Application of fluorescent and UV-Vis detection methods to profile antimicrobial activity of cassava tissues for an efficient *Agrobacterium*-mediated transformation

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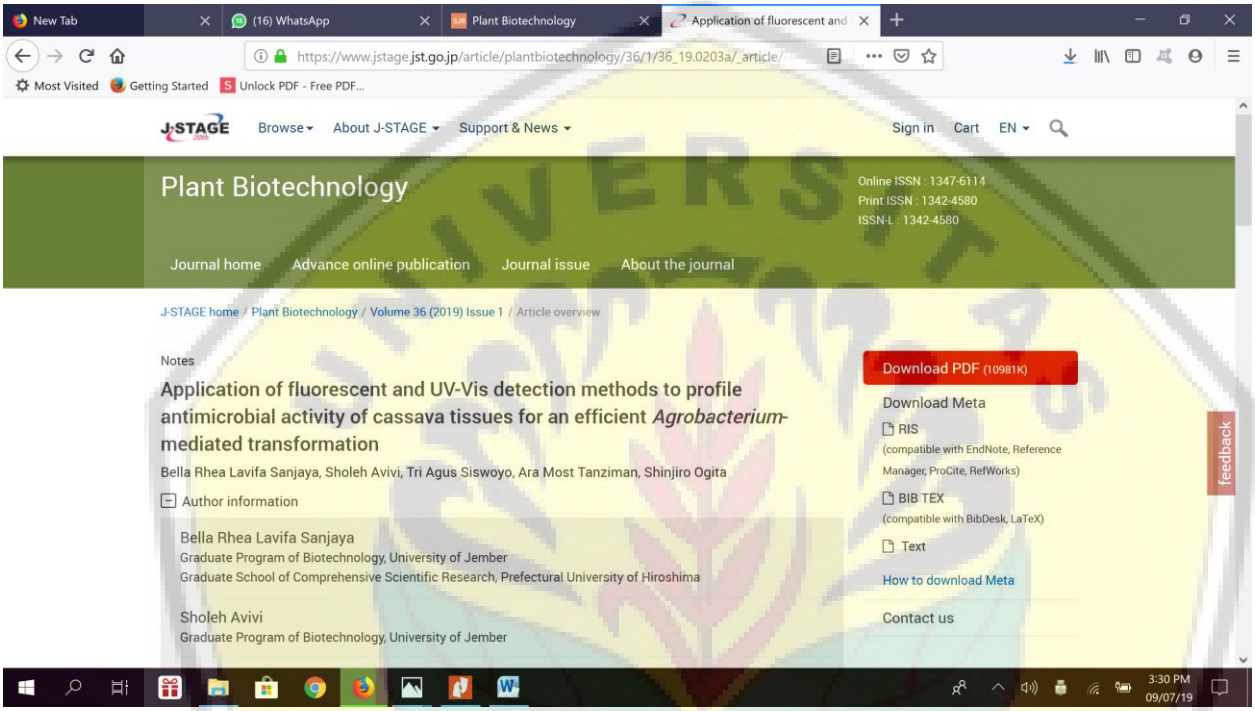

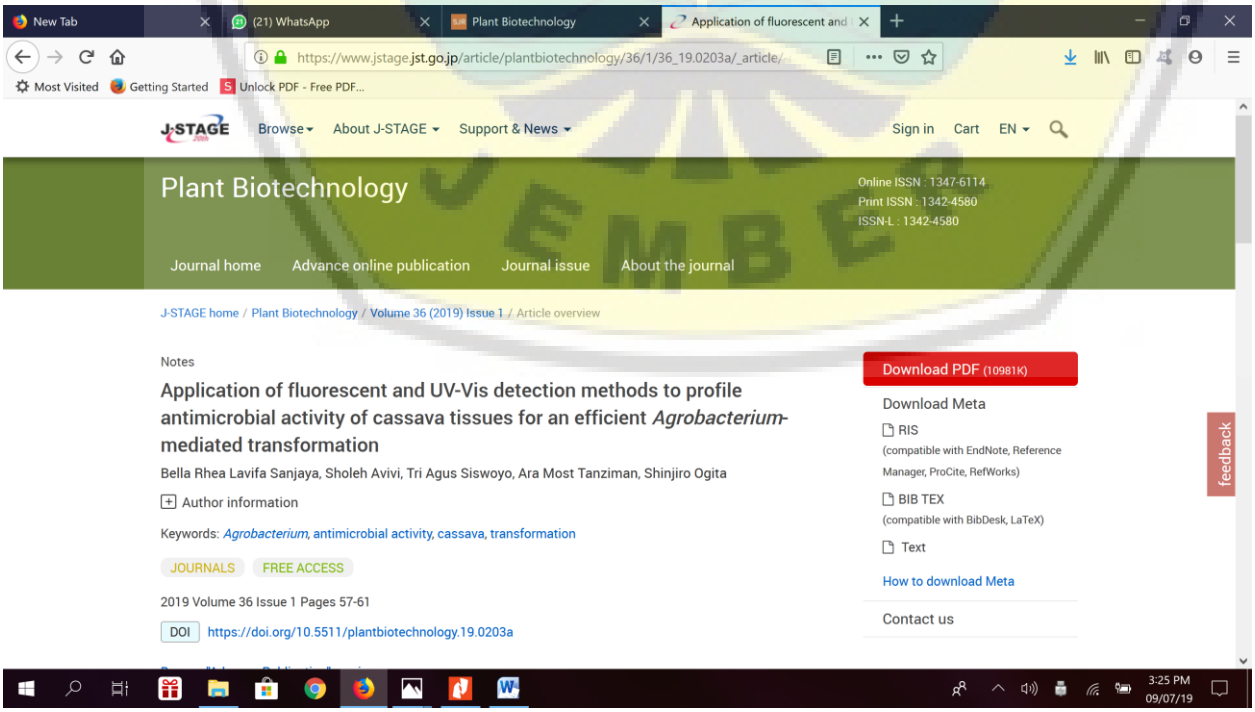

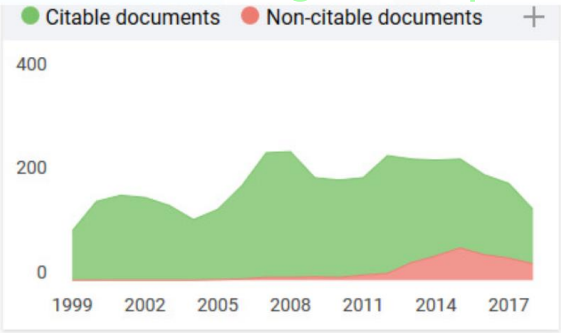

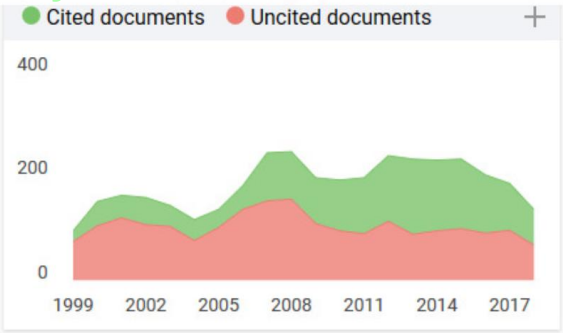

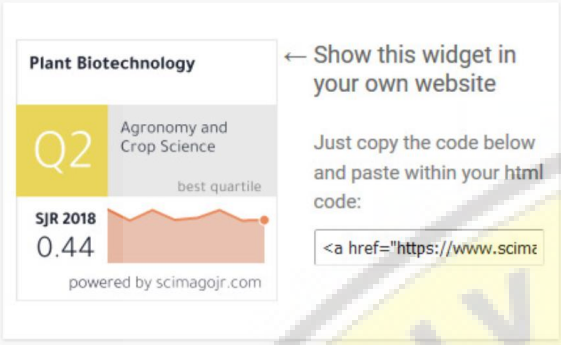

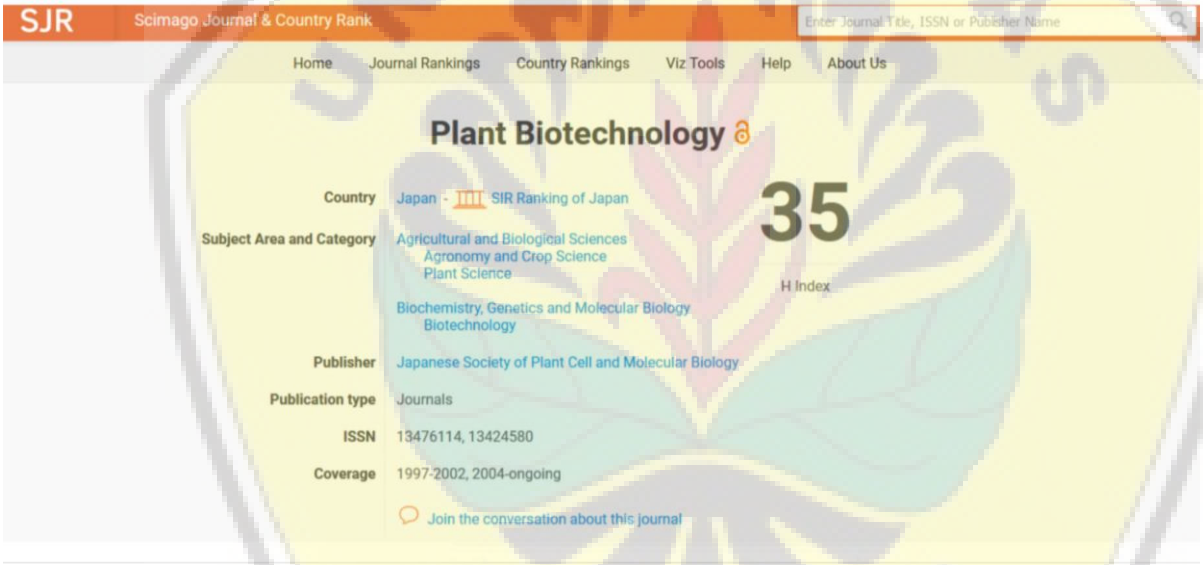

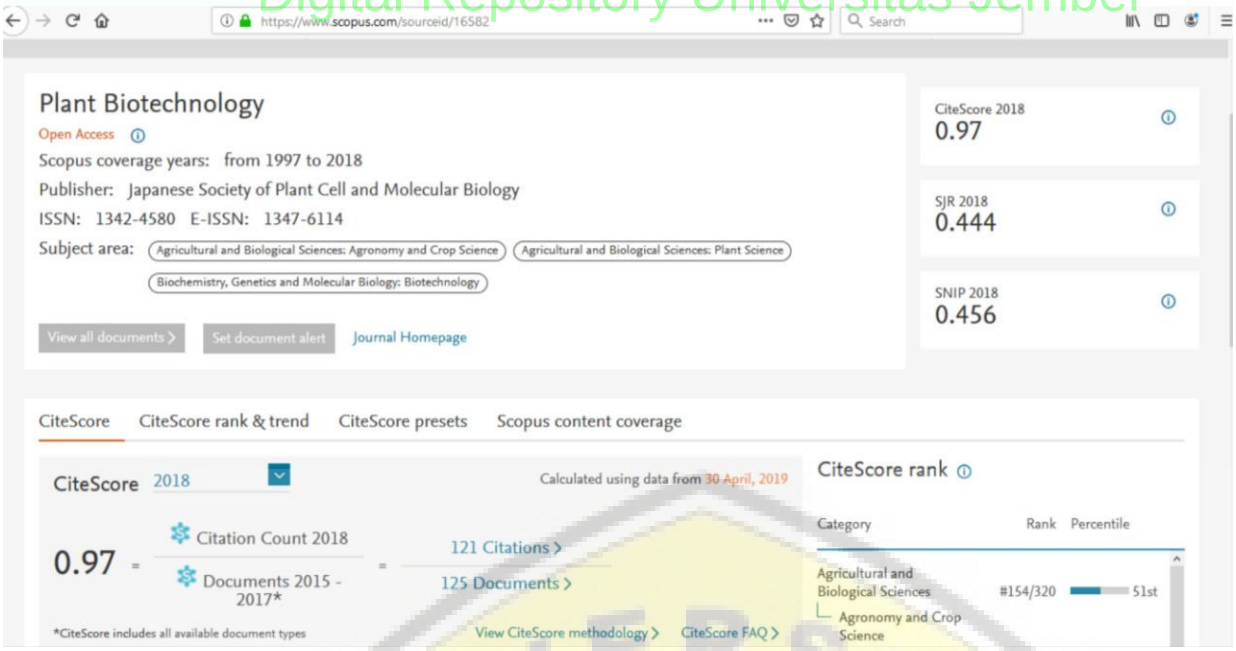

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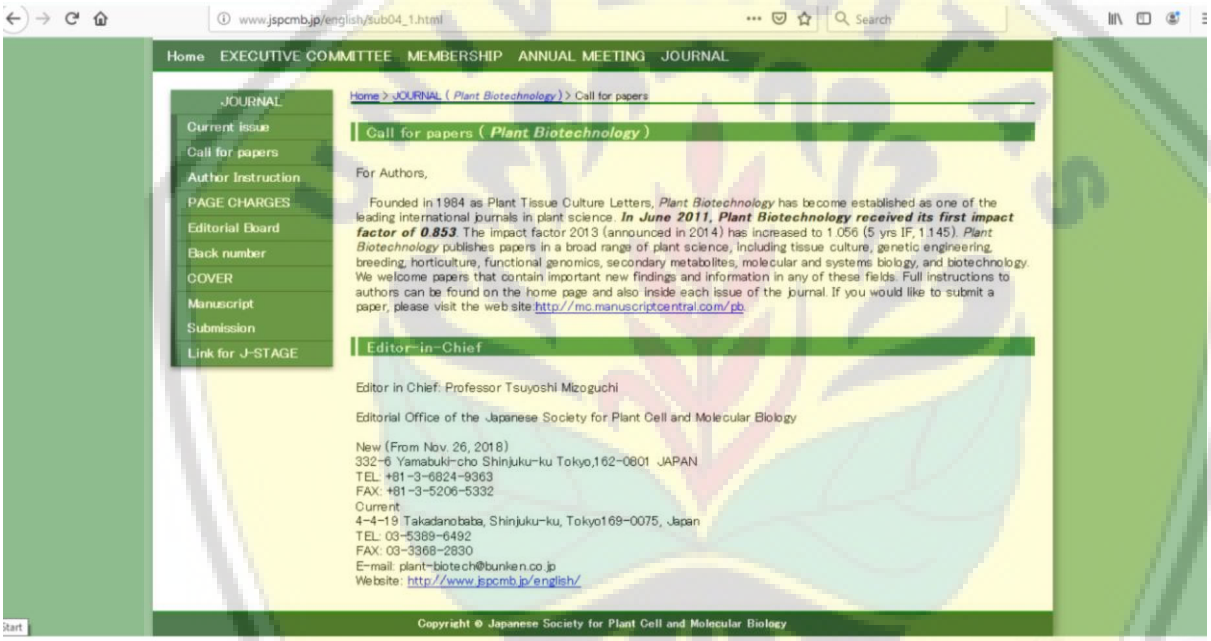

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Note

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Abstract The majority of tissue culture and transformation studies in cassava (*Manihot esculenta* Crantz) focus on the modification of conditions in order to establish a better protocol. Although this is a standard approach for making progress in genetic transformation technology for a target plant variety, serious difficulty still remains due to the limited applicability and adaptability of the given protocol. In the present study, we aim to develop a new concept that focuses on the development of simple but adaptable genetic transformation technology in cassava. In order to establish an efficient transformation protocol, two local edible cassava varieties, R-type, with a broad leaf with reddish petiole, and S-type, with a thin leaf with shiny greenish petiole, were obtained from Okinawa, Japan. Three detection methods, i.e., fluorescence microscopy, thin-layer chromatography (TLC) with detection under an ultraviolet (UV) illumination (254 nm) and light emitting diode (LED) illuminations (365 nm and 500 nm) without any staining, and a spectrum scanning (250–700 nm) by a microplate reader system were employed to identify a series of unique features of the petioles and leaves. Antimicrobial activity of methanol extracts from the tissues was also assayed. We succeeded in the transient expression of the *GUS* gene using cassava leaves and also established stable introduction of the *GUS* gene into three organogenic cassava calli by adapting *Agrobacterium*-mediated transformation. With all the findings, we have identified a flexible tool to create a better match between explants and *Agrobacterium* strains.

Key words: *Agrobacterium*, antimicrobial activity, cassava, transformation.

Cassava (*Manihot esculenta* Crantz) is a perennial woody shrub in the Euphorbiaceae family and is one of the most important sources of food and industrial raw materials in tropical and subtropical regions (Burns et al. 2010; FAO 2013). According to the latest statistics by FAOSTAT (FAOSTAT 2019), the Asian region, including Indonesia, has the second largest share of global production of cassava, i.e., 30.2% (average 66 M tons/year, 1994–2017), of which Indonesia contributes 19 M tons/year on average across the same period. Therefore, the importance of cassava in the agronomical economy of Indonesia has grown in recent years. Since one of the most significant aspects for ensuring the profitability of this valuable plant species is to control productivity and quality, several common and local varieties were collected from BALITKABI (the Indonesian government-based research center for various nuts and tubers). The collection of varieties were then maintained in a greenhouse of CDAST (Center for Development of Advanced Science and Technology), University of Jember, East Java, Indonesia. We investigated the morphological, physiological, and biomolecular traits of the collection to better understand key factors affecting their productivity and quality regulation.

Plant tissue culture and genetic transformation technologies are powerful tools for clonal propagation and breeding of target plants. Since the first genetic transformations of cassava using *Agrobacterium* were published in 1996 (Li et al. 1996), many progressive studies have been reported (e.g., Chavarriaga-Aguirre et al. 2016; Liu et al. 2011; Niklaus et al. 2011; Utsumi et al. 2017), the majority focusing on the modification of conditions to establish a better genetic transformation protocol. Although this is a standard approach to progress genetic transformation technology for the target variety, serious difficulty still remain in the generalization

Abbreviations: CIM, callus induction medium; GUS, β-glucuronidase; LED, light emitting diode; MS medium, Murashige and Skoog medium; Rf, retardation factor; TLC, thin-layer chromatography; UV, ultraviolet; Vis, visible.

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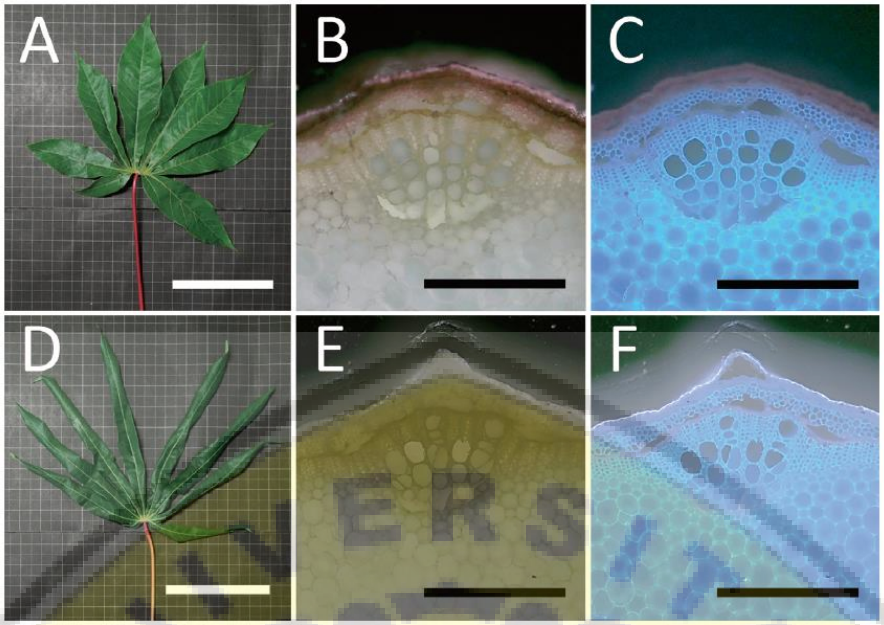

Figure 1. Morphological and histological characteristics of cassava varieties, R-type; (A)–(C) and S-type; (D)–(F). (A) and (D) are the morphology of leaves and petioles. Scales in (A) and (D) indicate 5 cm. (B), (C), (E), and (F) are cross sections of petioles. Scales in (B), (C), (E), and (F) indicate 50 μm. Sections were observed under bright-field illumination (B and E), and UV illumination (C and F), respectively.

and adaptability of given protocols. Therefore, we intend to develop a new concept that focuses on the further development of simple but adaptable genetic transformation technology to introduce superior traits to increase agricultural and/or industrial economic values of cassava.

The present study aims to improve the matching between plant materials and *Agrobacterium* strain. We aim to demonstrate the following: 1) to determine typical differences between the two local edible cassava varieties, R-type, which has broad leaf with reddish petiole, and S-type which has thin leaf with shiny greenish petiole; 2) to profile antimicrobial activity of both R- and S-type cassava, and; 3) to understand whether the antimicrobial activity of target tissues effects the efficiency of *Agrobacterium*-mediated transformation.

Fresh plant materials were collected from Okinawa Japan without any safety regulation. As shown in Figure 1, morphological and histological characteristics of petioles and leaves were observed using an inverted cell culture microscope (CKX53, Olympus). The epidermis layer of R-type petiole tissues which contained anthocyanin pigments did not show any specific signal in the outer cuticle layer, while a shiny whitish signal in the outer cuticle layer of S-type petioles could be seen under an ultraviolet (UV) light (U-FUW, excitation filter, 340–390 nm; extinction filter, 420 nm).

Methanol extracts (dried sample : 80% methanol = 1 : 9, v : v, heated at 40°C for 30 min with ultra-sonication) were prepared and analyzed by thin-layer chromatography (TLC silica gel 60 F254, Merck Millipore) in order to profile biochemical characteristics of cassava tissues. TLC plates (5×5 cm) were cut from the commercially available sheets. The methanol extracts were dotted in the plate (4 μl in total) and were developed in a closed chamber with a mobile phase (*n*-butanol : acetic acid : water = 4 : 1 : 5 v : v : v). The separated metabolites on the resulting plate were detected under a bright-field, UV (245 nm), and LED illuminations (365 nm and 500 nm) without staining, as shown in Figure 2. Orange to violet pigments were detected under a bright-field illumination (Rf values at around 0.37 to 0.53). Several UV absorbable metabolites could be identified as blackish spots (Rf values 0.21, 0.53, and at around 1.00) under 254 nm UV illumination, and light blue spots (Rf values 0.21 and 0.89) and dark-reddish spots (Rf value at around 1.00) under 365 nm LED illumination, respectively. Furthermore, some bright yellowish spots (Rf values at around 0.21) and reddish spots (Rf values at around 1.00) were also signalized under 500 nm LED illumination. The methanol extracts (200 μl in a well of 96-well microplate) were then overviewed as a spectrum scanning (250–700 nm) using a microplate reader system (Varioskan Flash, Thermo Scientific). As shown in Figure 3, similarities and/or differences for each spectrum profile were recognizable as peaked range of absorption wavelength at around 320–400 nm, 440–520 nm, and 640–680 nm. These were expected to indicate reference metabolites such as a group of aromatic hydrocarbons and a group of pigments

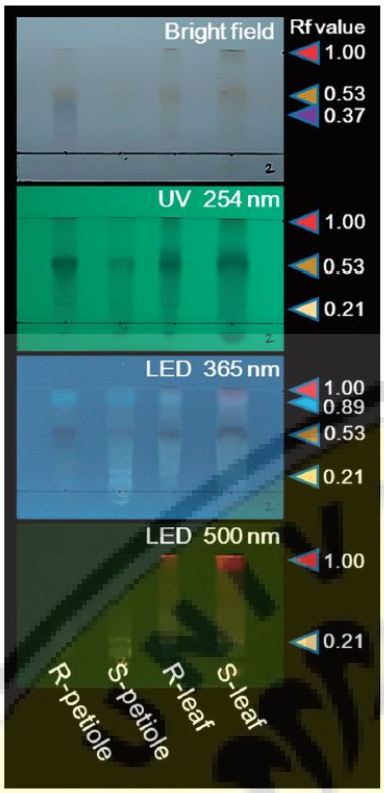

Figure 2. Separation and detection of methanol extracts of different strains and tissues of cassava. The developing solvent system is *n*-butanol/acetic acid/water (4:1:5 v:v:v).

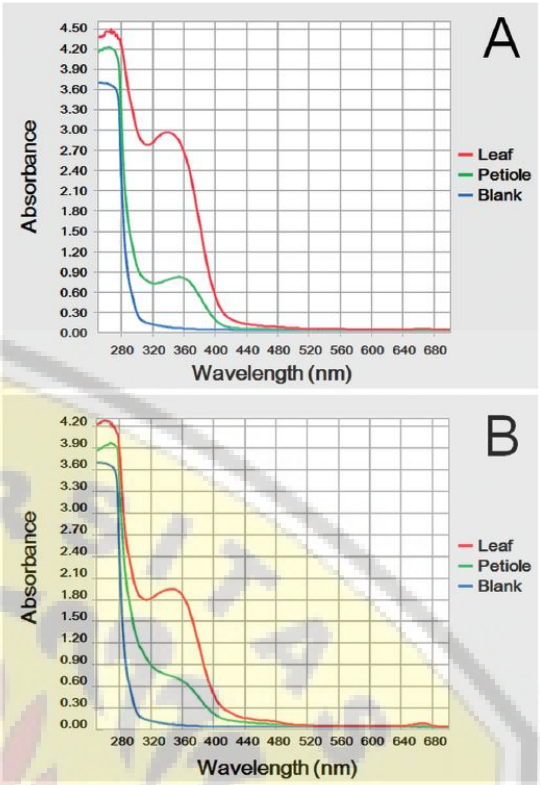

Figure 3. Spectrum profile (250–700 nm) of methanol extracts of different strains and tissues of cassava. (A) R-type, (B) S-type.

(flavonoids, carotenoids, betaines, and chlorophylls).

In order to identify further biochemical factors influencing the efficiency of tissue culture and transformation steps, another set of methanol extracts were prepared (fresh sample:80% methanol=1:2, v:v, heated at 40°C for 60 min). They were then applied to an antimicrobial activity assay. Briefly, four types of wild type bacterial strains, namely *Escherichia coli* (JM109), *Agrobacterium rhizogenis* (A13), and two *A. tumefaciens* (EHA101 and LBA4404), were preliminary cultured in liquid LB medium (polypeptone 10 g/l; yeast extract 5 g/l; sodium chloride 10 g/l) in the dark at 25°C on a rotary shaker at 200 rpm for 48 h. Approximately 4 μl of bacterial suspension was added to 200 μl of liquid LB medium in a well of a 96-well plate. After that, 4 μl of each methanol extract was added into the bacterial suspension in a well. In order to estimate the overall antimicrobial activity, the variation of bacterial cell density was measured every hour by using a microplate reader system. The antimicrobial activity of the additives was detected after 12 h (Figure 4A), and the overall effects were evaluated at the end of cell culture after 24 h as shown in Figure 4B. Leaf extracts showed an antimicrobial activity (ca. 80% inhibition in average) to bacterial growth of all strains tested while petiole extracts did not show any clear activity. Leaf extracts of the S-type had a stronger inhibition to bacterial growth, especially against EHA101 and LBA4404 strains, than that of the R-type.

Based on the findings above mentioned, we temporarily concluded that S-type cassava contained a series of unique biochemical profiles (see Figures 1–3) with higher antimicrobial activity in leaves (see Figure 4) and so is one of the recalcitrant varieties for tissue culture and genetic transformation experiments. Therefore, we focus mainly on the R-type cassava variety in the following experiments.

At first, transient expression of the *GUS* gene was assayed to determine the best matching between cassava leaves and *Agrobacterium* strains. The pBIH1-IG vector (Ohta et al. 1990), carrying the *intron-GUS* gene, was electroporated into two *A. tumefaciens* strains, both EHA101 and LBA4404. Briefly, abaxial side of cassava leaves were stamped in a circle shape by a cap of 1.5 ml Eppendorf tube as shown in Figure 5. Bacterial suspensions were dropped on the stamps for 3 days to provide infection. After that, GUS staining was applied for 24 h and then the chlorophyll in leaves were extracted with 70% ethanol for 24 h. We referred the

method (Ogita et al. 2011) for GUS staining with some modification. A positive blue color, ca. 76.0% in the infected area via transformed LBA4404 strain could be seen while ca. 20.8% in the infected area via transformed EHA101 were GUS-positive. As our estimation, LBA4404 was much more suitable for cassava transformation (3.6-fold difference, on average).

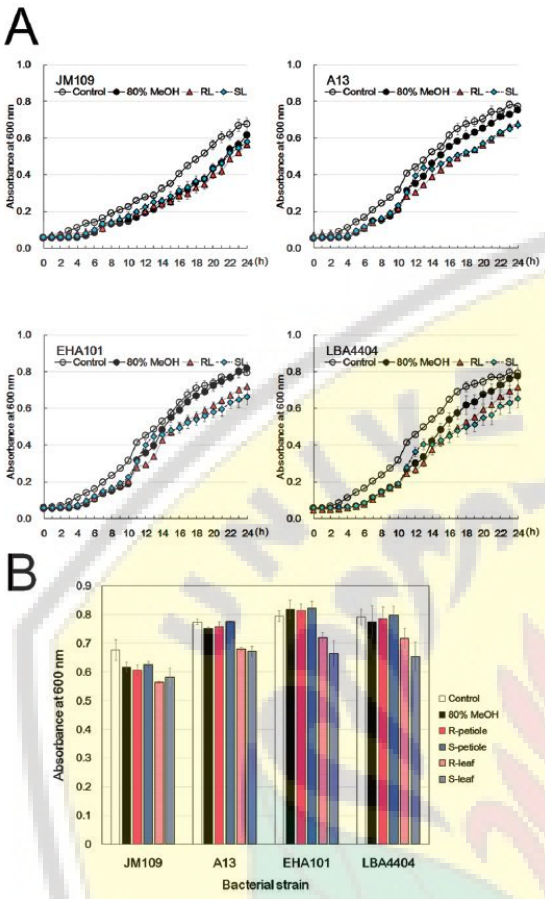

Figure 4. Antimicrobial activity of methanol extracts of different strains and tissues of cassava after 24 h. (A) Growth profile of each bacterial strain in LB medium containing R-, and S-leaf extracts. (B) Comparison of antimicrobial activity at the end of cell culturing after 24 h.

Second, in order to examine the efficiency of the stable expression of the *GUS* gene, we started callus induction from leaf, petiole, and juvenile node explants of both R- and S-type cassava varieties on a callus induction medium (CIM), i.e., Murashige and Skoog's MS medium (Murashige and Skoog 1962) containing 3 μM 2,4-D and 3 μM BA, solidified with 0.3% (v/w) gellan gum. All cultures were kept in the dark at 27°C. An initial sign of callusing from the cut edge of each tissue could be seen within 30 days. However, all the leaf explants from both cassava types scarcely browned and gradually died. The node of the S-type also tended to brown and was a very recalcitrant explant for maintaining a good callus (data not shown). Finally, three organogenic callus strains were obtained from node tissue of R-type, and petiole tissue of R- and S-type, respectively. These results suggest that there are some difficulties in handling S-type cassava tissues.

We then compared the stable expression level of the *GUS* gene by adapting *Agrobacterium*-mediated transformation into the three organogenic callus strains. Briefly, all callus strains used were inoculated with bacterial suspension and kept for approximately 5 min. After that, they were transferred to solid CIM in petri dishes for co-cultivation by maintaining the cultures in the dark at 27°C for 3 days. The selection of potentially transformed calli was completely within 3 weeks according to the method of Ogita et al. (2009), with some modification. The results were summarized in Table 1. As with the result from transient expression of *GUS* gene in leaf explants (see Figure 5), LBA4404 strain was much suitable for cassava callus in terms of the transformation efficiency, although the efficiency varied depending on the variety and the origin of each callus strain. For example, S-type calli originating from

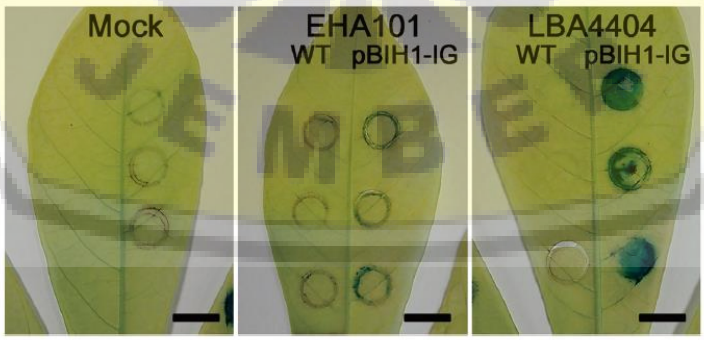

Figure 5. Transient expression of *GUS* gene assay of cassava leaves from the R-type strain. The pBIH1-IG vector (Ohta et al. 1990), carrying the *intron-GUS* gene, was electroporated into two *A. tumefaciens* strains, EHA101 and LBA4404. Both wild type (WT) and transformed (pBIH1-IG) bacterial strains were used.

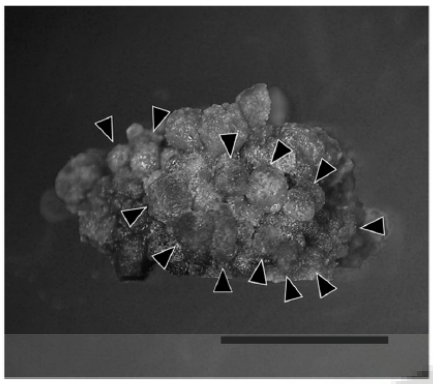

Figure 6. Stable *GUS* gene expression in cassava callus originated from petiole tissue of R-type cassava. Many meristematic primordia expressing *GUS* gene (arrowheads) were induced. Scale indicates 2.5 mm.

Table 1. Comparison of the transformation efficiency between cassava strain tissue and *Agrobacterium tumefaciens* EHA101 and *A. tumefaciens* LBA4404.

Type of callus tested	Transformation efficiency (%)	
	EHA101	LBA4404
Organogenic callus originated from node tissue of R-type	8.3	91.8
Organogenic callus originated from petiole tissue of R-type	55.6	77.8
Organogenic callus originated from petiole tissue of S-type	0	91.7

N=12 calli

petiole tissue showed a narrow adaptability for matching (EHA101 versus LBA4404, 0 to 91.7%), while R-type calli originating from petiole tissue had a wide adaptability (EHA101 versus LBA4404, 55.6 to 77.8%). Many meristematic primordia stably expressing the *GUS* gene were induced from typical transformed callus originated from petiole tissue of R-type cassava (Figure 6).

From all our findings, it is safe to say that the fluorescent and UV-Vis detection methods to profile antimicrobial activity will be a flexible tool for better matching between plant materials and *Agrobacterium* strains. This information will aid in the development of transformation technology for Indonesian cassava varieties. Related studies are now in progress.

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References

Burns A, Gleadow R, Cliff J, Zacarias A, Cavagnaro T (2010) Cassava: The drought, war and famine crop in a changing world. *Sustainability* 2: 3572–3607

Chavarriaga-Aguirre P, Brand A, Medina A, Prías M, Escobar R, Martinez J, Díaz P, López C, Roca WM, Tohme J (2016) The potential of using biotechnology to improve cassava: A review. *In Vitro Cel Dev Biol Plant* 52: 461–478

FAO (2013) Save and grow Cassava: A guide to sustainable production intensification. http://www.fao.org/3/a-i3278e.pdf

FAOSTAT (2019) Food and agriculture data. http://www.fao.org/faostat/en/#home

Li HQ, Sautter C, Potrykus I, Puonti-Kaerlas J (1996) Genetic transformation of cassava (*Manihot esculenta* Crantz). *Nat Biotechnol* 14: 736–740

Liu J, Zheng Q, Ma Q, Gadidasu KK, Zhang P (2011) Cassava genetic transformation and its application in breeding. *J Integr Plant Biol* 53: 552–569

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tabacco tissue cultures. *Physiol Plant* 15: 473–497

Niklaus M, Gruissem W, Vanderschuren H (2011) Efficient transformation and regeneration of transgenic cassava using the neomycin phosphotransferase gene as aminoglycoside resistance marker gene. *GM Crops* 2: 193–200

Ogita S, Kikuchi N, Nomura T, Kato Y (2011) A practical protocol for particle bombardment-mediated transformation of *Phyllostachys* bamboo suspension cells. *Plant Biotechnol* 28: 43–50

Ogita S, Usui M, Shibutani N, Kato Y (2009) A simple shoot multiplication procedure using internode explants, and its application for particle bombardment and *Agrobacterium*-mediated transformation in watercress. *J Plant Res* 122: 455–463

Ohta S, Mita S, Hattori T, Nakamura K (1990) Construction and expression in tobacco of a β-glucuronidase (*GUS*) reporter gene containing an intron within the coding sequence. *Plant Cell Physiol* 31: 805–813

Utsumi Y, Utsumi C, Tanaka M, Ha VT, Matsui A, Takahashi S, Seki M (2017) Formation of friable embryogenic callus in cassava is enhanced under conditions of reduced nitrate, potassium and phosphate. *PLoS One* 12: e0180736