A phenotyping method to predict β-carotene content of cassava tuberous root using ImageJ

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Abstract. Cassava (*Manihot esculenta*) tuberous roots are crucial in carbohydrate production, serving as a staple food and industrial material for decades. High β-carotene content is essential for nutrition and stress tolerance, conserving in yellow-fleshed cassava tuberous roots. However, the complex and costly laboratory analysis of the β-carotene content is an obstacle. This study aims to develop a simple and precise phenotyping method for predicting the β-carotene content of cassava tuberous root using grayscale analysis with ImageJ. This study was conducted at the Research Centre for Genetic Engineering, Bogor, in October 2021 for the training set and October 2022 for the validating set. The training set used six yellow and three white-fleshed cassava cultivars as genetic materials. Seven other white-fleshed cassava cultivars were added to the validating set. The grayscale analysis accurately distinguished the white and yellow-fleshed cassava cultivars, reflecting variation in the β-carotene content. This phenotyping method showed a stable result in the validating set. Additionally, a robust correlation was found between β-carotene content and grayscale values, showing the ability of grayscale analysis to predict β-carotene content. Thus, this method could be advantageous in a massive and early selection of the β-carotene content in segregated cassava populations.

1 Introduction

Cassava tuberous root (*Manihot esculenta*) is a critical carbohydrate crop globally, used by more than 500 million people, especially in Africa, Asia, and Latin America [1-3]. Currently, cassava tuberous root has become widely used as biofortified food to overcome malnutrition, notably in countries that use it as a staple [3-5]. The β-carotene is essential for human health because it is a precursor for provitamin-A [4]. Moreover, β-carotene content enhanced the capacity of cassava plants to intake and transport iron and zinc from the plant sources, rendering it not only to overcome vitamin deficiency but also iron and zinc deficiency, which is essential to defeat anemia [5]. Using cassava tuberous roots for

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biofortification was more profitable; hence, it produced yield and energy higher than other carbohydrate crops [1]. Thus, high β-carotene cassava was potentially used as a functional food to overcome malnutrition [5].

Another advantage of the yellow-fleshed cassava tuberous root is its higher tolerance to postharvest physiological deterioration (PPD), rendering a longer root shelf life than the white ones [5-9, 11]. The latest study by Beyene et al. [9] showed that the transgenic cassava plant with co-expression of *DXS* and *crtB* genes elevated biosynthesis of β-carotene about 15 to 20-fold and significantly extended the root shelf life until 11 days, which was less than two days in wild type. The natural β-carotene content of cassava was negatively correlated to PPD onset, increasing during storage until five days after harvest [10]. The antioxidant activity of β-carotene could reduce ROS compound accumulation in plants during stress [9-13]. Moreover, β-carotene was the first-line defense of the plant to quenching singlet oxygen as the most radical compound [13]. Thus, high β-carotene content is critical to increase plant stress tolerance [11-13].

Contrary to the vital role of β-carotene content, measuring β-carotene content is expensive and laborious due to its complexity as a biological compound [10]. Since a high β-carotene content is associated with the yellowness of cassava tuberous root, the color intensity of the cassava root could be used as an indirect trait for rapid selection [4-6]. While the qualitative method of color intensity scoring of the cassava root parenchyma showed a strong correlation with β-carotene [5, 6], its use for many samples in a breeding population selection was impractical [14]. Moreover, this method is prone to subjective assessment, depending on the capability of the surveyor [6]. Therefore, developing an objective and accurate method for predicting β-carotene content using cassava tuberous root images is essential [6, 14].

Enhancing a quantitative method through an image processing program offers a promising alternative for achieving more objective and straightforward measurement procedures. Mean grayscale measurement with ImageJ has proven effective for analyzing disease severity [15]. Consequently, assessing the correlation between the mean grayscale value and the β-carotene contents of cassava tuberous roots becomes essential.

This study aims to establish a practical and precise method for estimating β-carotene content based on root slice image processing using ImageJ software. The grayscale analysis enables quantitative values, resulting in more accurate measurements than the scoring method. Moreover, the simple equipment used in this method results in more practical methods than laboratory measurement. This new phenotyping method can precisely assess robust cassava tuberous root samples, eliminating the complexity and subjectivity in the recent β-carotene content measuring method.

2 Materials and Methods

1.1 Plant materials and experimental design

The cassava plants were grown at the BB Biogen Experimental Field in Depok, Indonesia, in collaboration with *Masyarakat Singkong Indonesia* (MSI). The cassava plants were harvested 11 and 10 months after planting, in October 2021 for the training set and October 2022 for the validating set. Cassava root samples were evaluated at the Research Centre for Genetic Engineering BRIN in Bogor, Indonesia. The experiment was meticulously designed as a randomized completely block design (RCBD) with a single factor of various cassava cultivars. Each cassava cultivar was treated as an experimental unit and replicated three times, ensuring the reliability and robustness of our findings. One to two tuberous roots per unit were used in the training and validating sets, respectively.

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The evaluation encompassed two tuberous root fleshed colors (i.e., yellow and white), reflecting the natural diversity of cassava. The training set was comprised of nine cassava tuberous root cultivars. Among these, three cultivars featured white-fleshed tuberous roots, namely cassava cv. Menti, Revita, and Roti. At the same time, the remaining six cultivars showcased yellow-fleshed tuberous roots, including cassava cv. Adira 1, Bokor, Carvita 25, Mentega 2, Nangka, and Ubi Kuning. The genetic diversity of the validating set is more extensive, adding seven white-fleshed cassava tuberous root cultivars: Adira 4, Adira 4 R, Apuy, Iding, Iding R, Kristal Merah, and Manggu. This validating set resulted in a rich collection of sixteen cassava germ-plasma, comprising six yellow and ten white-fleshed cassava cultivars, which underwent thorough evaluation in the subsequent year.

1.2 Grayscale value assessment for predicting β-carotene content

Cassava root slices from the proximal, middle, and distal ends were imaged on harvest day to estimate β-carotene content. Cassava root slices were captured using a flatbed scanner (CANON PIXMA MG2570S). Depending on root diameter, multiple cassava root slices (six to fifteen) were captured in a single scanning process. These recorded images were then analyzed using ImageJ software to obtain their respective grayscale values. The workflows for determining the grayscale value of cassava tuberous root parenchyma using ImageJ software are presented in Fig. 1, following well-established and proven procedures developed by [15].

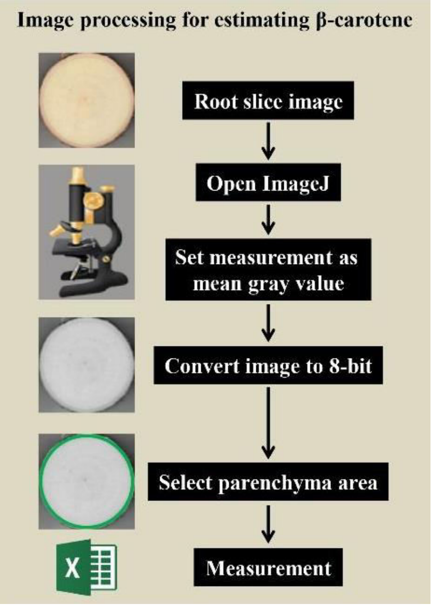

Fig. 1. Diagrammatic representation of image processing workflow using ImageJ for estimating β-carotene content using mean grayscale value.

1.3 β-carotene content analysis

Cassava cultivars Adira 4, Carvita 25, Menti, Roti, and Ubi Kuning, were selected for the β-carotene content analysis. The β-carotene content of cassava tuberous roots was assessed using the HPLC method [16]. The cassava tuberous roots were frozen using liquid nitrogen and then ground into a powder using a mortar and pestle. About 1.0–10.0 g of sample powder was homogenized in 1 mL NaCl and 30 mL of pyrogallol in ethanol. Add 3 mL of

KOH to the solution, incubate the mixed solution in a water bath at 56 ºC for 20 min, then cool in an icebox. Next, 23 mL of NaCl (1 %), 6 mL of 2-propanol, and 15 mL of ethyl acetate: n-hexane mix (1:9 v/v) were added to the solution. After that, the solution was centrifuged at 3,000 rpm for 5 min at 4 ºC, and this extraction process was repeated three times until the mixed solution became colorless. The upper layer of the solution was then transferred into a new tube (100 mL), evaporated at 40 ºC, and dissolved with 99.5 % ethanol. The dissolved fractions were injected into a 5-mL column using 99.5 % ethanol as the mobile phase for the HPLC analysis.

1.4 Validation of grayscale value assessment effectiveness

Sixteen cassava tuberous root cultivars were evaluated the subsequent year (by year replication) to validate the grayscale effectiveness in predicting β-carotene content. Image recording and processing workflow using scanner and ImageJ were the same as the previous experiment (Fig. 1). The grayscale value was assessed on cassava root slice images taken on the harvesting day. Follow-up evaluation was also conducted to validate the linear regression of the β-carotene content prediction using linear regression between grayscale and β-carotene from the previous experiment.

1.5 Statistical analysis

The grayscale values and β-carotene content were subjected to analysis of variance (ANOVA) and followed with Duncan's Multiple Range Test (DMRT) at a significance level of 5 %. Pearson's correlation and linear regression analyses measured the association between the grayscale values and the β-carotene content. The statistical analyses were performed using software developed by IRRI (http://bbi.irri.org/products), the Statistical Tools for Agriculture Research (STAR).

3 Results

The grayscale values of cassava root slice images, which showed significant differences among the white and yellow-fleshed cassava tuberous root cultivars (Fig. 2), have practical implications. These findings suggest that the grayscale value can be used to differentiate cassava tuberous roots based on their β-carotene content. Specifically, the cassava root slices images of yellow exhibited lower grayscale values than white-fleshed cassava cultivars (Fig. 2). The highest grayscale value of 221 is observed in cassava cv. Menti as white-fleshed root (Fig. 2 and 3). In contrast, the lowest grayscale value is identified in cassava cv. Ubi Kuning as yellow-fleshed root, with 199 (Fig. 2 and 3). Moreover, using a flatbed scanner for image recording offers another advantage, as multiple samples of root slices can be homogeneously recorded in a single scanning pass, as shown in Fig. 3.

Fig. 4A illustrates the β-carotene content of five selected cassava tuberous root cultivars: Adira 1, Carvita 25, Menti, Roti, and Ubi Kuning. As expected, the yellow-fleshed cassava tuberous root cultivars exhibit significantly higher β-carotene content than the white one (Fig. 4A). Specifically, the yellow-fleshed tuberous roots of cassava cv. Adira 1 and Ubi Kuning stand out with high levels of β-carotene content, approximately 0.70 ppm. In the medium levels of β-carotene content, cv. Carvita 25 (yellow-fleshed) and Roti (white-fleshed) show 0.42 and 0.34 ppm. In contrast, cv. Menti (white-fleshed) displays the lowest β-carotene content with 0.10 ppm (Fig. 4A), highlighting the significant differences among the cassava tuberous root cultivars.

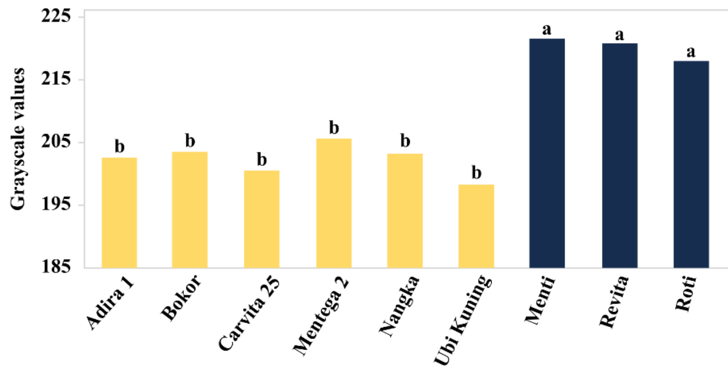

Fig. 2. The grayscale value of nine cassava tuberous root cultivars was evaluated at harvest. Lowercase letters above the bars indicate significant differences based on DMRT at a significance level of 5 %.

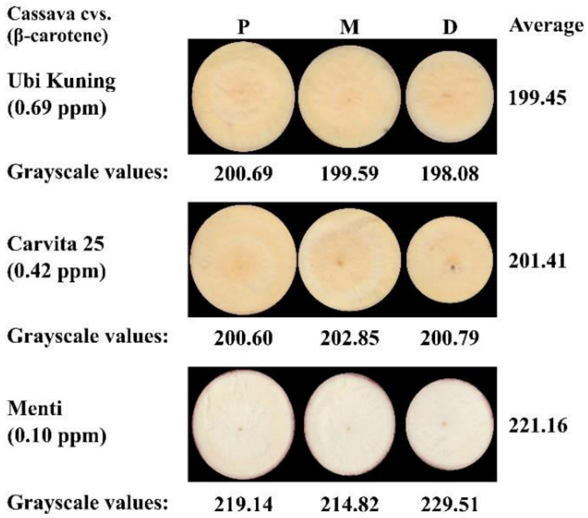

Fig. 3. Representative samples of cassava tuberous root slice images were recorded on harvesting day, and their grayscale values were determined using ImageJ. The root slice images illustrate a high (cv. Ubi Kuning), a medium (cv. Carvita 25), and a low (cv. Menti) of β-carotene content. The root slices were taken from the three positions of the cassava tuberous roots, such as P (proximal), M (middle), and D (distal) ends.

Our linear regression analysis establishes a significant negative association between the grayscale values and the β-carotene content (Fig. 4B) and offers a potential practical application. As the grayscale values of the cassava root slice images decrease, the β-carotene content increases (Fig. 4B). This intriguing finding suggests that grayscale values of root slice images could be a promising tool for predicting the β-carotene content, as demonstrated by the robust and negative coefficient of Pearson's correlation of −0.84 ($p < 0.01$) and a high coefficient determinant of 0.715 (Fig. 4B). Furthermore, Fig. 2 presents root slice images of three cassava cultivars, namely Ubi Kuning, Carvita 25 and Menti, representing low, medium, and high grayscale values. Interestingly, the β-carotene content of these cassava cultivars showed a clear inverse relationship with their grayscale values. This practical implication led us to use linear regression for the validation set, which involved sixteen cassava cultivars the following year.

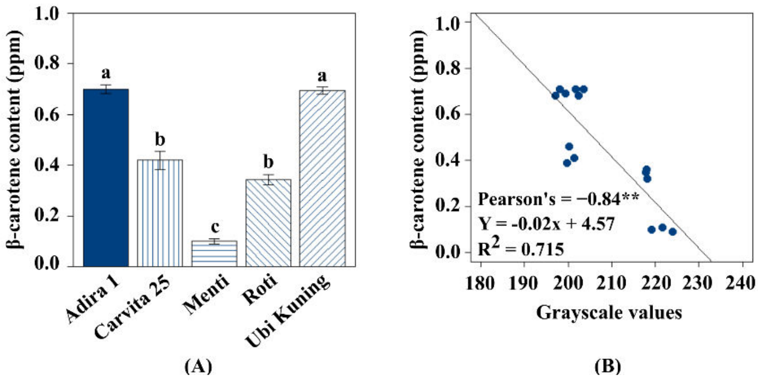

Fig. 4. (A) β-carotene content of tuberous roots from five cassava cultivars isolated at harvest. Lowercase letters above the bars indicate significant differences based on DMRT at a 5 % significance level. (B) Linear regression analysis between β-carotene content and the grayscale values of root slice images. Linear regression was conducted using five cassava cultivars, and each was replicated three times. ** shows significance at 1 % based on Pearson's method.

In the subsequent year evaluation, the grayscale values assessment is still practical for distinguishing the yellow and white-fleshed cassava tuberous root cultivars (Table 1). The yellow-fleshed cassava tuberous root cultivars consistently have grayscale values lower than the white-fleshed ones (Table 1). Moreover, the β-carotene content estimation using the previously determined linear regression (Fig. 4B) revealed a striking difference between yellow and white-fleshed cassava tuberous root cultivars in this validating set (Table 1). The β-carotene content of yellow-fleshed cassava tuberous root cultivars had similar values, slightly ranging from 0.50 to 0.52 ppm. In contrast, the white-fleshed cassava tuberous root cultivars are the lowest at 0.13 ppm on cv Menti and the highest at 0.24 ppm on cv Kristal Merah (Table 1).

4 Discussion

β-carotene content has numerous advantages for plant defence systems [5-13] and human diets [3-5]. Moreover, β-carotene content in cassava tuberous root could be a biomarker for selecting prolonged shelf-life cultivars as a critical trait in cassava industry [17]. While HPLC can accurately measure β-carotene content from cassava tuberous roots [10], this laboratory measurement is expensive, laborious, and challenging to apply to many samples in breeding populations [14]. Thus, cassava breeding for high β-carotene content necessitates an efficient approach of multiple lines in segregating populations [14]. As a result, a more dependable alternative to image processing that can precisely acquire huge samples simultaneously without fluctuating scoring results is advantageous.

The β-carotene content has high heritability, making it suitable for rapid cycling selection [18]. Moreover, β-carotene is a tetraterpenoid pigment compound usually expressed by yellow, orange, or red in plant organs [19]. In the cassava tuberous roots, the high β-carotene content strongly correlates with the yellowness level of parenchyma [4-6, 20]. Previously, scoring methods with different scales were used as selection criteria, such as white to orange scoring by 1–5 [5] and white to pinkish scoring by 1–9 [6]. These indirect measurements have been proposed and positively correlated with β-carotene content [5, 6]. However, there is a need for an accurate evaluation method to predict β-carotene content rather than relying on subjective and qualitative scoring methods [6, 14]. Image processing of cassava tuberous root slices with ImageJ may generate precise grayscale values for indirect β-carotene content assessment.

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Table 1. The grayscale values and estimation of the β-carotene content of sixteen cassava cultivars, recorded with a flatbed scanner on the harvesting date

Cassava cultivars	Tuberous root color	Grayscale value	Estimation of β-carotene content (ppm)
Adira 1	Yellow-fleshed	203.7 c	0.50 a
Bokor	Yellow-fleshed	202.8 c	0.51 a
Carvita 25	Yellow-fleshed	202.3 c	0.52 a
Mentega 2	Yellow-fleshed	203.6 c	0.50 a
Nangka	Yellow-fleshed	203.0 c	0.51 a
Ubi Kuning	Yellow-fleshed	203.2 c	0.51 a
Adira 4	White-fleshed	219.8 ab	0.17 bc
Adira 4 R	White-fleshed	218.1 ab	0.21 bc
Apuy	White-fleshed	219.1 ab	0.19 bc
Iding	White-fleshed	220.0 ab	0.17 bc
Iding R	White-fleshed	220.5 ab	0.16 bc
Kristal Merah	White-fleshed	216.4 b	0.24 b
Manggu	White-fleshed	219.6 ab	0.18 bc
Menti	White-fleshed	221.9 a	0.13 c
Revita	White-fleshed	219.7 ab	0.17 bc
Roti	White-fleshed	219.0 ab	0.19 bc

Note: A distinct lowercase letter in the same column indicates significant differences based on DMRT at a significance level of 5 %.

This study presents a novel phenotypic method that utilizes the mean grayscale value assessment, computed through ImageJ software, to predict β-carotene contents in yellow and white-fleshed cassava tuberous roots, as shown in Fig. 2 and Table 1. This innovative method can potentially revolutionize the assessment of numerous cassava samples, eliminating the subjectivity and complexity of such analyses. The investigation also provides compelling evidence of a robust and inverse relationship between the grayscale values and the β-carotene content (Fig. 4B). This discovery underscores the potential utility of employing grayscale value estimation using ImageJ as a game-changing alternative for screening β-carotene contents in segregated cassava tuberous root samples. The implications of these findings are substantial, promising significant advancements in cassava breeding methodologies and nutritional enhancement strategies, offering a brighter future for cassava production.

However, it is crucial to note that our study was limited to only two types of cassava cultivars: yellow and white-fleshed tuberous roots. This limitation highlights the need for a more extensive assessment of diverse cassava germ plasma, including white, yellow, orange, and pink-fleshed tuberous root, and their β-carotene content. The International Centre for Tropical Agriculture (CIAT) is already collecting such diverse germ plasma [5, 6]. This extensive assessment could potentially pave the way for substantial enhancements in the application of the proposed phenotyping method.

5 Conclusions

Our reliable use of grayscale values assessed with ImageJ software has significantly distinguished the yellow and white-fleshed cassava tuberous root cultivars, reflecting the differences in β-carotene content. Moreover, Pearson's analysis demonstrates a solid correlation between the grayscale values and the β-carotene content, albeit with a negative association. This method was consistently validated in subsequent years using larger samples (16 cassava tuberous root cultivars), offering a promising new approach for β-carotene content estimation. Thus, this study introduces a new phenotypic method for objectively and precisely predicting the β-carotene content of cassava tuberous root using grayscale values assessment. The established method could overcome the resource, time, and labor obstacle of selecting high β-carotene in large cassava tuberous root breeding populations.

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