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A new phenotyping technique for salinity tolerance at the reproductive stage in rice

Cecilia Diana C Palao[1], Celia B Dela Viña[1], Glenn B Gregorio[2] and Rakesh Kumar Singh*[2]

[1]University of the Philippines at Los Baños, Laguna, Philippines

[2]International Rice Research Institute, DAPO 7777, Metro Manila, Philippines

**E-mail: r.k.singh@irri.org*

ABSTRACT

Reproductive-stage salinity tolerance has been difficult to study due to the complexity of the trait and the lack of reliable stage-specific phenotyping techniques. A leaf-cutting technique was developed with the minimum number of leaves needed by the rice plant that will not significantly affect grain yield and yield components in order to standardize rice screening for reproductive-stage salinity tolerance. Salt stress equivalent to EC 10 dSm^{-1} was imposed to rice plants with trimmed leaves starting from boot leaf emergence up to 10 days in a pot experiment under controlled conditions. The stage-specific effect of salt stress was verified by observing saltsensitive (IR64) and salt-tolerant (IR4630-22-2-5-1-3) genotypes, as well as 201 F_2 plants derived from their cross. Leaf cutting before the booting stage efficiently directed the salt concentration to the reproductive stage and helped in discriminating the tolerant genotypes from the sensitive ones as evidenced by the low pollen viability and higher accumulation of toxic ions in the flag leaf of the sensitive genotype (IR64). The opposite was found true for the tolerant genotype (IR4630-22-2-5-1-3).

Key words: *rice, phenotyping technique, reproductive stage, salinity tolerance, pollen viability, salt stress*

Rice as a crop is generally regarded as sensitive to salt stress but most rice varieties exhibit variable reactions to salinity depending on the growth stage at which they have been exposed to saline conditions (Maas and Hoffman, 1977; Flowers and Yeo, 1981; Khatun *et al.,* 1995; Zeng and Shannon, 2000; Gregorio *et al,* 2002; Lisa *et al.,* 2004; Munns *et al.,* 2006; Singh and Flowers, 2010). It has also been reported, however, that the capacity of the rice plant to survive harsh saline environments at the vegetative stage is not actually correlated with its reproductive-stage tolerance (Mishra, *et al.,* 1998; Singh *et al.,* 2008, Moradi and Ismail, 2007; Rao *et al.,* 2008; Singh and Flowers, 2010). Salinity has in fact independent effects at these two critical stages, that is, tolerance at the seedling stage is not necessarily associated with tolerance at the reproductive stage, and vice versa. The reproductive stage seems to be more relevant for grain yield because it is within this stage that fertilization and formation of the seed occurs. It has long been recognized that salinity can cause sterility in rice, particularly if imposed during pollen development and fertilization (Jenks *et al.,* 2007); hence, high-yielding salt-tolerant rice varieties must possess reproductive-stage tolerance.

Although major QTLs for salinity tolerance have already been identified for seedling-stage tolerance (Bonilla *et al.,* 2002; Niones, 2004; Thomson *et al,* 2007; Singh *et al.,* 2010; Negrao *et al,* 2012), research on reproductive-stage salinity tolerance has progressed quite slowly, with only a few studies and small-effect QTLs described so far. This is probably due to the labor-intensive, time-consuming and difficult screening technique associated with phenotyping exclusively at the reproductive stage of rice *vis-à-vis* the seedling stage that has already been well standardized and is relatively easier, quicker, and straight forward. Because of the complexity of the trait, standardizing a phenotyping protocol at the reproductive stage alone without involving any other growth stage becomes another really complex problem.

Many physiological mechanisms operate in crop plants, including rice, that make them either tolerant of or sensitive to salinity stress. Organ-level partitioning such as leaf-to-leaf compartmentalization is one of the major tolerance mechanisms operating in rice under salinity stress as reported long ago by Yeo and Flowers (1982) and Yeo *et al.* (1985). Rice plant sensitivity during the reproductive stage is more critical when the plant enters the gametophytic stage; hence, the major sensitivity stage is about a week before and after anther dehiscence (Singh *et al,* 2008). If the rice plant is salinized targeting the reproductive stage to isolate a specific effect, we cannot salinize the plant much earlier without influencing the seedling and vegetative growth stages. Contrary to this, if the rice plant is salinized during the reproductive stage, it would already be too late. This is because, by the time salt reaches the youngest leaf, the flag leaf, and then inflorescence (reproductive organs) to ascertain whether the reproductive stage is tolerant or not, the plant would have already passed through the sensitive gametophytic stage and escaped the stress. Whatever results were obtained from such experiments would not be truly predictive of the effect of salinity at the reproductive stage. This could be due to partitioning and compartmentalization of salts as an escape mechanism through sequential physiological response to the influx of salt. This is because the rice plant transports the salt first to the older leaves, then to the younger leaves, and lastly to the inflorescence. Before the older leaves accumulate enough salt and start dying, the plant has already started diverting the salt to the next oldest leaf one after the other (Yeo and Flowers, 1982). In this way, old leaves act more or less like a sink for the toxicity of salt absorbed by the plant.

The aim of the study is to standardize a phenotyping protocol exclusively for reproductive-stage salinity tolerance. The hypothesis of this study was that cutting most of the old leaves of rice plants before salinization would force the plant to transport the salt in the remaining leaves and inflorescence much more quickly than with all the older leaves intact. By pruning of the older leaves, the rice plant will not have the ability to store the excess salt in the old leaves. But, the question now is, how many leaves are to be retained so that leaf removal does not significantly affect grain yield? This experiment was conducted to determine the minimum number of leaves needed by the rice plant that will not significantly affect grain yield and yield components and verification by observing the effect of stress on parents and derived mapping populations.

MATERIALS AND METHODS

The two *indica* rice genotypes used in this study were the salinity-tolerant IR4630-22-2-5-1-3 and salinity-sensitive IR64. Both were grown in normal soil using normal water. The experimental setups were kept in the NG02-02 screenhouse of the International Rice Research Institute, Los Baños, Laguna, Philippines, for the entire duration of crop growth.

Four small-scale setups were prepared for each genotype (IR64 and IR4630-22-2-5-1-3) for the leaf-cutting experiment: setups A, B, C and the control. Each setup made use of a plastic tray filled with ordinary tap water that could house the replicates and serve as a water bath to maintain the same water level. Six replicates were carried out per setup, that is, six perforated pots (15 cm in height, with 11 cm in diameter, and with 3-4 mm-diameter holes spaced 2 cm apart) were used as planting pots. Each perforated pot contained a plastic sieve bag filled with fertilized soil. The level of soil was about 1 cm above the topmost circle of holes. The soil was fertilized with 50, 25, and 25 mg N, P, and K, respectively, per kilogram of soil used. The water level in the plastic tray was kept level with the soil in the pots. Three pregerminated seeds were placed on the soil surface of each pot. Two weeks after seeding, the rice seedlings were thinned to one per pot; hence, each replicate involved a single plant grown until maturity. The water level in each of the plastic trays was raised to about 1 cm above the potted soil after thinning and this water level was maintained daily. Extra leaves were pruned when the flag leaf appeared. In setup A, all the leaves were cut except the flag leaf. Meanwhile, plants in setup B were trimmed to have only the flag leaf and the penultimate leaf while plants in setup C were pruned to have the top three leaves (including the flag leaf). The plants in the control setup remained untrimmed. Proper crop protection measures were employed as and when needed. Data on number of filled grains plant^{-1}, panicle length, and 100-seed weight were taken for each plant and the three setups were compared with the control.

The most optimum setup of leaf-cutting without a significant reduction in grain yield and component traits

was used to screen salt-sensitive IR64, salt-tolerant IR4630-22-2-5-1-3, and 201 F_2 plants derived from their cross at the reproductive stage in the same screenhouse (separate study on QTL analysis; details are not given). Three same-size (2 m x 0.8 m) concrete tanks were filled with ordinary tap water to house the F_2 plants and the parents were planted in perforated pots, serving as a water bath, similar to the conditions of the leaf-cutting experiment mentioned earlier. All plants were grown in regular water until the appearance of the flag leaf but were immediately shifted to saline water just when the flag leaf tip appeared. Salinization was done by dissolving NaCl into the water to raise the electrical conductivity up to 10 dS m^{-1}. The EC was checked regularly and maintained continuously for 10 days. All leaves of the plants were pruned immediately after shifting to saline water, leaving only two leaves based on the leaf-cutting experiment to direct the salt stress to the reproductive parts of the rice plant quickly and effectively rather than to compartmentalize the salt in older leaf tissues.

Spikelets were sampled for estimating pollen fertility and the parts from flag leaf were used for estimating ion concentration, *i.e.,* Na^+, K^+, and sodium-potassium ratio (Na:K ratio). Ion concentration and their ratio are known to be linked with salinity tolerance (Bay *et al.*, 1992; Cha-um *et al.,* 2009; Singh *et al.*, 2007; Qadar and Azam, 2007; Zhang *et al.*, 2010. Data on panicle length, 100-grain weight, and number of filled grains $plant^{-1}$ were also recorded.

At least five unopened spikelets per plant were sampled for pollen analysis. All six anthers were squashed and stained with 1% I_2KI to observe the number of fertile and sterile pollen based on four random fields of microscopic vision. Per cent pollen fertility was calculated using the following formula and the average for each sample tabulated:

$$\% \text{ pollen fertility} = \frac{\text{no. of fertile pollen}}{\text{total number of pollen observed}} \times 100$$

The flag leaf is quite big to accommodate in a test tube and, if it is sampled from the top, middle, or bottom portion, the ion concentration may vary due to ionic influx variation within the leaf (Flowers, personal communication). Therefore, each sample flag leaf was cut lengthwise and small portions from the base, lamina, and tip (Fig. 1) were placed together in screw-capped

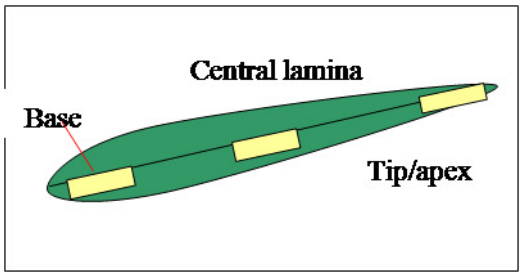

Fig. 1. Sampling pattern from flag leaf for ionic analysis.

bottles to be digested in 10 mL of 100 mM acetic acid in a water bath at 90°C for 2 hours. The digested individual leaf samples were oven-dried and weighed for the estimation of Na and K concentration. To analyze ion concentration, samples were diluted 10 times before using a PerkinElmer Analyst 300 atomic absorption spectrophotometer.

The ion concentration was calculated as follows:

Concentration of Na or K (mmol g^{-1}dwt) =

$$[C^* (d^*V/1000)]/dwt$$

where C is the atomic absorption spectrophotometer reading,

d is the dilution factor,

V is the extraction volume in ml, and

dwt is the oven dry weight of the sample in g.

Panicles from each plant were carefully laid down, individually measured (cm) from the pulvinus to the tip of the topmost grain using a ruler and then averaged.

Immediately after cutting and measuring of the panicles, fully formed seeds were manually removed from the panicles. The total number of fully formed seeds $plant^{-1}$ was noted and data represent number of filled grains.

Oven-dried seeds were weighed and adjusted to 14% moisture content. Since not all the plants had at least 100 grains, we extrapolated the test weight to 100-seed weight wherever plant produced less than 100 grains, as below:

100-grain weight (g) =

$$\frac{\text{weight of grains from one planting} \times 100}{\text{numbers of grain from that plant}}$$

RESULTS AND DISCUSSION

Two *indica* varieties of different reactions to salt stress (salt-tolerant IR4630-22-2-5-1-3 and salt-sensitive IR64) were tested to determine the extent of pruning that could be allowed for salt stress imposition at the reproductive stage. This preliminary study was carried out to know that until what extent, the act of leaf pruning does not significantly affect significantly the number of filled grains plant^{-1}, panicle length, and 100-grain weight of a rice variety.

For both varieties, the control (untrimmed plants) had the highest number of grains plant^{-1} while setup A had the lowest number of grains plant^{-1} (Table 1). In setup A, only the flag leaf was left on the plant, and the number of grains plant^{-1} for both genotypes in setup A significantly lower than the control. Plants in setups B and C where the top two and top three leaves were left respectively, did not vary significantly from the control. For IR4630-22-2-5-1-3, setup C was near the control for grains plant^{-1}, whereas, in the case of IR64, setup B showed the second-highest number of grains plant^{-1}.

Table 1. Average number of filled grains plant^{-1} of rice varieties IR64 and IR4630-22-2-5-1-3 grown under non-saline conditions.

Variety	Control	Setup A	Setup B	Setup C	LSD (5%)	LSD (1%)
IR64	451a	208b	428a	388a	82.80	114.40
IR4630-22-2-5-1-3	349a	247b	325a	346a	60.70	83.90

Lowercase letters indicate grouping (a,b)based on Duncan Multiple Range Test (DMRT) for significant difference from control at P<0.05, Control - untrimmed plant/no leaf cut
Setup A - only the flagleaf was left in the plant
Setup B - two leaves left (penultimate and flag leaves left)
Setup C - top three leaves left

Similarly for panicle length, only setup A significantly differed from the control and setups B and C. But control of IR4630-22-2-5-1-3 found similar to setup C only (Table 2).

For 100-grain weight, all setups except IR4630-22-2-5-1-3 with just the flag leaf left (setup A) did not vary significantly from the control (Table 3). For both genotypes, the lesser the number of leaves left on the plant, the lower the mean 100-grain weight observed.

Table 2. Mean panicle length (cm) of rice varieties IR64 and IR4630-22-2-5-1-3 grown under non-saline conditions.

Variety	Control	Setup A	Setup B	Setup C	LSD (5%)	LSD (1%)
IR64	22.05a	19.95b	21.35a	20.84a	2.09	2.91
IR4630-22-2-5-1-3	21.47a	17.97b	19.90b	21.22a	1.25	1.73

Lowercase letters indicate grouping (a,b) based on DMRT for significant difference from control at P<0.05
Control - untrimmed plant / no leaf cut
Setup A - only the flag leaf was left in the plant
Setup B - two leaves left (penultimate and flag leaves left)
Setup C - top three leaves left

Table 3. Mean 100-grain weight* (g) of rice varieties IR64 and IR4630-22-2-5-1-3 grown under non-saline conditions.

Variety	Control	Setup A	Setup B	Setup C	LSD (5%)	LSD (1%)
IR64	2.23a	2.08a	2.15a	2.18a	0.16	0.22
IR4630-22-2-5-1-3	2.19a	1.91b	2.24a	2.35a	0.27	0.37

* 100-grain weight was measured and extrapolated to 100 seeds (instead of 1000 seeds) because not all the treatments produced even 100 seeds; Lowercase letters indicate grouping (a,b) based on DMRT for significant difference from control at P<0.05
Control - untrimmed plant / no leaf cut
Setup A - only the flag leaf was left in the plant
Setup B - two leaves left (penultimate and flag leaves left)
Setup C - top three leaves left

Results from the current leaf pruning experiment were used for our further phenotyping work for salinity tolerance at the reproductive stage (separate experiment; details not given). All the old leaves of the two genotypes (IR64 and IR4630-22-2-5-1-3), as well as the individual plants of the F_2 mapping population derived from their cross were clipped bearing only two leaves (*i.e.* flag leaf and penultimate leaf) for the reproductive-stage phenotyping (data given for parents only as F_2s cannot have the control experiments). The average performance of the parents of a cross in terms of the most important traits to distinguish tolerant from sensitive parents is given in Table 4.

Pollen viability is the trait most affected by salinity during the reproductive stage. Sensitive genotypes lose their pollen viability much faster than tolerant genotypes (Khatun and Flowers, 1995b; Sarhadi *et al.*, 2012). This is probably due to the restriction mechanism for salt entry into the reproductive organs.

Table 4. Performance of the parents (IR64 and IR4630-22-2-5-1-3) when plants are pruned to have only the penultimate and flag leaves and are grown under salinized conditions.

Parameters		Mean of IR64 (Sensitive)	% decrease over non-stress	Mean of IR4630-22-2-5-1-3 (Tolerant)	% decrease over non-stress
Pollen fertility	Salt-stressed	20.3	78.9	82.6	14.8
	Control	96.3		96.9	
Number of filled grains	Salt-stressed	208	53.9	275	21.2
	Control	451		349	
Ion concentration in flag leaf					
Na (mM g^{-1} dwt)	Salt-stressed	0.245		0.09	
K (mM g^{-1} dwt)	Salt-stressed	0.67		0.93	
Na:K ratio	Salt-stressed	0.366		0.097	

Flag leaves of the parents as well as an F_2 mapping population were sampled after 10 days of salinization treatment (just from the time of appearance of the flag leaf). It was observed that IR64 (sensitive genotype) accumulated more than 2.7 times higher concentration of Na^+ in the flag leaf than IR4630-22-2-5-13 (tolerant genotype). Contrastingly, the tolerant genotype accumulated about 1.4 times higher K^+ than the sensitive genotype. Consequently, the Na/K ratio was much higher (about 3.8 times) in the sensitive genotype than in the tolerant genotype.

Filled grains $plant^{-1}$ has been found strongly associated with grain yields in rice and other crops (Abdullah *et al.,* 2002; Boonjung and Fukai, 1996; Casanova *et al.,* 2002; Thirumeni *et al.,* 2003; Uddin *et al.,* 2007). In the leaf-cutting experiment, the number of filled grains $plant^{-1}$ was least when only the flag leaf was left for both genotypes. Flag-leaf importance has been recognized in rice for a long time. It contributes 45-60% of grain yield (Enyi, 1962; Abou-Khalifa *et al,* 2008) but it has also been known that the penultimate leaf and the third upper leaf also contribute to growth and grain yield (Yoshida, 1981). However, whether the top two or the top three leaves would contribute more to grain yield for all rice genotypes cannot be generalized. Leaving the penultimate and the flag leaves for IR64 showed better yield than when the top three leaves were left but the opposite was found true for IR4630-22-2-5-1-3. Hence, we preferred to keep only two leaves (flag leaf and penultimate leaf) for the mapping population so as to keep the sink size as low as possible to store excess Na^+ during its uptake.

Panicle length, an agronomic trait, has a highly significant and positive correlation with grain yield (Ramakrishnan *et al.,* 2006; Khan *et al.,* 2009; Calapit-Palao, 2010). In this experiment, results for panicle length were similar to grains $plant^{-1}$ results for IR64 but not for IR4630-22-2-5-1-3. This suggests that the response of the rice plant to leaf pruning may be genotype-specific, at least for characters such as grains per plant and panicle length.

Leaf cutting had an almost non-significant effect on 100-grain weight in any setup compared to the control for IR64. Only in setup A was IR4630-22-2-5-1-3 significantly different, similar to the inferences taken from the number of grains $plant^{-1}$ and panicle length. This could be because seed weight is a highly heritable trait (Chauhan, 1998) and hence will not change easily despite differences in the environment.

This preliminary experiment gives a clear indication that, if a rice plant's old leaves were pruned, leaving the flag leaf and one or two more leaves, number of filled grains $plant^{-1}$, panicle length and 100-grain weight will not be affected. In this way, one can salinize test plants just after the appearance of the flag leaf and prune the old leaves as per the result of this experiment. Whatever differences are observed in grain yield after extra leaf cutting could be attributed to the salinization done from the day of flag leaf appearance for about 10 days. This duration could vary depending upon the objective of the phenotyping studies. The 4-6-cm distance between the flag leaf and penultimate leaf is already established as a robust morphological marker for the uni-nucleate to early bi-nucleate stage in anther-

culture experiments (Yoshida, 1981; Bishnoi *et al.,* 2000; Raina, 1989; Silva and Ratnayake, 2009. Therefore, salt stress can be imposed a few days before pollen development (gametophytic) stage with leaf pruning. The salt-stress can be continued for 10 days, to keep appropriate stress at the reproductive stage to discriminate the tolerant and sensitive genotypes. Organ-level compartmentation, which is one of the major mechanisms of salinity tolerance in rice (Yeo and Flowers, 1982), has led us to do this experiment and appropriately modify the screening technique at the reproductive stage. Our major goal was to direct the toxic ions such as Na^+ to build-up the stress on the reproductive parts, which otherwise cannot be ensured within a short window of 10-15 days.

A study in soybean also supported our hypothesis. When older leaves were cut under salinity stress, yield and grain size decreased because salt accumulated in the remaining leaves of the plant and affected the yield (Hiroshi *et al.,* 2006). Cutting of the older leaves is a regular phenomenon in the basmati rice belt in India that does not affect yield. This practice has also continued as a means to avoid lodging due to tall plant stature.

Meanwhile, in the validation experiment, we found that pollen viability declined by almost 80% in the sensitive genotype (IR64) under stress while the tolerant genotype (IR4630-22-2-5-1-3) could withstand stress with a reduction in pollen fertility of just less than one-fifth of that of the sensitive genotype. Abdullah *et al.* (2002) also reported salinity-induced changes in floral characteristics, including reduction of pollen viability and accumulation of higher Na^+ and lower K^+ in the flag leaf and other leaves as well resulting into reduction in grain number and weight. Indeed, number of filled grains is the ultimate measure of reproductive-stage salinity tolerance. The effect of pollen viability is clearly evident from the number of filled grains in sensitive and tolerant genotypes after imposing salinity stress at the reproductive stage. The sensitive genotype had about 2.5 times more reduction than the tolerant genotype in the number of filled grains. Khatun and Flowers (1995a) salinized different genotypes 1 month after sowing irrespective of the flowering duration of the genotypes. They reported a drastic reduction in seed set when plants were grown in saline culture solution; however, genetic variation was observed among the five genotypes (Khatun *et al.,* 1995). It was suggested that toxic ions (Na^+ and Cl^-) *per se* are responsible for pollen viability and ultimately seed setting. Rao *et al.,* 2008 reported that higher floret fertility contributes to higher seed set and grain yield in tolerant genotypes, whereas higher spikelet sterility leads to poor seed set and lower grain yield in sensitive genotypes.

Though we did not measure the Na^+, K^+, and Na/K ratio in control (non-stress) parents, much literature suggests that Na, K, and Na/K are among the most reliable parameters for ascertaining the degree of salinity tolerance not only in rice but also in many crops (Lee *et al.,* 2003; Lisa *et al.,* 2004; Saleque *et al.,* 2005; Singh *et al.,* 2007, Zhu *et al,* 2001; Matsumura *et al,* 1998; Flowers and Yeo, 1988; Yeo, 1992, 1994, 1998; Yeo *et al,* 1990). Higher Na^+, lower K^+ accumulation, and higher Na/K ratio in plants indicate more sensitive genotypes and vice versa for tolerant genotypes, and this is what we observed in our experiment after salinization.

We also observed that leaf pruning avoided overcrowding of the plants and that resulted in significantly lower insect and disease pressure in pruned plants than in the controls, probably because of the clean canopy approach.

This leaf-pruning experiment was targeted to isolate the effects of salinity at the reproductive stage without invoking any other tolerance mechanism or affecting stage-dependent tolerance. Based on the results, it can be clearly inferred that cutting of old rice plant leaves before salinization during the booting stage does not affect the number of filled grains $plant^{-1}$, panicle length and 100-grain weight of rice significantly which are highly associated with ultimate grain yield. Hence, if salt stress is imposed on plants whose leaves are trimmed or pruned to the minimum required, the significant differences that might arise could be attributed to only salt-stress itself and the duration but not to the leaf pruning. The results also suggest that leaf pruning may be employed to grow plants with either the top three leaves or the top two leaves without significantly affecting yield since such plants behave like untrimmed plants. However, because the goal is to impose salt stress on the reproductive parts as quickly as possible in order to effectively differentiate tolerant from sensitive rice plants at the reproductive stage, it remains more appropriate to have a smaller sink for

the toxic salt accumulation by leaving only the penultimate and flag leaves. It is evident that clipping of the leaves as suggested for the individual F_2 plants of a mapping population and parents for phenotyping in QTL studies (details not provided) for reproductive-stage salinity tolerance helped in discriminating tolerant genotypes from sensitive genotypes. Saline stress (100 mM or 10 dS m^{-1}) imposition for 10 days at the time of the first appearance of the flag leaf could differentiate sensitive genotypes from tolerant ones clearly and this was evident from the low pollen viability and higher accumulation of toxic ions in the flag leaf of the sensitive genotype (IR64) while the opposite was found true for the tolerant genotype (IR4630-22-2-5-1-3). In addition, the leaf-pruning technique minimized pest and disease occurrence in the plants, especially by avoiding overcrowding of plants in the screenhouse.

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