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SUGARCANE COMMERCIAL CULTIVARS WITH DROUGHT STRESS TOLERANCE ON IN VITRO AND GREENHOUSE SCALES

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SUMMARY

Sugarcane (*Saccharum officinarum* L.) is a significant agricultural commodity, contributing approximately 75% of granulated sugar worldwide. In 2022, a decline in productivity occurred compared to 2019 and 2020. This prompted the development of a breeding program aimed to determine the optimum induction concentration of PEG-6000, identify sugarcane cultivars tolerant to drought stress, and analyze commercial sugarcane cultivars through molecular characterization of the *P5CS* gene. The research transpired at the PT Gunung Madu Plantations (PT GMP), Lampung, Indonesia. Three commercial cultivars, namely, GMP-3, PSJT-941, and GP-11, served as samples in this study. According to the analysis of variance, the results showed the optimum concentration for inducing drought stress was 20% with a treatment duration of five weeks on an in vitro culture. For the molecular scale using sugarcane genotypes in the greenhouse, the sugarcane cultivars with the potential for tolerance to drought stress were PSJT-941, GMP-3, and GP-11, respectively, as supported by sequencing results.

Keywords: Sugarcane (*S. officinarum* L.), drought stress, genotypes, greenhouse conditions, in vitro culture, molecular markers, PEG-6000

Key findings: In sugarcane (*S. officinarum* L.), the morphological observations, carried out at in vitro condition, showed the optimal concentration of PEG-6000 at 20% while at greenhouse condition, obtaining narrow leaf width characteristics for cultivars GMP-3 and PSJT-941, with the cultivar GP-11

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owning the wide leaves. Molecular markers identified for the three cultivars ran through sequencing and phylogenetic analysis, shared a close relationship with the research sample from Bogor, Indonesia.

INTRODUCTION

Sugarcane (*Saccharum officinarum* L.) is an important and valuable crop with a high sugar content in the stems, utilized as raw material for the sugar industry (Windiyani *et al*., 2022). Moreover, sugarcane can be useful for paper introduction and animal feed. Sugarcane is a recognized agriculture commodity, producing over 75% of granulated sugar globally (Mahfut *et al*., 2023b) and plays a significant role in the economies of various countries through sugar exports (Dlamini, 2021). However, fluctuations in productivity have surfaced, with a decline in 2022 compared to 2019 and 2020 in Indonesia (Mahfut *et al*., 2024a; 2024b).

The decrease in sugarcane productivity is due to several aspects, including reduced sugarcane area (Sukmawati *et al*., 2021; Aritonang *et al*., 2024; Nuraini *et al*., 2024; Simamora *et al*., 2024), plant pests and diseases (Mahfut, 2020; 2021; Mahfut *et al*., 2020b; 2020c; 2021b; Anbiya *et al*., 2024; Septiana *et al*., 2024), and unavailable superior sugarcane cultivars tolerant to drought stress (Meiriani *et al*., 2020). Sugarcane's initial cultivation phase requires relatively more water, which decreases as the plant mature (Banerjee *et al*., 2015). Past findings reported 14 sugarcane genotypes with treatment of drought stress showed diverse responses to marked dryness with symptom leaf roll to use reduced transpiration and uptake sun rays with decline in chlorophyll rate (Tippayawat *et al*., 2023). Drought grip is one of the crucial factors and barrier in sugarcane production (Mahfut *et al*., 2024a; 2024b; 2024c).

PT Gunung Madu Plantations (GMP) is a sugar company striving to increase sugar production through developing superior varieties tolerant to drought stress (Mahfut *et al*., 2023a; 2023c; 2023d). In improving the productivity and fulfilling the country’s sugar requirement, the sugarcane genotypes breeding program initiative began to develop drought tolerant cultivars under the field conditions at the PT GMP. It mainly uses in vitro culture with Polyethylene Glycol (PEG-6000) in liquid medium and molecular markers through DNA isolation under drought stress conditions in a greenhouse.

The use of PEG-6000 in vitro culture simulates real drought stress conditions because plant do not absorb the PEG. Only root cell wall cellulose is penetrable by PEG with a heavy molecule of 3500 (Mahfut *et al*., 2025). The identification with molecular markers employed DNA extraction and separation from cellulose and other cell material. The third stage was to separate the DNA from other components and get its pure form. The DNA testing proceeded with quantity and quality tests. Quantity test continued through spectrophotometer by comparing A260 nm and A280 nm. The presented study sought to determine the optimum concentration of PEG-6000 for screening genotypes and identify the drought stress-tolerant cultivars at PT GMP.

MATERIALS AND METHODS

Plant material

The promising research, conducted both in vitro and in greenhouse scales, happened during 2021 and 2023, respectively. The breeding material included plantlets of the sugarcane (*S. officinarum* L.) cultivars, GMP-3, PSJT-941, and GP-11, with in vitro cultivation using Murashige Skoog medium liquid with PEG-6000 solution treatments at four concentrations (0% [control], 15%, 20%, and 25%). For the greenhouse scale, samples were six-month-old bud chips, while for the molecular characterization of the P5CS gene, the study used a 27-day-old leaf sample collected after treatment in the greenhouse. Both the in vitro and greenhouse studies used the complete random design (CRD) with five replications.

Morphological characterization

Morphological characterization on the in vitro scale ran for five weeks after the application of PEG-6000. Data recording continued every seven days, with observations on the dry plantlets in percentage. At five weeks after the PEG application, observations progressed on the volume and length of roots, relative water content, and drought sensitivity index. Morphological observations on each parameter followed the method of Putra *et al*. (2024a; 2024b) and Mahfut *et al*. (2023b; 2024a).

Morphological characterization on the greenhouse scale commenced five weeks after planting and by treating with holding water for six days. Observations continued on leaf characteristics, leaf midribs, and moisture content. Leaf characters consist of curved blades, width, color, triangle color, ears, and leaf position. The leaf midrib consists of position, area width, distance between the tops of the area, density of the dorsal spine, looseness nature of the leaf midrib, and the color of the leaf midrib. Moisture content analysis started by taking soil samples at three planting locations at a depth of 10–15 cm, and weighing as much as 10 g of soil.

Molecular characterization

The initial stage of DNA isolation began by taking a sample of 27-day-old sugarcane leaf using CTAB following the method of Asadudin *et al*. (2024); Mahfut *et al*. (2024b); Putera *et al*. (2024). The quantitative test continued using nanodrop spectrophotometer. Amplification of the P5CS gene transpired using three specific primers at each annealing temperature, namely, P5CS1 (55 °C), P5CS2 (59.2 °C), and P5CS3 (60 °C).

Statistical analysis

The data obtained from morphological characterization underwent quantitative analysis through statistical test using analysis of variance (ɑ = 5%). By getting the significant differences, the Real Difference Smallest ($RDS_{0.05}$) test was effective for the comparison of means. Data obtained through molecular characterization incurred descriptive assessment, with the scores attained by calculating Polymorphism Information Content (PIC) values. Meanwhile, entering the sequencing results into BLAST at the NCBI for alignment used the Clustal W Alignment BioEdit and MEGA V.11.0.11, used to present the phylogenetic tree. Phylogenetic analysis of each branch continued, engaging the Unweighted Pair Group Method and Arithmetic Mean (UPGMA) bootstrap-1000 (Mahfut *et al*., 2020a; Sari *et al*., 2024).

RESULTS AND DISCUSSION

Morphology characterization on in vitro scale

Dried plantlets (%)

In sugarcane (*S. officinarum* L.), the results revealed the PEG with higher concentration emerged with the higher percentage of the produced dry plantlets. Apart from concentration, the duration of PEG application in the planting medium also had a significant influence, particularly 3–5 weeks after application (Table 1). In the third week, the highest percentage of dry plantlets reached 53% at a PEG concentration of 25%. In the fourth week, it reached 69%, and in the fifth week, it reached 92%. At the PEG concentration of 20% for four weeks, it caused plantlets to dry by 85.84%. This shows increasing PEG concentration will increase dry plantlets. Aristya *et al*. (2020) reported an increase in the percentage of dry plantlets can be evident with higher PEG concentrations in sugarcane, which then causes a decrease in the osmosis process in the cells.

Root volume, length, and relative water content

The one-way analysis of variance displayed the parameters of root volume and root length for the sugarcane cultivars PSJT-941, GMP-3, and

Table 1. Dry plantlets (%) with addition of PEG-6000 in three sugarcane cultivars at the PT GMP on in vitro scale.

Cultivars	PEG concentration (%)	Percentage of dry plantlets at the other week		
		III	IV	V
PSJT-941	0	0.22^{e}	0.44^{cde}	0.59^{d}
	15	0.27^{cde}	0.51^{bcd}	0.69^{cd}
	20	0.33^{bc}	0.53^{bc}	0.86^{ab}
	25	0.33^{bc}	0.67^{a}	0.92^{a}
GMP-3	0	0.31cd	0.40^{def}	0.43^{e}
	15	0.39^{b}	0.48^{cd}	0.58^{d}
	20	0.34^{bc}	0.64^{ab}	0.76^{bc}
	25	0.53^{a}	0.69^{a}	0.87^{ab}
GP-11	0	0.25^{de}	0.31^{f}	0.34^{e}
	15	0.27^{cde}	0.33^{eph}	0.42^{e}
	20	0.31^{cd}	0.35^{eph}	0.42^{e}
	25	0.29^{cd}	0.41^{def}	0.44^{e}
$RDS_{0.05}$		0.0750*	0.1224*	0.1238*

Table 2. Root length and volume of plantlets of three sugarcane cultivars at the PT GMP on in vitro scale.

Cultivars	PEG concentration (%)	Root length (cm)	Root volume (ml)
PSJT-941	0	5.00^{a}	4.67^{bcd}
	15	4.33^{ab}	3.00^{cd}
	20	4.00^{ab}	3.00^{cd}
	25	3.33^{b}	2.33^{d}
GMP-3	0	3.33^{b}	8.67^{a}
	15	4.00^{ab}	7.33^{ab}
	20	3.33^{b}	6.00^{abc}
	25	3.67^{b}	5.00^{bcd}
GP-11	0	1.33^{c}	2.00^{d}
	15	2.00^{c}	3.00^{cd}
	20	2.00^{c}	1.67^{d}
	25	1.67^{c}	1.67^{d}
	$RDS_{0.05}$	1.2064*	3.4491*

GP-11, showing the genotypes had a noteworthy effect on the manifestation of these traits (Table 2). However, the PEG concentrations and their interaction with sugarcane cultivars exhibited no considerable influence. Sugarcane cultivars resistant to drought stress maintained higher water content, essential for the continuity of metabolic processes (Renfiyeni *et al*., 2019). A PEG with the control treatment (concentration of 0%) gave the highest water content value for the cultivars compared with the PEG concentration of 15%, 20%, and 25%. N'zi *et al*. (2023) stated genotypes with tolerance to drought stress conditions will produce higher growth than the sensitive genotypes. The three sugarcane cultivars with PEG (25%) provided relatively lower water content values than other concentrations (Table 3). This is because the PEG concentration of 25% contains concentrated levels of PEG-6000, causing lesser water needs and retaining the water content below.

The outcomes further disclosed for the three sugarcane cultivars, it indicated the cultivar GMP-3 has the relatively higher water content than the two other cultivars PSJT-941 and GP-11. Relative water content is a variable of plant resistance in facing drought stress because it describes the water status and turgor pressure of leaf cells, especially when sugarcane plants experience a decrease in water potential.

Sugarcane cultivars that suit the characteristics of the growing environment will enhance productivity (Leanasawat *et al*., 2021). According to Mahfut *et al*. (2023), a plant's quick response to drought stress is closing its stomata. Then, in the long term, the roots will extend searching a water source. According to Sajid *et al*. (2023), root volume served to determine whether a plant is stressed by drought. In contrast to the length of the roots, which becomes longer when experiencing a stressed condition, the stressed root volume will become lower because the stressed roots have less water content.

Drought sensitivity index

The drought sensitivity index is one of the indices used to assess a downward variation in yield caused by a suboptimal environment compared with an optimal environment (Fatimah *et al*., 2023). A low drought sensitivity index indicates the genotype tested under suboptimal conditions did not decrease significantly. The results of the average drought sensitivity index calculation supported to determine the drought tolerance, showing the three sugarcane cultivars to be moderate (0.1 to 0.62); however, the one approaching the sensitive phase was the cultivar PSJT-941 with PEG (25%) induction (0.62) (Table 3). Past findings enunciated drought sensitivity traits can divide into two groups, viz., moderate ($0.5 < ISK < 1.0$), and sensitive ($ISK \geq 1.0$) (Jardine *et al*., 2021).

Morphology characterization on the greenhouse scale

Morphological observations of sugarcane genotypes also occurred at the greenhouse scale through the leaf and midrib traits and engaging the principal component analysis (Table 4). Results of the principal component analysis comprised grouping into three main components. Based on the morphological characteristics, the three sugarcane commercial cultivars (GMP-3, PSJT-941, and GP-11) at PT GMP underwent scrutiny, noting the following observations. For leaf blade and width, cultivar GMP-3 has a moderately curved leaf blade with narrow leaves (< 4 cm), and the cultivar PSJT 941 also has a curved leaf blade with narrow leaves, indicating a similar leaf blade characteristic as in the genotype GMP-3. However, its color is darker green. The cultivar GP-11 exhibits a less curved leaf blade with medium-width leaves (4–5 cm), distinguishing it from the two other cultivars.

On leaf and leaf triangle color, both sugarcane cultivars GMP-3 and GP-11 have green leaves, while the cultivar PSJT 941 has a darker green leaf. However, GMP-3 stands out with its purple-colored triangular leaf compared with the green-triangular leaf of the two other cultivars. In leaf ears and position, the cultivar GMP-3 has weak leaf ears, whereas the cultivar PSJT 941 has strong, eared leaf. Meanwhile, the genotype GP-11 has leaf ears with medium strength. All the sugarcane cultivars have upright leaf ears. For field feather position and density, the cultivars GMP-3 and PSJT-941 have feathered leaf midribs that fall, while GP-11 is hairless, indicating a unique characteristic in the cultivar GP-11. The cultivar GMP-3 has a heavy field feather density, PSJT-941 has a low density, while the cultivar GP-11 remains hairless, further highlighting its distinctiveness. For the loose nature of leaf sheath, the cultivar GMP-3 leaf sheath easily curled, contrasting with the hard-curled leaf sheath in the cultivar PSJT-941, and the currently described nature of GP-11 leaf sheath. On leaf midrib color, cultivars GMP-3 and PSJT-941 share a green leaf midrib. The genotype GP-11 has a purple midrib.

The observed results on morphological characteristics of sugarcane genotypes treated with drought stress based on leaf type and leaf shedding characteristics showed leaves will roll up in response to drought stress in cultivars with narrow leaf types. Meanwhile, cultivars with wide leaf types experience dryness on the shoots and spread to the top of the leaves in the sugarcane crop (Misra *et al*., 2020). Mahfut *et al*. (2021a; 2024a) also explained cultivar GP-11 stands out with its medium-width leaves, purple leaf midrib, and hairless field feather characteristics, making it distinct from

Table 3. Relative water content and drought sensitivity index of three sugarcane cultivars at the PT GMP on in vitro scale.

Cultivars	PEG concentration (%)	Water content	Drought sensitivity index
PSJT-941	0	26.00[ab]	0
	15	22.67[ab]	0.37
	20	15.67[b]	0.53
	25	8.67[b]	0.62
GMP-3	0	50.33[ab]	0
	15	31.67[ab]	0.17
	20	28.00[ab]	0.33
	25	24.00[ab]	0.44
GP-11	0	15.67[b]	0
	15	70.67a	0.1
	20	16.67[b]	0.15
	25	17.00[b]	0.18
	$RDS_{0.05}$	48.526	3.4491*

Table 4. Morphological observations in three sugarcane cultivars at the PT GMP on a greenhouse scale.

Morphological traits		GMP-3	PSJT-941	GP-11
Leaf	Curved Leaf Blade	$^1/_3$ - ½ leaves	< ½ leaf	< $^1/_3$ leaf
	Leaf Width	< 4 cm (narrow)	< 4 cm (narrow)	4-5 cm (Medium)
	Leaf Color	Green	Dark green	Green
	Leaf Triangle Color	Purple	Green	Green
	Leaf Ears	Weak	Eared same (3 times the width) category strong	2-3 times the width (medium)
	Position Leaf Ears	Upright	Upright	Upright
Leaf Midrib	Field Feather Position Back	Fall down	Fall down	Hairless
	Field Feather Area Width Back	> ¼ width midrib leaf	< ¼ width of leaf midrib	Hairless
	Distance to the Peak of the Bulu Field Area Back	> 1 cm	Hairless	Hairless
	Field Feather Density Back	Heavy	Seldom	Hairless
	Loose Nature of Leaf Sheath (Curly)	Easy	Hard	Currently
	Leaf Midrib Color	Green	Green	Purple

the two cultivars GMP-3 and PSJT-941. Sugarcane cultivars GMP-3 and PSJT-941 share several similarities; however, these genotypes differed in leaf triangle color and leaf ear strength.

Moisture content (%)

The findings expressed moisture content (%) on greenhouse scale after six days were 14.64%, 13.98%, 13.47%, 13.00%, 12.76%, and 11.83%. Factors influencing the increase and decrease in moisture content include bulk density, organic matter, meso- and micropores, and dust and clay particles. As the value of bulk density (g/cm^3), organic matter (%), micropores (%), and mesopores (%) increases, the percentage of available moisture will also increase (Cui *et al*., 2017). The soil weight has the greater influence on raising moisture content. However, the influence of dust and clay particles was negative; thus, every increase in the percentage of dust and clay will ensure a decrease in the percentage of available moisture (De-Vries *et al*., 2016). The percentage of moisture content (< 20%) can inhibit the growth of sugarcane genotypes because sugarcane needs sufficient moisture to grow well. Insufficient moisture content can cause sugarcane to experience drought and difficulty in absorbing the nutrients required for plant growth.

The results showed during the six-day treatment of drought stress on the greenhouse scale, the moisture percentage experienced a decline until the sixth day, reaching 11.83%. The moisture rate has a significant influence on plant growth, the ability to germinate, and the beginning of growth at the rooting stages. In sugarcane crops, the early days of growth are very crucial and need an adequate water intake, and the roots become capable to grow with good absorption of nutrients to the stem (Songsri *et al*., 2019).

Molecular characterization

The DNA quantity test on isolated DNA pellets took place to know the DNA purity and concentration. The DNA purity calculation ensued through a comparison of A_{260} and A_{280}, which showed the values of 1.8–2.0 (Aristya *et al*., 2020). The DNA purity (< 1.8) indicates protein and UV contamination (Mahfut *et al*., 2015; 2020a), and the DNA purity (> 2.0) signifies the contamination of chloroform and phenol. However, in research, the purity ranges from 1.82 to 1.93, with a concentration range from 630 to 2,074 ng/µL. One of the factors affecting low-level DNA purity is the method of grinding samples. If the grinding sample is not too strong, then destroying other components will not be complete, and the proteins and carbohydrates dissolve. Another influencing factor for low-level of DNA purity is the method of taking DNA samples inside a tube (Iskandar *et al*., 2014).

Using three pairs of specific primers, i.e., P5CS1, P5CS2, and P5CS3, the P5CS gene amplification showed specific bands of 564, 981, and 821 bp, respectively. Based on band analysis, only the primer P5CS3 underwent further analysis and gave a clear and thicker band (Figure 1). The sequencing analysis indicated the sugarcane cultivars PSJ-941, GMP-3, and GP-11 sequences were 401, 471, and 164 bases, respectively. Molecular characterization based on sequencing analysis provided the percent content of pyrimidine bases (< 30%), indicating the plants' drought stress and mutation conditions. The percentage and total content of nitrogen bases in each variety varied in values. In cultivar GMP-3, it was evident that the total nitrogen bases were 148, with the highest percentage of cytosine (37.6%) and the lowest was thymine/uracil (5.9%). The cultivar GP-11 has a total of 163 bp of nitrogen bases, with the utmost base content of adenine (34.5%), and the lowest was thymine/uracil (10.3%). Meanwhile, the cultivar PSJT-941 showed the same percentage with the sample from Bogor, Indonesia, for cytosine (18.8%) and guanine (22.1%). The mutation process causes an effect and variation in the amino acids of the research samples and other comparison isolates. The percentage of the total amino acids in sugarcane reaches 97%. Drought stress causes the production, distribution, and metabolism of the amino acid proline to increase, as well as, the production of asparagine and glutamine will enhance in plant roots (Raza *et al*., 2023).

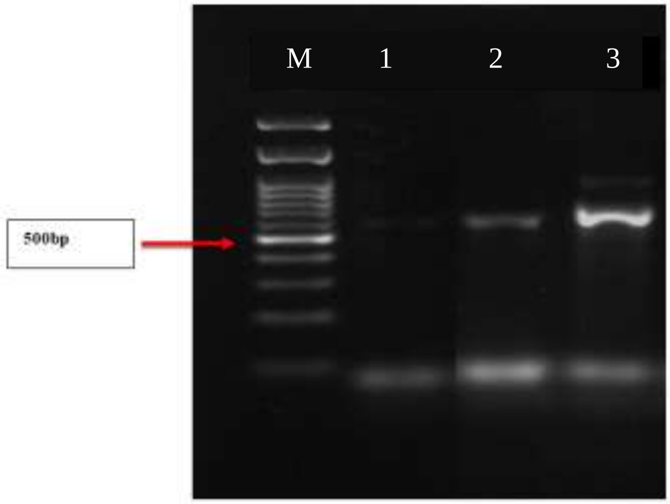

Figure 1. The visualization of P5CS gene application using specific primers P5CS3 in three sugarcane cultivars at the PT GMP. M = Marker (1 kb), 1 = GMP-3, 2 = GP-11, 3 = PSJT-941.

The homology analysis on BLAST displayed a percent identity similarity of 99.02% with the isolate from Bogor, Indonesia (KF-178300). The alignment of nucleotide sequences showed existing nucleotide differences between the sugarcane cultivars GMP-3, GP-11, and PSJT-941 due to mutation. After analyzing the alignment between the three cultivars, the occurring mutations underwent calculations. The Cultivar GMP-3 gave the highest number of mutations, namely, deletion, transversion, and transition (22, 10, and six times, respectively). The presence of mutations can influence the genetic variation in the cultivar (Polihito *et al*., 2022). The research samples implied cultivar GMP-3 experienced many mutations, with the dominance of deletion mutation. The mutation process caused an effect and change in amino acids in the research isolate compared with other comparison isolates (Perez *et al*., 2021).

The mutation process causes an effect and changes in the amino acids. The research samples showed the sugarcane cultivar PSJT-941 experienced relatively low mutation compared with the other cultivars. Dominance of transversion and translation mutations resulted in the cultivar GP-11. The mutation process had an effect and variations in the amino acids in the research isolate compared with other comparison isolates. The cultivar PSJT-941 has not experienced numerous mutations. The same result was consistent with the past research, which disclosed PSJT-941 was one of the superior cultivars for growth percentage, plant height, high vigor ability, number of leaves, and number of tillers (Iskandar *et al*., 2014).

The comparisons transpired with the isolate from Bogor, Indonesia (KF-178300) to enable phylogenetic and genetic distance analysis (Figure 2). Phylogenetic tree reconstruction showed cultivars GMP-3 and GP-11 have the maximum similarity compared with the cultivar PSJT-941. Evaluation of the phylogenetic tree used the bootstrap analysis of 1000 replications, as categorized into strong (> 85%), moderate (70%–85%), weak (50%–69%), and poor (< 50%). The greater genetic similarity values indicate the closer genetic distance between cultivars. The level of kinship and similarity depends on the type, basic nature, and interpretation of the character coefficients used (Polihito *et al*., 2022).

CONCLUSIONS

In sugarcane (S. officinarum L.), the morphological characterization of drought stress on in vitro, the optimal concentration of PEG-6000 (20%) resulted from categorizing

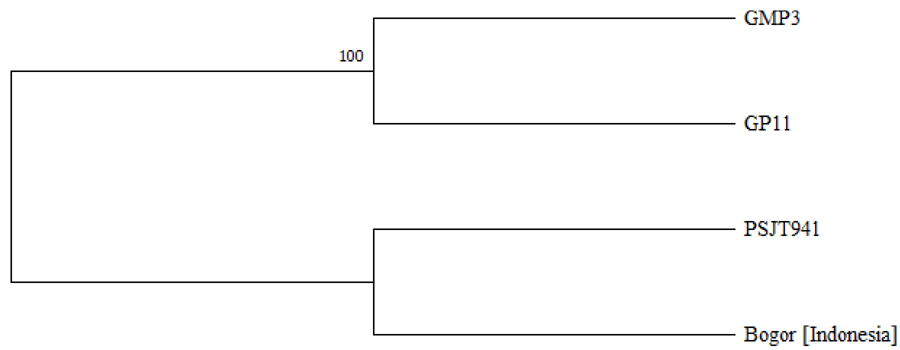

Figure 2. Phylogenetic tree of the three sugarcane cultivars at the PT GMP.

root length parameters, root volume, dry plantlets' percentage, and drought sensitivity index. The sugarcane cultivar GP-11 showed sensitivity to drought stress. The morphological characterization on the green house scale helped obtain the narrow leaf width characteristics for cultivars GMP-3 and PSJT-941, whereas the cultivar GP-11 owned wide leaves. The molecular analysis of the P5CS gene revealed cultivar PSJT-941, a variety with minimal mutation, shares a close relationship with the research sample from Bogor, Indonesia. Cultivars PSJT-941 and GMP-3 proved as drought-tolerant cultivars, while cultivar GP-11 was drought-sensitive.

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