Development of image-based phenotyping for selection characters of rice adaptability on the seedling salinity screening

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Abstract. Development of adaptability rice under salinity stress needs effective and selective methods in the screening process. The seedling screening method is a general method used in salinity screening. However, this screening method often uses conventional observation in its screening process. This observation is rated that has a high error level. Therefore, the development of a digital approach through image-based phenotyping expected could minimize the error in the adaptability screening. This study was designed with a nested randomized complete group design, where replications were nested in a stressful environment. The environment in this study was normal (0 mM NaCl) and salinity stress (120 mM NaCl). The genotype used consisted of 8 genotypes which were repeated three times. The number of characters observed was nine image-based phenotyping. The results of this study showed that green percentage, the 3rd leaf length, and total area were the selection characters of image-based phenotyping under seedling salinity screening. Besides that, the used adaptability index in salinity screening became a good approach in considered and distinguished tolerance responses among varieties, especially to Pokkali (tolerant control variety) and IR 29 (sensitive control variety). Based on this study, the application of image-based phenotyping recommended in the screening process of line adaptability under salinity stress.

1. Introduction
Rice development in Indonesia needs to consider the climate change. The problem of climate change impact change the plant growth environment from optimal to sub-optimal [1]. Sub-optimal environment will have an impact on decreasing crop productivity, including in rice plants. One of the sub-optimal environments that have an impact on rice productivity is the salinity environment [2]. According to Rad et al. [3], the salinity environment with an electrical conductivity (EC) as 6 dS m⁻¹ can decrease half the yield potency of a variety and the increase EC up to 120 dS m⁻¹ could impact on crop failure. Therefore, the development of adaptive variety rice under salinity stress is an efficient approach to solve the rice yield problem in saline lands or coastal areas.

Salinity is a stress that has high complexity. This stress is able to induce several other stresses for plants, apart from toxicity stress [4]. In general, other stresses that can be induced by salinity stress are physiological drought stress, ion homoestatic stress, and oxidative stress [5]. These causes the tolerance
mechanism for this stress to be relatively diverse at different levels [5,6]. Therefore, it is important to develop a screening method for the adaptability of rice in salinity stress. This development will later facilitate the screening process for adaptive rice lines under salinity stress.

Lines screening against stress is highly dependent on the screening method, the screening phase, the selection environment, and the selection characters. One of the methods widely reported in rice salinity screening is the static hydroponic method in the seedling phase with a concentration of 120 mM NaCl [7,8]. However, the selection character used is relatively qualitative and subjective [9]. This is in stark contrast to the nature of salinity tolerance which is relatively quantitative because it is influenced by many genes [9,10]. Therefore, a more precise approach is needed in assessing salinity tolerance trait. One good precision approach to use is the image-based phenotyping approach. This concept will produce image characters related to the salinity tolerance trait [11,12,13]. This approach has been carried out by several researchers on rice salinity stress [11,14,15]. However, this approach, image-based phenotyping in Indonesia, is still underdeveloped. In addition, this approach uses high-quality camera technology. Therefore, the development of simple image-based phenotyping is important in Indonesia. The objective of this study was to determine the character selection based on the image-based phenotyping approach to rice tolerance under the seedling salinity screening.

2. Materials and methods

The study was conducted in green house at Perumahan Dosen Universitas Hasanuddin, Makassar, South Sulawesi in July – August 2020. This study was designed with a nested randomized complete block design, where replicates were nested in the stress environment. The stressed environment in this study was normal (0 mM NaCl) and saline (120 mM NaCl). The genotypes used consisted of 8 genotypes (Inpari 34 Agritan Salin, Inpari 29, IR 29, IR 20, Salumpiki, Pokali, Ciferang, Jeliteng) repeated three times. Each culture tank represented one environment in one replication, so there were 6 culture tanks. Each experimental unit consisted of four rice seeds

2.1 Procedure.

2.1.1 Nursery. The salinity test in early vegetative phase was carried out on nutrient culture according to the method of Edgane et al. [16] which has been modified. The nursery was carried out for 7 days after germination in a nursery container. After that, the seedlings were transplanted into nutrient culture solution. Each seedling was floated in hydroponic media with a Styrofoam tray. Each styrofoam has a hole with a diameter of 18 mm and a space distance among the holes of 5 cm x 4 cm. The seedlings were rolled with a thin foam sheet before placing them in the nutrient culture solution. The nutrient culture solution used was 8 mL/L AB mix nutrient solution and each tank has solution capacity as 8 L. The solution per tank was changed every week. Stress induction was performed at 10 days after transplanting to the treatment tank. The stress was applied gradually to avoid osmotic shock, the first stage of NaCl was given 50% of the stress concentration, after three days the solution concentration was increased to 120 mM NaCl. NaOH or HCl 1 N were given every two days to maintain the solution pH in the range of 5.5-6.5. The observations made included image-based phenotyping characters, namely the first leaf length 1, the second leaf length, the third leaf length, shoot area, percentage of shoot green area, red, green, blue, red green ratio (R/G).

2.2 Image capture.

Image captures were taken at 14 days after stress application with Canon EOS 1200D camera. The plant shoot put manually into the portable photo studio with studio size 75 cm x 75 cm x 75 cm. The background used was a white background with two 8 watts LED lighting in a studio inside. The camera capture used 5.6 F-stop, 1/160 sec Exposure time, and ISO 800 without flash. As for, the capture position was on the top hole of the studio. Then, the image results were analyzed by Fiji software.
2.3 Data analysis.
All image-based phenotyping data were analyzed first by analysis of variance. Characters that were significantly influenced by interactions effect were selected as selection characters. The selection characters were further analyzed by the relative decrease analysis of all varieties. Relative decrease and phenotypic data at saline conditions were standardized to equalize the variance between the two data populations. The results of standardization from the two data populations were combined to form an adaptability index for each character with following formula:

\[ \text{Adaptability index} = \text{standardization of saline phenotypes} - \text{standardization of relative decrease}. \]

The adaptability indices of the selected characters were distributed into a 3D plot. The 3D plot analysis used the concept of heatmap on RStudio software with plot3D package [17]. The results of the 3D analysis were used as the basis for identifying groups among varieties.

3. Results and discussion
The results of the analysis of variance (table 1) show that the differences in the growing environment had a significant effect on all Image-based phenotyping characters, except for the green character. These results also indicate that the source of genotype diversity has a significant effect on the 2nd leaf length, the 3rd leaf length, the 4th leaf length, shoot area, and the percentage of shoot green area. Meanwhile, the interaction effect of Genotype x Environment only has a significant effect on the 3rd leaf length, the shoot area, and the percentage of shoot green area.

In general, the use of ANOVA plays a very important role in the assessment of tolerance traits, in particular the genotype and environmental interactions effect [9]. Significant interactions can describe the different responses between varieties to different growing environments [18]. This difference becomes the basis for the assessment of tolerance between tolerant and sensitive genotypes to differences in stress levels between growing environments [19,20]. Therefore, characters significantly influenced by interactions can be used as character selection in assessing the tolerant traits of plants. Based on the ANOVA table, the characters that can be used as selection characters for salinity stress are the 3rd leaf length, shoot area, and the percentage of shoot green area.

| Characters                  | Genotype (G) | Environment (E) | G xE | Error | Vg  | Vp  | R (%) | CV (%) |
|----------------------------|--------------|-----------------|------|-------|-----|-----|-------|--------|
| The 2nd leaf length        | 559.42**     | 2591.61*        | 34.77| 58.14 | 87.44| 88.65| 98.63 | 17.5   |
| The 3rd leaf length        | 681.26**     | 2862.73**       | 53.11| 22.40 | 104.69| 105.16| 99.56 | 11.7   |
| The 4th leaf length        | 671.58**     | 1644.20**       | 17.48| 30.32 | 109.02| 109.65| 99.42 | 16.0   |
| Shoot area                 | 21635.94**   | 187097.72**     | 5152.19**| 1336.80| 2747.29| 2775.14| 99.00 | 30.8   |
| Percentage of shoot green area | 6.66**    | 237.94**        | 5.56 | 1.77  | 0.00297| 0.00344| 86.30 | 18tr   |
| Red                        | 743.01       | 14285.07*       | 345.39| 613.89| 66.27| 79.06| 83.82 | 28.5   |
| Green                      | 477.57       | 3411.79         | 655.63| 638.18| 0.00 | 13.30| 0.00  | 23.6   |
| Blue                       | 226.72       | 6233.07**       | 165.20| 128.78| 10.25| 12.93| 79.26 | 20.8   |
| Red/green                  | 0.04         | 0.22            | 0.04 | 0.04  | 0.00 | 0.00 | 32.51 | 23.5   |

Notes: Vg = genetic variance, Vp = phenotypic variance, R = repeatability, CV = coefficient of variance.

The repeatability results (table 1) show that all characters have high repeatability, except for the green image character and the red/green ratio. Meanwhile, the image blue character was the only character that has a repeatability value below 80% among high repeatability characters. In general, repeatability has the same concept as heritability. However, this concept applies to populations with stable genetic constitutions. The concept of repeatability can be interpreted as the probable percentage...
of genotype can produce the same genetic response when the population was repeated in the same environmental conditions [19]. Based on the repeatability results in table 1, the characters with real interactions have very high repeatability. This indicates that the 3rd leaf length, shoot area, and the percentage of shoot green area genetically reflect the tolerance level of the selected population.

The results of the relative decrease, the phenotypic response to stress, and the adaptability index are shown in table 2. Relative decrease characteristics indicated that Inpari 34, IR20, IR29, and Jeliteng were characters that tend to have positive relative decrease values for the three selection characters. On the other hand, the Ciherrang, Inpari 29, Salumpikit and Pokkali varieties had a relatively negative decrease value. As for based on the phenotypic characters of plants in saline conditions, the Inpari 29, Pokkali, and Salumpikit varieties had positive phenotypic standardization values. In contrast, Ciherrang, Inpari 34, IR 20, IR 29, and Jeliteng had negative standardization values. The results of the two standardizations were combined into the adaptability index. Based on the results of the adaptability index, Inpari 29, Pokkali, and Salumpikit had a consistently positive adaptability index, while Inpari 34 and Jeliteng had a consistently negative adaptability index.

The response of plants to salinity stress has many variations. This depends on the plant's mechanism in minimizing damage due to salinity stress [6]. In general, the relative decrease is an indicator to determine the plant tolerance trait [7,21]. However, the plant phenotype when stressed can also be used as an indicator for assessing tolerance to their adaptability [9]. Plants relatively adapted have above-average growth traits, even though these plants have a high relative decrease [19]. Based on this, combination of two concepts is considered to be a good approach to the tolerance screening process. This assessment will be more precise than just focusing on one approach. The result of this combination was expected to be able to select plants with the low relative decrease and high phenotypic responses above the selection population mean so that the growth of these genotypes is relatively stable in various stressful environments. Therefore, the formation of the adaptability index formulation was carried out by reducing the value of the phenotype standardization in a stressed environment with its relative decrease. Meanwhile, the use of this standardization plays a role in preventing the dominance of variety between the two approaches.

### Table 2. Adaptability Index of selection character.

| Genotype | RD standardization | 120 mM NaCl standardization | Adaptability Index |
|----------|-------------------|-----------------------------|-------------------|
|          | 3LL | SA | PSGA | 3LL | SA | PSGA | 3LL | SA | PSGA |
| Ciherrang | -0.07 | 0.58 | -0.99 | -2.58 | -0.99 | 0.85 | -2.50 | -1.57 | 1.85 |
| Inpari 34 | 2.95 | 1.44 | 3.16 | -0.79 | -1.20 | -3.11 | -3.74 | -2.65 | -6.27 |
| Inpari29 | -4.61 | -5.74 | -2.05 | 1.60 | 0.34 | 2.08 | 6.21 | 6.07 | 4.13 |
| IR20 | -1.81 | 1.05 | 0.41 | -1.46 | -2.72 | -0.61 | 0.36 | -3.78 | -1.02 |
| IR29 | -2.65 | 2.46 | 3.70 | -1.33 | -2.84 | -3.67 | 1.33 | -5.31 | -7.37 |
| Jeliteng | 3.18 | 3.04 | 1.58 | -3.25 | -1.05 | -1.50 | -6.44 | -4.09 | -3.08 |
| Pokkali | 2.23 | -1.10 | -4.86 | 4.70 | 4.93 | 4.91 | 2.47 | 6.03 | 9.77 |
| Salumpikit | 0.78 | -1.75 | -0.93 | 3.10 | 3.54 | 1.04 | 2.32 | 5.29 | 1.97 |

Note: RD = relative decrease, 3LL = The 3rd leaf length, SA = shoot area, PSGA= Percentage of shoot green area.

The 3D plot results show that there were four main groups (figure 1). The first group consisted of Pokkali and Inpari 29 as the adaptive group. The second group consisted of Salumpikit and Ciherrang as a moderate adaptive group. The third group consisted Jeliteng and IR 20 as the less adaptive group. Meanwhile, the last group consisted Inpari 34 and IR 29 as a sensitive group.

3D plot analysis is a visualization method that can combine three variance direction in one image [12]. This will facilitate the assessment of the overall tolerance response of the genotype toward its selection character in the seedling salinity screening. Based on a combination of three characters selection, the concept of image-based phenotyping is considered capable of differentiating control
tolerant varieties (Pokkali) from sensitive varieties (IR 29). According to Anshori et al. [22], a good screening method for stress is a method that can distinguish between tolerant and sensitive controls in a distinctive way. However, in this study, the Inpari 34 saline agritant variety was considered a sensitive plant, whereas Inpari 29 was considered a tolerant variety. This result was also reported by Anshori et al. [9] in a seedling salinity screening against double-haploid rice lines. If the descriptions of Inpari 34 and Inpari 29 varieties are compared with the results of this study, Inpari 34 Salin Agritan is considered to be a tolerant variety for the seedling salinity screening and Inpari 29 is considered a tolerant variety for submergence stress [24]. It may be due to the different selection environmental conditions used so that Inpari 34 Salin Agritan experienced a decrease in tolerance response when tested in this study. In addition, this phenomenon occurs due to a change in the nature of Inpari 34 Salin Agritan in the process of reproduction. Therefore, the phenomenon of responses to Inpari 34 variety needs to be analyzed more deeply with a more precise approach. However, overall the results of the study can be concluded that the use of image-based phenotyping can be recommended for the salinity screening process in the seedling phase. However, the development of the concept of image-based phenotyping needs to be further developed to increase the precision of the selection.

Figure 1. 3D plot of three adaptability index and salinity tolerance clustering of eight varieties. 3LL = the 3rd leaf length, SA = shoot area, PSGA= Percentage of shoot green area.

4. Conclusion
Shoot area, percentage of shoot green area, and the 3rd leaf length characters are image-based phenotyping characters that can be used as selection characters in rice seedling salinity screening. Pokkali and Inpari 34 varieties are assessed as tolerant and adaptive varieties in the salinity phase of the seedlings, while Inpari 34 and IR 29 were sensitive varieties. The use of image-based phenotyping is recommended as an approach to screening rice tolerance at the seed phase salinity stress.
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