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Article

Morphological Responses of *Hakonechloa macra* (MAk) to Drought and Salinity Stress *In vitro*

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Abstract: Using in vitro tissue culture methods, the present work analyses the capacity of the grass species Hakonechloa macra (MAk) to withstand abiotic stresses associated with drought and salinity. The drought stress was simulated through the addition of PEG at various concentrations osmotically (with respect to water) to create different levels of water deficits. The salinity stress was created by adding NaCl to the standard MS medium in order to determine how changes in osmotic pressure affect plant growth. The results show that the morphology of the sample can respond in various ways. There was a significant and negative relationship between the concentration of stress agents (salt) and vegetative growth indicators; tiller number, branch length, and tuft fresh weight. Significant reductions of the root system efficiency were also recorded. Rooting percentages decreased from 76% in the control sample to 0% at high levels of stress agents (1% NaCl), thus indicating that there is extreme sensitivity of the root system to osmotic pressure and ionic toxicity. As a result of this study, there has been a steady increase in the rate of tissue necrosis and a corresponding decrease in the rate of survival of tissues as well, with a total of 30 percent having been reduced from the maximum amount of applied stress experienced. According to this study, Hakonechloa macra has very limited morphological defensive mechanisms that can be used to defend itself from stressful surroundings and therefore should be given very careful consideration with respect to growing in very harsh drought or salt-affected environments.

Keywords: Tissue culture, Drought stress, Salinity, PEG, NaCl, Morphological indicators, *Hakonechloa macra*

Introduction

Abiotic stressors like cold, salinity and drought create substantial global crop losses and represent significant challenges to plants, their essential functions and how they grow/productivity (especially given the present climate) [1], [2]. In terms of agricultural land, salinity currently affects 6% of total land area, while around 20% of irrigated land is salty [3], [4].

Plants can show changes to drought and salinity through the morphological, physiological, biochemical and molecular changes they undergo. The way that plants respond to stress (e.g. drought and/or salinity) is complex and can be seen at the molecular level (e.g. analysis of transcriptomes) as there are changes in gene expression [5], [6], specifically regarding the expression of genes encoding for antioxidant enzymes.

Osmotic agents such as sucrose, mannitol, sorbitol or poly(ethylene glycol) PEG can be added to culture media to induce osmotic stress to facilitate studies on drought stress responses [7], [8]. Poly(ethylene glycol) is often used to induce water deficit conditions, because PEG will not pass through the outer boundary of the cell wall [9], and PEG molecules with molecular weights greater than approximately 3000 will not be taken up [10]. As a non-ionic, inert osmotic agent, PEG can lower the water potential of plant nutrient solutions without being absorbed or adversely affecting plant growth in a toxic manner [11].

Increased use of in vitro cultural methods to test different plant species for drought resistance is becoming more prevalent [7]. There are many advantages associated with the use of in vitro selections, such as rapidity and the minimal amount of plant material, which support the rapid propagation of selected genotypes [12]. In addition, in vitro selections are made under controlled conditions, which help to identify differences in growth and developmental responses [13]. Based on a number of studies [14], the results of greenhouse tests of drought resistance in genotypes are generally consistent with the results from field experiments.

To better understand how plants respond to drought and salt stress, tissue culture experiments can be used to clarify cellular pathways for drought and salt tolerance and to evaluate PEG and NaCl tolerant cell lines [15], [16]. A large body of research has been done to develop salinity tolerant turf grass species. Comparing results from Roy and Chakraborty's & Qanbar Asker et al studies show that Imperata cylindrica [4], [17], Digitaria ciliaris and Cynodon dactylon exhibit the highest degree of salt tolerance among grasses that have been evaluated. According to Daba et al, five levels of salinity (0 – 20 dSm-1) were applied to evaluate growth of three genotypes of Chloris gayana. The most salt-tolerant genotype, ILRI-6633, was identified as the best performer of the three genotypes evaluated [18].

Henschke stated that joint growth of Briza media and Deschampsia cespitosa at low salinity levels (5 g·dm-3) was negatively affected [19]. At higher levels of salinity (15 – 60 g·dm-3), the growth of Spartina pectinata 'Aureomarginata' was also greatly affected. As one of the ornamental grasses being studied at low salinity levels, Hakonechloa macra (Poaceae: Arundinoideae) has been of great interest. Hakonechloa macra has been reported to exhibit shade tolerance and relatively slow growth rates [20], [21], making it a useful model for studying adaptive responses to multiple types of environmental stresses [22]. While limited research has been reported regarding the in vitro response of Hakonechloa macra to joint osmotic and salinity stress, researchers will benefit from conducting further studies on the adaptability of Hakonechloa macra to combined osmotic and salinity stress. As a result of this study, we are going to determine how tolerant Hakonechloa macra is to different amounts of osmotic and salinity stress in vitro (in plant tissue culture). We will be using polyethylene glycol (PEG) to simulate osmotic stress, and sodium chloride (NaCl) as the salinity source. Additionally, we will be measuring the impact of these stress treatments on important morphological parameters to better understand how the plant adapts under controlled environments.

Materials and Methods

The Department of Ornamental Plants, Dendrology, and Landscape Architecture at the University of Life Sciences in Lublin will conduct experiments to test various grasses. The explants for the project are tufts of Hakonechloa macra (MAk) obtained from established tissue cultures.

The effect of polyethylene glycol PEG and sodium chloride NaCl on Hakonechloa macra MAk was assessed using a system of seven tufts (new uniform microshoot samples, measuring between 1.5 - 2cm) grown in each of the flasks containing MS medium [Murashige and Skoog, 1962], and supplemented with vitamins B1 - 0.1 mg.dm-3; B6 - 0.5 mg.dm-3; PP - 0.5 mg.dm-3; glycine - 2.0 mg.dm-3; sucrose - 30g.dm-3; agar - 6.75g.dm-3; benzyladenine BA - 2.5 mg.dm-3; indol-3-butyric acid IBA - 0.1 mg.dm-3.

The concentrations of Polyethylene Glycol (PEG) including; 0.5%, 1%, 2%, 4%, and 8% (or 0.5, 1, 2 + 4, and 8 g/L), will be calculated by the number of grams per litre of the compound to add 0.5%, 1%, 1.5%, or 2% (or 86.2, 172.4, 258.6, 344.8, 431.0, or 517.2 mM) of NaCl sodium chloride to the previous formula, control the pH of the total mixture of media (i.e. without PEG and NaCl) to a pH of 5.7-5.8 using 0.1 N HCl and 0.1 N NaOH by adding 6.75 g of agar per litre of total liquid before autoclaving for 24 minutes at 121°C with a pressure of 1 bar.

Three flasks containing 7 microshoots (21 total shoots) were placed under 22 degrees celcius during the day and 20 degrees celcius at night, with a light intensity of 30µmol·s-1·m-2 and a 16 hour light/dark period. After 6 weeks of cultivation in tissue culture, several characteristics were measured: no. of tufts; % alive and damaged; no. of shoots; average length (mm) and weight (mg); no. of leaves; % rooting; no. of roots; average length (mm) and weight (mg) of roots.

Experiments were set up in a Completely Randomized Design (CRD) with 21 replications of each treatment. The data was statistically analyzed using SAS version 8.2, 2001 (Little et al., 2002) and means compared with Duncan's multiple range tests at the 5% sign level.

Results and Discussion

The effect of polyethylene glycol (PEG) on the morphological growth parameters of *Hakonechloa macra* cultured in vitro is presented in Table 1. The results showed that increasing PEG concentrations significantly affected all measured traits. The number of tufts decreased with increasing PEG concentration. The highest value (3.28) was recorded in the control Fig 1, followed by 0.5% PEG (2.57), while all higher PEG levels (1–8%) significantly reduced tuft formation, showing the lowest values.

Similarly, the number of shoots was highest at 0.5% PEG (6.09), followed by the control (5.52), while the lowest number (3.42) was observed at 1% PEG. Treatments with 2%, 4%, and 8% PEG showed intermediate values without significant differences among them.

Table 1. Effect of different concentrations of polyethylene glycol (PEG) on the Morphological growth indicators of *Hakonechloa macra* induced in vitro Culture

(PEG) %	No. of Tuft	No. of Shoots	shoots length (mm)	No. of leaves	weight (mg)
0	3.28 a	5.52 ab	32.28 a	19.09 ab	162.38 a
0.5	2.57 b	6.09 a	24.08 b	21.47 a	156.71 a
1	1.71 c	3.42 c	21.09 b	11.38 c	91.33 b
2	1.90 c	4.33 bc	22.73 b	15.52 bc	128.71 ab
4	1.85 c	4.23 bc	19.79 b	14.09 bc	110.52 b
8	1.76 c	4.47 bc	21.45 b	15.57 bc	127.57 ab

The similar letters on values do not differ significantly according to Duncan's multiple test at a 5% probability level.

The number of leaves was significantly influenced by PEG levels. The highest number of leaves (21.47) was obtained at 0.5% PEG, followed by the control (19.09), whereas the lowest value (11.38) was recorded at 1% PEG. Other treatments showed lower values.

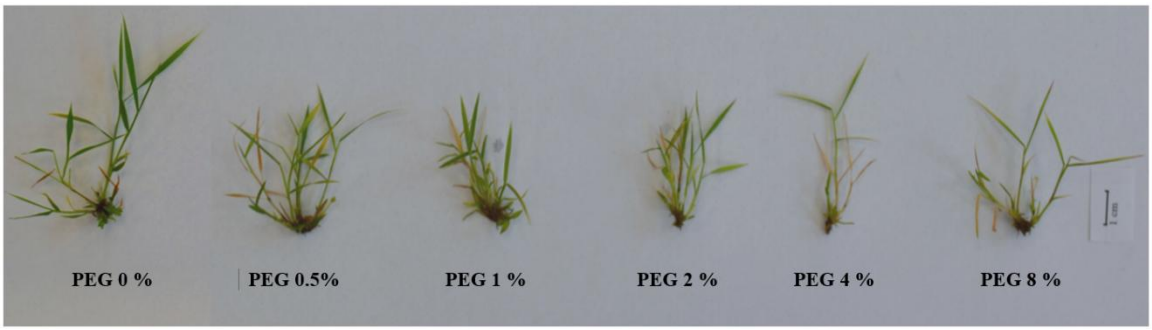

Figure 1. Microcuttings of *Hakonechloa macra* after 6 weeks of cultivation on MS medium supplemented with PEG *in vitro.*

Shoot length was highest in the control (32.28 mm), which differed significantly from all PEG treatments. All PEG concentrations (0.5–8%) caused a reduction in shoot length, with no significant differences among them.

The highest fresh weight (162.38 mg) was recorded in the control treatment (0% PEG), which was statistically similar to 0.5% PEG (156.71 mg). A significant weight reduction was observed at 1% PEG (91.33 mg), while moderate values were recorded at 2%, 4%, and 8% PEG concentrations.

Generally, Low PEG concentration (0.5%) either maintained or improved certain growth parameters, while higher concentrations (≥1%) harmed the morphological traits of *Hakonechloa macra..*

Effect of polyethylene glycol (PEG) on the Survival and necrosis rate of *Hakonechloa macra*

The relationship between the two variables is clearly shown in the graphs of the two variables: the percentage of plant survival rate and percentage of necrosis affecting plants due to increasing concentrations of polyethylene (PEG). The correlation between the concentration of PEG and tissue viability shows there is a clear negative relationship between concentration of PEG and plant survival rate, while at the same time, there is a direct relationship between the concentration of PEG and tissue necrosis percentage.

From the decrease in tissue viability, it can be determined that as the concentration of PEG increases, the tissue viability begins to exhibit a slow, steady decline (100% viability at level one to 70% viability at level six).

The necrosis rate was increasing from Level 1 to Level 6, 0% (either assumed or only a small amount of necrosis, because it could not be quantified at Level 1) to 30% (at Level 6). Since 70% of plant samples remained viable at the greatest concentration used, the linear nature of the necrosis rates suggests that it would be possible to completely degrade the biological system of the plant produced in culture by increasing the concentration beyond this point. Figure 2 shows perfect complementarity (100%) between survival and necrosis rates at all levels; thus, the experimental results indicate that PEG was the direct cause of damage and could be quantified with great precision..

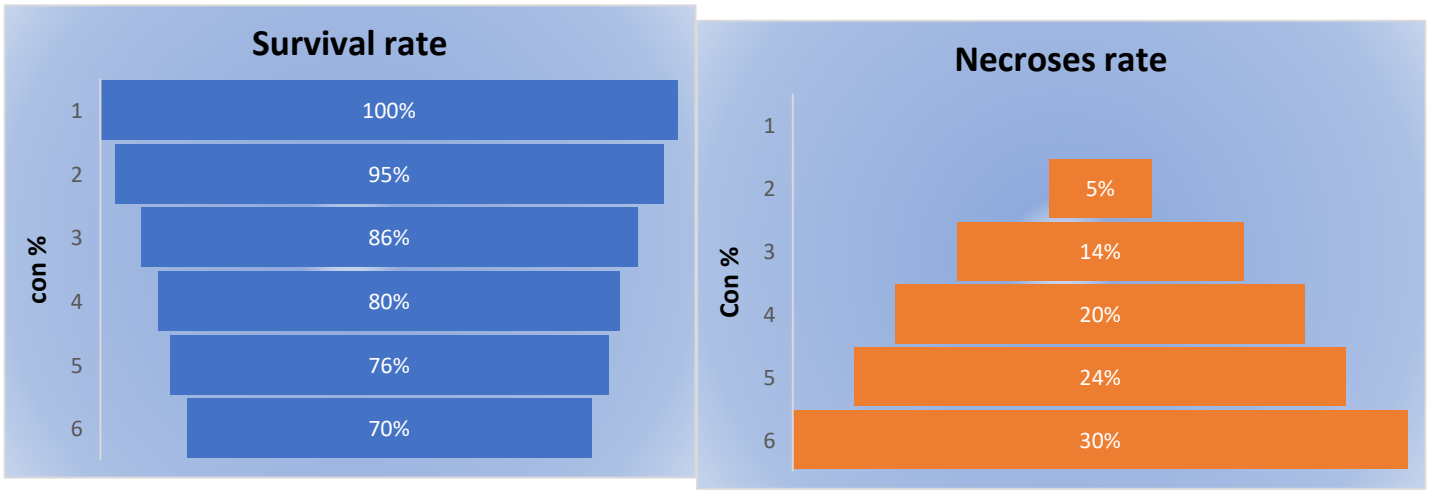

Figure 2. Effect of different concentrations of polyethylene glycol (PEG) on the Survival and necrosis rate indicators of *Hakonechloa macra* induced in vitro.

Effect of polyethylene glycol (PEG) on the Rooting growth indicators of *Hakonechloa macra*

The results of the second table demonstrate how low PEG concentrations affect rooting parameters of Hakonechloa macra grown in vitro and thus, the results of the experiment provide insight into PEG concentrations and their impact on these variables. As stated previously, the root system is much more responsive to osmotic stress than was the case with the shoot system. The rooting success percentage declined sharply and significantly as PEG concentration increased.

The rooting rate at the control level (0% PEG) was 76%, a high rooting rate for the species studied. As the total concentration of PEG reached 0.5%, this rooting rate dropped significantly to 43%. Both the 1% and 2% concentrations of PEG completely inhibited the tissue's ability to produce roots, yielding only approximately 5% rooting.

Concerning the number of roots (No. Root), the mean No. roots per plant decreased very significantly and gradually over time. The No. roots for the control (0.00%) sample was 2.47, which decreased to 1.28 No. roots at a concentration of 0.5%, and by the time the concentrations were above 0.5% the No. roots were nearly zero (0.095). The mean Length of Roots (mm) in the control (0%) sample was measured as 0.04 mm, while at the 0.5% concentration level, it was measured as 3.57 mm. The mean Weight of Roots (mg) was measured as 1.47 mg for the control, and dropped to 0.47 mg at a 0.5% concentration, with mean weights at higher concentrations being 0.021 mg.

Table 2. Effect of different concentrations of polyethylene glycol (PEG) on the Rooting growth indicators of *Hakonechloa macra* induced *in vitro*.

(PEG) %	Percentage of rooting %	No. of roots	length of root (mm)	weight of root (mg)
0	76 % a	2.47 a	0.04 a	1.47 a
0.5	43 % b	1.28 b	3.57 b	0.47 b
1	5% c	0.095 c	0.23 c	0.021 b
2	4.8% c	0.095 c	0.35 c	0.021 b
4	19% d	0.52 bc	0.35 bc	0.32 b
8	0 % e	0.00 c	0.00 c	0.00 b

The similar letters on values do not differ significantly according to Duncan's multiple test at a 5% probability level.

These data show that the level of water stress can significantly reduce the shoot lengths of wheat accessions. As the increased concentration of PEG in the solution was increased from control to 8%, every accession had an overall decrease in their shoot length. This study's findings agree with other studies, which have reported that when moisture stress was increased, both shoot and root systems reacted similarly with significant growth retardation compared to the control conditions in laboratory environments with moister conditions [23], [24].

Plants adaptively adjust their morphology in order to prevent water loss through evaporation (transpiration) during periods of drought. The amount of adjustment that occurs depends on both the intensity and duration of the drought [25].

Drought stress might be to blame for the reduction in root length; therefore, it makes sense that the study in question would show higher values than previous studies involving mechanisms of drought resistance. These finding also correlate with previous results obtained from Iranian almond seedlings [50]. Root systems that are long will help provide a seedling with a mechanism of drought resistance since they allow a seedling to reach deeper into the soil. Larger diameter xylem vessels and reduced axial resistance to hydraulic flux can work together with root systems to improve overall water acquisition efficiency during periods of limited water supply.." [23], [26].

Effect of Sodium Chloride (NaCl) on the Morphological Growth Indicators of *Hakonechloa macra*

Data from Table 3 indicate that high levels of NaCl have an adverse effect on several measures of plant morphology in Hakonechloa macra plants that were grown in vitro. The plants were not very tolerant to salinity (0.5% NaCl), and while there was no statistically significant difference in the number of shoots or weight of the plants relative to the controls (0%) at that concentration, "shoot length" and "number of tufts" showed highly significant negative impacts. A NaCl concentration of 1% is considered to be the threshold at which all parameters began to show significant negative impacts. For example, the number of leaves dropped from 19.09 (in the control) to 7.81, and the weight of the plants at this concentration was approximately 50% less than the controls (79.19 mg). With respect to the point of complete inhibition of growth (2% NaCl and higher), it was observed that plants had completely stopped growing (0) at all concentrations tested (2%, 4%, and 8%). Figure 3.

Table 3. Effect of different concentrations of Sodium Chloride (NaCl) on the Morphological growth indicators of *Hakonechloa macra* induced in vitro.

(NaCl) %	No. of tuft	No. shoots	length of shoots (mm)	No. leaves	weight (mg)
0	3.28 a	5.52 ab	32.28 a	19.09 ab	162.38 a
0.5	2.00 b	5.28 a	23.90 b	15.57 a	164.48 a
1	1.52 b	3.38 b	16.52 c	7.81 b	79.19 b
2	0.00 c	0.00 c	0.00 d	0.00 c	0.00 c
4	0.00 c	0.00 c	0.00 d	0.00 c	0.00 c
8	0.00 c	0.00 c	0.00 d	0.00 c	0.00 c

The similar letters on values do not differ significantly according to Duncan's multiple test at a 5% probability level.

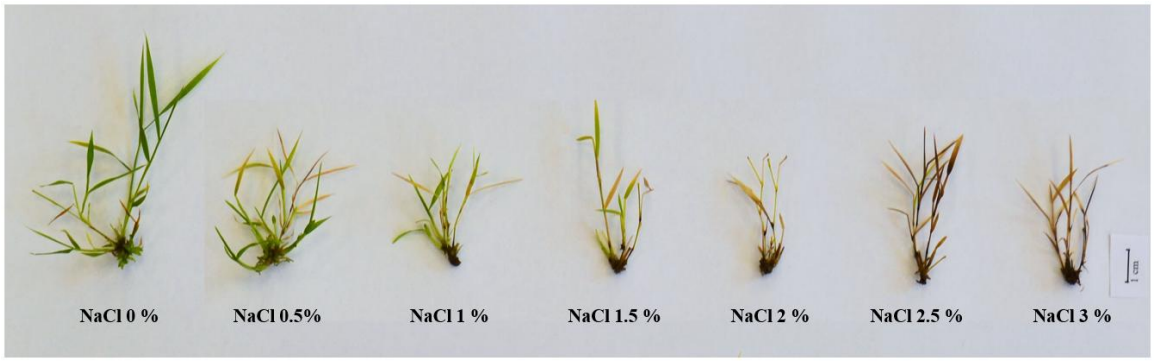

Figure 3. Microcuttings of *Hakonechloa macra* after 6 weeks of cultivation on MS medium supplemented with NaCl *in vitro.*

Effect of different concentrations of Sodium Chloride (NaCl) on the Survival and Necrosis rate indicators of *Hakonechloa macra*

The graphs below illustrate the impact of various levels of salinity found as sodium chloride (NaCl) on the viability of Hakonechloa macra grown in vitro with the use of two methods of measurement. The plant maintained excellent levels at these lower concentrations until the higher levels were reached. The control group (Control - 1) had a 100% survival rate. At 0.5% concentration (NaCl - 2), recovery was similar to that of the control group, with a survival rate of 100%. When the level increased to that of 1% (NaCl - 3), the plant experienced a 25% decline in recovery.

Concerning Tissue Necrosis Rates (Necrosis Rate): Level 1 and Level 2 had a necrosis rate of 0%. This confirms that no tissue had signs of degradation or programmed cell death as reflected in the survival rates. Level 3 (1% NaCl) had a necrosis rate of 25%. The necrosis rate increased significantly at this level, indicating that an additional 25 cells would die as a result of exposure to 1% NaCl compared to the Level 2 exposure to 0% NaCl..

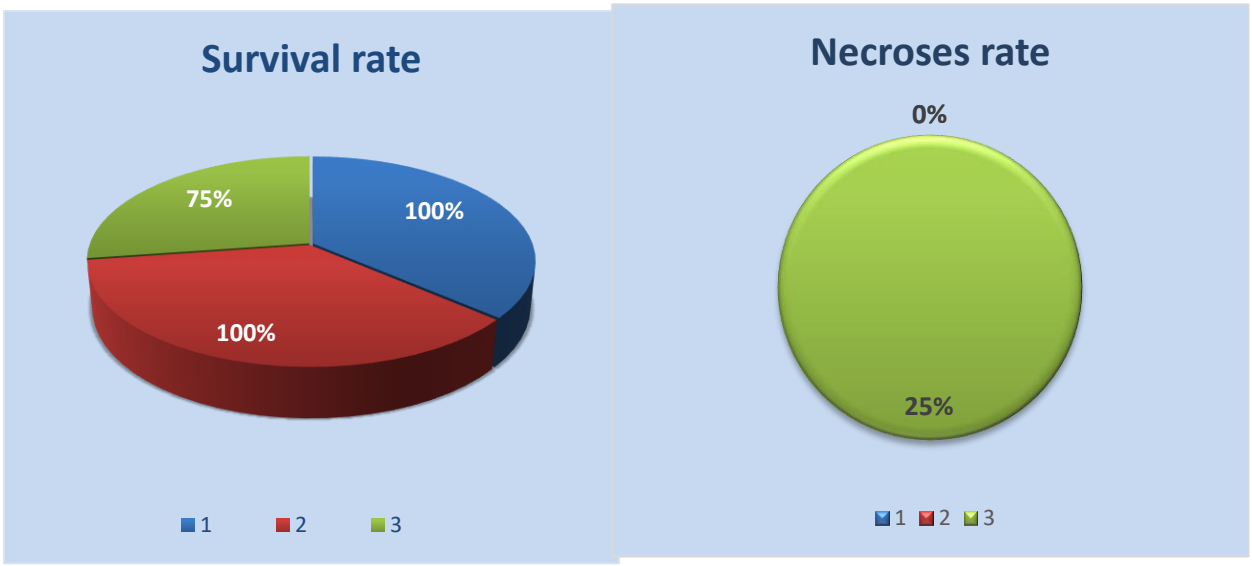

Figure 4. Effect of different concentrations of Sodium Chloride (NaCl) on the Survival and Necrosis rate indicators of *Hakonechloa macra* induced *in vitro*. (1: control, 2: 0.5%, 3: 1%)

Effect of different concentrations of Sodium Chloride (NaCl) on the Rooting Growth Indicators of *Hakonechloa macra*

The data presented in this table show the effect of sodium chloride (NaCl) concentrations on the rooting growth indicators of in vitro cultured *Hakonechloa macra* plants.

Rooting percentage (%): The rooting percentage showed a sharp and non-linear deterioration with increasing medium salinity. In the control sample (0% NaCl), the rooting percentage was 76%. When the concentration was raised to 0.5%, the percentage dropped to 33.3%, meaning a loss of more

than half of the plant's ability to produce roots. At a concentration of 1%, the plant's ability to root was completely lost (0%).

Number and length of roots (No. of roots & Length): The average number of roots decreased significantly from 2.47 in the control to 0.61 at 0.5% concentration, then to complete zero at 1% concentration. Regarding length, a numerical increase in length was observed at 0.5% concentration (1.26 mm) compared to the control (0.04 mm).

Root fresh weight (mg): A massive decrease in root biomass occurred. The weight dropped from 1.47 mg (at 0%) to 0.09 mg (at 0.5%), a reduction of over 93%.

Table 4. Effect of different concentrations of Sodium Chloride (NaCl) on the Rooting growth indicators of *Hakonechloa macra* induced in vitro**.**

(NaCl) %	Percentage of rooting %	No. of roots	length of root (mm)	Weight of root (mg)
0	76 % a	2.47 a	0.04 a	1.47 a
0.5	33.3 % b	0.61 b	1.26 b	0.09 b
1	0 % c	0.00 b	0.00 b	0.00 b
2	0 % c	0.00 b	0.00 b	0.00 b
4	0 % c	0.00 b	0.00 b	0.00 b
8	0 % c	0.00 b	0.00 b	0.00 b

The similar letters on values do not differ significantly according to Duncan's multiple test at a 5% probability level.

Numerous studies conducted by scientists and academic institutions have reported that there is a relationship between the amount of salt in the soil and different indicators of plant growth. When the amount of salt in the soil becomes too high, the ability of seeds to germinate, as well as the ability of existing plants to produce new plants will decline dramatically. The weight of both the plant and its root system will also suffer; therefore, the plant's chlorophyll levels will decrease as well [18], [27], [28], [29], [30]. In the recently completed study of three separate varieties of wheat, the researchers discovered that the regeneration rate and average weight (fresh) were inversely related to the amount of NaCl used.

In addition, using levels of photosynthetic pigments (which are greatly affected by environmental factors like salinity) as an indicator is a significant factor in assessing plant productivity under conditions of environmental stress; hence, the importance of using photosynthetic pigments to determine the effectiveness of plant growth as an indicator of environmental conditions and provide adequate data for assessing plant productivity [8], [20], [31], [32].

Salt stress causes leaf stomata to close, resulting in a decreased ability to take up carbon dioxide (CO_2) and, thus, reduced photosynthetic rates. Additionally, salt stress disrupts the regular flow of electrons through chloroplasts and creates a condition of over-reduction in the electron transport chain, leading to an increase in the amount of reactive oxygen species (ROS) produced [33], [34], [35].

Many studies have provided evidence that a number of plant species activate their antioxidant defense mechanisms when they are under salt stress to counteract the potentially destructive effects of these reactive types of species on them [35], [36], [37], [38], [39]. The group's author identifies three major types of antioxidative enzyme systems: SOD, CAT, and POD, which are most effective at scavenging free radicals, and therefore protecting cells from the oxidative damage associated with free radical attack [40], [41].

The osmotic stress reduces the energy content of the water available in soils, which in turn causes osmotic pressure to be applied to the cells of natural plants [42]. This osmotic pressure has caused negative morphological changes to the root system in terms of how long, dense and structure

the roots are than normal; further limiting their ability to absorb both water and nutrients and creating a state of physiological drought for plants to be unable to grow and ultimately die when exposed to very high levels of osmotic stress [43], [44].

Salinity is an abiotic stressor that allows the plant to enter a state of oxidative stress at the level of the cell (i.e. excessive amounts of reactive oxygen species [ROS]) [45], [46]. These molecules can cause extensive structural damage, such as the modification of a nitrogenous base in nucleic acids, the formation of cross-linages between the strands and within the strands of nucleic acids and the formation of covalent bonds with proteins that lead to double-strand brake (DSB) in nucleic acid [47], [48]. Other destructive effects of ROS include lipid peroxidation which destroys the structure and stabilser of cellular plasma membranes (increasing their permeability and fluidity), and degrades both functional and structural proteins thereby compromising vital cellular process [30], [49], [50], [51].

Conclusion

According to this research study, the growth and development of the ornamental grass species H. macra were adversely affected by elevated levels of osmotic PEG and NaCl salinity in vitro. For example, significant correlations were observed between bud formation, leaf number, and fresh weight, as well as an inverse correlation to the intensity of stress from PEG and NaCl in the tissue culture medium.

The root system was more susceptible than the shoot proliferation system. Rooting was highly impaired at low stress levels and completely inhibited at high levels of NaCl (1%) illustrating that root development is sensitive to osmotic pressure and ionic toxicity.

In addition, increased levels of stress resulted in more tissue necrosis and reduced survival rates. Conversely, some degree of tolerance was noted with the use of moderate PEG concentrations. The plant has a few adaptive responses, including using fewer leaves to decrease moisture loss.

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