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SALINITY RESISTANCE AND ABILITY OF N2-FIXING RHIZOBACTERIA ISOLATES TO IMPROVE RICE SEEDLING GROWTH UNDER SALINITY STRESS

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ABSTRACT

Salinity is the main limiting factor that inhibits the growth of rice plants significantly. N2-Fixing Rhizobacteria halotolerant able to increase the growth of cave rice plants to support plant productivity. This study was conducted to test PA and KP2 isolates to grow and increase Inpari rice plant growth at salinity stress (0, 4, 8, and 12 dSm-1). This study used GT (Growth time) with three replications to identify isolates growth ability on salinity stress as first stages. The second stage was to determine isolates ability to improve rice seedling growth under salinity stress using a randomized block design with twelve treatments and three replications. The result showed that all isolate could grow and tolerate salinity stress up to 12 dSm-1 and growth faster under salinity stress after 48 hours from inoculation. PA isolate grew 42.92 % while KP2 isolate was 57.80 % faster at 12 dSm-1 compared to non-saline condition. The result also showed that KP2 isolate give the best performance at 4 dSm-1 significantly increasing plant height 50.45%, plant height 183.88% compare to without inoculation on the same level salinity. Therefore, that ideal salinity for KP2 isolates to improve rice seedling growth under salinity stress was 4 dSm-1.

Keywords: Growth time, Inpari 79 Unsoed , N2-fixing rihizobacteria, salinity resistance .

1. INTRODUCTION

Land conversion and climate change have reduced land area and rice productivity in Indonesia. This is in accordance with [1] which shows that the area of rice land decreased from 11,377,934 ha in 2018 to 10,677,887 ha in 2019 and rice productivity decreased from 5.2 t ha^{-1} in 2018 to 5 .1 t ha^{-1} in 2019. The declining land area has resulted in the need for extensification efforts by utilizing sub-optimal land such as coastal areas that have high salinity. Climate change is causing the ice in the north and south poles to melt, resulting in rising sea levels. Rising sea levels increase seawater intrusion up to 20 km from the coastline which can be a serious problem, especially in Java, because Java Island is a national rice barn with an area of irrigated land of 3.3 million ha (42.5%) of the irrigated land area national, and 407,594 ha on the coast [2], [3].

High soil salinity damages soil structure, reduces aeration, permeability, nitrogen availability, and increases soil osmotic potential, causing physiological drought in plants [4]. In saline ecosystems, nitrogen deficiency generally occurs as a result of inhibition of the nitrogen cycle by microbes because high salt concentrations can result in plasmolysis in saline intolerant microbes [5]. The high accumulation of Na^+ and Cl^- in the soil also reduces the availability of nutrients such as K^+, Ca^{2+}, Fe^{2+}, and Mg^{2+} due to competition with Na^+ and Cl^- in the process of nutrient

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uptake by plant roots [6]. This problem is exacerbated by the characteristics of rice plants which are generally relatively sensitive to salinity stress with a threshold that causes a decrease in yield between 1.9 - 3 dSm^{-1} and if the EC is above 3 dSm^{-1} it will cause a decrease in the yield of 12% for every 1 dSm^{-1}. Therefore, under stress conditions of 6 dSm^{-1} the decrease in rice yield reached 50% [7]. Therefore, efforts are needed to overcome the deficiency of N in saline soils and increase the tolerance of rice plants to increased salinity. Under salinity stress, the concentration of the solution outside the roots was higher than inside the plant roots due to the presence of excess Na^+ and Cl^- ions, resulting in a decreased ability of the roots to absorb water due to osmotic stress [8]. A decrease in the rate of water uptake will reduce the rate of photosynthesis and increase respiration so that the sugar produced by photosynthesis cannot be used to support plant growth [9].

Rhizobacteria that live in the rhizosphere have the ability as a biofertilizer such as N_2-fixing from the atmosphere catalyzed by the nitrogenase enzyme. Biological nitrogen fixation is important because nitrogen is a necessary macronutrient for plant growth and is abundant in the atmosphere, but cannot be absorbed by plants. This is because atmospheric nitrogen is in the form of N_2 in the atmosphere. Therefore, atmospheric nitrogen needs to be converted into ammonia by rhizobacteria through nitrogen fixation. Rhizobacteria that have the ability to fix N_2 include *Rhizobium trifolii*, *Bradyrhizobium* sp., *Sinorhizobium meliloti*, *Azotobacter agilis*, *Azotobacter chroococcum*, *Azotobacter vinelandii* and *Klebsiella pneumoniae* [10].

Rhizobacteria isolated from saline ecosystems have the ability in nitrogen fixation. [4] stated that rhizobacteria such as *Azotobacter* sp. isolated from saline soil ecosystems had the ability to increase plant height and root length of rice. This is because these bacteria have the ability to convert free nitrogen in the atmosphere into ammonia needed by plants [11]. The research showed that *Pseudomonas stutzeri* ISE12 has the ability to increase plant resistance to salinity stress, because it has the ability to reduce stress due to salinity stress which is indicated by a decrease in proline concentration in plants (increases with salinity to balance osmotic pressure) thereby significantly increasing the number of leaves, dry weight (50, 150, and 300 mM NaCl), and significantly reduced the proline content in plant roots at 300 mM NaCl [12]. Research by Habib on rice plants in saline soils showed that Rhizobacteria could significantly improve the performance of rice plants in saline soils compared to plants that were not inoculated by N_2-fixing microbes *Bacillus* sp. and *Citrobacter* sp [13].

2. MATERIAL AND METHODS

The research was carried out in July-August 2021 at the Soil Biology Laboratory and Greenhouse Faculty of Agriculture, Padjadjaran University. Isolates that were used came from rice plant rhizosphere from Cilamaya, Karawang, West Java, Indonesia. This study used GT (Growth time) with three replications to identify isolates (PA and KP2) growth ability on various salinity stress (0, 4, 8, and 12 dSm^{-1}) as first stages. The second stage was to determine isolates (PA and KP2) ability to improve rice seedling growth under various salinity stress (0, 4, 8, and 12 dSm^{-1}) using a randomized block design with three replications.

2.1 Materials

In this research, there are several tools and materials used. Salinity resistance assay used Ashby mannitol consist of (10 g mannitol, 0.2 g K_2HPO_4, 0.2 g $MgSO_4.H_2O$, 0.1 g $CaSO_4.2H_2O$, and 5

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g $CaCO_3$) per litre, NaCl, petri dish, erlemeyer (100 and 250 mL), micropipet (100 µL and 8000µL) with microtips, test tube 10 mL, Laminar air flow, Autoclaf. Bioassay used Inpari Unsoed 79 Agritan seed, parchment paper, NaCl, test tube 100 mL, venier calipers, measuring tube, label and Fahraeus media nitrogen free consist of (0,132 g $CaCl_2$, 0,12 g $MgSO_4.7H_2O$, 0,1 g KH_2PO_4, 0,075 g $Na_2HPO_4.2H_2O$, 5 mg Fe-sitrat, dan 0,07 g $MnCl_2.4H_2O$, $CuSO_4.5H_2O$, $ZnCl_2$,H_3BO_3, dan $Na_2MoO_4.2H_2O$) per litre [14].

2.3 Salinity Resistance Assay

Determination of salinity resistance of rhizobacteria isolates (PA, and KP2) using Ashby mannitol liquid media with salinities of 0, 4, 8, and 12 dSm-1. Calculation of the growth of isolates using the direct calculation method with several stages. Ashby mannitol media (10 g mannitol, 0.2 g K_2HPO_4, 0.2 g $MgSO_4.H_2O$, 0.1 g $CaSO_4.2H_2O$, and 5 g $CaCO_3$) per litre, made up to 50 ml for each treatment [15]. The addition of NaCl salt was carried out at the same time as mixing the media material with a weight of 0.7913 g L^{-1} for a salinity of 4 dSm^{-1}, 1.9113 g L^{-1} for a salinity of 8 dSm^{-1}, and 4.5469 g L^{-1} for a salinity 12 dSm^{-1}. Weight of salt addition and media formula Ashby Mannitol was obtained from the calculation of the standard curve of media salinity. Pure cultures of rhizobacteria isolates that had been incubated for 3 days were inoculated as much as 10% of the volume of the medium (by suspending the culture in 5 mL of sterile 0.85% NaCl Solutions) into 50 mL of Ashby mannitol which was placed in a 250 mL Erlenmeyer for each treatment and stored in a gyratory shaker. at 115 rpm at room temperature 23-26 ^{O}C [16]. Measurements were made immediately after inoculation to determine the initial population and every 24 hours for 4 days after inoculation with a dilution factor at intervals of 10^{-4} to 10^{-7} using the Total Plate Count method and calculated by the following formula:

$$GT = \frac{t}{n}$$

$$n = \frac{(logn_t - logn_o)}{log_2} \quad (1)$$

Annotation:

GT : Generation time at logarithmic phase

n : generation number

n_t : Cell density at sampling time t

n_0 : cell density at treatment time

t : time (hour) between sampling time

2.4 Biological Assay

The N_2-fixing rhizobacteria bioassay was carried out with Fahraeus media nitrogen free ((0,132 g $CaCl_2$, 0,12 g $MgSO_4.7H_2O$, 0,1 g KH_2PO_4, 0,075 g $Na_2HPO_4.2H_2O$, 5 mg Fe-sitrat, dan 0,07 g $MnCl_2.4H_2O$, $CuSO_4.5H_2O$, $ZnCl_2$,H_3BO_3, dan $Na_2MoO_4.2H_2O$) per litre [14]. The media was then sterilized using an autoclave at 121 ^{o}C for 15 minutes at a pressure of 1 atm. The addition of salt was carried out during the media making process by adding NaCl as much as 2.380 g L^{-1} for a salinity of 4 dSm^{-1}, 5.050 g L^{-1} for a salinity of 8 dSm^{-1}, 7.721 g L^{-1} for a salinity of 12 dSm^{-1}. Biological tests were carried out by using a 100 mL test tube filled with 90 ml of saline liquid Fahraeus media and adding a liquid culture of isolate with an inoculation age of 72 hours on non-saline Ashby mannitol media (10 g mannitol, 0.2 g K_2HPO_4, 0.2 g $MgSO_4.H_2O$, 0.1 g

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$CaSO_4.2H_2O$, and 5 g $CaCO_3$). Observations were made every seven days for 21 DAI (Day after Inoculation) on variables such as root length, plant height, number of leaves, while observations of dry weight were carried out on day 28.

The inoculation of rhizobacteria in rice plants was carried out in several stages. Inpari Unsoed 79 Agritan was chosen because it belongs to the salinity tolerant variety [17]. Rice seeds were sterilized by immersion using 0.2% $HgCl_2$ for 2 minutes and followed by sterilization with 70% alcohol for 2 minutes and rinsed with sterile distilled water and then germinated. The sown rice seeds were soaked in distilled water for 24 hours at room temperature to break seed dormancy and stimulate growth hormone 25 ^oC [18]. Each tube containing 90 mL of Fahraeus media was given 10 mL of liquid culture of halotolerant N_2-fixing rhizobacteria isolates at 10^9 $CFUmL^{-1}$ and then planted in Fahraeus media that had been prepared previously with one rice plant for 100 mL test tubes. Maintenance is carried out in the form of adding Fahraeus saline media according to treatment if needed until it reaches the 100 mL limit that has been made in the test tube. After that, each treatment was placed in the greenhouse of the Soil Biology Laboratory, Padjadjaran University to be observed (number of leaves, plant height, and root length, every seven days until the plant was 21 days after inoculation [4].

2.5 Data Analysis

This research has two stages. First stage was carried out with descriptive quantitative design using GT (generation time) calculation from three replication data to asses rhizobacteria salinity resistance. Second stage used randomized block design with three replications then data was analyzed using SPSS 25 continued with Tukey's range test $p \leq 0.01$.

3. RESULT AND DISCUSSION

3.1. Salinity Resistance Assay

Microbial salinity resistance is the ability of microbes to continue to grow and divide cells in an environment that has abiotic stress in the form of excess salt content. Resistance describes the ability of microbes to avoid structural changes in the community when stress occurs, both abiotic and biotic biotik [19]. Salinity resistance is one of the characters that need to be considered in the selection of N_2-fixing rhizobacteria isolates to support the growth of rice plants in a saline environment because if the selected isolates do not have resistance to salinity, the proliferation of isolates will not be optimal, which has an impact on the carrying capacity that is less than optimal. One method to determine the resistance of N_2-fixing rhizobacteria isolates is to perform a resistance test on N-free media such as Ashby mannitol [15] which is treated with the addition of salt in the form of NaCl at various levels in dSm^{-1} units as shown in Table 1

Table 1: N_2-fixing rizobacteria growth at various salinity

Isolates	N_2-fixing rizobacteria population (10^6 CFU mL^{-1})					Growth Time at logarithmic phase
	Salinity levels (dSm^{-1})	0 hour	24 hours	48 hours	72 hours	

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PA	0	981	2,920	13,500	12,800	10.95
PA	4	169	9.7	208	152	5.39
PA	8	167	13.8	134	151	7.33
PA	12	131	18.3	269	137	6.25
KP2	0	573	6,160	14,900	14,500	18.91
KP2	4	238	15.8	167	139	6.94
KP2	8	128	15.9	125	179	8.26
KP2	12	127	14.6	125	129	7.98

Data were obtained from three replication

Table 1 showed that increasing salt content decreases the GT (Generation time) or the time required to duplicate two isolates of halotolerant N_2-fixing rhizobacteria in the logarithmic phase. It can be seen that in the first 24 hours there was a decrease in the population of rhizobacteria in saline conditions, whereas in non-saline conditions the bacteria did not experience a population decline. Population decline is thought to be an adaptation phase for inoculants on saline media. In this phase the inoculant cells adapt to salinity stress conditions in the form of excess Na^+ and Cl^- ions in the solution which causes an increase in solution potential because the solution outside the cell is more concentrated than inside the bacterial cell, resulting in osmotic stress [20]. then, the cell density of rhizobacteria in various salinity media increased at 48 hours. PA and KP2 isolates showed the increase of GT value due to salinity increase. This condition showed that isolate has adapted to salinity and able to grow faster compared to non-saline condition. Visual observation showed that there was size reduction in colony, but there was more colonies at petri dish in saline condition compared to non-saline condition based on total plate counting. Salinity stress on microbes induces the production of secondary metabolites such as EPS to stabilize the solution potential because EPS has the function of binding cations such as Na^+ in NaCl and reducing bioavailability, therefore microbe could growth after adapting under stress conditions [19].

3.2 Bioassay on Inpari Rice in Various Salinity

3.2.1. Effect N_2-Fixing Rhizobacteria on Root Length

The average root length used in the bioassay of rice plants was 37.67 mm and observations were made every 7 DAI (day of inoculation) to 21 DAI. This observation aims to determine effect N_2-fixing rhizobacteria on root growth of Inpari Unsoed 79 at various salinities as showed at Table 2.

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Table 2: Effect of N_2-fixing rizobacteria on Inpari rice root length 7-21 DAI at various salinity

Treatment	Root length (mm)		
	7 DAI	14 DAI	21 DAI
Cb0	78.70 c	108.17 f	122.50 e
Cb1	42,03 ab	71.27bcd	79.03 cd
Cb2	35,30 a	41.10 a	41.77 a
Cb3	40.60 ab	43.47 a	44.73 ab
PAb0	49.70 ab	72.37 bcde	81.17 cd
PAb1	54,03 ab	88.73 def	103.40 de
PAb2	38.87 ab	53.13ab	56.57 abc
PAb3	53,67 ab	59.17 abc	60.93 abc
KP2b0	52.83 ab	82.60 cde	107.43 de
KP2b1	60,27 ab	97.43 ef	118.90 e
KP2b2	44,87 ab	69.30 bcd	73.03bc
KP2b3	53,67 ab	58.33 abc	62.63 abc

Different letters over bar of indicates significant difference in treatment according to Tukey's multiple range test $p \leq 0.01$), C (control), PA isolates, KP2 isolates, b0 (0 dsm^{-1}), b1 (4 dSm^{-1}), b2 (8 dSm^{-1}), b3 (12 dSm^{-1})

Table 2 showed that root growth KP2 at salinity level 4 dSm^{-1} has the nog significant different with control at non-saline condition. This result showed that, inoculation N_2-fixing rhizobacteria could improve root growth at 21 DAI in 4 dSm^{-1} salinity level. The roots growth were varied from first week to third weeks. At first week control without salinity treatment has the highest value of root length, but at 14 DAI the conditions was changed, showed with there is no significant different between control without salinity which has root length 108.17 mm compared to isolate KP2 at 4 dSm^{-1} salinity level which has root length 97.43 mm. the trend at control showed that the increase of salinity decrease rooth length. Salinity stress changes physiological and metabolic processes that are influenced by severity and duration so that it could inhibit plant growth [21]–[24]. Excess Na^+ in the growing medium reduces the uptake of K^+ by the roots. This is because potassium and Na have the same transport mechanism, namely the K+ transporter which consists of genes and proteins such as the HKT (histidine kinase transporter) and the NHX (Na^+/H^+ antiporter) which functions to remove excess Na^+ into the vacuole from the planting medium into the plant [25]. In the early stages of salinity stress, the process that inhibits plant growth is reverse osmosis followed by ion poisoning [21], [22]. During the initial phase of salinity stress, root absorption capacity decreases and water loss from leaves increases as a result of osmotic stress and excess salt accumulation in the growing media and plant tissues, so it can be categorized as hyperosmotic stress. Osmotic stress in the early days of salinity stress causes physiological changes such as membrane disturbances, nutrient imbalances, weakening of Reactive oxygen species (ROS) detoxification, decreased photosynthetic activity, and stomata opening [22], [26]. Salinity stress also causes hyper-ionic stress due to excess accumulation of Na^+ and Cl^- ions in plant tissues that are in direct contact with growing media with high salt concentrations. Excess Na^+ and Cl^- in plant tissue disrupts physiological processes such as

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inhibition of K^+ ion absorption which inhibits plant growth and development and causes plant death [21].

3.2.2. Effect N-Fixing Rhizobacteria on Plant Height

The average plant height used in the rice bioassay was 8.87 mm and observations were made every 7 DAI to 21 DAI. This observation aims to determine one of the growth components of Inpari Unsoed 79 rice plants at various salinities and BFHNR inoculation treatments as shown in Table 3.

Table 3: Effect of N_2-fixing rizobacteria on Inpari rice plant height 7-21 DAI at various salinity

Treatment	Plant height (mm)		
	7 DAI	14 DAI	21 DAI
Cb0	98.27 bc	111.77 b	116.73 b
Cb1	43.20 a	77.27 a	81.90 a
Cb2	24.40 a	63.33 a	65.13 a
Cb3	44.03 a	51.73 a	53.03 a
PAb0	141.27 e	204.87 e	210.00 de
PAb1	114.27 cd	184.03 de	204.57 de
PAb2	86.57 b	153.70 cd	169.90 c
PAb3	91.00 bc	118.20 b	168.70 c
KP2b0	100.77 bc	153.73 cd	170.37 c
KP2b1	137.00 de	200.83 e	232.50 e
KP2b2	107.57 bc	177.20 cde	192.00 cd
KP2b3	104.47 bc	152.3 c	168.70 c

Different letters over bar of indicates significant difference in treatment according to Tukey's multiple range test $p \leq 0.01$), C (control), PA isolates, KP2 isolates, b0 (0 dsm^{-1}), b1 (4 dSm^{-1}), b2 (8 dSm^{-1}), b3 (12 dSm^{-1})

Table 3 showed that plant height growth inoculated with KP2 isolates at salinity level 4 dSm^{-1} has significantly higher compared with control at non-saline condition. This result showed that, inoculation N_2-fixing rhizobacteria could improve root growth significantly at from 7 to 21 DAI in 4 dSm^{-1} salinity level. At 7 and 14 DAI KP2 isolate at 4 dSm^{-1} salinity has no significant different with PA isolates at 4 dSm^{-1} salinity with plant height 137.00 mm and 141.27 mm respectively at 7 DAI and 200.83 and 204.87 mm at 14 DAI. Then, at 21 DAI KP2 at 4 dSm^{-1} salinity has higher value even though not significantly different compared to PA both at 0 dSm^{-1} and 4 dSm^{-1} salinity level. This indicated that giving isolates to rice plants can increase water efficiency, photosynthesis, and stomatal opening [27]. Therefore, after 21 DAI KP2 at 4 dSm^{-1} has the best plant height compared to control booth with same salinity and with non-saline conditions. Plant height inoculated with KP2 at 8 dSm^{-1} lower compared to KP2 at 4 dSm^{-1} which indicated there was a decrease which indicated that the salinity level had exceeded the salinity tolerance limit, resulting in a decrease in microbial performance in supporting plant growth [28]. This is because salinity is a factor that inhibits the nitrification process so that it can reduce the carrying capacity of bacteria to plants [29]. Plant height parameter have a positive

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effect on plant resistance to inundation conditions (generally occurs in saline rice field ecosystems on tidal land) because it causes the leaf surface to be on the water surface and can get sunlight and CO_2 (photosynthesis), as well as O_2 for respiration, especially maintaining oxygen supply on plant roots through aerenchyma [30]. This is following the research of Shamin which stated that from 35 colonies there were only five Azotobacter isolates that were tolerant to 6% NaCl and 2 isolates tolerant to 10% NaCl [28].

3.2.3. Effect N_2-Fixing Rhizobacteria on Leave Number

Observation of the number of leaves per plant used in rice bioassays was carried out every 7-21 DAI. This observation was aimed to determine one of the growth components of Inpari Unsoed 79 rice plant showed at Table 4.

Table 4: Effect of N_2-fixing rizobacteria on Inpari rice leave number 7-21 DAI at various salinity

Treatment	Leave number		
	7 DAI	14 DAI	21 DAI
Cb0	2.33	3.00 bc	3.00
Cb1	1.67	2.00 a	3.33
Cb2	1.67	2.00 a	2.00
Cb3	1.00	2.33 ab	2.67
PAb0	2.00	3.00 bc	4.00
PAb1	2.00	3.00 bc	3.67
PAb2	2.00	3.00 bc	3.67
PAb3	2.00	3.00 bc	3.00
KP2b0	2.67	3.33 c	3.33
KP2b1	2.33	3.00 bc	3.33
KP2b2	2.00	3.00 bc	4.00
KP2b3	2.33	3.00 bc	3.33

Different letters over bar of indicates significant difference in treatment according to Tukey's multiple range test $p \leq 0.01$), C (control), PA isolates, KP2 isolates, b0 (0 dsm^{-1}), b1 (4 dSm^{-1}), b2 (8 dSm^{-1}), b3 (12 dSm^{-1})

Table 4 showed the number of leaves at 7- 21 days after inoculation (DAI). At 7 DAI there is no significant effect N_2-fixing rhizobacteria on leave number improvement ant Inpari rice plant. But there was tendency that KP2 at non saline condition gave better growth with mean leave number 2.67, then at 14 DAI KP2 at non-saline condition gave highest leave number, but there was no significant different at 4, 8, and 12 dSm^{-1} salinity level. Then, at 21 DAI generally there was no significant effect of treatment to leave number production. This is showed that salinity is a stress salinity is the main limiting factor for plant growth [31]. Also, salinity stress affects plant health by reducing phosphorus availability, inducing osmotic stress, triggering ethylene production, mediating Na^+ and Cl^- toxicity, and causing reactive oxygen species (ROS) that inhibit plant growth [32]–[34].

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4. CONCLUSION

The research conclude that all isolate could grow and tolerate salinity stress up to 12 dSm^{-1} and growth faster under salinity stress after 48 hours from inoculation. PA isolates grew 42.92 % faster while KP2 was 57.80 % both at 12 dSm^{-1} compared to control. The result also showed that KP2 isolates give the best performance at 4 dSm^{-1} significantly increasing plant height 50.45%, plant height 183.88% compare to without inoculation on the same level salinity. Therefore, that ideal salinity for KP2 isolates to improve rice seedling growth under salinity stress was 4 dSm^{-1}.

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