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Research Article

Salt Tolerance, Morphological and Anatomical Responses of *in vitro Indigofera zollingeriana* Miq. Seedling

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Abstract

The species belonging to genus Indigofera with high nutritional value and tolerance against abiotic stresses are widely distributed in the tropics to the subtropic areas world over. In this study, two years old stored seeds of *I. zollingeriana*, a potential forage used in Indonesia, were evaluated for their tolerance to salinity stress. In the first step, the morphology and anatomy of *I. zollingeriana* seedlings under *in vitro* salinity stress level of 20-120 mM NaCl were investigated after 14 days. In the second step, the 3 days old seedlings were transferred to several concentrations of NaCl (140-300 mM) to estimate Ld_{50} (lethal dose). Several concentrations of gibberalic acid (GA_3) were applied to the previous estimated Ld_{50} (228 mM NaCl) medium for alleviating seedlings damage. It was estimated that the increasing concentration of NaCl caused reduction in the evaluated plant growth parameters and changed anatomy of the root and stem cross sections. The 100% mortality of the seedlings was noted after4 weeks on the medium containing 300 mM NaCl. Treatment of seedlings with <0.25 mg L^{-1} GA_3 +228 mM NaCl (Ld_{50}) in the culture medium was effective to reduce root damage for 4 weeks. Treatment of seedlings with >2.5 mg L^{-1} GA_3 + 228 mM NaCl (Ld_{50}) showed adverse effects in controlling damage by necrosis and blackening of roots and stems.

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Keywords
Abiotic stress, exogenous hormone, ındigofera, seed, salinity

INTRODUCTION

The genus *Indigofera* in the family fabaceae has ~750 annual, biennial, or perennial species that are highly tolerant to several abiotic stresses. It has been reported that the Indigofera's leaves are used for obtaining forage, natural indigo dye, pharmaceuticals and cosmetics. *Indigofera zollingeriana* Miq and other species belonging to this genus are important forage crops that are cultivated or grow naturally in Indonesia and other countries. They show tolerance to abiotic stresses like drought (high), light floods, acidic soils, salinity (tolerant to moderate) (Skerman, 1982; Hassen et al., 2007), high herbage yield and nutritional components (Abdullah and Suharlina, 2010; Herdiawan and Sutedi, 2012; Herdiawan et al., 2013). Poor seed germination of 24-35% has been noted due to seed coat thickness (Abdullah 2012, 2014; Abdullah et al., 2016). Successfull improving of low seed germination of *I. zollingeriana* stored seeds has been reported by using hot water, mechanical and chemical scarification by imbibing with water and sandwiching seeds in filter papers or on agar under *in vitro* culture conditions (Maesaroh and Ozel, 2019; Maesaroh and Demirbağ, 2020). Salinity stress is a major abiotic stress limiting growth and development of plants, damaging many crops in many areas including Indonesia which has about 0.44 million ha of saline lands (Mulyani et al., 2010). Karolinoerita and Yusuf (2020) have reported that areas near coasts are highly prone to salinization. The *I. zollingeriana* with high protein content is considered suitable for cultivation and improving the soil structures due to its tolerance against salinity and could be recommended for forage production in sub-optimal areas as they adapt well in these areas. Various methods are being applied to reduce negative impact on plants in response to salinity stress. Adding Ca and hydrogen peroxide have ameliorated and enabled cultivation of statice and triticale in these areas (Akad and Ozzambak, 2013; Demirbaş and Balkan, 2018). Moghaddam et al. (2020) has reported the succesfull use of salicylic acid on *Lathyrus sativus* under salt stress conditions and for improving seed germination, seedling growth, and physiological traits. The application of exogenous ascorbic acid has positive impact on enhanching chlorophyll A, the total chlorophyll contents and reduction of antioxidant enzymes level, malondialdehyde and hydrogen peroxide contents of maize under salinity stress (Doğru and Torlak, 2020). Abiotic stresses alter phytohormones level which involve the mechanisms of plant tolerance or susceptibility led to decreased plant growth (Morgan, 1990; Eyidoğan et al., 2012; Iqbal et al., 2012). The endogenous gibberalic acid play an important role in regulating the signal pathways, in seed germination, plant growth and decrease in response to salinity stress (Iqbal et al., 2012). There is limited information on *I. zollingeriana*'s salt tolerance. Therefore, the it is a need to identify the level of its salt tolerance responses on morphological and anatomical features of the plant. The study aimed to observe morphological and anatomical responses, determine salt tolerance levels and effect of adding exogeneous GA_3 for improving its ability on *I. zollingeriana* seedlings under *in vitro* salinity stress conditions.

MATERIAL and METHODS

Seed germination

The 2 years stored seeds were primarily scarified by sandpaper followed by scarification with sulphuric acid. Thereafter these seeds were soaked in sterilized distilled water for 3 days by continuous shaking (Maesaroh and Ozel, 2019).

Salt treatment

In the first experiment, the 3 days germinated seeds were transferred to MS medium containing different contentrations (20, 40, 60, 80, 100, 120 mM) of NaCl and control. The plant length, total leaves and roots of growth seedlings as growth parameters were observed under

concentrations of NaCl induced treatments after 14 days. Thereafter, the seedlings stem anatomy was noted under the microscope.
In the second experiment, the 3 days germinated seeds were transferred to MS medium containing (140, 180, 220, 260, 300 mM) contentration of NaCl. The lethal dose 50 (Ld_{50}) was determined from these treatments of NaCl. The 0.1, 0.2, 0.25, 2.5, 5.0, 10 and 15 mg L^{-1} concentrations of GA_3 were added to the above mentioned Ld_{50}.

Statistical analysis

Each treatment of the first experiment containing 25 explants was equally distributed into 5 replicates. The average of plant growth parameters as plant length, root length and number of roots was measured in triplicate. These data were analyzed by comparing means using IBM SPSS 24 program for the Windows 10. The comparison among the means was made by Duncan's Multiple Range Test (DMRT). The regression formula for determining Ld_{50} was obtained by comparing means.

RESULTS and DISCUSSION

Seed germination

Seed germination was carried out using methodology of Maesaroh and Ozel (2019) by shaking the sterilized seeds of *I. zollingeriana* in liquid medium containing sterilized bidistilled water with seed germination of 80%.

Salt stress treatments

Adding different concentratns of NaCl had variable effect due to salinty stress on the germinating *I. zollingeriana* seedlings under *in vitro* culture. These showed reduction of plant growth parameters (Table 1) depending on the concentration of salt in the treatment. The plant height significantly decreased in range of to 1.7-6.2 cm and the longest plants were noted on the untreated control treatment. The increasing concentration of NaCl was also followed by reduction of primary root length and induction of total number of lateral roots. The results of the study are in agreement with Budaklı Çarpıcı and Erdel (2016) and Moghaddam (2020) who notedthat increasing NaCl concentrations ends up with decreasing of plant length and root system of grass pea. There were non significantly important effects on total leaves while leaf size reduced slightly at higher concentrations of NaCl in agreement with Nadir et al. (2018) who reported change in number, colour and shape of leaves and plant height of *I. zollingeriana* treated under nursery conditions treated with salinity stress. Therefore, the reduction in number of leaves and their size compared to control after using 20 mM salt might be due to the accumulation of salt ions. The media containing 20 mM NaCl did not have a visible negative effect on growth and development of plants and supported by Munns and Tester (2008) who categorized that saline soil as equivalent to approximately 40 mM NaCl. No visible root injury in uptaking water containing 20 mM NaCl and have no visible effect on photosyntesis process.

Table 1. Morphological responses of *I. zollingeriana* under *in vitro* salt stress condition after 14 days

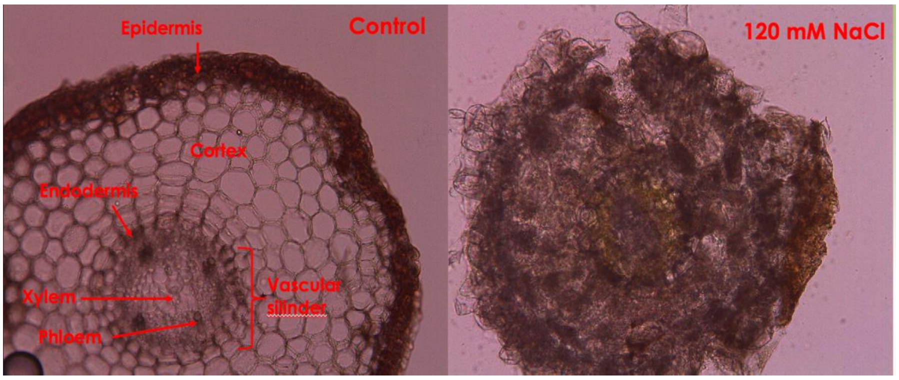
Treatment (mM NaCl)	Stem length (cm)	Number of leaves	Root	
			Primary length (cm)	Latera root number
Control	6.2a	3	3.0a	10a
20	5.2b	3	2.7b	6.7b
40	3.4c	2	2.0c	4.3c
60	2.7d	2	1.2d	3.3cd
80	2.2e	2	1.1d	2.3de
100	1.9f	2	0.5e	2.0e
120	1.7f	2	0.5e	0.7f

Means not followed by same letter within a column differ significantly at $P<0.05$

The reduction of water intake followed by increasing salts intake affected photosynthesis procces, resulted in reduction of plant growth parameters as leaf size, stem length, root length of *I. zollingeriana* at an earlier stage and caused root blackout, stem shringking and leaves mottling and necrosis at latter stages due to accumulation of ions (Picture 1). The salinity stress caused by adding various concentration of NaCl to MS medium carries both ionic toxicity and osmotic stress in *I. zollingeriana* seedlings in agreement with Tanveer and Shabala (2018). Accumulation of salts followed by ionic toxicity causes osmotic stress leading to root system damage, decrease in the plant ability to uptake water which cause water deficit and decrease in leaf turgor and stomata closure ending up in reduced photosynthesis percentage (Chaves et al., 2009; Karan and Subudhi, 2012).

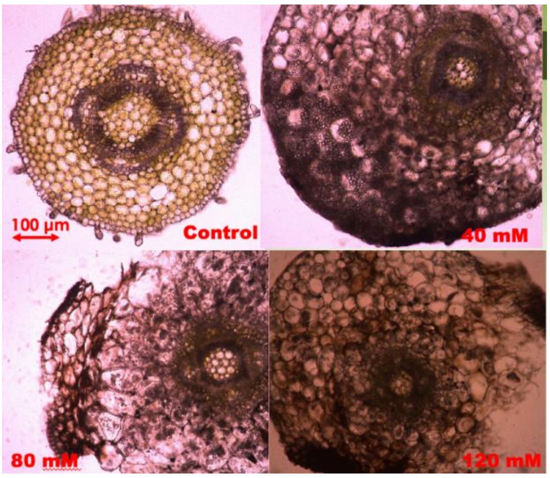
Picture 1. Root damage, stem shrinking and leave necrosis of *I. zollingeriana* seedling after transfer to media containing 100 mM (5) and 120 mM (6)

The increasing concentration of NaCl created physiological and anatomical changes in the body structure of *I. zollingeriana*. At the first, the high Na^+ and Cl^- in the medium negatively affected roots system that showed the injury of *I. zollingeriana* roots at 120 mM NaCl stress level (Picture 2). The lateral root and root hair, epidermis and cortex damage disorder increased with the absorption of water and minerals their transport to the center of roots (vascular) that led to the imbalance in water and mineral uptake in agreement with Byrt et al. (2018).



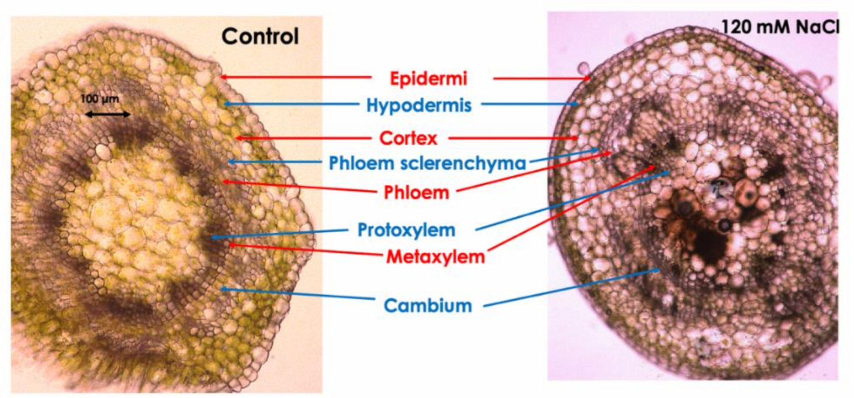
Picture 2. Root anatomy of *I. zollingeriana* control and treated by 120 mM

There is no cell damage on hypocotyledonous and epicotyl stem of *I. zollingeriana* when they were treated with a liquid without salts (control treatment). The increasing NaCl concentrations caused damage of hypocotyledonous stems (Picture 3). They also showed the salt accumulation on the stem cortex layer at 40-120 mM NaCl at early stage seedlings. The high levels Na^+ and Cl- absorption disturbed the absoption of essential nutrients which are needed for plant growth in agreement with Wakeel (2013) who declared that the Na^+ presence in medium affected K^+ uptake by plants. The leaves accumulate extreme amount Na^+ ions that limit photosynthesis and transpiration as physiological responses.



Picture 3. Anatomy of hypocotyledonous stem of *I. zollingeriana* at different concentration of NaCl

The blackout of xylem and other part of epicotyl stem indicated that xylem, a vascular bundle, which transport water and nutrition from root to other part of plant were damaged (Picture 4). The epidermis thickness and diameter decreased by 120 mM NaCl. Carcamo et al. (2012) reported that the restricted cell division and growth under salinity stress ocontributes to reduction of epidermal thickness. The salinity also reduced xylem vessels to minimize the hydraulic conductivity in agreement with Junghans et al. (2006), whereas pith area increased as defense response againt salt stress in accordance with Parida et al. (2016). There are non significant effect on other parts thickness. It is suggested that there is difference in anatomical changes between halophytes and glycophytes for adapting to salinity conditions (Atabayeva et al., 2013).



Picture 4. Anatomy comparation of epicotyl stem of *I. zollingeriana* between control and 120 mM NaCl treatment

Gibberellic acid (GA_3) treatment

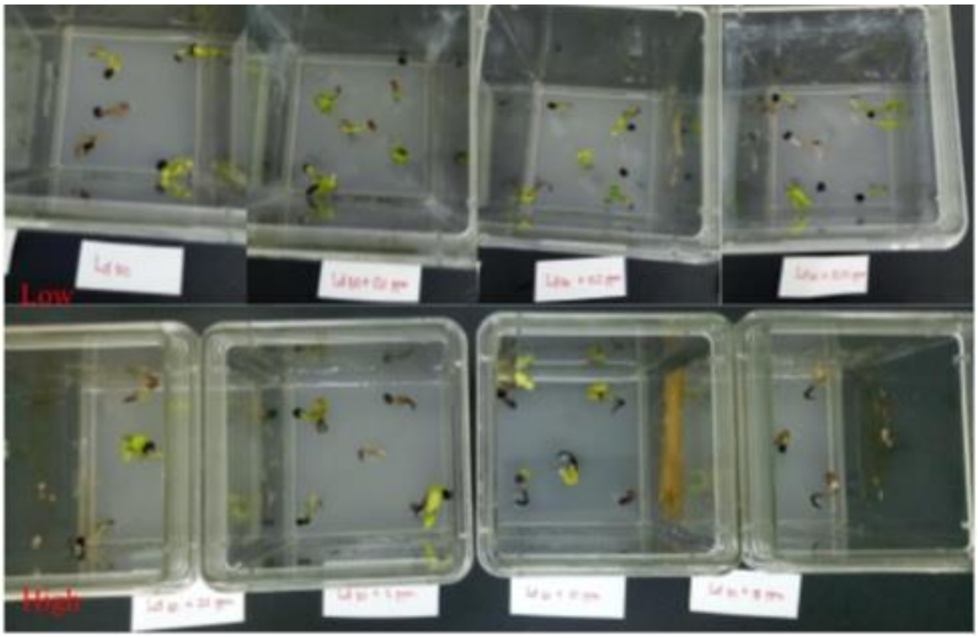
Table 2 showed the growth percentage of *I. zollingeriana* seedlings after 4 weeks transfer to MS medium containing various concentration of NaCl. The plant showed 100% growth and mortality after 4 weeks of treatment on the medium containing 140 mM NaCl and 300 mM NaCl, respectively. It was found that estimated LD_{50} as 228 mM of NaCl by **y = -0.5833x + 183** regresion. The stunted plants were observed at high concentratons of NaCl. The increasing to high concentration of NaCl may be attributed to high osmotic and ionic stress which causes physiological and anatomical changes leading to stunted plant growth or death in agreement with Munns and Tester (2008), Rahnama et al. (2010) and James et al. (2011).

Table 2. Growth percentage of *I. zollingeriana* seedling after 14 days transfer

Concentration of NaCl (mM)	Growth percentage (%)
140	100
180	73.33
220	60
260	40
300	0

The <0.25 mg L^{-1} concentration of gibberelic acid (GA_3) had no significant effect on the plant growth, *I. zollingeriana* seedlings could be protected until 4 weeks after transfer to medium containing 228 mM of NaCl (Ld_{50}) that showed no injury (black out) on roots (Picture 5). While the seedlings showed browing after a few days even after treatment with >2.5 mg L^{-1} concentration of GA_3 and caused seedling death after 4 weeks of culture. Zhu et al. (2019) reported that the low gibberellic acid (GA_3) can enhance water uptake and stimulate sweet sorghum germination at lower salinity conditions, while the contrary effects are shown by high concentratrion of GA_3 application at higher salinity stress. Kim et al. (2010) reported that abiotic stresses condition as drougt and salt stress enhance ABA production leads to stomatal closure, gene expression change and adaptive physiological responses. The adding exogenous GA could decrease the ABA production during salt stress conditions in agreement with Liu and Hou (2018) who noted the opposite regulation between ABA and GA in the plant growth regulation mechanisms.The germinated seedlings of *I. zollingeriana* showed 100 % growth on the medium containing 140 mM of NaCl (Table 2) and leaves damage was noted on the medium containing 100 and

120 mM of NaCl (Picture 1). According to Australia Agriculture and Food (2019), *I. zollingeriana* could be classified to tolerant plant due to sodium and chloride concentration in agreement with Nadir et al. (2018).



Picture 5. Effect of GA_3 concentration on *I. zollingeriana* seedling on LD_{50} medium under *in vitro* culture

CONCLUSION

The *I. zollingerana* could be suggested as moderately tolerant plant to salinity stress due to less morphological and anatomical changes in plant anatomical structure under *in vitro* conditions. The increasing of NaCl affected negatively on the observed plant growth parameters. Their anotomy changes can be considered as adaptation mechanism of these plants. The GA_3 can protect the germinated seedlings of these plants in this study partially or for a short duration only. The advanced studies against abiotic stresses could be developed to check the range of duration showing plant tolerance. The using of other methods could be considered to protect and improve the plant growth of *I.zollingeriana* at higher salinity stress level to decrease yield losses.

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