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Morphological characteristics and electrophoretic profiling is a primary tool to investigate Nickel (Ni) stress tolerance in *Cenchrus ciliaris*: A Cholistan desert grass

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Abstract

Abiotic stresses are one of important ecological factors that disturb the normal process of growth and development in plants. Our environment is going to change rapidly so it is very important to protect plants from such ecological hazards. The first effect of these stresses is alteration in morphology of a plant. Secondly proteins metabolism of plants are also affected very rapidly after exposing them to any kind of stress. Theses morphological and protein changes are important to assess the tolerance capabilities of a plant and also give a clue about the types of internal changes that occur in plants. There are many kinds of abiotic stresses and heavy metal stress is one of them. Heavy metals are important micronutrients but their excess amounts are toxic for plants. *Cenchrus ciliaris* a perennial grass from Cholistan desert was exposed to Nickle metal stress in different concentrations (0.3, 3, 10, 20mg/L) growing hydroponically to gain insight into the morphological changes and changes in plant total protein. Our results showed remarkable changes in plant morphology and electrophoresis pattern during stress that would be very important in our further studies on molecular characterization under abiotic stress. Results indicate that *Cenchrus ciliaris* has the ability to grow in moderate amounts of Ni but higher concentrations are toxic and lead to death.

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Introduction

Weather of our surroundings is changing rapidly. These progressive changes in environment alter the ecological requirements of land plants (Abdallaha *et al.,* 2014). Drought, salinity, temperature, light, ozone (O_3), carbon dioxide (CO_2) stress and heavy metal stress are known as ecological stresses and is a major cause of crop loss (Shah *et al.,* 2001; Watanabe and Lam, 2009). Land plants are static inhabitants so disincentive to these inhabitants is due to environmental stresses that restrain their growth and development (Zheng *et al.,* 2015). Many ecological stresses are due to anthropogenic influences (Klaus and Heribert, 2004) and heavy metal stress is one of them that cause a serious threat to terrestrial biota especially plants are more prone to these because of their static habitat (Pinto *et al.,* 2003). It is well known that heavy metals as micro-nutrients are essential for plants to carry on their life cycle.

In plants a wide range of reactions take place in respond to these stresses that result in morphological changes (Nouri and Komatsu, 2013). In the form of growth arrest, senescence and also influence energy synthesis processes (Maksymeic, 2007). Usually metals are affective for plant's metabolic processes but only when present in bioavailable forms and its excessive amount become lethal to plants (Valko *et al.,* 2005) (Nagajyoti and Sreekanth, 2010) bioavailable forms of metals become toxic only if stress remains continuous for a prolonged period it may lead to metabolic changes in cells that result in reduced growth and sometimes cause plant death that is undesirable (Chinnusamy *et al.,* 2004). Plants try to survive under such conditions and as a result morphological and molecular changes take place in response to toxicity and to generate tolerance. These are called stress-specific adaptive responses of plants (Kosova *et al.,* 2011).

Nickel is recognized as a trace element and an essential component of plant growth (Lopez and Magnitskiy, 2011). It is known as a Ni metal having dual characteristics because it influences plant growth by playing a role in urea hydrolysis (Yusuf *et al.,* 2011).

It is also very necessary for certain enzyme activities, nitrogen metabolism and various other biochemical, physiological and growth processes (Yusuf *et al.,* 2011). Nickel at low concentration enhances the plant growth (Ashraf *et al.,* 2011) but it is phytotoxic at high concentration due to its toxic effects on various metabolic processes in plants.

Proteomics technologies are gaining interest to advance our knowledge in plants against abiotic stress tolerance (Hussain *et al.,* 2013). Proteins such as the enzymes required for biosynthesis of various osmoprotectants, stress tolerance proteins, chaperones, and detoxification enzymes are generated in response to stress and are likely to function by protecting cells are being focused (Floris *et al.,* 2009; Mithöfer *et al.,* 2004). Ni toxicity in plants affects various biochemical mechanisms by interacting with important biomolecules like protein. As a result different stress responsive proteins appeared in cell to maintain cellular functions as well as specific morphological symptoms also appeared in plants that tell the internal physiological disturbance (Seregin and Kozhevnikova, 2006). The expression of some new protein and inactivation of some existing protein in cells under stressful condition told us about the adaptive strategy of a plant. Changes in protein profile in cell are not a single step. A broad range of mechanisms have involved when protein profile of a cell has changed i.e. it may be changed at the level of DNA or RNA and may be at translation level and at post translation level. Regulatory mechanism has also changed under such condition. It can be understand in a simple way that proteins that act as primary players change whole internal story of a cell. Toxicity of Ni in plants depends on many different parameters like plant species, plant organ and growth stages (Valko *et al.,* 2005).

There is some evidence about tolerance of *Cenchrus ciliaris* to some abiotic stress factors (Franklin *et al.,* 2006; Griffa *et al.,* 2010) but in this research our focus was on Ni metal to investigate the influence of its low and high concentrations on *Cenchrus ciliaris* growth.

Though Ni metal is beneficial for plant growth and metabolism at low concentration but it affect plant at a specific concentration that need to be assessed and it was also observed that Ni toxicity in plant do specific responses in morphology that showed initial and primary effect of toxicity.

The present study was designed to find out Ni stress effect on *C. ciliaris* by examining morphological changes because little is known about the toxic levels of Ni metals in *Cenchrus ciliaris*. Secondly we aimed to check the physical barrier like changes in protein profile of *Cenchrus ciliaris* under Ni stress because these changes in protein can be helpful to understand the some basic effect of Ni stress on plant growth. In future our focus is on Plants that's showed ability to tolerate stress. We are also interested to explore the molecular mechanism involved in stress adjustment.

Materials and methods

Site of experiment

C. ciliaris grass is found growing in Cholistan because of its natural tendency to tolerate many drastic conditions like abiotic stress factors (Arshad *et al.,* 2007; Arshad *et al.,* 2008).

Samples of *C. ciliaris* were collected from Cholistan Institute of Desert Studies (CIDS), The Islamia University of Bahawalpur and identified through physical examination by a botanist. The solvents and chemicals used were purchased from different registered companies (Merck, Sigma, Fermentasetc).

Plant collection

C. ciliaris was collected from fields of Cholistan institute of desert studies during spring and autumn seasons.

Cutting and Sterilization of stubs

C. ciliaris stubs (approximately of equal length about 5cm) were used to regenerate plant and were made from specific parts having uncut eyes. Surface sterilization of stubs was done for 20min. in 1% (v/v) sodium hypochlorite (NaOCl), followed by 70% ethanol then sterilized stubs were washed several times with distilled water.

Growth Conditions and Regeneration of plant

Surface sterilized stubs were regenerated in Hoagland solution hydroponically in plastic pots (Hoagland, 1920; Hoagland and Arnon, 1950). Plant regeneration process was initiated in March in natural environment and climatic changes were recorded periodically.

Duration of experiment

Cenchrus ciliaris is a grass and become mature in a short span. Plant was grown for a period of 45 days. Stress was applied when plants were mature (after one month) and was harvested after two week because at high concentrations the plant was starting pale near to dead.

Experimental design during Ni treatment

A single set of germination experiments comprised of 30 plants and performed in duplicate. Every set of experiment was consisting of two treatment groups. Group I was given no treatment (0 mg/L) (designated as control) Group II received Ni metal ($NiCl_2$ solution) treatment (0.3 mg/L, 3 mg/L, 10 mg/L, 20 mg/L) and was designated as test group. The dates and temperature during each experimental step were also recorded.

Nickel (Ni) treatment

A 1000 mg/L stock solution of $NiCl_2$ was prepared. To impose Ni stress test concentrations i.e. 0.3, 3, 10 and 20 mg/L were used. Volume of Hoagland Solution i.e. 6 liter was maintained in each pot to avoid variation in each treatment. Hoagland solution without Ni was used as control (0 mg/L).

Scheme of study

Nickel (Ni) stress levels				
0 mg/L	0.3 mg/L	3 mg/L	10 mg/L	20 mg/L

Morphological parameters

Morphological parameters were noted at the time of harvesting to check the effect of treatments. Following morphological parameters were noted after applying stress. Flag leaf area, No of tillers per plant, Length of shoot, Awn color, No of spikes, Internodes on the main stem. Node on the main stem, Maximum length of main root, Main root health, Leaf color, Position of leaf, Root branches, Flag leaf length, Flag leaf width and plant health.

Statistical Analyses

Statistical Analyses (analysis of variance (ANOVA)) of all data were done by using statistix 8 software.

Cutting of plant

All plant parts were cut into small pieces with the help of scissor to make grinding easy. Protein was extracted in extraction buffer using a homogenizer.

Extraction of protein

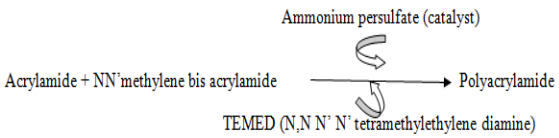
The plant samples (5 g) were taken in pre-chilled 50ml tubes kept on ice. The plant material was homogenized in a known volume (6 ml) of the phosphate buffer pH 7 containing PMSF, Triton x-100 and EDTA, for protein extraction. The sample tubes were kept on ice during homogenization to avoid any damage to enzymes at higher temperature. The homogenized materials were filtered through cheese cloth to remove debris. Filtered extracts were centrifuged at 16000 RPM at 4 °C for 20 minutes. The clear supernatants were collected in labeled eppendorfs and pellets were discarded.

The aliquoted supernatants were preserved at -70 °C for further analysis. Total protein was estimated with Bradford reagent (fermantas) by using 0.1 M phosphate buffer (Bradford 1976; Stoscheck 1987).

Sodium dodecyl sulfate Polyacrylamide gel electrophoresis (SDS-PAGE)

SDS PAGE is the most widely used electrophoresis technique to separate protein by mass. This is because the ionic detergent (SDS) denatures and binds to proteins to make them evenly negatively charged.

When a current is applied, all SDS-bound proteins in a sample will migrate through the gel toward the positively charged electrode (Steinberg *et al.,* 1996).



Preparing the Gel Mix and Pouring of gel

Gel mix 11% was prepared by mixing all of the components except 10% APS and TEMED in the required amounts to make up a specific volume of the gel mix for the resolving and 5ml for the stacking gel and kept on ice.

Required amounts of 10% APS and TEMED were added and thoroughly mixed to the gel mix just before pouring. n-butanol was poured on poured gel mix After polymerization of the resolving gel the n-butanol layer was removed, the gel top was washed with distilled water followed by pouring of the stacking gel mix containing 10% APS and TEMED and insertion of the comb in the poured stacking gel mix. The stacking gel was allowed to polymerize for a further 30–45 minutes.

Sample preparation for Electrophoresis

Extracted proteins were resolved on an 11% Polyacrylamide gel. Electrophoresis conditions were optimized as follows. A known volume of the protein extract containing 70 µg total proteins in case of leaf, stem and root samples were mixed with 6 µl of 5X loading buffer.

The mixture was diluted with phosphate buffer pH 7, heated in a boiling water bath for 5min, cooled, briefly centrifuged and loaded in a single well of the Polyacrylamide gel. Compositions percentage of the gel for stacking and resolving gels are given in the appendix.

Sample Loading and Electrophoresis

After polymerization the comb was removed carefully and the gel was placed in the electrophoresis chamber containing tris-glycine buffer in upper and lower buffer chambers. Wells were washed immediately with de-ionized water to remove unpolymerized acryl amide. A tracking dye, Bromophenol blue, was used to ascertain movement of the protein components. 30 µl sample was loaded in a well with the help of micropipette. The gel was run at 120 V for 2 hours and 45 minutes at RT. When the dye bands had moved to the opposite end of the column, the power was switched off, the gel was removed and stained with dye i.e., Comassiee blue for 4 hours. Protein bands were observed after distaining the gel in disdainer.

Result and discussion

When a plant is subjected to any biotic or abiotic stress, the first observed response is a change in morphology with a consequent decrease in metabolism. Changes in morphology are a fundamental step to study plant response to any kind of stress that may be biotic and abiotic.

In our work we used four Ni concentrations to check its effect on plants growth by observing their morphological responses. *Cenchrus ciliaris* plants were grown in Hoagland solution and stress was applied by using different concentration of Nickel metal i.e. (0.3 mg/L, 3 mg/L, 10 mg/L, and 20 mg/L). Plant absorbs Ni in the same way as other metals i.e. passive diffusion and active transport.

The ratio between the active and passive inputs depends on Ni^{2+} concentration in the nutrient solution. At low Ni^{2+} concentration active transport and at higher concentration the role of passive transport mechanism occurred (Seregin and Kozhevnikova, 2006).

These stress levels (ranges) were first determined through literature and then a pilot project was done to check the toxic range that causes death of *C. ciliaris*. It was seen that 30 mg/L $NiCl_2$ causes plant death. Our growth conditions were in accordance to our experimental design in which we were going to check the effects of stress and absorption of Ni metal on plant morphology and metabolism (Table 1).

Results showed that regenerated plants grew to full bloom with fresh green color in the absence of any applied treatment but as stress increased a change in plant growth was seen that shows *C. ciliaris* absorbs Ni, however every set of plant behave differently in different treatments of Ni.

The optimum temperature range for *C. ciliaris* plant was 20 ^oC to 37 ^oC and we grew this plant in that season having optimum temperature that was suitable for its growth (Table 1).

This temperature range was also suitable for Ni absorption. Literature review also proves that suitable temperature range for Ni absorption is 23^oC to 30^oC and at low temperature Ni^{2+} absorption is considerably reduced from the nutrient solution (Hasanuzzaman *et al.*, 2013; Jackson, 2007).

In the present work the pH of Hoagland solution (without adding $NiCl_2$) was 6.5 because metals are in general more soluble at low pH and consequently their phyto availability increases and it was studied that acidification may increase the essential metal availability for plants (Solymosi and Bertrand, 2012).

It was also observed that pH of Hoagland solution increased with increase in $NiCl_2$ concentration which showed that addition of $NiCl_2$ increase the basicity of nutrient solution and it was studied that accessibility of Ni usually declines at higher pH values of the soil solution due to the formation of low-soluble complexes. Ni is translocated from roots to shoots in several forms with citrate and malate complexes (Seregin and Kozhevnikova, 2006).

Our results showed that when *Cenchrus ciliaris* absorbed Ni^{2+} metal and transported it to its different tissues it caused inhibition of growth. This growth inhibition affect showed that our plant was affected by Ni stress and it has lethal effect on plant growth. From these results conduction of metal and nutrient solution was also observed in *C. ciliaris*.

When we study literature to find out the reason of growth inhibition it was described in there that growth retardation under Ni metal stress is due to reduced plasticity of cell walls that results from cell wall lignifications.

Such lignifications and reduced cell wall plasticity hinders mitosis and chromosomal aberrations (Almeida *et al.*, 2007). Morphological changes occurs at various levels of growth but we noted morphological changes at the time of harvesting after applying stress (Ni) to check the effect of different stress level with reference to control (0mg/L) because Ni excess can trigger decreased absorption of micronutrient (Ca, Mg, Fe, K) (Solymosi and Bertrand, 2012).

Table 1. Chronology of Plants grown hydroponically under Nickel metal stress.

	Start of the experiment	Sprouting 1st root	Sprouting 1st shoot	Sprouting 1st year	Maturity	Metal Treatment	Harvesting
		March			April		May
	14	17	18	3rd	12	15	1st
E. M Temp °C	27.22	28	28	19	17	16	19
D. Temp °C	36.12	37	36	35	34	34	35
N. Temp °C	31.1	31	31	22	20	19	21

E.M= Early morning, D. Temp = Day Temp, N. Temp= Night Temp.

According to our results flag leaf length and Flag leaf area was almost same at 0mg/L and 0.3 mg/L and it was maximum at 3 mg/Land then decreased gradually (at 10 mg/L and 20 mg/L) but flag leaf width increase at 20 mg/L but this increase was still less than observed at 3 mg/L.

Form these results it was noticed that this increase in flag leaf area at 3 mg/L Ni metal concentration was due to increase in flag leaf length but width was almost similar in each treatment.

Statistical analysis also showed the significant differences (Fig. 1, 2, 3). Literature proved that nickel in lower amounts caused a noticeable increase in plant growth. However plant health showed a decreasing trend at increasing concentration of nickel (Misra *et al.*, 2010).

In our study an increase in flag leaf area at 3 mg/L might be due to the growth conditions because *Cenchrus ciliaris* was grown in hydroponic culture so water availability was in excess because it was studied that leaf growth was more sensitive to water stress.

It has also been reported that water shortage mostly reduced leaf growth and in turn the leaf areas in many species of plant like *Populus* soybean and many other species like wheat (Munzuroglu and Geckil, 2002).

Mostly it was seen that Ni stress decreased leaf area because the volumes of intercellular spaces and palisade and sponge mesophyll decreased (Seregin and Kozhevnikova, 2006). When statistical analysis of these parameters was done it was seen that flag leaf length and area was 96% dependent on each other, and have highly significant +ve correlation.

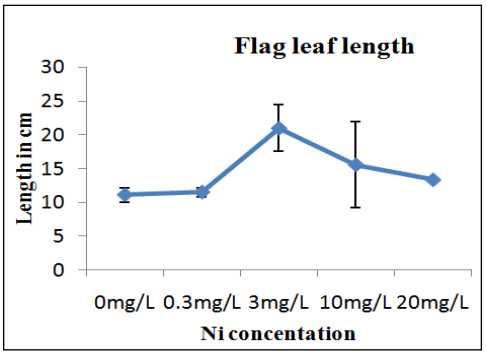


Fig. 1. Change in Flag leaf length of *C. ciliaris* under different conc. of Ni at p≤0.05

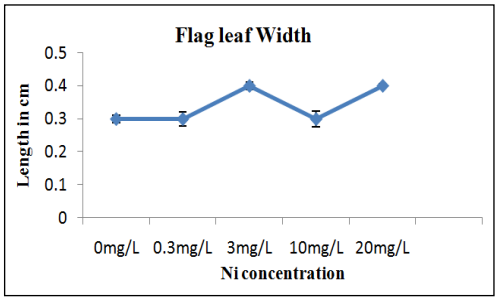


Fig. 2. Change in Flag leaf width of *C. ciliaris* under different conc. of Ni at p≤0.05

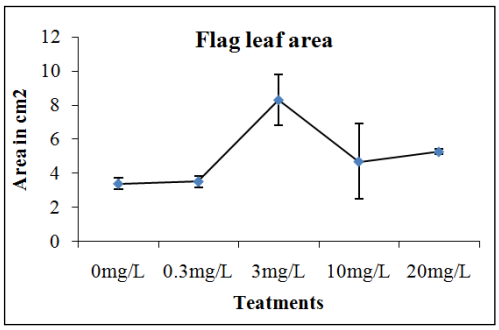


Fig. 3. Change in Flag leaf area of *C. ciliaris* under different conc. of Ni at p≤0.05

Results showed that shoot length at 0.3 mg/L $NiCl_2$ concentration was almost same as at 0 mg/L (control) and then it gradually decreased with increasing $NiCl_2$ concentration (3 mg/L, 10 mg/L, 20 mg/L).

Plants at 3 mg/L and 10 mg/L $NiCl_2$ concentration had almost same shoot length but decrease in shoot length was very remarkable in plants exposed to 20 mg/L $NiCl_2$ treatment (Fig. 4). These results were in accordance with already reported literature. Such type of changes has also been observed in various other plants under Ni metal stress e.g. high concentration of Ni^{+2} showed stunted growth of soybean seedlings and leaves (Hussain *et al.,* 2013). When a plant absorbs excess amount of Ni metal, it interacts directly or indirectly with plastid lipids, pigments, proteins, and important cofactors required for photosynthesis. They may replace another functional metal within enzymes or in key molecules such as chlorophyll (Solymosi and Bertrand, 2012). In plants mostly 10 µMNi^{+2} does not lead to significant alterations in shoot growth except for a slight increase in fresh mass. In contrast, 200µM Ni causes inhibition of shoot growth, and chlorophyll contents (Gajewska *et al.,* 2006). Since stomatal conductance and the transpiration rate remains unaffected by low Ni^{2+} treatment (Llamas *et al.,* 2008), low dose of Ni stimulated the growth performance of pea seedlings in contrast to its inhibitory role at high doses (Kumar Tewari *et al.,* 2004). These results were in accordance with our study on *Cenchrus ciliaris*.

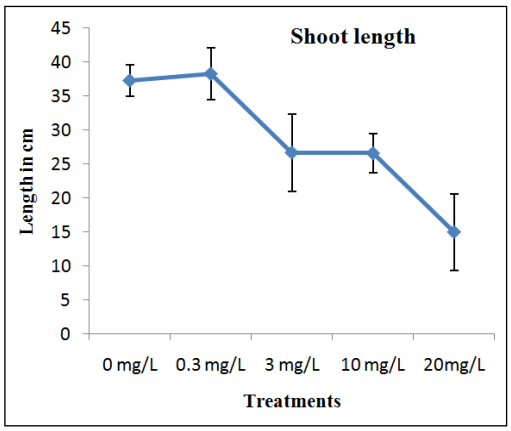


Fig. 4. Change in shoot length of *C. ciliaris* under different conc. of Ni at p≤0.05

It was also observed in our study that main root length showed a decreasing trend with increase in $NiCl_2$ concentration (Fig. 5). This was because the roots which have a direct contact with nutrient solution having Ni showed reduced growth.

Statistical analysis of our results also proved that main root length, shoot length and number of spikes have +ve correlation. In some plants like legumes and grasses, small amounts of Ni were essential for root nodule growth and hydrogenase activation but excess amount decreased the root growth (Ahmed and Ashraf, 2012; Seregin and Kozhevnikova, 2006). Inhibition of root growth was an early effect of heavy metal toxicity in plants especially in species which accumulate Ni more deeply in their roots (Seregin and Kozhevnikova, 2006) and generally all plant types showed these symptoms (Huillier *et al.,* 1996). The root growth of onion was inhibited under 100mm Ni concentration. The inhibition of root growth was also seen in maize and it was due to the higher levels of Ni found in maiz roots**.** A possible mechanism of Ni toxicity in maize was inhibition of carbohydrate transport from leaves to roots as suggested by the accumulation of starch in the leaves. This decrease in carbohydrate supply in roots show reduced mitotic activity in root tips. Thus the first effect of Ni was a direct inhibition of the mitotic activity in roots (Huillier *et al.,* 1996). Ni inhibit mitotic activity by 80% which may reduce radical length (Huillier *et al.,* 1996). In 2002 it was reported that Ni metal stress decreased shoot and root growth and reduction in leaf area in plants (Hussain *et al.,* 2013).

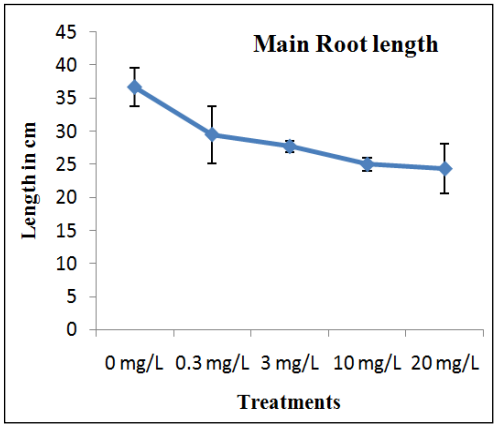


Fig. 5. Change in main root length of *C. ciliaris* under different conc. of Ni at p≤0.05

Under Ni stress conditions nutrient equilibrium gets disturbed in conducting tissues of main stem and stem become tilted and weak. Tillers which are lateral shoots emerging from the axils of the true leaves at the base of the main stem of the plant have great agronomic importance.

Tillers growth depends upon the growth and stability of main stem. Once a tiller has developed three or more leaves, it becomes nutritionally independent of the main stem and forms its own root system. In *C. ciliaris* when number of tillers was noted it was observed that no. of tillers was almost same in control plant (0mg/L) and plants exposed to 3 mg/L $NiCl_2$ stress but in other treatments (at 0.3 mg/L, 10 mg/L and 20 mg/L) it decreased as compared to control (Fig. 6).

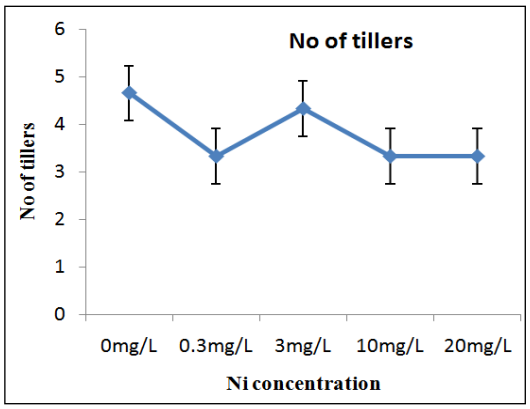


Fig. 6. Change in number of tillers of C. ciliaris under different conc. of Ni at $p \leq 0.05$

It was noted that in *C. ciliaris* the no. of tillers was going to decrease with increasing stress of Ni metal except at 3 mg/L treatment. The reason might be that the main stem showed variable length and it was tilted so new sprouted tillers could not be supported by main stem ref. Amazingly the number of tillers at 3 mg/L is almost similar to control 0 mg/L which indicates that at this Ni concentration plant might has developed tillers production as an adaptive strategy to cope with high quantity of Ni absorbed by roots. when number of spikes per plant of *C. ciliaris* was noted it was observed that no. of spikes in control plant (0 mg/L) was maximum than all treatments but in 3 mg/L $NiCl_2$ treatment, stressed plants has maximum than other treatments (at 0.3 mg/L, 10 mg/L and 20 mg/L). Here it decreased gradually and are also less than control (Fig. 9). Spike growth depends upon no. of tillers and growth e.g. all tillers produce spikes in wheat (Farooq *et al.,* 2008). It was found in literature that Cu stress caused the reduction in number of tiller number of tillers and the number of spikes per plant (Farooq *et al.,* 2008) because of floret death at the terminal and basal ends of the spike during stem extension (Maqbool *et al.,* 2015).

When we examined the no. of nodes and internodes in *C. ciliaris* plants under various stress treatments it was found that at 3 mg/L Ni metal stress the no. of nodes and internodes was maximum and even greater than control plants (0 mg/L).

Other treatments showed same number of nodes like control (0 mg/L) i.e. three (3) (Fig. 7,8). Statistical analysis proved that shoot length and No. of internodes, and nodes are 30% dependent on each other and was non-significant but No. of nodes and inter nodes was 100% dependent on each other and have +ve correlation and was highly significant. During the study it was noticed that *C. ciliaris* under Ni^{+2} metal stress showed a gradual decrease in plant color, plant health, spikes color, root health and root branches (Table 2). It was studied from literature that no. of nodes and internodes depends on the length of main stem but in some cases (sea grass- *Halophila ovalis*) plants shows shorter stem as compared to the control but number of nodes are not affected and remains same (Ambo-Rappe *et al.,* 2011).

Number of nodes and internodes also depend upon plant health specially stem heath and cell membrane stability. If plant is stable under any type of stress it shows that plant whole metabolism is not disturbed and nutrient uptake is in balance state so shorter stem also develop more nodes and internodes to start earing (Aown *et al.,* 2012). During our study a gradual decrease in plant color, plant health, spikes color, root health and root branches was observed (Table 2).

Studies on plants proved that Chlorosis is a common symptom of toxicity of heavy metals including Ni^{+2}. Decrease in chlorophyll content in tissues of metal treated plants was due to disturbances in the synthesis of this pigment as well as its increased degradation. It has been suggested that decline in chlorophyll content in shoots of Ni-treated wheat plants may result mostly from its enhanced degradation (Brahim and Mohamed, 2011). The decline in chlorophyll content in plants exposed to heavy metals stress such us Cu was believed to be due to: (a) inhibition of enzymes associated with chlorophyll biosynthesis and (b) inhibition of uptake and transportation of other metal elements such as Mn, Zn and Fe by antagonistic effects.

In the fresh leaves of maize, the concentration of chlorophyll content decreased with increased concentration of Ni^{+2} from 20 to 100 µM (Hussain *et al.,* 2013).

Table 2. Morphological Parameters of *Cenchrus ciliaris* under Nickel stress.

Parameters	Ni concentration				
	0 mg/L	0.3 mg/L	3 mg/L	10 mg/L	20 mg/L
Root branches	Many	Much	Normal	Very less	Very less
Root health	Good	Very Good	Good	Normal	Weak
Color of plant	Dark green	Fresh green	Fresh green	Yellowish light green	Yellowish light green
Plant health	Very good	Good	Good	Normal	Weak

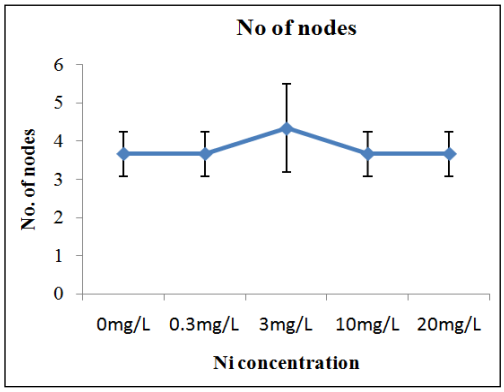


Fig. 7. Change in number of nodes of *C.ciliaris* under different conc. of Ni at p≤0.05

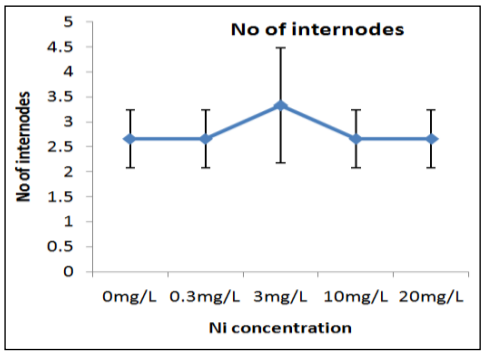


Fig. 8. Change in number of internodes of *C. ciliaris* under different conc. of Ni at p≤0.05

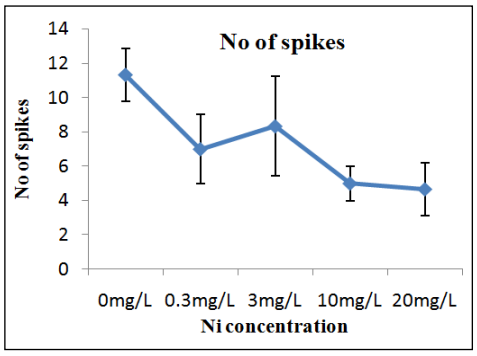


Fig. 9. Change in number of spikes of *C.ciliaris* under different conc. of Ni at p≤0.05

Variation in electrical conductivity was observed. Water showed extremely less E.C that was near to standard value of E.C. Compared to control, EC increased in all stress treatments applied but maximum activity was observed at 20 mg/L concentration. 0.3 & 10 mg/L $NiCl_2$ containing Hoagland solution showed almost the same EC value. EC of Hoagland solution at 3 mg/L was minimum. Overall an increasing trend in E.C was seen from 3 mg/L to 20mg/L concentration respectively (Fig. 11).

Increasing trend in E.C showed that *C. ciliaris* has taken Ni stress (Fig. 10) that causes membrane degradation leading to leakage of ions (Baccouch *et al.,* 1998). Hoagland solution at 3 mg/L Ni^{+2} showed less E.C as compared to 0.3 mg/L that shows at these concentrations a change take place in plant internal processes but plant occupy this level of stress but membrane damage occur but at 3 mg/L some other mechanism become active and ratio of membrane damage is less.

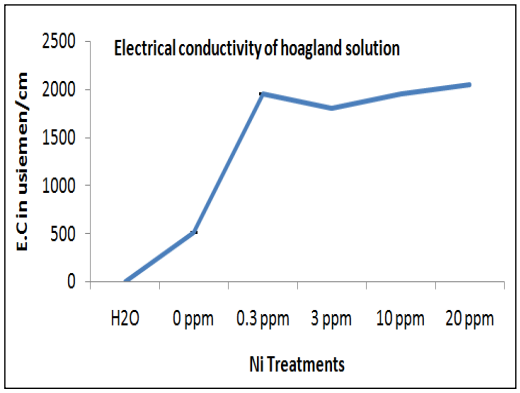


Fig. 10. Change in Electrical conductivity of Hoagland solution after applying different concentrations of Ni

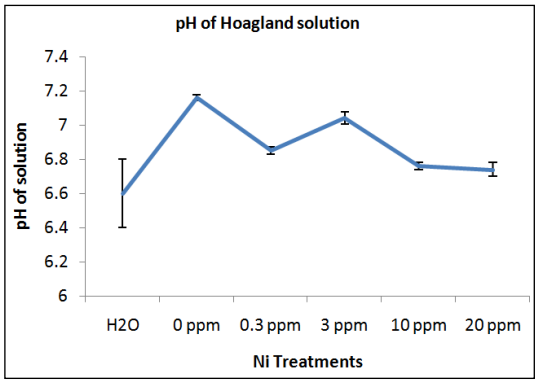


Fig. 11. Change in pH of Hoagland solution by after applying different conc. of Ni

pH of Hoagland solution was measured after completion of experiment and a slight decrease in pH was observed by increasing stress (concentration). Distill H_2O showed neutral pH. By adding different concentration of $NiCl_2$ in Hoagland solution a slight decrease in pH was observed Compared to control, pH of Hoagland solution decreased in all test treatments ($NiCl_2$ treatments) but at 3 mg/L stress treatments the pH was comparatively more than all other treatments (0.3, 10 and 20 mg/L (Fig. 10). Decreasing trend in pH shows that *C. ciliaris* was absorbing Ni metal at their all treatments (Fig. 11). From this study we have concluded that *C. ciliaris* can withstand moderate amount of Ni i.e. 3 mg/L however morphological changes tells that some metabolic changes occur in our plant. This was more cleared when change in total protein pattern was checked by SDS PAGE. Electrophoretic pattern in different tissues of *C. ciliaris* showed that with increasing Ni^{+2} stress, protein expression decreases (Fig. 12, 13). Our results showed that tissues of *C. ciliaris*, has prominent bands between molecular mass i.e., 200-100 kDa, 70-60 kDa and 50-40 kDa respectively (Fig., 12, 13). In stem some additional bands appeared in between 25 kDa -15 kDa. In root the results were not conclusive as the amount of protein was less however a band of molecular weight 30 kDa was very prominent (Fig. 12, 13). This shows that stress halted the cellular life of a plant and appearance of new protein bands give multiple clues i.e., some stress reliever proteins like heat shock proteins (Prasad *et al.,* 2001; Wang *et al.,* 2004) and metal othionein may be involved because these are low molecular, cysteine-rich, and metal-binding proteins (Clemens *et al.,* 1999; Huang and Wang, 2010; Sheng *et al.,* 2007) and in our plants under study, appearance of bands of 15, 20, 30, 35, 45, 40, 55 kDa make our hypothesis strong about metal othionein and heat shock proteins literature proves that plants that produce more metal othioneins and heat shock proteins has more tolerance against oxidative stress by inducing expression of antioxidant enzymes (Emamverdian *et al.,* 2015; Grennan, 2011). Secondly, some transporter proteins (Kramer *et al.* 2007) may be involved that help to transport more Ni to *C. ciliaris*.

It means some metabolic changes take place that need to explore. In future our focus is to explore the nature of these proteins to find out the stress mechanism in *C. ciliaris*. From this study we have concluded that *C. ciliaris* can withstand moderate amount of Ni i.e. 3 mg/L however morphological changes tells that some metabolic changes in our plant.

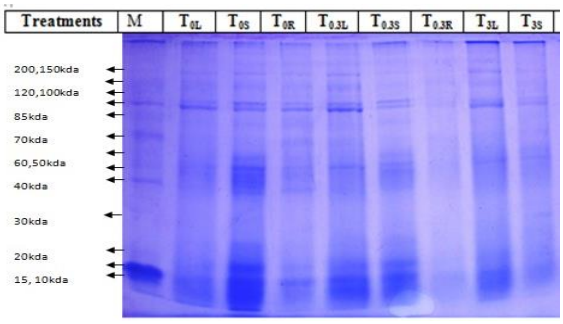


Fig. 12. Electrophoretic pattern of protein in various parts of *C. ciliaris* plants grown hydroponically under Ni stress (ranges 0 mg/L to 3 mg/L).

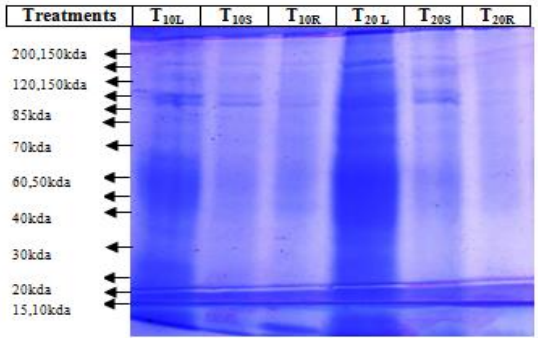


Fig. 13. Electrophoretic pattern in various parts of *C. ciliaris* plants grown hydroponically under Ni stress (ranges 10 mg/L to 20 mg/L).

Toxicity level by measuring morphological parameters of *Cenchrus ciliaris* under Ni metal stress.

No.	$NiCl_2$ stress (mg/L)	Observation	Tolerance/Toxicity
1	0 mg/L	Plant was fully groomed with green spikes and leaves. Strong and long roots, straight long shoots	Control plant
2	0.3 mg/L	Plant was green, spikes were light green, strong and long roots, straight long shoots	Highly tolerant (nontoxic)
3	3 mg/L	Brown tips, white spikes, better growth of roots and shoots	Tolerant (less toxic)
4	10 mg/L	Plant was yellowish green, spikes were white, weak roots	Moderately tolerant(toxic)
5	20 mg/L	Yellow tips, tilted growth, white spikes, inhibited root and shoot growth	At risk (very toxic)

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