µM Trolox in the percentage of inhibition and Trolox equivalents, respectively in response to 400 mg L^{-1} Nano-TiO_2 on the 5th day of cultivation (Fig. 5A-B).

Exposure to nanomaterials or metals produces over production of free radicals or reactive oxygen species in plant cells. The physiological response of plant cells can be scavenged and detoxified in free radicals or reactive oxy-gen species by the induction of enzymatic and non-enzymatic antioxidants substances for plant protection process [40, 41]. Therefore, plant cells produce and accumulate bioactive compounds and plant metabolites as potential antioxidants substances such as phenolic com-pound, flavonoids, anthocyanins, etc. in response to scavenge the overproduced of free radicals or reactive oxygen species for plant proliferation [42, 43].

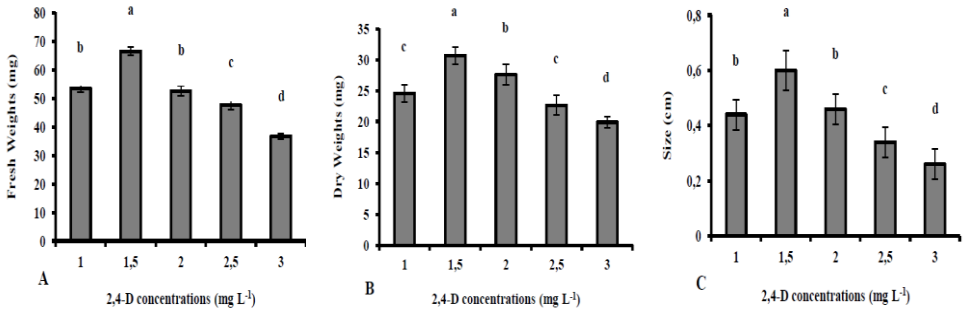

Fig. 1. The effect of 2,4-D on the callus formation of fresh weight (A), dry weight (B) and size (C) in rice callus exposed to 2,4-D (0, 1, 1.5, 2, 2.5, 3 mg L^{-1}) different concentrations on the 5th week of culture. Data are the means of five replication experiments ± SD. Treatment with at least one letter the same are not significantly different according to Duncan's Multiple Range Test ($P \leq 0.05$).

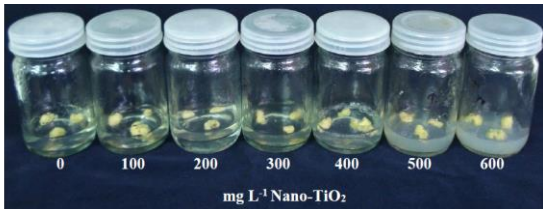

Fig. 2. Callus formation derived from in vitro cultured on the callus induction medium supplemented with 1.5 mg L^{-1} 2,4-D and Nano-TiO_2 (0, 100, 200, 300, 400, 500 and 600 mg L^{-1}) different concentrations on the 5th week of culture.

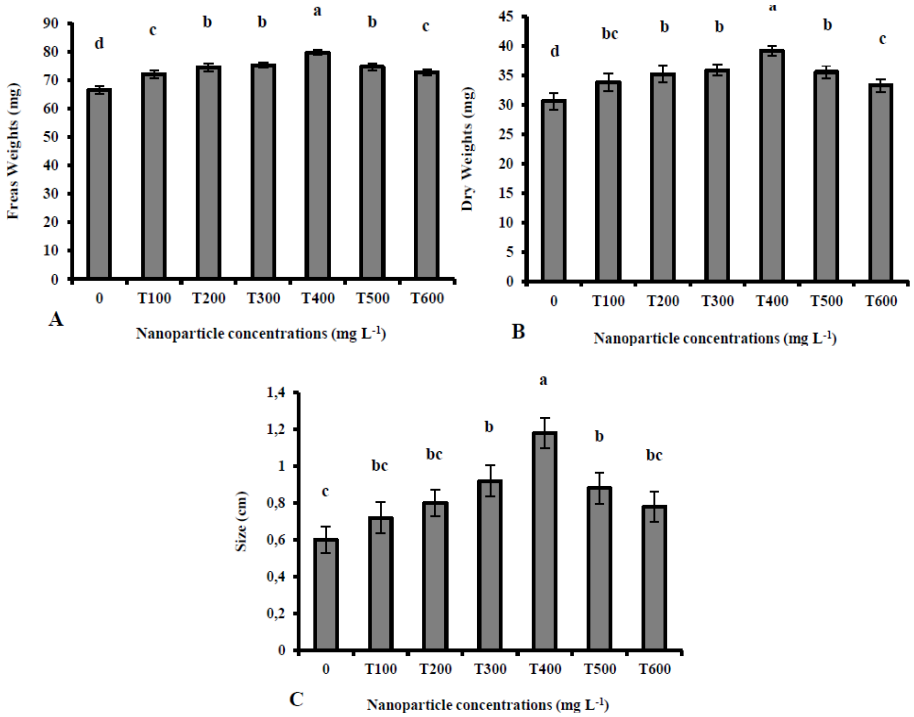

Fig. 3. The effect of Nano-TiO_2 on the callus formation of fresh weight (A), dry weight (B) and size (C) in rice callus exposed to Nano-TiO_2 (0, 100, 200, 300, 400, 500 and 600 mg L^{-1}) different concentrations on the 5th week of culture. Data are the means of five replication experiments ± SD. Treatment with at least one letter the same are not significantly different according to Duncan's Multiple Range Test ($P \leq 0.05$).

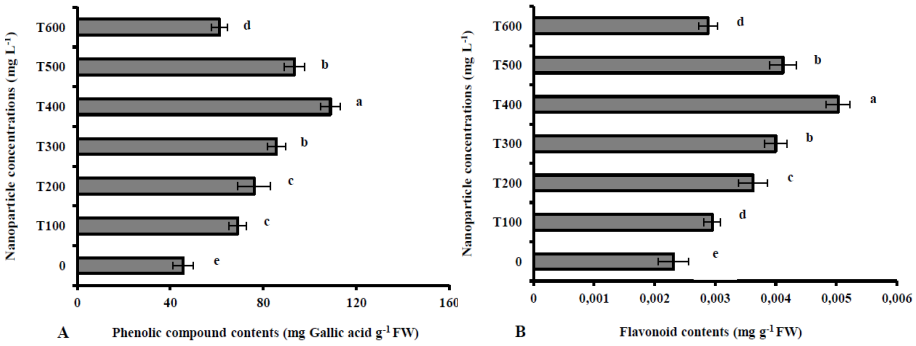

Fig. 4. The effect of Nano-TiO_2 on the callus extract of total phenolic compounds (A) and flavonoid (B) contents in rice callus exposed to Nano-TiO2 (0, 100, 200, 300, 400, 500 and 600 mg L^{-1}) different concentrations on the 5th week of culture. Data are the means of five replication experiments ± SD. Treatment with at least one letter the same are not significantly different according to Duncan's Multiple Range Test ($P \leq 0.05$).

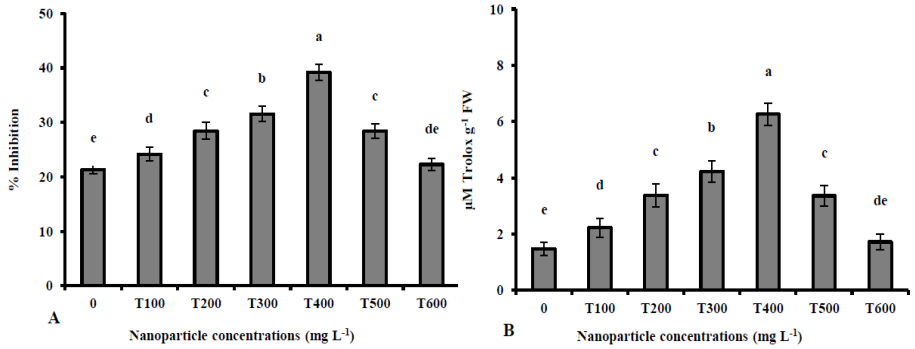

Fig. 5. The effect of Nano-TiO_2 on the callus extract of % Inhibition (A) and Trolox equivalents (B) levels in rice callus exposed to Nano-TiO_2 (0, 100, 200, 300, 400, 500 and 600 mg L^{-1}) different concentrations on the 5th week of culture. Data are the means of five replication experiments ± SD. Treatment with at least one letter the same are not significantly different according to Duncan's Multiple Range Test ($P \leq 0.05$).

4. Conclusions

The callus formation, plant metabolites and antioxidant activity of callus and callus extract from *indica* rice cv. Pathumthani were investigated. The high levels of total phenolic compounds, flavonoid and antioxidant activity from callus extract when exposed to Nano-TiO_2 have shown positive indication that a functional supplementary product could be improved from agriculture, food and cosmetic application in the further research.

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