



RESEARCH ARTICLE

The effect of chemical-induced mutagenesis in *indica* rice variety IR50404 for blast disease caused by the *Magnaporthe grisea* in Vietnam

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Abstract

Indica rice variety, IR50404 is the most-grown rice in Vietnam's Mekong Delta. It is susceptible to multiple blast diseases, making it an ideal genotype for identifying pathological trait mutations. Chemically induced mutant lines were derived from IR50404 by treating seeds with ethyl methanesulfonate (EMS) (0.1 and 0.125%), methyl salicylate (MeSA) (0.1 and 0.125 mM), and their combinations. Chlorophyll, proline, and protease enzyme inhibitors were examined for disease tolerance in mutant rice-based plants. Blast resistance (41.6%) and protease inhibition (30.4%) were highest in T5 (0.1% EMS + 0.1 mM MeSA). Proline (3.91 g/mL) and chlorophyll (8.52 g/mL) concentrations were significantly higher after 0.125% EMS treatment than in the untreated control. In addition, a high rate of protease inhibition (30.4%) was improved after treatment with 0.125% EMS + 0.125 mM MeSA. Results showed that blast resistance was improved by 0.1% EMS and MeSA. This also has implications for the breeding of plant varieties other than rice.

Keywords: Blast disease, chlorophyll a and b, EMS, MeSA, proline, protease

Introduction

Rice blast disease, caused by the fungus *Pyricularia grisea* Sacc [syn: *Magnaporthe grisea* (Hebert) Barr], is one of the most damaging fungi to rice production (Truc et al. 2019), with up to 50% yield losses (Yang et al. 2024). According to Alvin et al. (2015), yield losses of 5 to 10%, 8, and 14% were recorded in India in the 1960s and early 1970s, South Korea in the mid-1970s, and China in the 1980 and early 1981. In particular, a loss of 50 to 85% caused by the disease in the Philippines has been reported (Alvin et al. 2015). Similarly, Khatun et al. (2021) observed that rice blast caused up to 98% of the crop loss in Bangladesh. The use of resistant varieties is an efficient method to control this disease. However, the current resistance of varieties controlled by the commonly used genes, frequently deteriorates after only a few years of cultivation (Fair and Tor 2014). This is the case because virulent races have begun to appear.

The IR 50404 rice variety originated at the International Rice Research Institute (IRRI) and was imported into Vietnam in early 1990. It is grown mainly by farmers in the Mekong Delta and is the best-known rice variety in this area, especially in Tien Giang province. The IR 50404 rice variety is used by many people because of its low price and moderate income. The seeds are gourd-shaped with the desirable characteristic of blooming after cooking. Over the years, the

government has advised farmers to limit the use of IR 50404 due to high infection with brown plant hopper (BPH) and blast disease. However, farmers prefer IR 50404 due to the potential to produce large quantities with three crops per year, because of its short life cycle, yield stability and ease of cultivation. This means that new approaches to improving disease resistance in this variety are required.

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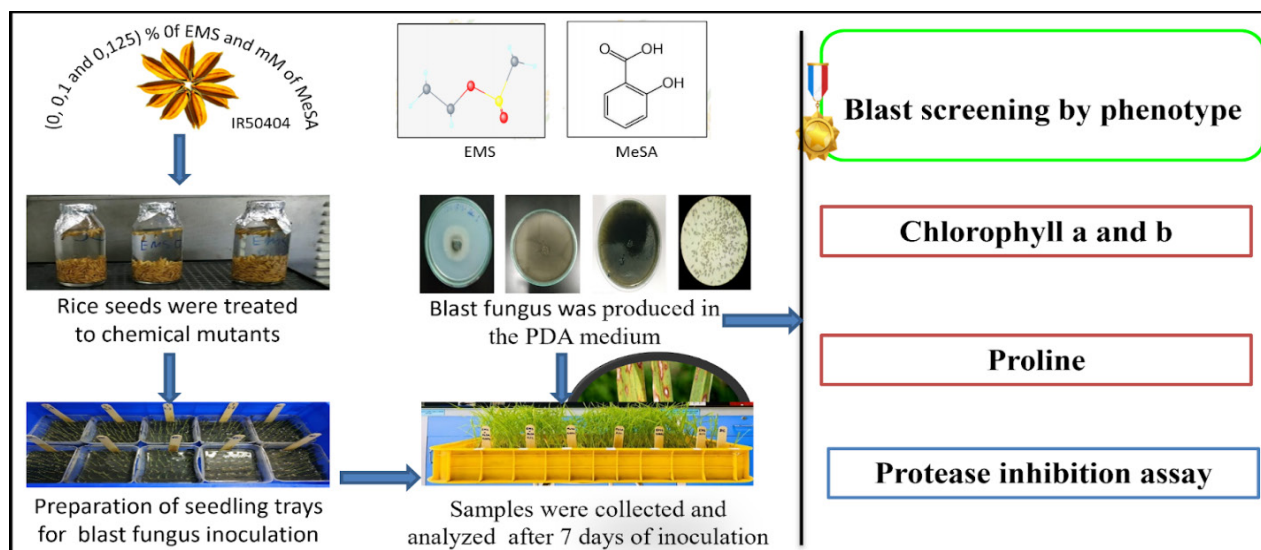


Fig. 1. Layout of experimental design

Numerous mutation methods have been employed in crop breeding and chemically induced mutations are often used. Targeted chemical mutation, using ethyl methanesulfonate (EMS), causes a point mutation (More 2022). EMS mutation was successful in enabling the development of early flowering in spring rape (*Brassica napus*) (Thurling and Depittayan 1992), and herbicide resistance in soybean (Sebastian et al. 1989). EMS has been utilized in sugarcane mutant breeding to improve abiotic stress resistance (Masoabi et al. 2023). EMS is highly effective in inducing leaf phenotypic variation, decreasing fruit size, and maximizing disease resistance in tomatoes (Yudhvir 1995). Methyl salicylate (MeSA) has been used to stimulate an increase in the activities of enzymes, specifically the protease enzymes involved in plant disease resistance mechanisms that are effective against insects and pathogens in plants (Zhu and Park 2005). Studies have shown that MeSA can protect tomato plants from cold and pests (Ding et al. 2002).

Proteins are one of the most abundant macro molecules in living systems and serve various functions, including structure, metabolism regulation, and defence (Mohanty et al. 2014). Proteins transported to the vacuole may also change shape, for example, the consequence of the luminal acidic pH, exposing cleavage sites to active proteases. Proteases play key roles in plant growth, development, and protection (Balakireva and Zamyatnin 2018). Activating proteases that degrade pathogens entering through injured tissues or amplifying pathogen protease inhibitors are two ways to use proteases (Müntz 2007). Mutagens like EMS and MeSA and their combination could increase protease enzyme activity against blast resistance in rice. To create a new IR50404 line with increased blast resistance via induced chemical mutation, the present study examined the effects of EMS and MeSA on protease enzyme production and blast

disease resistance.

Materials and methods

In the present study, the indica rice variety IR 50404 was used and seeds were obtained from the Genomic Research Institute and Seed (GRIS) of Cuu Long Delta Rice Research Institute, Vietnam. The steps undertaken in the experiment are given in Fig. 1.

Fungi source and chemicals induced

The blast fungus was isolated from wild rice by Truc et al. (2019) and used for the screening of the mutant lines to analyze the disease reaction. IR 50404 seed stock was surface sterilized by soaking it in 0.1% NaOCl for 30 minutes and then washing it in distilled water. The seeds were then treated with 0.1 and 0.125% by EMS, 0.1 and 0.125 mM by MeSA, and their combination for 5 hours. For the treatment with mutagen, 100 seeds were treated with each of the different doses and combinations, T0 (control untreated), T1 (0.1% EMS), T2 (0.125% EMS), T3 (0.1 mM MeSA), T4 (0.125 mM MeSA), T5 (0.1% EMS + 0.1mM MeSA), and T6 (0.125% EMS + 0.125 mM MeSA). The seeds germinated after treatment in the trays under greenhouse conditions.

Phenotyping for the blast disease

The experiment was conducted in a randomized block design with three replication for each treatment. The 3-week-old juvenile-stage plants were grown at room temperature. To ensure severe blast infection, inoculation with the fungus *Pyricularia grisea* Sacc [syn: *Magnaporthe grisea* (Hebert) Barr] was achieved by spraying the plants with a suspension of 10^5 spores/mL at 5 mL/plant. After inoculation, the rice plants were incubated in the dark at room temperature. The scoring for the blast disease was

after 7 days of inoculation. Each plant was scored for infection type (generally 0–2 resistant, 3–5 susceptible) according to Hayashi and Fukuta (2009).

The resistance and susceptibility frequencies resulting from the treatments with *P. grisea* were calculated according to the following equation:

$$\text{Frequencies (\%)} = \frac{R/S}{T} \times 100$$

where,

R = Number of resistant plants (with a score of 0–2)

S = Number of susceptible plants (with a score of 3–5)

T = A total number of plants

Chlorophyll a and b

Fresh leaves (1 g) from resistant and susceptible samples of each treatment were cut and crushed with a mortar pestle to make a homogeneous mixture, and 20 mL of 80% acetone and 0.05g of MgCO₃ were added. In a refrigerator at 4°C, this mixture was incubated for 4 hours before centrifugation at 500 rpm for 5 minutes. Transferred to a 100 mL volumetric flask, the supernatant was adjusted to 100 mL with 80% acetone. The solution's absorption at 649 and 665 nm was measured against an 80% acetone control using a UV-vis spectrophotometer (Arnon 1949). Formula followed is given here under:

$$\text{Chlorophyll a} = 13.70 \times A_{665} - 5.76 \times A_{649} \text{ } \mu\text{g/mL}$$

$$\text{Chlorophyll b} = 25.80 \times A_{649} - 7.60 \times A_{665} \text{ } \mu\text{g/mL}$$

$$\text{Chlorophyll total} = 6.10 \times A_{665} + 20.04 \times A_{649} \text{ } \mu\text{g/mL}$$

Estimation of proline in rice seedlings after screening for blast fungi

Proline content was estimated as described by Chinard (1952) with some modifications. For each treatment, 100 mg of finely ground leaves from resistant and susceptible samples were homogenized with 500 μL of 3% sulfosalicylic acid. The solution was centrifuged at 6000 rpm for 5 minutes. Next, 100 μL of the supernatant extract was added to 500 μL of reaction solution of 100 μL of 3% sulfosalicylic acid, 200 μL of glacial acetic acid, 200 μL of acidic ninhydrin [(1.25 g ninhydrin, 30 mL glacial acetic acid, 20 mL orthophosphoric acid 6M)]. The solution was incubated at 96°C for 1-hour before being stopped in ice-cold water for 5 minutes. Toluene (1-mL) was added to the mixture and vortexed for 20 minutes, and the sample was allowed to stand for 5 minutes. The absorbance of the reaction was measured at 520 nm using a UV-vis spectrophotometer. Calibration curves were constructed using DL-proline with concentrations of 2, 4, 6, 8, and 10 mg/mL. The amount of free proline was evaluated using a standard curve and expressed as mg/mL of fresh samples.

Protease extraction and inhibition assay

A protease assay was carried out in a total volume of 1-mL by mixing 67 μL of bovine trypsin (0.125 mg/mL; dissolved in 1 mM HCl), 67 μL of 0.9% NaCl, 536 μL of Tris buffer (200 mM Tris; 20 mM Calcium chloride, pH 7.8) and 330 μL of Na-Benzoyl-DLArginine-P-NitroAnilide (BAPNA) (1-mg/1 mL) as a substrate. A blank was used by adding 67 μL of 1 mM HCl instead of trypsin. In the protease inhibition assay described by Divya et al. (2014), 25 μL of plant extract was pre-incubated with 67 μL of trypsin (0.125 mg/mL) for 10 minutes, followed by the addition of 67 μL of NaCl (0.9%) and 511 μL of Tris buffer. The reaction was started by adding 330 μL of Na-Benzoyl-DLArginine-P-NitroAnilide (BAPNA) (1 mg/1 mL). Proteolytic activity was measured by the continuous spectrophotometric rate determination method using a UV Spectrophotometer, by recording the increase in absorbance at 405 nm for 5 minutes. All assays were done in duplicate and the percentage of inhibition was calculated by taking the activity in the presence of the enzyme alone as 100%.

Data analysis

The percentages of resistant and susceptible rice seedlings after blast infection were calculated. An analysis of variance (ANOVA) was used. All analyses were performed by SAS 9.1 (SAS Institute). The *p-values* of less than 0.05 were considered significant. Mean values and standard deviation (SD) were obtained from 3 replicates for chlorophyll a, b, proline, and protease inhibition and analyzed using Duncan's multiple range tests.

Results and discussion

Effects of EMS and MeSA treatments on blast resistance in the indica rice variety IR 50404 by phenotype

Rice blast resistance and genetically controlled protection are complex. Despite knowledge of a large collection of rice genes, the molecular responses of rice plants to physiological stress, especially in blast fungus interactions, remain unknown. Mutations can be used to study genes, which are the building blocks of plant growth and development and produce raw materials for genetically improving economic crops (Adamu and Aliya 2007). Mutants can help explain plant defence mechanisms by providing genetic diversity for gene discovery (Nogué et al. 2016). The results showed that the plants treated with EMS and MeSA and their combinations had different levels of blast resistance and were more resistant than the untreated control (T0) at the seedling stage are shown in Fig. 2. Treatment T6 had the lowest resistance rate of 20.5% when compared to mutation-treated treatments, but it was higher than treatment T0 (18%) while treatment T5 (0.1% EMS + 0.1 mM MeSA) had the highest resistance to blast disease at 41.6%, displaying high effectiveness against the disease. Yudhvir (1995)

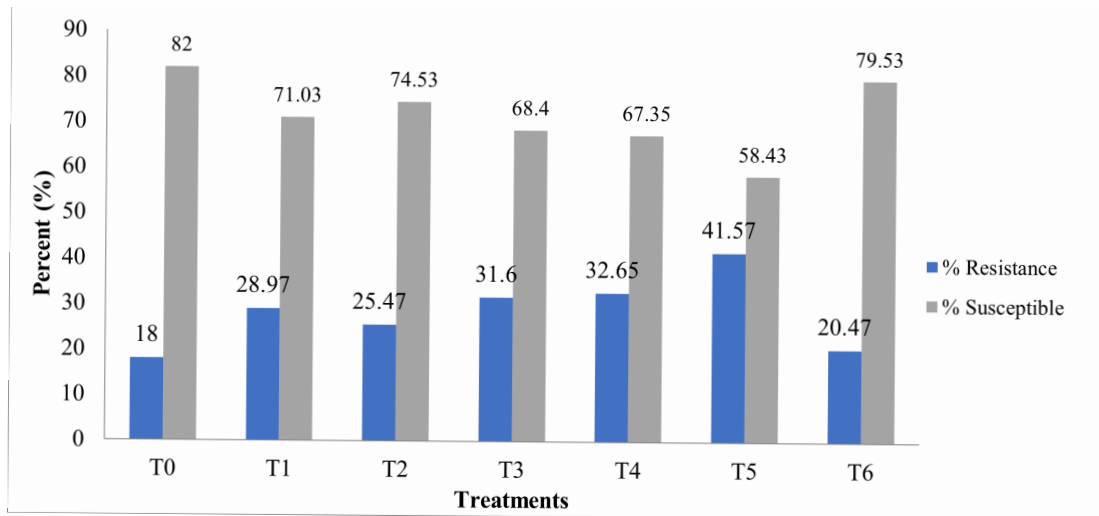


Fig. 2. Effects of EMS and MeSA-induced application on the phenotype of IR50404 rice after screening with blast fungus. Note: T0 (control), T1 (0.1% EMS), T2 (0.125% EMS), T3 (0.1 mM MesA), T4 (0.125 mM MeSA) T5 (0.1% EMS + 0.1 mM MeSA, T6 (0.125% EMS + 0.125 mM MeSA)

demonstrated that EMS can induce leaf shape, fruit size reduction, and maximum disease resistance in tomatoes. Recently, this mutagen has achieved satisfactory results in the breeding of Fusarium-head-blight-resistant wheat plant (Chhabra et al. 2021) and smut-resistant sugarcane strains (Dalvi et al. 2021). 0.175% EMS also induced blast-resistant clones in rice varieties (Rakshit 2010). Meanwhile, MeSA was utilized to increase the activity of enzymes involved in plant disease and insect resistance (Zhu and Park 2005; Kalavaini et al. 2021; Gondor et al. 2022). Many studies have shown that MeSA increases tomato plant resistance to cold and pests (Ding et al. 2002; Conboy et al. 2020). However, there is a limited study using EMS and MeSA combinations. In this study, rice exhibited the highest disease resistance when treated with 0.1% EMS + 0.1 mM MeSA. This suggests that the combined effect of EMS and MeSA on rice increased resistance to blast disease.

Effects of EMS and MeSA treatments on chlorophyll a and b

Changes in chlorophyll have been reported due to certain host-pathogen interactions such as wheat - *P. oryzae*,

Pucciniatriticina, and *Blumeria graminis* f. sp. *tritici* (Mandal et al. 2014; Yang and Luo 2021), rice - *Monographella albescens* (Tatagiba et al. 2015), maize - *Stenocarpella macrospora* (Bermúdez-Cardona et al. 2015), and soybean - *Colletotrichum truncatum* (Dias et al. 2018). The results showed that treatment T2 had the highest chlorophyll a and b extracted from disease-resistant rice plants (8.52) and was significantly different and higher than treatment T0 (5.94) (Table 1). On the other hand, the content of chlorophyll a, extracted from infected plants, was significantly higher than that of treatment T0. Treatment T2 had the highest concentration of chlorophyll a and b extracted from infected plants (5.34). In addition, the results recorded in Table 1 also showed that the chlorophyll a and b extracted from the resistant rice plants was always higher than the chlorophyll a and b extracted from the infected plants. In particular, the content of chlorophyll a, extracted from disease-resistant and infected rice plants of EMS + MeSA treatments, was always higher than that from T0. Giri and Apparao (2011) found different chlorophyll mutants in pigeon peas (*Cajanus cajan* L.) after EMS treatments: chlorina, xantha, albina, and striata. Nair and Gayathri (2022) also observed an increase

Table 1. Effects of EMS and MeSA-induced application on chlorophyll

Treatments	Chlorophyll a (µg/mL)		Chlorophyll b (µg/mL)		Chlorophyll a, b (µg/mL)	
	Resistance	Susceptible	Resistance	Susceptible	Resistance	Susceptible
T0	2.99 ± 0.08 ^{cd}	1.03 ± 0.06 ^c	2.94 ± 0.25 ^c	1.59 ± 0.13 ^c	5.94 ± 0.23 ^d	2.89 ± 0.09 ^c
T1	3.93 ± 0.06 ^b	2.89 ± 0.80 ^a	2.69 ± 0.03 ^c	2.31 ± 0.40 ^b	6.62 ± 0.04 ^c	5.20 ± 0.39 ^a
T2	4.76 ± 0.72 ^a	2.27 ± 0.05 ^b	3.52 ± 0.57 ^b	3.07 ± 0.20 ^a	8.52 ± 0.26 ^a	5.34 ± 0.27 ^a
T3	3.67 ± 0.03 ^b	2.04 ± 0.04 ^b	3.93 ± 0.04 ^b	2.09 ± 0.02 ^{bc}	7.60 ± 0.08 ^b	4.11 ± 0.04 ^b
T4	1.99 ± 0.10 ^e	1.27 ± 0.02 ^c	2.97 ± 0.18 ^c	1.63 ± 0.21 ^c	4.99 ± 0.32 ^e	2.89 ± 0.24 ^c
T5	2.75 ± 0.08 ^d	1.83 ± 0.08 ^{bc}	3.71 ± 0.16 ^b	2.43 ± 0.19 ^b	6.49 ± 0.25 ^c	4.23 ± 0.28 ^b
T6	3.45 ± 0.03 ^{bc}	2.02 ± 0.07 ^b	4.41 ± 0.16 ^a	2.43 ± 0.74 ^b	7.85 ± 0.19 ^b	4.47 ± 0.64 ^b

Mean ± Standard deviation. Values in columns with similar letters are not significantly different ($p < 0.05$).

Table 2. Effects of EMS and MeSA-induced application on proline content trait of rice variety, IR50404 after screening with blast fungus

Treatments	Proline content ($\mu\text{g}/\text{mL}$)	
	Resistance	Susceptible
T0 (no induced)	2.05 ± 0.03^e	1.61 ± 0.01^f
T1 (0.1% EMS)	2.59 ± 0.02^d	2.41 ± 0.02^b
T2 (0.125% EMS)	3.91 ± 0.13^a	2.26 ± 0.03^c
T3 (0.1 mM MeSA)	2.85 ± 0.02^b	2.13 ± 0.01^d
T4 (0.125 mM MeSA)	2.56 ± 0.03^d	2.09 ± 0.02^e
T5 (0.1% EMS + 0.1 mM MeSA)	2.70 ± 0.01^c	2.14 ± 0.01^d
T6 (0.125% EMS + 0.125 mM MeSA)	2.82 ± 0.01^b	2.50 ± 0.02^a

Mean \pm Standard deviation. Values in columns with similar letters are not significantly different ($p < 0.05$).

in the frequency of chlorophyll mutants with 0.51% of EMS treatment in cowpeas. Khan and Tyagi (2010) identified four chlorophyll mutants, albina, xantha, chlorina, and viridis, in soybeans treated with gamma rays and EMS. Gamma rays and ethyl methanesulfonate were also used by Kumar et al. (2003) on Lima bean (*Phaseolus lunatus* L.) seedlings (EMS).

Effects of EMS and MeSA treatments on proline

Table 2 shows that a significant difference ($p < 0.05$) was observed in the proline content in rice leaves among the control and T₁, T₂, T₃, T₄, T₅, and T₆ after blast fungus inoculation between resistant and susceptible plants. The proline content of extracts from disease-resistant rice plants for treatment T₂ reached the highest level (3.91 $\mu\text{g}/\text{mL}$) and the lowest was for T₀ (2.05 $\mu\text{g}/\text{mL}$). The proline content of extracts from blast-infected rice leaves of treatments T₁; T₂; T₃; T₄; T₅, and T₆ was significantly higher than that of the control, T₀. Of these, treatment T₆ (0.125% EMS + 0.125 mM MeSA) had the highest proline content of 2.5 ($\mu\text{g}/\text{mL}$) and treatment T₀ had the lowest proline content of 1.61 ($\mu\text{g}/\text{mL}$). Viruses, bacteria, and pathogens were found in tissues infected with proline (Radwan et al. 2007). According to the present findings using EMS + MeSA improved rice blast resistance by increasing the content and activity of proline, which improves plant disease resistance.

Effects of EMS and MeSA treatments on inhibiting protease activity

Results for the ability of extraction from rice leaves to inhibit proteases are presented in Table 3. The protease inhibition capacities of rice blast-resistant T₁, T₂, T₃, T₄, T₅, and T₆ were significantly different and higher than that of the control, T₀. The protease inhibition capacity of T₅ (0.1% EMS + 0.1 mM MeSA) was the highest (88.9%) and treatment T₆ was the lowest (33.3%). Results for the ability to inhibit protease of infected rice plants of T₅ (0.1% EMS + 0.1 mM MeSA) and T₆ (0.125% EMS + 0.125 mM MeSA) were the highest (30.4%)

Table 3. Effects of EMS and MeSA-induced application on protease inhibition of IR50404 rice after screening with blast fungus

Treatments	Enzyme protease inhibition (%)	
	Resistance	Susceptible
T0 (no induced)	80.25 ± 0.27^b	26.75 ± 0.31^b
T1 (0.1% EMS)	75.58 ± 0.31^c	4.56 ± 0.32^e
T2 (0.125% EMS)	49.51 ± 0.55^f	22.92 ± 0.48^c
T3 (0.1 mM MeSA)	61.38 ± 0.63^d	14.05 ± 0.18^d
T4 (0.125 mM MeSA)	57.65 ± 0.79^e	26.13 ± 0.24^b
T5 (0.1% EMS + 0.1 mM MeSA)	88.90 ± 2.25^a	30.43 ± 0.78^a
T6 (0.125% EMS + 0.125 mM MeSA)	33.28 ± 0.31^g	30.43 ± 0.24^a

and treatment T₁ was the lowest (4.6%). Protease mutation was also one of the important criteria to determine the effectiveness of the mutagens. MeSA was used to help resist cold and insects (Ninkovic et al. 2021) and has been used in tomato plants (Ding et al. 2002). Several beneficial mutations of EMS have been identified for various traits such as drought tolerance, salinity, pesticides, and bacterial blight resistance (Mohapatra et al. 2014). Induced treatments combining EMS and MeSA also influenced the suppression of protease activity in host plants when compared to the control treatment. This shows that 0.1% of EMS + 0.1 mM of MeSA treatments increased the protease inhibitors in the leaf tissue, which helps the plants resist the disease.

The present study found that rice seeds treated with 0.1% EMS + 0.1 mM MeSA produced blast disease resistance. The 0.1% EMS treatment also improves biochemical processes like chlorophyll content and proline reaction. The findings will strengthen the methodology for selecting rice varieties using chemical mutation to improve the disease resistance and facilitate breeding of new varieties. The development of blast fungus-resistant rice mutant lines allows effective control while reducing the use of chemicals in production, supporting current trends toward safe sustainable, and environmentally friendly agriculture.

Author's contribution

Conceptualization of research (PTTH, RJH); Designing of the experiments (PTTH, RJH); Contribution of experimental materials (TMT); Execution of field/lab experiments and data collection (TMT); Analysis of data and interpretation (TKT); Preparation of the manuscript (PTTH, RJH, TKT, TMT).

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