Development of rice mutant tolerance to salinity through combination of gamma rays irradiation and *in-vitro* selection

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**Abstract.** Global warming caused a large rise in global sea level poses many threats, especially to a country like Indonesia. One of the threats that affected rice field located adjacent to coastal area is increase level of soil salinity. Inpari 34 and Inpari 35 varieties tolerant to salinity at seedling phase cannot perform well when planted in salinity prone area where the increase in salinity affected by the up and down of sea surface occurred during the planting season. The aims of this experiment were to obtain putative salinity-tolerant mutant (M1) at vegetative and generative phase from Inpari 34 and Inpari 35 through combination of gamma rays irradiation and *in-vitro* selection. The study consisted of several activities, namely embryogenic callus induction, mutation induction by gamma-ray irradiation, callus *in-vitro* selection by NaCl, regeneration of selected callus, and plantlet acclimatization and greenhouse grow-out. In this experiment 62 and 54 putative salinity-tolerant mutant lines obtained from Inpari 34 and Inpari 35, respectively.

**Keywords:** mutant, *in vitro* selectin, Inpari 34, Inpari 35, salt tolerant.

1. **Introduction**

Efforts to increase rice production in Indonesia can be done by utilizing marginal land, such as saline land [1]. Worldwide, saline land area reaches 800 million hectares or 6% of the total land area in the world [2]. In Indonesia, the potential for saline land is 440 thousand hectares, most of which are tidal land located in Sumatra, Java, Madura, Sulawesi, Maluku and Papua [3]. The area of saline land in Indonesia can continue to grow, especially in coastal areas [1]. This land can be further developed for agricultural purposes [4] [5]. The rice fields of the Pantura region (north coast of Java Island) which is national rice bran have experienced seawater intrusion which causes a high level of salinity to affect the chemical properties of soil, disrupting rice growth and reducing yield [6]. In addition to seawater intrusion that happened slowly for a long time, salinity can be caused unexpectedly due to natural events such as the tsunami that hit Aceh in 2004 [7].

High salinity gives osmotic stress on the ground [8]. In the later stages, salinity stress will have an ion stress effect [9]. Osmotic effect, namely the decrease in osmotic potential of soil solutions thereby reducing water availability [8]. The high concentration of salt in the soil disrupts the capacity of the roots to extract water; this condition causes partial cell dehydration and loss of cell turgor due to reduced water potential in the cell [10]. The next effect of toxicity is an increase in ionic concentrations that are toxic to plants. This ionic stress effect is a major factor that can inhibit rice growth [11].

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One solution to the problem of salinity can be to use an approach using salinity tolerant varieties with high yield potential [12]. This approach looks more promising at a more economical and socially acceptable cost [12]. The large potential of saline land in Indonesia makes it possible to plant saline tolerant rice so that it can increase national rice production [1].

The use of saline soils for rice farming requires varieties that are tolerant to stress conditions [12]. Tolerant local varieties generally have low productivity [13], so it is necessary to assemble new varieties of rice that are tolerant to salinity with high productivity. In-vitro culture techniques combined with mutation induction are one of the alternative methods to obtain new characters, which are not available at existing germplasm sources [14]. The combination of mutation induction and in vitro culture can increase plant tolerance to salinity [15]. This method has been applied to how many rice varieties to obtain tolerant salinity stress plants [16].

Inpari 34 and Inpari 35 varieties are new superior varieties that are tolerant of salinity in the Seed phase [17]. To increase the resistance of Inpari 34 and Inpari 35 varieties to salinity in the next phase of growth can be done with genetic mutations by using gamma-ray radiation combined with in vitro selection. This has been done on the Cihera and Inpari 13 varieties and produces several numbers of rice that are tolerant of NaCl up to 150 mM [18]. The aim of this study was to obtain 100 putative (M1) salinity tolerant from Inpari 34 and Inpari 35 variety from in vitro selection.

2. Methodology

The plant material used was Inpari 34 and Inpari 35 variety. In this experiment consisted of several stages of activity namely embryogenic callus induction, mutation induction by gamma-ray irradiation and in vitro callus selection, callus regeneration as a result of selection and acclimatization of putative mutant plantlets.

2.1. Callus induction

The explants used were zygotic embryos isolated from ripe seeds. Seeds are sterilized in laminar flow using sterilized ingredients, including alcohol and NaOCl. The embryos were then planted on callus induction media, namely MS + 24D (1, 3 and 5 mg/l) + Casein Hirolisate 3 g/l. In one bottle, 10 explants were planted. Bottles that have been planted with explants were then incubated in the culture shelf in the dark by being covered using a black cloth. The room temperature was set to 24 - 25°C. The variables observed were the percentage of embryonic callus formation, as well as visual callus. Callus induction is performed repeatedly in order to obtain embryonic callus in large quantities.

2.2. Mutation induction by gamma-ray irradiation and in vitro selection callus

Embryogenic callus formed was given irradiation treatment. The gamma-ray irradiation dose given is 20-30 Gy. Callus which has been given mutation treatment is then transferred to the same medium for callus induction for 4 days. Callus which has been treated with mutations by gamma-ray irradiation, transferred to callus proliferation media, added NaCl as much as LC50 value for each variety. Callus from the selection medium after 2 weeks was cultured on the same medium with the same NaCl content for 2 weeks. Selection is carried out for 2 periods and each period for 2 weeks. The variables observed are the number of browning callus.

2.3. Regeneration of callus as a result of selection

Living callus then transferred to the shoot regeneration media, namely MS + BA (3, 5, 7 mg/l) + Zeatin (0, 0.1, 0.3 mg/l) + Proline 100 mg/l. The variables observed were percent regenerated callus forming whole plantlets in each rice variety used.

2.4. Acclimatization of putative mutant plantlets

The plantlets obtained were acclimatized by means of the resulting plantlets being removed from the agar media and cultured on ordinary water media at room temperature. After one week the plantlets
were transferred to Yosida's media until new roots were formed. The plantlets were then planted in sludge media. The variables observed were percent of living plantlets from each rice variety.

3. Results and discussion

3.1. Callus induction
In the second week after planting, explants in the form of zygotic embryos began to respond. It was indicated by the explants began to swell and form callus. Explants cultured on 2,4-D media are generally able to form callus.

Table 1 depicts the increasing of 2,4-D concentration to 5 mg/l can reduce the ability of explants to form callus and lower the percentage of embryogenic callus. In addition, the 3 mg/l 2,4-D was found as the optimum concentration to form callus in explants of Inpari 34 and Inpari 35 varieties. Its callus characterized as nodular callus, crumbly, and yellowish-white in color. This is an embryogenic callus characteristic that can be regenerated to form shoots [19].

| 2,4-D (mg/l) | Inpari 34 | Inpari 35 |
|-------------|----------|----------|
| % forming callus | % embryogenic callus | % forming callus | % embryogenic callus |
| 1 | 52 | 26 | 58 | 39 |
| 3 | 97 | 68 | 94 | 72 |
| 5 | 79 | 31 | 91 | 53 |

3.2. Mutation induction by gamma-ray irradiation and callus in vitro selection
The embryogenic callus was cultured on MS media and then irradiated according to LD50. The value of LD 50 for Inpari 34 rice callus was 22.53 while for Inpari 35 variety rice was 24.36. After irradiation the callus transferred to callus induction media to recover callus conditions and detect contamination. Furthermore, the callus was selected on media containing NaCl at the LC50 concentration. The LC50 value of NaCl for Inpari 34 varieties of rice callus was 92.6753 and Inpari 35 was 98.78. The mutant callus selected on NaCl media (at the LC 50 concentration) produced a salinity tolerant mutant plantlet. The same method has also been applied to callus varieties Inpari 13 and Ciherang which produce salinity tolerant lines [20].

In one week, the callus on the media selection began to brown and the structure became softer. Callus cells then began to show damage due to radiation stress and NaCl poisoning from the selection media. This happens because cells that are intolerant of stress stress will experience osmotic stress or salt poisoning so cells brown and lysis [8]. The callus response of each variety to NaCl is quite diverse. In Inpari 34 varieties, the percentage of browning callus at 66.50 - 75.65 % and Inpari 35 varieties were 58.75 - 69.29 % (table 2).

3.3. Regeneration of callus as a result of selection
At the 6th week after culture planted in regeneration media, some green spots begin to appear. In the eighth week, shoots have begun to formed. For the parameters of the number of adventitious shoot in callus Inpari 34 varieties, the medium that contained 3 mg/l of BA and 0.1 mg/l of Zeatin gave the best response, in detail, 85% of the callus was able to form buds with an average number of shoots is 2.3 (table 3). The same thing happened to callus varieties Ciherang and Inpari 13 where the combination of BA and Zeatin gave the best response to the formation of shoots [18].

In Inpari 35 rice callus, a medium containing 3 mg/l BA combined with 0.1mg/l Zeatin was able to induce 90% shoot formation. With the number of shoots of 2.6. This treatment is the best treatment for shoot induction in Inpari 35 variety of rice callus (table 4). Increased concentrations of BA (5 and 7
mg/l) and Zeatin (0.3 mg/l) resulted in a lower number of shoots produced. This is because growth regulators which are added at higher concentrations will inhibit the formation of shoots [21].

Table 2. *In vitro* selection of mutant callus on media containing NaCl with LC50 concentration.

| Radiation intensity | varietas Inpari 34 | varietas Inpari 35 |
|---------------------|-------------------|-------------------|
|                     | number of callus  | number of browning callus (%) | number of callus  | number of browning callus (%) |
| I                   | 200               | 133               | 66.50               | 150               | 103               | 68.67               |
| II                  | 220               | 160               | 72.73               | 120               | 77                | 64.17               |
| III                 | 210               | 144               | 68.57               | 170               | 112               | 65.88               |
| IV                  | 190               | 132               | 69.47               | 140               | 97                | 69.29               |
| V                   | 230               | 174               | 75.65               | 160               | 94                | 58.75               |

Table 3. Regeneration of embryogenic callus to form buds of Inpari 34 varieties.

| Zeatin concentration (mg/l) | BA concentration (mg/l) | 0 | 3 | 5 | 7 |
|-----------------------------|-------------------------|---|---|---|---|
|                             | percent of shoot | number of shoot | percent of shoot | number of shoot | percent of shoot | number of shoot |
| 0                           | 0           | 0         | 5           | 0.5         | 20           | 0.6         | 5         | 0.1       |
| 0.1                         | 15          | 0.15      | 85          | 2.3         | 20           | 0.2         | 25        | 0.25      |
| 0.3                         | 25          | 0.55      | 15          | 0.15        | 10           | 0.15        | 0         | 0         |

Table 4. Regeneration of embryogenic callus formed shoots of Inpari 35 rice varieties.

| Zeatin concentration (mg/l) | BA concentration (mg/l) | 0 | 3 | 5 | 7 |
|-----------------------------|-------------------------|---|---|---|---|
|                             | percent of shoot | number of shoot | percent of shoot | number of shoot | percent of shoot | number of shoot |
| 0                           | 0           | 0         | 10          | 0.1         | 25           | 0.5         | 10        | 0.2       |
| 0.1                         | 10          | 0.15      | 90          | 2.6         | 15           | 0.15        | 25        | 0.35      |
| 0.3                         | 35          | 0.65      | 20          | 0.3         | 15           | 0.25        | 10        | 0.15      |

From the table 3 and table 4, it can be seen that the best medium for regenerating callus forming shoots in Inpari 34 and Inpari 35 is MS Media which added BA 0.3 mg/l and Zeatin 0.1 mg/l. This formulation is used to regenerate callus to form shoots. The callus used was mutant callus that had been irradiated with gamma rays at LD50 concentrations and selected in NaCl media at LC50 concentrations. From these activities obtained 132 callus from 34 and Inpari varieties 113 callus from Inpari 35 which can form buds (table 5). The resulting shoots are salinity tolerant mutants because they can grow on NaCl media but the controls are not. The same thing has been done on Ciherang, Inpari 13 and Inpara 3 varieties [18].

3.4. Acclimatization of putative mutant plantlets

Acclimatization is an important stage especially in plants produced by somaclonal diversity, because generally plantlets derived from somaclonal diversity and in vitro selection of roots have different
structures so that the ability at the time of adaptation at the time of acclimatization is not the same. The percentage of acclimatization success is usually very low, so the treatment and environmental conditions during acclimatization greatly determine the success of acclimatization. Acclimatization is carried out with plantlets cultured on an ion free air medium until the plantlets are capable of removing roots (figure 1), then proceed to the mud media which is seeded for seeding. After the plantlets are able to produce 3-4 new leaves, they are transferred to the bucket. Acclimatization is done in stages to increase the percentage of plantlets that live, because in vitro plantlets are less adaptive to low humidity [22].

**Table 5.** Callus regeneration that has been mutated with gamma rays and selected on NaCl media.

| varietas  | number of selected mutant callus | number of callus forms shoot | average number of perennial shoots |
|-----------|----------------------------------|-----------------------------|-----------------------------------|
| Inpari 34 | 307                              | 132                         | 1.1                               |
| Inpari 35 | 257                              | 113                         | 0.9                               |

**Figure 1.** Acclimatization of in vitro shoots of rice on ion-free water media.

In table 6, it can be observed that 83 plantlets originating from Inpari 34 variety have a number of live plantlets which are equal to 62. For Inpari 35 varieties the number of acclimatized plantlets is 89 with 54 plantlets living in them (table 6.) These mutants must be tested in a greenhouse and salted field to determine the stability of salinity tolerance and yields before being released as salinity tolerant varieties [23].

**Table 6.** Acclimatization of putative somaclonal mutants.

| varieties  | the number of acclimatized plantlets | the number of plants remains green |
|------------|--------------------------------------|-----------------------------------|
| Inpari 34  | 83                                   | 62                                |
| Inpari 35  | 89                                   | 54                                |

**4. Conclusion**

From this study, 62 numbers of somaclonal mutants derived from Inpari 34 and 54 p numbers of somaclonal mutants originating from Inpari 35 variety rice were obtained. The mutant plants obtained were candidates for salinity tolerant lines to be tested in the field. Selected mutants need to be tested in saline land to see the stability of salinity tolerance in the vegetative and generative phases as well as its yield.

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