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Effects of salt stress on germination and growth in *Centaurium erythraea* Rafn. and *Triticum aestivum* L.

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Introduction

One of the major environmental factors known for limitating plant growth and productivity is salt stress, particularly in arid and semi-arid regions. Salinity causes a two-fold effect on plants: the salt in the soil solution decreases the availability of water for the roots (osmotic stress) and the salt taken up by the plant can be toxic above certain levels inside tissues (ionic stress) (M u n n s et al., 1995).
NaCl has been reported to be an abiotic compound which significantly reduces growth rate, especially in salt sensitive plants. One of the causes for growth rate decreasing is the consumption of ATP by H+ ATPase that pumps protons to the environment in order to maintain the ionic balance inside the cell membrane (Khan and Rizvi, 1994).

Germination is a crucial part of plant life histories. The ability of their seeds to germinate in presence of a high salt concentration in the soil is therefore essential for the survival and progenies of these species. In saline habitats, seed germination takes place after high precipitation, i.e., under conditions of reduced soil salinity (K h a n and R i z v i, 1994).
Under oxidative stress conditions such as salinity, drought and low or high temperature, plants secrete some active oxygen species, which are harmful to plant growth due to their deleterious effects on the subcellular components and plant metabolism, leading to oxidative destruction of cells. Active oxygen species lead to the deterioration of membrane phospholipids, causing cell to burst and cytoplasm to come out (Mishra and Choudhuri, 1999). In addition to these ionic and osmotic components, salt stress, like other abiotic stresses, also leads to oxidative stress through an increase in reactive oxygen species (ROS), such as superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\bullet}$) (Alscher et al., 1997, Mittler, 2002 and Neill et al., 2002). These ROS are highly reactive and alter the normal cellular metabolism throughout oxidative damage to lipids, proteins and nucleic acids (McKersie and Leshem, 1994,Alscher et al., 1997 and Imlay, 2003). Chlorophyll is also damaged by ROS, which leads to its bleaching. To mitigate the oxidative damage initiated by ROS, plants have developed a complex defense called antioxidative system, including low-molecular mass antioxidants as well as antioxidative enzymes, such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (GPX) and glutathione reductase (GR) (McKersie and Leshem, 1994 and Noctor and Foyer, 1998).
As a mechanism to maintain the osmotic potential, plant cells increase the concentration of organic compounds in the cytoplasm. One of this molecules is proline, a high water-soluble amino acid which forms multimers with a hydrophilic and hydrophobic side. The main

function of proline multimers is to attach to poorly soluble proteins during water removal to prevent them from denaturing (Beck et al. 2002).
In this study, we have tested the effects of salt stress on two different species of plants with two different levels of salt tolerance: *Centarium erythraea* Rafn. and *Triticum aestivum* L.
Centaurium erythraea Rafn. (common centaury) is an annual or biennial medicinal plant belonging to the Gentianaceae family. The species inhabits dry grassland, scrub, and mountain habitats (M e l d e r i s, 1972). Natural habitats of *C .erythraea* are damp grassy or sandy soils. In former Yugoslavia, it is widespread along the Adriatic Coast (Siler et al., 2007).
Triticum aestivum L. is a genus of the family Poaceae, commonly known as the grass family. Above all cultivated wheats, it is economically by far the most important. It is described as mid-tall annual or winter annual grass with flat leaf blades and a terminal floral spike consisting of perfect flowers. We have decided to use this plant in our experiment because it's a common agricultural specie and it has a high growth rate allowing us to obtain enough biomass in a limited period of 13 days.

Materials and methods

Centarium erythraea seeds collected from four different locations, Vrban (L1), Vlasina (L2), Igalo (L3), and Herceg Novi (L4), were used in this experiment. The seeds from all four locations were sterilized by placing them for five minutes in (5%) bleach and then washing them with distilled water. NaCl concentrations of 600mM, 400mM, 200mM, 100mM, 50mM, and 25mM were prepared and distilled water was used as the control. All the solutions including the distilled water contained the fungicid Nystatin at a concentration of 500 mg/L. For the treatment, 30 seeds were counted and placed in petri dishes containing sterilized double layer filter paper. In each petri dish around 0.5mL of the treatment solutions was added. The experiment was done in duplicates. The seeds were left to incubate under artificial light for 5 days after which they were counted on a daily basis together with the number of seeds that have germinated. The seeds were retreated with the solutions when the filter paper on which they were growing started to dry. Growth and germination rate were monitored every 24h under 8-35x magnification.

Common commercially bought wheat seeds (*Triticum aestivum*) have been sterilized and placed in triplicate inside sterile Petri dishes covered with a two-layer filter paper in order to measure and compare their growth under salt stress conditions. All the seeds have been washed with distilled water and superficially sterilized with a two minute immersion in bleach; a final rinse was then given one more time in dH2O to take away all the bleach remainings and avoid inside seed damage. The seeds have been grown under three different salt concentrations of NaCl: 50mM (C1), 100mM (C2),150mM (C3) and distilled water has been used as control (0mM (K)). Filter paper was moisturized depending on the dryness, with 3ml of the corresponding concentration. Seeds were left to grow in the dark. Growth and germination rate were monitored every 24h.
After 72h seeds were translocated to 150ml parafilm-covered graduated jars filled with relative solutions and afterwards placed inside grow-boxes in order to maintain their optimal growth conditions.

Proline extraction protocol:

In order to extract and measure proline content, 0.2g of biomass per concentration was taken from the shoots of the germinated seeds, homogenized using mortar and pestle in a 5ml (3%) sulfosalicylic acid solution and afterwardes filtred through Watman #2 filter paper; 1ml of the filtrate has been taken then for further analysis inside test tubes. Ninhydrin and glacial acetic acid (1ml/each) were added and the whole concentration has been incubated for 60min at the temperature of 100°C. After the incubation period, 2ml of toluene was added and everything has been vortexed for 15-20sec. Chromophore formed a supernatant layer on the aqueous part. Finally the absorbance could be measured using a spectophotometer setting toluene as blank.

In order to determine the proline concentration out of the absorbance of the spectophotometer a standard curve was constructed using given proline concentrations of: (1, 3, 4, 8, 12, 16μg/ml).

Confocal microscopy:

In order to localize the Na+ ions in *Centaurium erythraea* 3ml of CoroNa green staining was used for 6 seeds taken from each concentration. The seeds were first rinsed in distilled water to remove all the possible salt remainings on their surface caused by the saline solutions watering. They were afterwards incubated in dark conditions at room temperature for 60min on a shaker to guaratee an effective binding between the dye and the Na+ ions.
After these first steps, seeds were rinsed with distilled water again to remove all the excessive CoroNa accumulations. Applying a gentle friction, the seed's coating could have been removed, exposing the embryo and allowing a confocal microscope analysis.

Images were obtained using an Olympus IX81 confocal microscope. Samples were excited with a 488 nm laser and the emitted signal was recorded at 516 nm.

Results

The graphs underneath show the germination and growth rates of *Centaurium erythrea* and *Triticum aestivum* seeds developing under various salt stress concentrations. Spectophotometry observed proline content is also displayed.

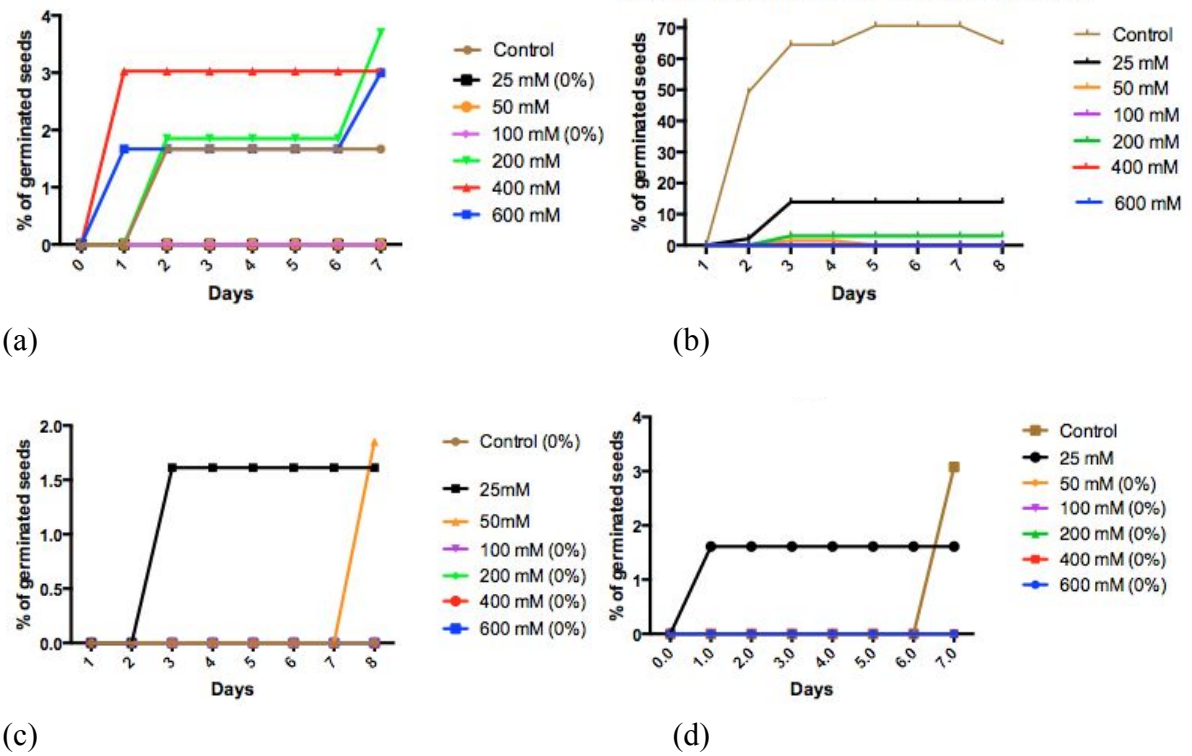

Figure 1: Germination dinamycs of *Centaurium erythraea* of the 4 different locations. (a) Vrban (L1), (b) Vlasina (L2), (c) Igalo (L3), and (d) Herceg Novi (L4).

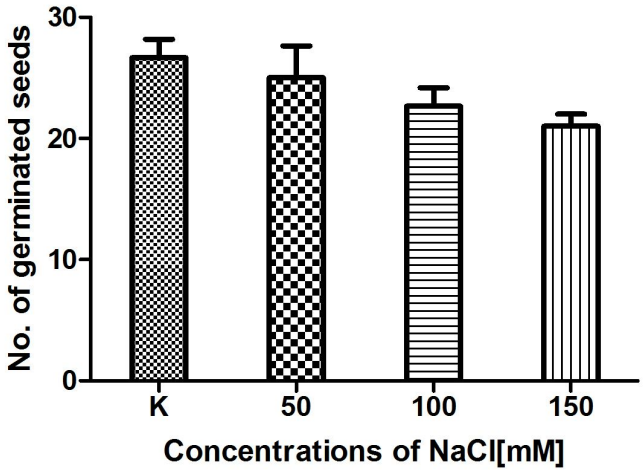

Figure 2: Germinations of *Triticum aestivum* seeds after 72h.

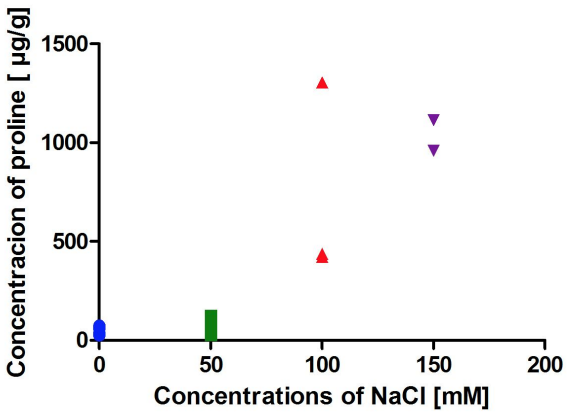

Figure 3: Proline content in *Centaurium erythraea* seeds after growing then under salt stress.

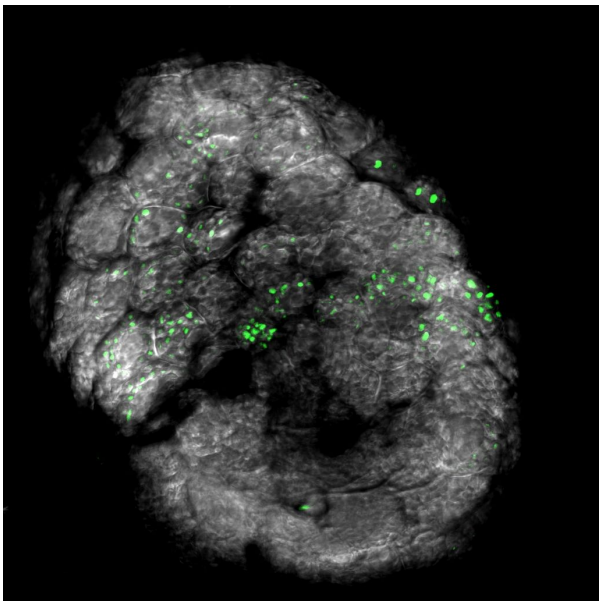

Figure 4: image of *Centaurium erythraea* embryon obtained by confocal microscopy. Na+ accumulations visible in green thanks to CoroNa dye.

Discussion

The stress that saline media induces to plant cells results in the decrease of its growth. The main reason for it is that the energy is consumed for keeping the osmotic and saline balance between the cytoplasm and the extracellular space instead of focusing it on development and plant growth. The ATP-H+ pump and the synthesis of metabolites take up most of the cell resources that would usually be used for growth and therefore adversely affecting germination.

The effect is confirmed in this experiment's results. A significant decrease in the germination number is observeted for Wheat (we need the numbers to calculate the percentage).

In *Centaurium*, germination rates are so low that results cannot be significant. Only L2 exhibits coherent results; in its control group around 70% of the total seeds have germinated on time. Comparing this number with the salt stressed samples, whose germinations barely reach 10%, it can be easily infered that the presence of salt in the media is resposible for this decrease, especially considered the fact that the germination percentage drastically drops with the salt concentrations increasing.

On the contrary, in L1, L3 and L4, the remaining *Centaurium* ecotypes, germinations don't even reach 5% out of the single sample's total, making these results statistically unsignificant. Contributing furthermore to the statistical untreatableness, irregularities with some control groups have been observated; L3 in particular hasn't germinated and control L1 germinations showed up lower from its salt treated samples.

Very low general germination rates might have occured due to several reasons: oldness, death or seeds dormancy. The last hypothesis arises as the most supported one since when on the 12th day gibberellic acid was added in order to stimulate germinations, L1 and L4 have shown a germination increase proving that in those cases seeds were alive but required more time for development.

Regarding proline extraction data, we have faced some challenges due to the slow wheat growth. In the most salt-concentrated groups (100mM and 150mM) biomass was not sufficient to allow 3 extractions per each replicates, constituting a problem to the standard deviation especially for the 100mM samples. These consequences were caused by a non-random relocation of seedlings during the biomass cutting procedure, grouping accidentally more developed seedlings apart from less developed ones and some errors comitted during the extraction. However, a trend towards proline increase in higher salt concentrations was observed, explaining proline being synthesised by the cell as a protection mecanism from salt damage.

At last, for obtaining conclusive data a repetition of the experiment should be performed, including more replicates for each ecotype and expanding the overall time.