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Research Article
Sodium Metabisulfite Suppresses Stoichiometry of Phenolic Compounds in Oil Palm Leaves for Flow Cytometric DNA Content Analysis

[1]Theera Srisawat, [2]Yaowapa Sukpondma, [3]Kanokrat Keawchai and [3]Parinuch Chumkaew

[1]Faculty of Innovative Agriculture and Fisheries, Prince of Songkla University, Surat Thani Campus, Surat Thani 84000, Thailand
[2]Division of Physical Science, Center of Excellence for Innovation in Chemistry, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla 90110, Thailand
[3]Faculty of Science and Industrial Technology, Prince of Songkla University, Surat Thani Campus, Surat Thani 84000, Thailand

Abstract

Background and Objective: Nuclear lysis buffer containing sodium metabisulfite produces more efficient and accurate results from flow cytometric (FCM) analysis of a woody plant, but most studies have focused on only LB01 buffer. The present study aims to develop new nuclear lysis buffer used for flow cytometric analysis of oil palm. **Materials and Methods:** Phenolic contents in oil palms (three cultivars) were measured and evaluated for inhibition using Folin-Ciocalteu (FC) and High-Performance Liquid Chromatography (HPLC) analysis. Concentrations of sodium metabisulfite (0-20 mM) were modified with LB01-based buffer. The modified buffers were evaluated for effects on FCM analysis of oil palm. The repeatability of each experiment was performed for three replicates. **Results:** The phenolic compound found in both unopened leaves (UOL) and fully-opened leaves (FOL) of all seedling stages was tannic acid. Sodium metabisulfite (20 mM) was found to be an effective inhibitor of tannic acid by reducing the negative effects of tannic acid released from oil palm leaves, giving a high FL (276.30 channel units) and YF (0.35 nuclei s^{-1} mg^{-1}) and the lowest CV (5.60%) for histograms. The LB01 led to satisfactory results in terms of DF and YF and the modified buffer containing 10-20 mM sodium metabisulfite was usually the most appropriate buffer for FCM analysis of oil palm leaves (2C DNA content = 3.7 pg). **Conclusion:** This study demonstrated the function of sodium metabisulfite, suggesting potential utility in oil palm DNA content analysis by FCM. Further experiments are needed to improve the buffer for oil palm tissue so that it can be applied directly to a wide variety of investigations.

Key words: 2C-DNA value, flow cytometry, phenolic content, oil palm, sodium metabisulfite

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Corresponding Author: Theera Srisawat, Faculty of Innovative Agriculture and Fisheries, Prince of Songkla University, Surat Thani Campus, Surat Thani 84000, Thailand

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Time-consuming molecular biology techniques have been popular in analyzing DNA from many plant species in many advanced breeding programs[1]. Flow cytometry (FCM) was developed for confirmation of the resultant offspring individuals, but only a few protocols are specific for some plant species cultured for food. Regarding the oil palm, many studies have published FCM measurements of *Elaeis* species[2-4]. Since phytochemicals in oil palms include phenolic compounds[5], the FCM results may differ by procedure or by equipment. Key factors affecting the success of FCM analysis of plant samples are the choice of the nuclear lysis buffer and consequent fluorochrome intercalation into the DNA double helix[6]. Because of different procedures, the parental species genomes of oil palm and their interspecific hybrids are controversial[4].

Interference effects of phenolic compounds may be responsible for some errors in FCM analyses[7]. Understanding how phenolic compounds affect oil palm genome size/DNA content results has become a serious issue in relation to oil palm breeding. There is no best method or nuclear lysis buffer that could completely eliminate the effects of phenolic compounds on nuclei fluorescence[6]. The LB01[8] is the most suitable and capable of isolating good quality nuclei from many plant species[9,10]. The woody plant buffer (WPB) keeps consistent position of G0/G1 histograms with the similarity of DNA content (3.7-3.8 pg) to its phenolics-free embryo. The WPB buffer contains a reducing agent namely sodium metabisulfite and is specified for woody species. Therefore, to suppress stoichiometric error caused by phenolic compounds, the type and amount of phenolic compounds in oil palm leaves could be evaluated and the common nuclear lysis buffer could be modified to be appropriate specifically for oil palm.

MATERIALS AND METHODS

Plant materials: One-year-old seedlings of oil palm cv. Hybrid Tenera (Suratthani 1, Deli x Calabar, 2: Deli x La Me and 7, Deli x EKONA) were obtained as plant samples from the Suratthani Oil Palm Research Center, Surat Thani Province, Thailand.

Extraction: Phenolic and tannin contents in unopened leaves (UOL) and fully opened leaves (FOL) of oil palm were extracted followed by Narayanaswamy and Balakrishnan[11]. After the extraction, the elute was filtered using a Whatman No. 1 filter paper prior to evaporating until the crude extract was obtained.

Analysis of types and amounts of phenolics: Total phenolic content analysis of oil palm UOL and FOL extracts was done by the Folin-Ciocalteu (FC) Method[12]. The results were expressed in gallic acid and tannic acid equivalents as milligrams per milliliter standard equivalents. Gallic acid (Sigma, G7384), tannic acid (Sigma, 403040), apigenin (Sigma, 42251) and luteolin (Sigma, 72511) were used as the standards. Folin-Ciocalteu reagent that is phenol reactive (Sigma-Aldrich, E9252) and sodium carbonate (J.T. Baker, 2024) were used.

High-Performance Liquid Chromatography (HPLC) analysis of phenolic content: Qualitative and quantitative data of phenolic components in UOL and FOL of oil palm were determined by using HPLC. Twenty milliliters of gallic acid (100 mg L^{-1}) and tannic acid (1,000 mg L^{-1}) were dissolved in methanol and used as standard. Chromatographic experiments were conducted on an Agilent 1200 series DAD HPLC system using a C18 Hypersil ODS (5 µm particle size) (Supelco) column (250×4.0 mm). The solvents used to develop the gradient were 0.1% of acetonitrile (A) and H_3PO_4 (Fisher Scientific) phosphoric acid (B). The solvent gradient in volumetric ratios of solvents A and B was as follows: 0-45 min, 8A/ 92B, 45-50 min, 22A/78B, 50 min and 8A/92B. Detection was performed using 280 nm excitation whereas the flow rate was set to 1.0 mL min^{-1}. Concentration of each phenolic compound was calculated based on the retention times and calibration constants for every wavelength.

Analysis of phenolic compound suppression: To inhibit phenolic compound effects *in vitro*, 5-20 mM sodium metabisulfite was used as chemical inhibitor added to UOL and FOL extracts. Then, quantitative data of entire phenolic contents in UOL and FOL extracts were collected using HPLC.

Applying sodium metabisulfite and flow cytometry to determine the DNA content and genome size of oil palm seedlings: Oil palm seedlings had been cultivated for three years. Seeds of maize (*Zea may* CE-777)[13] for use as an external reference plant were kindly provided by Dr. Jaroslav Dolezel (Institute of Experimental Botany, Olomouc, Czech Republic).

For FCM, approximately 50 mg of oil palm leaves were prepared and chopped in 1 mL of LB01 buffer and in modified LB01 plus by 10-30 mM sodium metabisulfite. The following parameters were analyzed, the relative fluorescence intensity of PI-stained nuclei (FL), half G0/G1 peak coefficient of variation (%CV), debris factor (%DF) and yield intact nuclei factor (YF)[7]. The DNA contents and haploid genome of oil palm samples were estimated[14].

Statistical analysis: The mean values were subjected to ANOVA using SPSS 11 software (IBM Corp., Armonk, New York, USA). Tukey's Test was used for multiple means comparison at a 5% significance level. Phenolic content, all FCM parameters, DNA content and genome size are expressed as Mean±SD.

RESULTS AND DISCUSSION

Total phenolic content analysis of one-year old oil palm leaves: The UOL and FOL from one-year-old oil palm seedlings were compared using absorbance measurement at 765 nm. The comparison of total phenolic contents showed remarkable differences by cultivar and by stage of the leaves. The main group of phenolic compounds found in oil palm leaves was tannins. Total tannin content in oil palm leaves had the rank order FOL>UOL (Table 1). Phenolic compounds are reported to be present in oil palm leaves[15] and consequently lead to further effects on stoichiometric oxidation in FCM for plant analysis by affecting the staining of nuclei with fluorescence dye[16]. According to our results, the total phenolic content in oil palm leaves differed between UOL and FOL. However, the types of and total phenolic components in oil palm leaf samples were comparatively low relative to prior studies. This might be due to organic solvents and methods for quantitative and qualitative analyses differing from those of earlier studies. In previous works, the tannic acid amount has been reported as 165 mg/g/DW accounting for 26.57% of quantitative analysis of oil palm leaves[17]. In this study, types and amounts of phenolic compounds in oil palm leaves (found to be tannins and tannic acid) ranged within 23-78 mg L^{-1} of standard solution, respectively.

HPLC analyses of phenolic components in leaves of one-year-old oil palm: The results revealed that apigenin and luteolin flavonoids were not detected in extracts of UOL or FOL oil palm leaves. For gallic acid and tannic acid, the standard peaks were observed at 3 and 10 min into the chromatogram, respectively. Representative chromatograms of the standard gallic acid and tannic acid were shown in Fig. 1a-b. The 0.1% acetonitrile and phosphoric acid used in the ratio of 8:92 (v/v) was found to give sharp peaks of gallic acid and tannic acid standard. The HPLC chromatograms of UOL and FOL extracts of oil palm seedlings revealed the presence of tannic acid (t_R = 15.88 min for B and t_R = 16.39 min for C, respectively) (Figures not shown). Concentrations of the two phenolic compounds measured and calculated based on similar retention times as the standards were shown in Table 2. The HPLC is the preferred technique for the qualification and quantification of phenolic compounds[18], while the chromatogram results depend on many factors. The HPLC coupled with other novel techniques gives high sensitivity and the outputs are more reliable than from HPLC only[19]. Tahir *et al.*[20] reported the quantities (as mg g^{-1}) in oil palm leaves of apigenin and luteolin derivatives using LC-ESI-MS/MS by series of types and amounts for both phenolic compounds. According to our results, no gallic acid, apigenin, or luteolin was found in the leaves of oil palm, but there was only tannic acid. Thus, other novel applications should be used that are simple, rapid, environmentally friendly and comprehensive.

For phenolic compounds suppression, as sodium metabisulfite level was increased, the observed concentration of tannic acid continuously decreased (Table 3). The overall tannic acid content was suppressed by up to 50%, especially in the FOL extract and three concentrations of sodium metabisulfite were subjected to further FCM analyses.

Table 1: Total phenolic contents in leaf extracts of one-year-old oil palm seedlings determined by using the Folin-Ciocalteu Method

	Total phenolic content (μg mL^{-1})		Tannin content (μg mL^{-1})	
Cultivar	UOL	FOL	UOL	FOL
ST1	715.50	781.03	614.77	1,196.59
ST2	229.31	1,612.00	443.18	1,720.00
ST7	294.83	1,024.14	281.82	1,601.14

ST1, ST2 and ST7: Oil palm cultivars Suratthani 1, 2 and 7, UOL: Unopened leaves and FOL: Fully opened leaves

Table 2: Yields of phenolic compounds obtained from UOL and FOL of one-year-old oil palm seedlings representing cultivars Suratthani 1, 2 and 7

Leaf/cultivar	Apigenin content (mg L^{-1})	Luteolin content (mg L^{-1})	Gallic acid (mg L^{-1})	Tannic acid (mg L^{-1})
UOL/ST1	0	0	0	62.217±1.147
FOL/ST1	0	0	0	31.778±0.618
UOL/ST2	0	0	0	78.532±1.073
FOL/ST2	0	0	0	85.649±3.427
UOL/ST7	0	0	0	80.539±0.426
FOL/ST7	0	0	0	23.917±1.498

Values are given as mean and standard deviation of the mean (SD), UOL: Unopened leaves and FOL: Fully opened leaves

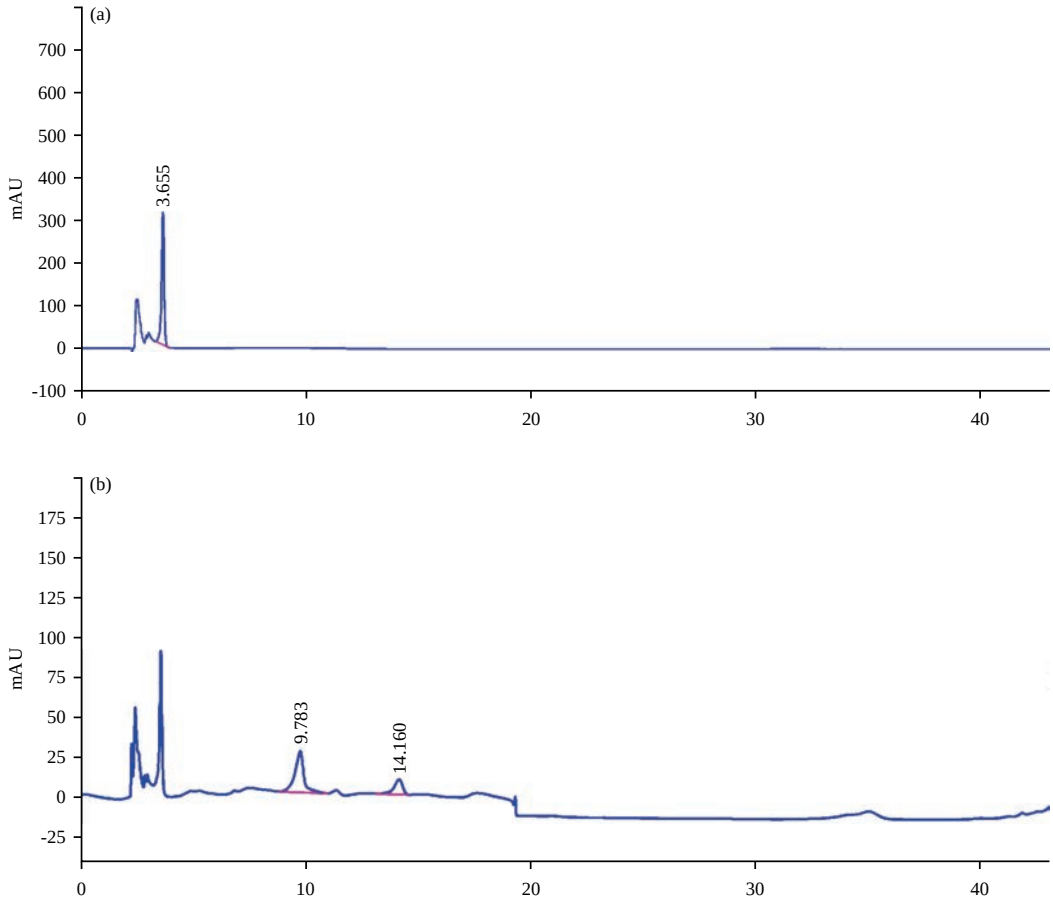

Fig. 1(a-b): Typical HPLC chromatograms of (a) Gallic acid and (b) Tannic acid, using 0.1% phosphoric acid and acetonitrile in gradient flow

Table 3: Concentration of tannic acid in UOL and FOL extracts of three-year-old oil palm seedlings representing cultivar Suratthani 7 after treatments with sodium metabisulfite

	Tannic acid (Mean±SD) (mg L^{-1})	
Sodium metabisulfite (mM)	UOL	FOL
0	24.48±1.51	50.12±0.31
5	21.88±1.53	38.90±1.60
10	19.43±0.07	43.79±1.68
15	19.08±0.47	38.40±1.71
20	18.34±0.13	27.75±2.46

Values are given as mean and standard deviation of the mean (SD), UOL: Unopened leaves and FOL: Fully opened leaves

Analysis of flow cytometric histogram quality: We reasoned that if oil palm leaves contain phenolic compounds, adding sodium metabisulfite to the buffer could suppress the phenolic compounds' effects, giving accurate and reliable histograms. The efficiency of the modified buffers was considered via the FL and YF values (larger is better) and the CV (smaller is better) (Table 4). Although DF was higher in the FCM histogram, this histogram quality did not affect DNA content measurements. The FL and CV seemed to affect G0/G1 histogram position more with a modified buffer having sodium metabisulfite than YF and DF (Fig. 2). The increased FL was suitable as a reliable parameter to distinguish histogram positions by a concentration of sodium metabisulfite and this is the main criterion for DNA content calculation.

Our results confirmed that sodium metabisulfite in WPB buffer influenced the lysis buffer to avoid browning in the FCM estimations in all *Elaeis guineensis* leaf samples[10]. Browning of lysis buffer is undesirable as it degrades buffer properties and interferes with PI staining[16]. This might be related to polyphenol oxidase (PPO), which catalyzes the oxidation of several phenols into o-quinones[21]. Sodium metabisulfite is a reducing agent, it can react with o-quinone and form diphenols, so the polymerization of o-quinone into a coloured pigment-or browning-can be prevented[22-23]. Thus, it is essential to complement the staining dye with DNA and PI-staining inhibitor.

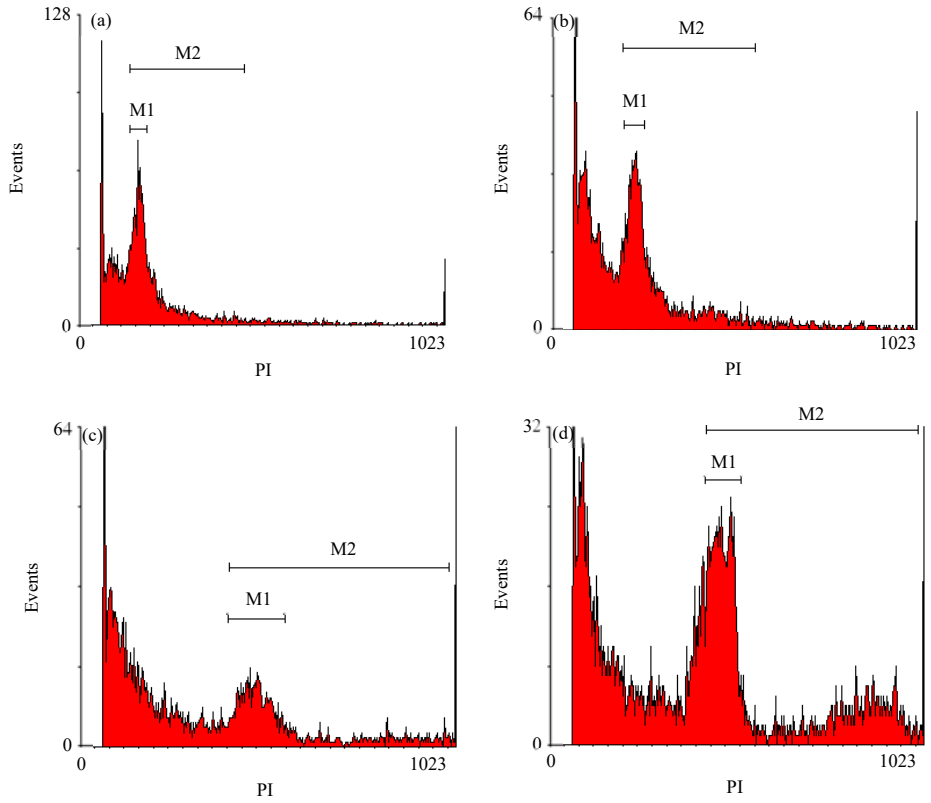

Fig. 2(a-d): Histogram of three-year-old *E. guineensis* cv. Tenera Suratthani 7 analyzed by using LB01 and modified LB01 buffer with gating (M1 represents G0/G1 phase, applied to calculate DNA content, whereas M2 represents G0/G1, S and G2/M phases, applied to draw region of intact nuclei, (a) LB01, (b) LB01+10 mM sodium metabisulfite, (c) LB01+20 mM sodium metabisulfite and (d) LB01+30 mM sodium metabisulfite

Table 4: Effects of LB01 and modified LB01 lysis buffers on FCM parameters analyzed from histogram of tested leaf extracts from three-year-old *E. guineensis* cv. Tenera Suratthani 7

	FCM parameters (Mean±SD)			
Nuclear lysis buffers	FL (channel unit)	CV (%)	DF (%)	YF (nuclei s^{-1} mg^{-1})
LB01	190.73±19.06^{b}	8.95±1.16^{b}	42.31±3.20^{a}	0.45±0.23^{a}
LB01+10 mM sodium metabisulfite	276.30±37.41^{a}	5.60±0.94^{a}	60.48±6.28^{c}	0.27±0.05^{ab}
LB01+20 mM sodium metabisulfite	263.41±9.43^{a}	10.57±1.66^{b}	49.55±1.93^{b}	0.35±0.10^{a}
LB01+30 mM sodium metabisulfite	301.70±12.30^{a}	8.58±1.60^{b}	69.56±1.42^{d}	0.06±0.02^{b}

Values are given as mean and standard deviation of the mean (SD), Means for the same nuclear lysis buffers followed by the different letters are statistically different according to the multiple comparisons Tukey's Test p<0.05

Table 5: Effects of LB01 and modified LB01 lysis buffers

Nuclear lysis buffer with additive	FL2 Channel *Z. mays* CE777	2C-DNA content (pg) *E. guineensis*	Genome size (bp)
LB01	384.66	2.67±0.04	1305.63
LB01+10 mM sodium metabisulfite	442.16	3.79±0.55	1853.31
LB01+20 mM sodium metabisulfite	469.97	3.75±0.20	1833.75
LB01+30 mM sodium metabisulfite	476.17	3.99±0.46	1951.11

2C-DNA content values are given as mean and standard deviation of the mean (SD), (1): FL2 channel of reference standard (*Z. mays* CE777), (2): 2C-value estimates of three-years old *E. guineensis* cv. Tenera Suratthani 7 and (3): Genome size of plant sample

DNA content analysis of a three-year-old oil palm tree: We found that the effects of sodium metabisulfite concentration in tannic acid suppression can be attributed to the remaining quantity of tannic acid (decreased by 50% in FOL). Since sodium metabisulfite caused an increase in FL and a decrease in CV, while LB01 based buffer responded with a decreased FL and an increased CV, the nuclear DNA content was increased with the dose level of sodium metabisulfite. We hypothesized that the expected DNA content of a three-year-old oil palm tree might be similar to the DNA content of oil palm embryos

(3.7 pg)[10] that are free from phenolic compounds. The mean 2C DNA content of three-years old *E. guneensis* cv was shown in Table 5. Tenera Suratthani 7 from the Suratthani Oil Palm Research Center ranging from 3.7 pg (10 and 20 mM sodium metabisulfite) to 3.9 pg (30 mM sodium metabisulfite). Thus, genome size of oil palm had values ranging from 1800 to 1900 bp.

There are many factors affecting FCM estimation, such as components of lysis buffer, type of dyes, chopping procedure and DNA PI-staining inhibitor[24]. Although the modified LB01 buffers result in high values of FL and give consistent PI-histograms, high YF and low DF were found for the LB01 based buffer. Interestingly, after modifying with 10 mM sodium metabisulfite, the buffer gave the lowest CV at 5%. In general, the CV of the peaks generated in the G0/G1 phase when below 3% is considered acceptable[25]. The FOL from three-year-old oil palm trees having higher levels of tannic acid than in young or UOL was collected. It is known that the tannic acid[26] in FOL could affect FL2-histograms giving elevated CV. The range 3.7-3.8 pg of DNA contents in oil palm cv. Tenera has been reported[2,3,27] and the expected DNA content in three-year-old oil palm cv. Tenera is expected to be similar to the previous results.

Although modifying with sodium metabisulfite showed a negligible effect on DF and YF, higher FL and lower CV influenced the consistency of histograms enabling improved accuracy in DNA content estimates. Only two levels tested (10 and 20 mM sodium metabisulfite) exhibited acceptable YF (0.27 and 0.35 nuclei s^{-1} mg^{-1}) compared to 0.45 nuclei s^{-1} mg^{-1} of LB01 based buffer, suggesting that very high level of sodium metabisulfite was not a good choice for FCM buffer of oil palm. Similar to current findings in oil palm, the DNA content estimated as 3.7-3.9 pg also falls into the short range as described by previous authors[2-4,27].

CONCLUSION

This study demonstrated the presence of tannic acid in oil palm leaves, which could interfere with FCM resulting in histogram errors affecting DNA content and genome size estimates. The optimization of the FCM protocol was supported by modifying the most suitable/popular lysis buffer with 10-20 mM sodium metabisulfite for plants rich in phenolic compounds, to obtain improved accuracy in DNA content and genome size estimates by FCM.

SIGNIFICANCE STATEMENT

This research documents information on significant modified reducing agent for oil palm FCM analysis, which was analyzed from the exhibited phenolic compounds after suppressed by the reducing agent. Here, we explore an existing of the optimum FCM criteria based on the hypothesis of "if phenolic compounds released from oil palm tissue and appeared in the lysis buffer solution, verifying and suppressing the phenolic compounds could help an effort for development of suitable nuclear lysis buffer for producing FCM peak/2c-DNA content of oil palm". Our method of analysis provides the fact on the present of tannin while information of tannin inhibition can effectively be used for application in the specific lysis buffer for oil palm FCM analysis.

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