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Evaluation of salt stress and molecular analysis of genetic variation of Iraqi rice cultivars

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Abstract. *Mandal AM, Alhasnawi AN, Jasim H, Mohamad A. 2019. Evaluation of salt stress and molecular analysis of genetic variation of Iraqi rice cultivars. Biodiversitas 20: 3309-3314.* The salinity-stress casts an inhibitory effect on rice, which is not only complex but also a key reason for impeding plant growth as well as decreasing crop productivity. A helpful method to improve our understanding in this space would be to make a direct comparison pertaining to the response to salinity by considering two closely related cultivars, i.e. Yassimen and Anber-33, which demonstrate varying levels of salt-tolerance. Exposing rice seeds to high concentrations of 200mM NaCl stress resulted in considerable impact on shoot, chlorophyll content and root growth. The growth of both cultivars was considerably decreased due to salt toxicity. A range of differences was seen in terms of growth parameters due to interaction between cultivars and salt levels. It can be said that the various cultivars vary in terms of their salt tolerance with regards to seedling growth and seed germination. Based on the results from the Inter-simple sequence repeats (ISSR) data, cultivar-Anber-33 had 27 bands in total, while for cultivar-Yassimen, it was 25. These results demonstrated that ISSR markers proved to be a valuable method for determining genetic variation, fingerprinting, classification, and identification of Iraqi rice cultivars.

Keywords: Chlorophyll, genetic analysis, ISSR markers, morphological variations, salt toxicity

INTRODUCTION

Genetic potential pertaining to soil physical, crop cultivar, biotic stresses, and chemical characteristics impacts the development, growth and yields of crops (Subash et al. 2011). More than half of the global population considers rice to be a key staple crop. Historically, for many breeding programs, enhancing yield was the key import target. In the past half-century, two big leaps of rice yield have been recorded, mainly due to genetic enhancement: producing hybrids by employing heterosis and decreasing the height of the plant by employing semi-dwarf gene to improve harvest index (Chen et al. 2010). However, amongst many challenges faced in rice production, salinity is deemed as common abiotic stress pertaining to agriculture production (Wang et al. 2013). Many countries face issues in agriculture strengthening due to salt-affected soils that are serious hindrances. In semiarid and arid regions, sodicity and salinity are grave issues, usually caused by adverse climatic conditions as well as mismanagement of agricultural lands by using brackish and saline water for irrigation (Ahmad et al. 2013). In susceptible rice cultivars, complete submergence speeds up the degradation process of chlorophyll content versus in tolerant ones (Damanik et al. 2010).

To improve the yield of food, it is important to enhance the crops' tolerance against various abiotic stresses as well as expand their adaptation to different environments, within the limited land area and natural resources in tandem with changing environments. The key goal of modern plant biotechnology now is to enhance crops in terms of their abiotic stress tolerance (Chen et al. 2010).

It is known that plants make use of various strategies to minimize salt injury, and characteristically, these relate to adjusting the water potential and, secondly, sequestering and/or excluding the excess salt out from the metabolism sites in the cells, especially organelles such as mitochondria, chloroplast, etc. due to their vulnerability to oxidative damage. Furthermore, if salt is retained in excess, it becomes more injurious to plants that are exposed to high illumination, and a high oxidative burst/exposure is induced with the decrease in the molecular oxygen through highly energized electrons (via the electron transport chain), which is then converted into some over-oxidized moieties commonly known as ROS. Generation of ROS, such as O_2^-, $OH^\bullet$, and H_2O_2, in excess induces rapid cell damage by activating a chain of reactions. This results in making the plant more susceptible to oxidative damage as well as prone to salt that impacts the tissues (Ghosh et al. 2011). For these radicals, the chief production areas in the plant cells are situated in the electron transfer chain pertaining to chloroplast and mitochondria as well as peroxisome. Certain protection mechanisms are developed by plants to protect against harmful effects caused by ROS. One of the major antioxidant defense systems for cultivars

are the antioxidant enzymes (Sen et al. 2011). Only few rice cultivars are submergence tolerant, while most are not (Damanik et al. 2012).

The genetic variations pertaining to different crop plants help in offering a valuable tool to choose cultivars that possess desirable traits (Danai-tambhale et al. 2011). Inter-simple sequence repeats (ISSRs) are regions that are present in the genome flanked SSR sequences. Numerous amplification products can be produced via PCR amplification of these regions by employing a single primer, which can also be utilized as a dominant multi-locus marker system to evaluate genetic variation pertaining to different organisms. ISSR-fingerprinting is popular due to its associated lowcost and ease of use as well as methodologically less demanding versus other dominant markers, which makes it an optimum genetic marker for using ISSRs (Ng et al. 1994). ISSR amplification has been deemed a valuable technique to swiftly identify cultivars as well as determine genetic variability that could exist amongst plant varieties. The genetic fingerprinting method has been considered to be effective and also helps to define the large numbers of plant accessions that were held in national and international germplasm centers (Alhasnawi et al. 2019). This study was aimed at determining the impact of various concentrations of salt stress on chlorophyll, growth parameters and molecular analysis pertaining to genetic variation by considering two Iraqi rice cultivars.

MATERIALS AND METHODS

Plant materials and culture conditions

From the research center for rice in Al-Mishikhab, obtaining of two rice (*Oryza sativa* L.) cultivars: Yassimen and Anber-33. Germinating seeds grown in aqueous solution in a laboratory were assessed in five different levels of salinity (0, 50, 100, 150, and 200 mM NaCl). For each cultivar, treatment of ten seeds was done with NaCl. In this study, three replications with ten samples per each replication were applied.

Growth parameters and chlorophyll content

Post 1 week, seedling separation into root and shoot lengths (cm), which were recorded on a metric scale. Post 2 weeks, separation of the seedlings was done into shoots and roots fresh and dry (mg plant^{-1}) matter for shoots and roots as well as number of roots were measured. Goodwin (1977) methods were employed to measure the content of chlorophyll.

Statistical analysis

One-way analysis of variance was done for the experiment along with SAS-programmer. Data presentation was done as means per group ± standard errors of the means, which were determined by employing. Significant differences among the mean values of treatments were determined using Duncan's Multiple Range Test (DMRT) that was calculated a ($P \leq 0.05$).

ISSR primer and PCR-amplification

Plant Genomic DNA Extraction Mini Kit (50 preps), FavorPreo^{TM}, FAVORGEN, BIOTECH CORP. was employed to isolate genomic DNA from two cultivars-(Yassimen and Anber-33). In this study, the employed ISSR primers were synthesized based on Alpha DNA Canada. Initial screening of 7 ISSR primers was done, and all 7 demonstrated discernible and bright bands, which were employed for the analysis (Table 1).

One Taq Quick-Load 2X Master Mix with Standard Buffer was employed to conduct ISSR amplification reactions. Dilution of DNAs was done for ISSR-PCR along with TE buffer so as to achieve a concentration of 25 µg µL^{-1}, by employing a weighted marker with 0.8% agarose gels. For each accession, scoring of reproducible ISSR products was done manually for band presence (1) or absence (0). A binary qualitative data matrix was done after the scoring. The Polymorphism Information Content (PIC) values for each ISSR markers were estimated by determining the frequency of alleles per locus according to (Chesnokov et al. 2015).

RESULTS AND DISCUSSION

As per ANOVA, a significant difference persisted amongst growth response ($P \leq 0.05$) pertaining to rice (*Oryza sativa* L.) for two cultivars Yassimen and Anber-33, at various salt concentrations viz. five different levels of salinity (0, 50, 100, 150, and 200 mM NaCl) (Figures 1, 2, 3).

Table 1. Primer sequence (5' - 3'), temperature, and length (mers) by seven ISSR markers used in this study

Primer		Primer Sequence (5' - 3')	Temperature	Length (mers)
ISSR1	$(AC)_8G$	ACACACACACACACACG	48.5	17
ISSR2	$(AC)_8Y$	ACACACACACACACACY	47.1	17
ISSR3	R $(ACA)_5$	RACAACAACAACAACA	42.2	16
ISSR4	$(CA)_8DT$	CACACACACACACACADT	48.3	18
ISSR5	$(GT)_8C$	GTGTGTGTGTGTGTGTC	48.3	17
ISSR6	$(GACA)_5$	GACAGACAGACAGACAGACA	48.3	20
ISSR7	G $(CA)_8$	GCACACACACACACACA	48.3	17

Note: Y= C, T; R = A, G; D = A, G, T

The data demonstrated analysis of variance to determine the impact of various NaCl-stress concentrations on plant height to Anber-cultivar seedlings, while a less value of 200 mM was demonstrated with NaCl treatment (Figure 1.A). Salinity caused a significant reduction of the roots length pertaining to Anber-cultivar, compared to the non-saline control and a less value if 150 and 200 mM was demonstrated with NaCl treatment (Figure 1.B). The controls showed the maximum roots length and plant height pertaining to Anber-cultivar seedlings. For other growth parameters, based on ANOVA, a significant difference was seen amongst fresh and dry weight pertaining to shoots and roots, while the number of roots decreased with rising in NaCl-concentration for Anber-cultivar seedling (Figure 1.C).

For the entire studied parameters, a very high significant NaCl stress was seen in the 'plant height and roots length' of Yassimen cultivar. The plants had less height and their root lengths were between 150 and 200 mM with NaCl treatment, versus other treatments. The control showed the highest values to 'plant height as well as roots length' for Yassimen cultivar (Figure 2.A-B).

In the current research, (0, 50, 100, 150, 200 mM) NaCl-stress was applied exogenously, which observed a considerable decrease amongst fresh and dry weight for shoots and roots as well as the number of roots. For shoots and roots as well as the number of roots, minimum values of 200 mM NaCl were seen, versus the control (without salt) that yielded the maximum values with Yassimen-cultivar (Figure 2.C).

Based on these results, NaCl stress concentrations were found to cast greatest significant impact on the decrease in content pertaining to total chlorophyll from 4.23 mg/g (control) to 2.63 mg/g (50 mM), 2.16 mg/g (100 mM) and 1.2 mg/g (150 mM), and 0.7 mg/g (200 mM), correspondingly, with NaCl on Anber-cultivar seedling (Figure 3.A, C).

Figures (3.B, C) show the mean values of variance analysis that has been applied to the data, which demonstrated a very high significant total chlorophyll as well as NaCl-stress impact, 3.8 mg/g (control) to 2.91 mg/g (50 mM), 2.47 mg/g (100 mM), 1.8 mg/g (150 mM) and 1.2 mg/g (200 mM), correspondingly, with NaCl on Yassimen-cultivar.

Table 2. Summary of PCR amplification; Total amplified bands and Approximate size range (bp) of seven ISSR markers per PCR amplification on two rice cultivars (Anber-33 cv and Yassimen cv)

Primer		Anber-33 cv		Yassimen cv		PIC value
		Total amplified bands	Approximate size range (bp)	Total amplified bands	Approximate size range (bp)	
ISSR1	$(AC)_8G$	6	400-1600	-	-	0.75
ISSR2	$(AC)_8Y$	5	300-1000	5	300-1000	0.25
ISSR3	R $(ACA)_5$	7	400-1500	8	300-1500	0.25
ISSR4	$(CA)_8DT$	4	300-1300	-	-	0.75
ISSR5	$(GT)_8C$	3	200-500	10	200-1800	0.53
ISSR6	$(GACA)_5$	1	100-100	1	100-100	0.50
ISSR7	G $(CA)_8$	1	100-100	1	100-100	0.00
Total		27		25		3.03
Average		3.85		3.57		0.43

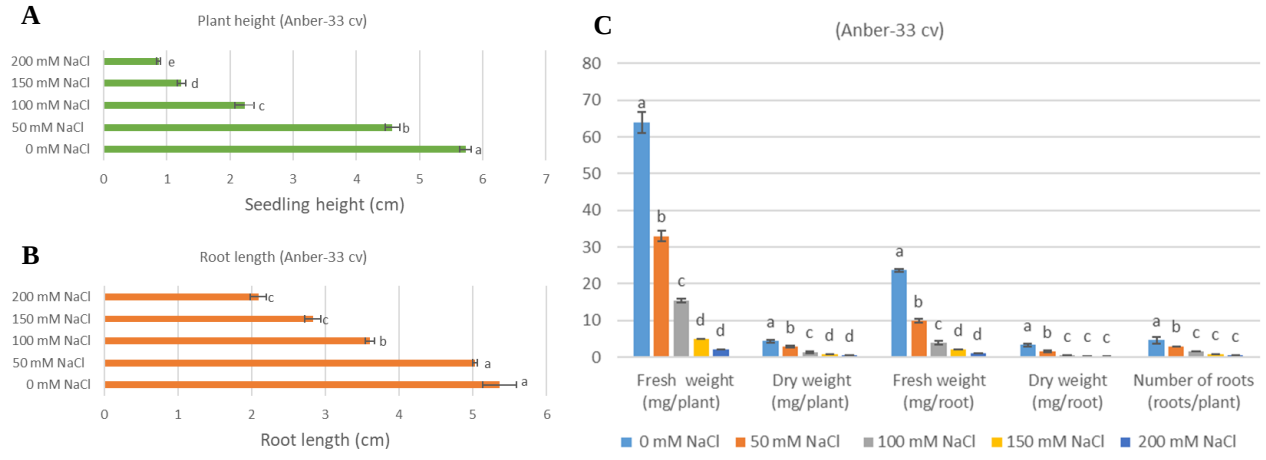

Figure 1. Effect of different concentrations of NaCl on plant height (cm), shoot fresh weight (mg plant^{-1}), shoot dry weight (mg plant^{-1}), root fresh weight (mg), root dry weight (mg), and number of roots on rice cultivar (Anber-33)

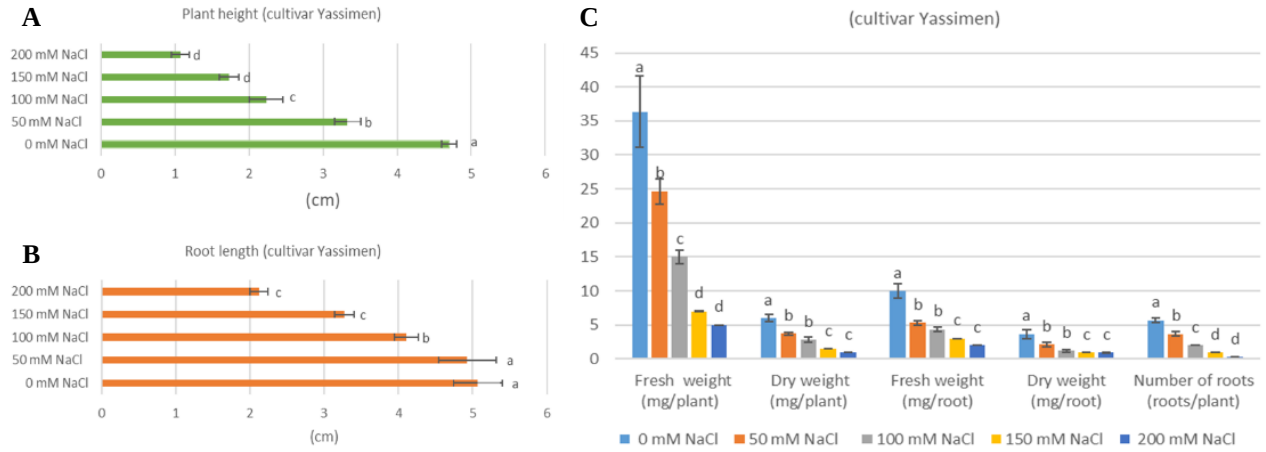

Figure 2. Effect of different concentrations of NaCl on plant height (cm), shoot fresh weight (mg plant^{-1}), shoot dry weight (mg plant^{-1}), root fresh weight (mg), root dry weight (mg), and number of roots on rice cultivar (Yassimen)

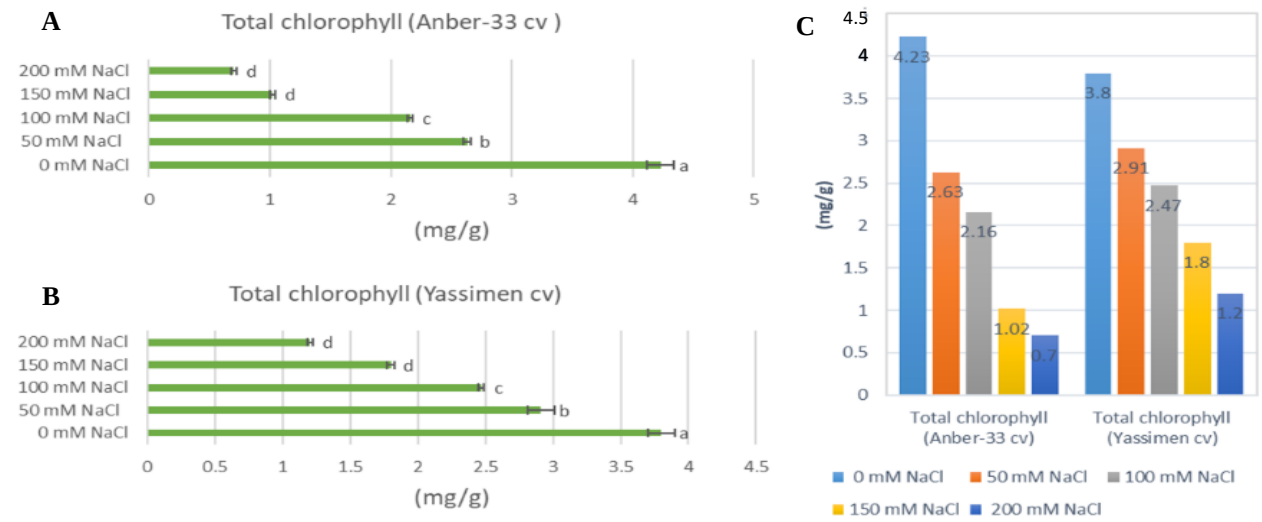

Figure 3. Effect of different concentrations of NaCl on total chlorophyll (two rice cultivars; Anber-33 cv and Yassimen cv)

PCR assays employing the 7 primers were chosen for ISSR amplification (Figure 4 and Table 2). Investigation of DNAs was done derived from the bulks pertaining to each of the two rice cultivars Yassimen and Anber-33. The same table lists out the total number of amplified bands, wherein obtaining of approximate size range (bp) is done per each primer as well as cultivars.

Based on our data, the total number of amplified products was 27 (with an average of 3.85 bands per primer). The amplicons size ranged from 400-1,600 bp and yielded 6 bands for primers ISSR1. The amplicons size ranged from 400-1,600 bp, ISSR2 primer yielded 5 bands. The amplicons size ranged from 400-1,500 bp and yielded 7 bands for primers ISSR3. The amplicons size ranged from 300-1,300 bp and primers ISSR4 yielded 4 bands. For ISSR5 primer, 3 bands were yielded by the amplified bands along with a molecular size ranging from 200-500 bp. In the results pertaining to ISSR6 and ISSR7; the amplified bands yielded 1 band with a molecular size of almost 100 bp with DNA Anber cultivar.

Amplified products in total were 25 (with an average of 3.57 bands per primer). Based on our data, the primers ISSR1 revealed no amplification in Yassimen cv. The number of amplified bands that was yielded by employing ISSR2 primer showed 5 bands with amplicons size ranging from 400-1,600 bp. For primers ISSR3, the amplicons size ranged from 300-1,500 bp and yielded 8 bands. The primers ISSR4 demonstrated no amplification. In ISSR5 primer, 10 bands were yielded by the amplified bands along with a molecular size ranging from 200-1,800 bp. Based on the results pertaining to ISSR6 and ISSR7; the amplified bands yielded 1 band that had a molecular size of almost 100 bp with DNA Yassimen cultivar.

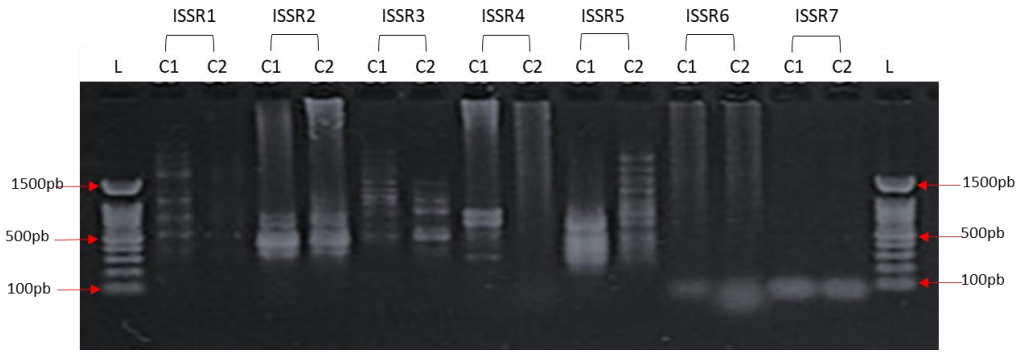

Figure 4. Seven ISSR amplification products obtained from the two rice cultivars (C1; Anber-33 cv and C2; Yassimen cv)

In our research, in evaluating the polymorphism information content (PIC) for the dominant each ISSR markers. The average PIC value of the 7 ISSR markers was (0.43). The maximum value is 0.75. Note, that for the ISSR1 (AC)8G and ISSR4 (CA)8DT markers with equal distribution in the population the PIC values are higher comparison low PIC value for the ISSR primer (0.00). Note, that for the ISSR7 G $(CA)_8$ (Table 2).

Discussion

Based on these investigations, applying various NaCl stress levels was done post-germination in order to assess variations pertaining to physiological, morphological and genetic characteristics of rice two cultivars Yassimen and Anber-33. These results signify that salt stress had a higher inhibitory impact on shoot growth versus root. Similar responses could also be observed in rice cv 'Yamahoushi (Sathish et al. 1997)

Salinity has an impact on seedling growth and influences the metabolism because of low external water potential resulting in osmotic inhibition of water availability, nutritional imbalance due to such ions and toxic impacts of salt ions (Mishra et al. 2013). The physiological mechanisms that were associated with the decrease in plant growth caused by salinity include reduction in photosynthetic activity, molecular responses, turgor pressure reduction, oxidative stress and impacts of accumulated salts on metabolic activities (El-Bastawisy 2010).

The current global challenge pertains to protecting crops against damages caused by salinity. Osmotic/ionic stress results due to high salinity (e.g. increased concentrations of Cl^- and Na^+ in the soil solution). The response of plant growth towards salinity occurs in two phases: (i) a rapid osmotic phase, which commences right away after increase in salt concentration near the roots to a threshold level, which leads to considerable decrease in the rate of shoot growth and (ii) a slower ion-specific phase, which commences with the build-up of salt concentrations to toxic levels in old leaves, and subsequently their death (Rai et al. 2010).

The length decrease, when exposed to salt stress conditions, may be a strategy to minimize water flow from roots to soil, while maintaining lower soil osmotic potential versus roots (Aroca et al. 2011). Salinity tolerance could be enhanced with reduced root and increased shoot growth, with the action of limiting the flux of toxic ions to the shoot while delaying the onset of tolerance threshold simultaneously. In the salt-tolerant rice cultivar, this factor could possibly aid in salinity tolerance (Pattanagul et al. 2008). A key criterion for monitoring plant growth in stress studies is to determine the levels of photosynthetic pigments that are impacted by environmental factors like salinity (Sen et al. 2011). It has been previously shown that a difference exists for plant in terms of reduction of chlorophyll when exposed to saline conditions (Taylor et al. 2001).

The photosynthetic capacity of a leaf relies on physiological properties like Rubisco activity, chlorophyll content and photosystem efficiency. Decrease in the plant's chlorophyll content occurs in tandem with a lower efficiency of PSII and senescence. Another screening parameter is leaf injury for determining salinity tolerance, which could be measured based on premature chlorophyll loss, membrane damage or damage caused to the photosynthetic apparatus. However, these methods are limited to discriminating between genotypes that tolerate moderate or low salinities (Rai et al. 2010).

One bulk for each cultivar was employed to perform DNA analyses. As bulk samples pertaining to DNA were employed in the production of ISSR, amplification could be performed for a mixture of sequences possessing various degrees of homology with the primer. This research found that there exists a relationship between genetic variability and rice cultivars. ISSR primers are regarded as an efficient determination tool to perform further molecular studies for specific rice species genetically, and this has been recorded for all varieties of rice (Mohamad et al. 2017). To successfully conduct ISSR analysis, it is important that pairs of simple sequence repeats occur in a short distance (in base-pairs), which is amplified via a PCR reaction that yields a band that can be resolved with agarose gels or standard polyacrylamide. The ISSR-technique finds another key application in rice, i.e. assessment of genetic diversity. Since large numbers of DNA-fragments per reaction are amplified by the ISSR technique, which signifies multiple loci from across the genome, it is regarded to be an optimum method for fingerprinting rice

varieties as well as a helpful substitute to hybridization-based or single-locus methods (Alhasnawi et al. 2015). The high values of differentiating ISSR were present because of comparatively higher levels of marker ratio, marker index and average PIC based on the diverse nature of the germplasms examined. The ISSR primers amplified microsatellite regions that were potentially polymorphic (Alhasnawi et al. 2015).

In conclusion, the study showed halophyte of rice two cultivars Yassimen and Anber-33 to be very similar at the genome level, but demonstrated extreme differences with regards to their response to NaCl-stress. The results pertaining to the current investigation corroborate the importance of salt stress-induced changes in shoot, chlorophyll, root growth and germplasm diversity pertaining to rice seedling by employing dominant DNA marker like ISSR-markers, which are regarded as a powerful tool to understand genetic variation, generate fingerprinting keys and possess the potential to detect cultivar-specific markers pertaining to rice cultivars.

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