



THE EFFICACY OF HOT WATER TREATMENTS AGAINST *FUSARIUM FUJIKUROI*: THE FUNGAL AGENT OF BAKANAE DISEASE

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Abstract: Rice is one of the most grown agricultural products in the world. It is the most preferred food item in the Turkish diet. One of the most important fungal diseases of rice is Bakanae disease. It is a seed-borne and complex disease caused by the *Fusarium* species. *Fusarium fujikuroi* is the most virulent and widespread species. The excessive use of fungicides has raised concerns such as a decrease in the fungicide sensitivity of *F. fujikuroi* in the world. For this reason, alternative methods are being investigated to control the disease. In this study, the therapeutic effect of hot water treatment on contaminated seeds was investigated. Trials were carried out under *in vitro* and *in vivo* conditions, to determine the effects of hot water treatment on the germination rate of rice seeds. Hot water treatments at 55 °C and 57 °C were the most effective treatments against pathogen. However, pathogen was not inhibited at 50 °C. The lowest disease severity was determined at 57 °C (2.5%) and this was followed by hot water treatments at 55 °C (22.33%) and 52 °C (77.30%) *in vivo* tests, respectively. No disease symptoms were observed in the negative control plants. According to evaluations, the disease incidence decreased when treatment temperature was increased, resulting in a slightly reduced germination rate.

Keywords: Rice, Bakanae disease, *Fusarium fujikuroi*, Hot water treatment, Seed germination

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1. Introduction

Rice (*Oryza sativa* L.), whose center of origin is estimated to be India and China in Southeast Asia, is an important cereal of the Poaceae family (Crawford and Shen, 1998; Gnanamanickam, 2009). It is one of the oldest cultivated plants that can germinate in water and its roots can utilize oxygen in the water. It has an important role in human nutrition with its protein, carbohydrate, minerals, and vitamins in the world (Çevik, 2011). Rice is one of the most grown agricultural products in the world with a cultivation area of 167.132.623 ha and a production amount of 782.000.147 tons. Countries such as China, India, and Vietnam supply a significant part of the rice produced in the world (FAO, 2020). It is cultivated on 130.042.4 ha and total rice production is 940.000 tons in Türkiye. The rice is preferred to be cultivated by Turkish farmers due to its high yield (8218.8 kg ha⁻¹), thus the rice growing area in Türkiye increases (TUIK, 2020).

Rice is one of the most preferred staple food of Turkish society, so it is imported at increasing rates since our domestic rice production is not adequate. In most regions in Türkiye, rice is cultivated in fields where other crops cannot grow and therefore alternative farming cannot be done. The crop pattern of rice is not changed and disease

agents continue their vitality in the soil. Rice cultivation is carried out in the same field for at least five years in a row, even 20-25 years. Therefore, disease, pests, and weeds increase over time and these biotic factors cause significant yield losses in rice production (Sade et al., 2011). Plant residues and soil may also affect the spreading of pathogens. Previous studies indicated that the agents of disease can survive for months in rice seed and also plant residues and soil (Mishra et al., 1989; Dodan et al., 1994; Sunder and Satyavir, 1997; Manandhar, 1999).

One of the most important biotic factors affecting rice yield is fungal disease. It has been reported that Bakanae disease causes yield losses of up to 70% in epidemic areas (Hajra et al., 1994; Singh et al., 1996; Batsa and Manandhar, 1997). The disease has been identified as the most destructive disease of rice, causing significant economic yield and quality losses in most of the countries where rice production has been done in the past 20 years (Khan et al., 2000; Carter et al., 2008; Zainudin et al., 2008; Karov et al., 2009; Rabbi and Ali, 2011; Jeon et al., 2013; Gupta et al., 2014; Kim et al., 2015; Chen et al., 2016).

Bakanae disease is caused by *Fusarium* species within the



Gibberella fujikuroi species complex. Studies have reported that *F. fujikuroi* Nirenberg, *F. proliferatum* (Matsushima) Nirenberg, *F. sacchari* Buttler, *F. subglutinans* (Reinking) Wollenworth, *F. verticillioides* (Sacc.) Nirenberg and *F. andiyazi* Marasas are the most virulent species (Amoah et al., 1995; Desjardins et al., 2000; Zainudin et al., 2008; Amatulli et al., 2010, Pra et al., 2010; Choi et al., 2018; Egerci et al., 2020a, Egerci et al., 2020b). The previous studies reported that *F. fujikuroi* is the most virulent and widespread species among these species complex and that gibberellin produced *F. fujikuroi* increases disease severity (Desjardins et al., 2000; Malonek et al., 2005; Zainudin et al., 2008; Amatulli et al., 2010; Ora et al., 2011; Bashyal et al., 2016, Egerci et al., 2020a).

The disease is a seed-borne pathogen and it develops systemically within the rice plant. The symptoms of the disease may emerge within about a month after sowing (Webster, 2004). The seed either does not germinate at all or then most of the seeds turn brown and rot with their sprout. The most common symptoms are thin, yellowish-green and elongated seedlings, chlorotic leaves, root, and crown rot. In addition, early ripening can cause symptoms such as empty husk (glumes) and white ear formation (Coşçu and Karaca, 1983; Desjardins et al., 2000; Webster, 2004; Zainudin et al., 2008, Egerci et al., 2020a). All of these symptoms are described as Bakanae syndrome.

Bakanae disease caused by *F. fujikuroi* is a widespread disease that causes yield and product losses particularly in Balıkesir and Çanakkale provinces of Türkiye (Egerci et al., 2020a). The most of the rice farmers in Türkiye separate some of their product grown as the seed which they use in the next production year, the disease continues to increase year by year causing serious yield losses because the disease is seed-borne. Moreover, *F. fujikuroi* has a high degree of genetic variation and the risk of creating an epidemic has increased with the spread of the disease in recent years. Seed treatments with chemical fungicides are the most common control practice for this disease in the world. However, the excessive use of fungicides has raised concerns such as a decrease in the fungicide sensitivity of pathogens. Previous studies have reported that the increasing occurrence of resistant strains of *F. fujikuroi* to fungicides is posing a new risk. For this reason, alternative methods of control are being investigated in the world to manage the disease (Kim et al., 2010; Matic et al., 2017).

To prevent this disease by producing permanent solutions has become important. For these purposes, the therapeutic effect of different temperatures of hot water on infected seeds was investigated in this study. To evaluate the effect of hot water treatment on the disease, *in vitro* and *in vivo* experiments were carried out to determine the effects of these treatments on the germination rate of rice seeds.

The objectives of the the study were to (i) investigate the therapeutic effect of different temperatures of hot water

on infected seeds and (ii) determine the effects of these treatments on the germination rate of rice seeds.

2. Materials and Methods

2.1. Preparation of the Inoculum

F. fujikuroi isolate which had been identified previously with morphological and molecular methods (Acc. no: MN091843) and determined to have high virulence and is widely common in the region was used in the study (Egerci et al., 2020a). The PCR amplification of the TEF-1 α gene region was carried out by using EF1/EF2 primers described by O'Donnell et al. (1998). The fungal isolate was incubated on PDA medium for 7-10 days in the incubator at 25 \pm 2 $^{\circ}$ C. The isolate of *F. fujikuroi* grown on PDA was flooded by adding 5 ml of sterile distilled water containing 1-2 drops of Tween 80 per liter. The resulting suspension was sifted through a double layer of cheesecloth to remove mycelium, spore count was performed under the microscope (Olympus, Stream 2.1) and a spore suspension with a density of 1x10⁶ spores/ml was prepared (Egerci et al., 2020b).

2.2. Hot water Treatment of Seeds

The seeds of the Baldo rice variety, which is known to be susceptible to the disease, were sterilized with 1% sodium hypochlorite for 45 seconds. The seeds were soaked in sterilized distilled water for 24 hours and then in spore suspension of 1x10⁶ spore/ml density for 48 hours. Inoculated seeds were kept in hot water at 50 $^{\circ}$ C, 52 $^{\circ}$ C, 55 $^{\circ}$ C and 57 $^{\circ}$ C for 15 minutes in the hot water bath and allowed to dry on filter paper. The seeds used for negative (healthy) control were kept in sterile distilled water for 72 hours. Isolation of pathogen from treated and nontreated seeds was carried out under *in vitro* conditions with 5 replications and by placing 5 seeds per PDA plates. The plates were allowed to incubate at 24 \pm 2 $^{\circ}$ C for 7 days in the incubator under 12 hours light / dark conditions.

Pot tests were also conducted under *in vivo* conditions to determine the effect of hot water treatment of infected seeds. The experiments were carried out in plastic pots (22x14x9 cm) containing sterilized soil which was autoclaved twice; plastic pots were kept in a growth chamber at 24 $^{\circ}$ C and 80% relative humidity, with 12 hours of light. The experiment was laid out in randomized design with 5 replications. Ten seeds were planted in each 5 plots. Irrigation was performed 3 times a day and 3 g ammonium sulphate was given to each tray at the 15th-day interval. After sowing the seeds, the disease evaluation was made on the 30th day by using the Ooi (2002) disease scale (Table 1). The plants' tissues were used for the fungal re-isolation as to Koch's postulates, after scoring. Statistical analyses were performed using the SPSS 16 (IBM Corp., Armonk) software package. Data were scored and analyzed by analysis of variance (ANOVA) implemented in SPSS to determine the significant variation within and among the treatments. Data means were treated using the least significant difference test at P=0.05 level.

2.3. Seed Germination Test

Germination rates of rice seeds treated to hot water were determined under *in vitro* and *in vivo* conditions. *In vitro* tests were performed using the Blotter method. According to the ISTA (International Rules for Seed Testing) rules, a filter paper moistened with sterile distilled water was placed in each petri dish and 100 seeds were placed on it, and the rice seeds were covered with another moistened filter paper. The tests were carried out with 4 replications. The germination rates were calculated at the end of the 7th day by leaving the seeds to germinate in the incubator at 24 ± 2 °C and 12 hours of lighting.

In vivo tests were arranged in randomized design and carried out in a growth chamber at 24-26 °C and 80%

relative humidity, 12 hours of lighting conditions. Experiments of the *in vivo* tests were carried out in plastic pots containing sterilized soil. The disease evaluation was carried out when the plants were 2 mm tall on the soil surface. After the seed germination rates (%) were determined, statistical analyses were performed using the SPSS 16 (IBM Corp., Armonk) software package (equation 1). Data were analyzed by analysis of variance (ANOVA) implemented in SPSS to determine the significant variation within and among the treatments. Data means were treated using the least significant difference test at P =0.05 level.

$$\text{Germination rate} = \frac{(\text{number of germinated} \times 100)}{\text{total number of planted seeds}} \quad (1)$$

Table 1. Symptoms of rice plants were scored basing on disease scale 0–4

Disease scale	Disease symptoms
0	Healthy and uninfected plants (no external symptoms)
1	Normal growth but leaves beginning to show yellowish–green
2	Abnormal growth, elongated, thin and yellowish-green leaves; seedlings also shorter or taller than normal
3	Abnormal growth, elongated; chlorotic, thin and brownish leaves; seedlings also shorter or taller than normal
4	Seedlings with fungal mass on the surface of infected plants or died

3. Results

3.1. Hot Water Treatment of Seeds

Isolation of pathogen from treated and nontreated seeds were transferred to PDA plates and incubated at 24 ± 2 °C for 7 days in incubator under 12 hours of light/dark conditions. The results from seeds treated with hot water are summarized in Table 2. The highest disease severity rate of 100% was observed on positive control and the treatments at 50 °C and 52 °C. This was followed by the treatments at 55 °C (36%) and 57 °C (8%). There was no mycelial development in the negative control seeds (healthy). Statistical analysis showed that there was a difference between treatments (Table 2).

Table 2. The disease severity on seeds treated with different hot water temperatures

Hot water treatments (°C)	Disease severity (%)
50	100.0 a
52	100.0 a
55	36.00 b
57	8.00 c
Positive Control	100.0 a

*There is no statistical difference between the values indicated by the same letter (P=0.05).

Pot trials were carried out under *in vivo* conditions to evaluate the effect of hot water treatments on disease development and evaluation of which were made on the 30th day according to the Ooi (2002) disease scale. The disease severity (%) obtained in pot tests from seeds

treated with hot water is given in Table 3.

According to the results of *in vivo* tests, the highest disease severity was determined in the treatment at 50 °C (85.45%). This was followed by hot water treatments at 52 °C (77.30%), 55 °C (22.33%) and 57 °C (2.5%), respectively (Table 3). The disease symptoms were observed within 30 days of rice seed sowing and accordingly, typical symptoms of Bakanae diseases were observed on seedlings. *F. fujikuroi* caused pale green to yellowing of foliage, chlorotic leaves, and necrotic roots on plants. No disease symptoms were observed in the negative control plants. The statistical difference was found between the treatments (Figure 1).

Table 3. The disease severity (%) on seeds treated with hot water *in vivo* tests

Hot water treatments (°C)	Disease severity (%)
50	85.45 a
52	77.30 b
55	22.33 c
57	2.50 d
Positive Control	85.50 a

*There is no statistical difference between the values indicated by the same letter (P= 0.05).

3.2. Seed Germination Test

The germination rates of rice seeds subjected to hot water treatment were determined by the Blotter method and pot tests under *in vivo* conditions. In the Blotter method, germination rates were calculated at the end of

the 7th day. In pot tests, the evaluation was carried out, when the plants on the soil surface were 2 mm tall.

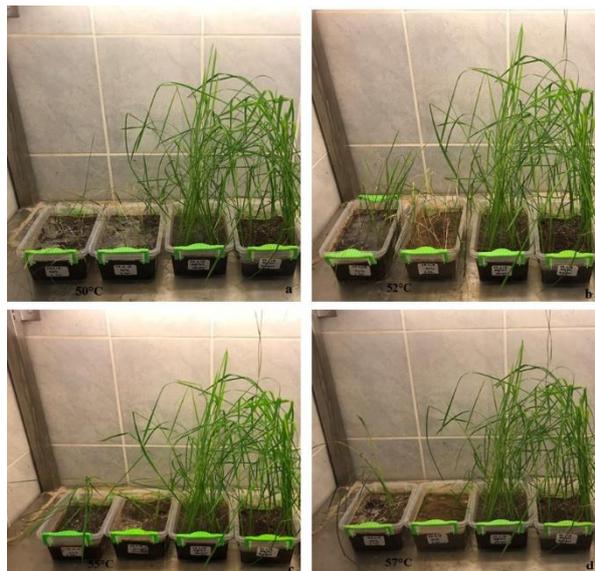


Figure 1. The efficacy of hot water treatments on infected seeds and compared control (healthy) plants a) Comparison of hot water application at 50 °C with control (healthy) plants, b) Comparison of hot water application at 52 °C with control (healthy) plants, c) Comparison of hot water application at 55 °C with control (healthy) plants, d) Comparison of hot water application at 57 °C with control (healthy) plants

In Table 4, the seed germination rates (%) obtained in the Blotter method and pot trials are given. As a result of the Blotter method, the highest seed germination rate of 86.25% was observed in the hot water treatments at 50 °C, followed by 52 °C (75%), 55 °C (40%) and 57 °C (9%),

respectively (Table 4). There was a statistical difference between the treatments. In pot trial tests, the highest germination rate was observed in hot water treatments at 50 °C (74%). This was followed by 52 °C (64%), 55 °C (36%) and 57 °C (8%), respectively. There was no statistical difference between the 50 °C and 52 °C treatments. In the blotter and pot trial tests, seed germination percentages decreased as the temperature increased.

4. Discussion

Rice is one of the most important agricultural products with rich nutrient contents in the world. Due to the high amount of average yield of rice production in Türkiye, rice producers have increasingly preferred to cultivate rice in recent years, thus an increase in rice cultivation areas. It has been reported in the world that Bakanae disease causes significant yield and product losses in areas where the epidemic occurs (Singh et al., 1996; Batsa and Manandhar, 1997). In Türkiye, the situation of this disease is very serious. Eğerci et al. (2020a) reported that *F. fujikuroi* is widespread in Balıkesir and Çanakkale provinces of Türkiye. The low-level usage of certified seed in rice production, separation of part of the harvested product as seeds for subsequent production, and use of rice crops from abroad as seeds play an important role in the spread of this disease in Türkiye. The management of Bakanae disease is difficult due to the genetic mutation potential of *Fusarium* spp. and also an epidemic character of the disease (Serefica and Cruz, 2009). For this reason, alternative methods (hot water applications, resistant varieties, etc.) are being investigated in the world to control the disease.

Table 4. The seed germination rates (%) in the blotter method and pot trials

Hot water treatments (°C)	The seed germination rates (%)	
	Blotter Method	Pot Trials
50	86.25 b	74.00 c
52	75.00 c	64.00 c
55	40.00 d	36.00 d
57	9.00 e	8.00 e
Negative Control	95.75 a	100.0 a
Positive Control (Contaminated)	85.75 b	98.00 b

*There is no statistical difference between the values indicated by the same letter (P = 0.05).

In this study, the therapeutic effect of hot water treatments which is an alternative control method was investigated in the 'Baldo' rice variety, which is known to be sensitive to Bakanae disease. According to the studies conducted under *in vitro* and *in vivo* conditions were evaluated, it was determined that the most ineffective hot water treatments at 50 °C and 52 °C. Disease severity rates were determined at almost the same rate as the positive control. The disease severity values are significantly decreased in the treatments of hot water at 55 °C and 57 °C, on the other hand the seed germination

with the lowest rates was determined. Miyasaka et al. (2000) reported that the disease was decreased in seeds kept for 15 minutes in hot water application at 60-62 °C, but seed germination decreased, too. Fukuda et al. (2013), determined the effect of hot water treatment on the germination of rice seeds by treating seeds of the "Bekoaoaba" variety for 10 minutes at 50-60 °C. They found that the germination rate decreased significantly, but the disease was prevented. In a different study, the seeds showing the signs of rice root rot disease were treated for 15 minutes with hot water at 57 °C, and it was

determined that seed infection was significantly prevented. No negative effect on seed germination was reported (Wolf, 2002).

In previous studies, it was observed that both disease outbreak rate and seed germination rates decreased when temperatures increased. Rocha (1984) determined the effect of hot water treatments on the quality of rice seeds belonging to different varieties in this study. The treatments done at 52 °C for 15 and 30 minutes and at 57 °C for 15 minutes did not have any negative effects on the seed quality. In the application performed at 57 °C for 30 minutes, it was determined that the quality of the seeds was diminished. Likewise, Permana et al. (2017), reported that among Japanese varieties 'Koshihikari' rice variety was the most resistant to hot water treatments and that even at 70 °C. The seed germination was observed in this rice variety at a rate of 76%, whereas hot water treatment reduced the germination rate in other varieties. They suggested that it may be due to the difference in sensitivity to temperature and tolerance levels among varieties.

In conclusion, the effectiveness of different temperatures of hot water against Bakanae disease in rice seeds was investigated in this study. Tests were carried out in order to evaluate the effect of hot water treatment on the disease, and also to determine the effects of these applications on the germination rate of treated rice seeds. It was determined that hot water application significantly reduced the disease rate, but the temperature negatively affected seed germination. The results obtained from hot water applications are light for future studies.

Author Contributions

Y.E. (50 %) and P.K.T. (50 %) conceived and designed the study. Y.E. (100 %) performed the trials. Y.E. (50 %) and A.U.M. (50 %) analyzed the data and wrote the manuscript. All authors reviewed and approved final version of the manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

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