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Eco-physiological Response of *Solanum nigrum* to Cd and Ni Stress under Hydroponic Conditions

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SOLANUM nigrum L. was transplanted from soil to hydroponic culture system with Hoagland's solution to study its eco-physiological response to Cd and Ni toxicity. The plants were exposed to 10 or 25mg/L Cd or Ni over 7 days before harvesting for analysis. The findings of this study provided better understanding of stress adaptation in *S. nigrum* under Cd and Ni stress. The photosynthetic pigments and activities of antioxidant enzymes were estimated in the plant leaves. Chlorophyll content and carotenoids decreased progressively with increasing concentrations of both metals. Activities of the antioxidant enzymes POD, APX, CAT, and SOD in the leaves increased significantly by exposure to the high levels for both metals. The plant responses was varied with both metal and tested concentrations, reflecting the potentiality of using *S. nigrum* as phytoremediator of Cd and Ni polluted water in hydroponic culture system.

Keywords: Antioxidant enzymes, Oxidative stress, Phytoremediation, Plant pigments, Stress tolerance.

Introduction

Aquatic habitats, especially fresh water ecosystems, are more contaminated than other environments because of water use for processes of industrial and residential waste disposal (Demirak et al., 2006; Fernandesa et al., 2007). Because of the possible harmful environmental effects of contaminants, this situation has become an environmental issue of major importance.

Physiological processes can be disrupted by heavy metals such as carbon assimilation decrease, inhibition of chlorophyll synthesis, generation of oxidative stress (Benavides et al., 2005; Gratão et al., 2008; Yusuf et al., 2011; Hussain et al., 2013; Selem, 2019). Excessive heavy metals are known to reduce photosynthetic apparatus performance and promote reduction in photosynthetic pigments includingchlorophyll and carotenoids production. (Fargasová, 2001; Ali et al., 2017).

More than 450 species of plants are referred to as hyperaccumulator plants. Such species, typically raise the problem of low biomass yield and growth rate (Verbruggen et al., 2009). *Solanum nigrum* has been reported to be growing in polluted sites around the world as hyperaccumulating of heavy metals. It also characteristic of being a fast-growing, easy to adapt and has greater shooting biomass than most hyperaccumulators of metals (Marques et al., 2007).

As the phytoextraction using hyperaccumulators is seen as an innovative and competitive technique, the physiological responses of *S. nigrum* are important to be known in this highly toxic HM

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to provide a basic understanding of the biological mechanisms of metal hyperaccumulation and tolerance (Verbruggen et al., 2009; Wei et al., 2006). The oxidative stress caused by heavy metals in plants can occur through the production of active oxygen species (AOS) such as hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\bullet}$) and superoxide radicals ($O_2^{\bullet-}$) (Qureshi et al., 2005; Ali et al., 2016; Rizwan et al., 2016; Ahmad et al., 2017). Such oxygen species induce lipid peroxidation, inactivation of the enzyme and destroy DNA structures, this reduces growth and productivity and that caused death of the plant (Dat et al., 2000; Gratão et al., 2005; Wang & Zhou, 2006).

Antioxidant enzyme regulation provides additional defensive capabilities for plants against oxidative stress and generating AOS (Sun et al., 2007). Therefore, hyperaccumulators have an active tolerance strategy for heavy metals related to the expression of antioxidant enzymes under heavy metal stress (Boominathan & Doran, 2003). Increased stress tolerance in metal-exposed plants is often associated with higher levels of antioxidants, particularly the increased activity of antioxidant enzymes, meanwhile, among the key antioxidant enzymes, superoxide dismutase stimulates the dismutation of $^{\bullet}O_2^-$ to H_2O_2 and O_2, and forms, the first line of antioxidant defensive (Fidalgo et al., 2011, 2013; Gomes-Junior et al., 2006; Gratão et al., 2008). Different antioxidant enzymes, mainly found in peroxisomes, reduce the accumulation of H_2O_2 in cells. Peroxidase enzyme (POD) contributes in lignin biosynthesis and convert H_2O_2 to H_2O is extremely important for plant respiration as well as H_2O_2 may be scavenged by Catalase (CAT) or peroxidases such as ascorbate peroxidase (APX) enzymes (Sun et al., 2007; Gajewska & Slodowska, 2008; Hamed & Abd Elgawad, 2018). The previous phytoremediation studies on *S. nigrum* were carried out on plants grown in soil. This study was designed to treat the species phytoremediation potentiality in hydroponic culture as an important asset for its future use in treating waste water for further use in raising hydroponic crops. The main objective of this study is to assess the potential effect of Cd and Ni on photosynthetic pigments and antioxidant enzymes activity in *S.nigrum* raised in hydroponic culture.

Materials and Methods

Plant materials and growth conditions

Seeds of *Solanum nigrum* L. were collected from a wild population growing in River Nile bank of Sohagcity. The seeds were germinated in Peat moss nutrient media for 2 weeks until they reached vegetative growth 3-5cm high. The seedlings were transferred to plastic pots with clay-sandy soil for 2 months where they develop suitable biomass. Plants were then up-rooted by careful washing soil particles using tap water followed by distilled water before transformation to the hydroponic system (Fig. 1). The plants were kept to acclimatize for 3 days before heavy metal treatment (Cowgill & Milazzo, 1989).

Fig. 1. Photo of the hydroponic system, the treatment solutions in the upper water tank (inlet) transferred through the three pipes into the lower water tank controlled by a timer switch. The solution in the lower tank was drained to the upper water tank to complete a water cycle [There were one water cycles every 24 hrs].

The water temperature in the hydroponic system was 22 ± 2°C during the experimental time and the ambient air temperatures were 25°C day (14hrs) and 15°C at night. The light intensity of the day time was 1950 lux neon light. A ventilation system was designed to circulate the ambient air.

Based on the preliminary test results, initial concentrations (control, 10, 25mg/L) of the tested metals Cd and Ni were applied separately forevery metal. Nickel solution was prepared from Ni $(NO_3)_2.6H_2O$ and Cadmium solution from Cd $(NO_3)_2.4H_2O$ (Sigma, St. Louis, MO). The plant and water samples were collected for consecutive 1, 3, 5, 7 days. The reagents were dissolved in 20 LHoaglandnutrient solution (Hoagland & Arnon, 1938). Bidistilled water was used to achieve the appropriate contamination level in the hydroponic system.

Photosynthetic pigments

For determination of chlorophyll and carotenoids, the leaves of *S. nigrum* were extracted from 0.5g of fresh leaves in 85% aqueous acetone. The leaf samples were grinded, centrifuged for 10min at 5,000rpm, the supernatant was completed to 10mL with the aqueous acetone and the absorbance was measured at 452.5, 644, and 663.6nm using spectrophotometer (Perkin Elmer Analyst 400, USA). The data were calculated according to Lichtenthaler & Wellburn (1983) and expressed as mg/g fw.

Antioxidant enzymes

Extraction and assays of antioxidant enzymes

Enzyme extraction was performed by grinding 0.5g of fresh leaves at 4ºC in 3mL extraction buffer. The extraction buffer contain 50mM sodium phosphate buffer (pH 7), 1% (w/v) polyvinyl pyrrolidone (PVP) and 0.1mM NaEDTA. The extract was subjected for centrifugation at 13000rpm for 20min at 4ºC. The supernatants obtained were used to determine the enzyme activity.

Peroxidase enzyme (EC 1.11.1.7) activity was determined by mixing supernatants (0.05mL) with assay mixture (2.950mL) as described by MacAdam et al. (1992). Assay mixture containing 50mM Sodium, potassium phosphate buffer (pH 6.8), 5mM guaiacol and 0.3mM H_2O_2. Oxidation of guaiacol was spectrophotometrically measured at 470nm. The activity was determined in terms of µM of oxidized guaiacol min^{-1} g^{-1} fresh weigh at 25± 2ºC (Zhang, 1992).

Catalase enzyme (EC 1.11.1.6) activity was spectrophotometrically measured according to Aebi (1984). The activity of CAT enzyme was estimated by H_2O_2 consuming leading to a decrease in absorbance at 240nm for 1min. The reaction solution contain 50 mM sodium, phosphate buffer (pH 6.8), 20mM H_2O_2, and 0.1mL enzyme extract. One unit of CAT is defined as the quantities of enzyme needed to decompose 1µmol min^{-1} of H_2O_2 under the assay conditions.

Ascorbate peroxidase, enzyme (EC 1.11.1.11) activity was measured as Nakano & Asada (1981). APX activity was determined by using 3mL assay media. It contained 50mM sodium, phosphate buffer (pH 6.8), 0.5mM ascorbate, 0.1mM H_2O_2, 0.1mM NaEDTA and 0.05mL enzyme extract. There was a decline in absorbance at 290nm for 1min resulted from the reduction in ascorbate concentration and the activity of the enzyme was measured using the extinction coefficient for ascorbate.

Superoxide dismutase enzyme (EC 1.15.1.1) was measured according to Giannopolitis & Ries (1977) the activity of the enzyme was measured. SOD's ability to reduce the photochemical reduction of nitro blue tetrazolium (NBT) was determined for its activity determination. The reaction was performed in 3mL reaction solution contains 50mM sodium phosphate buffer (pH 6.8), 2mM riboflavin, 13mM methionine, 75mM NBT, 100mM Na-EDTA and 0.05mL enzyme extract. SOD activity was known as the quantity of enzyme that inhibit the photoreduction of nitro blue tetrazolium.

Data analysis

Data were subjected to statistical analysis using Minitab 17.0, where One-Way ANOVA followed by comparisons between means was employed. Linear regression was carried between each parameter and the concentrations of the metal and used to indicate the response of the plant.

Results

Photosynthetic pigments

The changes in the photosynthetic pigment chlorophyll and carotenoids in the leaves of *S. nigrum* treated with Cd and Ni are shown in Figs.

2 and 3. Compared to the control, leaves of plants treated with 10mg/L and 25mg/L of Cd exhibited a significant decrease in Chl. a content of plants. The content of Chl. b and carotenoids decreased with time and Cd concentration increase in the nutrient medium as shown by the determinant coefficient (R^2) and Spearman's coefficient (r) values (Table 1). The Inhibitory effect of high level Cd (25mg/L) was more pronounced on Chl. b and carotenoids than Chl. a when compared with the lower concentration 10mg/L. Compared with the control, Chl. a, b and carotenoids content in *S. nigrum* exhibited a similar response with a gradual decline at the two level of Ni concentration treatment. The maximum decline was attained after 7 days in the two tested concentrations as appear in R^2 and r values in Table 1. Chlorophyll b decreased with increasing Ni concentration in the nutrient medium ($P< 0.05$), while the content of chlorophyll a and carotenoids showed insignificant response to Ni concentration from 10mg/L to 25mg/L ($P> 0.05$).

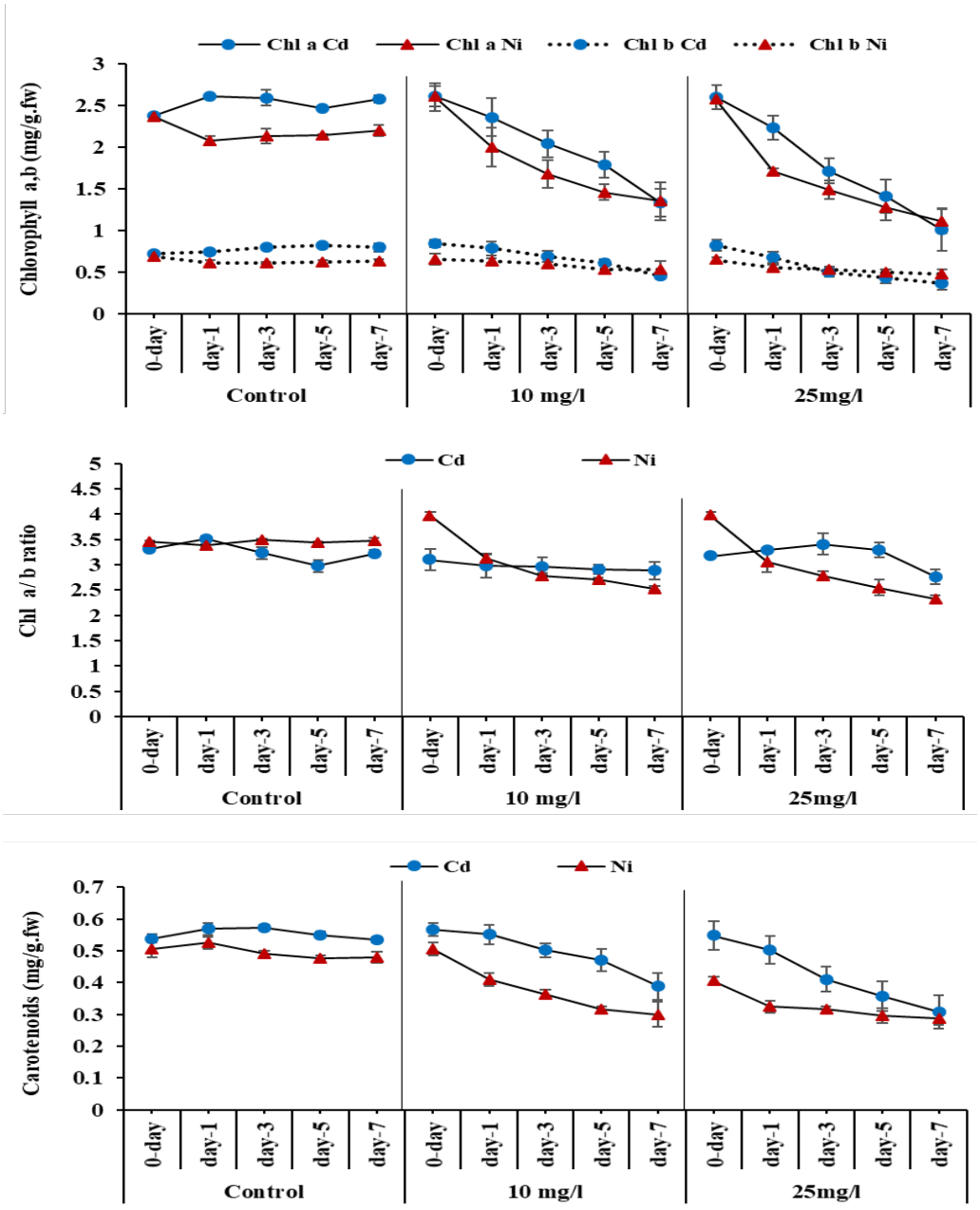

Fig. 2. Effect of Cadmium and Nickel treatment on photosynthetic pigments, chlorophyll a, chlorophyll b, Chl. a/b ratio and carotenoids in *S. nigrum* leaves.

TABLE 1. Photosynthetic pigments responses (mg/g.fw) and antioxidant enzymes activity (Unit/mg fw.min) of the tested enzymes in *S. nigrum* treated for 7 days with different concentratios of Cd and Ni (0, 10, 25mg/L) under hydroponic system (n=9).

Pigments & enzymes	Control		10mg/L		25mg/L		Regression equation	
	Cd	Ni	Cd	Ni	Cd	Ni	Cd	Ni
Chl. a	2.52 (0.09)	2.18 (0.01)	2.03 (0.16)	1.82 (0.17)	1.79 (0.17)	1.63 (0.09)	Y =2.447 -0.028 X R^2= 90.3 , r= -0.95 *P*> 0.05	Y =2.13-0.02140X R^2=91.2 , r= -0.95 *P*> 0.05
Chl. b	0.77 (0.04)	0.63 (0.03)	0.67 (0.05)	0.59 (0.06)	0.55 (0.06)	0.54 (0.03)	Y =0.774 -0.0087 X R^2= 99.6 ,r= - 0.99 *P*< 0.05	Y= 0.6322-0.003551X R^2 =99.8 , r= -0.99 *P*< 0.05
Chl a/b	3.25 (0.19)	3.45 (0.05)	2.97 (0.08)	3.03 (0.57)	3.19 (0.25)	2.94 (0.64)	Y = 3.153 - 0.0013X R^2=0.4, r= -0.06 *P*> 0.05	Y = 3.366 - 0.01936 X R^2=16.0, r= -0.4 *P*> 0.05
Carot	0.55 (0.01)	0.49 (0.02)	0.49 (0.02)	0.37 (0.02)	0.42 (0.04)	0.34 (0.01)	Y = 0.551 – 0.0051X R^2= 99.7 , r= -0.99 *P*< 0.05	Y=0.4732-0.005657X R^2=82.3 , r= -0.90 *P*> 0.05
POD	722.5 (95.41)	591.17 (67.50)	1317.05 (291.80)	677.68 (95.14)	2288.4 (387.81)	796.528 (155.95)	Y = 460+61.8X R^2= 99 r= 0.99 *P*< 0.05	Y =592.9 + 8.2 X R^2 = 99.9, r= 1 *P*< 0.05
CAT	0.00356 (0.0005)	0.00335 (0.0003)	0.00848 (0.0022)	0.00779 (0.002)	0.0156 (0.002)	0.0136 (0.002)	Y = 0.004+0.0004 X R^2= 100, r =1 *P*< 0.05	Y =0.004+0.0004 X R^2=99.9, r =0.99 *P*<0.05
APX	3.16 (0.17)	0.568 (0.05)	5.355 (1.41)	0.973 (0.14)	8.827 (1.96)	1.11 (0.29)	Y = 3.1 + 0.2 X R^2= 100, r=1 *P*< 0.05	Y = 0.64 +0.02 X R^2=85.7, r=0.92 *P*> 0.05
SOD	23.35 (3.71)	10.09 (0.43)	35.35 (12.60)	18.25 (1.93)	50.15 (17.44)	32.29 (4.95)	Y = 23.9 + 1.1 X R^2= 99.7, r=0.99 *P*< 0.05	Y =9.8 +0.89 X R^2=99.9, r =0.99 *P*< 0.05

Numbers are mean ± SD (values between bracets).

The ratios of chlorophyll a/b were higher in the control than those in the treated growth medium (Fig. 2). The chlorophyll a/b ratio in Cd concentration of 10 mg/ L significantly decreased with time ($P< 0.05$). Alternatively, that ratio was increased in the higher Cd concentration of 25mg/L ($P> 0.05$). The chlorophyll a/b ratio in growth medium treated with Ni decreased significantly ($P< 0.05$) in the higher concentration 25mg/L with time.

Antioxidant enzymes

The activity of Superoxide dismutase (SOD) in *S. nigrum* increased significantly with the increasing concentration of Cd. The Cd treatments caused an increase in Peroxidase (POD), Ascorbate peroxidase (APX) and Catalase (CAT) activities with the time of the experiment duration (Fig. 4).

The enzymes activity increased with the increased Ni concentration, whereas CAT and SOD showed decrease in activity at the seventh day of the experiment of treatment (Fig. 4). The POD, CAT, and SOD activity increased with increasing Ni concentration ($P< 0.05$) compared with the control (Table 2). Alternatively, APX activity did not show an increase with the increase of Ni concentration. The R^2 and r values confirm these correlations as shown in Table 1.

Discussion

The results of this study reflected the response of *Solanum nigrum* to Cd and Ni stress and their effects on pigment content and enzyme activity. As reported by Chandra & Kulshreshtha (2004) and Qian et al. (2009), Cadmium-induced decreasing impact on chlorophyll and carotenoids content may be explained by its inhibitory effect on pigment biosynthesis enzymes directly or through the substitution of the central Mg ion. Also decreased chlorophyll a and chlorophyll b content in response to Cd results from the inhibition of chlorophyll biosynthesis resulting in senescence (Fang et al., 1998). These finding

are in agreements with El-Khatib et al. (2011), who reported that the leaves of *Ceratophyllum demersum* and *Myriophyllum spicatum* under Cd stress showed decreased in their chlorophyll and carotenoid contents. Hence, chlorophyll pigments tend to be one of the main causes of plant heavy metal injury. This event reduced, synthesis of chlorophyll may also be due to the lack of access of essential nutrients for the plant's physiological activities (Fidalgo et al., 2011; Liu et al., 2013), where the rate of photosynthetic CO_2 fixation decreases at high concentrations of heavy metal (Gao et al., 2010). This pigment decrease may also be due to inhibition of enzymes responsible for the chlorophyll synthesis (Cenkci et al., 2010; Pourraut et al., 2011). As reported by Bhattacharya et al. (2000), Cadmium was found to inhibit photosynthesis due to inhibition of chlorophyll synthesis. The decreased chlorophyll a/b ratio with time indicates that the absorption band of mixed pigments extends toward the green part of the system (Fahmy et al., 1990). This means that the plant maximizes its photosynthetic activity in response to toxic metal stress.

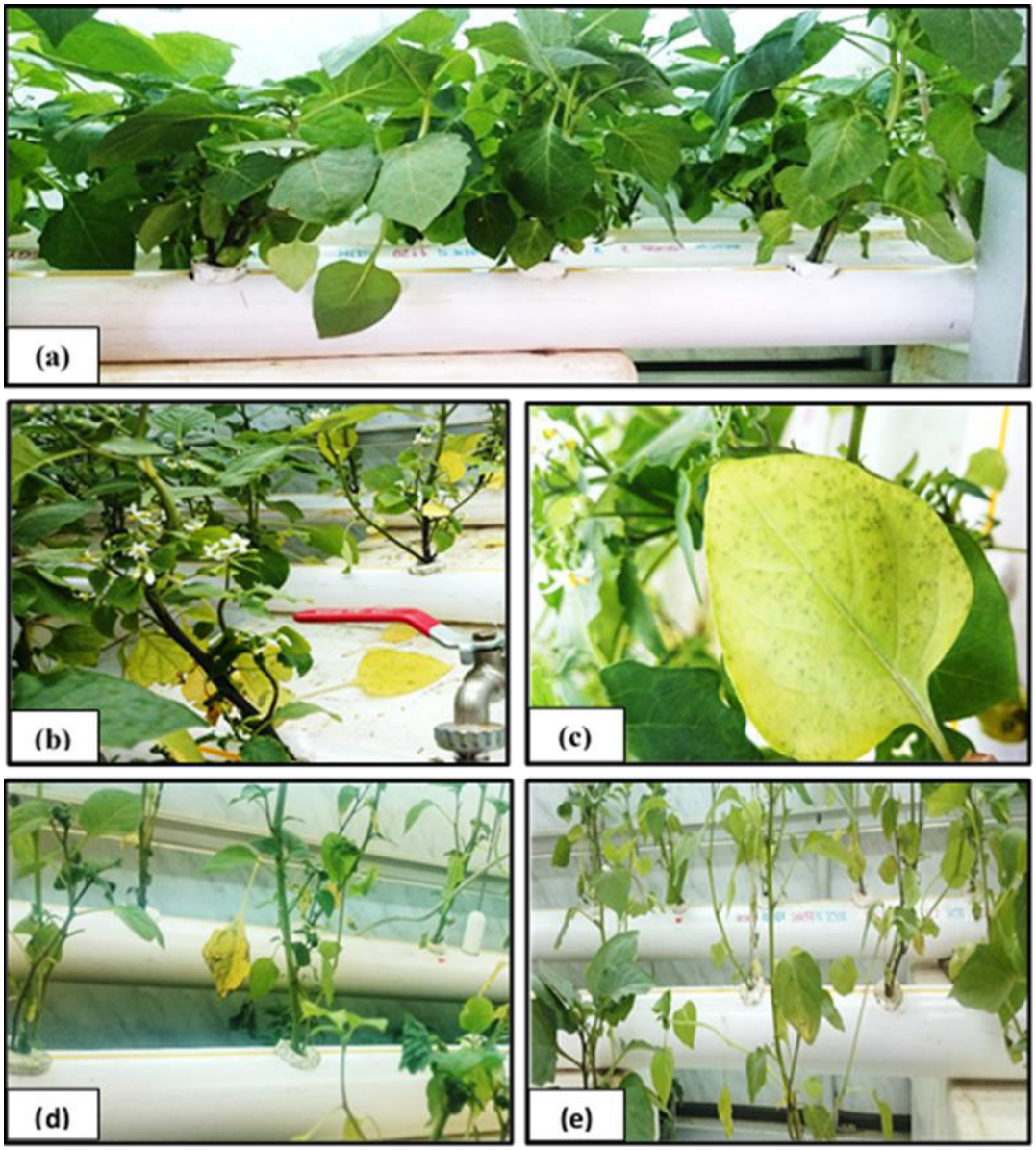

Fig. 3. Morphological variations in *S. nigrum* under heavy metal treatment; (a) control, (b and c) visual chlorosis, mosaic and leaf falling under Cd stress, and (d and e) visual chlorosis under Ni stress.

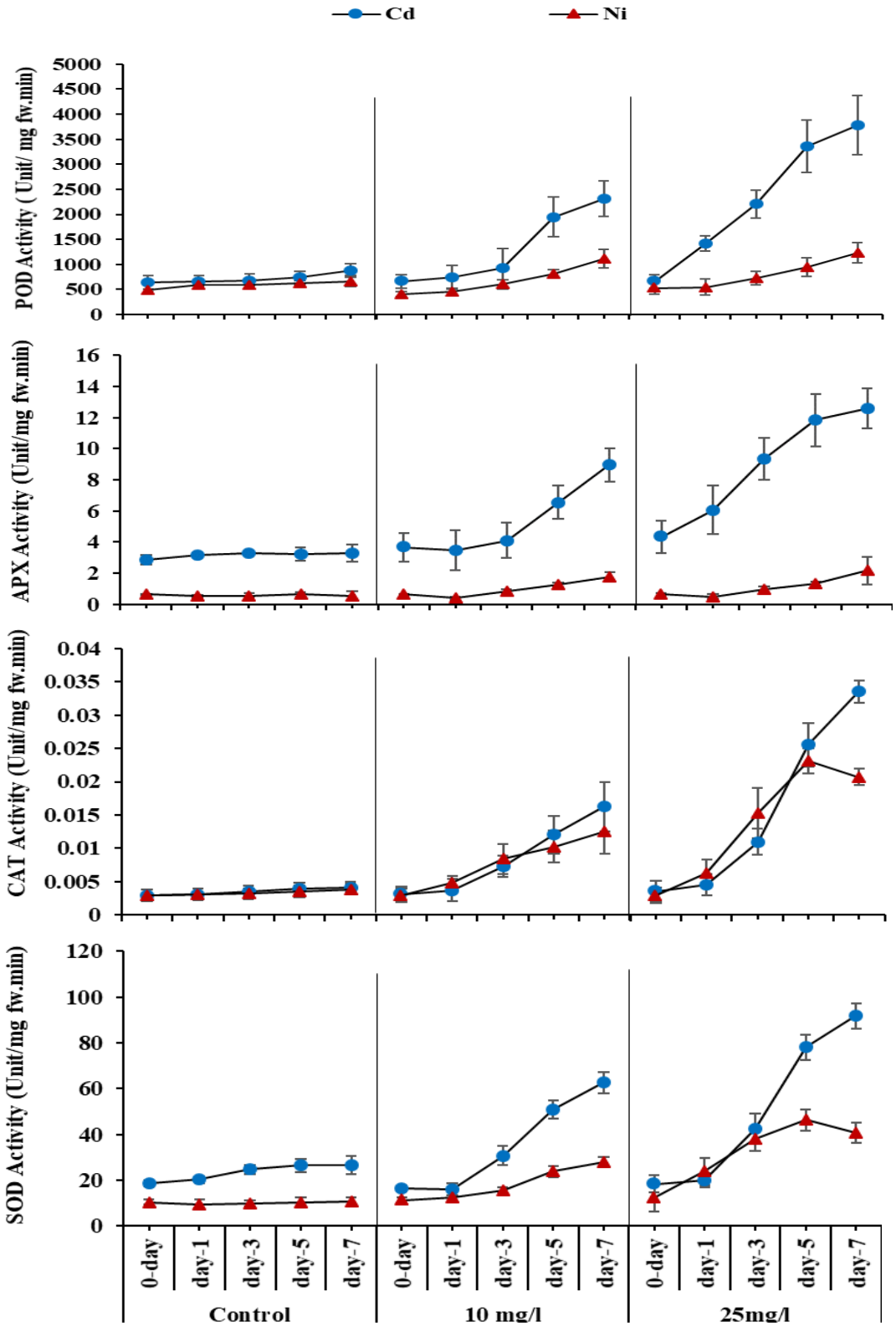

Fig. 4. Effect of Cadmium and Nickel treatment on antioxidant enzymes activity, POD, APX, CAT and SOD of *S. nigrum.*

TABLE 2. Person's correlation matrix of the studied parameters with concentration of metals in plant tissue durig the experiment duration (n=9).

Metals	Treatment (mg/L)	Chl. a	Chl. b	Carotenoid	POD	APX	CAT	SOD
Cd	10	-0.95***	-0.94***	-0.95***	0.88***	0.93***	0.94***	0.97***
	25	-0.97***	-0.95***	-0.97***	0.98***	0.98***	0.96***	0.95***
Ni	10	-0.86***	-0.83***	-0.81***	0.96***	0.98***	0.94***	0.94***
	25	-0.78**	-0.77***	-0.79***	0.96***	0.95***	0.70**	0.63*

***Correlation is significant at the 0.001 level ,**Correlation is significant at the 0.01 level ., *Correlation is significant at the 0.05 level.

Reduction in the levels of photosynthetic pigments, including carotenoids, on exposure to biotic or abiotic stressors was observed in many other species (Lau et al., 2006), where metals may affect carotenoids synthesis depending on metaltype and concentration (Singh & Tewari, 2003).

Because Cd is a redox active metal, oxidative stress is caused by Cd toxicity, which may occur through production of ROS (Gratão et al., 2005). Plants enhance the antioxidant enzymes to scavenge ROS and prevent oxidative damage superoxide dismutase, Catalase, ascorbate peroxidase and peroxidase (Kanazawa et al., 2000). In contrast, Ni may not directly produce ROS, as it is not a redox-active metal. Ni can indirectly interfere with the responses of an antioxidant system (Ahmad et al., 2010; Gajewska & Slodowska, 2008; Gill & Tuteja, 2010). Even so, the results suggest that the activity of the antioxidant enzymes under Cd stress is higher than their activity under Ni stress.

High SOD activity associated with plant stress tolerance because it neutralizes the activity of superoxide radical that is over produced under metal stress. SOD is one of the stress-resistance enzymes, which can catalyze the conversion of two O_2^- radicals to H_2O_2 and O_2 (Foyer & Noctor, 2000). Previous studies agree with these findings where Cd stress leads to an increase in SOD activity and this event was often associated with the increased plant tolerance (Deng et al., 2010).

The POD has many physiological functions including its role in lignin biosynthesis, building up a physical barrier against toxic heavy metals, participation in plant photosyn thesis, respiration, auxin metabolism, and virus resistance. Also, POD is considered as one of the key enzymes in the ROS removal, where its extra- and intracellular forms participate in the breakdown of H_2O_2. Elevation in POD activity in *S. nigrum* treated with Cd suggests its role in the H_2O_2 detoxification (Dey et al., 2009). Enhancing POD activity in plants treated with excess Cd may result in either peroxidant damage to the thylakoid membrane or reduced auxin and protein content in tissues of plants (Sandman & Boger, 1980; Sun et al., 2007).

Among the H_2O_2-destructive enzymes, Cd and Ni enhanced the activity of CAT and APX. The activity of CAT is essential for plant antioxidant defence system and cell protection (Shigeoka et al., 2002; Pandey, 2008; Xu et al., 2012). The present results are in agreement with the results on the other metal-accumulators *Thlaspi caerulescens* and *Brassica juncea* (Wang et al., 2008).

Eventually, the synthesis of antioxidant enzymes plays an important role in scavenging reactive oxygen species as an enzymatic protection system (Sharma et al., 2012). The results showed that *S.nigrum* has an effective tolerance strategy to Cd, suggesting its strong internal detoxification mechanisms are a vital feature of Cd-hyperaccumulators (Boominathan et al., 2003).

Depending on the concentration, Ni seems to have dual effect as stimulating or inhibitory enzyme activities in plant tissues. Thus, another common mechanism of Ni toxicity is the increased activity of peroxidase, ascorbate oxidase, and catalase and superoxide dismutase with the increased concentration due to the formation of reactive oxygen species in plant cells, as well as elevating the activity of the antioxidant enzymes which protect plant cells against ROS toxicity (Schickler & Caspi., 1999; Seregin & Ivanov, 2001). But the activity of CAT and SOD enzymes was inhibited in the seventh day at 25mg/L of Ni treatment causing decline in

cell metabolism due to the inhibition of enzyme activities (Seregin & Kozhevnikova, 2006). Also, the decline in antioxidant enzyme activities may result from accumulation of the reactive oxygen species, which may cause severe plant damage (Seregin & Kozhevnikova., 2005; El-Khatib et al., 2014).

Conclusion

In conclusion, the present results suggest that the exposure of *S. nigrum* to high levels of Cd and Ni induce an appositive response by the antioxidant system. Various physiological mechanisms are involved as defense strategy against heavy metal accumulation effects. *S. nigrum* tolerates higher metal accumulation by modifying the antioxidant defense system. Our finding showed that the four major enzymes SOD, POD, CAT, and APX play an important role in the detoxification / elimination of ROS induced by Cd and Ni, helping to alleviate the magnitude of stress onthe plant. These adaptive features of *S.nigrum* against the toxicity of Cd and Ni recommends its potential use in phytoremediation of polluted water in aquaculture agricultural systems.

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الاستجابات الفسيولوجيا- بيئية لنبات عنب الديب المعامل بعنصري الكادميوم والنيكل تحت ظروف الزراعة المائية

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لقد تم استخدام نبات عنب الديب وهو من النباتات البرية الأرضية لغرض دراسة التأثيرات الفسيولوجيا – بيئية تحت ظروف زراعة مائية معاملة بعنصري الكادميوم والنيكل. اتضح أن الاستجابة الفسيولوجيا- بيئية تعتبر آلية دفاع مناسبة يستخدمها النبات للتعامل مع الإجهاد المفروض من قبل تركيزات مختلفة لكل من عنصري الكادميوم والنيكل. لذلك تم قياس التغير فى أصباغ التمثيل الضوئي ونشاط الأنزيمات المضادة للأكسدة فى أوراق نبات عنب الديب النامي تحت تركيزات 10، 25 مليجرام لكل لتر من الكادميوم والنيكل لمدة 7 ايام. وأظهرت النتائج ان محتوي الكلوروفيل أ وب انخفض تدريجيا مع زيادة تركيزات العنصرين اما صبغ الكاروتين انخفض ولكن بمعدل اقل وهذا ما يؤكد دوره كمادة مضادة للاكسدة تعمل على حماية النبات، كما اظهرت النتائج عن زيادة كبيره في نشاط الإنزيمات المضادة للأكسدة فى الأوراق. اوضحت هذه الدراسة تنوع استجابة نبات عنب الديب مع كل من العناصرالثقيلة وتركيزاتها مما يعكس ملائمته للاستخدام فى عملية المعالجة البيولوجية للمياه الملوثة بهذه العناصر. تساعد نتائج هذه التجربة على فهم افضل لكيفية تكيف النبات تحت اجهاد كل من عنصري الكادميوم والنيكل.