Cyanide acclimation in willow (*Salix babylonica* L.), a prospect for the phytoremediation of cyanide

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Abstract

Phytoremediation counts as a major method for future which essentially needs resistance to contaminating agents. We pretreated the plants (*Salix babylonica*, as a model plant in phytoremediation of polluted waters) by sodium cyanide (0, 3 and 5 mg CN^- L^{-1}) to induce resistance with regard to acclimation and then examined their resistance to higher concentrations of cyanide. Accordingly, some of the resistance- related physiological parameters were measured. The results showed that the pretreatment increased the resistance up to 3 folds more than toxicity threshold. It induced the superoxide dismutase activity and ion leakage from roots. Dehydrogenase activity, reducing capacity of the roots and chlorophyll was decreased in pretreated plants. Results also showed that no difference was seen between the control and pretreated plants in the uptake of cyanide from media. The induced resistance via cyanide acclimation could be attributed to physiological responses such as higher activities of antioxidants and not to prevention of cyanide uptake into the cells.

Key words: Acclimation, Cyanide, Phytoremediation, Pretreatment, *Salix babylonica*

Introduction

Cyanide is used in a host of industrial processes such as gold mining, electroplating and production of adhesives, pigments and paints. In addition to its industrial production, it is produced as a byproduct in some industries such as coal coking, iron, steel and aluminum manufacturing and petroleum refining. The produced cyanide is finally introduced into the nature in different forms, causing lethal effects and an imbalance in nature. There are several processes to remove or detoxify cyanide, mostly including of natural scavenging, alkaline chlorination and oxidation through using hydrogen peroxide (Reviewd by Dzombak *et al.*, 2006). Since removing cyanide from wastewater by using chemical processes is complicated and costly, and it also produces new chemicals, attention has paid to biological methods such as phytoremediation. Based on a globally accepted definition, phytoremediation is the use of vascular plants, algae and fungi to metabolize or sequester contaminants, or to induce contaminant breakdown by micro-organisms in soil (Salt *et al.*, 1998; McCutcheon and Schnoor, 2003). One of the most important advantages of phytoremediation is that the final residuals are just the biomass of living organisms, carbon dioxide and methane.

There are some limitations in using plants for phytoremediation. For example, the selected plants must be highly resistant to pollutants (Salt *et al.*, 1998). So, the most important characteristic of plants when used in phytoremediation of cyanide is having high resistance to it. Higher plants are oxygenic organisms that use four mitochondrial complexes for respiration. The complex IV or cytochrome oxidase, is the main place of inhibitory effect of cyanide on respiratory electron transport chain. Inhibition of complex IV results in a high decrease in ATP production which finally causes cell death.

Cyanide affects plant cells, which could be measured accurately, through metabolism of the cells via inhibition of electron transport chains in mitochondria and chloroplasts, and also through changing the activities of different metalloproteins by binding to their metal groups. One of the enzymes that are inhibited by cyanide is Cu/Zn superoxide dismutase (Cu/Zn SOD). A decrease in the levels of ATP in cells causes lowers integrity in membranes and induces electrolyte leakage from the cells (Ebbs *et al.*, 2006). In addition, changes in the production and consumption of reducing agents are the reasons for cellular oxidative stress (Dietz, 2010; Grene, 2002). Occurrence of oxidative stress in the cells which have been treated by cyanide is the main damaging reason in cells (Borowitz *et al.*, 2006). Indeed, the main effects of cyanide on cells could be categorized as follows (reviewed by

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Borowitz *et al*., 2006):

a) Cellular acidosis via occurrence of a condition similar to hypoxia (Eisler, 1991) in which metabolism changes from aerobic to anaerobic. b) Increment of cytosolic calcium via release of calcium from intra- and extracellular reservoirs. c) Oxidative stress: high cytosolic calcium could activate cyclooxygenase enzyme that produces ROS (Shou *et al*., 2000); inhibition of electron transport chain causes leakage of electrons, transferring them on oxygen and production of ROS. d) Effects of cyanide on mitochondria: inhibition of complex IV prohibits the roles of mitochondria in both ATP production and acting as a calcium reservoir (Solomonson, 1981; Khodorov *et al*., 2002). In processes through which cyanide causes cell death mitochondrial permeability transition pores are opened and cytochrome c penetrates into the cytosol after decrease in ATP production which leads to cell death. e) Interaction in the action of the other enzymes: cyanide irreversibly binds to the succinate dehydrogenase as a non-competitive inhibitor (Puri, 2006).

Plants can basically tolerate higher concentrations of cyanide through an additional mitochondrial electron transport chain called alternative oxidase (AOX). In addition, plants are able to metabolize cyanide by different metabolic pathways such as cyanoalanine pathway (Miller and Conn, 1980). This pathway is present in all plants and acts coordinately with the pathway of ethylene production (Tittle *et al*., 1990). Cyanide is produced as a byproduct of the ethylene biosynthesis pathway with a ratio of 1:1. Therefore, it should be removed and metabolized immediately after right production. The presence of such properties in plants qualifies them as potentially efficient cyanide scavengers. In addition, some plants produce and accumulate cyanogenic compounds (cyanogenic glycosides) and have acquired increased tolerance capacities through evolution (Lechtenberg and Nahrstedt, 1999) although such a higher tolerance has not been proved to exist in high concentrations of exogenic cyanide (Ebbs *et al*., 2006; Yu *et al*., 2004).

An increase in AOX activity, other tolerance parameters and metabolizing capacity are crucial for an efficient cyanide phytoremediation. Acclimation is one of the ways through which plants acquire higher resistance against environmental stresses (Taiz and Zeiger, 2002; Hirt and Shinozaki, 2004). This phenomenon affects the resistance of plants against different environmental stresses such as cold, heat, and lack of access to nutritional elements and chemicals (with artificial or natural sources). As a result, plants should continually be exposed to a non-lethal intensity of the stressor. Changes in the pattern of genes expression and metabolites induce higher resistance (Desikan *et al*., 2003). The acquired resistance could be ten times higher than the intensity of used stressor (Dietz, 2010; Taiz and Zeiger, 2002).

The aim of this study was to determine whether the pretreatment of plants by non-lethal concentrations of cyanide could increase plants resistance to high concentrations of exogenous cyanide via acclimation. Pretreatment and acclimation of a lethal factor could help plants in a more affective resistance which implies an effective method of proper phytoremediation. Indeed, in this study we were going to increase resistance of plants. In studies on phytoremediation of cyanide, different species of willow have been considered as model plants because they usually grow fast and produce high biomass in both root and shoot. In addition, they adapt well to different environments (such as different local micro-climates in Iran), have a high rate of uptake and translocation from root to shoot and can be propagated easily (Ebbs *et al*., 2006; Bushey *et al*., 2006).

Materials and Methods

Preparation of cuttings and culture media: Fifteen to 20 cm cuttings were prepared from semi-woody 2-year old branches of a *Salix babylonica* tree in late spring, 2011. Cuttings were placed in perlite in half of their length from base, watered with tap water and kept in a growth chamber with a light regime of 16:8 (light : dark) and constant temperature of 24°C. Rooting of the cuttings occurred after 25 days. A hydroponic culture was used to treat the plants. The cuttings were transferred into the light proof vessels containing 10 L of a modified Hoagland solution which consisted of 0.5 mM $CaNO_3$, 0.5 mM $MgSO_4$, 0.1 mM KH_2PO_4, 0.5 mM KNO_3, 0.005 mM FeEDDHA (Ferric ethylenediamine–di-2-hydroxyphenylacetate), 0.01 mM H_3BO_3, 2 μM $MnCl_2$, 0.2 μM $ZnSO_4$, 0.2 μM $CuSO_4$ and 0.1 μM Na_2MoO_4. The pH of the media was adjusted to 5.8.

The solutions were aerated every 3 hours periodically and subsequently exchanged every 10 days. Evaporation was compensated daily by adding distilled water to the solutions. The plants were kept in these situations for more growth and adaptation to the greenhouse conditions. The following experiments were performed in greenhouse conditions during the late spring and summer. The range of temperature during the day was 25-36°C and 22-25°C at night.

The pH of solutions was adjusted to 5.8 but after treatment of plants by cyanide, it was readjusted to 7.8 to reduce evaporation of cyanide which occurs in lower pH. Since cyanide forms complexes with different metals and the complexes are not taken up easily by plants, the concentration of culture solutions were reduced 1/10 to diminish complex formation after treatment of plants with cyanide. In all of experiments, the employed form of cyanide was NaCN.

Pretreatment and treatment: To examine cyanide resistance threshold, the plants were treated by six concentrations of cyanide (0, 3, 5, 10, 15 and 20 mg $CN^- L^{-1}$) in 4 replicates, each containing two plants. Pretreatment of plants was performed by non-lethal concentrations of cyanide (3 and 5 mg $CN^- L^{-1}$) for 2 weeks. Since some of the added cyanide is evaporated

and some is taken up by plants, pretreatments were repeated every 48 hours by adding cyanide into the cultures. To determine the effect of the gradually increased cyanide concentration on resistance, the plants were treated by using concentrations which were increased every 48 hours up to 10, 12, 15, 20, 25, 30, 50, 75 and 100 mg $CN^- L^{-1}$.

Measurement of different physiological parameters: After pretreatment of plants for 2 weeks, we measured different parameters, including leaf chlorophyll concentration, oxygen consumption by roots, electrolyte leakage from roots, reducing capacity of root extract, relative dehydrogenase activity and superoxide dismutase activity of roots.

Chlorophylls a and b alongside with total and carotenoid concentrations were measured based on a spectrophotometeric method using absolute methanol (Lichtenthaler and Buschmann, 2001).

Measurement of oxygen consumption: We cut one gram fresh weight of root and washed it twice with distilled water and then transferred it into the test tubes containing 45 ml of distilled water simultaneously. Dissolved oxygen was measured every 2 hours using an oxygen meter (WTW with an electrode model Cellox 325, Germany). The oxygen meter was calibrated before use based on the instruction of the company.

Measurement of electrolyte leakage: After harvesting, 1 gram FW of root from each pretreatment was subjected to desorption using an ice cooled desorption solution containing 5 mM $CaSO_4$ and 5 mM Na_2EDTA, pH 5.8, for 10 minutes and then it was washed two times by double distilled water. The roots were then transferred into the test tubes containing 45 ml of double distilled water and shaken very gently (50 rpm). The electrical conductivity (EC) of the water was measured every 2 hours using an EC-meter (Elmetron, Poland).The EC-meter was calibrated based on the instruction of the company.

Reducing capacity test of root extracts: The reducing capacity of the roots was measured based on the FRAP test (Ferric reducing antioxidant power; Benzie and Strain, 1999). One gram FW of root was ground by using an ice cooled mortar and pestle and liquid nitrogen. Three mls of 50 mM phosphate buffer (pH 6) was added and after more grinding, the extracts were centrifuged in 12000 g at 4°C for 10 minutes. Throughout all of the steps, we kept the temperature below 4°C. The test was performed using TPTZ (2,4,6-tri(2-pyridyl)-1,3,5-triazine). Reduction of Fe^{3+} to Fe^{2+} by reducing agents of the extract at low pH caused an intense blue color after formation of TPTZ-Fe^{2+} complex with an absorption peak at 593 nm. A standard curve was obtained by using different concentrations of $FeSO_4$. The standard curve was linear between 10 and 100 μM $FeSO_4$.

Measurement of dehydrogenase activity: Dehydrogenase activity was measured according to Kittock and Law (1968). Two-tenth gram FW of root was submerged in 1 ml of 1% TTC (2, 3, 5 three

phenyltetrazolium chloride) solution for 2 hours. The roots were then submerged in 3 mls of methyl cellosolve (2-methoxyethanol) solvent and gently shaken for 6 hours until the roots became white. The solution was separated and centrifuged at 10000 g for 10 minutes. Finally, the absorption measured at 480 nm and the results were expressed as percent of the control plants.

Measurement of the superoxide dismutase (SOD) activity: We measured the activity of SOD according to Giannopolities and Ries (1997). The reaction solution contained 50 mM buffer phosphate, pH 7.8, 13 mM methionine, 75 μM NBT (ρ-nitrobluetetrazolium chloride), 2 μM riboflavin and 0.1 mM EDTA. The reaction started by adding 100 μL of root extract as the aforementioned preparation. One thousand μL of reaction solution and 100 μL root extract were mixed and put under florescent light with the intensity of 30 μ mole photon $m^{-2}s^{-1}$ for 15 minutes. By using dark samples as blank, the absorption was measured at 560 nm. Based on definition, preventing half of reaction in converting NBT (yellowish) to Formazan (gray-bluish) is defined as one unit of SOD activity.

Measurement of cyanide in medium: A colorimetric method was used to measure cyanide in medium (Gouldenet *et al.*, 1972). One ml of 1 M NaOH and 1 ml of 0.4% chloramine T was added to 5 mls of sample. The mixture was kept for 2 minutes and then 1 ml of pyridinebarbituric acid was added to each sample. The mixture was kept for 5 minutes, during which a purple color appeared. Light absorption at 578 nm was measured and the concentration of cyanide in medium was determined by using a standard curve (at the range concentration of 0.02 - 0.2 mg $CN^- L^{-1}$).

Statistical analyses: The experiments were performed in a complete randomized design. To determine the statistically significant differences between the means, multiple comparisons were made by one-way ANOVA and Tukey's HSD test. To determine the statistically significant effect of pre-treatment of cyanide on survival of plants, a Cox regression survival analysis was carried out considering three time ranges of 0, 24 and 48 h. Cox Regression builds a predictive model for time-to-event data. The model produces a survival function that predicts the probability that the event of interest has occurred at a given time t for given values of the predictor variables. The SPSS software (version 16 for Windows; SPSS Inc., Chicago, IL, USA) was used for statistical analyses. The reported data included means of 4 replicates ± SD.

Cautions: After the end of the experiments, the cyanide-contaminated solutions, such as nutrient solutions, were gradually exposed for being consumed by plants. After 1 week, the concentrations of cyanide in the solutions were measured and in case no cyanide was detected, they were poured out.

Results
Resistance threshold to cyanide: Treating the plants

by different concentrations of cyanide showed that toxicity occurred at 10 mg CN^- L^{-1} or more. Acute toxicity symptoms including drying of aerial parts in 24 hrs. without losing green color, browning of the roots and changing the color of root meristems from white to gray were observed. At lower concentrations (3 and 5 mg CN^- L^{-1}), aerial parts were not dried but at 5 mg CN^- L^{-1}, and moderate symptom of graying root meristems was observed. At 5 mg CN^- L^{-1}, some of the plants showed light yellowing of leaves that finally led to their becoming green again. Indeed, No toxicity symptom was observed at 3 mg CN^- L^{-1}, and 10 mg CN^- L^{-1} was considered as lowest toxic concentration of CN^- for the plants.

Effect of pretreatment on survival of plants after treatment by toxic concentration of cyanide: The results showed that survival in the pretreated plants was significantly higher than the control plants (table 1). Statistical model by Cox regression showed significant effect of pretreatment on survival of plants (chi-square = 20.8, df = 2, P < 0.001).

Effect of gradual increase of cyanide treatment on survival of plants: After pretreatments for two weeks, the plants were treated by cyanide through which the concentrations were increased each 24 h. The results showed that after increasing cyanide concentration to 20 mg CN^- L^{-1}, all pretreated plants by 5 mg CN^- L^{-1} and 75% of pretreated plants by 3 mg CN^- L^{-1} survived (Table 2). By increasing the concentrations to 30 and 75 mg CN^- L^{-1}, 50% of plants were still alive in pretreated plants by 3 and 5 mg CN^- L^{-1} respectively. Finally, when treating by 100 mg CN^- L^{-1}, all of plants died.

Measurement of chlorophyll and carotenoids: Results showed that the concentrations of Chlorophylls *a* and *b* and total chlorophyll significantly decreased in the pretreatment of 5 mg CN^- L^{-1} (P <0.05; Fig 1). No statistically significant difference was observed between control and pretreated plants by 3 mg CN^- L^{-1}. The concentration of carotenoids between control and pretreated plants was not different.

Oxygen consumption: Oxygen consumption by detached roots showed an increase in 5 mg CN^- L^{-1} pretreated plants. Oxygen consumption by these plants was 27% more than the control group plants (Fig 2).

Electrolyte leakage: No significant difference was detected between the two concentrations of cyanide pretreatment but significant differences were observed between the control plants and the pretreated plants by 3 or 5 mg CN^- L^{-1} (Fig 3).

Assessment of reducing capacity of root extract (FRAP): The results showed that reducing capacity of root extracts in the pretreated plants (both concentrations) was significantly lower than the control plants (P <0.05). There was also a significant difference between the two concentrations of pretreatments (Fig 4).

Dehydrogenase activity of roots: Pretreating the plants by two concentrations of cyanide (3 and 5 mg CN^- L^{-1}) caused a significant decrease in dehydrogenase activity when compared to the control plants but there was no difference between both groups of pretreated plants (Fig 5).

Superoxide dismutase activity: The results showed that pretreatment of plants by both concentrations of 3 and 5 mg CN^- L^{-1} significantly increased SOD activity in roots. Increased SOD activity in plants pretreated by 5 mg CN^- L^{-1} was higher than those of 3 mg CN^- L^{-1} (Fig 6).

Effect of pretreatment on cyanide uptake: No effect of pretreatment was observed in cyanide uptake by plants. The results showed that more than 99% of added cyanide to the medium was taken up by plants during 24 hrs. by all of plants.

Discussion

Cyanide is a relatively simple chemical but has wide range and multi aspect effects on oxygenic organisms. Basically, the effects of the agents that widely disturb the homeostasis in organisms are complicated; so, it could not be easy to discuss the observed phenomena. The most important effect of cyanide is its ability in ceasing ATP production in mitochondria via inhibition of electron transport chain. Depletion of cells from ATP strongly affects metabolism, membrane function and integrity, etc which finally leads to loss of homeostasis in cells.

One of the important characteristics of plants is acclimation to the unfavorable environmental condition. It happens when a plant is exposed to a non-lethal intensity of stressor. In these situations, the mechanisms that normally keep the cellular homeostasis will be stimulated which results in having a more resistant plant (Taiz and Zeiger, 2002). For example, in salt acclimation, the most important process is that ion homeostasis components act more strongly and more efficiently. In this case, the cells will be able to let additional ions enter into the cells (Quintero et al., 2000).

Plants show acclimation to hypoxia; accordingly, production of ATP will be in minimum and in special periods and concentration of NADH increases. In similar situations such as inhibition of mitochondrial complex IV by cyanide, electrons transfer on oxygen from other pathways and produce superoxide radicals (Grene, 2002) which then convert to hydrogen peroxide by superoxide dismutase. The produced hydrogen peroxide is the major agent for sensing of the presence of oxidative stress via redox status in the cells (Pastori and Foyer, 2001). Redox status and also oxidative stress factors (such as H_2O_2) in cells affect the expression of some gene families which code catalase and superoxide dismutase (Kliebenstein et al., 1998; Mittler et al., 2004). Indeed, a cascade of reactions occurs which increases plant's resistance against unfavorable situation which causes oxidative stress. Therefore, the increased superoxide dismutase activity that was observed in this study could be attributed to occurrence of oxidative stress in the presence of cyanide. Increased resistance to oxidative stress is one of the reasons for increased

Table 1. Effect of pretreatment of cyanide on resistance against lethal concentration of cyanide (10 mg CN⁻ L⁻¹) in *Salix babylonica* after 24 and 48 h. The data are survived plants as percent.

Time	Cyanide pretreatment (mg CN⁻ L⁻¹)		
	0	3	5
24	0	75	100
48	0	75	100

Table 2. Survival of plants treated by gradually increased cyanide concentrations. The plants were pretreated by 0, 3 and 5 mg CN⁻ L⁻¹ preliminary for 2 weeks. The shown data are survived plants as percentage.

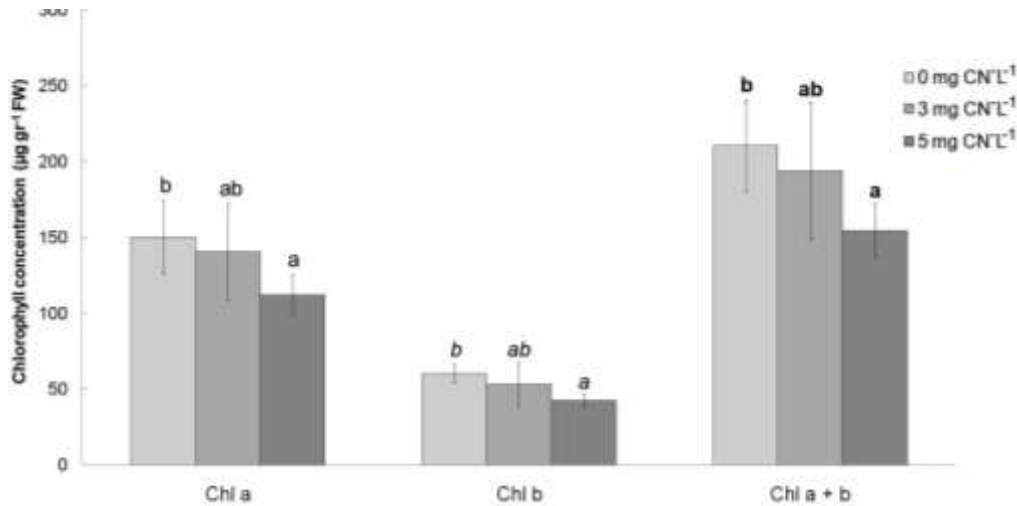| Cyanide treatment (mg CN⁻ L⁻¹)	Cyanide pretreatment (mg CN⁻ L⁻¹)		
	5	3	0
10	100	75	0
12	100	75	0
15	100	75	0
20	100	75	0
25	50	50	0
30	50	50	0
50	50	25	0
75	50	25	0
100	0	0	0

Fig 1. Effect of cyanide pretreatments (0, 3 and 5 mg CN⁻ L⁻¹) in hydroponics on chlorophyll concentration in leaves of willow. Different letters in each series (normal letters for chlorophyll *a*, italicized letters for chlorophyll *b* and bold letters for total chlorophylls *a* and *b*) indicates statistically significant difference based on ANOVA (Tukey's HSD; *P* <0.05). Bars represent arithmetic means ± SD of *n* = 4 replicates, each containing 2 plants.

resistance against cyanide when plants are treated by salicylate (Ghasemi and Mokhtari, 2013).

Oxidative stress responses in plants are needed for resistance against environmental stressors (Bowler and Fluhr, 2000). Redox balance in a cell is related to the relative rates of production and scavenging ROS (Grene, 2002). Stimulators which increase ROS production or decrease antioxidant activities could upset redox balance and cause oxidative stress. Oxidative stress affects cells in different ways such as damaging cell compartments like membranes or changing redox potential (Schafer and Buettner, 2001). The evidence from the current study, including decrease in chlorophyll, more activity of SOD and ion leakage (the well-known effects of oxidative stress), demonstrated the occurrence of oxidative stress by pretreatment of

cyanide.

To start acclimation and appropriate responses, plants should sense changes in environmental conditions. Moreover, transferring of messages to response elements and finally plant's response via causing change in biochemical processes are needed to confront stress (Hirt and Shinozaki, 2004). In first step, specific (Ha *et al*., 1999) or general sensors are needed. In the case of absence of a known specific sensor, metabolic situations of cells change gradually and finely tuned adjustments change by increase in the stressor intensity. Resetting the balance occurs after activation of some regulatory mechanisms. Responses of plants to some stressors such as natural or artificial substances and changes in nutrient availability occur through this mechanism (Wang *et al*., 2004). One of the most
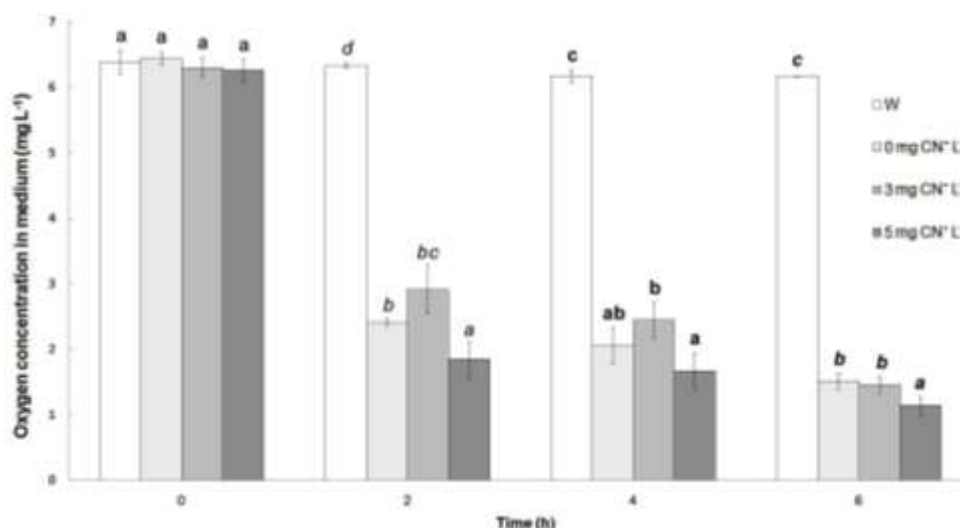
Fig 2. Oxygen consumption by detached roots of pretreated (0, 3 and 5 mg CN⁻ L⁻¹) willow plants. W, water without added root. Different letters in each time series (normal letters for the time of 0, italicized letters for 2h, bold letters for 4h and bold italicized letters for 6 hrs.) indicates statistically significant difference based on ANOVA (Tukey's HSD; *P* <0.05). Bars represent arithmetic means ± SD of *n* = 4 replicates, each containing 2 plants.
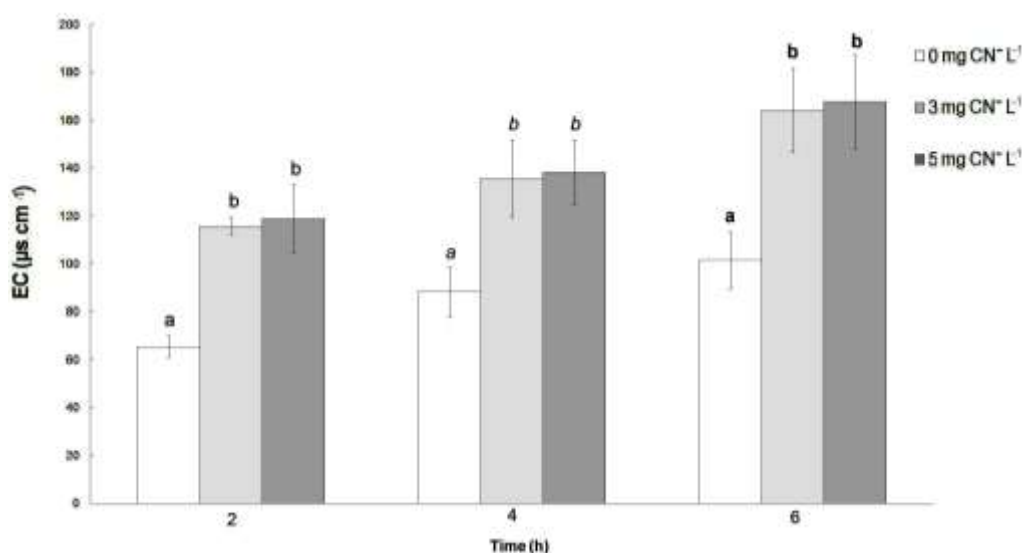

Fig 3. Electrolyte leakage from detached roots of willow plants pretreated by different concentrations of cyanide (0, 3 and 5 mg CN⁻ L⁻¹). Different letters in each time series (normal letters for 2 hrs., italicized letters for 4 hrs. and bold letters for 6 hrs.) indicates statistically significant difference based on ANOVA (Tukey's HSD; *P* < 0.05). Bars represent arithmetic means ± SD of *n* = 4 replicates, each containing 2 plants.

important responses is regulation of redox and antioxidant defense of the cells (Dietz, 2010). Redox status of the cells is important in acclimation responses such as light acclimation and development of plastids and mitochondria (Wagner *et al*., 2008; Dietz, 2008). Production of more ROS in stresses is accompanied by change in ratios of NADH/NAD⁺ and NADPH/NADP⁺ toward more reduced or oxidized status (Dietz, 2008). There are some additional pathways for oxidation of accumulated reduced NAD (P) H in plant cells such as mitochondrial external NAD(P)H dehydroganases and alternative oxidases (AOXs).

AOXs contain two groups, AOX1 and AOX2, which strongly respond to environmental stresses (Clifton *et*

al., 2006). The results of the current study showed increase in oxygen consumption by pretreated plants. While cyanide inhibits oxygen consumption by mitochondrial complex IV, it could be concluded that a higher respiratory function of pretreated plants has been performed by activity of AOX which has been induced by cyanide via change in redox status at the presence of cyanide in medium.

Observations in this study indicated that the reducing capacity of the cells was decreased. In other words, reducing agents such as NAD (P)H were decreased. As noted before, more activity of AOXs could be counted as a reason. In addition, cyanide also inhibits reducing agent producing pathways such as
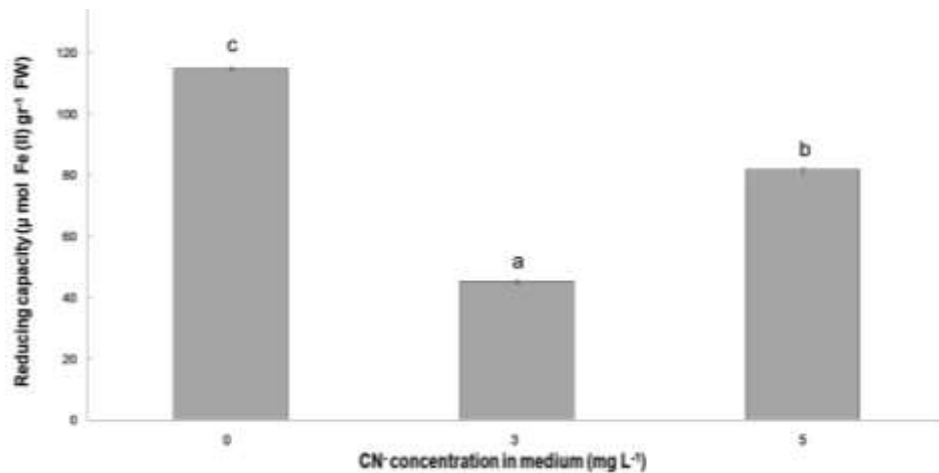
Fig 4. Reducing capacity of root extract of willow plants pretreated by different concentrations of cyanide (0, 3 and 5 mg CN⁻ L⁻¹). Different letters indicates statistically significant difference based on ANOVA (Tukey's HSD; $P < 0.05$). Bars represent arithmetic means ± SD of $n = 4$ replicates, each containing 2 plants.
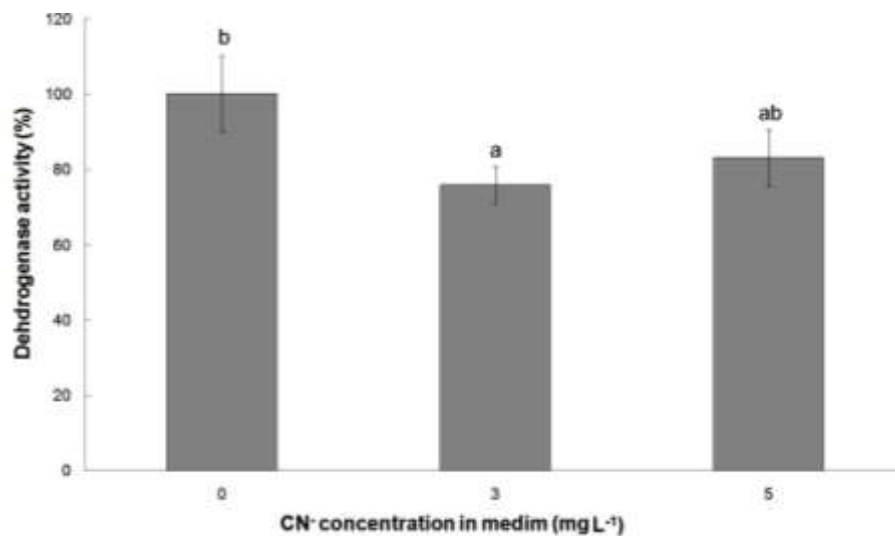

Fig 5. Effect of cyanide pretreatment (0, 3 and 5 mg CN⁻ L⁻¹) on root dehydrogenase activity in willow. Different letters indicates statistically significant difference based on ANOVA (Tukey's HSD; $P < 0.05$). Bars represent arithmetic means ± SD of $n = 4$ replicates, each containing 2 plants.

glycolysis and tricarboxylic acid cycle (Puri, 2006). Decrease in dehydrogenase activity, which is a major utilizer of NADH in the cells, was observed in this study. Indeed, an increased AOXs activity and decreased production of reducing agents in parallel to inhibition of dehydrogenase activity have reduced the reducing potential of the cells.

Conclusion
In this study, we observed an increased resistance to cyanide in pretreated plants. Resistance could be gained via different reasons: preventing the penetration of cyanide into the cells, activation of cyanide resistant pathways such as AOXs and activation of the pathways which metabolize cyanide. Nonetheless, we observed no difference between pretreated plants and the control plants in uptake of cyanide. Therefore, the first option did not occur during pretreatment. In summary, the effects of the presence of cyanide in medium (even in

sublethal concentrations) including decrease of chlorophyll content, increase in electrolyte leakage and activation of SOD were observed in this study. These effects could occur via a mild oxidative stress in the presence of cyanide. Decrease of ATP production in the presence of cyanide for a long term of 2 weeks decreased membrane integrity.

For a deeper investigation of cyanide acclimation and cyanide phytoremediation, it will be useful to study the activity of cyanide metabolizing pathways such as cyanoalanine pathway in pretreated plants. In addition, hormone and nutritional treatments in parallel to pretreatment could have synergic effects on cyanide resistance.

Acknowledgment

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