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TITANIUM DIOXIDE (TiO_2) NANOPARTICLES INDUCED CALLUS INDUCTION AND PLANT REGENERATION OF *INDICA* RICE CULTIVARS (SUPHANBURI1 AND SUPHANBURI90)

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Efficient plant regeneration systems are useful for *indica* rice using micropropagation and/or genetic transformation. Nanomaterials provide applications and opportunities in various fields of life science and agriculture technology. In this research, *indica* rice cultivars 'Suphanburi1' and 'Suphanburi90' (*Oryza sativa* L. ssp. *indica*) were used to investigate. Seeds were examined to evaluate callus induction and plant regeneration frequency. Culture of sterilized-seeds on callus induction medium supplemented with 50 mg L^{-1} of TiO_2 nanoparticles induced a high frequency of callus induction and content of biomass accumulation. Embryogenic calli were transferred to plant regeneration medium supplemented with 10-50 mg L^{-1} TiO_2 nanoparticles. The highest frequency of plant regeneration and the ratio of the number of seedlings to the number of regenerated calli were produced on plant regeneration medium supplemented with 40 mg L^{-1} of TiO_2 nanoparticles. The regenerated shoots induced roots on NB medium without plant growth regulators and the plantlets were established in soil. The results may be used for nanomaterial applications of rice and/or other plants for its further improvement.

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Keywords: Callus induction, Indica rice, Micropropagation, Plant regeneration, TiO_2 nanoparticles

1. Introduction

Rice (*Oryza sativa* L.) is the important crop and widely cultivated crop in the world and Thailand. Cultivated rice belongs to the genus Oryza, family Gramineae (Poaceae) and the two major subspecies, *indica* and *japonica*. About 90% of the two major rice subspecies is produced and consumed in Asia [1, 2]. The production of rice cultivation is decreasing due to biotic and abiotic stresses. The elevation of the growing global population is higher than the production of rice. In the future, the amount of grain will be inadequate to the needs of the population [3]. Plant tissue culture is a basic technique which develops micropropagation and genetic transformation for plant improvement. Regeneration frequency of *indica* rice has been limited and less effective than *japonica* rice [4]. The successful applications of rice tissue culture depend on rice species, different types of explants, culture conditions, culture method, and compositions and supplementary medium [5-7].

Nanotechnology is the model technology and interdisciplinary science has been applied in various fields such as electronic, cosmetics, materials, and agricultures, especially TiO_2 nanoparticles have been used in a variety of applications. Various manufacturers are incorporating TiO_2 nanoparticles into many consumer products and have been shown to be productive in efficient improvement of their products [8]. In agricultural field, nanotechnology has been studied and utilized nanoparticles for the bio-system control such as antimicrobial effectiveness, phytopathogens, food development, and agricultural productivity [9-11]. Many researches indicate that difference of plant responses caused by metal nanoparticles exposure and related to the

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characteristics of metal nanoparticles [12, 13]. TiO_2 nanoparticles in agricultural applications allow for enhanced the plant growth, plant productivity and metabolites [14-16]. Moreover, Thailand is an agricultural country and there are a lot of rice cultivation and farmers. In agricultural field, few researches have been focused on nanomaterials-enhanced micropropagation solutions by plant tissue culture technique, especially *indica* rice.

In this research, we used the seeds of two *indica* rice cultivars including Suphanburi1 and Suphanburi90, have been widely used in the cultivation of agriculturists, to determine the effect of various concentrations of TiO_2 nanoparticles that added to the callus induction medium and regeneration medium on the regeneration efficiency.

2. Experimental

2.1. Plant materials and sterilized conditions

The seeds of two *indica* rice cultivars (*Oryza sativa* L. cv. Suphanburi1 and Suphanburi90) were obtained from Pathumthani Rice Research Center, Ministry of Agriculture and Cooperatives, Thailand. The mature seeds were dehusked and surface sterilized with 70% (v/v) ethanol for 2 min. Then, the dehusked seeds were sterilized with 5% (v/v) commercial bleach for 30 min with shaking and followed by 30% (v/v) commercial bleach for 30 min with shaking. The sterilized-seeds were rinsed thoroughly 5-6 times with sterilized distilled water and placed on sterilized filter papers in a petri dish.

2.2/ Effect of TiO_2 nanoparticles on the efficiency of callus induction

The dried seeds were cultured on NB medium [17] supplemented with 500 mg L^{-1} glutamine, 300 mg L^{-1} casein hydrolysate, 30 g L^{-1} sucrose, 2 mg L^{-1} 2,4-D and 8 g L^{-1} agar (the callus induction medium; [18]). TiO_2 nanoparticles (average size 47.21 ±9.71 nm; Evonik Degussa GmbH, Germany) were added to the callus induction medium at various concentrations (0-60 mg L^{-1}). The particle size and size distribution of TiO_2 nanoparticles was determined by particle analyzer (Delsa™Nano C; Fig. 1). The medium's pH was adjusted to 5.6-5.8 with 1 M HCl or 1 M NaOH. The cultures were incubated at 25 ±2 ºC in darkness for the callus induction. In the experiment, size of callus, dry weight of callus, percentages of callus induction frequency were evaluated after three weeks.

After three weeks, the induced-calli were transferred to sterilized filter papers in a petri dish for callus desiccation and incubated at 25 ±2 ºC in darkness. After one week, the desiccated-calli were transferred to the regeneration medium [18]. The cultures were incubated at 25 ±2 ºC under light conditions with a 16-h photo period (1000 lux) for the plant regeneration. The percentages of green spots, plant regenerations and the ratio of the number of seedlings to the number of regenerated calli were evaluated after four weeks.

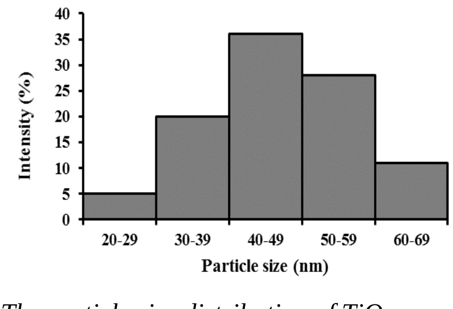


Fig. 1. The particle size distribution of TiO_2 nanoparticles.

2.3. Effect of TiO_2 nanoparticles on the efficiency of plant regeneration

The appropriate concentrations of TiO_2 nanoparticles from callus induction section were used. The desiccated-calli were transferred to the regeneration medium [18] supplemented with various concentrations (0-50 mg L^{-1}) of TiO_2 nanoparticles. The medium's pH was adjusted to 5.6-5.8 with 1 M HCl or 1 M NaOH. The cultures were incubated at 25 ±2 ºC under light conditions with a 16-h photo period (1000 lux) for the plant regeneration. The percentages of green spots, plant regenerations and the ratio of the number of seedlings to the number of regenerated calli were evaluated after four weeks.

2.4. Statistical analysis

Statistical analyses were conducted using SPSS (SPSS for Windows version 15, SPSS Inc., Chicago, USA). The experiments were arranged in a Completely Randomized Design (CRD) as a single factor experiment with five replicates (n=5). The level of significance was evaluated from the analysis of variance (ANOVA). Duncan's New Multiple Range Test (DMRT) was used to compare the mean values.

3. Results and discussions

Recently, the agricultural technology seems to be one of the main users of metal nanoparticles, especially ZnO and TiO_2 nanoparticles. The phytotoxic and genotoxic potentials of TiO_2 nanoparticles have been several presented in different species of plants [19, 20]. The efficiency of plant tissue culture and plant regeneration is influenced by several components, such as supplementary substances, culture conditions, plant growth hormones, etc. Advanced technology based on nanomaterials is applied to many fields, including electronics, sensors, biomedicines, agriculture, waste water treatment and food processing [21-23]. Few researches have been studied on nanomaterial effects of callus induction and plant regeneration frequency in rice. This research investigated a current study using TiO_2 nanoparticles and their effects on physiological and differentiation responses in plants. In this research, we evaluated the callus induction and plant regeneration frequency of two *indica* rice cultivars (Suphanburi1 and Suphanburi90). TiO_2 nanoparticles were studied for enhancing the callus induction and plant regeneration frequency.

Compact and yellowish calli were induced from embryo of two *indica* rice cultivars (Suphanburi1 and Suphanburi90) after 1-3 weeks of callus induction (Fig. 2A). All concentration treatments induced embryogenic callus ranging from 89.76% to 94.67%. The result showed that the high concentrations of TiO_2 nanoparticles caused a reduction in the size, weight and the percentages of callus induction. The callus induction medium containing 50 mg L^{-1} TiO_2 nanoparticles presented the highest size (1.14 and 1.08 cm), dry weight (11.87 and 11.56 mg), and percentage of callus induction (94.67% and 93.25%) of Suphanburi1 and Suphanburi90 cultivars with satisfactory embryogenic characteristics when compared to other treatments (0, 10, 20, 30, 40 or 60 mg L^{-1} TiO_2 nanoparticles; Table 1). The lowest size (0.87 and 0.82 cm), dry weight (10.35 and 9.67 mg), and percentage of callus induction (90.36% and 89.47%) of Suphanburi1 and Suphanburi90 cultivars was obtained on the callus induction medium containing 60 mg L^{-1} TiO_2 nanoparticles.

In addition, the percentages of green spots, plant regenerations and the ratio of the number of seedlings to the number of regenerated calli was recorded in the induced-calli which transferred to the regeneration medium (without TiO_2 nanoparticles) after four weeks. The induced-calli of Suphanburi1 and Suphanburi90 cultivars from the callus induction medium containing 50 mg L^{-1} TiO_2 nanoparticles showed the highest percentages of green spots (94.11% and 93.57%), plant regenerations (61.89% and 60.25%) and the ratio of the number of seedlings to the number of regenerated calli (3.11 and 3.06; Table 2).

Table 1. Effect of TiO_2 nanoparticles in the callus induction medium on the size, dry weight, and percentages of calli in two indica rice cultivars (Oryza sativa L. cv. Suphanburi1 and Suphanburi90).

TiO_2 nanoparticles (mg L^{-1})	Size of callus (cm)		Dry weight of callus (mg)		Callus induction (%)	
	Suphanburi1	Suphanburi90	Suphanburi1	Suphanburi90	Suphanburi1	Suphanburi90
0	0.89 ±0.02b	0.85 ±0.03b	10.45 ±0.12b	9.87 ±0.13b	90.53 ±1.02d	89.76 ±1.12d
10	0.91 ±0.02b	0.89 ±0.04b	10.55 ±0.11b	9.98 ±0.12b	91.24 ±1.11c	90.67 ±1.02c
20	0.92 ±0.03b	0.90 ±0.03b	10.60 ±0.12b	10.05 ±0.12b	92.44 ±1.12b	91.22 ±1.02b
30	0.92 ±0.04b	0.91 ±0.02b	10.67 ±0.10b	10.08 ±0.13b	92.59 ±1.05b	91.56 ±1.02b
40	0.94 ±0.02b	0.92 ±0.02b	10.69 ±0.13b	10.09 ±0.14b	92.82 ±1.04b	91.67 ±1.02b
50	1.14 ±0.03a	1.08 ±0.04a	11.87 ±0.15a	11.56 ±0.16a	94.67 ±1.01a	93.25 ±1.02a
60	0.87 ±0.04b	0.82 ±0.02b	10.35 ±0.14b	9.67 ±0.13b	90.36 ±1.14d	89.47 ±1.02d

Values are presented the means of three replicates ±S.D. (n=5). Means on the same column with the different superscripts are significantly different ($P < 0.05$).

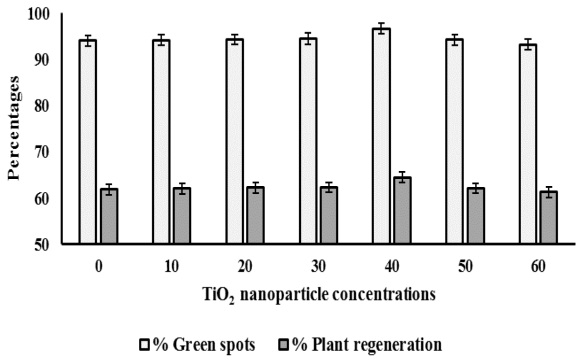
Table 2. Effect of TiO_2 nanoparticles in the callus induction medium on the percentages of green spots, plant regenerations and the ratio of the number of seedlings to the number of regenerated calli in two indica rice cultivars (Oryza sativa L. cv. Suphanburi1 and Suphanburi90).

TiO_2 nanoparticles (mg L^{-1})	Green spots (%)		Plant regenerations (%)		Ratio of the number of seedlings to the number of regenerated calli	
	Suphanburi1	Suphanburi90	Suphanburi1	Suphanburi90	Suphanburi1	Suphanburi90
0	91.45 ±1.13c	90.89 ±1.12b	60.12 ±1.11b	58.44 ±1.14b	2.77 ±0.04b	2.67 ±0.03b
10	92.27 ±1.12b	90.95 ±1.11b	60.24 ±1.12b	58.55 ±1.13b	2.80 ±0.02b	2.70 ±0.03b
20	92.53 ±1.11b	91.15 ±1.14b	60.29 ±1.13b	58.57 ±1.15b	2.82 ±0.03b	2.71 ±0.04b
30	92.67 ±1.13b	91.27 ±1.15b	60.33 ±1.14b	58.67 ±1.13b	2.84 ±0.03b	2.74 ±0.05b
40	92.78 ±1.21b	91.44 ±1.13b	60.53 ±1.15b	58.73 ±1.16b	2.88 ±0.06b	2.77 ±0.08b
50	94.11 ±1.15a	93.57 ±1.14a	61.89 ±1.13a	60.25 ±1.13a	3.11 ±0.05a	3.06 ±0.06a
60	91.33 ±1.14c	90.85 ±1.15b	60.02 ±1.12b	58.14 ±1.13b	2.72 ±0.04b	2.65 ±0.05b

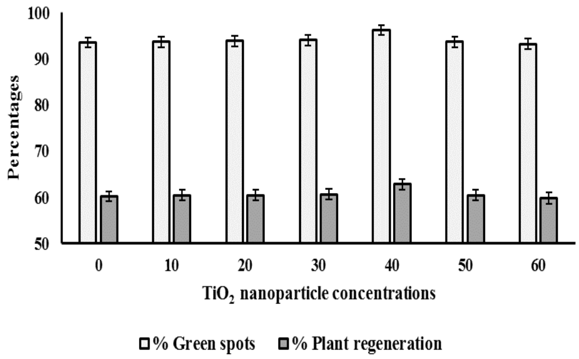
Values are presented the means of three replicates ±S.D. (n=5). Means on the same column with the different superscripts are significantly different ($P < 0.05$).

In order to study the TiO_2 nanoparticles for obtaining plant regeneration frequency, the induced and desiccated-calli were incubated in the regeneration medium supplemented with various concentrations of TiO_2 nanoparticles for 4 weeks. Green spots and plant regeneration were variably developed from the induced and desiccated-calli and were visible within 2-4 weeks (Fig. 2B and 2C). The various concentrations of TiO_2 nanoparticles were added to the regeneration medium were significantly differentiated in terms of the percentages of green spots, plant regenerations and the ratio of the number of seedlings to the number of regenerated calli (Fig. 3 and 4). The highest percentages of green spots (96.67% and 96.25%), plant regenerations (64.50% and 62.89%) and the ratio of the number of seedlings to the number of regenerated calli (3.67 and 3.50) of induced-calli from the regeneration medium supplemented with 40 mg L^{-1} TiO_2 nanoparticles were observed in Suphanburi1 and Suphanburi90 cultivars, respectively when compared to other treatments (0, 10, 20, 30, 50 or 60 mg L^{-1} TiO_2 nanoparticles; Fig. 3 and 4).

Fig. 2. Callus induction (A) from seed explants after three months of culture on the callus induction medium supplemented with 50 mg L^{-1} TiO_2 nanoparticles. Green spots (B) and plant regenerations (C) from callus induction after four months of culture on the plant regeneration medium supplemented with 40 mg L^{-1} TiO_2 nanoparticles.

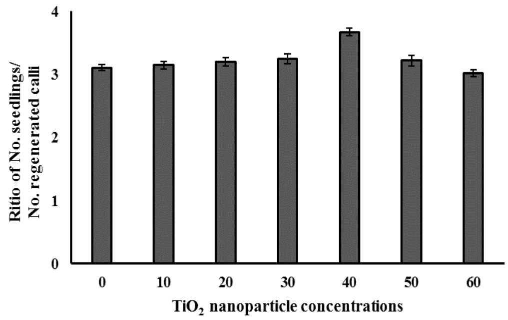


a)



b)

Fig. 3. Effect of TiO_2 nanoparticles in the regeneration medium on the percentages of green spots and plant regenerations in two indica rice cultivars [Oryza sativa L. cv. Suphanburi1 (a) and Suphanburi90 (b)]. Data are presented the mean ±S.D. (n=5). Means on bars with the different superscripts are significantly different ($P < 0.05$).



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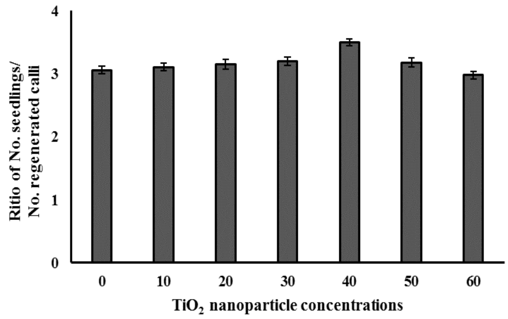

b)

Fig. 4. Effect of TiO_2 nanoparticles in the regeneration medium on the ratio of the number of seedlings to the number of regenerated calli in two indica rice cultivars [Oryza sativa L. cv. Suphanburi1 (a) and Suphanburi90 (b)]. Data are presented the mean ±S.D. (n=5). Means on bars with the different superscripts are significantly different ($P < 0.05$).

In this research, we succeeded in obtaining the application of TiO_2 nanoparticles for high frequency callus induction and plant regeneration from mature seed derived embryogenic callus cultures in order to use them for micropropagation and genetic transformation in further experiments. TiO_2 is one of the most commonly used nanomaterials for plants and has been applied in many plant species causes high biocompatibility [24]. The stimulatory effect of nanomaterials has been reported to plant growth, gene expression, and metabolites [25-27]. Whereas, the applications of nanomaterials for micropropagation in plant tissue culture techniques are still very limited especially in *indica* rice. The present research revealed that, 50 mg L^{-1} and 40 mg L^{-1} TiO_2 nanoparticles were found optimum concentrations for callus induction and plant regeneration, respectively in Suphanburi1 and Suphanburi90 cultivars. The callus induction as well as plant regeneration frequency could be regulated by supplementary substances in culture medium [28, 29]. The endogenous compositions of medium could be induced the expression of gene and controlled the differentiation of plant cell and tissue [30, 31]. The previous researches showed that the differentiation of *indica* rice was more complicated than that of *japonica* rice in tissue culture techniques because of various varieties of *indica* rice cultivars [32, 33]. The results of this research were similar to Chutipaijit S. [18] that presented the high percentages of plant regeneration in *indica* rice cv. KDML105 cultured on 25 mg L^{-1} TiO_2 nanoparticles. TiO_2 has proven to be effective in inducing the gene expression of photosynthetic complexes, nitrogen metabolism and plant hormones metabolism [34-36]. These biosynthesis pathways could be enhance cell division, plant growth and differentiation of plant cells that involved in development and multiplication of shoots [37-39].

4. Conclusions

In conclusions, the results were observed that the optimum concentration of TiO_2 nanoparticles has significantly enhanced the percentages of callus induction and plant regeneration of *indica* rice cultivars (Suphanburi1 and Suphanburi90 cultivars). This research showed a high-efficiency medium supplemented with TiO_2 nanoparticles for *in vitro* micropropagation from mature seeds of Suphanburi1 and Suphanburi90 cultivars. Culture medium supplemented with 50 mg L^{-1} and 40 mg L^{-1} TiO_2 nanoparticles were selected as the best media composition for callus induction and plant regeneration sections, respectively. Future studies should also application in micropropagation of various plant species and investigation of the interaction between metal nanoparticles and plant cell differentiation.

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Compliance with Ethical Standards

The authors declare that they have no conflict of interest.

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