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Using standard screening protocol and effect of salinity stress on plant growth, pigment, ion fluxes and antioxidant enzyme of *Azolla* spp. in pot culture

Hari Narayan, Tapas Chaudhary, Sangita Kumari, Ravindra Soni and Upendra Kumar

Abstract
Salinity is one of the most serious problems to agricultural productivity. The aquatic fern *Azolla* is used as an effective bio-inoculant in rice paddy production. Result indicated that two species of *Azolla* chosen based on survivability which is *A. microphylla* (salt-tolerant) and *A. rubra* (salt-sensitive) in 80 mM NaCl. Although using standard screening protocol is better than hydroponics condition due to its provide natural condition for screening of *Azolla* plant. Although the DT was lower in *A. microphylla*, under salt stress (80 mM NaCl) the total biomass increased as characterized by RFC, RGR, DW and ARL in comparisons with *A. rubra* which had comparatively higher DT. Similarly, the MC reduced sharply with rise in salt concentration in *A. rubra*. Under salt stress condition soil pH and EC was reduces by *Azolla* plant but highly reduces by *A. microphylla* contrast *A. rubra*. After 15 days, reductions in chl a and b and carotenoid contents were found to be associated with both the strains, however with rise in salt concentrations, significant increase in anthocyanin content was observed in both the strains. The concentrations of antioxidant enzymes such as SOD, APX, CAT and proline and depicting the antioxidant mechanisms in this species play major role for salt tolerance. MDA content was increased with rise in salt concentration in both strain of *Azolla*. Presence of NaCl in the nutrient medium Na^+ and Ca^{2+} increased in plant cell at different salt stress, while it reduced the cellular K^+ level in both species when rise salt concentration.

Keywords: *Azolla*, salt stress, antioxidant enzyme, plant pigment, ion fluxes

1. Introduction
Increasing soil salinity is a serious threat to the growth and development of plants. Application and non-judicious use of fertilizers, loss of forest cover and improper water usage have resulted in an increase in the salinity level. The plant growth is untenable in soils that contain too much salt. It has been estimated that globally 0.5 to 1.0% of the irrigated area is being lost every year due to salinity. Increase in the soil salinity leads to significant reduction in crop production. Salinity induced decrease in the yield in some areas was around 97%. It is therefore important to understand the salinity tolerance of an argonomically important organism like *Azolla* which helps in sustaining the productivity and improving the fertility of the soil. Salinity leads to a wide variety of physiological and biochemical perturbations in plants leading to decrease in growth, uptake of mineral nutrients, photosynthesis, water relations, respiration, protein synthesis and nucleic acid metabolism.
The nitrogen fixing aquatic fern *Azolla* is found floating on the surface of water bodies and has a fast doubling time of 5–10 days. *Azolla* plant is an extremely small sporophyte having a rhizome which is horizontal (0.5 to 7 cm in diameter) and densely arranged branches and overlapping leaves. The single leaf has a thin ventral and thick dorsal lobe and cavities present in the dorsal lobe house the symbiotic cyanobacteria (Peters and Mayne, 1974) [53]. *Azolla* is an effective green manure and biofertilizer for flooded crops particularly rice. Inoculation of *Azolla* improved the physico-chemical properties of the soil due to release of nutrients and organic matter. *Azolla-Anabaena* system is thus important for the improvement of nitrogen use efficiency in rice crop (Yao *et al.*, 2018) [83]. The exploitation of *Azolla* as biofertilizer is limited by increasing salinity which diminishes its growth and productivity. However, very few reports are available on the impact of salinity on ferns. Salinity tolerance in pteridophytes has been addressed but few pteridophytes exhibit considerable salinity tolerance.

Salinity stress causes the disruption of cellular and metabolic machinery by causing osmotic and ionic stress which ultimately affects plant growth and development.

The stress response mechanism includes avoidance and tolerance to stress. In stress avoidance, a number of protective mechanisms are employed by the plants to protect them from the adverse impact of the stress. Enhanced production of reactive oxygen species (ROS) in the cells is another serious impediment for the normal metabolism under salinity stress. An increase in the levels of ROS lead to oxidative stress and cause damage to the biomolecules such as proteins, lipids, carbohydrates, DNA and adversely affect the cellular metabolism. Plants have developed appropriate mechanisms to scavenge the ROS through the coordinated action of enzymatic as well as the nonenzymatic antioxidants. Epigenetic changes and modified expression of specific genes have been reported in response to salinity stress conditions. High concentration of salts exerts a negative effect on the plant metabolism and growth due to accumulation of Na^+ and Cl^- ions and osmotic stress. Maintaining the ion homeostasis is important to regulate the excess Na^+ ions which is toxic and disrupts the enzyme activities and interfere with the K^+ ions. Enhanced production of reactive oxygen species (ROS) in the cells is another serious impediment for the normal metabolism under salinity stress.

Therefore, the present study was evaluate the effects of salt stress on the activity of antioxidant enzymes, ion leakage nutrient content and soil pH and EC in two species of *Azolla* plants (*A. microphylla* and *A. rubra*) which are commonly found in the paddy fields in order to better understand their differences in salt tolerance.

2. Materials and method

2.1 Study location and protocol for screening *Azolla* under salinity

Experiments were conducted at the microbiology net house (20.52ºN latitude and 85.83ºE longitude) at the ICAR-National Rice Research Institute, Cuttack during rabi season (Nov-Feb, 2019). For the experiment, each small plastic pot (30.48 cm diameter) was drilled using a drilling machine to create holes evenly spread to the side walls of the pot. Each hole was around 0.5 cm in diameter and 2 to 3 cm gap was maintained between two successive holes. A nylon mess was placed at the bottom of the perforated pot, then a thick layer followed by a medium and thin layer of gravel was placed at the bottom of the pot one after another. A layer of sand was placed on the top of the gravel, one perforated pipe (Piezometer) put into the soil, with its opening outside the soil field (Plate 3.1 and 3.2). Then all pots put into the plastic tub (height 0.30 m and diameter 0.50 m), so that water could pass into the pots through the bottom. Water inside the piezometer was obtained from saturated soil. The salinity level of the saturated soil extract inside this piezometer checked using a hand-held pH-EC meter. Salinity levels of 80 and 120 mM NaCl were maintained along with control (0 mM NaCl) throughout the growing period or *Azolla* selected for this experiment.

2.2 Inoculation of *Azolla* spp

Fresh *Azolla* (0.44-0.50g) were surface sterilized using 0.1% mercury chloride (HgCl2) and then washed with distilled water for 4-5 times and inoculated in the pots which have been kept inside a water tub filled with 19L water. The water level in the plastic tub was maintained at 3-5 cm above the soil surface of the perforated pots and salinity level was maintained at 80 and 120 mM NaCl with a control (0 mM NaCl). The water level in the plastic bath maintained at 5-3 cm above the soil surface of the perforated pots. In pot culture only *Azolla* was inoculated after treatment of different level (0, 80 and 120 mM) of NaCl.

2.3 Determination of relative frond count, frond length, frond width and fresh biomass of *A. microphylla* and *A. rubra* under salt stress

Relative frond count (RFC), frond length and frond width were analysed after 15 days of uniform growth of *A. microphylla* and *A. rubra* in IRRI media under different NaCl treatments with respect to control (growth under NaCl-free IRRI media) by using following formulas.

$$RFC = (Nt-N0)/N0 \quad (1)$$

N0 = Total initial number of fronds inoculated, Nt = Total number of fronds on the 8th days of inoculation.

Frond length and width of *Azolla* under different treatments were measured by manual scale calculated the by following formulas.

For determination of *Azolla* biomass productivity, fresh *Azolla* biomass was collected after 8 days of NaCl stress inoculation and fresh biomass weight was recorded after blotting.

2.3. Determination of relative growth rate and doubling time

The plant samples were collected after 8daysrinsed with double distilled water, blotted using filter paper and recorded fresh weight immediately. The sample was dried using hot air oven at 60°Cin order to obtain a constant weight. Relative growth rate (RGR) was calculated based on the weight by using following method.

$$RGR = (\ln Dw_2 - \ln Dw_1)/ t_2 - t_1 \quad (2)$$

Where, lnDw1 and lnDw2 were natural logarithm of initial fresh weight of *Azolla* (0.22 g) and final fresh weight of *Azolla* (after 15 days of growth), respectively. At times $t_2$and t_1, Dw_2 and Dw_1 were the weights.

$$DT = \ln 2/RGR \quad (3)$$

Where, DT: doubling time and expressed asd^{-1}.

2.5 Moisture content

Determination of moisture content of *Azolla*, after 15 days inoculation, first taken fresh weight of sample then sample were keep in autoclave for 24 hrs in 60 ºC. When dry sample was constant in weight then final weight was taken.

2.5.1 Estimation of pigments from *Azolla* (chlorophyll a, b, carotenoid and anthocyanin)

15 day sold *Azolla* was harvested from each treatment and then blotted on filter paper. Samples were ground with 80 % acetone in cooled mortar and pestle and kept overnight at 4°Cunder dark conditions. Centrifugation of the samples was done for 5minat 4°C at 10000 g and supernatants was

collected in a new tube and the process was repeated till it becomes colorless. At 645 nm and 663 nm the reading of the solution was taken, where solvent acetone was kept as blank chl *a* and *b* concentrations were estimated by using the following formula:

Chl *a*: $12.7(A_{663}) - 2.69\ (A_{645})$ (4)

Chl *b*: $22.9\ (A_{645}) - 4.68\ (A_{663})$ (5)

The value of chl *a* and chl *b* were derived in mg g^{-1} fresh weight (FW) of *Azolla* fronds.

Carotenoid content was also extracted in 80 percent of acetone by using spectrophotometer (Evolution™ 300 UV-Vis, US) as per method of Lichtenthaler (1987) [31]. Take fresh *Azolla* tissue and add 80% acetone and grind it well in a mortar and pestle. Now centrifuge it at 3000rpm for 10 mins. Take the supernatant and discard the pellet. Now read the OD values at 470 nm in UV-spectrophotometer.

Azolla anthocyanin was extracted by blending samples (0.2 g) kept in dark at 4°C for 2 days with 50 mL of 1% HCl (v / v) in methanol. Then the extract was filtered and spectrophotometer was used to measure anthocyanin at two different absorbance that is 530 nm and 657 nm.

2.5.2 Estimation of pH and Electric conductivity

Among these, pH, and EC/TDS are usually measured on the experimental spots by using portable pH meter, EC/TDS meter (Poratble Multimeter. HACH Portable case far pH, pH/ISE Conductivity & DO Meter).

2.12. Estimation of lipid peroxidation

Lipid peroxidation was measured as the amount of MDA produced by the thiobarbituric acid (TBA) reaction, as described by Dhindsa *et al.,* (1981) [86]. Fresh leaves (0.5 g) of control and NaCl treated-plants were homogenized in 2 ml of 20% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 3500 g for 20 min. 1.5 ml of 20% TCA containing 0.5% (w/v) TBA and 100 mL 4% butylated hydroxyl toluene in ethanol were added to 0.5 ml of the aliquot of the supernatant. The mixture was heated at 95 °C for 30 min and was then quickly cooled in ice. The extracts were centrifuged at 10,000 g for 15 min and the absorbance was measured at 532 nm. The value of non-specific absorbance at 600 nm was subtracted. The concentration of MDA was calculated using an extinction coefficient of l55 $mM^1\ cm^1$.

2.8. Nutrient determinations

Azolla grown under different treatments were washed and blotted on filter paper to determine the Ca^+, Na^+ and K^+ ions. A mixture (1:1, v / v) of HNO_3: $HClO_4$ was used for digestion of the sample (0.5 g each) in a boiling water bath for 30 min, Ca^+, Na^+ and K^+ were evaluated using a flame photometer (Association of Official Analytical Chemists, 1984).

2.9. Estimation of electrolyte leakage

Electrolytic leakage (EL) was analysed in 15 days old *Azolla*, harvested from each treatments as per methodology of Dionisio-Sese and Tobita (1998) [17]. Fresh *Azolla* (0.2 g) were blotted and were chopped into small parts (5 mm) and kept in test tube containing 10 mL deionized water. At 32 ºC the sample was incubated in a water bath for 2h and the first electrical conductivity (EC 1) of the sample was recorded. Later the sample was autoclaved at 121 ºC for 20 min and cooled at 25 ºC. All the electrolytes were expelled from the sample and recorded the last electrical conductivity (EC 2).

The electrolytic leakage was determined using the

EL = (EC1/EC2) × 100 and expressed as %.

2.10. Determination of superoxide dismutase (EC 1.15.1.1) activity

Superoxide dismutase (SOD) activity in 15 days old *Azolla* was determined as per methodology of Stewart and Bewley (1980) [69]. The fresh *Azolla* plant (0.1 g fresh weight) was crushed into fine powder in liquid nitrogen and the sample was blended in 50 mM potassium phosphate buffer (10 ml) having pH 7.5 and for 10 min the sample was centrifuged at 4 °C in 9000 g. SOD activity was determined by measuring its potential to inhibit the photochemical depletion of nitro-blue tetrazolium (NBT). These reaction mixtures included 0.3 mL (sample) enzyme extract in 100mM phosphate buffer (pH 8.0), 200 mM methionine, 3mM EDTA and 2.25 mM NBT, Na_2Co_3 added 0.1 mL and distilled water 0.6 mL. Followed by addition of 2 mM riboflavin to the sample mixture in order to initiate chemical reaction and the sample was placed at a distance 30 cm apart from the fluorescent tube for 10 min and the absorbance was determined at 590 nm.

2.11. Determination of ascorbate peroxidase (EC 1.11.1.11) activity

Ascorbate peroxidase (APX) activity in 15 days old *Azolla* was determined as per methodology Nakano and Asada (1984) [46]. In an ice-cold homogenization buffer the *Azolla* plants (0.1 g fresh weight) were crushed using cooled morter and pestle. Reaction mixture containing 1 mL having pH 6.1 phosphate buffer (50 mM), 0.5 Mlguiacol (96 mM), 0.8 mL distilled water, 0.5 mL of H_2O_2 (0.5%) added the time of absorbance and 0.2 mL of the enzyme extract. Take absorbance at 470 nm. One enzyme unit determined the amount of enzyme necessary to decompose 1 µmoL ascorbate per milligram of protein per minute at 25 °C.

2.12. Estimation of protein

Azolla protein was determined as per methodology of Bradford assay (1976), taken standard observation at 595 nm absorbance. Samples of *Azolla* (100 - 300 mg fresh) are powdered with liquid nitrogen, using pre-cooled mortars and pestles. *Azolla* proteins are extracted by blended in the described cold 0.05 M Tris buffer. A small quantity (0.05 g) of the antioxidant polyvinyl polypyrollidone (PVPP) is added to each sample during the blend procedure, keep tube in ice. Homogenates are transferred to cold centrifuge tubes (2 ml Eppendorf tubes) and then was centrifuged at 14,000 - 19,000 for 20 min. at 4 °C. After centrifugation, clear supernatants can be used immediately for the protein assay at 595 nm, or frozen at -20 °C and used later.

2.13. Estimation of proline

Proline content was determined as per methodology of Bates *et al.,* (1973) [11]. Proline was extracted from the 500 mg fronds with10 mL sulphosalicyclic acid (3%) and centrifuge at 15000 rpm at 15 min. 2 mL extract was mixed with 2 mL of a mixture of glacial acetic acid and orthophosphoric acid (6 M) (3:2, v/v) and ninhydrin (2 ml). After 1 h of incubation at

100 °C in water bath, the test tubes were cooled for 30 min and 4mLtoluene was added. Absorbance was determined spectrophotometrically at 520 nm, and the proline content was determined using standard curve.

3. Result

3.1 Effect of salinity on growth in pot culture

ANOVA has shown significant varietal growth effect in pot culture. Significantly (0.05 %) RFC in *A. rubra* reduces after 15 days of inoculation (DOI) in 80 and 120 mM NaCl and no further development was observed at greater levels salt (NaCl). However, *A. microphylla* was found to be salt (NaCl) tolerant at 80 mM NaCl but with considerably reduced RFC, at 120 mM salt concentration there was no growth in *A. microphylla*.

Under salt (NaCl) stressed condition (80mM NaCl) the RFC (number d^{-1}) of *A. microphylla* was significantly decreased by 1.85 (80 mM) as compared to control (3.1), while the RFC of *A. rubra* was checked in80 and 120 mM survived only in control condition (Table 1). The ARL (cm) of *A. microphylla* after 15 days was significantly decreased at 80 mM (1.57) as compared to control and while in *A. Rubra* ARL sharply reduced in 80 mM (0.34) as compared to control (2.01) (Table 1). The production of dry biomass (t hac^{-1}) of *A. microphylla* was greatly reduced by 80mM (0.10) compared to the control (0.14). However, dry biomass of *A. microphylla* higher (0.10) in 80 mM compared to *A. rubra* (0.03). DT (t hac^{-1}) of *A. microphylla* (13.72) was longer in 80 mM as compared to control (4.22). However, DT of *A. rubra* was significantly higher (44.24) in 80 mM compared to control (5.82) (Table 1). RGR (g g^{-1} fresh *Azolla*) of *A. mirophylla* was significantly decreased (0.05) in 80 mM as compared to control (0.17) (fig. 1). Similarly RGR of *A. rubra* was significantly higher (0.01) in 80 mM compared to control (0.12) (Table 1). MC (%) of *A. mirophylla* was significantly decreased (93.01) in 80 mM as compared to control (94.79). Similarly, MC content in *A. rubra* was significantly reduces (84.45) in 80 mM as compared to control (93.82). Frond length and width (cm) of *A. mirophylla* was significantly decreased (0.80 and 1.04) in 80 mM as compared to control (1.02 and 1.56) (fig. 1). Similarly, frond length in *A. rubra* was significantly reduces (0.60 and 0.44) in 80 mM as compared to control (0.91 and 0.68). After 15 days of inoculation *A. microphylla* was unable to tolerate salt (NaCl) stress at 120 mM and *A. rubra* in at 80, 120 mM. At 120 mM, the *A. microphylla* plant looked like yellow, brown and gets degraded. Similarly, *A. rubra* looked like yellow, brown and get degraded at 80 mM and 120 mM (Table 1).

3.2 Effect of salinity on pigments in pot culture

When *A. microphylla* and *A. rubra* exposed to salt (NaCl) stress, showed a decreased amount of *chl a* and *b* and carotenoid content. Compared to *A. microphylla* significant reduction in *Chl a* and *b* of *A. rubra* was observed. At 80mM in *A. microphylla chl a* (mg mL^{-1}) was (2.93) was significantly decreased compared to control (3.08) (Table 2), but *chl a* was significantly higher in *A. microphylla* (2.93 and 3.08) as compared to *A. rubra* (2.63 and 2.81) under 80 mM NaCl and controlled condition. At 80mM*Chl b* (mg mL^{-1}) in *A. microphylla* was significantly decreased (0.85) as compared to control (0.89) but *chl b* was significantly higher in *A. microphylla* (0.85 and 0.89) as compared *A. rubra* (0.73 and 0.77) under 80 mM NaCl and controlled condition. Similarly, carotenoid content (mg g^{-1}fm) was significantly decreased when NaCl concentration increased and reduction of carotenoid content was higher in *A. rubra* (2.59 and 1.32) compared to *A. microphylla* (3.50 and 2.88) at 0and 80 mM (Table 2). With enhanced concentration of NaCl, anthocyanin pigment (g kg^{-1}) has risen considerably. Anthocyanin content of *A. microphylla* was higher in 80 mM (0.88) as compared to controlled condition (0.56), but anthocyanin content was significantly higher in *A. microphylla* (0.88) as compared to *A. rubra* (0.76) under 80 mM NaCl.

3.3 Ion fluxes

Greater extent of electrolytic leakage (%) was significantly observed at 80 mM in *A. microphylla* (30.26) compared to under controlled condition (22.38) (Table 2) but *A. rubra* was sensitive to 80 mM (27.29) of NaCl concentration as compared to control (15.49). EL affected the cell membrane due to NaCl.

3.4 Soil EC and pH

Treatment of different level of NaCl (0, 80 and 120 mM), soil EC was significantly reduced (0.53, 7.77 and 10.83 respectively) by *A. microphylla* as compared to treatment of *A. rubra* (0.69, 8.40 and 11.11respectively). Similarly, soil pH was significantly reduced by *A. microphylla* (7.53, 6.37 and 6.42) as compared to *A. rubra* (7.31, 6.44 and 6.37) at 0, 80 and 120 mM (Table 2).

3.5. Antioxidant enzymes

The two species of *Azolla* showed a substantial distinction in SOD content (units mg^{-1} fwt^{-1}) as a result of increased concentration of NaCl. SOD activity was significantly increased (0.67) in *A. microphylla* when subjected to an increased in NaCl levels at 80 mM as compared to control (0.21), similarly result found in *A. rubra* (0.24 and 0.32) at 0 and 80 mM, but higher SOD activity in *A. microphylla* than *A. rubra* (Table 3).

In *A. microphylla* APX activity (unit g.fwt.$^{-1}$ min^{-1}) was higher in at 80 mM (45.86) compared to control (34.02), but similarly result found in *A. rubra* (28.91 and 34.48)at 0 and 80 mM. Another antioxidant enzyme CAT (µmol h_2O_2 reduce mg^{-1} protein m^{-1}) was significantly increased (5.65) in *A. microphylla* at 80 mM as compared to control (3.28), but similarly found in *A. rubra* (2.45 and 3.57) at 0 and 80 mM (Table 3). Due to salt stress (NaCl), the distinct reaction of antioxidant enzymes like SOD APX and CAT indicates the role of oxidative stress as a part of *Azolla* plant environmental stress.

3.6 Osmotic stress

Salinity increased proline content (µ g g^{-1}) at different salt (NaCl) concentrations 80 and 120 mM NaCl in *Azolla* species (fig. 4C). Under 80 mM NaCl stress conditions, the highest proline content was observed in *A. microphylla* at 80 mM NaCl (34.16) as compared to control (21.23) at 0 and 80 mM (Table 3), similarly result was observed in *A. rubra* (20.27 and 30.84) at 0 and 80 mM.

3.7 Lipid peroxidation

Salinity increased MDA content at different salt (NaCl) concentrations 0 and 80mMNaCl in *Azolla* species. Under 80mMNaCl stress conditions, the highest MDA content was observed in *A. microphylla* at 80 mM (1.23) as compared to

control (0.93) (Table 3), similarly result was observed in *A. rubra* (0.87 and 1.22) at 0 and 80 mM.

3.8 Na^+, K^+ and Ca^{2+} content estimation

In this study, the highest Na^+ content (µmol mg dry wt^{-1}) was significantly increased observed in *A. microphylla* (36.16) exposed at 80mM as compared to control (26.59), similarly result was observed in *A. rubra* (25.49 and 105.75) at 0 and 80 mM. In case of K^+ content *A. microphylla* exposed to 80 mM NaCl the K^+ content was significantly increased (36.31) when NaCl concentration was increased as compared to control (22.58) (Table 3) but in case of *A. rubra* at 80 mM (13.11), K^+ content was decreased as compared to control (20.37). The Ca^{2+} content (µmol mg dry wt^{-1}) increased when salinity (NaCl) concentration was increased. Ca^{2+} content was significantly higher in *A. microphylla* (42.76) in 80 mM as compared to control (20.64) (Table 3), but similarity result found in *A. rubra* (14.22 and 36.83) at 0 and 80 mM NaCl.

4. Discussion

Many studies indicated that salinity is an important abiotic factor that reduces plant development and manufacturing (Parida and Das, 2005) [50]. However, strategies are required to increase salinity tolerance of these species in order to better exploit them for future use as bio-inoculants. With several experiments conducted by various researchers in order to find the best salt tolerant species of *Azolla*, the minimizing growth rate was observed in *A. pinnata* with change in different NaCl treatments like when the concentration was increased from 10 to 20 mM in the nutrient medium, indicating that 20 mM is the critical salt level for plants that did not thrive against salinity (Rai and Rai, 2000; Mishra and Singh, 2006) [55, 38]. In some cases the growth of *A. filiculoides* was reduce significantly by salt level above 10 mM NaCl whereas *A. pinnata* survived well in 30 mM but again the growth was almost completely reduce at 40 mM NaCl (Masood *et al.*, 2006) [36]. Inhibition of growth and down regulation of proteins related to energy metabolism and photosynthesis in *A. microphylla* due to salinity (Thagela *et al.*, 2017) [71]. Major causes of reduction of growth may be cause to osmotic injury or severe toxicity of ions due to salt entry (Nandwal *et al.*, 2000; Shrivastava and Kumar, 2015) [47, 64]. Excess salt reduces leaf water potential that results in reduced nutrient uptake and water by the plant (Baccio *et al.*, 2004) [10]. Salinity reduced biomass production of all *Azolla* species except *A. microphylla*, having highest resistance to salinity followed by *A. rubra*, but *A. pinnata* could not survive in high salt levels (Majumdar *et al.*, 1993) [35]. With respect to different levels of NaCl (0, 5, 100, and 150 mM), the relative growth rate (RGR) and biomass production decreased, and this decrease was found to increases the duplication of the control condition (Mostafa and Tammam, 2012) [43]. With increased salt stress condition the average root length of *A. microphylla* reduced due to water deficiency and reduced mineral uptake (Thagela, 2017, Rai and Rai, 2003) [71, 58]. The relative growth rate (RGR) of *A. filiculoides* was decreased when NaCl (30, 60, 90 and 120 mM) increased in the nutrient solution. Doubling time (DT) and relative growth rate (RGR) were decreased when *A. caroliniana* was treated with different levels of NaCl (0, 50, 100, and 150 mM) (Mostafa and Tammam, 2012) [43] due to inhibition of cell division of *Azolla*. Salt can directly have an impact on pigment content of the plant, like at higher concentrations of NaCl particularly 30 and 40 mM, the chl a/b ratio was significantly lower, and at lower NaCl concentrations such as 10 and 20 mM the chl a/b ratio was high, and significantly the chl a/b ratio was higher in control conditions (Masood and Abraham, 2006) [36] and reduction of chlorophyll (Chl) and carotenoides under salt stress can cause reduction in photosynthesis activity (Francisco *et al.*, 2002; Thagela *et al.*, 2018) [20, 72]. Example of such cases is found in *A. pinnata* where the amount of chlorophyll and carotenoides decreases in different salinity stresses (25, 50, 75 and 50 mM) with reduced photosynthetic pigment efficiency (Panda *et al.*, 2006) [49]. The reduction of Chls in salinity is related to photo inhibition or formation of reactive oxygen species (Kato and Shimizu 1985) [27]. Previously reports were found that due to salinity treatment decrease in chlorophyll content in *Azolla* occurs (Rai and Rai, 2003, Gomes *et al.*, 2017) [58, 22]. The impact of salt stress in *Azolla* fronds was found to be significant. Observed that the when increasing NaCl concentration the content of chlorophyll and carotenoid was reduced (Panda *et al.*, 2006) [49]. The impact of salinity on the antioxidant content has shown that pigment has accumulated in the form of flavonoids and anthocyanins in all plant areas. Anthocyanin is a flavonoid compound whose accumulation in plant exposed to salt stress has been largely documented under salt stress (Van Oosten *et al.*, 2013) [34]. Anthocyanin yield occurs in response to salt stress but not in response to other stresses like low phosphorous, high light, high temperature, or drought stress and other abiotic stresses. Electrolytic leakage (EL) increased with a rise in various salt concentrations in *A. microphylla*, *A. mexicana* and *A. filiculoids* (Parida and Das, 2005; Singh *et al.*, 2008) [50]. Increased solute leakage may be due to increased membrane permeability or a lack of membrane. The treatment of *Azolla* in 50 mM NaCl the percentage of solute leakage has the value 97 % higher as compared to control (Mostafa and Tammam, 2012) [43]. Solute leakage also increased with increasing level of salt stress. Significantly higher levels of electrolyte leakage percentage were observed in *A. filiculoides* as compared to *A. pinnata* exposed to NaCl. However, in *A. pinnata* at higher NaCl levels between 30-40 mM electrolyte leakage significantly increases (Masood *et al.*, 2006) [36] due to external membrane damage. The potential of stressed and adopted *Azolla* to maintain ion level under salt stress condition was observed with significant variance (Rai and Rai, 2000) [55]. Modulating ion transporters included in homeostasis has been observed to have salt stress (Tester and Davenport, 2003; Zhu, 2003) [70, 12]. An increased in Na^+ content of *A. microphylla* was found to be 95.7 % with direct exposure whereas with pre-exposed condition the internal Na^+ content of *A. microphylla* raised by 38.8 %. The direct exposer of *Azolla* to 60 mM NaCl showed increased Na^+ content than control and *A. filiculoids* have produced slightly more sodium in the 60, 90 and 120 mM NaCl treatments than those in the *Azolla* in controlled conditions (Mostafa and Tammam, 2012) [43]. Conversely, salt stress reduced K^+ concentration significantly (Mostafa and Tammam, 2012) [43] and the K^+ concentration in pre-exposed plants increased over the control value as well as the plants directly exposed. (Rai, 1999; Masood *et al.*, 2006) [59, 36]. Enzyme function can be inhibited due to unfavourable ratio of K^+ to Na^+ (Greenway and Munns, 1980) [23]. When NaCl induces reduction cellular K^+ content leading to salt toxicity in plants, K^+ was found responsible for salinity tolerant and K^+ is important factor for maintaining the turgor within the

plants cell. In *A. pinnta* exposed to 60 mM NaCl, the concentration of Ca^{2+} increased by 27% compared to control. Thus, with the rise in Na^{+} concentration, the intracellular Ca^{2+} level also increases sharply, which in turn promotes its attachment to the SOS3 protein. Together with SOS proteins, Ca^{2+} modulates homeostasis of the intracellular Na^{+}. Excessive accumulation of Na^{+} and Cl^{-} ions in salt affected ecosystems can also interfere with the uptake of essential macronutrients leading to nutritional imbalance in plants. Proline functions as a free radical scavenger against protein denaturation and prevents membrane degradation (Reddy *et al.*, 2004; Chris *et al.*, 2006) [60, 15]. Proline helps in osmo-regulation and defense protein dehydration and plays an important role in stressed condition by enzymatic regulation (Rontain *et al.*, 2002) [61]. Proline accumulates in greater numbers than all other amino acids because of the intensity of salinity (Ashraf and Harris, 2004) [8]. The diverse response of antioxidant enzymes such as SOD and APX due to NaCl stress indicates the function of oxidative stress on *Azolla* as a component of environmental stress. Salt tolerant plants appear to have a defense mechanism through the induction of SOD activity against salt-induced free radical development (Hernandez *et al.*, 1993) [25]. SOD activity increased significantly due to NaCl treatment (0, 4, 9 and 13) (33.34, 48.60 and 54.42 unit mg protein^{-1}). However, the rise in SOD operation was even higher in pre-exposed plants as compared to directly exposed plants. Higher SOD activity is an indicator of an efficient detoxification of the superoxide radicals, Salt-tolerant plant showed salt-mediated free radical manufacturing safety mechanism by mediating SOD concentration (Hernandez *et al.*, 1993) [25]. Reported in *A. pinnata* 12–80 % increase in SOD activity due to exposure to NaCl (10–40 mM) with *A. pinnata* had a substantially higher APX level at all levels of NaCl (10, 20, 30 and 40 mM) (Masood *et al.*, 2006) [36]. Salinity treatment showed an improvement in SOD and APX function and an improvement in antioxidant enzymes showed growth in salinity sensitivity to salt tolerance (Shalata *et al.*, 2001; Mostafa and Tammam 2012) [63, 43] and antioxidant enzyme profile as a basis to assess the salinity induced toxic effects in *A. microphylla* (Abraham 2010) [1]. Improvement of antioxidant defense system and sustainability in ion homeostasis are the key components in mitigating the adverse impacts of salinity (Singh *et al.*, 2015) [54]. Variations in antioxidant levels may be a guide for modulating ROS scavenging processes and transduction of ROS signals (Foyer *et al.*, 1997; Mittler, 2002) [19, 40].

5. Conclusion

The present study shows that the negative effect of salinity (0, 80 and 120 mM) on morpho-physiological, ion fluxes, antioxidant enzyme (SOD, APX CAT and MDA) activities, proline accumulation, nutrient profiling in tolerant (*A. microphylla*) and susceptible (*A. rubra*) species of *Azolla*. Result indicated that salinity reduces plant cell growth, production of plant pigment and antioxidant enzyme of *A. rubra*. Soil pH and EC was higher reduces by *A. microphylla.* Accumulation of proline, MDA and antioxidant enzyme was higher in *A. microphylla* for better growth and survivability.

6. References

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