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Effects of Titanium Dioxide nanoparticles on salinity tolerance of rice (*Oryza sativa* L.) at the seedling stage

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ABSTRACT

This study aimed to evaluate the effects of titanium dioxide nanoparticles on the salinity tolerance of rice. The effects of five nano titanium dioxide concentrations (0 mg/L, 25 mg/L, 50 mg/L, 75 mg/L, and 100 mg/L) on the physiological and biochemical parameters of rice were evaluated. The results showed that among three rice varieties (ST24, ST25, OM18), only ST25 grew in a better manner with the application of TiO_2 nanoparticles and the optimal concentration of TiO_2 nanoparticles was 50 mg/mL. It increased the shoot height by 20.07% and the survival rate of rice compared to the control. These growth-promoting effects were simultaneous with increased levels of chlorophyll, carotenoid and proline. The activities of antioxidant enzymes were improved. While activities of enzymes catalase and peroxidase increased significantly, no change in the activities of ascorbate peroxidase was observed. Finding of this study showed that titanium dioxide nanoparticles increased the salinity tolerance of rice by promoting the photosynthetic and anti-oxidative processes in rice seedlings.

1. INTRODUCTION

Soil salinity is one of the detrimental abiotic stresses that affect plant growth and crop production worldwide. Among important crops, rice (*Oryza sativa* L.) plays an important role in providing food for about half of the world's population and has the third largest production in the world after maize and wheat. At the physiological level, soil salinity disturbs ionic and water homeostasis, while at the cellular level, it leads to enhanced accumulation of reactive oxygen species (ROS) (Abdel et al., 2017; Hasanuzzaman et al., 2021). The increased ROS level disturbs the cellular redox homeostasis, leading to oxidative damage. However, most of these studies are limited to lab scale, and their applicability under natural field conditions still needs validation. To complement such genetic approaches, chemical priming has emerged as an alternative strategy for enhancing the abiotic stress resistance of plants (Abdel et al., 2017; Thakur et al., 2019).

Nanotechnology offers a significant advantage in the field of chemical priming as the effectiveness of chemicals can be enhanced in their nano form, thus reducing their environmental load (Khan et al., 2017). Titanium dioxide photocatalyst is a well-known and well-researched photocatalyst due to its interesting properties, which include stability, non-toxicity, biocompatibility, and optical and electrical properties (Nyamukamba et al., 2018). TiO_2 is one of the major engineered nanoparticles produced in

large quantities by the cosmetics, chemical, and agricultural food industries (Liu et al., 2014). TiO_2 can be considered a plant stimulant that activates different defence mechanisms involved in plant tolerance against various abiotic stress factors (Gohari et al., 2020). Hence, this study was conducted to examine the exogenous applications on rice leaves of titanium dioxide nanoparticles in order to evaluate the efficacy of the elevation to rice growth in salinity conditions. The influences on chlorophyll and carotenoid content, proline content, and activities of antioxidant enzymes (APX, CAT, and POD) were also investigated.

2. MATERIALS AND METHOD

2.1. Plant materials and treatment

Source of nano particles: TiO_2 powder was purchased from XFNano company (USA) with the sizes from 15 to 25 nm.

Seed preparation: Seeds of four rice varieties (IR29, OM18, ST24, and ST25) were used. Rice seeds were treated in hot water (54^{o}C) for 10-20 minutes, then they were soaked in clean water for 24 - 48 hours and drained. Next, the seeds were incubated for 36-48 hours until they grew into seedlings.

Screening for salt concentration: Germinated seeds were planted in plastic cups (9 seedlings per cup) filled with washed sand on the half strength of Hoagland's nutrient solution for 7 days before adding salt. Then, 10 days rice seedlings (ST24, ST25, OM18, IR29) was tested with six levels of NaCl salt test (0‰, 2‰, 4‰, 6‰, 8‰ and 10‰) in the vegetative stage for 21 days in a net house with natural conditions. The seedlings were harvested, and the data was recorded after 21 days of salt treatment to select the salt concentration for following experiments.

Screening for TiO_2 concentration: Germinated seeds were planted on the half strength of Hoagland's nutrient solution for 7 days before adding the selected salt concentration. Subsequently, these seedlings were sprayed with 5 different concentration of titanium dioxide nanoparticles every three days (0 mg/mL, 25 mg/ mL, 50 mg/ mL, 75 mg/ mL and 100 mg/ mL dissolved in 0.1% Tween 20) (Abdel Latef et al., 2017; Gohari et al., 2020; Shah et al., 2021) on leaves immediately after adding salt. The optimum nanoparticles concentration was selected for following experiments.

Measurements of Plant Growth: The shoot elongation (cm) was measured by a ruler. The survival percentages and scores were evaluated according to the method described by Gregorio et al. (1997).

2.2. Effects of TiO_2 nanoparticles on biochemical parameters of rice

Plant treatments: Once the rice seeds were geminated (follow the incubation steps of salinity experiment), the seedlings were planted on holes (10 seedlings per holes) punched on a foam which was inserted a tray containing Yosida solution (Yoshida, 1976) for 7 days before adding salt. In this experiment, the seedlings were treated with selected NaCl and TiO_2 concentration. The nanoparticles were applied three times on the fifth, eighth, and eleventh day after salt treatment. The sample was collected after 24 hours of each spray time for biochemical analysis.

Chlorophyll and carotenoids content: Chlorophylls and carotenoids were estimated by the spectrophotometric method according to Minh et al. (2016). Chlorophyll and carotenoid were expressed as µg/g FW.

Proline content: Proline content was determined spectrophotometically at 520 nm. Proline concentration is expressed as mg/g fresh weight (Bates et al., 1973).

Enzyme Extraction and Assays: An amount of 0.1 g leaves was homogenized with 1800 µL of phosphate buffer (pH 6.8) and 200 µL of 1 mM ethylenediaminetetraacetic acid (EDTA). The mixture was centrifuged for 20 min at 15,000 rpm, and the upper portion was kept at −20 ◦C for further measurement. APX activity was evaluated using the method described by Nakano et al. (1981) with several changes. The absorbance was recorded on spectrophotometer at 29 nm for 3 minutes. The APX activity was calculated using the extinction coefficient (2.8 $mM^{-1}cm^{-1}$) and expressed as unit/min/g FW. The activities of enzyme catalase (CAT) was accounted by calculating the quantity of decomposed H_2O_2 according to the method of Damanik et al. (2010). The absorbance was recorded on spectrophotometer at 240 nm for 60s. The CAT activity was calculated using the extinction coefficient 6,93 x 10^{-3} $mM^{-1}cm^{-1}$ and expressed as unit/min/g FW. Peroxidase (POD) activity was measured at 420 nm by the guaiacol method describe by Herzog (1973) with some changes. The POD activity was calculated based on

the extinction coefficient (25 mM^{-1} cm^{-1}) and shown as unit/min/g FW.

2.3. Statistical analysis

Obtained data were expressed as means ± standard deviation (SD). The data were subjected to One-way Analysis of Variance; statistical package for social sciences (SPSS) software for windows version 20, followed by Duncan test to compare the significance of differences between means. The results were considered significant at $P< 0.05$.

3. RESULTS AND DISCUSSION

3.1. Effects of salinity on the growth parameters of rice

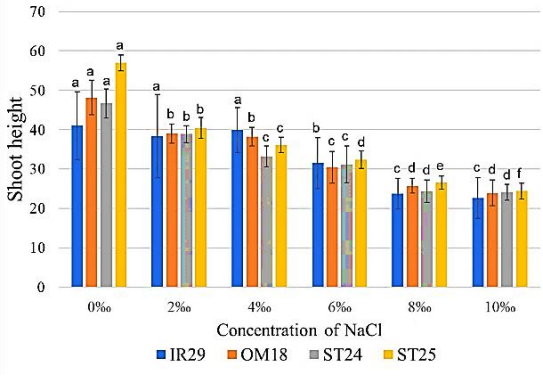

Figure 1. Effects of salt concentration on shoot height

Different letters in same column with the same colour indicate significantly difference at p < 0.05

The data in Figure 1 show that the shoot height of rice varieties decreased when the salt concentration increased. The shoot height of OM18, ST24, and ST25 reduced at the concentration of 2‰ by 18.92%. 16.72%, and 28.96%, respectively. At the concentration of 10‰, an approximately 50% reduction was observed in the shoot height of all the examined rice varieties. Experimental results on the reduction of plant height under salinization gave similar results to the experiment by Rahman et al. (2017).

For the salinity tolerance level, all four rice varieties recorded normal growth and development (salinity tolerance level equals 1) at the concentration 0‰ (Table 1). The highest salinity level was 9 – very contaminated was recorded at salt concentrations of 8‰ and 10‰ in all rice varieties OM18, ST24, and ST25. The survival rate of OM18 decreased at the concentration 6‰ with 33.33%. Whereas the figure for ST24 and ST25 fell by 33.33% and 22% respectively at the lower concentration of 4‰. At the highest salt concentration (10‰), all the rice varieties had the lowest survival rate with 22% of OM18 and 0% of ST24 and ST25. Experimental results agree with the study of Munns and Tester (2008), which shows that high salt concentration often leads to growth arrest and even death of plants. The decrease in plant height and survival rate in rice varieties under salinity were different, which could be explained by the different genotype differences in the four rice varieties (Hasanuzzaman et al., 2009). The NaCl concentration selected to use for the following experiments in this experiment is 6‰ since, at this concentration, the survival rate of all the rice varieties was about 50%.

Table 1. Effects of salt concentration on the survival percentage and SES score of rice.

Varieties	Salt treatment (‰)	Survival percentage (%)	SES score
IR29	0	100	1
	2	33.33	7
	4	0.00	9
	6	0.00	9
	8	0.00	9
	10	0.00	9
OM18	0	100	1
	2	100	1
	4	100	3
	6	66.67	5
	8	33.33	7
	10	22.22	7
ST24	0	100	1
	2	100	3
	4	66.67	5
	6	55.56	7
	8	0.00	9
	10	0.00	9
ST25	0	100	1
	2	100	3
	4	77.78	5
	6	55.56	7
	8	22.22	7
	10	0.00	9

3.2. Effects of TiO_2 nanoparticles on plant growth and biochemical parameters

3.2.1. Effects of TiO_2 nanoparticles on rice growth

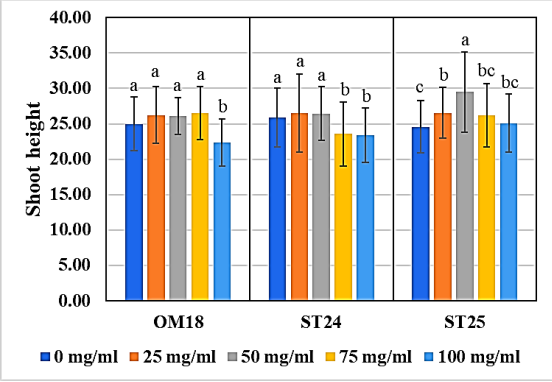

Figure 2. Effects of TiO_2 nanoparticles on shoot height.

Means with different letters indicate significantly difference at $p < 0.05$

The data in Figure 2 and Table 2 present the effects of five different concentrations of TiO_2 nanoparticles on the shoot height, survival rate and SES - score of three rice varieties. Regarding OM18 and ST24, there were no enhancements in the shoot height, survival rate, and SES score in the treatments sprayed with TiO_2 nanoparticles compared to the control. In addition, higher concentrations of TiO_2 nanoparticles can decrease the shoot height of these rice varieties. To be more specific, 100 mg/mL TiO2 nanoparticles reduce the shoot height of OM18 by 13.64%, whilst the decrease of shoot height of ST24 can be observed at the concentration of 75 mg/mL, at roughly 9%. There was an opposite pattern in the shoot height, survival rate, and SES score of ST25 at the treatment of 50 mg/mL TiO_2 nanoparticles. The shoot height increased by 20.07%, the survival rate grows by 11,11% and the SES score decreased from 7 (sensitive) to 5 (moderately tolerant). The increase in plant height when using nano TiO_2 could be because TiO_2 enhanced the absorption rate of macronutrients and micronutrients, improved plant growth characteristics, and reduced the negative effects of salinity by affecting photosynthesis and absorption of essential elements (Rahneshan et al., 2018). In addition, according to Rui et al. (2018), the photocatalytic properties of TiO_2 nanoparticles can help increase photosynthesis and ultimately improve plant growth. Therefore, TiO2 nanoparticles can improve the rice growth of ST25 and mitigate the negative effects of salt stress.

Table 2. Effects of salt concentration on survival percentage and SES score of rice

Varieties	TiO_2 treatment (mg/L)	Survival rate (%)	SES-score
OM18	0	51.11	7
	25	53.33	7
	50	62.22	7
	75	68.89	7
	100	48.89	7
ST24	0	53.33	7
	20	55.56	7
	50	60.00	7
	75	48.89	7
	100	42.22	7
ST25	0	48.89	7
	25	60	7
	50	66.67	5
	75	57.78	7
	100	53.33	7

The rice varieties ST25 and the TiO_2 nanoparticles concentration of 50 mg/mL were selected to use for the following experiments because of the better growth under salt stress of ST25 when it is sprayed with TiO_2.

3.2.2. Effects of TiO_2 nanoparticles on biochemical parameters of rice.

a. Chlorophyll and carotenoid contents

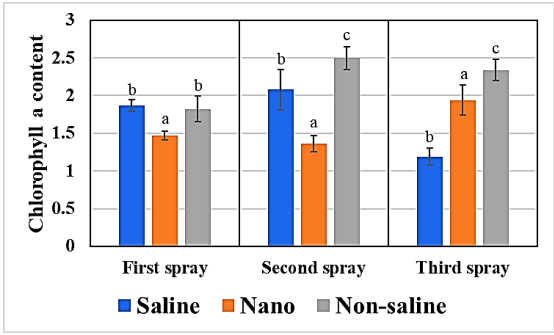

Figure 3. Effects of TiO_2 nanoparticles on chlorophyll a content

Means with different letters indicate significantly difference at $p < 0.05$

Figures 3, 4 and 5 present the changes in pigment content of different treatments at distinct spraying times. In the first spray, the data after 24 hours of spraying showed that there was no difference in the content of pigments (chlorophyll and carotenoid) between the saline and the control treatments. In the second spray, the chlorophyll content in the saline control treatment was reduced by 16.8% compared

to the non-saline treatment after 24 hours of nanospray. By the third nano-spray, 50%, 28.33%, and 50.47% were the percentages of reduction of chlorophyll a, chlorophyll b, and carotenoids, respectively, after 24 hours of nano-spray between saline and non-saline treatments. Therefore, the influence of salinity on the pigment (chlorophyll a) content started from the second nanospray and the effect of salinity increased with the time of salinization. In the nano treatment, the pigment content after 24 hours in nanospray 1 and 2 decreased by 21.39% and 34.6%, respectively, compared to the saline treatment. In the third spray, the pigment content was 63.02% (chlorophyll a), 33.7% (chlorophyll b), and 62.26% (carotenoid) higher than in the saline treatment. The above results show that nano can increase the content of pigments in plants.

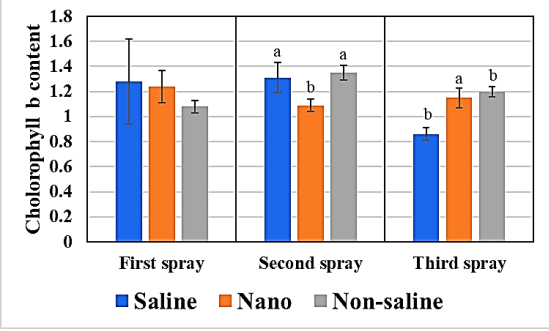

Figure 4. Effects of TiO_2 nanoparticles on chlorophyll b content

Means with different letters indicate significantly difference at $p < 0.05$

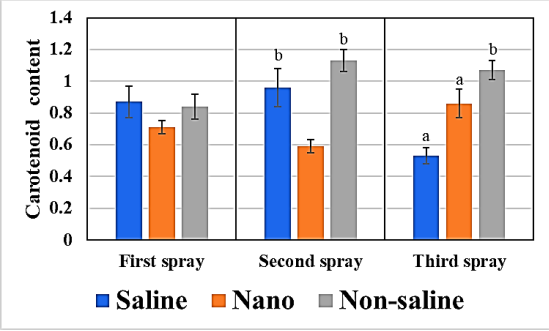

Figure 5. Effects of TiO_2 nanoparticles on chlorophyll a content

Means with different letters indicate significantly difference at $p < 0.05$

The results of the reduced pigment content because of the influence of salinity were similar to previous studies (Amirjani, 2010; Rahman et al., 2017). The decrease in photosynthetic pigments observed under saline conditions could be due to decreased biosynthesis or more likely increased degradation of pigments because of intracellular ROS accumulation, or dysfunction in stomatal opening and closing and the instability of pigment-protein complexes under saline conditions. Salinization leads to pigment degradation by the accumulation of toxic ions in chloroplasts and ROS-induced oxidative stress in plants (Rahneshan et al., 2018). According to Ashraf and Harris (2013), Na^+ accumulation reduces the content of precursors of chlorophyll biosynthesis (such as glutamate and 5-aminolevulinic acid) and thereby disrupts chlorophyll biosynthesis under saline conditions. Carotenoids are pigments that have several functions in plants, in addition to their direct role in photosynthesis, they are also involved in protective mechanisms of oxidative stress (Gill et al., 2010). Thus, the reduction of carotenoids under adverse conditions is associated with depletion of β-carotene and formation of zeaxanthin, which appears to be associated with protection against photosynthetic inhibition (Sharma & Hall, 1991).

b. Proline content

Table 3. Effects of TiO_2 on proline content

Treatment	Proline content (µg/mg FW)		
	1st spray	2nd spray	3rd spray
Nano	8.01[a]	9.41[a]	12.64[a]
Saline	5.82[b]	7.56[b]	9.04[b]
Non-saline	6.38[b]	6.09[c]	6.05[c]

Different letters in same column indicate significantly difference at $p < 0.05$.

The data from Table 3 showed that the proline content in the saline treatment was higher than that in the non-saline treatment in the second and third sprays by 24.14% and 49.42%, respectively. In the nano treatment, the proline content in three spray times was 8.01 µg/mg FW; 9.41 µg/mg FW, and 12.64 µg/mg FW, respectively. The value in the third spray was the highest compared to the saline and non-saline treatment (table 3). The proline content in the nano treatment at three spray times increase 37.63%, 24.42%, and 39.82%, respectively, compared with the saline control. This result is similar to the study reporting the effect of nano TiO_2 on legumes (*Vicia faba* L.) (Abdel Latef et al., 2017). The mechanism for proline accumulation is that salinization closes the stomata leading to reduced CO_2 fixation, leading to a decrease in carbon in the Calvin cycle (Lawlor et al., 2002). As a result, there is no more $NADP^+$ electron

acceptor involved in photosynthesis, so glutamate will accept electrons from $NADPH_2$ to biosynthesize proline to regenerate $NADP^+$ for photosynthesis (Wang et al., 2007). Experimental results show that when applying nano TiO_2, the high accumulation of proline under saline conditions can partly explain the higher tolerance of plants to salinity. Hence, the use of nano TiO_2 increased proline accumulation to make rice more resistant to salinity stress.

c. Antioxidant enzyme assay

Table 4. Changes of ascorbate peroxidase (APX), catalase (CAT), and peroxidase (POD) activities after TiO_2 nanoparticles treatment

Enzyme	Treatment	Enzyme activity (U/min/g FW)		
		1st spray	2nd spray	3rd spray
APX	Nano	2.88	2.38[ab]	1.91
	Saline	3.75	2.57[a]	1.81
	Non-saline	2.87	1.42[b]	0.65
CAT	Nano	13.00[a]	3.80	4.51
	Saline	5.23[b]	12.57	4.24
	Non-saline	4.64[b]	10.99	1.77
POD	Nano	4.65[b]	5.44	16.78[a]
	Saline	7.40[a]	3.92	12.44[b]
	Non-saline	3.78[b]	3.74	6.39[c]

Different letters in same column indicate significantly difference at $p < 0.05$

Ascorbate peroxidase (APX), which uses ascorbic acid as a reducing agent in the first step of the glutathione ascorbate cycle, is one of the most important peroxidases involved in H_2O_2 detoxification and increased membrane stability and CO_2 fixation (Asada, 2006). Table 4 shows APX enzyme activity through three sprays of nano titanium dioxide under saline conditions. The APX enzyme activity in the saline treatment was not different from the non-saline treatment. Meanwhile, many reports show that APX activity is increased by salt treatment (Oidaira et al., 2000). The results of APX enzyme activity did not change under saline conditions in this experiment, similar to the previous studies Karim et al. (2012) and Sanoubar et al. (2020). According to Mittova et al. (2004), the increase in APX enzyme activity is associated with higher levels of H_2O_2 production in the cell. Therefore, unchanged APX enzyme activity under saline conditions may be related to the intracellular H_2O_2 concentration. Sanoubar et al. (2020) explained that the stability of the APX enzyme could be because H_2O_2 formation is not a major salt stress-induced process in radish. Therefore, the same can happen in ST25 rice. At all three times of nano TiO_2 spraying, APX enzyme activity in nano treatment was not different from that of saline treatment. APX enzyme activity was not affected by nano titanium dioxide under saline conditions.

Catalase (CAT) is found mainly in leaf peroxisomes, where it functions mainly to remove H_2O_2 formed during photomolecular reactions or during β-oxidation of fatty acids in glyoxysomes (Dat et al., 2000). The experimental results showed a constant trend of CAT enzyme at all three times of nano TiO_2 spraying, similar to the APX activity experiment in the saline treatment (Table 4). The experimental results are similar to the study on the effect of salinity on mint (*Mentha haplocalyx* Briq.) by (Zhou et al., 2021). This study reported that saline conditions had no effect on CAT activity. Or at the time of nano-spray, the rice plants had not yet activated the oxidative stress response mechanism. Therefore, at the first spray, the CAT activity in the nano treatment of 13 U/min/g FW increased about three times that of the saline and non-saline treatments, which may not be related to the plant's response to salt stress. Moreover, similar to APX enzyme, CAT enzyme activity was not affected by nano titanium dioxide under saline conditions because data in nano treatment showed no difference in enzyme activity between nano treatment and saline treatment.

Regarding POD, it plays a role in the metabolism of chloroplast-generated H_2O_2 (Polash et al., 2019). At the first and second sprays, in general, POD activity in the nano treatment was lower than that of the saline treatment and there was no difference compared with the non-saline treatment. This may be because at this stage titanium dioxide enhances other salinity response mechanisms in rice to reduce the effects of oxidative stress, so the rice does not need to increase POD enzyme activity. However, the highest POD enzyme activity (16.78 U/min/g FW) in the nano treatment at the third spray increased by 34.89% compared to the saline treatment and 162.60% compared with the non-saline treatment. From there, it was shown that nano titanium dioxide also has the ability to enhance the activity of the POD enzyme to help rice plants better tolerate saline conditions. Salt stress increases the production of ROS, and plants have used a system to control ROS levels with the participation of

antioxidant enzymes. Among them, APX, CAT, and POD are the main antioxidant enzymes in plants (Mittler, 2002). The enzyme activity test results showed that nano titanium dioxide had no effect on APX enzyme activity. However, nano TiO_2 enhances the activity of CAT and POD enzymes to reduce ROS content. The ability of nano titanium to enhance CAT and POD enzyme activities in tomato plants was investigated and reported by (Tiwari et al., 2017). According to (Tiwari et al., 2017) nano TiO_2 increased intracellular ROS production leading to an increase in CAT and POD enzyme activities in response to oxidative stress and maintaining redox function in plants. In addition, the study by Abdel et al. (2017) on beans also gave similar results, nano titanium dioxide has the ability to enhance the antioxidant system including APX, CAT, and POD enzymes of beans and helps reduce the damage of salinity. Thereby, the use of nano titanium dioxide can reduce the effects of oxidative stress caused by saline conditions and help rice plants grow better under saline conditions.

4. CONCLUSIONS

Nano titanium dioxide increased chlorophyll and carotenoid content and increased proline accumulation. Regarding antioxidant activities, titanium dioxide nanoparticles have no effect on APX activity, but increases CAT and POD enzyme activities. Thereby, the application of nano titanium dioxide has positive effects to help rice plants better tolerate saline conditions.

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