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Physiological Response and Detection of *Inh2* Gene in Dieng Red Potato (*Solanum tuberosum* L.) Affected by Frost

Wa Ode Kamillah*, Hermin Pancasakti Kusumaningrum, Rejeki Siti Ferniah , Azalia Puspa Herida, Garinda Linggar Nasansia

Department of Biology, Faculty of Science and Mathematics, Diponegoro University, Semarang 50275, Indonesia

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Abstract

In this study, the response of Dieng red potato plants to frost exposure was evaluated physiologically by testing electrolyte leakage and chlorophyll content, and molecularly by detection of *Inh2* gene. The physiological response shows an increased electrolyte leakage rate and a significant decrease in chlorophyll content in plants exposed to frost compared to those grown in normal temperatures. Sequencing of *Inh2* gene identifies four homologous *Solanum tuberosum* alleles. The difference in the order of *Inh2* lies in the gap of 12 bp. Phylogenetic analysis shows that *inh2* gene is in a cluster with INH2α*C allele and has evolved slowly from a common ancestor with *S. lycopersicum*. This is the first study on the physiological mechanisms of frost tolerance and the sequencing of *Inh2* gene in Dieng red potatoes. Hopefully, this study will provide useful information for the breeding of low-sugar Dieng red potatoes.

Keywords: Dieng, electrolyte leakage, chlorophyll, *Inh2*, frost

1. Introduction

Potato (*Solanum tuberosum* L.) includes thousands of varieties that vary in size, shape, pigment, and other characteristics. One of the pigmented potatoes that has been widely cultivated is red potato plant. The glycemic index (GI) value of potato tubers varies widely and depends on the type, storage and serving. Red potato tubers have a lower GI value than other types of potato tubers. The low GI value in red potato tubers reaches 56 when served by boiling and consumed when cold (Eleazu, 2016). Red potato tubers also contain potential nutritional sources in the form of carbohydrates, minerals, vitamins C and B_1 (Beals, 2019). Therefore, choosing red potato tubers to serve as diet food is the best choice. Furthermore, there are pre-harvest factors that affect red potato tubers, namely the temperature of their growing period. Potato plants are very sensitive to frost, so temperature is one of the factors that affect the productivity of red potatoes.

Potato plants cannot tolerate low temperatures and will immediately show symptoms of freezing damage (Che *et al.*, 2020). Potato plants are not able to tolerate the formation of ice in its tissues, either extracellular or intracellular. Leakage of electrolytes from cells often occurs after frost injury (Arvin and Donnelly, 2007; Rooy *et al.*, 2017). Another damage that occurs after an electrolyte leak is a reduction in photosynthesis. Chlorophyll content of plants has a good correlation with the ability of photosynthesis. The reduction in chlorophyll contents in potato plants has been shown to slow down the rate of photosynthesis (Harb and Lahham, 2013; Li *et al.*, 2021).

Potato plants exposed to low temperatures will also cause the accumulation of reducing sugars, namely glucose and fructose or cold-induced sweetening, CIS (Datir *et al.*, 2019). Acid invertase activity was the most significant factor in determining the accumulation of fructose and glucose (Stein and Granot, 2019). Acid invertase activity can be controlled post translation by invertase inhibitors. Invertase inhibitor is of two types, the apoplastic invertase inhibitor encoded by *Inh1* gene and the vacuolar invertase inhibitor encoded by *Inh2* gene (Datir, 2020). According to Liu *et al.* (2010), the interaction between acid invertase and vacuolar invertase inhibitor may play an important role in controlling reducing sugars.

Understanding the response of potato plants to frost is essential for the development of cold-resistant crops. Red potatoes used in this research come from the Dieng. This is because air temperature in Dieng during the dry season (June-August) is very low and can reach freezing point in the morning, thus causing frost. The objectives of this study was to determine the physiological response of red potato plants to the amount of electrolyte leakage and chlorophyll content based on its resistance after exposure to frost and to analyze the sequence and compile the phylogenetic tree of *Inh2* gene of Dieng red potato plants. The results of this study are expected to be used as supporting data and a first step to breed cold-tolerant red potato plants so that they can be used as diet food by producing low-sugar potatoes through the mechanism of potato plants in inhibiting the formation of reducing sugar.

* Corresponding author. e-mail: no crossponding auther.

2. Materials and Methods

The materials used in this study were a tuber and leaves of Dieng red potato obtained with permission from the Kejajar District Agricultural Extension Center. For determination of electrolyte leakage and chlorophyll content, plants were divided into three groups. Group one, a tuber was planted in a pot and grown at normal temperature 24/31° C (day/ night temperature) in conditions of long days (16 hours of light) and a relative humidity of 65- 95%. In the other two groups, plant leaves were taken directly in the field, namely in the Dieng area, and had been exposed to natural frost in the early hours. The two groups exposed to frost were taken at different altitudes, namely 2.110 m above sea level on the hill and 2.064 m above sea level in the valley. Leaf samples taken were immediately put in liquid nitrogen, stored in a styrofoam box filled with ice and testing was carried out immediately. For *Inh2* gene detection, healthy red potato leaves were selected, one month old, and very young leaves were taken from the shoot tips. The samples were put in a perforated ziploc bag and put into a styrofoam box filled with ice.

2.1. Determination of Electrolyte Leakage

The method for determining electrolyte leakage follows the method of Campos *et al.* (2003). Potato leaves were cut into discs with an area of 0.5 cm^2 in each group. The first group came from plants grown at normal temperatures, and the other two groups from plants that grew in nature and were exposed to frost. The leaf discs were then placed in 25 mL of demineralized water, incubated with a room temperature shaking incubator at 200 rpm for two hours. The electrolyte conductivity value C1 is measured with a conductivity meter (ExStih II, EC500, Extech, US). The sample was then heated in an oven for 30 minutes and cooled. The electrolyte conductivity value C2 was measured. Conductivity measurements are carried out 3-5 repetitions. The results of electrolyte leakage are expressed in relative conductivity (C%).

2.2. Determination of Chlorophyll Content

The method of determining chlorophyll content follows the method Liang *et al.* (2017). Potato leaves were prepared 100 mg from each group. The first group came from plants grown at normal temperatures and the other two groups from plants that grew in nature and exposed to frost. The leaves were crushed and homogenized at 10 mL of 80% acetone. Samples were centrifuged for 15 minutes at 10,000 rpm. The supernatant formed was then used to measure the absorbance at 633 nm and 645 nm against the solvent blank (acetone) using a spectrophotometer (UH5300 Spectrophotometer, Hitachi, Japan) with three repetitions. The concentrations of chlorophyll a, chlorophyll b and total chlorophyll were calculated using the following equation from Arnon (1949).

2.3. Genomic DNA Isolation

Genomic DNA isolation from 0.5 g of red potato leaves. Genomic DNA was isolated using the modified Doyle and Doyle (1987) CTAB method. The quantity of DNA was determined using the Nanodrop 2000 Spectrophotometer (Thermo Scientific).

2.4. DNA Amplification

PCR Mix compositions were made in 50 µL. DNA was amplified using *Inh2* forward primer (5′-CCTTCATCAACTTCTCATTTCTTC-3'), *Inh2* reverse primer (5′-GTGCATTGAACGGCAAATTA-3′) (Datir *et al.*, 2019). PCR mix is made with Mytaq HS Red Mix as much 50 µL starting with mixing 25 µL Mytaq HS Red Mix; the forward and reverse primers each are 3 µL; DNA template, 2 µL; and ddH_2O, 15µL. The PCR conditions used were 30 seconds of pre-denaturation at 98° C, 15 seconds of denaturation at 98° C, 30 seconds of annealing at 55° C, 30 seconds of elongation at 72° C, and 10 minutes of post-elongation at 72° C carried out in 35 cycles. The PCR product was then subjected to electrophoresis 1% agarose (1x TAE buffer), stained with florosafe and visualized with Gel documentation XR+ system (Bio-Rad).

2.5. DNA sequencing

Analysis of potato DNA sequences using the company 1st BASE and Genetics Science services. The results of the sequences were then analyzed to compare the similarities of sequences available to GenBank through the Basic Alignment Search Tool (BLAST) on NCBI. The putative mRNA and protein sequence using MEGA X and Geneious Prime software. Phylogenetic analysis uses the molecular application ClustalX to align the sample sequences with other organism sequences available on the NCBI website and the MEGA X application to construct phylogenetic trees.

3. Result

3.1. Electrolyte Leakage

In this study, potato plants exposed to frost were taken in two different places, namely at an altitude of 2,100 m above sea level i.e. on a hill and 2,064 m above sea level, i.e. in a valley. Potato sampling in two different places was based on a research done by Chung *et al.* (2006) that cold air from the slopes will descend the slopes and settle in the valley. As a result, frost zones will tend to accumulate at the bottom of the valley. So, it is expected that potato plants that grow in valleys will show higher electrolyte leakage due to the amount of frost compared to potato plants that grow on hills with little frost.

The conductivity test showed that the leaf electrolyte leakage increased due to exposure to frost as shown in Figure 1. There is a significant difference at normal temperature, and the average electrolyte leakage value is around 39,4%. Potato plants exposed to frost in the valleys showed an increase in electrolyte leakage of 96,4%, while potato plants in the hills only reached 85,8%.

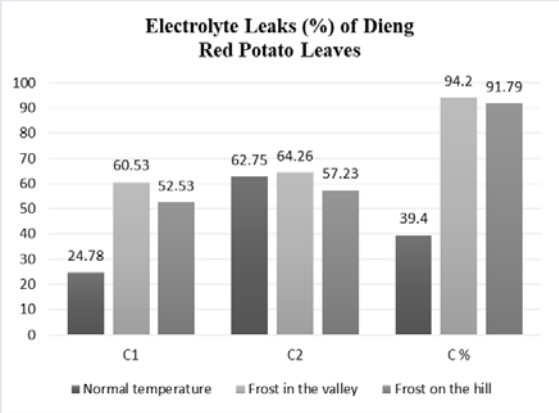

Figure 1. Results of electrolyte leakage (%) leaves of Dieng red potato plant leaves due to frost exposure, and plants not exposed to frost. Information: C1= initial conductivity value, C2 = conductivity value after heating and C% = relative conductivity value

3.2. Chlorophyll content

Plants optimize light absorption and photosynthesis through adequate chlorophyll content. Stress at low temperatures is known to affect photosynthesis and reduce the ability of plants to absorb light (Liu *et al.*, 2013). The results of determining the chlorophyll content of potato plants in Figure 2 exposed to frost both in the valley and the hill showed low chlorophyll contents compared to those of potato plants that were not exposed to frost; namely chlorophyll contents reached 7,21 mg.g^{-1}FW. Chlorophyll contents were seen to decrease in potato plants in the valley, namely 1,01 mg.g^{-1}FW compared to those in the hill, namely 1,79 mg.g^{-1}FW.

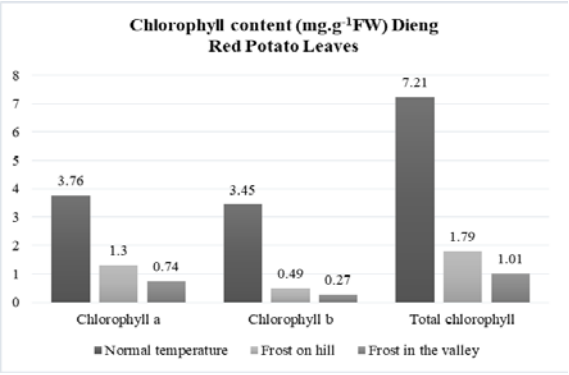

Figure 2. Results of chlorophyll content (mg.g^{-1}FW) leaves of Dieng red potato plant leaves due to exposure to frost, and plants not exposed to frost

3.3. The Inh2 Gene

The amplification results show good DNA quality with clear bands. The agarose gel showed an invertase inhibitor PCR amplicon of about ± 600 bp (Figure 3). The size of the potato *S. tuberosum inh2* gene varies widely. The result of DNA amplification in this study is by the report of Datir *et al.* (2019) and Liu *et al.* (2010). Therefore, the actual size of the *Inh2* gene in this study needs to be proven by means of amplified *Inh2* sequencing.

The purpose of *Inh2* gene sequencing is to determine the sequence of nucleotide bases and the size of the gene. The sequencing results are then combined, namely the unification of the primary forward *Inh2* and reverse *Inh2* primary sequences to obtain a complete sequence. The results of the second contig of *Inh2* primers showed that the length of the nucleotide base of the *Inh2* gene was 630 bp (Figure 4). The sequencing results were then aligned using the NCBI Blast Alignment Search Tool (BLAST) to determine the percentage of homology of the red potato *Inh2* gene base sequence obtained with the *Inh2* gene sequence database contained in the GenBank (Table 1). The BLAST results show that there are 11 *Solanum* species that have a maximum of 613 nucleotides out of 630 nucleotides.

Probability of mRNA and protein prediction were analyzed using MEGAX and Geneious Prime. The INH2 sequences of Dieng red potatoes were aligned and compared with our identified *Inh2* homology (Table 1). The next analysis is to compile a phylogenetic tree which aims to determine the kinship of a species, the amino acid sequences of the *Inh2* homologs identified by us (Table 2). The ClustalX software is used for aligning sequences and the software for reconstructing phylogenetic trees. The phylogenetic tree formed in Figure 5 consists of four species (Table 2), namely Dieng red potato, *S. tuberosum*, *S. lycopersicum* and *Manihot esculenta* as an outgroup. Dieng red potatoes are in the same clad with *S. tuberosum* for INH2α* C. Dieng red potato along with *S. tuberosum* and other wild potatoes appear to have ancestry from *S. lycopersicum* (Slugina *et al.*, 2020). Genetic distance shows the kinship relationship between each sequence. The smaller the number of genetic distances, the more linked the sequence is. The genetic distance results of *Inh2* gene showed that Dieng red potatoes were closely related to the *S. tuberosum* INH2alpha* C and INH2alpha* D alleles with a genetic distance of 0.054 and 0.060. This was further followed by S. *tuberosum* INH2alpha* A, INH2alpha* B.

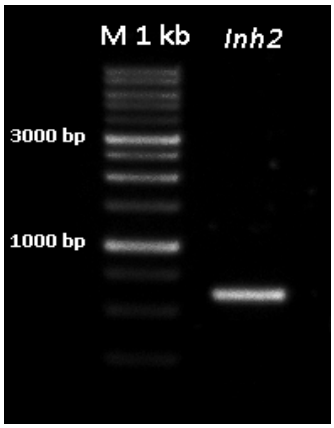

Figure 3. Visualization of PCR products from the Dieng red potato plant of *Inh2* geneDescription: Line 1; Marker 1 kb; Line 2; Dieng red potato *Inh2* gene

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  1 ccttcatcaa tttctcattt tttcaatttt caaaaaaaaa aagttaaaaa aatggagaaa
 61 tttattcccc atattgatgc taatcaccaa tttggcactc aacaacgata acaacaacaa
121 caacaataat tataatctca tacactcaac gtgtagggag accccatatt actccctatg
181 tctcaccacc ctacaatccg atccacgtag taacgaggtt gagggtgatg atgccatcac
241 caccctaggc ctcatcctgg tggacgcggt gaaatcaaag tccatagaaa taatggaaaa
301 aataaaagag ctagagaaat cgaaccctga gtggcgggcc ccacttagcc agtgttacgt
361 ggcgtataac gccgtcctac gagctgatgt aacggtagcc gttgaagcct taaagaaggg
421 tgtccctaaa tttgctgaag atggcatgga tgatgttgtt gttgaagcac aaacttgtga
481 gtatagtttt aattattata ataaatttgga ttttccaatt tctaatttga gtagggaaat
541 aattgaacta tcaaaagttg ctaaatccat aattagaatg ttttttagaa gggggaaaaa
601 aaagtttggg gtttaatttg ccgttcaatg
```

Figure 4. The sequence of *Inh2* gene in Dieng red potato plant

Table 2. BLAST results of Dieng red potato plant *Inh2* gene on NCBI

Organism	Description	Query coverage	Percent identity	Accession
S. tuberosum	*Inh2alpha*C*	100%	97,30%	FJ810208
S. tuberosum	*Inh2alpha*D*	99%	97,29%	FJ810209
S. tuberosum	*Inh2alpha*B*	99%	95,44%	FJ810207
S. tuberosum	*Inh2alpha*A*	99%	95,30%	FJ810206
S. lycopersicum	*Inh2*	92%	88,85%	NM_001329220

Table 3. Predictions of amino acid composition, molecular weight and isoelectric point of Vacuolar Invertase Inhibitor

Organism	Description	Amino acid	Molecular weight	Isoelectric point	Accession
Dieng Red Potato	*Inh2*	179	20.28	5.3	
S. tuberosum	*Inh2α*A*	181	20.51	4.57	ACO35697
S. tuberosum	*Inh2α*B*	180	20.37	4.69	ACO35697
S. tuberosum	*Inh2α*C*	178	20.14	4.57	ACO35697
S. tuberosum	*Inh2α*D*	178	20.13	4.47	ACO35697
S. lycopersicum	*Inh2*	175	19.90	5.00	ACO35697

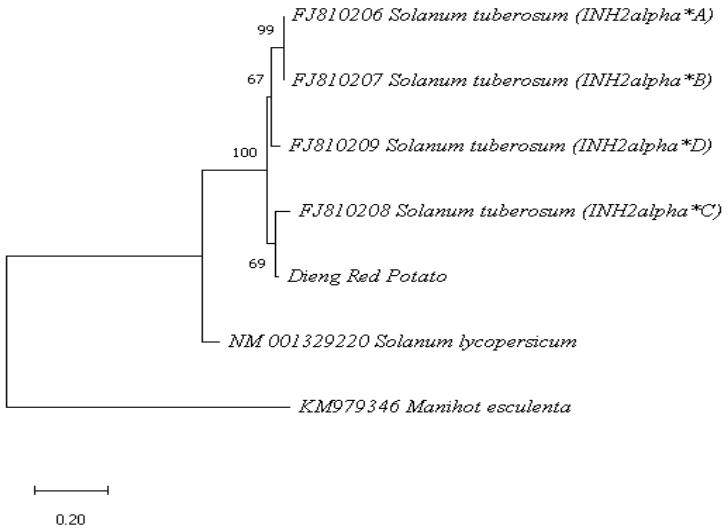

Figure 5. Phylogenetic tree of Dieng red potato *Inh2* gene

Table 4. Genetic range of Dieng red potato *Inh2* gene with several *Solanum* comparators from NCBI.

No	Species	1	2	3	4	5	6	7
1	*S. tuberosum Inh2alpha*A* (FJ810205.1)							
2	*S. tuberosum Inh2alpha*B* (FJ810207.1)	0,000						
3	*S. tuberosum Inh2alpha*C* (FJ810208.1)	0,123	0,123					
4	*S. tuberosum Inh2alpha*D* (FJ810209.1)	0,060	0,060	0,118				
5	*M. esculenta* (KM979346.1)	1,543	1,543	1,530	1,507			
6	*S.lycopersicum* (NM001329220.1)	0,270	0,270	0,280	0,298	1,373		
7	Dieng Red Potato	0,073	0,073	0,054	0,060	1,596	0,254	

4. Discussion

In this study, Dieng red potatoes affected by frost were analyzed physiologically and molecularly. Physiologically, electrolyte leakage and chlorophyll content were estimated. When plants experience frost stress, the cell membrane structure will be damaged. The level of damage to the cell membrane can be observed from the electrolyte leakage value using a conductivity meter. Relative conductivity is an effective indicator to evaluate the responsiveness of plants to low temperature stress (Liu *et al.*, 2013). Potatoes exposed to frost cause the cell membrane to change from a relatively liquid state to a more rigid state, resulting in reduced membrane permeability. For such experiments, the plants are subjected to frost compared to normal temperatures. The relative electrolyte leakage of leaves increases rapidly in the presence of frost as shown in Figure 1. Research on electrolyte leakage in potato plants due to low

temperatures was started by Lindstrom and Carter (1985). Temperatures -4 ° C indicates a large number of electrolyte leakage (80-90%) for each potato crop compared to controls stored at 0° C. In 2007, Arvin and Donnelly reported that the average value of potato electrolyte leakage due to cold stress was 61,9% for several cultivars of S. *tuberosum* and reached 47,9% for wild potato species. Plasma membrane is considered to be the site of the main attack when cold injury occurs (Huang *et al.,* 2014). As a result, lipids in the plasma membrane will undergo a transition phase. Liquid crystals or the formation of liquid into a gel or solid will affect the membrane permeability when the temperature drops (Huang *et al.,* 2014; Al-Shuneigat *et al.,* 2015).

Injury due to low temperatures also causes a decrease in chlorophyll content thereby affecting plant photosynthesis (Liu *et al.,* 2013). In plants that are cold tolerant, a decrease in photosynthesis will affect cold acclimation and prevent the attainment of maximum freezing tolerance. This is because the cold acclimation process requires a lot of energy due to changes in metabolism resulting from exposure to low temperatures. Potato plants are very sensitive to frost and cannot tolerate coldness (Chang *et al.,* 2014). As a result, there will be a decrease in chlorophyll content (Figure 2). The decrease in chlorophyll content of leaves exposed to frost in the valley was higher than that of the leaves on the hill. This is suitably described Chung *et al.* (2006) that the valley floor area accumulates more frost, so that the presence of frost causes the accumulation of chlorophyl to be low. Results of determination of potato chlorophyll content in this study are in line with research published previously by Li *et al*. (2021) which showed a similar reduction in chlorophyll content after exposure to low temperatures. The results of research by Li *et al.* (2021) showed that the total chlorophyll content decreased with increasing treatment duration, indicating that chlorophyll synthesis was inhibited during low temperatures.

Inh2 gene encodes the vacuolar invertase inhibitor protein that plays a role in inhibiting the activity of invertase acid. The BLAST results show the maximum percent identity is 97,30%, and the query coverage is 100%. Dieng red potato *inh2* gene along with the alleles INH2alpha* A, INH2alpha* B, INH2alpha* C, and INH2alpha* D of the species *S. tuberosum* shows similarities to the sequence starting at base 42. The difference in the next *Inh2* sequence lies in the gap of 12 bp. Analysis of the predicted protein sequences in Dieng red potatoes showed 178 amino acid residues with a calculated molecular mass of 20.28 kDa and an isoelectric point of 8,866. The INH2 protein prediction of *S. tuberosum* did not show different variations; INH2alpha*A (181aa, 20,51 kDa), INH2alpha*B (180aa, 20,37 kDa), INH2alpha*C (178aa, 20,14 kDa), INH2alpha*D (178aa, 4,47 kDa) with isoelectric point values between 4,47 to 4,69. The high isoelectric point value in Dieng red potatoes is due to the presence of amino acid groups, namely lysine, arginine and histidine which are incompatible with *S. tuberosum*. The isoelectric point value is strongly influenced by the ionized amino acid groups, namely arginine, aspartate, cysteine, histidine, glutamate, lysine, and glutamate (Mohanta *et al.,* 2019). The point of electricity of a protein is very important in understanding the biochemical function of proteins. Phylogenetic tree shows groupings with a bootstrap value of 10000 (Figure 5). The *Inh2* gene was first sequenced in the tobacco plant (Greiner *et al.,* 1998). Currently, the *Inh2* gene has been identified in many plants, such as *Arabidopsis* (Link *et al.,* 2004), corn (Bate *et al.,* 2004), soybeans, sweet potatoes, rice, and tomatoes (Rausch and Greiner 2004; Tymowska-Lalanne and Kreis, 1998). The *Inh2* gene is found in all parts of potato plant. During cold storage on potatoes, vacuolar invertase inhibitors accumulate in CIS-resistant cultivars rather than susceptible cultivars. Increasing the amount of vacuolar invertase inhibitor can contribute to suppressing the activity of invertase acid and preventing the cleavage of sucrose (Datir *et al.,* 2019).

5. Conclusion

Dieng red potato is a crop that is sensitive to frost. Frost injury causes changes in membrane fluidity and decreased photosynthetic capacity. This study shows that the exposure to frost led to increase in electrolyte leakage and decreases leaf chlorophyll levels. Plants that have been exposed to frost in the valley and on the hill show no significant value for physiological changes. This study provides an understanding of the physiological responses of Dieng red potato plants after exposure to frost on a field scale. Further research should include other physiological parameters on a laboratory scale.

The *Inh2* gene can be found in the Dieng red potato plant and evolutionary analysis shows that *Inh2* Dieng red potato is in the same clad as *S. tuberosum*, so its role is very important in inhibiting invertase acid activity and regulating sucrose metabolism. Studying the diversity of *Inh2* gene sequences is very important, especially Dieng red potatoes. This is because the demand for Dieng potatoes will increase every year so that a good cultivar is needed, especially one that can cope with stress due to frost. It is hoped that our research results contain information that can be used further by plant breeders to develop good potato cultivars.

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