Cluster heatmap for detection of good tolerance trait on doubled-haploid rice lines under hydroponic salinity screening

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Abstract. The development of salinity tolerant varieties can be done by using doubled haploid technology. In the determination of the salinity tolerance trait, hydroponic screening at the seedling stage is the most common screening method. However, it needs improvement on the assignment of the tolerance traits. Cluster heatmap analysis has been reported to select lines by combining various data. The objective of the study was to select tolerant doubled-haploid rice lines to salinity stress at the seedling phase and evaluate the use of cluster heatmap analysis by combining tolerance score and growth characters. The study was conducted in a greenhouse using a nested factorial design (repetition nested in NaCl concentrations), with 3 replications. The plant materials used consisted of 62 genotypes and NaCl concentration levels consisted of 0 (1 dS/m) and 120 mM (13.3 dS/m). Hydroponic cultures used a Yoshida solution. Each container contained 14 experimental units and 2 units of check i.e. sensitive and tolerant controls. Observations was carried out on tolerance scoring and growth parameters. The results showed that tolerant and moderate genotypes have a lower pattern of decline than sensitive genotypes, especially in shoot-related characters. Based on the cluster heatmap analysis, there were 38 doubled haploid rice lines considered good tolerance traits under saline condition. The cluster heatmap assignment was considered more selective than only focused on tolerance score, especially in negative selection to sensitive lines.

1. Introduction

Rice production increase is the main priority for food sufficiency in Indonesia. However, climate change causes rice production became stagnant and decrease. The climate change induces some negative environmental impact which influenced the plant growth and the yield [1], one of which is salinity.

Salinity is an abiotic stress associated with salt content found in the soils and the plants. The salinity effect is more serious if the salt content consisted of Na⁺ ion [2]. The high content of Na⁺ ion can induce toxic stress in plants [3]. In addition to toxicity, salinity from Na⁺ also stimulates some stresses, such as physiological drought (osmotic stress), homeostatic ion, and oxide stress [4],[5],[6]. It causes plant growth and its yield sharply decreases. Therefore, salinity stress became one of the main abiotic stresses in Indonesia, especially in coastal where rice is planted in significant areas.
The salinity problem can be solved by the development of new salinity tolerant variety through doubled-haploid technology. The technology has an advantage in shortening the lines’ obtainment so that it accelerates the development of new variety [7]. Safitri [8] developed 56 doubled-haploid rice lines produced from crossing between the salinity tolerant variety and commercial variety. Therefore, its lines screening for salinity tolerance need to be done.

Seedling phase screening is the most common screening for salinity stress [9], [10], [11]). The seedling phase has better heritability than the reproductive phase under salinity stress [12]. Besides, seedling phase screening is assessed to be faster [10], effective [9] and minimize the environmental effect by controlled treatment [13]. However, the disadvantage of the screening is to focus on tolerance scores as it is considered being less representative. Salinity tolerance is a quantitative trait with the discovery of Saltol QTL[14]. Therefore, the involvement growth characters in the assessment of salinity tolerance to better considered.

The combination of the score and growth character approach can be analyzed together with the dendrogram [15]. However, the disadvantage of dendrogram analysis is the identification of why the grouping occurs is less clear. Its weakness can be solved by the cluster heatmap analysis. The cluster heatmap analysis can combine genotype grouping and character grouping in the same dimension by visualizing the difference in color intensity [16]. It can provide information related to the characteristics of a grouping simply [17]. Therefore, the use of cluster heatmaps for both approaches is expected to produce a more accurate selection of salinity tolerance in the doubled-haploid rice lines. The objective of the study is to select the tolerant doubled-haploid rice lines to salinity stress at the seedling phase and evaluate the use of cluster heatmap analysis on the combined approach of score and growth character.

2. Materials and Methods

Seedling phase screening was carried out in nutrient culture using a modified method of Egdane et al [18]. The material used in the study consisted of 56 doubled-haploid lines derived from anther culture, 4 commercial varieties (Ciherang, Inpara 5, Inpari 29, and Inpari 34 Salin Agritan), Pokkali as tolerant check variety and IR 29 as sensitive check variety. The study was conducted at ICABIOGRAD greenhouse, Cimanggu, Bogor, West Java (6°34’26.2” S, 106°47’07.5” E) in April - June 2017. The maximum temperature and humidity in the greenhouse was 41.25 °C and 90.33%, respectively. The minimum temperature and humidity was 25.72 °C and 39.18%, respectively.

2.1 Experimental design and procedure

The experiment was arranged in a randomized complete block design (RCBD) of nested factorial, the repetition nested in NaCl concentrations (0 (1 dS m⁻¹) and 120 mM (13.3 dS m⁻¹) ). The genotype used consisted of 62 genotypes. The experiment was repeated three times. Each culture tank consisted of 10 genotypes and 2 check genotypes repeated 2 times so that the total experimental unit was 504 experimental units found in 36 treatment tanks. Each experimental unit consisted of four rice seedlings.

The nursery was carried out up to 7 days after germination, then the seedlings were transferred to nutrient culture media. Seedlings on nutrient culture media were floated with styrofoam trays. Each styrofoam has a hole with a diameter and a spacing of 18 mm and 5 cm x 4 cm, respectively. Seedlings planted in styrofoam holes were rolled with thin foam sheets so the seedlings can be floated well on nutrient culture media. The nutrient culture media used was Yoshida media with a volume of 17 L per tank replaced every week. Salinity stress induction was carried out 13 days after transfer to the treatment tank. Stress was done in stages to avoid osmotic shock, the first stage of NaCl treatment given was 60 mM NaCl, then after two days, it was increased to 120 mM NaCl. The solution of NaOH or HCl 1 N were applied every two days to maintain the pH of the solution in the range of 5.0-5.1. The main observation was the scoring of tolerance to salinity symptoms following the evaluation standards of Egdane et al. [18]. Meanwhile, other character observations were seedling height, longest root
length, number of tillers, number of leaves, shoot fresh weight, root fresh weight, shoot dry weight, and root dry weight.

2.2 Data Analysis
Scoring data on each genotype were analyzed based on Friedman's analysis to determine the median of tolerance. Friedman’s analysis was carried out using Minitab software version 14. Each genotype was grouped based on the tolerance scoring and the responses were identified on each character. The response of characters in the tolerant group was compared to moderate and sensitive groups to find out the different patterns. Data were analyzed with cluster heatmap analysis to find out how the grouping of genotypes and the specific patterns of grouping was based on all observational characters. This analysis uses color intensity to distinguish the strength or degree of each genotype against a character [16].

3. Results and Discussion

3.1 Screening and Grouping of Salinity Tolerance to Doubled-haploid Rice Lines Based on Scoring Analysis
The results of doubled-haploid rice lines screening through Friedman’s analysis showed that 24 genotypes in 3-4 interval, 20 genotypes in 4-5 interval, and 16 genotypes above of 5 (Table 1). The results of the analysis have kind data as interval data. The modification in the determination of the salinity tolerance level has to be done and appropriated with the grouping of Edgane et al. [18]. Based on the grouping, lines in 3-4 intervals were categorized as tolerant, lines in 4-5 intervals were considered as moderate and lines above 5 values were considered as sensitive. It can be an early basis to identify different response patterns from different tolerance groups to all rice growth characters under salinity stress.

3.2 Character Responses of Doubled-haploid Rice Lines Grouping In Seedling Salinity Screening
NaCl treatment has a different response compared to the normal condition. The difference was shown by a relative decrease in all growth characters (Table 2). Bhowmik et al. [9] dan Ali et al. [10] had reported that the salinity caused a relative decrease to all seedling characters in hydroponic screening. However, the relative screening among the tolerance group commonly has different responses on each character. Al-Amin et al. [19] also stated that the salinity stress gave the various response degree to rice depended on its tolerance trait. It proved that the growth characters were suitable as the basis for the determination of the salinity tolerance trait determination.

The tolerant group has a lower relative decrease than the other tolerance groups. The lower of the relative decrease was correlated to the higher character average responses under salinity stress. It proved that the tolerant genotype would minimize salinity effect [20] so that the genotype still maintained good growth under salinity stress. Meanwhile, the sensitive genotype has the highest relative decrease in all characters. However, its relative decrease of root length has the same value to the moderate group and its ratio was considered low. It indicated that both tolerant and sensitive genotypes tried to minimize the negative effect of salinity on root growth so that plants could maximize water absorption when exposed to salinity stress.

Tolerant and moderate groups were not significantly different in almost all growth characters (Table 2). The largest average difference between the two was found in the number of tillers (26.32%). However, the difference was the same as the difference between tolerant and sensitive. Low differences between the tolerant and moderate groups were found in the characters of root length and shoot height. It indicated that both groups, when experiencing salinity, still maintained the growth of root height and shoot length. De Leon et al. [21] had reported that the least-square means (LSM) between the two groups on the relative decrease in root length was not significant. They may have adaptation mechanisms in maintaining their growth. However, in general, the tolerant group was
higher in value than the moderate group. Therefore, tolerant and moderate genotype can be grouped as good tolerance genotype.

Tolerant and sensitive groups have large differences in all characters. The largest difference between the tolerant and sensitive group was the shoot fresh weight, whereas the lowest difference was the number of leaves. It proved that the sensitive genotypes were not efficient to keep the water balance so that its growth decreased under stress. In addition, the shoot related characters were more significantly different than the root related characters in detecting good tolerant genotype (Table 2). It indicated that the good tolerant and sensitive genotypes have the same behavior under the saline condition on the root related characters [22]. Therefore, the fresh weights and shoot related characters were the most important character groups of rice under salinity stress [23]. However, the simultaneous analysis of this study needed to include all characters. It could be more informative and objective in early study.

Table 1. A salinity solerance grouping of doubled-haploid rice lines

| R | Genotype   | Median | Group | R | Genotype   | Median | Group |
|---|------------|--------|-------|---|------------|--------|-------|
| 1 | Pokkali    | 3.00   | Tolerant | 32 | HS1-35-1-7 | 4.87   | Moderate |
| 2 | HS4-15-1-9 | 3.00   | Tolerant | 33 | HS4-11-1-72 | 4.87   | Moderate |
| 3 | HS4-15-1-24 | 3.00 | Tolerant | 34 | HS4-11-1-73 | 4.87   | Moderate |
| 4 | HS4-15-1-62 | 3.00  | Tolerant | 35 | HS1-35-1-5  | 5.00   | Moderate |
| 5 | HS4-15-3-17 | 3.00  | Tolerant | 36 | HS1-35-1-6  | 5.00   | Moderate |
| 6 | HS4-15-3-30 | 3.00  | Tolerant | 37 | HS4-11-1-75 | 5.00   | Moderate |
| 7 | HS1-35-14  | 3.27   | Tolerant | 38 | HS4-15-1-23 | 5.00   | Moderate |
| 8 | HS4-15-1-22 | 3.27  | Tolerant | 39 | HS4-15-1-27 | 5.00   | Moderate |
| 9 | HS4-15-3-4  | 3.27   | Tolerant | 40 | HS4-15-1-29 | 5.00   | Moderate |
| 10 | HS4-15-1-15 | 3.33  | Tolerant | 41 | HS4-15-2-9  | 5.00   | Moderate |
| 11 | HS4-15-1-25 | 3.33  | Tolerant | 42 | HS4-15-3-6  | 5.00   | Moderate |
| 12 | HS4-15-1-26 | 3.33  | Tolerant | 43 | HS4-15-3-29 | 5.00   | Moderate |
| 13 | HS4-15-1-63 | 3.33  | Tolerant | 44 | HS4-15-3-32 | 5.00   | Moderate |
| 14 | HS4-15-1-70 | 3.33  | Tolerant | 45 | HS4-15-1-1  | 5.00   | Moderate |
| 15 | HS4-15-2-6  | 3.33   | Tolerant | 46 | HS4-11-1-70 | 5.40   | Sensitive |
| 16 | HS4-15-3-8  | 3.33   | Tolerant | 47 | Inpari 34 Salin Agritan | 5.40 | Sensitive |
| 17 | HS4-15-3-26 | 3.33  | Tolerant | 48 | HS4-15-1-43 | 5.43   | Sensitive |
| 18 | HS1-35-1-10 | 3.37  | Tolerant | 49 | HS1-35-1-13 | 5.60   | Sensitive |
| 19 | HS4-15-1-64 | 3.37  | Tolerant | 50 | HS1-28-1-5  | 6.33   | Sensitive |
| 20 | HS1-35-1-15 | 3.40  | Tolerant | 51 | HS4-11-1-71 | 6.33   | Sensitive |
| 21 | HS4-11-1-2  | 3.40   | Tolerant | 52 | HS4-15-1-47 | 6.57   | Sensitive |
| 22 | HS4-11-1-30 | 3.40  | Tolerant | 53 | HS1-35-1-8  | 6.63   | Sensitive |
| 23 | HS4-15-1-6  | 3.40   | Tolerant | 54 | HS1-35-1-9  | 6.63   | Sensitive |
| 24 | HS4-15-1-28 | 3.40  | Tolerant | 55 | HS4-15-3-5  | 6.77   | Sensitive |
| 25 | HS4-15-2-4  | 3.43   | Tolerant | 56 | HS1-35-1-4  | 6.97   | Sensitive |
| 26 | HS4-45-1-66 | 4.33  | Moderate | 57 | HS4-11-1-36 | 7.40   | Sensitive |
| 27 | HS4-11-1-1  | 4.40   | Moderate | 58 | Inpara 5    | 8.23   | Sensitive |
| 28 | HS4-11-1-74 | 4.40  | Moderate | 59 | Ciheraug   | 8.33   | Sensitive |
| 29 | HS4-15-1-16 | 4.40  | Moderate | 60 | HS17-33-1-8 | 8.40   | Sensitive |
| 30 | HS 17-3-1-1 | 4.40   | Moderate | 61 | HS17-21-1-7 | 9.00   | Sensitive |
| 31 | Inpari 29   | 4.40   | Moderate | 62 | IR 29       | 9.00   | Sensitive |

Noted: R= rank
Figure 1. Cluster heatmap analysis to doubled-haploid rice lines toward means of characters under saline condition (S) and its relative decrease (R). (1 = root dry weight, 2 = total of biomass dry weight, 3 = shoot fresh weight, 4 = total of biomass fresh weight, 5 = shoot height, 6 = shoot dry weight, 7 = number of leaves, 8 = number of tillers, 9 = root length, 10 = root fresh weight, SKR = scores)
Table 2. A Means of doubled-haploid rice lines to salinity tolerance screening in hydroponic culture

| Character | Tolerant | Moderate | Sensitive | General |
|-----------|----------|----------|-----------|---------|
|           | 120 mM RD | 120 mM RD | % CT | 120 mM RD | % CT | 120 mM RD | % CT |
| NL        | 10.2 ± 1.5 | 9 ± 1.0 | 11.76 | 8.7 ± 1.3 | 0.20 | 14.71 | 9.4 ± 1.4 | 0.13 |
| NT        | 1.9 ± 0.5 | 1.4 ± 0.33 | 26.32 | 1.4 ± 0.4 | 0.30 | 26.32 | 1.6 ± 0.5 | 0.11 |
| SH (cm)   | 45.73 ± 2.72 | 41.8 ± 2.93 | 8.59 | 33.6 ± 6.6 | 0.50 | 26.53 | 41.36 ± 5.25 | 0.39 |
| RL (cm)   | 18.84 ± 1.96 | 17.4 ± 2.05 | 7.64 | 15.8 ± 2.89 | 0.20 | 16.14 | 17.53 ± 2.27 | 0.16 |
| SFW (g)   | 1.63 ±0.28 | 1.32 ± 0.25 | 19.02 | 0.87 ± 0.24 | 0.74 | 46.63 | 1.37 ± 0.37 | 0.62 |
| RFW (g)   | 0.98 ± 0.14 | 0.83 ± 0.16 | 15.31 | 0.60 ± 0.14 | 0.58 | 38.78 | 0.85 ± 0.20 | 0.47 |
| BFW (g)   | 2.62 ± 0.41 | 2.16 ± 0.38 | 17.56 | 1.47 ± 0.37 | 0.69 | 43.89 | 2.22 ± 0.56 | 0.57 |
| SDW (g)   | 0.53 ± 0.1 | 0.26 ± 0.06 | 51.21 | 0.19 ± 0.05 | 0.63 | 42.42 | 0.27 ± 0.06 | 0.52 |
| RDW (g)   | 0.10 ± 0.02 | 0.08 ± 0.02 | 20.00 | 0.06 ± 0.01 | 0.56 | 40.00 | 0.08 ± 0.02 | 0.50 |
| BDW (g)   | 0.43 ± 0.11 | 0.34 ± 0.07 | 20.93 | 0.26 ± 0.05 | 0.62 | 39.53 | 0.35 ± 0.08 | 0.51 |
| N         | 24          | 20        | 16       | 16       |

Notes: RD = relative decrease, % CT = percentage of tolerant comparison, NL = number of leaves, NT = number of tillers, PH = shoot height, RL = root length, SFW = shoot fresh weight, RFW = root fresh weight, BSW = total of biomass fresh weight, SDW = shoot dry weight, RDW = root dry weight, BDW = total of biomass dry weight.

3.3 Grouping of Doubled-haploid Rice Lines Based on Cluster heatmap Analysis

Dendrogram analysis was one of the grouping methods based on similarity or dissimilarity among genotypes in a current population [15]. The grouping could be useful if the specific characters in the group have been known. Cluster heatmap or clustergram could cover the disadvantage of dendrogram cluster. The analysis combined two dendrograms in the same dimension attached to heatmap analysis [24]. The combination produced a relationship pattern among genotypes and characters in color intensity which was easy to understand [17]. The higher of the color intensity indicated that the genotype has higher responses than the others in a current character [16]. The approach has been conducted by Anshori et al. [25] to identify the agronomic potential of doubled-haploid rice lines. Therefore, the analysis could be applied in the detection of the good tolerance trait under salinity screening.

Cluster heatmap analysis showed that there were two big genotype groups having a reverse orientation to character responses (Figure 1). The first group (I) which consisted of 22 genotypes was categorized as a less tolerance group. The group had a large relative decrease and low phenotype value under saline condition. The second group (II) which consisted of 40 genotypes was categorized as a good tolerant group. The group had a low relative decrease and good phenotype value under salinity stress. The analysis showed the difference pattern by color intensity between less and good tolerance trait under saline condition. It was considered a good and easy approach to detect tolerant genotypes in the early study. If the result of the analysis was compared to the median scoring, there were 5 moderate genotypes were grouped as less tolerance trait. Based on all results, the cluster heatmap analysis was considered more effective and selective than the scoring method in selecting tolerant doubled-haploid rice lines.

4. Conclusion

The good tolerant genotype has a lower relative decrease pattern than the less tolerance or sensitive genotype under salinity stress. The fresh weight characters, especially shoot related characters are more important to detect good tolerance traits than the other characters. Based on the cluster heatmap analysis, there were 38 doubled haploid rice lines considered as tolerant genotypes under saline condition. The cluster heatmap assignment was considered more selective than only focused on tolerance score so that it is especially useful in negative selection to sensitive lines.
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