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# Analysis of Drought Stress Effect on Inpari Germination: Survival Method

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## ABSTRACT

Drought stress using mannitol can inhibit the germination of rice variety seeds. These studies typically produce time-to-event data and censored observation. Survival analysis techniques are valuable for accounting for these non-germination events, as they describe how germination probability changes over time based on the likelihood of seed development. Until now, there have not been survival studies regarding rice germination affected by drought stress in Indonesia. Thus, we investigated the germination probability of three rice varieties (Inpari 19, Inpari 32, and Inpari 49) under drought stress using survival analysis. The seeds were germinated in 0%, 2%, 4%, 6%, and 8% concentrations of mannitol and evaluated daily over 14 days. Our results demonstrated that higher mannitol concentrations significantly decreased the germination percentage and delayed germination time. The survival rates varied significantly between different mannitol concentrations, highlighting the adverse effects of drought stress. However, there was no significant difference in the probability of seed germination among the varieties treated with 2% mannitol. Among the varieties studied, Inpari 19 is more likely to be drought-resistant compared to Inpari 32 and Inpari 49. It is based on the highest germination percentage, shortest germination time, and highest probability of germination compared.

## 1. Introduction

Complex interactions between physiological and environmental factors influence seed germination. Environmental conditions such as light, salinity, seed burial depth, pH, water stress, and temperature play substantial roles in initiating and inhibiting seed germination (Humphries *et al.* 2018; Javaid *et al.* 2022). Water, as the main abiotic factor, can limit seed germination and early seedling growth. Germination is the most vulnerable stage in the life cycle of plants because of high seed mortality (Liu *et al.* 2023). The viable seeds that do not experience the set of environmental conditions suitable for germination may lose their ability to germinate (Silveira *et al.* 2014). Seed germination begins with water absorption (imbibition) and terminates with radicle emergence (Liu *et al.* 2019). Drought stemming from water stress may negatively impact plant regeneration, growth, and survival because seeds reduce the osmotic potential that limits their metabolism (Bhatt *et al.* 2022).

Rice is sensitive to drought conditions during germination and the early phases of seedling growth. The rice variety Inpari is a new high-yielding variety suitable for planting in irrigation fields (Thamrin *et al.* 2023). This variety is widely cultivated in Indonesia, so varieties that are adaptive to global environmental issues are needed. The screening of drought-tolerant rice varieties is important for global environmental issues, including climate change resilience, food security, and sustainable agriculture. Screening drought stress resistance in the germination phase can provide earlier information on drought-tolerant

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rice varieties. Several studies on plant responses to drought stress use osmotic solutions regarding seed germination have been reported, including studies of rice (*Oryza sativa*). Islam *et al.* (2018), Liu *et al.* (2019), and Purbajanti *et al.* (2019) use PEG-6000 solution, while Dash & Swain (2015), Shobbar *et al.* (2012) and Veronica *et al.* (2022) applied mannitol solution as drought stress treatment.

Germination study is usually concerned with time-to-event assessment. This study generates a binary (yes/no germination) response variable, where data is collected as cumulative counts over time and consists of modeling the time to response for each seed in the sample (Romano and Stevanato 2020). Many studies of time-to-event methods of seed germination only apply the model of the cumulative seed germination curve empirically (Pêgo *et al.* 2012; Moura *et al.* 2019; Suriyasak *et al.* 2020), which is used to interpret pattern and performance germination over time. On the other hand, germination studies naturally produce time-to-event data that contain interval censoring (Onofri *et al.* 2018), which may have limitations, especially when seeds do not germinate until the end of an experiment. The germination time of ungerminated seeds is known as right censored data.

The germination curve often shows a complex pattern that presents difficulties in interpreting and analyzing results statistically (Talská *et al.* 2020). This sigmoid curve does not consider seed ungerminated until the end of an experiment, which is called censored observations. Therefore, this conventional method is not optimal to deal with the censored data problem (Onofri *et al.* 2010). The survival analysis can examine changes over time to specified events (germination times) and accommodate censored response time data (Etikan *et al.* 2018). The probability of seed germinating after a specific time that is relevant to survival probability can be estimated nonparametrically using the Kaplan-Meier method. In addition to estimating survival probability, it is frequently used to compare germination time to different treatments that are applied.

Until now, there has been no time-event assessment on rice germination in Indonesia with considering viable seeds that have not germinated at the end of experiment (censored observed). The study of rice germination in Indonesia only focused on the percentage of germinated seeds in each randomization unit (petri dish) at each assessment time. No report on survival studies regarding rice germination affected by drought stress in Indonesia is available. Therefore, this study aims to investigate the germination probability of rice variety Inpari seeds as affected by various drought stress using survival analysis.

## 2. Materials and Methods

### 2.1. Seed Samples

Seed samples were obtained from the Indonesian Center for Rice Research. Seeds of three Inpari varieties used in this study were Inpari 19, Inpari 32, and Inpari 49, all from the stock seed class. The samples used were not empty seeds and were equally sized. Details of the Inpari variety seed samples in this experiment are in Table 1.

### 2.2. Drought Stress Treatment

Drought stress treatment was given through the addition of mannitol. All three varieties of Inpari were germinated in a petri dish (150 mm) coated with 3 sheets of filter paper and moistened with 10 ml of 2%, 4%, 6%, and 8% of mannitol solution, and aquades (distilled water) (0% of mannitol) as a control. Different concentrations of mannitol were used to stimulate osmotic potential (MPa) calculated by Van't Hoff's formula through an interpolation approach from the study (Cabral *et al.* 2014). The osmotic potential of mannitol concentrations 0%, 2%, 4%, 6%, and 8% were 0 MPa, -0.03 MPa, -0.05 MPa, -0.08 MPa, and -0.11 MPa, respectively. Mannitol solution (2 ml) was added daily to maintain the humidity of the germination medium. The experiment was organized following a completely randomized design with four replicates. Each petri dish replicate consisted of 25 seeds, making a total of 100 seeds per treatment. The dishes were placed in a seed germinator at a temperature of 25°C and a relative

Table 1. Phenotype description of Inpari seed samples (IAARD 2022)

| Variety | Planting recommendations | Grain form (l/w) | Grain color | Planting age (days) |
|---|---|---|---|---|
| Inpari 19 | irrigated and rainfed rice fields at an of 0-600 MSAL | > 3.0 | yellow | 104 |
| Inpari 32 | lowland rice fields up to an altitude of 600 MASL | 2.1-3.0 | clear yellow | 120 |
| Inpari 49 | irrigated rice fields at an altitude of 0-600 MSAL | 2.1-3.0 | straw yellow | 112 |

humidity of 95%. The germination period lasted 14 days (ISTA 2018).

### 2.3. Data Collection

The study was carried out in the Seed Technology and Production Laboratory of Jember University, Indonesia (8°9'53.85"S and 113°42'59.3"E). The experiment was conducted to examine the parameters of time to event (germination) in three Inpari varieties under drought stress of mannitol. In this study, a seed was considered to have germinated when the emerging radicle reached a length of 2 mm and appeared healthy (Onwimol *et al.* 2016). The number of germinated seeds was recorded daily over 14 days, with daily counting and removal of germinated seeds from the petri dish (Sousaraei *et al.* 2022). The assessment of the germination rate was calculated using Mean Germination Time (MGT) and Median Germination Time ($t_{50}$). MGT in days, is the average length of the day required for maximum time germination of a seed, with the following formula (Aravind *et al.* 2022):

$$MGT = \frac{\sum_{i=1}^{k} n_i t_i}{\sum_{i=1}^{k} n_i}$$

$n_i$ is the number of seeds germinated at the time $i$, $t_i$ is the time from the start of the experiment to the ith observation, and k is the time of the last germination. The $t_{50}$ is the time to reach 50% of maximum germination, with the following formula (Aravind *et al.* 2022):

$$t_{50} = t_i + \frac{\left(\frac{N+1}{2} - n_i\right)(t_j - t_i)}{n_j - n_i}$$

$N$ is the final number of germinated seeds, $n_i$ and $n_j$ are the total numbers of seeds germinated in adjacent counts at the time $t_i$ and $t_j$ respectively, when $n_i < (N+1)/2 < n_j$. Germination probability was used to describe the time-to-event germination of the Inpari varieties. The number of non-germinated seeds was recorded, coded "1" for Germinated seeds and "0" for ungerminated seeds until the end of the experiment (right-censored observation).

### 2.4. Data Analysis

Germination data (MaxG, MGT, and $t_{50}$) were analyzed with Kruskal Wallis to compare the rank of MaxG, MGT, and $t_{50}$ among treatments followed by the Dunn test. Survival analysis was applied to estimate the rate of failure or hazard of seed not germinating. The Kaplan-Meier S(t) was used to estimate the probability of individual survival up to the t-time (survival function) nonparametrically. The general equation of the survival function used to shape Kaplan-Meier's survival curve (McNair *et al.* 2012):

$$S(t) = \prod_{j=1}^{s}\left(1 - \frac{d_j}{n_j}\right)$$

$d_j$ is the number of seeds that germinate in each interval of time $j$, and $n_j$ is the number of seeds at risk of germination in the same interval. Furthermore, the presence or absence of differences between survival curves was tested with Log Rank. This test determines the significant difference between seed germination survival curves based on variety and mannitol concentration. Then, we evaluated whether survival curves differed statistically among categories with the Cox proportional hazard (PH) model. This model estimates PH for the event not occurring (Messick and Hoagland 2018). The PH assumptions were tested with a goodness-of-fit approach using residual Schoenfeld. If the PH assumption was not met, a stratified Cox PH model was applied. Statistical analysis was performed using R studio software with germination metrics (Aravind *et al.* 2022) and survival (Therneau *et al.* 2024) packages.

## 3. Results

### 3.1. Germination

After 14 days of sowing, 1192 out of 1500 seeds have germinated. There were still ungerminated seeds in the three varieties, which are referred to as censored observation. Thus, Inpari 19, Inpari 32, and Inpari 49 were censored by 5.2%, 35.8%, and 20.6%, respectively.

Figure 1 shows the cumulative germination determined on five different drought stress treatments for each Inpari variety. The seed did not germinate simultaneously, and germination rates varied with each mannitol concentration treatment. The fastest germination process was observed on the aquamarine curve (0% mannitol) and the lowest on the green curve (8% mannitol). The first seeds of Inpari varieties germinated two days after sowing. The median time to germination was three days in Inpari 19, six days in Inpari 32, and four days in Inpari 49.

Out of 100 seeds of Inpari 19, 62% germinated within the first three days under the 6% mannitol treatment (Figure 1A). For Inpari 32, daily germination percentages under the 6% mannitol treatment were low (Figure 1B). The first germination appeared on the 4th day after sowing, with a germination percentage of 4%. Seeds of Inpari 32 failed to germinate in the lowest potential osmotic of mannitol treatment, with a cumulative germination rate of 8% by the end of the observation period. Inpari 49 seeds showed few germinations initially under 8% mannitol treatment, but germination increased over the subsequent days, reaching the highest rate by the end of the 14 days (Figure 1C).

## 3.2. Radicle Emergence Time

MaxG, MGT, and $t_{50}$ for each petri dish were analyzed using Kruskal-Wallis due to non-normality and inhomogeneity of experimental errors in the data. The results indicated a significant effect of mannitol concentration on MaxG, MGT, and $t_{50}$ for each Inpari variety studied (Table 2). According to the Dunn test, there were no significant variations between the 0%, 2%, and 4% mannitol treatments for MaxG. Generally, the selected Inpari varieties had germination rates of less than 30% at 8% mannitol. The highest MaxG percentage was found under control conditions, while the lowest was observed at 8% mannitol. The Inpari 19 seeds showed the highest MaxG in 2%, 6%, and 8% concentrations of mannitol compared to other varieties, but it showed similar results with Inpari 49 under 4% mannitol.

The germination time was significantly delayed by drought treatment for all studied Inpari varieties. The Dunn test detected no significant variations between the 6% and 8% mannitol treatments for MGT and $t_{50}$. Regarding MGT, the highest MGT value showed the average value of the length of time required for maximum germination of the fastest Inpari seed. Under the 8% mannitol, the longest time taken to germinate was by Inpari 32 at 13.57 days. The number of days required to reach 50% germination under the

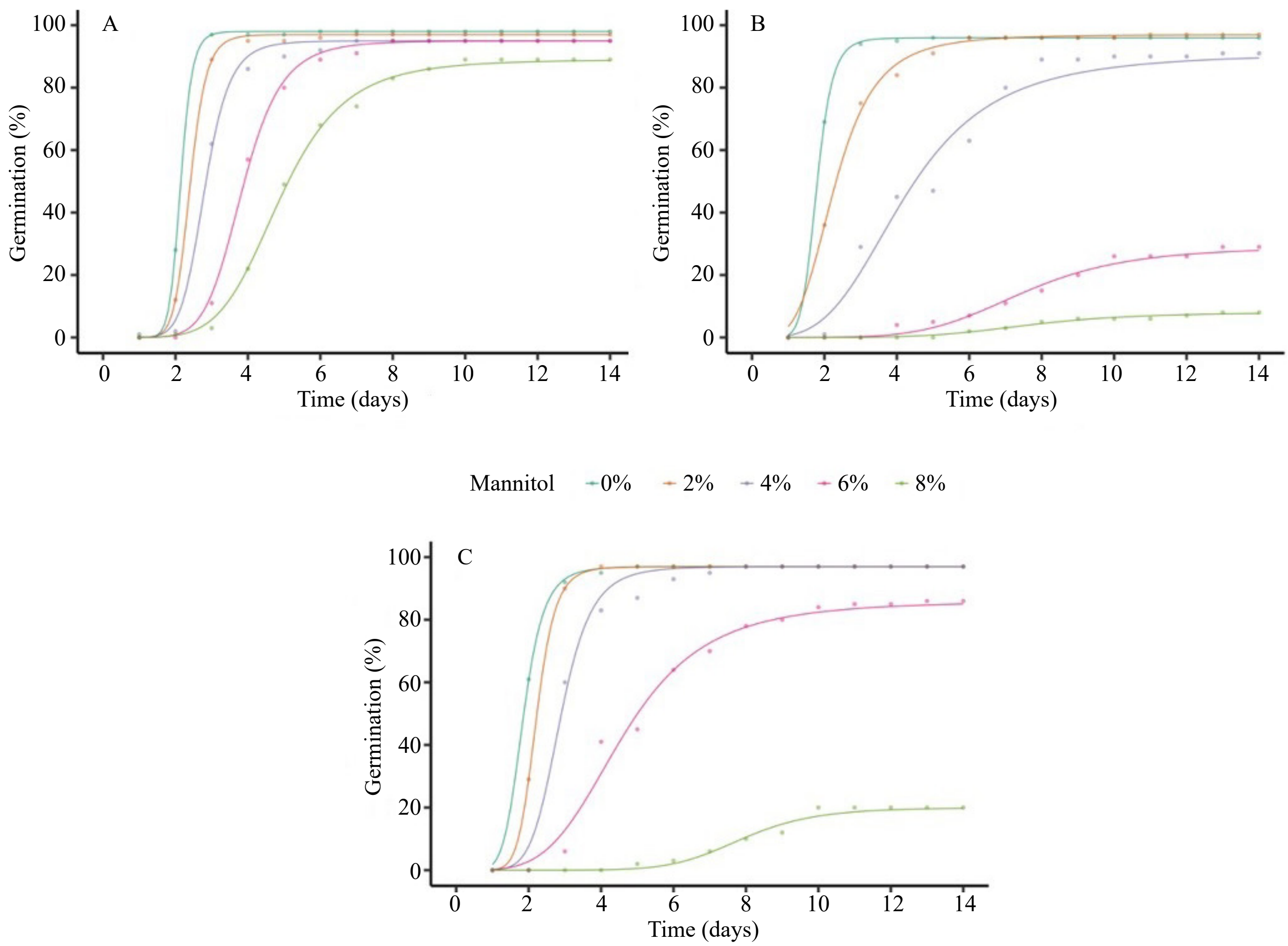


Figure 1. Cumulative germination of 100 seeds for (A) Inpari 19; (B) Inpari 32; (C) Inpari 49 at five different concentrations of mannitol. The dots represent observed cumulative values, and the lines represent fitted curves of each mannitol concentration

Table 2. MaxG, MGT, and $t_{50}$ of Inpari variety seeds under different mannitol concentrations

| Variety | Mannitol (%) | MaxG (%) | MGT (days) | $t_{50}$ (days) |
|---|---|---|---|---|
| Inpari 19 | 0 | 69 (15.25) a | 2.96 (3.25) a | 2.31 (3.00) a |
| | 2 | 77 (16.75) a | 3.34 (7.12) a | 2.51 (6.38) a |
| | 4 | 60 (11.12) ab | 4.03 (9.88) ab | 2.75 (10.12) ab |
| | 6 | 46 (6.88) bc | 5.02 (13.75) bc | 3.89 (14.50) bc |
| | 8 | 27 (2.50) c | 6.58 (18.50) c | 5.26 (18.50) c |
| P-value | | 0.001 | 0.003 | 0.002 |
| Variety | Mannitol (%) | MaxG (%) | MGT (days) | $t_{50}$ (days) |
| Inpari 32 | 0 | 69 (18.50) a | 2.78 (3.25) a | 1.76 (2.50) a |
| | 2 | 39 (12.75) ab | 3.43 (6.00) a | 2.56 (7.00) a |
| | 4 | 28 (12.25) ab | 5.96 (10.25) ab | 4.71 (10) ab |
| | 6 | 6 (5.88) b | 12.31 (15.25) b | 11.57 (16.50) b |
| | 8 | 2 (3.12) b | 13.57 (17.75) b | 11.28 (16.50) b |
| P-value | | 0.003 | 0.002 | 0.002 |
| Variety | Mannitol (%) | MaxG (%) | MGT (days) | $t_{50}$ (days) |
| Inpari 49 | 0 | 61 (14.50) a | 2.79 (3.50) a | 1.87 (2.50) a |
| | 2 | 61 (14.38) a | 3.11 (5.62) a | 2.37 (6.75) a |
| | 4 | 60 (14.12) a | 4.00 (10.38) ab | 2.78 (10.25) ab |
| | 6 | 35 (7.00) ab | 6.76 (14.50) b | 4.92 (14.50) b |
| | 8 | 8 (2.50) b | 12.87 (18.50) b | 11.55 (18.50) b |
| P-value | | 0.002 | 0.002 | 0.001 |

Mean ranks in parentheses with the same letter do not significantly differ according to the dunn test ($p>0.05$). MaxG is maximum germination, MGT is mean germination time, $t_{50}$ is median germination time

most stressful condition was 5.26 days for Inpari 19, 11.28 days for Inpari 32, and 11.58 days for Inpari 49. Increasing mannitol concentration generally reduced the germination rate and delayed germination time.

### 3.3. Survival Analysis

Generally, the seed survival rate under 8% mannitol treatment in all varieties decreases slowly and remains above the other curves, indicating the highest probability of seeds not germinating under this condition. Seeds in the control treatment (0% mannitol) have the highest survival probability compared to other mannitol concentrations. Figure 2A presents that the survival curve of Inpari 19 seed decreases sharply on the 3rd day for 0%, 2%, and 4% mannitol, with the probabilities of germination being 0.97, 0.89, and 0.62, respectively. In contrast, at 6% and 8% mannitol concentrations, the probability of germination on the 3rd day is much lower, at 0.11 and 0.03.

For Inpari 32 seeds, the survival curves for 6% and 8% mannitol treatments are close, though the 8% treatment curve shows longer germination times and a lower probability of germination. The survival probability for 6% and 8% mannitol stabilizes after thirteen days at 0.29 and 0.08, respectively (Figure 2B). For Inpari 49, the probability of germination treated with 6% mannitol on the 13th day is 0.86, while the 8% mannitol treatment does not reach 50% final germination within the study period (Figure 2C).

Kaplan-Meier survival estimates for the Inpari seed germination under different mannitol concentrations are presented in Figure 3. Inpari 32 seeds exhibit slower decreases in survival curves compared to Inpari 19 and Inpari 49 under mannitol treatments. Many Inpari 32 seeds remain ungerminated or survive as seeds for 14 days, showing a high probability of not germinating. Inpari 19, however, has the lowest probability of not germinating among the varieties. The survival curves for the three Inpari varieties given 2% mannitol are closely aligned, suggesting no significant difference in survival times among them under this treatment.

Table 3 presents the log-rank test results of three Inpari varieties and five mannitol concentrations. All p-values <0.05 indicate significant differences in survival curves among the Inpari varieties and mannitol concentrations, except for the 2% mannitol concentrations. The PH assumption test revealed no violations for the 4% mannitol treatment (p-value= 0.4), allowing the use of the Cox PH model for this treatment. Stratified Cox PH models were used for other treatments to identify differences in survival curves. All comparisons within the same germination of the Inpari variety were significant (p-value < 0.05),

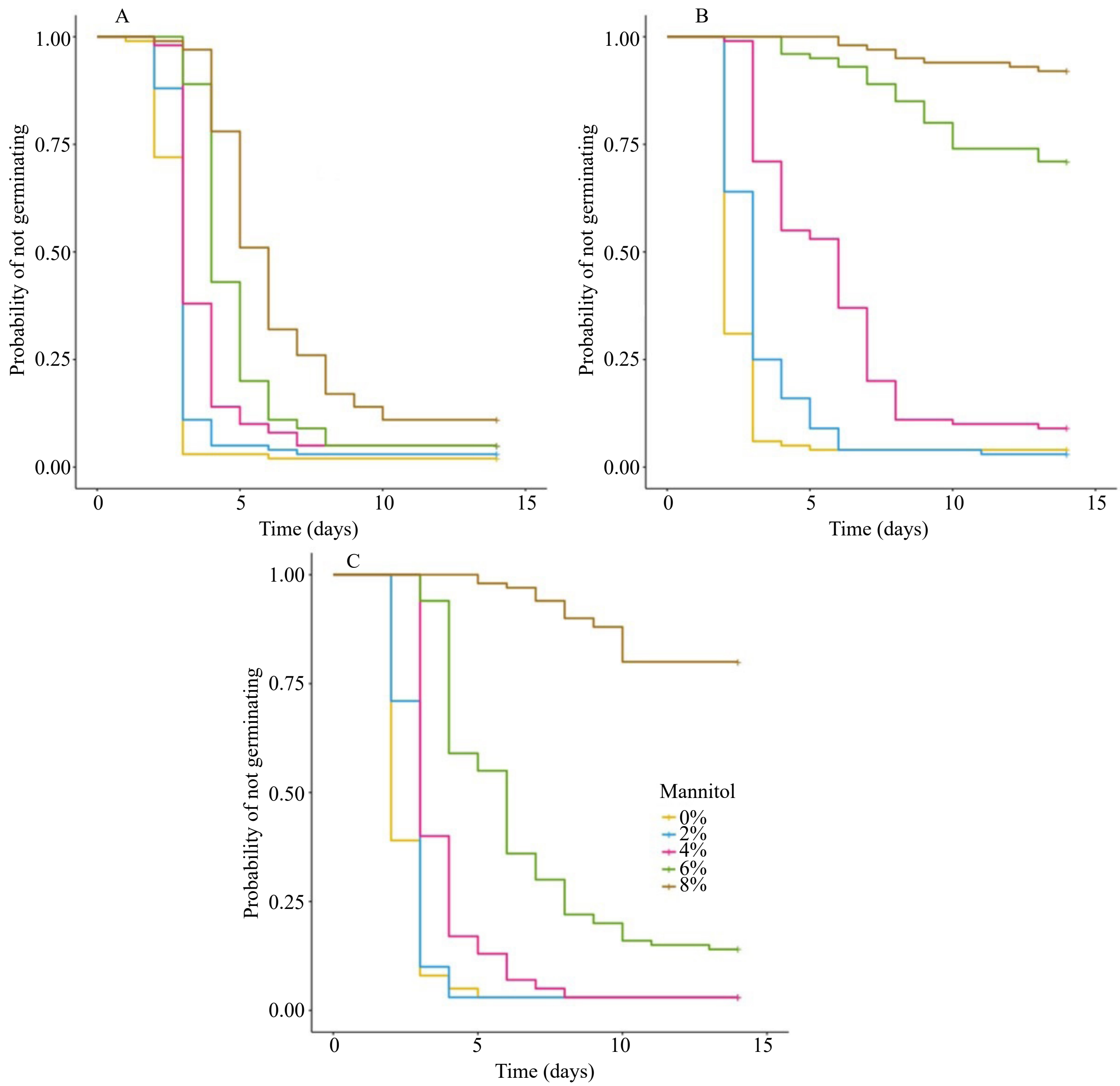


Figure 2. Kaplan Meier estimation curve of germination for (A) Inpari 19, (B) Inpari 32, and (C) Inpari 49 at five different concentrations of mannitol. The final cross represents a censored observation

though some between mannitol concentrations were not significantly different.

Table 4 presents the hazard ratios for different Inpari seed germination survival rates under the same mannitol concentrations. In the case of 0% mannitol, a significant difference exists only between Inpari 19 and Inpari 32 seeds, with Inpari 19 seeds being 1.51 times more likely to germinate. No significant differences are observed between Inpari 19 and Inpari 49 at 0% and 4% mannitol treatments. However, significant differences are observed under 6% and 8 % mannitol treatments. Specifically, Inpari 32 seeds are 6.54 times more likely to germinate than Inpari 49 seeds at 6% mannitol and 2.66 times more likely at 8% mannitol.

## 4. Discussion

Mannitol, an ideal material to stimulate arid soil, has impacts such as excess or lack of water for plants (Maksimovic *et al.* 2020). Drought stress influences the physiological and biochemical processes of plants, especially in the early stages of plant life (Możdżeń *et al.* 2015). Dhakal & Subedi (2020) reported that drought stress induced by mannitol decreased the rate of germination

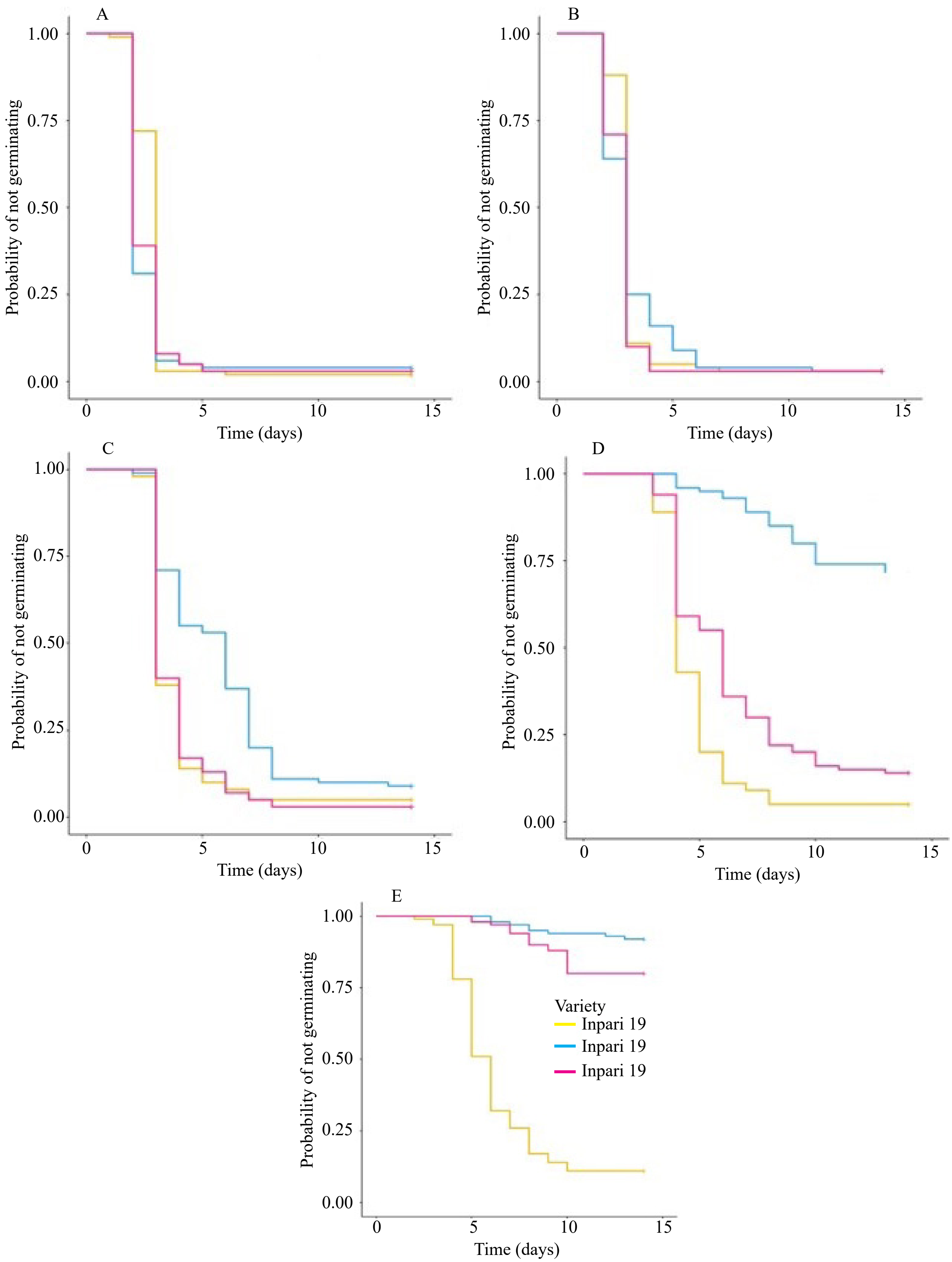


Figure 3. Kaplan Meier estimation curve of germination for (A) 0% mannitol; (B) 2% mannitol; (C) 4% mannitol; (D) 6% mannitol; (E) 8% mannitol at three different varieties of Inpari. The final cross represents a censored observation

Table 3. The result of the log-rank test

| Treatment | Chi-square | p-value |
|---|---|---|
| Variety | | |
| Inpari 19 | 219 | $< 2 \times 10^{-16*}$ |
| Inpari 32 | 502 | $< 2 \times 10^{-16*}$ |
| Inpari 49 | 420 | $< 2 \times 10^{-16*}$ |
| Concentration | | |
| 0% mannitol | 18.10 | $1 \times 10^{-4*}$ |
| 2% mannitol | 3.20 | 0.20 |
| 4% mannitol | 36.60 | $1 \times 10^{-8*}$ |
| 6% mannitol | 163 | $< 2 \times 10^{-16*}$ |
| 8% mannitol | 246 | $< 2 \times 10^{-16*}$ |

*p-value significant by test (α = 0.05)

Table 4. Hazard ratios from the Cox PH Model and Stratified Cox PH model for Inpari seeds between varieties

| Mannitol (%) | Comparison | Hazard ratio | p-value |
|---|---|---|---|
| | Inpari 19-Inpari 32 | 1.51 | 0.0044* |
| 0 | Inpari 19-Inpari 49 | 1.14 | 0.0718 |
| | Inpari 32-Inpari 49 | 0.89 | 0.4310 |
| | Inpari 19-Inpari 32 | 0.46 | $2.78 \times 10^{-7*}$ |
| 4 | Inpari 19-Inpari 49 | 0.98 | 0.8170 |
| | Inpari 32-Inpari 49 | 2.23 | $1.22 \times 10^{-7*}$ |
| | Inpari 19-Inpari 32 | 0.08 | $< 2 \times 10^{-16*}$ |
| 6 | Inpari 19-Inpari 49 | 0.73 | $4.11 \times 10^{-5*}$ |
| | Inpari 32-Inpari 49 | 6.54 | $<2 \times 10^{-16*}$ |
| | Inpari 19-Inpari 32 | 0.04 | $<2 \times 10^{-16*}$ |
| 8 | Inpari 19-Inpari 49 | 0.29 | $<2 \times 10^{-16*}$ |
| | Inpari 32-Inpari 49 | 2.66 | 0.0194* |

*p-value significant by test (α = 0.05)

and postponed germination time. Similar findings in this study show that the germination percentage of Inpari variety seeds in the control treatment is higher than that of mannitol treatment. The high mannitol concentration (8% mannitol) reduced the germination percentage of Inpari 19, Inpari 32, and Inpari 49 to 9%, 88%, and 68%, respectively, after 14 days of germination compared to the germination percentage of Inpari seeds germinated under control conditions (0% mannitol). Germination percentage at higher concentration mannitol or lower potential osmotic is always much slower, and vice versa. Our result is in line with previous studies reported by (Maksimovic *et al.* 2020) that drought stress using mannitol negatively influences seed germination percentage on pea and squash seeds. Additionally, mannitol-induced drought stress can affect germination, seedling growth traits, physiological parameters, and phytochemical content in squash landraces (Saadaoui *et al.* 2023).

In this study, drought stress not only affects seed germination but also increases mean germination time and time required to reach 50% germination. At the highest drought stress conditions (-0.11 MPa), the survival probability of Inpari 19 is 0.89, and Inpari 32 is 0.08 after 14 days, while Inpari 49 completely dies. Inpari 19 has the shortest mean and median germination time. High survival and survival probability of Inpari 19 under severe drought stress indicate that the variety can tolerate water deficit compared to other varieties. Research by Jauhari *et al.* (2020) also found that the productivity of Inpari 19 does not differ significantly from Inpago 9, which is upland rice when planted in rain-fed rice fields. This confirms that Inpari 19 is a tolerant variety of drought. Inpari 19 is derived from BP342B-MR-1-3 and BP226E-MR-76 lines. The potential yield is around 9.5 tons/ ha, with a rather sticky, soft texture and amylose content of 18% (Thamrin *et al.* 2023). This study suggests that stronger recovery capability contributes to developing drought-tolerant rice cultivars.

Drought stress can inhibit seed germination entirely under high-stress conditions, while Bhatt *et al.* (2022) and Lu *et al.* (2022) note that seeds can recover quickly and exhibit high germination percentages once the stress is alleviated. A study by Violita & Azhari (2021) stated that the rice variety's germination significantly reduced with the increased concentration level of osmotic solutions, which can be used to determine tolerance and susceptibility in germination level. Overall, drought stress has a significant impact on seed germination parameters, affecting the timing and efficiency of this critical stage in plant development.

Drought stress significantly affects rice germination through various morpho-physiological and biochemical mechanisms. The impact of drought stress on rice germination includes inhibition, chlorophyll content reduction, and oxidative stress (Bhandari *et al.* 2023). Drought stress inhibits seed germination in rice, leading to slower growth rates and reduced root and shoot length (Fatimah *et al.* 2023). Moreover, drought stress results in seeds with lower chlorophyll content, which affects photosynthesis and overall plant growth (Syamsia *et al.* 2018). In addition, it causes stomatal closure, reducing the rate of photosynthesis and affecting plant development. Drought stress induces the generation of reactive oxygen species, leading to oxidative stress in plants. This oxidative damage can result in lipid peroxidation, protein oxidation, DNA damage, and cell death (Seleiman *et al.* 2021).

In conclusion, drought stress simulated by mannitol treatment decreases germination percentage and increases germination time in all Inpari varieties due to the lack of water availability in the media. There are no different responses for MaxG, MGT, and $t_{50}$ in 0%, 2%, and 4%

mannitol treatments. The survival method in this study provides useful information about the germination potential in seeds with drought. From the result of the present study, it can also be concluded that with increasing levels of mannitol, the probability of not germinating is adversely affected in all Inpari variety seeds. Based on the log-rank test, there is a difference in survival time between mannitol concentration categories. However, there is no different response time for each Inpari variety treated with 2% mannitol. Inpari 19 is considered a tolerance variety to drought in the germination stage. This variety has a better ability for osmotic adaptation than other varieties of this study. Also, the osmotic pressure of -0.05 MPa (mannitol 4%) is the limit of drought stress tolerance for Inpari seeds in this study. Further analysis is needed to obtain their tolerance mechanism for breeding purposes. This study has limitations where germination is carried out in the laboratory using petri dishes. Therefore, our study did not know the possibility of survival of rice germination in the field. It would be more interesting if further research could conduct longitudinal studies related to the survival rate of rice seeds in the field.

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