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Screening protocol standardized for identification of salinity - tolerant *Azolla* strains

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Abstract
Salinity-tolerant *Azolla* is generally screened under hydroponics condition but its applicability under *ex situ* and *in vivo* is very challenging mainly because of stress heterogeneity, presence of other soil-related stresses and the significant influence of environmental factors such as temperature, relative humidity and solar radiation. Therefore, the present study aimed to standardise a suitable, rapid and efficient protocol to screen salt-tolerant *Azolla* for the first time by using already developed salinity-screening protocol for rice cultivars. Two species of *Azolla* (*Azolla microphylla* and *Azolla rubra*) and two rice varieties (Luna sankhi and IR- 64) were used as experimental material in this modified protocol where dimension of pots and quantity of soil and gravels have been reduced without altering its composition ratio. Two saline treatments were imposed by adding sodium chloride (80 mM and 120 mM NaCl) in pots and results were analyzed with respect to control (0 mM NaCl). By using the modified protocol, the observed parameters like soil electrical conductivity, soil pH, growth rate and antioxidant enzymes (superoxide dismutase, catalase and ascorbate peroxidase) showed the similar trend of results in *Azolla* with respect to rice. Finally, our study revealed that the modified salinity-screening protocol worked well for *Azolla* and it could be employed further for the identification of salt-tolerant *Azolla* strains in very efficient manner.

Keywords: *Azolla*, salinity**,** screening protocol

1. Introduction
Salinity is one of the most vicious abiotic stresses and it impacts 20% of irrigated land and 2% of the dry land of the cultivable area (Munns and Tester, 2008) [1]. Soil salinity reduces plant growth, production, destruction of cell and metabolic machinery, accumulation of toxic ions Na^+ and Cl^-, nutritional and oxidative stress imbalances in various agricultural crops including *Azolla* (Flowers and Colmer, 2008; Isayenkov, 2012; Deinlein *et al.*, 2014; Muchate *et al.*, 2016; Isayenkov and Maathuis, 2019) [2, 3, 4, 5, 6].

Azolla is a free-floating atmospheric nitrogen (N) fixer water fern and a known biofertilizer for low land rice crop (Kumar *et al.*, 2019, 2020, 2021; Kumar and Nayak, 2019) [7, 8, 9, 10], however, under salinity stress, its overall growth was hindered (Sadeghi *et al.*, 2013; Bhuvaneshwari and Singh, 2015) [11, 12]. *Azolla* may save up to half of the rice crop's N requirement and could add 40-60 kg N ha^{-1} (Kannaiyan, 1994; Kumar *et al.*, 2021) [13, 9]. However, salinity causes a major troubles it affects the N-fixation rate of *Azolla*-cyanobionts (Rai *et al.*, 2001) [14].

Though a considerable amount of study on the inhibitory impact of salinity on *Azolla* has been done previously (Masood *et al.*, 2006) [15], but to improve its salinity tolerance in the field, very few attempts have been made till date. Screening under field conditions is difficult due to stress heterogeneity, presence of other soil-related stresses and the significant influence of environmental factors such as temperature, relative humidity and solar radiation (Rao *et al.*, 2008) [16]. These complexities, together with the degree of control of salinity, cause difficulties in developing and using reliable methods of screening voluminous materials.

Hydroponics-based salt screening of *Azolla* was done so far (Asghar *et al.*, 2018) [17], however, it is suitable mainly under laboratory condition, hence, standardization of a robust screening protocol, particularly to screen salt-tolerance *Azolla* to address its applicability in the field was need of the hour. Therefore, the present study was an attempt to standardise a suitable, rapid and efficient protocol to screen salt-tolerant *Azolla* by using already developed protocol for screening rice cultivars under salinity (Chattopadhyay *et al.*, 2018) [18]. We expected that the developed salt-screening protocol in rice could also work well in *Azolla* as both grow better under aquatic environment.

2. Material and Method

2.1 Materials used

Two species of *Azolla* (*Azolla microphylla* and *Azolla rubra*) and two rice varieties (Luna sankhi and IR- 64) were taken as experimental materials for standardization of screening protocol under salinity condition. Drilled small plastic pots (30.48 cm diameter), soil (as a growing medium), different sizes of gravels (diameter:2-3 mm, 4-6 mm and 8-10 mm), nylon mess, perforated pipe (piezometer), large plastic tub (height 0.30 m and diameter 0.50 m and water capacity 30 L), sodium chloride (NaCl) and 0.1% mercury chloride ($HgCl_2$) were used in this experiment.

2.2 Study location and protocol for screening Azolla under salinity

Experiments were conducted at the microbiology net house (20.52º N latitude and 85.83º E 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 (Gregorio *et al.*, 1997; Bhowmik *et al.*, 2007; Chattopadhyay *et al.*, 2018) [19, 20, 18]. 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 (Fig. 1). Then all pots put into the plastic tub (height 0.30 m and diameter0.50 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* and rice cultivars selected for this experiment. The modified screening protocol for *Azolla* under saline condition in comparison with rice is mentioned in Table 1.

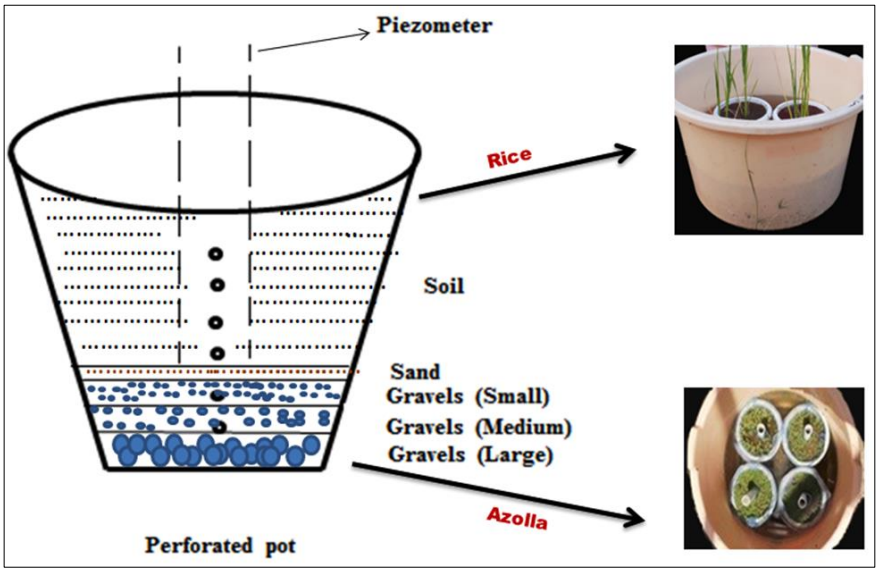

Fig 1: Comparative protocol of screening of rice and *Azolla* for salinity

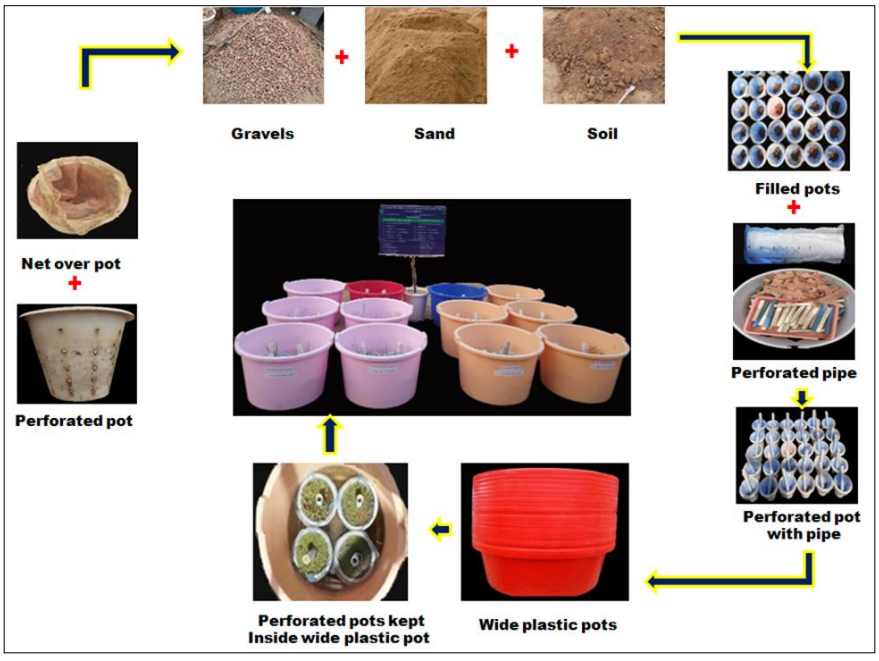

Fig 2: Protocol standardized for screening *Azolla* for salinity

Table 1: Modification screening protocol of *Azolla* for salinity in comparison with rice

Materials			Gravel size (mm)		
	Azolla*	**Rice#**		***Azolla***	**Rice***
Pot size (m)	0.15	12	Large	8-10	10-15
Water tank height (m)	0.30	0.5	Medium	4-6	6-8
Water tank diameter (m)	0.50	1	Small	2-3	2-3

* Modified protocol; #Chattopadhyay *et al.*, 2018.

2.3 Inoculation of Azolla and rice for saline-tolerance screening

Fresh *Azolla* (0.44-0.50 g) were surface sterilized using 0.1% mercury chloride ($HgCl_2$) and then washed with distilled water for four 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). Similarly, the surface sterilized 20-25 days of old rice seedlings were transplanted under suitable experimental conditions in perforated pots. In each pot, 10 mL of fertilizer solution of nitrogen (N, 100 kg ha^{-1}), phosphorous (P, 60 kg ha^{-1}) and potassium (K, 60 kg ha^{-1}) were added in the form of urea, single super phosphate and murate of potash, respectively. The salt stress was imposed from the beginning in *Azolla*. In case of rice the salt stress was imposed from 7 days after transplanting. At the time of rice planting, half of the quantity of N was applied and the remaining quantity was applied before panicle initiation.

2.4 Estimation of pH and electric conductivity

The pH and electric conductivity (EC) were measured on the experimental pots by using portable pH meter and EC/TDS meter.

2.5 Estimation of relative growth rate and dry biomass of Azolla

For determination of *Azolla* dry biomass productivity of the *Azolla* samples were collected after 15 days of its growth which rinsed with double distilled water, blotted using filter paper and recorded fresh weight immediately. The samples were 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.

$$RGR = (lnDw2 – lnDw1)/t2 - t1$$

Where, lnDw1 and lnDw2 were natural logarithm of initial fresh weight of *Azolla* and final fresh weight of *Azolla* (after 15 days of growth), respectively. At times t2 and t1, Dw2 and Dw1 were the weights.

2.6 Determination of superoxide dismutase (EC 1.15.1.1) activity

Superoxide dismutase (SOD) activity was determined as per methodology of Stewart and Bewley (1980) [21]. The fresh 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 7.5and for 10min the sample was centrifuged at 4 ℃ 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 100 mM phosphate buffer (pH 8.0), 200 mM methionine, 3 mM EDTA, 2.25 mM NBT, 0.1 mL Na_2CO_3 and 0.6 mL distilled water. Followed by addition of 2 mM riboflavin to the sample mixture in order to initiate chemical reaction and the sample was placed at a 30 cm apart from the fluorescent tube for 10 min and the absorbance was determined at 590 nm.

2.7 Estimation of catalase activity

Catalase (CAT) activity assay methodology was described by Aebi (1984) [22]. In a chilled mortar and pestle in an ice-cold homogenization extraction buffer (pH 8.0) used for CAT asses, plant (0.1 g fresh weight) were homogenized. The activity of catalase was determined by tracking the absence of hydrogen peroxide by measuring the absorbance reduction at 560 nm. The reaction was conducted in a reaction mixture containing 1.5 mL of reaction buffer (pH 7.0), 0.3 mL of enzyme extract, 0.5 mL of H_2O_2 and 0.8 mL of purified water added at the time of absorption. The amount of enzyme required for the decomposition of 1 µmol of H_2O_2 per mg of protein was calculated as one enzyme unit.

2.8 Determination of ascorbate peroxidase (EC 1.11.1.11) activity

Ascorbate peroxidase (APX) activity was determined as per methodology given by Nakano and Asada (1984) [23]. In an ice-cold homogenization buffer the 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 mL guiacol (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 mg of protein per minutes at 25 ℃.

2.9 Estimation of protein

*Azolla*protein was determined as per methodology for determination of assay by Bradford (1976) [24], taken standard observation at 595 nm absorbance. A sample of *Azolla* (100 - 300 mg fresh weight) was powdered with liquid nitrogen, using pre-cooled mortars and pestles. *Azolla* proteins was extracted by blended in the described cold 0.05 M Tris buffer. A small quantity (0.05 g) of the antioxidant polyvinyl polypyrrolidone (PVPP) was added to each sample during the blend procedure, keep tube in ice. Homogenates were transferred to cold centrifuge tubes (2 mL Eppendorf tubes) and then was centrifuged at 14000 - 19000 for 20 minutes at 4 ℃. After centrifugation, clear supernatants were used immediately for the protein assay at 595 nm, or frozen at -20 ℃ and used later.

2.10 Estimation of yield attributing traits of rice

Yield attributing characteristics plant height and panicle weight was calculated as per methodology of Bouslama and Schapaugh (1984) [25].

3. Result

3.1 Soil EC and pH in *Azolla* and rice under salinity

Soil EC (dS m^{-1}) in *A. microphylla* (7.77 and 10.83) and *A. rubra* (8.4 and 11.11) were increased proportionally at 80 and 120 mM NaCl compared to control (0 mM NaCl; 0.53 and 0.69, respectively) (Table 2). Similar trend of soil EC was also observed in both rice cultivars (Luna sankhi and IR64) (Table 2). Whereas soil pH was decreased in *A. microphylla* (6.37 and 6.42) and *A. rubra* (6.44 and 6.73) under 80 and 120 mM NaCl, respectively compared to control (7.35 and 7.31) (Table 2). Similar trend of soil pH was also observed in both rice cultivars (Luna sankhi and IR64) (Table 2).The reduction of soil EC and pH was more prominent in *A. microphylla* compared to *A. rubra*, whereas these parameters were more reduced in Luna sankhi than IR64 (Table 2).

Table 2: Physiological and biochemical changes in *Azolla* and rice plant under salt stress

Treatment	Soil EC				Soil pH				Plant growth				Yield attributing character			
	Azolla		Rice		*Azolla*		Rice		*Azolla* (RGR)		Rice (PH)		*Azolla* (DW)		Rice (PW)	
NaCl*	AM	AR	LS	IR-64	AM	AR	LS	IR-64	AM	AR	LS	IR-64	AM	AR	LS	IR-64
0	0.53^{c}	0.69^{c}	0.63^{c}	0.71^{c}	7.35^{a}	7.31^{a}	7.52^{a}	6.86^{a}	0.17^{a}	0.12^{a}	60.1^{a}	52.75^{a}	0.14^{a}	0.09^{a}	2.57^{a}	1.54^{a}
80	7.77^{b}	8.40^{b}	7.64^{b}	8.76^{b}	6.37^{b}	6.44^{b}	6.55^{b}	6.52^{c}	0.05^{b}	0.01^{b}	47.2^{b}	39.25^{b}	0.10^{b}	0.03^{b}	1.35^{b}	0.03^{b}
120	10.83^{a}	11.11^{a}	10.91^{a}	11.41^{a}	6.42^{b}	6.73^{c}	6.99^{c}	6.70^{b}								

*NaCl: sodium chloride (mM); RGR: relative growth rate (g g^{-1} fresh *Azolla*); PH: plant height (cm); DW: dry weight (t ha^{-1}); PW: panicle weight (g); AM: *Azolla microphylla*; AR: *Azolla rubra*; LS: Luna Sankhi (salt-tolerant rice cultivar); IR64: salt-sensitive rice cultivar.

3.2 Relative growth rate and yield attributes of *Azolla* and rice under salinity

Relative growth rate (RGR, g g^{-1} fresh *Azolla*) of *A. mirophylla* (0.05) and *A. rubra* (0.01) was significantly decreased in 80 mM NaCl than control (0.17 and 0.17, respectively) (Table 2). Similarly, reduced plant growth of two cultivars in rice was also observed (Table 2). Yield attributes like *Azolla* dry weight and rice panicle weight in selected species also showed significant reduction in 80 mM NaCl than control (Table 2).

3.3 Antioxidant enzyme of *Azolla* and rice

SOD activity (unit mg^{-1} fm) in *A. microphylla* and *A. rubra* was significantly increased in 80 mM NaCl (0.53 and 0.42, respectively) than control (0.29 and 0.26 respectively) (Table 3). Similarly, CAT (µmol H_2O_2 reduce mg^{-1} protein m^{-1}) APX activity (unit g.fwt.$^{-1}$ min^{-1}) were also increased significantly in *A. microphylla* (3.44 and 1.52) and *A. rubra* in 80 mM NaCl (2.85 and 1.49) as compared to control (Table 3). The similar trend of SOD (unit mg^{-1} fm), CAT (µmol H_2O_2 reduce mg^{-1} protein m^{-1}) APX activity (unit g fwt^{-1} min^{-1}) were also observed in both cultivars (Luna sankhi and IR64) of rice (Table 3).

Table 3: Antioxidant activities (SOD, CAT and APX) in *Azolla* and rice under salt stress

Treatment	SOD				CAT				APX			
	Azolla		Rice		*Azolla*		Rice		*Azolla*		Rice	
NaCl*	AM	AR	LS	IR-64	AM	AR	LS	IR-64	AM	AR	LS	IR-64
0	0.21^{b}	0.24^{b}	0.29^{b}	0.26^{b}	3.28^{b}	2.45^{b}	3.23^{b}	1.95^{b}	34.02^{b}	28.91^{b}	1.41^{b}	0.97^{b}
80	0.67^{a}	0.32^{a}	0.53^{a}	0.42^{a}	5.65^{a}	3.57^{a}	3.44^{a}	2.85^{a}	45.86^{a}	34.48^{a}	1.52^{a}	1.49^{a}

*NaCl: sodium chloride (mM); SOD: super oxide dismutase (unit mg^{-1} fm^{-1}); CAT: catalase (µmol H_2O_2 reduce mg^{-1} protein m^{-1}); APX: ascorbate peroxidase activity (unit g fwt^{-1} min^{-1}).

4. Discussion

The procedure for screening rice plants for salt tolerance has been well established and validated through number of experiments (Gregorio *et al.*, 1997[19]; Islam *et al.*, 2012) [26]. However, screening and validation of salt-tolerant *Azolla* are currently very limited; therefore, the present study highlighted the standardization protocol of salinity for rapid screening of *Azolla* under *ex situ* condition. Previously, a novel protocol was developed to identify salt-tolerant rice cultivars with high level of efficacy to maintain its tolerance at desired level of soil salinity (Chattopadhyay *et al.*, 2018; Bhowmik *et al.*, 2007) [18, 20].The similar protocol has been modified in the present study to screen *Azolla* strains for the first time and compared its efficacy with rice.

Azolla is used as N-fertilization in rice crops, but under saline soil its N-fixing efficacy is drastically reduced. Our findings showed the proportional increment of soil EC in saline-treated *Azolla* and the similar result was also observed in rice which indicated that modified protocol perform well in case of *Azolla*. Similar result also observed for soil pH in both *Azolla* and rice. Alteration of EC and pH provide good indicators of efficacy of this protocol (Asghar *et al.*, 2018) [18].Our result also suggested that longer duration salt tolerance studies in soil: stone (gravels) medium was comparatively better in terms of uniformity and homogeneity in maintaining the appropriate level of salt stress for a longer duration of study.

Salinity was negatively impacted plant growth and yield attributes which has been corroborated with our study as RGR and dry weight of both species of *Azolla* and plant growth and panicle weight of both cultivars of rice were significantly reduced under higher NaCl concentration (80 mM). However, the scavenging mechanism of radicals activated during the course of their metabolic activity, was found to be more robust in *A. microphylla* and Luna Sankhi in determining the innate capability of this *Azolla* species for withstanding salinity stress. Previous study also showed that reduced RGR was observed in *A. pinnata* with change in different NaCl treatments (Rai and Rai, 2000; Mishra and Singh, 2006) [27, 28]. Major causes of reduction of growth may be due to osmotic injury or severe toxicity of ions due to salt entry (Nandwal *et al.*, 2000; Shrivastava and Kumar, 2015) [29, 30]. Excess salt reduces leaf water potential that results in reduced nutrient uptake and water by the plant (Baccio, 2004) [31].

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. SOD activity increased significantly due to NaCl treatment. However, the rise in SOD operation was even higher in pre-exposed plants as compared to directly exposed plants. Previous study also reported that 12-80% increased SOD activity in *A. pinnata* due to exposure to NaCl (10-40 mM) (Masood *et al.*, 2006) [16]. Finally, the present study proved that the modified salinity protocol showed the same trend of result in *Azolla* with respect to rice. Thus, the present standardized screening protocol will be employed in the large scale study for the identification of salt-tolerant *Azolla* strain.

5. Conclusions

The present study concludes the similar trend of results for soil EC, pH, growth rate and antioxidant enzymes (superoxide dismutase, catalase and ascorbate peroxidase) in both *Azolla* species (*A. microphylla* and *A. rubra*) with respect to rice cultivars by using modified salinity-screening protocol. Thus, this protocol proved for the first time that it could serve effectively for screening and identification of salt-tolerant *Azolla* strains.

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