

The effect of Zinc oxide nano particles and Humic acid on morphological characters and secondary metabolite production in *Lilium ledebourii* Boiss

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Abstract

The effects of different concentrations of Zinc oxide nano particles (ZnONP; 0, 10, 25, 50, 75 and 100 mg L⁻¹) and Humic acid (HA; 0, 50, 100, 500 and 1000 mg L⁻¹) on root length and number, leaf length and number, bulb diameter, and chlorophyll, total phenol, anthocyanin and flavonoid contents were examined under *in vitro* conditions. Total phenol, flavonoid and anthocyanin contents were significantly ($P \leq 0.01$) affected. The maximum phenolic content of plantlets was obtained in the medium containing 75 mg L⁻¹ ZnONP. The highest anthocyanin content was observed in plantlets treated with HA at 100 mg L⁻¹. The highest flavonoid content measured at 270, 300 and 330 nm wavelengths were obtained with ZnONP at 25 mg L⁻¹, ZnONP at 25 mg L⁻¹ and HA at 100 mg L⁻¹, respectively. Explants treated with HA produced the highest root length, leaf length and number, bulb number and chlorophyll content in the media containing 500, 50, 50, 50 and 500 mg L⁻¹ HA, respectively. However, the media containing 50, 50, 50, 75 and 50 mg L⁻¹ ZnONP produced the highest root length, leaf length and number, bulb number and chlorophyll, respectively.

Key words: Humic acid, *Lilium ledebourii* Boiss,

Secondary metabolites, Zinc oxide nano particles.

INTRODUCTION

Secondary metabolites in plants are diverse biochemical substances with numerous biological functions. Major roles of plant secondary metabolites include plant protection from biotic and abiotic stresses. Plants can be unique sources of secondary metabolites for pharmaceuticals, food additives, flavors and other industrial substances. Accumulation of these secondary metabolites often occurs in plants subjected to stresses mediated by various chemical elicitors and signal molecules. Plant cell and tissue culture has been used as a means of producing secondary metabolites whose chemical synthesis is difficult (Zhao *et al.*, 2005). Moreover, the evolving commercial importance of secondary metabolites has resulted in an interest in the possibility of enhancing the production of bioactive plant metabolites by tissue culture technologies (Vanisree *et al.*, 2004).

Lilium ledebourii Boiss. (Family *Liliaceae*) is called Susan-e-Chelcheragh in Persian. It is the only endangered *Lilium* species in Iran. Wild populations are currently at risk of eradication because of irregular grazing and poaching (Azadi and Khosh-Khui, 2007).

L. ledebourii grows on the eastern slopes of the Alburz Mountains, where it is now protected in a small area of degraded beech forest around Damash village (Bakhshaie *et al.*, 2010; Azadi and Khosh-Khui, 2007). It is a perennial plant with lanceolate yellowish scales, a thick stem and erect leaves. Its flowers are white and large (Farsam *et al.*, 2003).

Little or no investigation has been carried out on the phytochemistry of this species. However, there are reports on the phytochemistry of other *Liliums*. The fresh and dried bulbs of *L. candidum* have been suggested useful in the treatment of gynecological disorders, ulcer, burns, injuries (Farsam *et al.*, 2003). Primary qualitative phytochemical tests showed steroidal saponins, phenolic glycosides, flavonoid alkaloids, and pyrroline-pyrrolidine alkaloids (Farsam *et al.*, 2003).

Humic acid (HA) has been recognized to have morphological and physiological effects on higher plants. However, relationships between HA structure and biological activity are not clear (Mora *et al.*, 2010). Positive influences of HA on plant growth and development have been reported. Yildirim (2007) reported that HA applied in the soil and sprayed on tomato plants, had increased product quality and quantity. In this context potential effects on antioxidant activity have received little attention (Aminifard *et al.*, 2012). Studies on lettuce and tomato revealed nutrient sources did not affect the total phenolic content. In contrast, flavonoids in tomatoes and antioxidants in strawberries were increased in organic crop management practices. As reported in one study, organic strawberries had higher levels of total phenolics, Ellagic acid, and flavonols compared to conventionally cultured strawberries (Aminifard *et al.*, 2012).

Flavonoids encompass a large sub-group of phenolic plant secondary metabolites that have *in vitro* antioxidant free radical scavenging activity (Mitchell *et al.*, 2007). They are produced through the phenyl propanoid pathway. The key enzyme that catalyzes their biosynthesis is phenylalanine ammonia-lyase. Stresses including nutrient deficiency, wounding, pathogens, and UV radiation are known to activate the biosynthesis of phenyl propanoid compounds (Mitchell *et al.*, 2007).

Environmental factors cause changes in the growth of medicinal plants, including the quantity and quality of secondary metabolites. These factors include culture medium micronutrients, ie. Zn. Nanoparticles (small size and large surface area) are considered ideal for delivering Zn fertilizer to plants (Parasad *et al.*, 2012).

Most studies have been carried out on the effect of Zn nanoparticles on the growth factors of plants, but there are no studies on the effect of Zn nanoparticles on the production of secondary metabolites. Zinc is an essential micronutrient and limited studies have shown its beneficial effect on essential oils as well as on plant growth. Zn is important as a metal component of certain enzymes and/or as a functional structural regulatory cofactor. It is essential for protein synthesis, photosynthesis, auxin synthesis, cell division, membrane structure and function, and sexual fertilization (Said *et al.*, 2010). Previous studies have shown that heavy metals can increase the activity of the antioxidant enzymes catalase (CAT) and ascorbate peroxidase (APX). As yet, there is no information on ZnO nanoparticle (ZnONP) effects on secondary metabolites (Hernandez-Viezcas *et al.*, 2011).

The present research investigated the effects of some elicitors at various concentrations on *in vitro* growth and secondary metabolites production in *L. ledebourii*. Using these elicitors can probably stimulate secondary metabolite synthesis, genetically and physiologically.

MATERIALS AND METHODS

Plant Material

Bulb scales obtained by *in vitro* micro-propagation in MS medium (Murashing and skoog, 1962) were used as source for plantlet production. Scales of *L. ledebourii* were separated and cultured in different concentrations of HA solutions (0, 50, 100, 500 and 1000 mg L⁻¹) and ZnONP (0, 10, 25, 50, 75 and 100 mg L⁻¹) in the MS medium. After six subcultures, plantlets were used for the evaluation of root length and number, leaf length and number, bulb diameter, and chlorophyll and secondary metabolites production. The experiment was a completely randomized design with six replicates. All cultures were maintained at 23 ± 2 °C and 16-hour photoperiod with light intensity of 2000 lux at plant level provided by cool white fluorescent lamps.

Chlorophyll content was measured by a SPAD chlorophyll meter. Leaf, root and bulb number counting was done by observation. Length was measured by a digital ruler.

Total phenolic content

Total phenolic content was determined using the Folin-Ciocalteu assay (Singleton and Rossi, 1965) with minor modification. Diluted extracts were directly assayed at 600 nm with gallic acid as the standard. Results were expressed as total phenols in micromoles of Gallic acid equivalents (GAE) per gr of fresh weight.

Table 1. Analysis of variance for effects of HA on root and leaf length, leaf and bulb number, chlorophyll, phenol, flavonoid (270, 300 and 330 nm) and anthocyanin contents.

Source of variations	df	Mean of square				
		Root length	Leaf length	Leaf number	Bulb number	Chlorophyll
HA	3	207.5*	986.72*	9.39*	3.793*	190.047*
Error	20	54.56	216.76	2.51	0.883	54.325
		Phenol	Flavonoid 270	Flavonoid 300	Flavonoid 330	Anthocyanin
HA	3	97.496**	0.294**	9.553**	0.513**	5.826**
Error	20	1.863	0.022	1.853	0.054	0.144

*, **: Significant at 5% and 1% probability level, respectively.

Total anthocyanin

Anthocyanin content was examined according to Masukasu *et al.* (2003). Frozen tissue (0.2 gr) was homogenized with acidic MeOH (MeOH and HCl at 99:1) and centrifuged at $12000 \times g$ for 15 min. The supernatant was kept in dark overnight. Anthocyanin content was determined as absorbance at 550 nm using the extinction coefficient of $33,000 \text{ cm}^{-1} \text{ M}^{-1}$ and was expressed as $\mu\text{M/g FW}$.

Total flavonoids

Total flavonoid content was determined according to Krizek *et al.* (1993). Frozen tissue (0.2 g) was homogenized with acidic EtOH (EtOH and HOAC at 99:1) and centrifuged at $12000 \times g$ for 15 min. The supernatant was held in an 80°C water bath for 10 min. After cooling, absorbance of flavonoids was at 270, 300 and 330 nm. Flavonoids content was determined by using extinction coefficient of $33000 \text{ cm}^{-1} \text{ M}^{-1}$ and was expressed as $\mu\text{M/g FW}$.

RESULTS AND DISCUSSION

Effects of HA

Analysis of variance indicated that different concentrations of HA significantly ($P \leq 0.01$) affected all characteristics (Table 1). Mean comparison by Duncan multiple range tests showed that the maximum root and leaf length, leaf number, bulb number and chlorophyll content was achieved in the media containing 500, 50, 50, 50 and 500 mg L^{-1} HA, respectively (Figure 2 A, B, C, D and E). The highest phenol content of plantlet was obtained in the medium containing 100 mg L^{-1} HA (Figure 2 F). However, all HA concentrations significantly ($P \leq 0.01$) improved total phenolic content in plantlets compared to control and no significant differences ($P \leq 0.05$) were found in 50, 500 and 1000 mg L^{-1} HA treatments (Figure 2 F).

All HA concentrations significantly ($P \leq 0.01$) improved anthocyanin content in the treated plantlets compared to control (Table 1). The highest content of anthocyanin was obtained from treated plantlets by HA at 500 mg L^{-1} (Figure 2 G). It is concluded that increasing HA concentrations higher than 500 mg L^{-1} decreased anthocyanin content. However, no significant differences ($P \leq 0.05$) were found in 100 and 500 and also 50 and 1000 mg L^{-1} HA treatments (Figure 2 G). All HA concentrations significantly ($P \leq 0.01$) improved flavonoid content in 270, 300 and 330 nm wavelengths compared to control (Table 1). The results also revealed that the highest concentration of flavonoid in 270, 300 and 330 nm wavelengths were obtained in the medium supplemented by 100 mg L^{-1} HA (Figure 2 H, I and J). The results of mean comparison showed that increasing HA concentrations higher than 100 mg L^{-1} caused a decrease in flavonoid content at all measured wavelengths (Figure 2 H, I and J).

Among various concentrations of HA, only 1000 mg L^{-1} treatments produced the lowest flavonoid content in plantlets at all measured wavelengths (Figure 2 H, I and J).

Effects of Zinc oxide nano particles

Analysis of variance indicated that the effects of various concentrations of Zinc oxide nano particles on root length and number, length of leaf, number of bulbs, chlorophyll, total phenol content, flavonoid and anthocyanin content significantly differed compared to the control (Table 2).

Mean comparison by Duncan multiple range tests showed that the maximum root length, leaf number and length, bulb number and chlorophyll content was obtained in the media containing 50, 50, 50, 75 and 50 mg L^{-1} Zinc oxide nano particles, respectively (Figure 1 D and E and Figure 3 A, B, C, D and E). Maximum

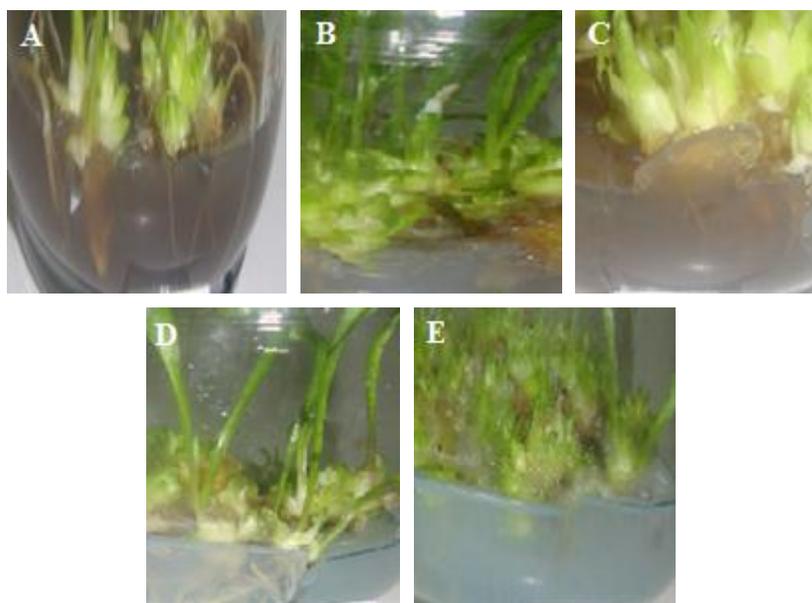


Figure 1. Effect of different concentrations of HA and ZnONP on growth factors of *Lilium ledebourii* (A: 500 mg L⁻¹ HA, B: 50 mg L⁻¹ HA and C: 100 mg L⁻¹ HA, D: 50 mg L⁻¹ Zinc oxide and E: 75 mg L⁻¹ Zinc oxide).

Table 2. Analysis of variance for the effect of ZnO on root and leaf length, leaf and bulb number, chlorophyll, phenol, flavonoid (270, 300 and 330 nm) and anthocyanin contents.

Source of variations	df	Mean of square				
		Root length	Leaf length	Leaf number	Bulb number	Chlorophyll
ZnO	5	342.56*	79.16*	1.944*	49.12**	335.75*
Error	30	12.05	5.32	0.494	1.39	26.67
		Phenol	Flavonoid 270	Flavonoid 300	Flavonoid 330	Anthocyanin
ZnO	5	85.941**	1.281**	28.32**	0.086**	0.503**
Error	30	0.892	0.05	2.88	0.02	0.042

*, **: Significant at 5 and 1 % probability level, respectively.

amount of phenolic content of plantlet was obtained in the medium containing 75 mg L⁻¹ Zinc oxide nano particles (Figure 3 F). However, all Zinc oxide nano particle concentrations significantly ($P \leq 0.01$) improved total phenolic content in plantlets compared to control. In case of total phenolic content in plantlets, no significant differences ($P \leq 0.05$) were found among 10, 25, 50 and 100 mg L⁻¹ Zinc oxide nano particles treatments (Figure 3 F).

The results also revealed that the highest concentrations of flavonoids measured at 270, 300 and 330 nm wavelengths were obtained with Zinc oxide

nano particles at 25 mg L⁻¹ (Figure 3 G, H and I). It is concluded that increasing Zinc oxide nano particles concentrations higher than 25 mg L⁻¹ decreased flavonoids content at all measured wavelengths (Figure 3 G, H and I).

The results also revealed that the highest concentrations of anthocyanin content of plantlet was obtained in the media containing 75 mg L⁻¹ Zinc oxide nano particles (Figure 3 J). However, all Zinc oxide nano particle concentrations significantly ($P \leq 0.01$) improved anthocyanin content in plantlets compared to control. In case of anthocyanin content in plantlets,

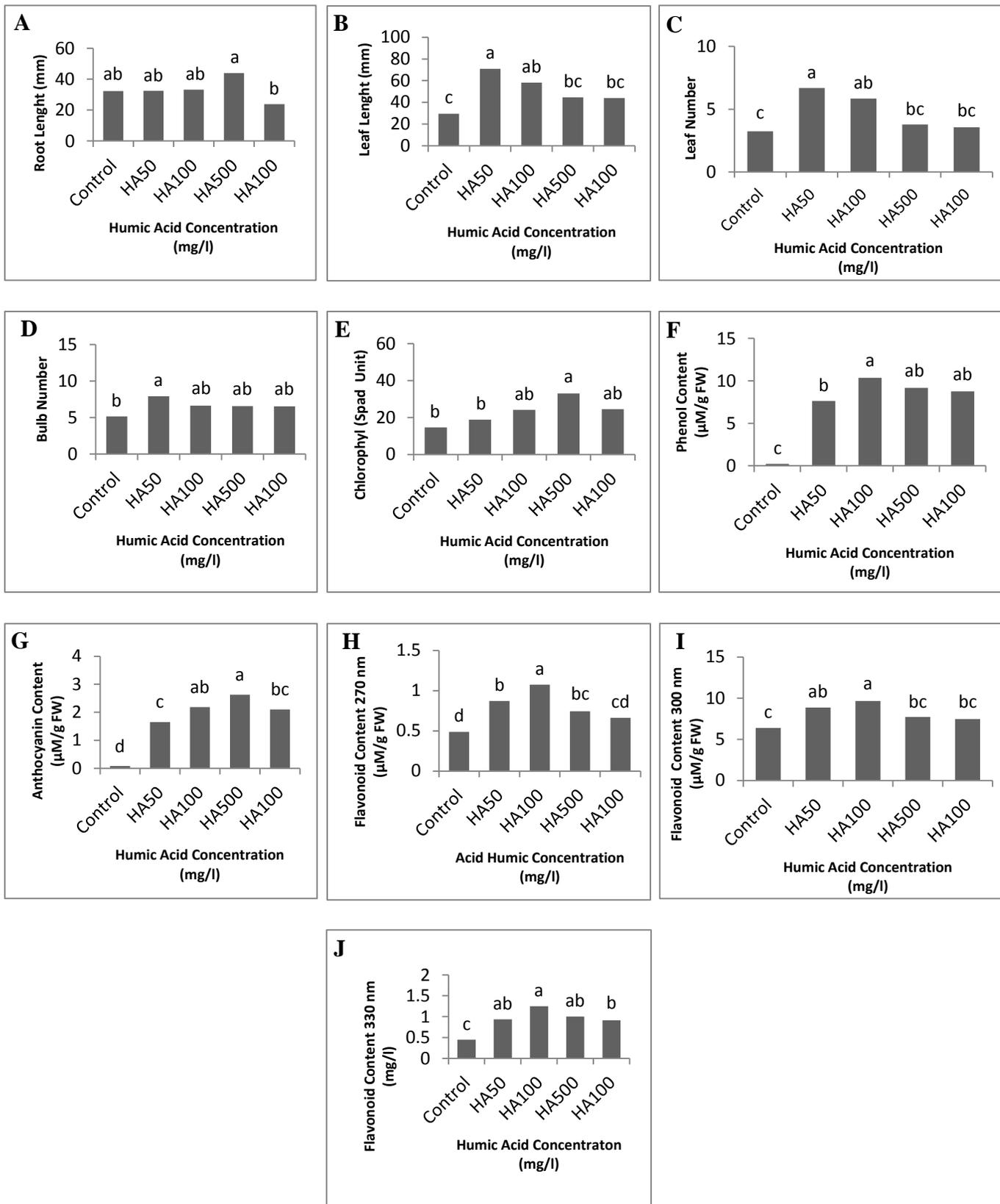


Figure 2. Effects of HA on root and leaf length (A, B), leaf and bulb number (C, D), chlorophyll (E), phenol (F), anthocyanin (G) and flavonoid (270, 300 and 330 nm) (H, I, J) contents.

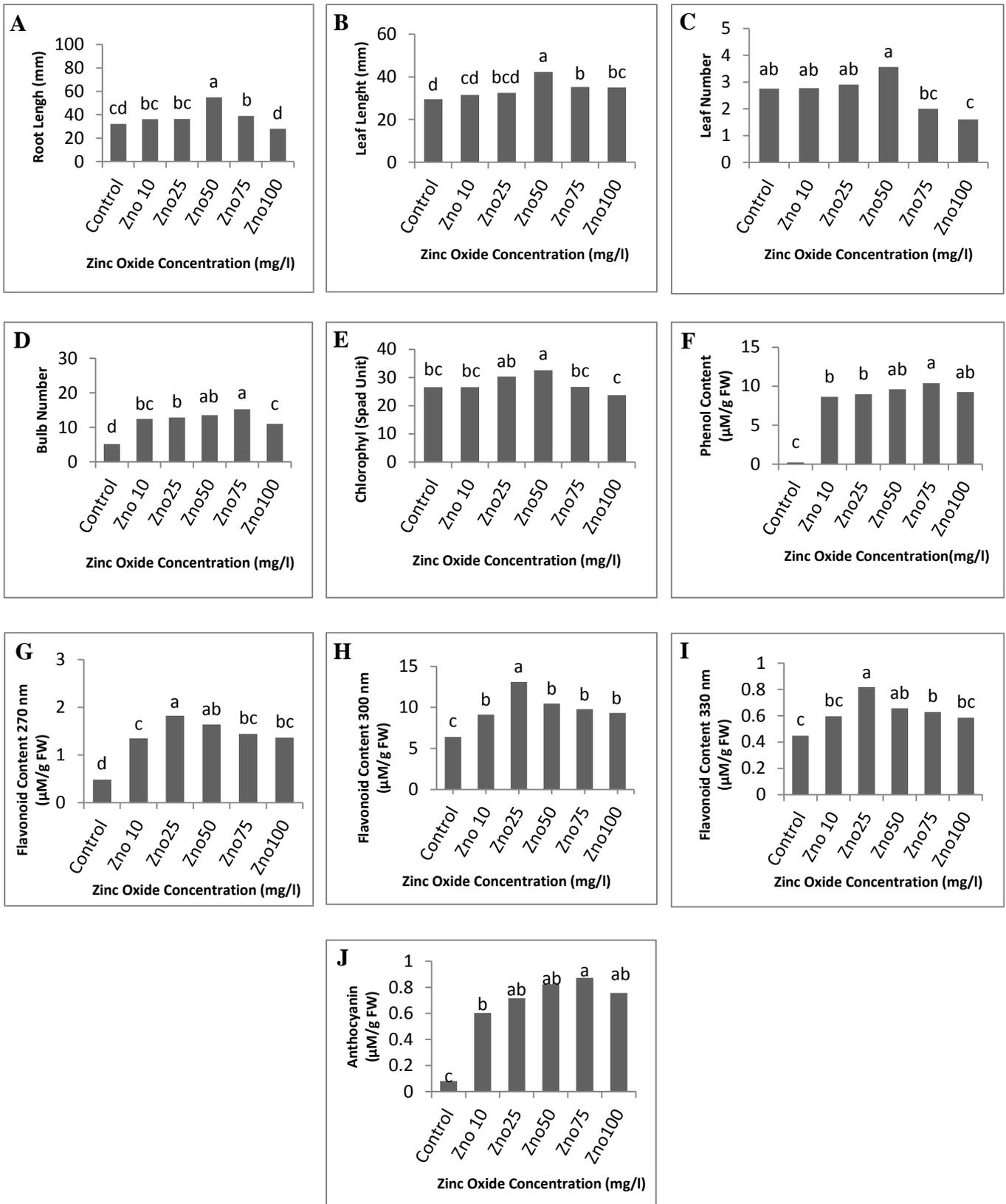


Figure 3. Effects of Zinc oxide nano particles on root and leaf length (A, B), leaf and bulb number (C,D), Chlorophyll (E), phenol (F), flavonoid (270, 300 and 330 nm) (G, H, I) and anthocyanin (J) contents.

no significant differences ($P \leq 0.05$) were found among 25, 50, 75 and 100 mg L⁻¹ Zinc oxide nano particles treatments (Figure 3 J).

The result of this study showed that the maximum root length was obtained in the medium containing 500 mg L⁻¹ HA. Bandiera *et al.* (2009) reported humic substances led to a reduction in root diameter and an increase in specific root length in radish (*Raphanus sativus*).

HAs have also been shown to be able to stimulate the proliferation of lateral roots along with the activation of plasmalemma and vacuolar H⁺-ATPases and tonoplast H⁺-PPase. In agreement, it has recently been shown that HAs could interact with root organic acid exudation and are able to change root area, primary root length, number of lateral roots and lateral root density (Zancani *et al.*, 2009).

The results of our experiment revealed that the highest leaf number and length was obtained from treated plantlets by HA at 50 mg L⁻¹. However, plant growth significantly decreased when the HA concentrations was increased. The same result was found in the study carried out by Atiyeh *et al.* (2002) which revealed that the general pattern of plant growth was increased in response to low concentrations of HA but decreased significantly when the exceeding concentrations of HA were applied. It is suggested that its mechanism might be hormone-like activities of HA, such as an auxin activity, since plant hormones such as auxin when applied at high concentrations (beyond an optimum level) could reduce the growth and development of plants. In a study carried out by Obsuwan *et al.* (2011) it was shown that humic substances enhanced the growth by increasing the uptake of micronutrients. So far, few studies have investigated the influence of organic management on bulb production in plants. Abdel- Razzak *et al.* (2013) reported that the application of HA on garlic increased bulb number. However, it seems that these growth responses were most probably due to the hormone-like activity of HAs.

In our study the highest chlorophyll content was obtained in the media containing 500 mg L⁻¹ HA.

The effect of HA on the availability of micronutrients has been given particular attention because of observed increases in the uptake rates of these nutrients following application of HA on *Fragaria ananassavar*: Camarosa (Ameri and Tehranifar, 2012). Humic substances have positive effects on plant physiology, so that HA at 20 ppm was effective in increasing chlorophyll content. It causes a reduction of Fe³⁺ to Fe²⁺ and made iron

chelates, which are readily available to the plants (Ameri and Tehranifar, 2012).

It is concluded that increasing of HA can improve phenol, anthocyanin and flavonoid contents.

Chassy *et al.* (2006) reported that HA significantly increased levels of soluble solids, flavonoids, total phenolics, and ascorbic acid in organic tomatoes compared to their conventional counterparts grown in model plots over a 3-year period. Strawberries grown organically had higher levels of antioxidants including total phenolic, Ellagic acid, and flavonols compared to conventionally grown strawberries (Amimifard *et al.*, 2012). Parandian and Samavat (2012) reported that HA significantly increased the amount of anthocyanin in *Lilium* flowers. Mitchell *et al.* (2007) reported that the levels of flavonoids increased over time in organically treated samples, whereas the levels of flavonoids did not vary significantly in conventional treatments.

The result of our experiment showed that the maximum root length was achieved in the medium containing 50 mg L⁻¹ Zinc oxide nano particles. In contrast, root length decreased at Zn concentrations higher than 50 mg L⁻¹ (Figure 3 A).

Luo *et al.* (2010) reported that the first visible damage, due to excessive Zinc, was on root growth caused by a reduction in cell division. Ghodake *et al.* (2011) reported that with increasing concentrations of the nano particles, the elongation of the roots was severely inhibited by Zinc oxide NPs toxicity in the cultured *Allium cepa* (onion bulbs). It was recently reported that the NPs may induce the formation of new and large-size pores and routes for the internalization of large NPs through cell walls (Ghodake *et al.*, 2011). Although, the molecular mechanism of the toxicity due to Zinc oxide NPs in the plant roots is not clear and requires further investigation. However, it should be closely related to the chemical composition, chemical structure, particle size, and surface area of the NPs (Ghodake *et al.*, 2011).

Our results showed that the highest leaf length and number was obtained in the treated plantlets by ZnO at 50 mg L⁻¹ (Figure 3 B and C). Mahajan *et al.* (2011) reported that with an increase in nano-ZnO concentration, the root and shoot growth of mung (*Vigna radiata*) and gram (*Cicer arietinum*) had increased. However, after certain concentrations, the growth of root and shoot was found to decline. Luo *et al.* (2010) reported that the biomass of plantlets obtained from cotyledons, hypocotyls of *Jatropha curcas* seedling, increased gradually up to 0.25, 0.5 and 0.5 mM Zinc, respectively and then decreased.

Therefore, in various plant species there are different growth reactions. Sugar beet shoot, fresh root and dry mass decreased progressively when Zn concentration in the nutrient solution increased (Sagardoy *et al.*, 2008).

Maximum number of bulbs was obtained in the medium containing 75 mg L⁻¹ Zinc oxide nano particles (Figure 3 D). Khalifa *et al.* (2011) showed that the foliar spraying of Zinc sulphate (4.5g/l) at all concentrations significantly increased growth parameters such as bulblets number of Iris plants and yield/plant compared to the control.

In our study, the maximum and minimum chlorophyll contents in plantlets were obtained in the media containing 50 and 100 mg L⁻¹ ZnO, respectively (Figure 3 E). In fact, by increasing ZnO concentration, chlorophyll content decreased and leaf toxicity symptoms were observed. It has been reported that Zinc inhibits Fe translocation and in the young leaf induces chlorotic leaves, showing Zn²⁺ toxicity (Rout and Das, 2003). Zinc toxicity symptoms include reduced yields and stunted growth, Fe-deficiency induces chlorosis through reductions in chlorophyll synthesis and chloroplast degradation (Martin, 2006).

Leaves of sugar beet (*Beta vulgaris* L.) treated with 50 and 100 µm Zn developed symptoms of Fe deficiency, including decrease in Fe, chlorophyll and carotenoid concentrations, increase in carotenoid/chlorophyll ratios. Excess Zinc also decreased the number of leaves and leaf area, leaf margins were distorted or wrinkled and leaves were rolled inwards and showed chlorosis symptoms (Sagardoy *et al.*, 2008).

The result of the present study revealed phenolic content and anthocyanin in *Lilium ledebourii* Boiss. Plantlets exposed to excessive Zinc, showed very slight and non significant growth by increasing Zinc concentrations to 75 mg L⁻¹. But flavonoid content in three wavelengths (270, 300 and 330 nm) increased by increasing Zinc concentrations from 0 to 25 mg L⁻¹. Nevertheless, beyond this concentration, the flavonoid content gradually decreased by increasing Zinc concentrations. There have been numerous reports on the induced accumulation of phenolic compounds and peroxidase activity in plants under stress. The roots of many plants exposed to heavy metals show high levels of phenolics. In plants, phenolic compounds such as flavonols and phenyl propanoids act as potential antioxidant compounds by donating electrons to guaiacol peroxidases (GPX) for detoxification of high amounts of H₂O₂ produced under stress conditions (Bartwal *et al.*, 2013).

Various studies have been carried out to explore the

effect of nanoparticles on the growth of plants but few studies were found to concentrate on the effect of nanoparticles on plants secondary metabolites. Zinc is one of the micronutrient of plants, and limited studies have been done on the beneficial effect of Zinc and other essential micronutrients on plant growth. However, it is well known to be necessary for plant growth and development. However, excessive Zn in plants can profoundly affect normal ionic homeostatic systems by interfering with the uptake, transport, osmotic and regulation of essential ions and results in the disruption of metabolic processes such as transpiration, photosynthesis and enzyme activities related to metabolism (Mahajan *et al.*, 2011). Zinc is a metal component of various enzymes or as a functional structural or regulatory cofactor and for protein synthesis, photosynthesis, the synthesis of auxin, cell division, the maintenance of membrane structure and function, and sexual fertilization (Said *et al.*, 2010). In contrast, Zn phytotoxicity also induces oxidative stress by generating free radicals and reactive oxygen species (ROS).

CONCLUSION

The present study showed the significant effects of HA and ZnO nanoparticles on *in vitro* multiplication and some secondary metabolite production in *L. ledebourii*. It was found that HA and ZnO might be used as elicitor. For this purpose, establishment of suspension cultures from this plant in future studies would be useful. Thus, our next investigation will focus on such components to evaluate secondary metabolite production.

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