REACTIVE OXYGEN SPECIES (ROS), TOTAL ANTIOXIDANT CAPACITY (AOC) AND MALONDIALDEHYDE (MDA) MAKE A TRIANGLE IN EVALUATION OF ZINC STRESS EXTENSION

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ABSTRACT

Reactive oxygen species (ROS) are generated in response to the various environmental stresses, and overproduction of ROS can lead to the lipid peroxidation, which often is monitored by measuring the malondialdehyde (MDA). Nevertheless, the extent of oxidative stress is partly dependent on the total antioxidant capacity (AOC) of plant cells. In many experiments, the assessment of oxidative stress is based on the measurement of ROS only or more commonly individual antioxidants, but a more reliable assessment of oxidative stress requires the simultaneous measurement of ROS, AOC and MDA levels. In this study, the effects of different ZnO concentrations were studied on the oxidant and antioxidant potentials, and also on MDA levels in tobacco plants. Moreover, the significant correlations were considered between the indices, which were evaluated via different methods. Results showed that both Zn deficiency and excess levels led to the increased ROS/H_2O_2 production, Chl a/b reduction, electrolytes leakage and lipid peroxidation, compared to the control. The inverse conditions were observed for the adequate ZnO amount (1μM) with the highest AOC level. ROS was significantly correlated with H_2O_2 (P<0.01, R= 0.8) and MDA (P<0.01, R= 0.6-0.8) but not with AOC. Only MDA-PhB method caused a significant correlation (P<0.01, R= -0.7) with the results from AOC methods. Unlike Chl$a$/Chl$b$, the electrolyte leakage was often significantly correlated with other stress indices. In general, a dependency to the used method was observed for finding the significant correlations among oxidative stress markers, and their simultaneous measurements were also suggested for the better interpretation of data.

Key words: Antioxidant Capacity, Malondialdehyde, Reactive Oxygen Species, Zinc Stress.

Abbreviations: AOC, Antioxidant capacity; Chl, Chlorophyll; DPPH, α, α-diphenyl-β-picrylhydrazyl; EC - Electrical conductivity; LiA, Linoleic acid; MDA, Malondialdehyde; ROS, Reactive oxygen species; PhB- Phosphate buffer; TBA, Thiobarbituric Acid; TCA, Trichloroacetic Acid; TiCl- Titanium chloride; Zn, Zinc; ZnO, Zinc oxide; XO, Xylenol orange.

INTRODUCTION

Zinc is an essential micronutrient and activates enzymes that are involved in the synthesis of certain proteins (Dal Corso *et al*., 2014; Dhanalakshmi *et al*., 2018). This element is involved in the formation of chlorophyll, zinc-chlorophyll complexes (Ngo and Zhao, 2007; Samreen *et al*., 2017) and some carbohydrates, in addition to the conversion of starches to sugars (Suzuki *et al*., 2012). Moreover, zinc is necessary in the metabolism of auxin, which helps with growth regulation and stem elongation (Weisany *et al.,* 2012). Zn deficiency affects plant growth and quality (Sagardoy *et al.,* 2009; Sharma *et al.,* 2009; Henriques *et al.,* 2012; Aslam *et al.,* 2014). Zinc is immobile, and deficiency results in new leaves that are smaller in size and often distorted with varying pattern of chlorosis (often interveinal). The normal concentration range of Zn in plant tissues is reported to be about 25-100 ppm (Campbell 2000; Obreza *et al.,* 2010). Zinc concentration in soils typically ranges between 10-300 ppm (Alloway 2004), and Zn is included

in *in vitro* growth media at concentrations of between 1.5×10^4-0.9 ppm (Saad and Elshahed, 2012).

The influences of different Zn forms, such as sulphate, chloride and nitrate have been widely investigated on plants (Cherif *et al.,* 2011; Daneshbakhsh *et al.,* 2013; Hafeez *et al*., 2013; Fontes *et al.,* 2014; Marichali *et al.,* 2014; Asad *et al.,* 2015). Nevertheless, the effects of Zn in the form of zinc oxide (ZnO) have been less studied on plants. This form has very important and unique properties, including high chemical stability, low electrical constant, high electrochemical coupling index, wide range of radiation absorption, and high photostability (Sánchez-Quiles and Tovar-Sánchez, 2014). Because of the mentioned properties, zinc oxide has many substantial applications in electronics, optoelectronics, laser and rubber technology, pharmaceutical and cosmetic industries (Broadley *et al.,* 2007; Kołodziejczak- Radzimska and Jesionowski, 2014). Because of its worldwide applications, ZnO can be accumulated in toxic amounts and lead to toxicity.

ZnO is a strong oxidizing agent and produces significant amounts of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), which consequently leads to hydroxyl radical production (Yu *et al.*, 2011). H_2O_2 can act as a multifunctional trigger, modulating metabolism and gene expression. Thus, it plays an important role in cell signaling, particularly in response to stress. However if present at high levels it can damage cells. H_2O_2 is more stable than other ROS and can easily traverse membranes. Hydroxyl radicals produced by high levels of H_2O_2, attack polyunsaturated fatty acids (PUFA) in membranes forming a reactive carbon radical that react with oxygen forming peroxyl radicals. This product causes self-amplifying generation of lipid peroxides leading to widespread damage and finally membrane disintegrations. Lipid peroxides decompose to form a range of aldehydes including MDA which is often used as an analytical marker of lipid peroxidation (Bhattachrjee, 2005; Packer and Cadenas, 2013).

Plants have an efficient antioxidant system for scavenging excess ROS (Boguszewska and Zagdańska, 2012; Yadav *et al.*, 2014) and preventing oxidative damage. Measurements of antioxidant levels are often used as an indirect measure of oxidative stress.

In general, for full evaluation of ZnO toxicity, three main components should be considered, including increments in ROS production, the consequent antioxidant responses, and the oxidative stress damage to the cellular components. Since, each component is related to the others, therefore, a single measurement of only one component, may not give a complete and realistic picture of the extent of stress. For example, ROS levels are highly dependent on the antioxidant capacity which is itself highly affected by the changes in ROS concentrations. Based on many researches it can be stated that, there is a relationship between the severity of stress and the stress response. Kranner *et al.*, (2010) believed that, together with increasing in stress intensity, the concentrations of antioxidants often display bell-shaped patterns, with raised concentrations in the alarm and resistance phases and a reduction in the exhaustion phase. Therefore, sampling in the alarm and the exhaustion phases may result in the same values for antioxidant concentrations, which cause an incorrect interpretation of the stress response. For this reason, measurement of damage levels can help to make a correct conclusion. Moreover, a certain relationship between the changes in components with the stress duration should not be ignored.

Given that the above mentioned, simultaneous measurement of ROS, AOC and probable oxidative damage, make a more reliable way to study the effect of stress. For the mentioned evaluations in this field, there are some simple and different methods which are being used by researchers; however the sensitivity of these methods could be different.

In the present research, H_2O_2 level, MDA content and total antioxidant activity were measured by using two methods, and also ROS levels and electrolyte leakage to understand more accurate oxidative effects of zinc oxide on tobacco and the compatibility of changes among the three above factors. We also studied the H_2O_2 standard curve for H_2O_2 and ROS measurement and showed that each method should be used in an especial range of substance concentration. Most ecological research has focused on the toxicity of ZnO nanoparticles on plants, but bulk ZnO toxicity could also be very important, because it has not been completely demonstrated that particle size per se has a decisive role in toxicity (Landa *et al.*, 2015).

In the present study, we showed the importance of simultaneous measurement of ROS/AOC/MDA under stress condition of ZnO application, and that the evaluation of the above indices by more than one method may be helpful for more accurate and a better interpretation of data. Furthermore, the influences of different concentrations of ZnO on tobacco seedlings were compared and discussed.

MATERIALS AND METHODS

Tobacco (*Nicotiana tobacco* L.) seeds were obtained from Isfahan Tobacco Company (ICT). Seeds were stored dry at 4°C in darkness prior to use. Seeds were sown in plastic pots filled with perlite and propagated in a controlled greenhouse with 14/10 hours day/night cycle and a range of 25–32°C temperature during the day and 20–23°C during the night. Photosynthetic active radiation was ~100 μmol m^{-2} s^{-1} measured at the top of the canopy.

After seed germination, the seedlings were irrigated with tap water every three days and 1/10 strength modified Johnson's nutrient solution (Siddiqi *et al.*, 1989) once a week. The pH of the nutrient solution was adjusted to 5.8.

After two months (at 4-leaf stage) seedlings were transferred to a hydroponic system with three plants in a 1.5 L pot per replicate. After 2 days, the nutrient solution was replaced with a modified Johnson nutrient solution containing different concentrations of ZnO (0.2, 1, 5 and 25 μM) instead of $ZnSO_4$. The nutrient solution without $ZnSO_4$ was made and different concentration of ZnO was added (pH of nutrient solution was adjusted 5.8±0.2). Johnson nutrient solution with $ZnSO_4$ was as control group. The solutions were aerated continuously and nutrient solution was changed once a week. Three replications were used for each treatment. After 21 days, the sixth leaves of plants were harvested, weighed and frozen in liquid nitrogen, and stored at –20ºC for subsequent biochemical analysis.

Total ROS and hydrogen peroxide levels: ROS content was monitored by using the xylenol orange method (ROS-XO), in which, ROS is reduced by ferrous ions in an acidic solution to form a ferric product the xylenol orange complexes (Bindschedler et al., 2001). Because of their instability, solutions were freshly prepared, 25 mM $FeSO_4$ and 25 mM $(NH_4)_2SO_4$ were dissolved in 2.5 M H_2SO_4. One ml of solution was added to 100 ml of 125 µM xylenol orange and 100 mM sorbitol. The reagent remained stable for 6-8 hours. 0.1 g of a leaf tissue was homogenized in 4 ml cold 50 mM sodium phosphate buffer, pH 6.8. The homogenate was centrifuged at 16,000 × g for 20 min at 4ºC and 100 µl of the supernatant was added to 900 µl of xylenol orange (sigma) reagent. The reaction mixture was incubated 30-60 min at room temperature before measuring the absorbance at 560 nm. Blank was contained 100 µl water with 900 µl of xylenol orange reagent. H_2O_2 (30%) was used as a standard and the serial dilutions were prepared by the reagent.

At the first quantification of H_2O_2 level in elicited tobacco leaves, it was specifically monitored using 10% titanium chloride in HCl assay (Chang and Kao 1998), and introduced as H_2O_2-TiCl method. Hydrogen peroxide was extracted by homogenizing 0.1 g leaf tissue with 4 mL of ice-cold 50 mM phosphate buffer, pH 6.5. The homogenate was centrifuged (Hettich universal 320 R, UK) at 16,000 g for 20 min at 4°C. To determine H_2O_2 levels, 2 mL of extracted solution was mixed with 0.2 ml titanium chloride 10% in HCl, and then centrifuged at 16,000 ×g for 20 min. The intensity of the yellow color of the supernatant was measured at 410 nm.

For the second measurement method of H_2O_2, 1 M KI was used (Zlatev et al., 2006) and was named (H_2O_2-KI). In this measurement, leaf tissue (0.1 g) was homogenized in ice bath with 4 ml of a cold 0.1 % (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged (12000 g, 15 min, 4ºC) and 0.5 ml of the supernatant was added to 0.5 ml of 100 mM potassium phosphate buffer (pH 6.8) and 1 ml of 1 M KI. The absorbance was read at 390 nm by spectrophotometer UV-1601 (Rayleigh, China).

Optimum H_2O_2 concentrations for the above three methods was identified and H_2O_2 content was expressed as µmol of H_2O_2 per gram of fresh weight of plant tissue.

Lipid peroxidation: The level of lipid peroxidation in tobacco leaves was determined by estimating the MDA content, which is known to be a breakdown product of lipid peroxidation, via two methods. In the first malondialdehyde evaluation method, (MDA-TCA), 0.1 g of a leaf tissue was homogenized in 2 ml 0.1% TCA. The homogenate was centrifuged at 10,000 × g for 20 min. To 500 µl of the supernatant, 2 ml of 20% TCA containing

0.5% TBA were added. The mixture was heated at 95ºC for 15 min and cooled immediately on ice. The absorbance was measured at 532 nm. The value for the nonspecific absorption at 600 nm was subtracted (Heath and Packer, 1968). The level of lipid peroxidation was expressed as micro molar of MDA formed using an extinction coefficient of 155 mM^{-1} cm^{-1}.

In the second method (MDA-PhB), 0.1 g of a tissue sample was homogenized in two ml ice-cold 50 mM phosphate buffer, pH 7.8. The homogenate was centrifuged at 16,000 × g for 20 min at 4°C. Two milliliter of 10% TCA containing 0.5% TBA was added to a 1 ml aliquot of the supernatant. The mixture was heated at 95ºC for 15 min and cooled immediately in an ice bath. The contents were centrifuged at 4000 × g for 15 min, and the absorbance was measured at 532 nm. The value for the nonspecific absorbance at 600 nm was subtracted. The level of lipid peroxidation was expressed as micro molar of MDA formed using an extinction coefficient of 0.155 µmol L^{-1} cm^{-1} (Wang et al., 2013).

Total antioxidant activity; DPPH assay: The total antioxidant capacity (AOC) or activity based on DPPH assay was evaluated according to Hussain et al., (2010) as AOC-DPPH. The principle of this assay was based on the changing in color of DPPH (α, α-diphenyl-β-picrylhydrazyl) solution from purple to yellow as the scavenging capacity of antioxidants. Briefly, 100 μL of methanolic tobacco leaves extract was added to 900 µL of 25 µM of DPPH in ethanol and allowed the mixture to stand in the dark at room temperature for 30 min. The absorbance of DPPH was evaluated by measuring the decrease of absorbance at 517 nm. Ethanol was used for blank. The inhibition percentage (I%) was computed by using a following formula:

I (%) = [(A control - A sample) / A control] × 100

Where, A control is the absorbance of the control (containing DPPH, plus methanol), and A sample is the absorbance of the test compound in sample (DPPH, methanol plus sample).

Total antioxidant activity; lipid peroxidation inhibition: The total antioxidant capacity was determined with using linoleic acid, according to the Yiğit et al., (2008) as AOC-LiA. Briefly, 150 µL of phosphate-buffered tobacco leaves extract were mixed with 400 µl of 0.02 M linoleic acid emulsion, contains an equal weight of Tween-20 in pH 7.4 adjusted to 5 ml with saline (0.02 M, pH 7.4) in a test tube and incubated in darkness at 40°C. The amount of peroxide was determined by measuring the absorbance at 500 nm after coloring with $FeCl_2$ and thiocyanate after 24 h incubation. Lower absorbance indicates higher antioxidant activity. To eliminate the solvent effect, the same amount of solvent used to prepare the solutions of test samples was added into the control test sample, which contains the linoleic acid emulsion. Measurements

of antioxidant activity were carried out for three sample replications, and values are the average of three replicates. This activity is given as percent lipid peroxidation inhibition which is calculated with the equation:

Lipid peroxidation inhibition (%) = [(A $_{control}$ - A $_{sample}$) / A $_{control}$] × 100

Where, A $_{control}$ is the absorbance of the control (containing linoleic acid, plus phosphate-buffer), and A sample is the absorbance of the test compound in sample (linoleic acid, phosphate-buffer plus sample).

Electrolyte leakage: Electrolyte leakage was measured by extravasations' conductivity (Elmetron, Poland) according to Bajji *et al.*, (2002). Twenty leaf discs (0.5 cm^2) per sample were cut and an initial electrical conductivity (EC$_i$) was taken at the beginning of assay. Then, the tubes containing the segments were kept at dark at room temperature (25°C). After 2 h of rehydration (EC$_f$) and ultimately boiling, the total electrical conductivity (EC$_t$) was evaluated. Electrolyte leakage was expressed as, (EC$_f$-EC$_i$)/(EC$_t$-EC$_i$) × 100.

Chl *a/b* content: Chlorophyll content was determined using the methods of Lichtenthaler and Buschmann, (2001). In this method, chlorophylls was extracted in pure methanol in 40°C the absorbance of extracts was measured at 665.2, 652.4, and 470 nm with spectrophotometer UV-1601 (Rayleigh, China).

Statistical analysis: The experiment was arranged as Completely Randomized Design (CRD) with three replications (n=3). The data were statistically analyzed by one-way analysis of variance (ANOVA) using SPSS (version 18) software and tukey test were used to test the significant differences between treatments. The correlations between parameters were evaluated using Pearson's correlation coefficients.

RESULTS

Total ROS and hydrogen peroxide levels: Our preliminary spectrophotometric screening, showed the different optimum ranges of H$_2$O$_2$ concentrations, for plotting the calibration curves in the different used methods for ROS/H$_2$O$_2$ evaluations. The appropriate concentrations of H$_2$O$_2$ for plotting the calibration curves were about 2-50 µmol, less than 5 mM and 2 mM, respectively for the methods ROS-XO (Fig. 1a), H$_2$O$_2$-TiCl (Fig. 1b) and H$_2$O$_2$-KI (data not shown). In the recent assay (H$_2$O$_2$-KI method), the higher concentrations than 2 mM could not be detected by the spectrophotometer, hence a saturation point for the OD to dose curve of H$_2$O$_2$ could not be observed.

According to Fig. 2a, 21 days after the exposure of plants to different ZnO levels, the highest ROS levels was observed at 25 µM, but the least amount was in 1

µM ZnO, which was the same as control. A nearly similar pattern with ROS content was observed for H$_2$O$_2$ content, when hydrogen peroxide was measured by both H$_2$O$_2$-TiCl and H$_2$O$_2$-KI methods (Fig. 2b and c). Although in contrast to the ROS pattern, H$_2$O$_2$ levels at 1 µM ZnO by both methods were lower than their relative controls (Fig. 2b and c). Controls treated with 0 µM ZnO and were included only 0.2 µM ZnSO$_4$. H$_2$O$_2$-TiCl method appeared more sensitive to the lower concentrations of H$_2$O$_2$, than those in H$_2$O$_2$-KI method.

Lipid peroxidation content: Figure 3-a and b, shows the changes in MDA content of tobacco leaves, which was measured by two methods (MDA-TCA and MDA-PhB). The results showed that compared with the control, MDA content was significantly (p<0.05) decreased in both methods at 1 µM ZnO, while it showed differences in other concentrations when was evaluated by both methods. The MDA content in both evaluations showed an incremental trend from 1 µM to 25 µM, while the results of the two methods were not the same at 0.2 µM (Fig. 3a and b).

Antioxidant activity: Two widely methods used in determination of the total antioxidant potential; total antioxidant activity measured via DPPH radical scavenging assay (AOC-DPPH) (Fig. 4a) and inhibition of lipid peroxidation evaluated by using linoleic acid methods (AOC-LiA), (Fig. 4b) were used in this study. The results showed significant inductions on the DPPH scavenging activity caused by 1 and 0.2 µM ZnO treatments and represented 83% and 21% induction, respectively in comparison with control plant (Fig. 4a). Five and 25 µM ZnO-treated plants had antioxidant activity similar to the control (Fig. 4a). A nearly similar pattern was observed for total antioxidant activity measured by AOC-LiA method (Fig. 4b), except the AOC contents were much lower in plants treated by 5 µM and 25 µM ZnO, than those evaluated by AOC-DPPH method. This suggests a great difference in the antioxidant capacity of treated plants at high concentrations of ZnO (upper than 1 µM), when those evaluated by both methods.

Chl *a/b* ratio: According to the results, 1, 5, and 25 µM ZnO treated tobaccos had a significantly lower Chl *a/b* ratio than control plant, while no significant difference (p<0.05) was observed between 0.2 µM ZnO treated plant and control (Fig. 5a).

Electrolyte leakage: Treatment of plants with 5 and 25 µM ZnO caused significant increases (p<0.05) in the percentage of EC in comparison to the control. But 1 µM ZnO treatment caused the minimum percentage of electrolyte leakage among the treated plants (Fig. 5b). Overall, high ZnO concentrations showed higher EC compared to the lower concentrations and control. Fig. 6, show a morphological comparison among tobacco plants

treated by different ZnO concentrations. Plant treated by 1 µM ZnO, was larger in size of leaves and length of roots, but 0.2 µM treated plant showed the smallest size of leaves and seedling, indicating limited growth.

Table 1 contains evaluation of correlations between the parameters. ROS level showed significant and positive correlation with H_2O_2 content ($p < 0.01$, $R = 0.8$) and also MDA level ($p < 0.01$, $R = 0.6$ and 0.8) which were evaluated by both methods. Antioxidant capacities (AOC-DPPH and AOC-LiA) were negatively correlate with H_2O_2 and MDA-PhB. AOC did not show a significant ($p < 0.05$) relation with ROS content, while EC had significant and positive correlations with ROS, H_2O_2 and MDA-PhB, but a negative correlation with both evaluated AOC ($p < 0.01$, $R = 0.7$ and 0.8). Chl a/b showed the significant correlation only with MDA-TCA ($p < 0.05$, $R = 0.56$).

Table 1. Correlations matrices showing relationships between indices of oxidative stress evaluated by different methods, in *Nicotiana tobacco* L, treated by 0.2, 1, 5 and 25 µM of ZnO.

	ROS-XO l	H_2O_2 -TiCl	H_2O_2 -KI	MDA-TCA	MDA-PhB	AOC-DPPH	AOC-LiA	Chl a/b	Electrolyte leakage
ROS-XO	1								
H_2O_2 -TiCl	0.830**	1							
H_2O_2 -KI	0.822**	0.896**	1						
MDA-TCA	0.668**	0.722**	0.587*	1					
MDA-PhB	0.835**	0.766**	0.807**	0.440	1				
AOC-DPPH	-0.456	-0.704**	-0.656**	-0.447	-0.739**	1			
AOC-LiA	-0.417	-0.566*	-0.572*	-0.192	-0.795**	0.918**	1		
Chl a/b	0.020	0.419	0.304	0.530*	-0.014	-0.471	-0.153	1	
Electrolyte leakage	0.702**	0.636*	0.692**	0.252	0.926**	-0.731**	-0.861**	-0.162	1

*. Correlation is significant at the 0.05 level and **. Correlation is significant at the 0.01 level.
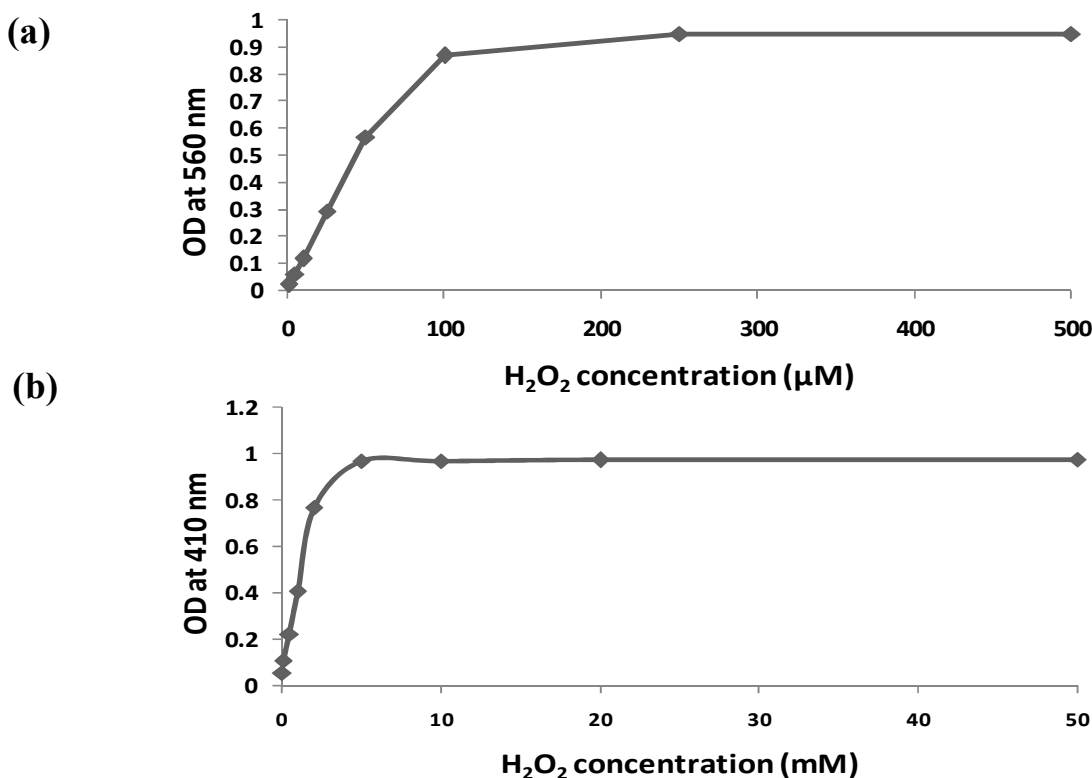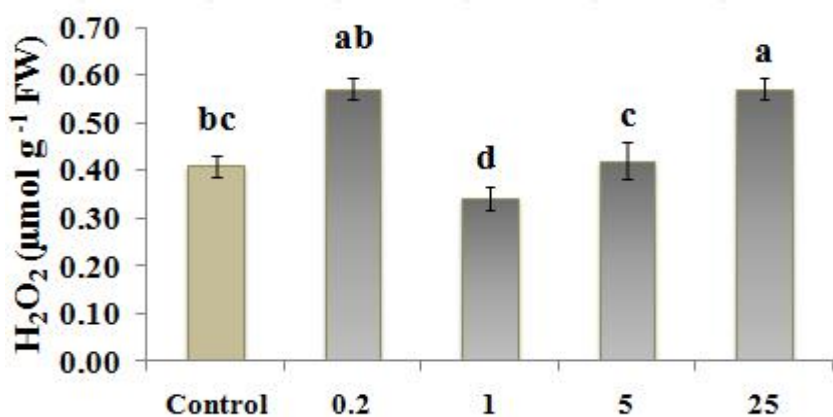

Fig. 1   Preliminary spectrophotometric screening; OD to dose curve for H_2O_2 obtained by ROS-XO method (a) and OD to dose curve for H_2O_2 obtained by H_2O_2-TiCl method (b). Values are the means of three replicates ±StD. Standard deviation bars are horizontal or covered into the points.
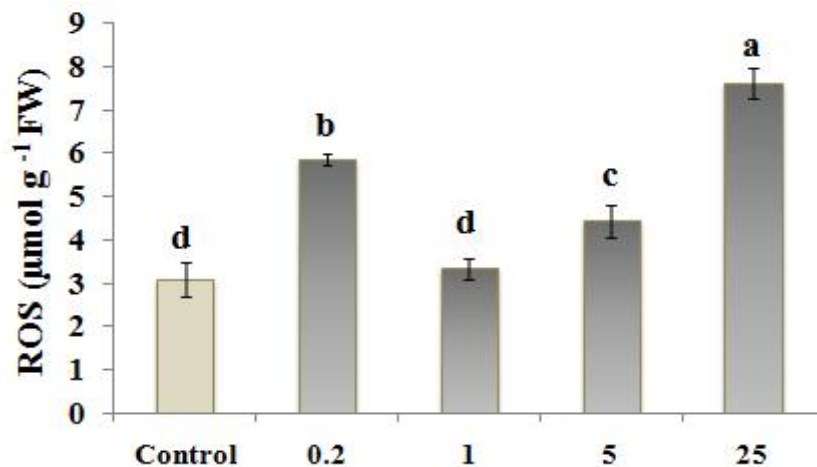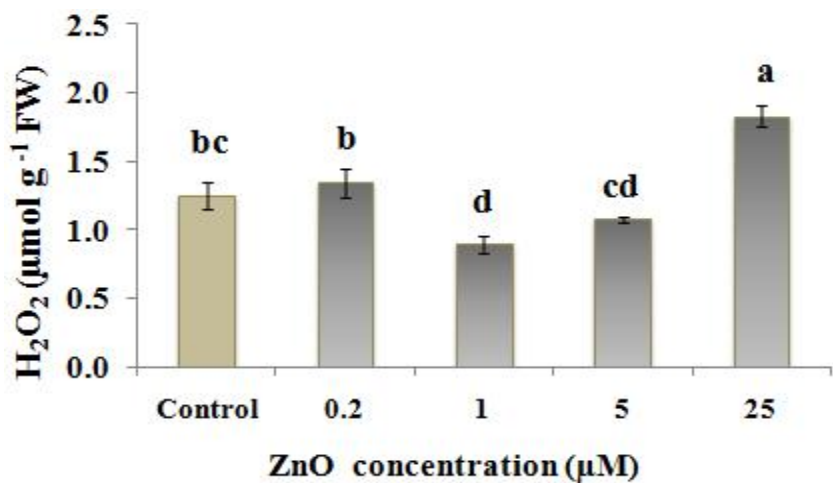
Fig. 2 ROS and H_2O_2 levels in *Nicotiana tobacco* L. treated with different concentration of ZnO (0, 0.2, 1, 5, 25 µM); (a) ROS levels evaluated by ROS-XO method; (b) H_2O_2 level evaluated by H_2O_2-TiCl method and (c) H_2O_2 level evaluated by H_2O_2-KI method. Values are the means of three replications ±StD. Bars indicate significant difference at $p < 0.05$ according to Tukey test.
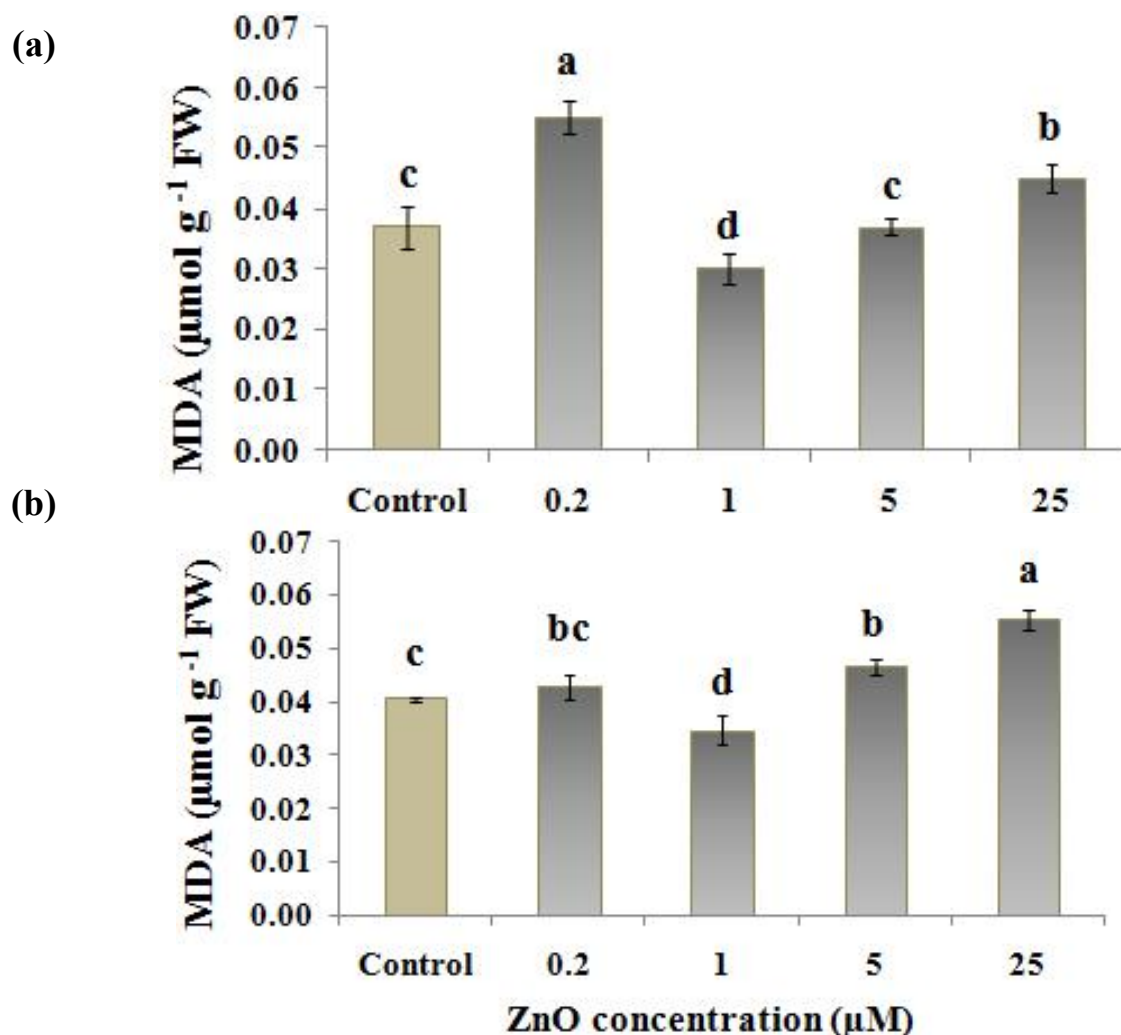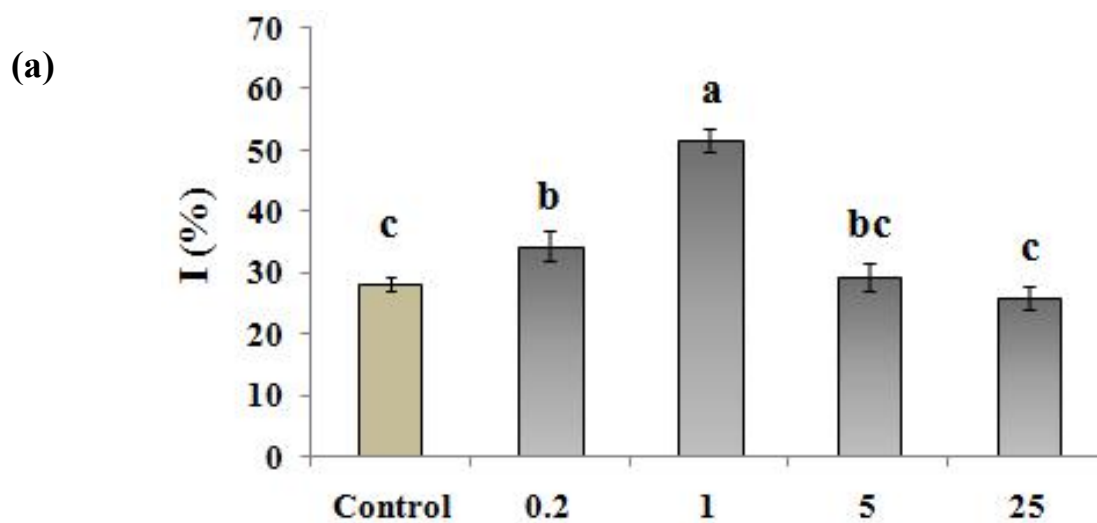
(a)

(b)

Fig. 3 MDA levels in *Nicotiana tobacco* L. treated with different concentration of ZnO (0, 0.2, 1, 5, 25 µM); (a) MDA content evaluated by MDA-TCA method and (b) MDA content evaluated by MDA-PhB method. Values are the means of three replications ±StD. Bars indicate significant difference at p<0.05 according to Tukey test.
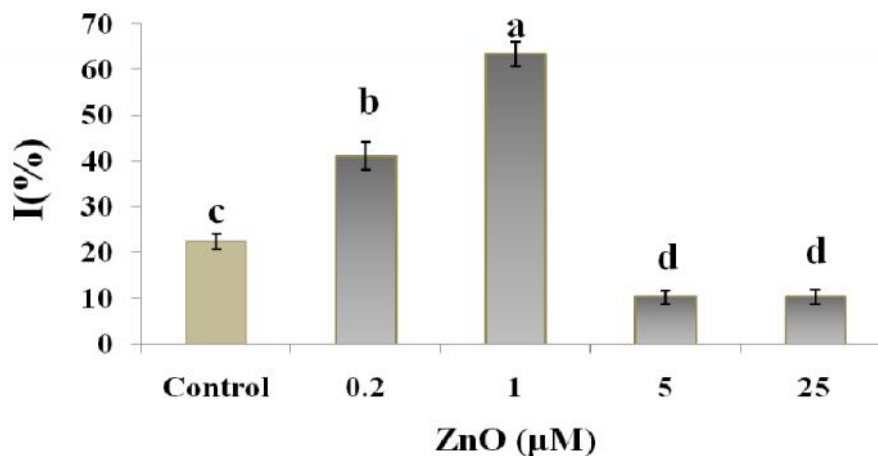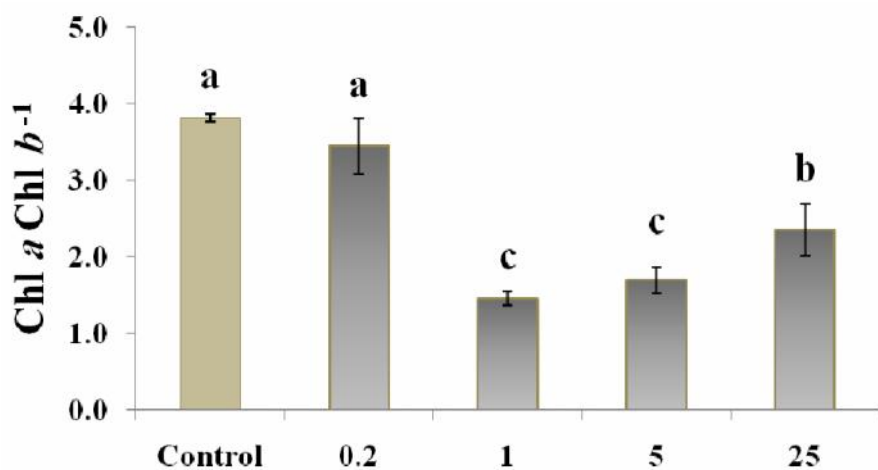
(a)

(b)

Fig. 4 AOC levels in *Nicotiana tobacco* L treated with different concentration of ZnO (0, 0.2, 1, 5, 25 µM); (a) total antioxidant capacity evaluated by AOC-DPPH method and (b) the inhibition of lipid peroxidation /linoleic acid evaluated by AOC-LiA method. Values are the means of three replications ±StD. Bars indicate significant difference at p<0.05 according to Tukey test.
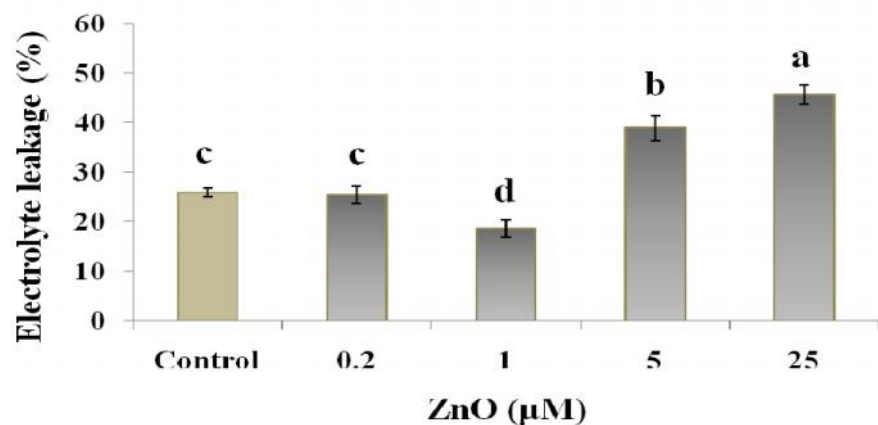
(a)

(b)

Fig. 5 Chl *a/b* and Electrolyte leakage percentage in *Nicotiana tobacco* L. treated with different concentration of ZnO (0, 0.2, 1, 5, 25 µM); (a) Chlorophyll *a/b* ratio and (b) Percentage of electrolyte leakage. Values are the means of three replications ±StD. Bars indicate significant difference at p<0.05 according to Tukey test.
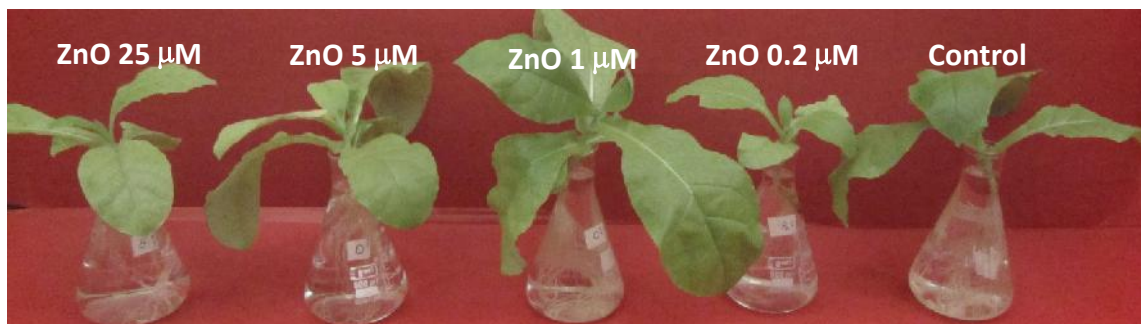
Fig. 6 *Nicotiana tobacco* **plants treated with different concentrations of ZnO (0, 0.2, 1, 5, 25 µM), on 21th days in hydroponic culture medium.**

DISCUSSION

ROS content, antioxidant capacity and extent of damage can be imagined as three corners of a triangle to show the important interrelations and intensity of stress in a plant cell. It is well known that metal stress as well as other environmental stresses leads to the overproduction of ROS/H_2O_2 in plants, which are highly toxic and reactive. Although antioxidant activities will be induced as a result, but depends on the intensity of oxidative stress, some damages may occur in the living cells (Tewari *et al.,* 2008; Gill and Tuteja, 2010; Michael and Krishnaswamy, 2014; Waszczak *et al.*, 2018).

According to the results, among the ZnO treated plants, 1 µM ZnO caused the lowest ROS level (measured by Xylenol orange, Fig. 2a) and also H_2O_2 content (measured by two titanium chloride and KI methods, Figs. 2, b) in cells. But the lowest and highest ZnO levels, 0.2 µM and 25 µM ZnO respectively, often caused a higher ROS/H_2O_2 content in tobacco plants. Tewari *et al.,* (2008) suggested that the deficiency or excess in Zn content, exacerbate the oxidative stress through the increased generation of ROS, and cause an interrupted redox homeostasis in the mulberry plants. An increase in ROS level, because of the deficiency or excess of Zinc content, has also been reported by other researchers (Reichman and Minerals 2002; Michael and Krishnaswamy, 2014).

In contrast to the Zn deficiency or excess levels, adequate levels (which seem to be about 1 µM in this study) are completely necessary for cell metabolism. Weisany *et al.*, (2012) reported that adequate zinc prevents the production of free oxygen radicals, and helps to limit lipid peroxidation rate, which lead to stabilizing the bio-membranes against the activated ROS.

The Zn deficient tobaccos (0.2 µM ZnO) had similar content of H_2O_2 to that in control (Fig. 2b and c) with a higher content of ROS compared to control (Fig. 2a). This suggests that other reactive oxygen species, such as singlet oxygen ($^1O_2$), nitric oxide (NO), superoxide anion radical (O_2^{-o}), hydroxyl radical (HO^o) and perhydroxyl radical (HO_2^o) might be produced and

involved (Rodgman and Perfetti, 2009). Control at these experiments was included only 0.2 µM $ZnSO_4$. Since the solubility of Zn in the form of sulfate is much more than that in ZnO form (Ollig *et al.*, 2016), it thus seems that 0.2 µM $ZnSO_4$ did not cause a deficiency similar to 0.2 µM ZnO. The differences in H_2O_2 content evaluated by different methods (Figures 1 and 2), can be related to the difference in absorbance of H_2O_2 at different wavelengths, in addition to the preparation methods (Junglee *et al.*, 2014; Zhang *et al.*, 2013).

Evaluation of MDA as a stress marker is one of the most widely accepted assays under the oxidative stress conditions (Grotto *et al.*, 2009). According to the results, MDA content was the highest at 0.2 µM treated tobacco, only when MDA-TCA method was used. (Fig. 3a). But evaluation of electrolyte leakage of leaf disks from 0.2 µM treated tobacco (Fig. 5) did not show any significant difference between 0.2 µM treated plant and control, showing membrane integrity in this treatment. This may suggest a more consistency of EC data with the results from MDA-PhB assay, than MDA-TCA method (Table 1). Therefore, other supplementary tests such as EC assay may help with the better interpretation of data.

DPPH assay and the inhibition of lipid peroxidation of linoleic acid are two determinant assays for evaluating the antioxidant activity of plant extracts (Yiğit *et al.,* 2008; Kedare and Singh, 2011). Total antioxidant activities measured by both methods (Fig. 4) showed the highest activities in the treated plants by 1 µM, which was consistent with the lowest ROS/H_2O_2 and also MDA content in this treatment. An induction in AOC at 0.2 µM (Fig.4a and b) seems to be a response to the high ROS levels in cell, which consequently could prevent the membrane damage and electrolyte leakage. However, the smallest size of seedling at this treatment (0.2 µM) compared to the other treatments refers to an inappropriate condition resulting from inadequate Zn amount for growth (Fig. 6). Zn deficiency causes the rapid inhibition of plant growth and development, which results in ROS production (Zhao and Wu, 2017).

In contrast, the low antioxidant activities in high ZnO (5 µM and 25 µM) treated plants compared with 0.2

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µM or 1 µM treated ones, caused a high MDA content (specially by MDA-PhB) (Fig. 3, b) which was confirmed by the increased EC as well (Fig. 5, b). A significant and negative correlation ($p < 0.01$, R= -0.7) has been found between the data from AOC and MDA-PhB methods (Table. 1).

When sets of data were considered (instead of a single treatment), and used for measuring the correlations (Table 1), AOC was not significantly correlated with ROS and MDA from MDA-TCA method. Concerning the relationship between antioxidants and other stress indicators some important points, such as changes in their concentrations with the intensity of stress (Kranner *et al.*, 2010), the distinct influences of different forms of metal (Moldovan *et al.,* 2010), the duration of stress (Chutipaijit, 2011), age of plant (Srivastava and Bhagyawant 2014) and kind of measurement method could be determinant.

In contrast to the electrolyte leakage that showed a significant correlation ($p < 0.01$, 0.05) with the most of the indices, Chl *a/b* was not often correlated (Table 1). There are some reports that Chl *a/b* could be an earlier response to the environmental stress than plant growth (Sun *et al.,* 2013). Therefore, it might be better to measure this factor at the initial stages of stress.

In general, our results showed that, for more reliable and accurate interpretation of data all three indices (ROS/AOC/MDA) should be evaluated, however some supplementary tests such as EC assay may also be helpful. Moreover, the correlation measurements among the indices revealed a dependency to the kind of method. This suggests that, sometimes evaluation of indices by more than one method may help with the better explanation and interpretation of data.

Conflict of interest: Authors have no conflict of interest.

Contribution of authors: MMH suggested the idea. MMT designed and performed the experiments. MMT made a draft. MMH rewrote the manuscript and revised it.

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