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Response of transgenic tobacco with P5CS gene expression to polyethylene glycol-induced drought stress

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Abstract. *Riduan A, Santoso D, Sudarsono. 2024. Response of transgenic tobacco with P5CS gene expression to polyethylene glycol-induced drought stress. Biodiversitas 25: 3974-3984.* Drought stress, a significant obstacle in plant growth and production, can be effectively addressed with the use of polyethylene glycol (PEG). This treatment has shown promise in screening plant germplasm responses to drought stress. The objectives of this experiment were to determine the effects of stress due to PEG treatment at 0%, 5%, or 10% concentration on growth, leaf proline content, and their correlation to stress responses in the T1 seedlings derived from five T0 transgenic Gombel Shili (GS) tobacco P5CS. Positive results of total nucleic acid PCR analysis in T1 seedling populations derived from each of the T0 plants indicated that the regenerated T0 plants were transgenic tobacco integrating P5CS transgene. Results of the experiment revealed that the effects of stress due to PEG treatment indicated stress due to PEG treatment (5% or 10%) reduced the growth plants of all tobacco plants. The stress sensitivity index categorized the T1 plants P5CS transgenic GS tobacco into tolerance, medium tolerance, and sensitivity against PEG-induced stress. Notably, the T1 plants P5CS transgenic GS tobacco exhibited better growth with higher plant height, leaf, biomass, and root dry weight compared to non-transgenic tobacco under stress and non-stress conditions. The over-expression of the P5CS gene led to a significant increase in leaf proline content after drought stress was observed in all transgenic tobacco compared to non-transgenic tobacco.

Keywords: Agrobacterium-mediated genetics transformation, PEG-induced drought stress, proline accumulation, sensitivity index, transgenic tobacco P5CS

INTRODUCTION

In general, drought stress can reportedly be a limiting factor in crop production (Aslam et al. 2022; Yahaya and Shimelis 2022). Drought stress can also inhibit plant growth (Yadav et al. 2020; Zaib et al. 2023) because it negatively influences various physiological processes such as leaf initiation and enlargement (Razi and Muneer 2021). Drought stress can also reduce the total leaf area of a plant and has a negative effect on the photosynthesis process due to decreasing the number of leaf chloroplasts and the rate of photosynthesis (Begna 2021).

Plants, in their remarkable resilience, adapt to reduce water loss through transpiration by decreasing leaf area (Beebe et al. 2013). This is a form of plant response to drought stress, a testament to nature's adaptability. While a decrease in leaf area may seem detrimental to the plant's photosynthesis area, it is a necessary survival strategy. Even under severe drought stress, plants can experience a decrease in fresh leaf weight of up to 12% and still persist (Popova et al. 2006).

Plants have developed a remarkable tolerance to drought stress through osmotic adjustment, a key mechanism that maintains their growth and development. This adjustment involves the osmotic potential of leaf and root cells and a decrease in stomatal density per leaf surface area (Sigit et al. 2022). By regulating tissue osmotic potential, plants can limit water loss through transpiration and maintain cell turgor pressure, ensuring they can absorb water even under severe drought-stress conditions. This adaptation is a testament to the ingenuity of nature (Rasool et al. 2015).

Proline content, a solute in osmotic adjustment, plays a crucial role in plant responses to drought stress (Laskari et al. 2022; Wang et al. 2022). This mechanism allows plants to maintain lower leaf and root water potential and maintain sustained cell turgor under drought stress conditions (Abid et al. 2018). These two abilities are indicators of plant traits that are tolerant to drought stress (Kokkanti et al. 2022). Based on this, the increase in leaf proline content under drought stress conditions can be used as a predictor of plant tolerance to drought stress (Rasool et al. 2015).

Δ1-pyrroline-5-carboxylate synthetase (P5CS) is a crucial regulatory enzyme involved in the proline biosynthetic pathway. Over-expression of P5CS in transgenic plants leads to proline accumulation and has been shown to enhance plant tolerance to drought stress (Sellamuthu et al. 2024). The creation of transgenic Gombel Shili (GS) tobacco, which incorporates the P5CS gene (P5CS transgenic tobacco), has yielded promising results. The analysis revealed that the P5CS transgenic tobacco obtained constitutively was able to express the P5CS gene and accumulate the highest proline in its leaf tissue. This suggests that the

P5CS transgenic tobacco is likely to exhibit tolerance to drought stress. However, to confirm this, further experiments are needed to assess the response of P5CS transgenic tobacco to drought stress need to be carried out.

The osmotic compound polyethylene glycol (PEG) is a reliable tool for reducing the water potential of the medium, effectively simulating drought stress conditions when added to the growing medium (Avci et al. 2017). PEG has been widely reported as an effective method for screening the response of plant germplasm to drought stress (Reyes et al. 2023). PEG has also been used to screen the response of various crops, including grapes, peanuts, sugarcane, soybean, and paddy, against drought stress (Cui et al. 2016; Meher et al. 2018; Sagar et al. 2020; Mishra et al. 2021). The results consistently demonstrate that there was a positive correlation between plant tolerance to stress due to PEG treatment and tolerance to drought stress), reinforcing the reliability of PEG as a drought stress simulator (Meher et al. 2018; Sagar et al. 2020).

This experiment generally aims to evaluate the effect of drought stress on the growth and development of T1 zuriat plants from the T0 generation P5CS transgenic GS tobacco. Specifically, this experiment aims to (i) test the effect of PEG watering on the growth and yield of T1 zuriat plants from T0 generation P5CS transgenic GS tobacco; (ii) analyze the accumulation of proline in the leaves of T1 zuriat plants from T0 generation P5CS transgenic GS tobacco under conditions stress by watering with PEG 6000 solution; and (iii) analyzing the relationship between leaf proline accumulation and growth and yield of T1 zuriat plants from P5CS generation T0 transgenic GS tobacco.

MATERIALS AND METHODS

Preparation of *Agrobacterium* culture and tobacco explants

The presence of the pBI-P5CS plasmid in *Agrobacterium* cells was analyzed by polymerase chain reaction (PCR). Plasmid pBI-P5CS was isolated from *Agrobacterium* cells using a method developed by Budiani et al. (2019). The plasmid isolated from *Agrobacterium* was used as a template for PCR using a pair of P5CS-specific primers (primer forward: 5'-CGGGGGTTCATGAAGGACG-3' and primer reverse: 5'-GAATCGTTAAACATTGTGGACC-3') and if the plasmid carries the P5CS chimeric gene it will produce an amplification product with size 2.4 kb. The reagent components for the PCR reaction consist of 2.5 µL PCR buffer 10×, 0.5 µL dNTP 10 mM, 1 µL each pair of forward and reverse primers 50 ng/µL, 0.2 µL Taq polymerase enzyme 2 U/µL, and 9.8 µl ddH_2O. The PCR reaction was carried out for 35 cycles. Each cycle had the following steps: DNA denaturation at 94°C for 1 minute, primer annealing at 55°C for 1 minute, and primer extension at 72°C for 3 minutes. The PCR results were separated using agarose gel electrophoresis in 0.5× Tris-EDTA (TBE) buffer solution and a voltage of 50 V for 1 hour. After staining with ethidium bromide, the agarose gel was photographed and documented.

Agrobacterium tumefaciens strain LBA4404, which was positive for carrying the binary plasmid pBI-P5CS (Figure 1), was refreshed in Luria Broth (LB) medium with the addition of the antibiotics 100 mg/L kanamycin, 20 mg/L rifampicin, and 8 g/L agar and grown in a room at 28°C for two days. The single colonies obtained were then inoculated into liquid LB medium (100 mL) with the addition of the antibiotic kanamycin 100 mg/L and rifampicin 20 mg/L, shaken using a shaker at 100 rpm for 24 hours, and the bacterial suspension was harvested by centrifugation. The bacterial precipitate was washed twice with MS medium (Murashige and Skoog 2006) without the addition of agarose (liquid MS) and resuspended in liquid MS medium with the addition of the antibiotic rifampicin 20 mg/L. The bacterial density was measured with a spectrophotometer, and the desired density was obtained by diluting the stock suspension until it reached a value of OD=1 (equivalent to a bacterial density of 109 cells/mL) using a liquid MS medium.

Leaf explants were prepared in vitro by germinating tobacco seeds cv. Sterilized Gombel Shili (GS) was grown in MS medium without plant growth regulators (MS) and with the addition of 8 g/L agar. Tobacco seeds were grown in MS medium for 3 months, and fully opened leaves were used as a source of explants.

Transformation of the P5CS gene and regeneration of transgenic plants

The introduction of the chimeric P5CS gene into the GS tobacco genome was carried out with the help of *Agrobacterium.* The tobacco transformation procedure was carried out following the method that had been used previously (Budiani et al. 2019). Tobacco leaf pieces (2×2 cm^2) were soaked in the prepared *Agrobacterium* suspension for 15 minutes, drained to remove excess liquid, and planted in regeneration medium (MR) consisting of MS base medium with the addition of 0.5 mg/L BAP and 8 g/L agar. Cocultivation of explant tissue that has been inoculated with *Agrobacterium* is carried out in MR medium for 2-3 days. After cocultivation, the leaf explants were washed using liquid MS medium with the addition of the antibiotic cefotaxime 300 mg/L and planted in MR medium with the addition of 8 g/L agar, the antibiotic cefotaxime 300 mg/L and kanamycin 100 mg/L (selective MR medium).

Shoots begin to grow from the explant after being planted in a selective MR medium for 1-2 months. After reaching a height of 5 cm, each shoot obtained was propagated in vitro into three parts. One part of the shoot was maintained in in-vitro conditions for conservation purposes, and two parts of the shoot were grown in MS medium for 2 weeks to form plantlets. One plantlet was maintained in vitro, and the other plantlet was planted in a plastic pot (250 mL) containing a sterile mixed medium of sand:compost (1:1) and acclimatized in a room with 100% air humidity and 1000 lux light for two weeks. After the acclimatization period, surviving T0 tobacco seedlings were transferred to the greenhouse and maintained for two weeks at a temperature of 28-35°C, humidity of 75-86%, and lighting of 90-110 lux. T0 tobacco seeds were transferred to plastic bags measuring 30×30x×30 cm^3 (polybags) containing 5 kg of

mixed medium soil:compost:sand (2:1:1), evaluated for growth, and maintained in a greenhouse until they produced T0:1 seeds (T1 zuriat seeds from T0 plants).

Total nucleic acid PCR analysis for the P5CS gene

The presence of the P5CS gene in the genome of each T0 transgenic GS tobacco candidate resulting from genetic transformation and in the T1 zuriat plants of each T0 plant was analyzed using total nucleic acid PCR with specific primers for the P5CS gene. The total genome of tobacco plants was isolated from leaves using the CTAB method developed by Amir et al. (2015) and used as a template for PCR. The reagent components and PCR steps were carried out as previously mentioned, except that the template used was total nucleic acid isolated from each plant tested, carried out using the method previously described. PCR reactions using the pBI-P5CS plasmid were used as meticulous controls (+), while PCR reactions with non-transgenic plant genomes as templates and PCR reactions without DNA templates were each used as two additional controls (-). The total nucleic acid PCR results were separated using agarose gel electrophoresis as previously described.

Transgenic tobacco seeds

T0:1 seed harvested from each T0 generation P5CS transgenic tobacco number (GS-1, GS-2, GS-3, GS-4, and GS-5) were grown in MS medium (Murashige and Skoog 2006) with the addition of 100 mg/L kanamycin. The number of seedlings that were able to grow from seed and that died in the medium with the addition of kanamycin was observed and used to determine the number of transgenes integrated into the genome of each T0 transgenic plant. Only T1 zuriat plant seeds of the T0 generation P5CS transgenic GS tobacco grown in a medium containing kanamycin were used in the experiments.

Preparing tobacco seedlings

Seedlings Zuriat T1 plants from transgenic GS tobacco P5CS generation T0 and non-transgenic GS tobacco were grown in plastic pots (250 mL) containing a mixture of soil:sand:manure (2:1:1) and acclimatized in a humidity-controlled room (100%) and illumination (1000 lux) for one week. After the acclimatization period, the tobacco plants were moved into plastic pots (500 mL) containing a mixture of charcoal husk:coco peat (1:1) and maintained in the greenhouse until they were 2 weeks old (14 days). Maintenance carried out includes watering until saturation every morning and evening.

Effect of PEG stress on growth and yield

After zuriat T1 plant seedlings from transgenic GS tobacco P5CS generation T0, which is resistant to kanamycin, and non-transgenic GS tobacco seedlings were watered with 5% or 10% PEG solution until 60 days after planting (DAP). PEG solutions with concentrations of 5% and 10% were prepared by dissolving PEG in distilled water with a ratio of 5 mg and 10 mg PEG in 100 mL of distilled water, respectively. The PEG solution is watered every two days at 20 mL per plant. Some other T1 transgenic plants were watered every day with water (PEG 0%) until harvest and served as controls.

The experimental unit consisted of 2 treatment factors, namely 6 tobacco seedlings (transgenic and non-transgenic) and 3 concentrations of PEG solution; each treatment was repeated 3 times. The experiment was conducted using a factorial treatment design and a randomized block environment design. Next, to determine the effect of treatment, an analysis of variance was carried out with a significance level of 5%. If the treatment has a significant effect, it will be continued with the Duncan multiple distance test at a level of 5%. Regression and correlation analyses were also performed between stress sensitivity index and leaf dry weight as well as proline content and root length.

Stress sensitivity index

The stress sensitivity index (S) is calculated by the formula developed by Dababat et al. 2015, namely: $S=(1-[Y/Yp])/(1-[X/Xp])$; Y and Yp: respectively are the average observed values for one particular genotype under drought stress conditions and non-stress conditions, while X and Xp are the average observed values for all genotypes under drought stress conditions and non-stress conditions. The S index is calculated using the variable dry weight of leaves per plant (gr). Based on the S index obtained, the transgenic tobacco plants tested were categorized as tolerant if $S < 0.5$, medium tolerant if $0.5 < S < 1$, and sensitive if $S > 1$.

Analysis of proline content

T1 zuriat plant seeds from transgenic GS tobacco P5CS generation T0 with or without PEG watering were harvested for leaves at 60 DAP and analyzed for proline content. Analysis of leaf proline content was carried out using a method developed by Abraham et al. (2010), namely quantitative measurement using the colorimetric method. The proline content of leaves of non-transgenic GS tobacco plants with or without PEG watering was used as a control.

RESULTS AND DISCUSSION

Transformation of the P5CS gene and regeneration of transgenic plants

Agrobacterium tumefacient used in genetic transformation has tested positive for carrying the pBI-P5CS plasmid based on PCR results. PCR amplification using a plasmid template isolated from *Agrobacterium* with specific primers for the P5CS gene produced a piece of amplified DNA with a size of 2.4 kb (Figure 1.A).

The introduction of the P5CS gene into the tobacco genome through genetic transformation with the help of selected *Agrobacterium* was carried out four times, with a total of 136 leaf explants transformed. Three of the four genetic transformations carried out only produced Kan^R shoots but failed to form plantlets due to contamination or death. In the 4th genetic transformation (Table 1), 10 Kan^R shoots were successfully regenerated, and 8 plantlets were gradually obtained. After a period of acclimatization, five T0 generation transgenic tobacco candidates were finally successfully obtained as a result of genetic transformation.

Of the five transgenic tobacco candidates (GS-1, GS-2, GS-3, GS-4, and GS-5) obtained, all were able to produce T0:1 seeds (Table 1). From the data obtained, the percentage of successful formation of shoots, plantlets, and transgenic tobacco candidates resulting from genetic transformation with the help of *Agrobacterium* was 22.8%, 5.9%, and 3.7%, respectively (Table 1).

Total nucleic acid PCR analysis for the P5CS gene

Total nucleic acid PCR analysis using the total nucleic acid template of T0 generation transgenic tobacco candidates and T1 zuriat produced a DNA band of 2.4 kb. A portion of the total nucleic acid PCR results for the P5CS gene is presented in Figure 1.B. Positive results from total nucleic acid PCR for the P5CS gene in T1 tobacco indicated that T0 tobacco no. GS-1, GS-2, GS-3, GS-4, and GS-5 are transgenic tobaccos that integrate the P5CS gene in their genome (P5CS transgenic tobacco).

Effect of PEG stress on growth and yield

All tobacco plants tested were able to grow normally on cocopeat and husk charcoal media. Next, 2 (two) plants died out of 270 plants tested (0.74%). However, this plant death was not caused by the conditions of the growing media or PEG treatment but rather by the plant adaptation process being less than optimal at the acclimatization stage from the culture room to greenhouse climatic conditions.

The results of the observations showed that watering the PEG solution at a concentration of 5% or 10% provided stress conditions for the tobacco plants tested. Watering 5 or 10%, PEG generally reduced plant growth of non-transgenic tobacco plants and T1 zuriat plants of P5CS transgenic GS tobacco (Figure 2).

Table 1. Development of tobacco leaf tissue cv. Gombel Shili is in various stages of genetic transformation with the help of *Agrobacterium* to become a transgenic plant. Genetic transformation to introduce the P5CS gene with the help of *Agrobacterium* was carried out in four experiments

No. exp.	Number of explants	Results of transformation with *Agrobacterium*:			Plants produce seeds
		Bud	Plantlets	Plant	
1.	24	5	- *)	-	-
2.	36	7	-	-	-
3.	48	9	-	-	-
4.	28	10	8	5	5
Total	136	31	8	5	5
P**	-	22.8%	5.9%	3.7%	3.7%

Notes: *[-] Contaminated, so plantlets and plants cannot be obtained. P** is the percentage of the total number of explants, calculated using the P formula=(number of responses/number of explants) ×100%.

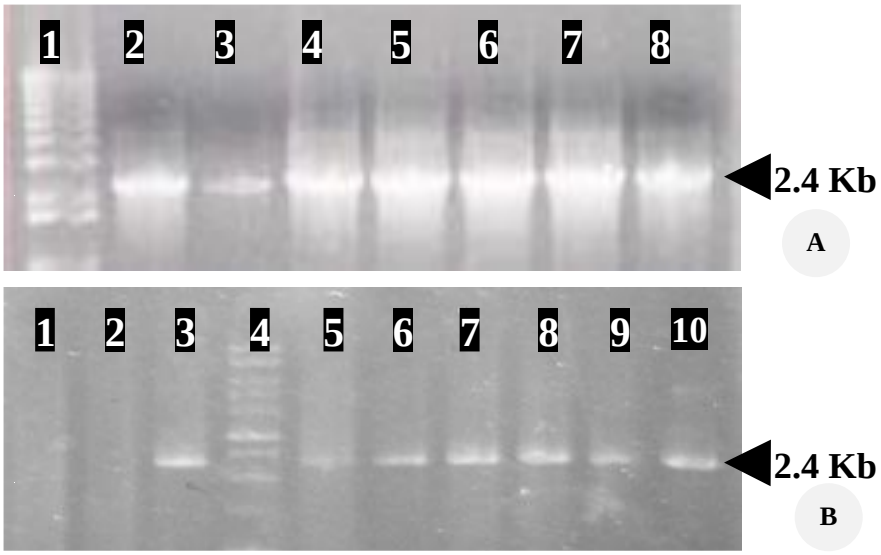

Figure 1. PCR analysis results and total nucleic acid PCR analysis, respectively, to confirm the presence of the pBI-P5CS plasmid in *Agrobacterium tumefaciens* isolate LBA4404 and the P5CS gene in the transgenic tobacco genome. A. PCR results to detect the presence of the pBI-P5CS plasmid with specific primers for the P5CS gene: 1: 1 kb ladder DNA marker; 2 and 3: PCR results with a plasmid template isolated from E. *coli* (positive control); and 4-8: PCR results with plasmid templates isolated from *A. tumefaciens* isolate LBA4404; B.Total nucleic acid yieldPCR to detect integration of the P5CS gene in the transgenic tobacco genome with 1: Non-transgenic tobacco genome template; 2: Without template (replaced with distilled water); 3: pBI-P5CS template; 4: 1 kb ladder DNA marker; and 5-10: PCR results with the T1 zuriat generation transgenic tobacco genome template from T0 plants numbered GS-1, GS-2, GS-3, GS-4, and GS- 5, as well as T0 plant number GS-4

Figure 2. Zuriat T1 plants from T0 generation P5CS transgenic GS tobacco and non-transgenic GS tobacco under conditions A. Without PEG; B. treated with 5% PEG; C. Treated with 10% PEG; No. 0: Non-transgenic GS-0, 1: Transgenic GS-1, 2: GS-2, 3: GS-3, 4: GS-4 and 5: GS-5

The 5% PEG watering treatment significantly reduced plant growth, but a greater decrease occurred in non-transgenic tobacco plants compared to T1 zuriat plants from the T0 generation P5CS transgenic GS tobacco except for the leaf number variable (Table 2; Table 3). On stress conditions by watering with 5% PEG solution, all T1 zuriat plants from the T0 generation P5CS transgenic GS tobacco that were tested showed mean plant height, number of leaves, and node length that was not significantly different. For leaf area, dry leaf weight, shoot dry weight, total dry biomass weight, and root dry weight variables, T1 zuriat plants from transgenic P5CS tobacco generation T0 number GS-2 showed the highest growth. In comparison, T1 zuriat plants from P5CS transgenic tobacco generation T0 number GS-2 GS-1, GS-3, GS-4, and GS-5 showed growth that was not significantly different (Table 2; Table 3).

Stress by watering with 10% PEG solution also significantly reduced the growth of all tobacco plants tested. Watering 10%, PEG can reduce plant height, node length, leaf area, and dry leaf weight, shoot dry weight and total dry biomass weight, and root dry weight of non-transgenic GS tobacco plants, which tend to be greater than T1 zuriat plants from T0 generation P5CS transgenic GS tobacco. However, for the number of leaves variable, the reduction in growth between P5CS transgenic and non-transgenic tobacco plants was not significantly different except for Transgenic GS-4 (Table 2; Table 3).

Stress by watering with 10% PEG solution caused all the T1 zuriat plants from the T0 generation P5CS transgenic GS tobacco to show growth that was not significantly different. Still, there was a tendency for the T1 zuriat plants from the T0 generation P5CS transgenic tobacco number GS-2 to show the highest growth than T1 zuriat plants from other T0 generation P5CS transgenic GS tobacco (GS-1, GS-3, GS-4 and GS-5) (Table 2; Table 3).

Specifically for the root length variable, stress by watering with 5% and 10% PEG solutions can actually lengthen plant roots in both T1 zuriat plants from P5CS generation T0 transgenic GS tobacco and non-transgenic GS tobacco. However, root elongation that occurred in T1 zuriat plants from transgenic GS tobacco P5CS generation T0 was significantly greater compared to non-transgenic GS plants (Table 2; Figure 3).

Stress sensitivity index

The sensitivity index (S) is a key component of our research process, calculated based on the dry leaf weight of tobacco plants. It guides us in categorizing the tolerance levels of the plants. For instance, non-transgenic GS tobacco plants are categorized as medium tolerant. In comparison, T1 zuriat plants from P5CS generation T0 transgenic GS tobacco are segregated into the categories of tolerant, medium tolerant, and sensitive to stress with solution watering. PEG 5% or 10% in the vegetative growth phase. Based on the S index in the population of T1 zuriat plants from transgenic GS tobacco P5CS generation T0, it was found that T1 zuriat plants from transgenic tobacco P5CS generation T0 number GS-2 had 2, 7 and 6 plants respectively, GS-3 had 4, 2 and 9 plants, GS-4 had 5, 4 and 5 plants and GS-5 had 3, 8 and 4 plants which were categorized as tolerant, medium tolerant and sensitive to drought stress by watering with 5% PEG solution while T1 zuriat plants from P5CS transgenic tobacco generation T0 GS-1 number only has 3 medium tolerant genotypes and 12 sensitive genotypes (Figure 4).

Table 2. The effect of watering polyethylene glycol (PEG) with concentrations of 0%, 5%, and 10% at the age of 15-60 DAP on shoot, roots, total biomass weight, and root length of T1 zuriat plants of transgenic GS tobacco P5CS generation T0 and non-GS tobacco transgenic

Tested variables and genotypes	Variable values on PEG watering concentration					
	0%		5%		10%	
Shoot dry weight (g):						
Non-transgenic GS-0	0.81	aE	0.46	bC	0.31	bB
Transgenic GS-1	1.79	aA	0.65	bB	0.56	bA
Transgenic GS-2	1.52	aB	0.91	bA	0.62	cA
Transgenic GS-3	1.02	aD	0.57	bBC	0.36	cB
Transgenic GS-4	1.18	aC	0.63	bBC	0.45	bAB
Transgenic GS-5	1.20	aC	0.68	bB	0.36	cB
Dry root weight (g):						
Non-transgenic GS-0	0.49	aC	0.33	abB	0.24	bB
Transgenic GS-1	1.09	aA	0.46	bB	0.37	bAB
Transgenic GS-2	1.02	aA	0.63	bA	0.51	bA
Transgenic GS-3	0.64	aB	0.43	bB	0.34	bAB
Transgenic GS-4	0.68	aB	0.50	bAB	0.41	bAB
Transgenic GS-5	0.64	aB	0.48	bAB	0.34	bAB
Total dry biomass weight (g):						
Non-transgenic GS-0	1.31	aD	0.78	bC	0.55	cC
Transgenic GS-1	2.88	aA	1.11	bB	0.93	bAB
Transgenic GS-2	2.54	aB	1.54	bA	1.13	cA
Transgenic GS-3	1.65	aC	1.01	bBC	0.70	bBC
Transgenic GS-4	1.86	aC	1.13	bB	0.86	bABC
Transgenic GS-5	1.83	aC	1.16	bB	0.71	cBC
Root length (cm):						
Non-transgenic GS-0	11.8	bB	13.6	abB	15.4	aC
Transgenic GS-1	17.9	cA	23.0	bA	25.7	aAB
Transgenic GS-2	14.2	bAB	24.3	aA	26.0	aAB
Transgenic GS-3	15.0	bAB	22.9	aA	24.3	aAB
Transgenic GS-4	16.9	bAB	20.3	bA	27.3	aA
Transgenic GS-5	15.0	bAB	21.5	aA	23.0	aB

Note: Mean data in rows with the same lower-case letters or columns with the same capital letters are not significantly different based on Duncan's multiple range test at α=0.05

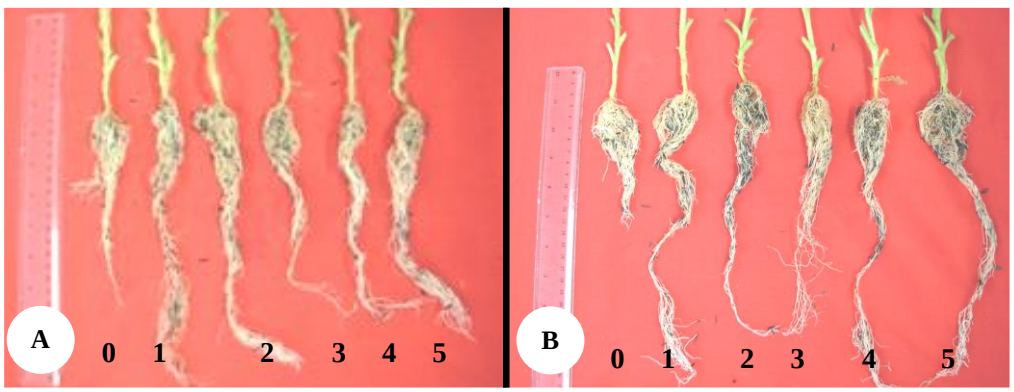

Figure 3. Zuriat T1 plant roots from T0 generation P5CS transgenic GS tobacco and non-transgenic GS tobacco under conditions: A treated with 5% PEG; B. treated with 10% PEG; No.0: Non-transgenic GS-0, 1: Transgenic GS-1, 2: GS-2, 3: GS-3, 4: GS-4 and 5: GS-5

Table 3. The effect of watering polyethylene glycol (PEG) with concentrations of 0%, 5%, and 10% at the age of 15-60 DAP on plant height, number, area, and weight of dry leaves and node length of T1 plants zuriat transgenic GS tobacco P5CS generation T0 and non-transgenic GS tobacco

Tested variables and genotypes	Variable values on PEG watering concentration		
	0%	5%	10%
Plant height (cm):			
Non-transgenic GS-0	11.0 aD	4.8 bB	3.4 cB
Transgenic GS-1	18.9 aA	8.9 bA	6.6 cA
Transgenic GS-2	17.8 aAB	8.5 bA	6.2 cA
Transgenic GS-3	16.0 aC	7.7 bA	5.8 cA
Transgenic GS-4	17.4 aBC	8.8 bA	6.6 cA
Transgenic GS-5	16.4 aBC	8.7 bA	5.6 cA
Number of leaves (sheets):			
Non-transgenic GS-0	7.4 aB	7.3 aA	7.8 aAB
Transgenic GS-1	8.3 aA	7.3 bA	7.9 aAB
Transgenic GS-2	8.1 aA	7.5 bA	8.5 aA
Transgenic GS-3	7.8 aA	7.5 aA	7.6 aB
Transgenic GS-4	8.1 aA	7.7 bA	7.7 bB
Transgenic GS-5	7.2 bB	7.1 bA	7.9 aAB
Leaf area (cm^2):			
Non-transgenic GS-0	162.8 aC	111.9 bC	87.3 bB
Transgenic GS-1	285.8 aA	160.9 bB	160.1 bA
Transgenic GS-2	294.3 aA	218.7 bA	166.4 cA
Transgenic GS-3	204.4 aB	144.0 bB	101.3 bB
Transgenic GS-4	226.1 aB	161.3 bB	128.6 bAB
Transgenic GS-5	241.5 aB	174.5 bB	104.0 cB
Dry leaf weight (g):			
Non-transgenic GS-0	0.41 aD	0.28 bC	0.22 bB
Transgenic GS-1	0.90 aA	0.40 bB	0.40 bA
Transgenic GS-2	0.74 aB	0.55 bA	0.42 cA
Transgenic GS-3	0.51 aCD	0.36 bBC	0.25 bB
Transgenic GS-4	0.57 aC	0.40 bB	0.32 bAB
Transgenic GS-5	0.61 aC	0.44 bB	0.26 cB
Node length (cm):			
Non-transgenic GS-0	1.73 aC	0.77 bB	0.52 bB
Transgenic GS-1	2.67 aA	1.43 bA	0.97 cA
Transgenic GS-2	2.57 aAB	1.32 bA	0.84 cA
Transgenic GS-3	2.40 aB	1.20 bA	0.88 cA
Transgenic GS-4	2.47 aAB	1.35 bA	1.01 cA
Transgenic GS-5	2.65 aAB	1.42 bA	0.82 cA

Note: Mean data in rows with the same lower-case letters or columns with the same capital letters are not significantly different based on Duncan's multiple range test at α=0.05

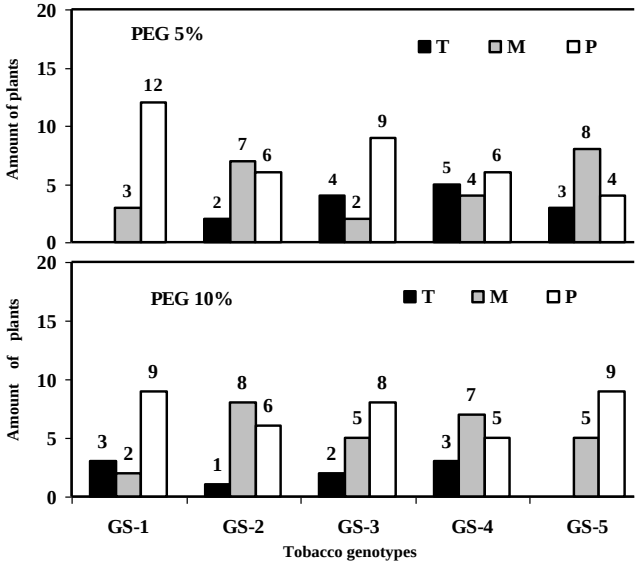

Figure 4. Frequency distribution of responses of T1 zuriat plants from T0 generation P5CS transgenic GS tobacco and non-transgenic GS tobacco to stress by watering with 5% or 10% PEG solution. P: Sensitive; M: Tolerant medium, T: Tolerant

Based on the S Index under stress by watering with 10% PEG solution, T1 zuriat plants from transgenic tobacco P5CS generation T0 number GS-1 had 3, 2, and 9 plants, GS-2 had 1, 8, and 6 plants, GS-3 had 2, 5, and 6 plants, and GS-4 had 3, 7, and 5 plants categorized as tolerant, medium tolerant, and sensitive, while T1 zuriat plants from transgenic tobacco P5CS generation T0 number GS-5 only had 5 medium-tolerant and 9 genotypes sensitive (Figure 4).

From the relationship between the sensitivity index value and the dry leaf weight of tobacco plants, it can be seen that the lower the sensitivity index value of the plant or the more tolerant the plant is to drought stress, the higher the dry leaf weight it has, both in the 5% PEG and 10% PEG solution watering treatments (Figure 5). From this correlation, it can also be seen that the non-transgenic tobacco plants, which are categorized as medium, are tolerant to 5% and 10% PEG watering, but the dry leaf weight they have is much lower compared to the T1 zuriat plants of the tolerant T0 generation P5CS transgenic GS tobacco and the medium tolerant against stress by watering with PEG solution.

The result of calculating the plant sensitivity index to stress by watering with 5% PEG or 10% PEG solution with dry leaf weight as a variable shows that non-transgenic GS tobacco plants are categorized as medium tolerant. However, T1 zuriat plants from P5CS generation T0 transgenic GS tobacco, despite being segregated into the tolerant category, show a promising potential, being both tolerant and sensitive medium. In general, T1 zuriat plants from the T0 generation P5CS transgenic GS tobacco still showed higher growth and yield compared to non-transgenic GS tobacco under stress conditions by watering with 5% and 10% PEG solutions.

Effect of PEG stress on proline content

Under non-stressful growing environmental conditions (without PEG), all T1 zuriat plants from P5CS transgenic GS tobacco T0 generation have shown a tendency to have higher proline content compared to non-transgenic GS tobacco plants. The effect of stress by watering with 5 and 10% PEG solutions generally increased the proline content in all tobacco plants tested (non-transgenic and transgenic). Still, statistically, a significant increase in proline only occurred in T1 zuriat plants from P5CS transgenic GS tobacco. T0 generation except number GS-1, while non-transgenic GS tobacco plants did not show a significant increase in proline content (Figure 6).

Even under stress-induced stress by watering with PEG solution, only T1 zuriat plants from transgenic P5CS tobacco generation T0 number GS-1 did not show a significant increase in proline content, but it was still higher compared to non-transgenic GS tobacco both under stress conditions by watering both 5% and 10% PEG solutions. As the severity of the drought stress increased, these T1 zuriat plants from the T0 generation P5CS transgenic GS tobacco tested showed higher proline content, with those stressed by 10% PEG solution exhibiting even greater proline content than under 5% PEG watering conditions. This once again highlights the superiority of transgenic plants in coping with drought stress (Figure 6).

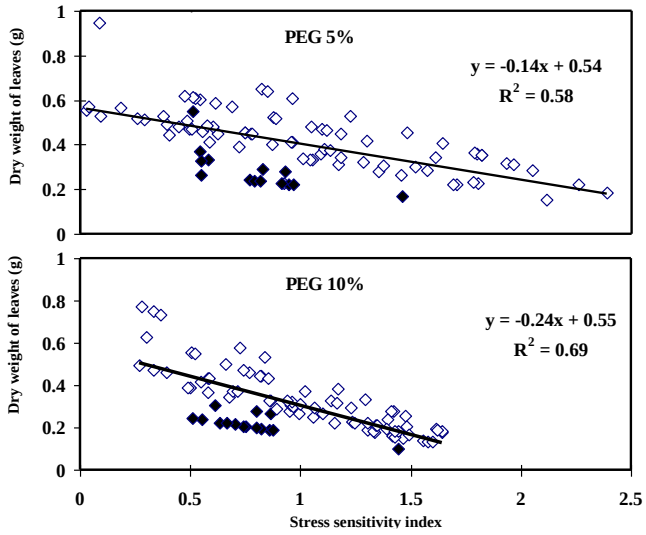

Figure 5. The relationship between stress sensitivity index values and watering with 5% or 10% PEG solution was calculated using dry leaf weight data from non-transgenic GS (♦) and transgenic tobacco (◊). A sensitivity index value ≤0.5 is categorized as tolerant, between 0.5 and 1 is categorized as medium tolerant, and >1 is categorized as sensitive

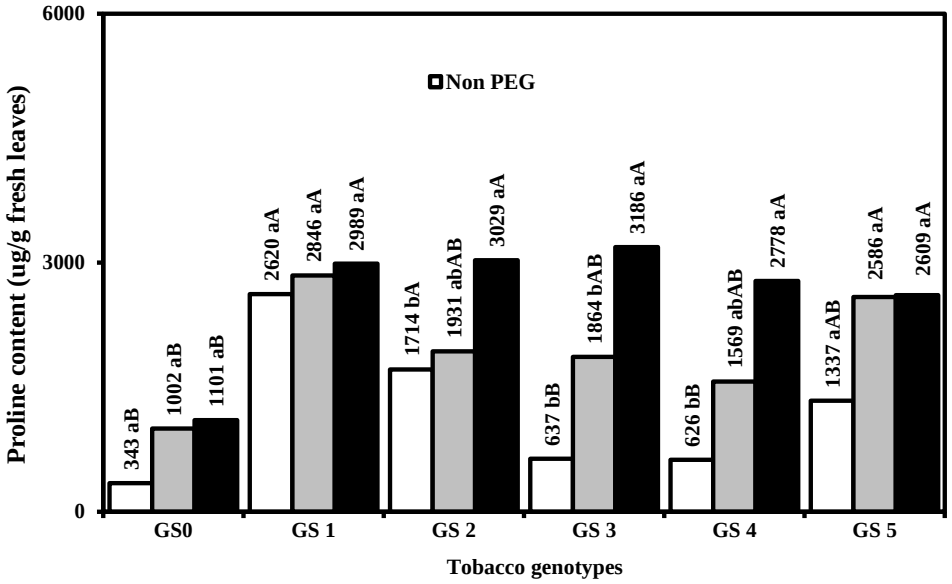

Figure 6. The effect of watering PEG 5 or 10% in the period 15 to 60 days after planting (DAP) on the total proline content of leaves T1 zuriat plants from transgenic GS tobacco P5CS generation T0. Leaf samples were taken at 60 DAP of the plant. Mean data for certain tobacco genotypes followed by the same lower-case letter or for certain stress treatments followed by the same capital letter are not significantly different based on Duncan's multiple range test at α=0.05

Under stress conditions with 5% PEG watering, non-transgenic GS tobacco plants had a proline content of 1002 µg/g fresh leaves, whereas T1 zuriat plants from the T0 generation P5CS transgenic GS tobacco had a significantly higher proline content, ranging from 1569 to 2846 µg/g fresh leaves. This trend was also observed under 10% PEG watering conditions, with non-transgenic tobacco plants having a proline content of 1101 µg/g fresh leaves and T1 zuriat plants from the T0 generation P5CS transgenic GS tobacco showing a proline content between 2609 to 3186 µg/g fresh leaves (Figure 6). This comparison clearly demonstrates the potential benefits of transgenic plants under stress conditions.

The results of the analysis of variance, there was a significant correlation between root length and leaf proline content between root length and leaf proline content under stress conditions with 5% and 10% PEG solution watering with p-value <0.05, namely 0.027 and 0.043; R2 values of 0.48 and 0.36 respectively (Figure 7). The higher the proline content of the leaves of the tobacco plants tested, the longer the roots produced by the plants, both under stress conditions by watering with 5% PEG and 10% PEG solutions. T1 zuriat plants from transgenic GS tobacco P5CS generation T0 showed a longer average root length with higher proline content compared to non-transgenic GS tobacco both under stress conditions with 5% PEG solution watering and 10% PEG solution watering conditions.

Discussion

The pBI-P5CS plasmid is a binary plasmid that carries the P5CS chimeric gene construct in its T-DNA. The P5CS gene is a gene encoding a key enzyme that plays a role in the proline biosynthesis process. The binary plasmid pBI-P5CS has been introduced into A. *tumefaciens* isolate LBA4404 cells which can transfer the T-DNA construct on the binary plasmid pBI-P5CS into the plant genome. The A. *tumefaciens* isolate LBA4404 cells used were proven to carry the pBI-P5CS plasmid based on positive results for PCR amplification using a pair of P5CS-specific primers and a plasmid template isolated from *Agrobacterium.*

In this experiment, five T0 generation transgenic tobacco candidates that were resistant to kanamycin were successfully regenerated into seedlings for 3 months. Positive results from total nucleic acid PCR analysis using P5CS specific primers and total nucleic acid templates from T0 and T1 generation plants (the zuriat of each T0 plant) prove that the five T0 tobacco numbers obtained are transgenic tobacco that integrates the P5CS gene. The T0 generation P5CS transgenic GS tobacco (GS-1, GS-2, GS-3, GS-4, and GS-5) grown in the greenhouse flowered and produced T0:1 seeds after 3.5 months.

Watering PEG solutions can do more controlled treatment of drought stress in plants, because in water, PEG can dissolve and can cause a homogeneous decrease in water potential, giving the impression of water stress for plants. Total mass or total molecular sub-units (-CH_2-O-CH_2-) in the PEG polymer chain is an important factor that controls water potential ψ_w. The free energy of H_2O can be proportionally reduced according to the length of the PEG polymer chain. The matrix strength of the ethylene oxide subunits in the PEG polymer probably controls the decline ψ_w caused by PEG. The H_2O molecule will be attracted to the oxygen atom of the ethylene oxide subunit via hydrogen bonds (Fonzo et al. 2019).

The negative impact of osmotic stress due to drought at a water potential of -0.01 to 0.5 MPa is that it can reduce the synthesis of cell walls, proteins, formation of protochlorophyll, and cell division (Salisbury and Ross 2009). Watering 5% and 10% PEG equivalent to osmotic potential (ψ_w) -0.03 and -0.09 MPa (Pawar and Veena 2020) significantly reduced the growth of tobacco plants, both non-transgenic tobacco plants and P5CS transgenic tobacco plants. This indicates that PEG watering can inhibit growth, as occurs as a result of drought stress treatment. From the results of previous research, it was reported that drought stress can reduce the total dry weight of vegetative organs and leaf area of beans (Seleiman et al. 2021), decrease fresh weight, chlorophyll content, and root vitality of passion fruit (Qi et al. 2023) and inhibit growth rice plant callus (Adrees et al. 2024).

There were differences in response to PEG watering between P5CS transgenic tobacco plants and non-transgenic tobacco plants. P5CS transgenic tobacco plants tended to show higher growth compared to non-transgenic plants due to 5% and 10% PEG watering treatment. Several P5CS transgenic tobacco plants are categorized as sensitive to 5% and 10% PEG watering but still show higher dry leaf weights than non-transgenic tobacco plants, which are categorized as medium tolerant to drought stress. This condition is thought to be closely related to the higher proline content of transgenic tobacco plants as a result of the expression of the P5CS gene compared to non-transgenic tobacco plants, both under stress (watered with PEG) and non-stress (without PEG) conditions.

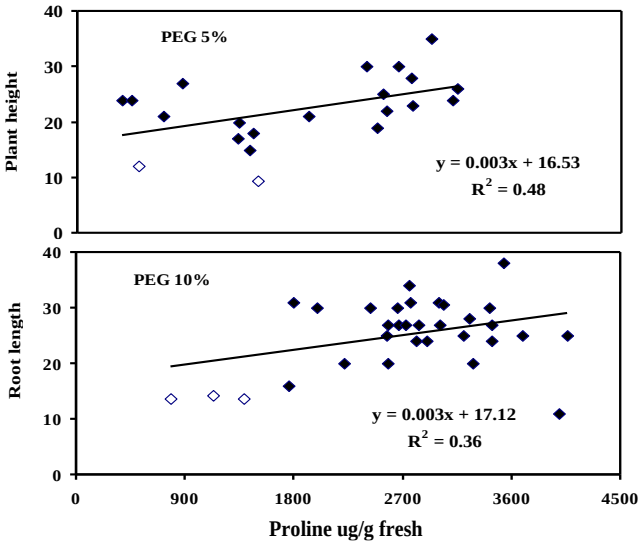

Figure 7. Regression between the index of sensitivity to drought and the variable root length of T1 zuriat plants from P5CS generation T0 transgenic GS tobacco under stress conditions with watering with 5% and 10% PEG solutions. Transgenic tobacco plants (♦) and non-transgenic (◊)

Research results by Xiaoyang et al. (2012) show that the P5CS gene in tomato plants is able to increase proline levels more than 6-fold in root and leaf tissue and is able to reduce the negative impact on growth due to drought stress. Transgenic tobacco plants were better able to maintain their growth under 5% and 10% PEG stress conditions, which also correlated with increased root length. T1 zuriat plants from transgenic GS tobacco P5CS generation T0 showed longer root length due to stress by watering with PEG solution compared to non-transgenic tobacco plants; this is also thought to be related to an increase in high leaf proline accumulation as a result of over-expression of the P5CS gene. The R2 values of 0.48 and 0.36 indicate a moderate positive correlation between the over-expression of the P5CS gene and the observed increase in root length. Thus, T1 zuriat plants from the T0 generation P5CS transgenic GS tobacco are thought to be able to absorb water and nutrients from the soil under stress conditions and can show better growth and reduce the negative impacts caused by stress by watering with PEG solution. This is also in line with the research results by Sabbioni et al. (2021) in rice plants introduced with the P5CS gene from moth bean (*Vigna aconitifolia*), which showed an increase in P5CS gene activity up to 9.8 times and proline content up to 252% and showed higher root length, shoot weight and root weight variables compared to control plants. According to Jian et al. (2021), one of the mechanisms of plant resistance to drought is through effective water absorption, manifested in the form of elongated, deep, and thick root morphology. In response to drought conditions, plants can also increase root growth to maximize water absorption (Culpepper 2019; Seleiman et al. 2021).

In conclusion, the results of research on the effect of stress due to PEG treatment showed that stress due to PEG treatment (5% or 10%) reduced plant height, node length, leaf area and dry leaf weight, leaves, shoots, biomass, and root dry weight of non-transgenic GS tobacco plants which tend to be greater than T1 zuriat plants from T0 generation P5CS transgenic GS tobacco. The stress sensitivity index calculated using leaf dry weight characteristics grouped T1 plants derived from P5CS transgenic GS tobacco into moderate tolerance, moderate tolerance, and sensitivity to PEG-induced stress. In comparison, non-transgenic GS tobacco plants had moderate tolerance. T1 plants derived from GS P5CS transgenic tobacco showed a response to more growth compared with non-transgenic tobacco under stress and non-stress conditions. A higher increase in leaf proline content after drought stress occurred in all transgenic tobacco, while a lower increase occurred in non-transgenic tobacco. Increased leaf proline content due to overexpression of the P5CS gene under 5% or 10% PEG was stress-induced drought correlated with the drought tolerance phenotype in T1 plants derived from transgenic GS tobacco.

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