Response of rice genotypes to zinc fertilizer detected using RAPD

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Abstract. Rice nutrition as a staple food in Asia is important for human metabolism and health. One of the global warming effects is micro element reduction in rice, including Zn. Fertilizer application of Zn(SO$_4$)$_2$·2H$_2$O was studied to investigate effect of Zn on some genotypes of local rice (white rice cv. Mansur, red rice cv. Cendana, sticky rice and black sticky rice) from Jatiluwih, Tabanan, Bali using RAPD marker. Experiments was conducted on plastic pot containing 2 kg soil enriched with Zn(SO$_4$)$_2$·2H$_2$O with concentration as followed 0.0 ; 2.5; 5.0 mg/kg soil. Each treatment consists of three replicates with 2 sub unit for each replicates. There were two seedlings per pot. Pots were placed in open field at Denpasar, Bali. Rice seedlings were grown until 8 weeks after planted. Seedlings were kept waterlogged during 8 weeks growth period. Random Amplified Polymorphic DNA (RAPD) marker was employed to detect polymorphism between treatments. Primers used were OPB12 and OPH1. RAPD showed that each genotype had different response to Zn treatment.

1. Introduction

Local cultivar of rice (Oryza sativa L.) is a special staple food that cultivated around Jatiluwih Village, Tabanan Regency of Bali Province, Indonesia. Farmers in this village planted four cultivars of rice based on the colour and taste, that is white rice cv. Mansur, red rice cv. Cendana, white sticky rice cv. ‘Ketan Tahun’ and Black sticky rice. Four types of rice from Jatiluwih are unique and require to be explored for its conservation. Germplasm of rice at Jatiluwih can be used for plant breeding and source of genetic conservation. As example, red rice cv Cendana has lower glicemic but higher in vitamin B and minerals compared to other rice cultivars [1]. Rice as staple food must contain standard nutrition, especially for baby and people at any young age.

Zinc (Zn) fertilizer is one of essential micronutrient that involve in plant growth. Based on study from [2], rice had high relative sensitivity to Zn deficiency. In fact, Zn nutrition on rice sampled from Jatiluwih area had analysed and Zn concentrations of young fully expanded leaf was very low and undetectable using ICP-E9000 [3]. USDA reported that Zn concentration on rice was 1.09 mg/100 g. Application of Zn(SO$_4$)$_2$ fertilizer combined with alternate watering and dry (AWD) technique increased rice yield and Zn concentration in rice grain [4]. Rice genotypes and time application of Zn fertilizer, during panicle initiation, can increase Zn translocation to grain [5]. Zn deficiency tolerant genotypes showed less symptom and higher dry mass [6].

Zinc fertilizer can effect plant growth on rice genotypes. Rice seedlings require Zn concentration in early young stage to proper growth until harvesting. The research aimed to investigate response of rice genotypes to Zn fertilizer.
2. Material and Methods

2.1. Plant material
Rice seeds of local rice from Jatiluwih were collected from farmer. There were four rice cultivars, that is rice cv. Mansur, red rice cv. Cendana, white sticky rice and black sticky rice. Rice seeds were sowed in plastic container for 3x24 hours to induce radicula growth, then soil was added to cover germinated seeds.

2.2. Growth condition
Seedlings were transplanted into plastic pots contain 2 kg soil at 14 days after germination and grown in the open field at Denpasar. Basic fertilizer was supplied with Diaminomethanal (NH$_2$)$_2$CO 175mg/kg soil; 70% was applied at planting and 30% at flower initiation, Triple Super Phosphate (TSP) 50 mg/kg soil and potassium chloride (KCl) 100 mg/kg soil. Media was watered for 2 weeks to homogenisation growth media. Zn fertilizer (Zn(SO$_4$)$_2$H$_2$O) was added to the growth media at 4 weeks after transplanting to plastic pot.

There were three concentrations of Zn(SO$_4$)$_2$. 2H$_2$O added to soil as Zn treatment, 0 as control; 2.5; 5.0 mg/kg soil. Four rice cultivars were treated with Zn treatment. The experiment was conducted using RCBD (Randomized Completely Block Design) with 3 replications and 2 experiment units for each treatment combination.

2.3. Zn analysis
Young fully expanded leaf blade from each main plant was cut for each treatment for Zn analysis in laboratory. A subsample of leaf (3-5 g) was heated at 500oC until dry and formed white ash, then dilute in 5mL of HNO$_3$ 1N and heated for 2-3 minutes. The solution was filtered using Whatmann No.41 into volumetric flask and added with HNO$_3$ 1N until volume become 50 mL. Blank solution was also prepared with HNO$_3$ 1N. Zn concentration was determined using MPAES (Microwave Plasma Atomic Emission Spectroscopy).

2.4. DNA extraction
DNA was extracted according to [7] with modification [8]. As much as 0.1 g of leaf sample were ground using mortar and 1 ml of CTAB buffer (d 2% w/v CTAB, 1.4 M NaCl, 50 mM EDTA, 100 mM Tris-HCl (pH 8), and 2% (v/v) 2-mercaptoethanol) was added and incubated at 65°C for 30 min. The mixture was centrifuge for 5 min at 14,000 rpm and supernatant was transferred to a new tube. Extraction was further done using chloroform:isoamylalcohol (24:1), and was centrifuge for 5 min at 14,000. The supernatant was collected and ¾ vol of cold isopropanol was added. The mixture was incubated at 4°C for 1 hour. After that, centrifugation was done to obtain pellet DNA and the pellet was washed with 70% ethanol and air dried. The DNA was resuspended in 100 µl H$_2$O.

Electrophoresis was done using 1% agarose gel in 1x TAE buffer to evaluate the quality and quantity of DNA. The gel was stained with ethidium bromide and visualised with Geldoc (EnduroTMGDS, Labnet Intl,Inc.)

2.5. PCR-RAPD analysis
Analysis of PCR-RAPD was done using two primers with 10 bp length, i.e., OPB12 (5’CCTTGAACGCA3’ and OPH1 (5’ GGTGCGGAA3’). (synthesized by 1st Base). Total PCR reaction was 20 µL which contained 1 µL template DNA, 1 x PCR buffer, 0.2 U taq polymerase (GoTaq Flexy DNA Polymerase – Promega), 200 µM dNTP, 2.5 mM MgCl$_2$, 1 µM primer).

The PCR cycle was as follow: 1 cycle pre-denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 37°C for 45 sec, and 45 sec extension at 72°C. The final cycle was done as final extension at 72°C for 10 min.

PCR products were separated using 1.5% agarose gel electrophoresis in 1 X TAE buffer. The gel was stained using Ethidium bromide and visualised using GelDoc.
3. Results and Discussion

3.1. Germination of rice seeds
Rice seeds started to germinate after 4 days of sowing. Rice cv. Mansur, sticky rice, red rice and black sticky rice seedlings grew differently whereas rice cv Mansur showed the slowest growth. Seeds germination effected by internal and external factors. Rice genotyped differed on germinations due to seed morphology. Seed germination of cereal assisted by energy from endosperm starch hydrolysed by enzyme of $\alpha$-amylase in layer of aleurone [8]. Room temperature and time of storage can influence the seed conditions. Condition of rice seeds germination was different (Figure 1).

![Figure 1. Seedlings of rice cultivars at 14 days after germinated](image)

3.2. Plant height
Plant height measured once a month since 0 weeks after transplanting into pots. The results were shown in Table 1. Addition of Zn fertilizer increase plant height. Zn has important role on metabolism in plants, especially on enzymatic reaction. Zn also involve on cell division.

Table 1. Plant height (cm) of rice cultivars after fertilized by ZnSO$_4$.2H$_2$O at 0, 4 and 8 weeks after transplanting

| Rice type          | Plant Height (cm) | ZnSO$_4$.2H$_2$O (mg/kg soil) |
|--------------------|-------------------|-------------------------------|
|                    | Plant age         | (0.0)                        | (2.5) | (5)  |
| Sticky rice        | 0                 | 14.30                        | 14.55 | 13.50|
|                    | 4                 | 23.83                        | 36.67 | 34.17|
|                    | 8                 | 33.33                        | 39.33 | 34.50|
| Red rice           | 0                 | 17.17                        | 16.92 | 16.92|
|                    | 4                 | 26.83                        | 37.50 | 24.67|
|                    | 8                 | 32.33                        | 45.00 | 26.00|
| Black sticky rice  | 0                 | 14.33                        | 18.58 | 19.00|
|                    | 4                 | 22.00                        | 34.67 | 32.67|
|                    | 8                 | 28.00                        | 45.50 | 44.33|
| Rice cv. Mansur    | 0                 | 12.42                        | 13.30 | 13.45|
|                    | 4                 | 23.33                        | 41.83 | 28.67|
|                    | 8                 | 38.83                        | 55.33 | 40.50|
At 8 weeks grown in the open field, plant height of rice was varied due to addition of Zn 2.5 mg/kg soil. Plant height of sticky rice increased by 6%, red rice 12.6%, black sticky rice 17.5% and rice cv. Mansur 16.5%. Each rice cultivar showed different response to Zn treatment.

Zn in rice grain and spikelet was supplied from phloem after mobilization from flag leaf and other leaves from plant [9]. Zn transportation in the stem is transferred by xylem to phloem. Sufficient of Zn supply can increase activity of enzyme and metabolism of auxin that promote apical growth of plants [10].

3.3. Zn concentrations on the last fully expanded leaf
White sticky rice and rice cv. Mansur showed enhancement of Zn concentrations in the leaf blade with the addition of Zn fertilizer (Figure 2). Different cultivar showed varied on Zn absorption from soil solution to upper part of plant. Zn usually transport actively in the form of Zn2+.

In contrast, supply of 5 mg ZnSO4.2H2O decreased Zn concentration on leaf blade of sticky rice, red rice and rice cv. Mansur.

![Figure 2](image)

**Figure 2.** Zn concentrations on the last fully expanded leaf of four rice cultivars at 8 weeks after transplanting

3.4. DNA extraction from leaf segment of the last fully expanded leaf
Result of DNA isolation shown in Figure 3. As can be seen in Fig 3, DNA were successfully extracted from 10 samples out of 12 samples. DNA from Sticky rice + ZnSO4.2H2O 5 mg/kg soil and Black sticky rice + ZnSO4.2H2O 0 mg/kg soil failed to be extracted. Several factors affected the success of DNA extractions such as the presence of polysaccharides, protein, tannin in leaves [11]. Moreover in cereals, there is rigid non cellulose compound in cell wall of leaves that make DNA extraction difficult [12]. The use of the last fully expanded leaf in this study may also contribute to the unsuccessful DNA extraction. In mature leaves, the quantity of polyphenol and polysaccharides are higher than that in younger leaves [13].

Smearing of DNA was observed in each sample. This indicated the degradation of DNA during extraction process. Liquid nitrogen may be essential in the grinding process of leaf sample.
Figure 3. Gel electrophoresis of DNA from rice leaf

Legend Note
- Rice + ZnSO$_4$.$2$H$_2$O 0 mg/kg soil
- Rice + ZnSO$_4$.$2$H$_2$O 2.5 mg/kg soil
- Rice + ZnSO$_4$.$2$H$_2$O 5 mg/kg soil
- Sticky rice + ZnSO$_4$.$2$H$_2$O 0 mg/kg soil
- Sticky rice + ZnSO$_4$.$2$H$_2$O 2.5 mg/kg soil
- Sticky rice + ZnSO$_4$.$2$H$_2$O 5 mg/kg soil
- Black sticky rice + ZnSO$_4$.$2$H$_2$O 0 mg/kg soil
- Black sticky rice + ZnSO$_4$.$2$H$_2$O 2.5 mg/kg soil
- Black sticky rice + ZnSO$_4$.$2$H$_2$O 5 mg/kg soil
- Red rice + ZnSO$_4$.$2$H$_2$O 0 mg/kg soil
- Red rice + ZnSO$_4$.$2$H$_2$O 2.5 mg/kg soil
- Red rice + ZnSO$_4$.$2$H$_2$O 5 mg/kg soil

3.5. RAPD pattern of rice DNA by primer OPB12

In this study, amplifications using OPB12 and OPH1 did not occur in all samples. Instead of distinct band of PCR product, smear patterns were observed in several samples. This may correspond to the low quality of DNA or improper PCR reaction.

Figure 4. Amplified RAPD patterns of genomic DNA of rice using primer OPB12

Legend Note:
- Rice + ZnSO$_4$.$2$H$_2$O 5 mg/kg soil
- Black sticky rice + ZnSO$_4$.$2$H$_2$O 0 mg/kg soil
- Black sticky rice + ZnSO$_4$.$2$H$_2$O 2.5 mg/kg soil
- Black sticky rice + ZnSO$_4$.$2$H$_2$O 5 mg/kg soil
- Sticky rice + ZnSO$_4$.$2$H$_2$O 2.5 mg/kg soil
- Black sticky rice + ZnSO$_4$.$2$H$_2$O 0 mg/kg soil
- Red rice + ZnSO$_4$.$2$H$_2$O 2.5 mg/kg soil
- Red rice + ZnSO$_4$.$2$H$_2$O 0 mg/kg soil
- Red rice + ZnSO$_4$.$2$H$_2$O 5 mg/kg soil

Different PCR-RAPD band patterns were observed between Black Sticky Rice, Sticky Rice and Red Rice. Zn treatments induced changes in PCR-RAPD pattern. As shown in Fig. 3 and Fig. 4, Black Sticky Rice (2.5mg ZnSO$_4$.$2$H$_2$O /kg soil) and Black Sticky Rice (5mg ZnSO$_4$.$2$H$_2$O /kg soil) exhibited different PCR-RAPD pattern. Using OPB12, the highest band (2160 bp) in Black Sticky Rice (2.5mg ZnSO$_4$.$2$H$_2$O /kg soil) did not appear in Black Sticky Rice (5mg ZnSO$_4$.$2$H$_2$O /kg soil).
Figure 5. Amplified RAPD patterns of genomic DNA of rice using primer OPH1

Legend Note:
- Rice + ZnSO$_4$.2H$_2$O 5 mg/kg soil
- Black sticky rice + ZnSO$_4$.2H$_2$O 0 mg/kg soil
- Black sticky rice + ZnSO$_4$.2H$_2$O 2.5 mg/kg soil
- Sticky rice + ZnSO$_4$.2H$_2$O 0 mg/kg soil
- Black sticky rice + ZnSO$_4$.2H$_2$O 5 mg/kg soil
- Black sticky rice + ZnSO$_4$.2H$_2$O 2.5 mg/kg soil
- Red rice + ZnSO$_4$.2H$_2$O 0 mg/kg soil
- Red rice + ZnSO$_4$.2H$_2$O 5 mg/kg soil
- Black sticky rice + ZnSO$_4$.2H$_2$O 2.5 mg/kg soil
- Red rice + ZnSO$_4$.2H$_2$O 2.5 mg/kg soil

In contrast to this research, previous finding suggested that 150 mg/L treatment of zinc sulphate to rice seedling for 10 days did not induce changes in PCR-RAPD pattern [14]. Other study reported that the application of natural phycomolecule-coated ZnO nanoparticles as fertilizer to cotton seedling at concentration of 25, 50, 75, 100, and 200 mg/l changed PCR-RAPD bands intensity but did not change band pattern [15].

Low concentration of Zn prevents plant DNA from oxidative damage by reactive oxygen species [16]. This may contribute to the changing of priming sites of RAPD primers, which leads to changes of PCR-RAPD patterns as observed in this research.

4. Conclusion
RAPD showed that each genotype had different response to Zn treatment.

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