ASCORBIC ACID PROLONGS CASSAVA TUBEROUS ROOT SHELF-LIFE THROUGH INCREASED ROS SCAVENGING CAPACITY

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ABSTRACT

The short shelf-life of cassava tuberous root limits storability to less than three days due to postharvest physiological deterioration (PPD). Postharvest treatments of cassava tuberous roots to delay PPD have been investigated using various chemical compounds. However, more affordable compounds for large-scale cassava harvest, such as ascorbic acid (AsA), are necessary. This study was conducted to examine the effectiveness of AsA in delaying PPD in cassava root slices and whole roots in the Postharvest Laboratory of IPB University, Indonesia, in September 2021. The results showed that soaking 2.5 % AsA for two hours substantially delayed PPD. Metabolic assessment of the root slice showed that AsA soaking markedly repressed H_2O_2 and enhanced POD activity. In cassava whole roots, AsA soaking also prolongs shelf-life up to ten days, which could solve the constraint in cassava storability before going into the processing lines. AsA also increased the tolerant responses in the intermediate and PPD-sensitive cultivars. Moreover, transcriptome analysis on the PPD tolerant cultivar revealed the significant role of the glutathione-ascorbate cycle in responding to oxidative stress during PPD. Therefore, using AsA as postharvest treatment could be an excellent solution to prolong cassava root's shelf-life on a large scale.

Key words: antioxidant, oxidative stress, peroxidase, postharvest treatment, root damage

INTRODUCTION

Cassava (*Manihot esculenta* Crantz.) tuberous root is an essential crop for food security in many developing countries (Ayetigbo et al. 2018). Moreover, cassava starch has multiple purposes as a raw material in various industries, such as paper manufacture, glue, pharmacy, polywood, and textiles (Parmar et al. 2017; Ayetigbo et al. 2018). Nowadays, cassava has also produced bioethanol as a renewable energy resource (Parmar et al. 2017). Unfortunately, cassava use as food and industrial raw materials is hampered by the short shelf-life of cassava roots due to postharvest physiological deterioration (PPD) (Zainuddin et al. 2018).

Cassava tuberous root has a limitation in storability due to PPD, resulting in blue, black, or brown root discoloration and wasting the root in a few days (Hu et al. 2016). Therefore, cassava tuberous roots should immediately be used once harvested; otherwise, PPD damages would make them unsuitable for processing (Hu et al. 2016; Parmar et al. 2017). Moreover, PPD also adversely affects some

physicochemical characteristics of cassava starch, which are critical for its industry use (Sanchez et al. 2013). Thus, PPD severely affects the cassava industry and socio-economic status in many producer countries (Prempeh et al. 2017). Unfortunately, cassava breeding for PPD tolerance stays challenging and takes a long time (Zainuddin et al. 2018). Hence, delaying PPD through postharvest treatment could temporarily solve cassava postharvest problems by prolonging cassava tuberous root shelf-life.

Effective postharvest treatments using chemical compounds to delay PPD have been reported, such as using melatonin (Ma et al. 2016; Hu et al. 2016; Hu et al. 2018), $CaCl_2$ (Hu et al. 2018), methyl jasmonate (Liu et al. 2019a), and ethanol (Liu et al. 2019b). However, those chemicals were only applied on a small experimental scale. Moreover, small farmers' accessibility to those chemicals might be difficult (Uchechukwu-Agua et al. 2015), and they are only suitable for high-value agricultural products (Tomlins et al. 2021). Therefore, a more accessible and affordable compound than those chemicals that delay the PPD of tons of cassava tuberous root every harvest is needed.

Ascorbic acid (AsA) is widely used as vitamin C in many countries, and its application for keeping product shelf-life is low-cost (Yin et al. 2022). Treatment of AsA has proven effective in keeping the postharvest quality of various horticultural crops (Sogvar et al. 2016; Guimarães et al. 2017; Barzegar et al. 2018; Zhao et al. 2019; Mohammadi et al. 2023). In those reports, AsA treatment in various horticultural crops reduced the metabolic rate (Guimarães et al. 2017), increased the ROS scavenging capacity, and reduced the ROS accumulation (Barzegar et al. 2018; Zhao et al. 2019) of the treated plant tissues. Hence, AsA could be used as an alternative compound to delay the onset of PPD damage.

In addition, the ROS scavenging mechanism has also been associated with delayed PPD regulation (Xu et al. 2013; Djabou et al. 2017), which also involves internal AsA content (Uarrota et al. 2016). Moreover, it has been shown that the ascorbate-glutathione (AsA-GSH) cycle plays an essential role in AsA biosynthesis and that its biosynthesis is activated during the wounding response in papaya (Li et al. 2022). In other studies, overexpression of an essential enzyme in the AsA-GSH cycle (i.e., *MeGPX*) substantially delays PPD in cassava (Vanderschuren et al. 2014). At the metabolic level, it also shows that the higher the tolerance to PPD, the more AsA content in cassava roots. Such results indicate the critical role of internal AsA in cassava tuberous roots' PPD-tolerant characters (Uarrota et al. 2016). In line with the internal AsA function, an external AsA treatment has also been reported to extend the shelf life of the in vitro cassava micro-tubers by ten days (Onejeme et al. 2023).

Although external AsA treatment has been reported to play a positive role in maintaining the postharvest quality of pineapple (Guimarães et al. 2017), chili (Barzegar et al. 2018), strawberry (Sogvar et al. 2016; Zhao et al. 2019), and anthurium (Mohammadi et al. 2023), it is not examined whether external AsA treatment could delay PPD in cassava tuberous roots. Therefore, this study sought to evaluate the effectiveness of AsA in delaying PPD damage of cassava tuberous roots. Subsequently, the role of AsA in delaying PPD responses at the phenotypic, metabolic, and molecular levels was investigated. The results of this research will aid in finding an affordable chemical compound for prolonging the storability of large cassava tuberous root quantities.

MATERIALS AND METHODS

Experimental design and plant materials. This research was conducted using a randomized complete block design. Metabolic analyses were undertaken in the laboratory using cassava root slice samples since they could be handled efficiently. The effectiveness of AsA on the whole cassava tuberous root was examined in the field study. The AsA concentration was optimized by testing the effects of soaking in two AsA concentrations (i.e., 2.5 and 5.0 %) to delay PPD in root slices and whole roots of cassava (Fig. 1A). Soaking in water (0 % AsA) was used as the control treatment. Furthermore, the effect of various cassava genetic backgrounds was evaluated using the optimum AsA concentration in the

previous step (Fig. 1B). This study was conducted in the Postharvest Laboratory, Department of Agronomy and Horticulture, IPB University, Bogor, Indonesia, in September 2021.

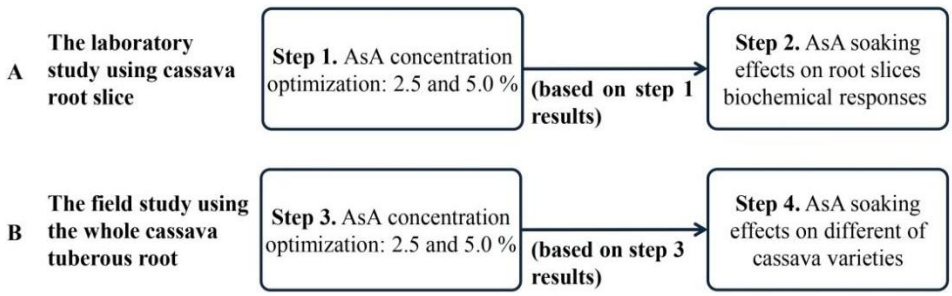

Figure 1. Stepwise implementation of the experiments to investigate the effect of ascorbic acid (AsA) soaking on cassava tuberous root shelf-life using (A) cassava root slices and (B) whole cassava roots.

A local cassava cultivar from Dramaga, Bogor, West Java, was used in this study, and the cassava farmers in Bogor have cultivated this local cultivar for years since it is a high-yielding genetic material. In addition, two advanced mutant genotypes (M_1V_7) derived from the irradiation of cassava cv. Gajah (GJ-10 mutant) and cassava cv. Malang-4 (ML-19 mutant) was also evaluated to investigate the genotype response to the AsA treatment.

Effectiveness of AsA to delay PPD in cassava root slices. The cassava tuberous root slices were soaked in either 0 (control), 2.5, or 5.0 % (w/v) of AsA solution for two hours. Subsequently, the excess AsA was removed from the treated cassava tuberous root slices using tissue papers. After treatment, the cassava tuberous root slices were incubated in Petri dishes for 0, 1, and 2 d. Each treatment used nine biological replications with one cassava root slice/replication.

The use of the AsA concentrations (i.e., 2.5 and 5.0 %) was based on a previous study by Sogvar et al. (2016) in the strawberry postharvest studies. Meanwhile, the selection of two-hour soaking is based on the previous three studies (Ma et al. 2016; Hu et al. 2016; Hu et al. 2018), which used melatonin to delay the PPD of both cassava root slices and whole roots. In these studies, cassava tissues were soaked for two hours to allow the infiltration of the applied compound into the treated inner layer tissues.

The PPD symptom evaluation was conducted using recorded images of the evaluated cassava root slices (SONY DSC-W810). The images were recorded during proper observation periods to determine the PPD symptoms. The extent of PPD symptoms was evaluated based on the damaged score values analyzed using ImageJ. In brief, the measurement is set as an area fraction on the analyze menu. The root slice image was converted to 8-bit, and then the parenchyma area was selected to adjust the threshold and measure the PPD symptom (Xu et al. 2013; Qin et al. 2017; Rahmawati et al. 2022). The image processing could distinguish the discolored from the fresh root areas, and the area fraction expressed in the percentage of the discolored area from the total root area was representative of PPD symptoms. The PPD symptoms among treatments were compared to determine differences in the effectiveness of delaying PPD. The effectiveness of AsA treatment was measured as reduced PPD symptoms in treated cassava tuberous root slices, and the most effective AsA concentration was selected and used in later analysis (Fig. 1A).

AsA effects on biochemical responses of cassava root slices. The AsA-treated root slice samples were prepared and analyzed for hydrogen peroxide (H_2O_2) according to Uarrota et al. (2016). The sampled cassava flour (ca. 1 g) was homogenized with 5 mL of 0.1 % (w/v) trichloroacetic acid (TCA). The

homogenized samples were centrifuged for 5 min at 4,000 rpm, and the supernatant (1 mL) was mixed with 1 mL of phosphate buffer (50 mM, pH 7.0) and 2 mL of potassium iodide (1 M). The absorbance of the mixture was read using a spectrophotometer (BEL Photonics, Spectrumlab D180, Brazil) at 390 nm to measure the H_2O_2 content, which was calculated through a standard curve and expressed as mmol kg^{-1}.

The cassava tuberous root slice samples (ca. 200 mg) were homogenized with 10 mL of phosphate buffer (0.1 M, pH 6.8) to measure peroxidase (POD) activity. The homogenized mixtures were centrifuged at 2 ºC and 17,000 rpm for 15 min. The supernatant (5 mL) was mixed with 250 mg of H_2O_2 (1 %) and 2.5 mL of pyrogallol (0.2 M). Subsequently, the POD activity was measured based on the mixture absorbance using a spectrophotometer at 420 nm. POD activity, measured as an increase in absorbance per unit time, was taken as one-unit enzyme activity (U) and expressed per kg protein (U kg^{-1}) (Kar and Mishra 1976).

Effectiveness of AsA soaking to delay PPD in whole cassava tuberous roots. The readily harvested, whole cassava tuberous roots were soaked in 2.5 or 5 % AsA in an air-tight chamber, and the air was vacuumed out from the box for two hours to ensure that AsA was absorbed into the cassava tuberous root. Soaking in water (0 % AsA) was the control treatment. After AsA treatment, the cassava tuberous roots were stored and evaluated at 5, 10, and 15 d. Five replicates were evaluated for each AsA concentration, comprising five cassava tuberous roots for each replicate.

The PPD symptoms were recorded at the proper observation periods (5, 10, and 15 d) by cutting the root slice samples (± 5 mm thickness) from the treated whole cassava tuberous root in four positions (i.e., at 25, 37.5, 50, and 75 % relative positions from the proximal end). The extent of PPD symptoms in the sampled cassava root slices was determined using the PPD damaged score as previously described.

The level of microbial infections was also observed for each of the treated cassava tuberous roots and classified into five classes (i.e., score 0 = no microbial infection, 1 = infection in 25 %, 2 = 37.5 %, 3 = 50 %, and 4 = 75 % relative positions from the proximal end). The presence and the level of microbial infection in the sample root slices were recorded in all treated whole cassava roots.

Dry matter content was calculated as the percentage of dried weight compared to fresh weight. About 40 g of fresh roots were chopped from the treated whole cassava tuberous roots and dried in an oven at 105 ºC overnight (Sanchez et al. 2013). The root weight was measured in every storage period and compared to the initial weight on the harvesting day to calculate the weight loss (Tomlins et al. 2021). The starch content was estimated using a specific gravimetry method using linear regression of $y = 96.65x - 71.60$ (Subekti 2018). Approximately 3 - 5 kg of cassava root samples were weighed in the air (wa) and the water (ww). Subsequently, the x value was calculated using the following formula:

$$x = \frac{wa}{wa - ww}$$

Note: wa = weight of cassava tuberous root in the air (kg), and ww = weight of cassava tuberous root in the water (kg).

Effectiveness of AsA to delay PPD in three cultivars of cassava tuberous roots. The genotype effects were evaluated on three cassava accessions (i.e., local cassava, ML-19, and GJ-10 advanced mutant). ML-19 was identified as PPD-sensitive in an earlier study (Rahmawati et al. 2022), while the PPD response of GJ-10 was unknown. In this evaluation, three cultivars of cassava tuberous root samples were soaked in 2.5 % AsA for two hours and subsequently stored for 10 d (Fig. 1B). PPD response evaluation was performed as previously described.

Statistical analysis. All PPD symptom data were transformed using Arcsine square before statistical analysis (Garcia et al. 2013). The data were analyzed using ANOVA, and the mean comparisons were made using HSD at $\alpha = 5$ %. All statistical analysis was conducted using STAR software developed by IRRI (http://bbi.irri.org/products).

Transcriptome profile of ascorbate-glutathione (AsA-GSH) metabolic pathway in PPD-tolerant cassava. The following year, transcriptome analysis was made using cassava cv. Adira-4, which is a PPD tolerant variety, to evaluate the significance of the glutathione metabolism pathway related to AsA synthesis and show the regulation of AsA on PPD tolerance cassava variety. Partial transcriptome evaluation results were presented to indicate the possible molecular evidence of AsA regulation in the PPD tolerance. The partial transcriptome analysis was used to illustrate the molecular role of AsA during PPD and complemented the PPD phenotypic symptoms and biochemical responses.

Total RNA was isolated using the standard procedures of the QUICK-RNA Miniprep Kit (R1055). RNA-Seq libraries were constructed using the Illumina Novaseq 6000 reagent kit and then sequenced using Illumina Novaseq 6000. The processing of transcriptome data was initiated with quality checks using FastQC (Ding et al. 2016). The clean reads were aligned to the cassava reference transcript version 8.1 (http://phytozome-next.jgi.doe.gov/) using SALMON (Patro et al. 2017). Subsequently, differentially expressed genes (DEG) were assessed with the DESeq2 package using the RStudio software (Love et al. 2014), with a significance level set of p-value < 0.05 and log2FC > 2/-2. The DEG analysis was assessed by comparing the transcriptome profile of the storage root (1 d) to the fresh root (0 d) as the control (1 d/0 d). Significant differences in gene expression were shown as a dynamic process of PPD, describing the changes from the initiated (the fresh roots) to the progress of the PPD processes (the 1 d stored roots). Furthermore, gene ontology (GO) enrichment and gene network were performed using STRING (Jensen et al. 2009).

RESULTS AND DISCUSSION

Effectiveness of AsA to delay PPD in cassava root slices. AsA soaking of cassava root slices delayed PPD. The representative photographs of root slice damages due to PPD at 1 and 2 d after treatment are presented in Fig. 2A. The PPD symptoms of AsA-treated cassava root slices reached 5.5 % in 5.0 % AsA treatment to 9.6 % in 2.5 % AsA at 1 d and up to 11.3 % at 2 d after treatment (Fig. 2B). Meanwhile, the PPD symptoms of untreated cassava root slices (control) reached over 50 % of the total root area at 2 d (Figs. 2A and B). There were no significant differences in PPD symptoms among cassava root slices treated with either 2.5 or 5.0 % AsA (Fig. 2B). Therefore, the 2.5 % AsA concentration was selected for subsequent PPD response evaluation of cassava root slices.

AsA effects on biochemical responses of cassava root slices. PPD is initiated when the cassava roots are detached from the plant and results in elevating reactive oxygen species (ROS) immediately after wounding (Zainuddin et al. 2018). The ROS compound acts as a messenger for the signaling pathway to mediate the defense mechanism by altering gene expression (Djabou et al. 2017). However, ROS could be toxic and harmful to plant cells, leading to high concentrations of oxidative stress (Singh et al. 2019). Oxidative stress has been indicated as the primary PPD inducer in previous studies, and it is followed by complex deterioration processes (Xu et al. 2013; Hu et al. 2016; Qin et al. 2017). Therefore, keeping ROS homeostasis in cassava roots after harvesting is vital for delaying PPD. ROS homeostasis is partially regulated by antioxidants, including AsA and peroxidase (POD) (Xu et al. 2013; Uarrota et al. 2016; Djabou et al. 2017).

A sudden increase of H_2O_2 in cassava tuberous roots contributes to PPD onset. Therefore, reducing H_2O_2 accumulation is essential to delay PPD in cassava tuberous roots (Xu et al. 2013; Vanderschuren et al. 2014). In this study, accumulation of H_2O_2 in the cassava tuberous root slices occurred since 1 d storage, indicating oxidative stress had started (Fig. 3A). At 2 d, the cassava root

slices soaked in 2.5 % AsA showed a lower H_2O_2 than the control (Fig. 3A). These results are similar to studies in strawberry (Zhao et al. 2019) and anthurium (Mohammadi et al. 2023) showing lower H_2O_2 in the AsA-treated tissues than the control. AsA is a compound that gives electrons to complete the catalytic process of H_2O_2 into malondialdehyde (MDA) and H_2O by ascorbate peroxidase (APX) (Hu et al. 2016). Hence, soaking cassava root slices in AsA should also enhance the internal catalytic processes to reduce H_2O_2 and thus could delay the PPD responses. Moreover, biosynthesis of internal AsA might be insufficient for plant defense under severe stress conditions. Therefore, external AsA is needed for optimal stress mitigation (Akram et al. 2017) to prevent PPD in cassava root slices.

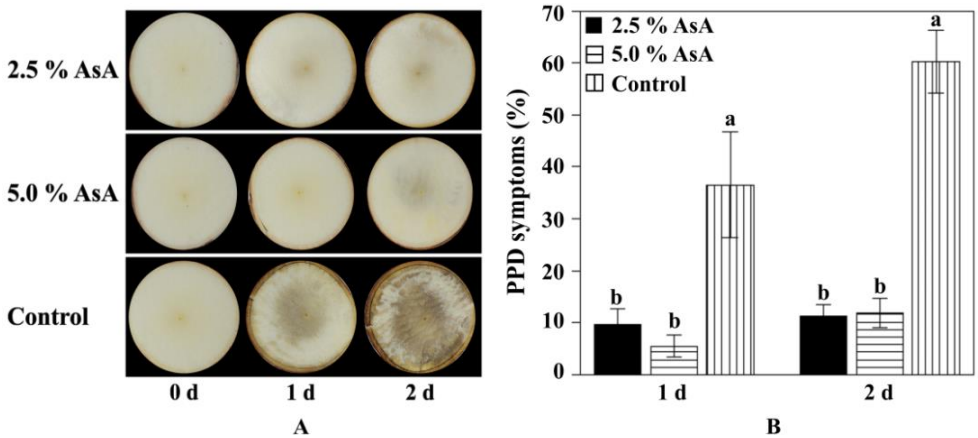

Figure 2. Effect of ascorbic acid (AsA at 2.5 or 5.0 %) soaking of cassava root slices on the PPD symptoms at 1 and 2 days (d) after treatments. Soaking in water (0 % AsA) was the control treatment. (A) The visual of PPD symptoms of the AsA-treated cassava root slices and (B) The percentages of PPD damage symptoms of the AsA-treated cassava root slices. The distinct lowercase letters above the bars in Fig. 2B indicated that the mean values between treatments for the same storage period (i.e., 1d or 2d after storage) differ significantly based on HSD at $\alpha = 0.05$.

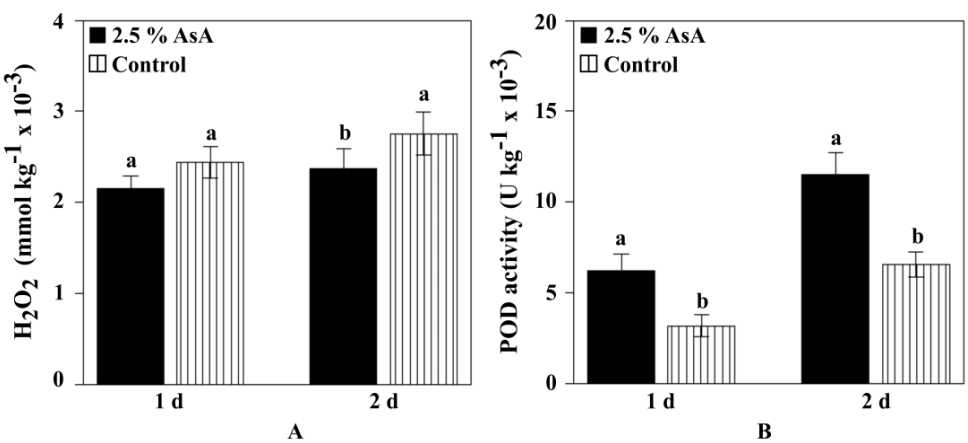

Figure 3. Effect of ascorbic acid (AsA, 2.5 %) soaking on biochemical responses of the treated cassava root slices at 1 and 2 d after treatment. (A) H_2O_2 accumulation and (B) POD activities. The distinct lowercase letters above the bars in Figs. 3A and 3B indicate that the mean values between treatments for the same storage period (i.e., 1d or 2d after storage) differ significantly based on HSD at $\alpha = 0.05$.

The POD activity in cassava root slices soaked in AsA was also higher than in the untreated control (Fig. 3B). POD is one of the critical enzymes for ROS scavenging, as shown by the 78 % up-regulation of POD genes during PPD (Wu et al. 2019). In the metabolic processes, POD changes H_2O_2 into H_2O (Hu et al. 2016). The increased level of POD activity from 1 to 2 d was sharper in the AsA-treated cassava root slices than in the control (Fig. 3B), suggesting that AsA stimulated other antioxidant activities. The increase in POD activities on the AsA-treated cassava root slices coincided with the low H_2O_2 content (Figs. 3A and B). Similar responses have been reported that AsA stimulated various antioxidant activities, such as ascorbate endogen, ascorbate peroxidase (APX), peroxidase (POD), superoxide dismutase (SOD), and catalase (CAT) (Berzegar et al. 2018; Zhao et al. 2019; Mohammadi et al. 2023). Moreover, these antioxidants are also desirable for delaying PPD in cassava tuberous roots (Xu et al. 2013; Hu et al. 2016; Qin et al. 2017).

ROS are free radical compounds that can cause cell damage, leading to plant cell death (Djabou et al. 2017; Singh et al. 2019). The decreased H_2O_2 after soaking AsA (Fig. 3A) might imply minor cell damage in cassava roots. Therefore, AsA-treated cassava roots can maintain freshness (low PPD symptoms) until 2 d compared to the control (Fig. 2A). Moreover, this study revealed increased POD activity due to AsA soaking that could contribute to H_2O_2 scavenging and delay PPD response.

Effectiveness of AsA to delay PPD in whole cassava tuberous roots The results showed that PPD symptoms increased during the five-day observation periods. However, the PPD symptoms between the AsA-treated whole cassava roots and the control treatments only significantly differ at 10 d after storage (Fig. 4A). At that storage period, the AsA-soaking treatment (at 2.5 or 5.0 % AsA) of whole cassava roots resulted in a low PPD symptom (< 8 %). In contrast, the control treatment resulted in higher PPD symptoms (> 10 %). Similar results have been reported by Onejeme et al. (2023), who showed that adding 100 mg L^{-1} AsA in tissue culture medium significantly prolongs the shelf life of cassava micro tuber to ten days. Moreover, there are no significant differences in PPD symptoms between either 2.5 or 5.0 % AsA treatments at 10 d storage (Fig. 4A). Therefore, the 2.5 % AsA concentration was selected for subsequent PPD response evaluation of whole cassava roots.

Cassava tuberous roots naturally become unpalatable in less than three days (Hu et al. 2016). Results of this study showed that AsA treatment prolongs the whole cassava root's shelf-life for up to ten days, an enormous improvement for the cassava industry. Moreover, the low PPD symptom also indicates the inhibition of secondary metabolite oxidation (browning). The low browning process in PPD tolerant cultivars can maintain the starch gel clarity (Sanchez et al. 2013). Hence, AsA treatment could become an alternative postharvest treatment of cassava to prevent PPD, prolong processing time, and maintain product quality during root storage, which farmers need (Prempeh et al. 2017). Moreover, the low cost of AsA allows its large-scale application for tons of cassava tuberous root every harvest to delay PPD, eliminating the cost restriction of CaCl2, melatonin, methyl jasmonate, and ethanol (Uchechukwu-Agua et al. 2015; Tomlins et al. 2021).

AsA can not prevent starch loss and microbial infection of the whole cassava roots (Figs. 4B and D), probably because it only acts on ROS scavenging. The cassava root respiration still occurs after harvest and during storage, converting starch to sugar (Sanchez et al. 2013). Moreover, defense responses against pathogen attacks differ from the PPD defense mechanisms (Djabou et al. 2017). Therefore, AsA-induced mechanisms resulting in decreased PPD symptoms might not be able to protect cassava roots from microbial attack, especially at 15 d. The AsA treatment has no significant effect on the dry matter content of cassava tuberous roots during storage (Fig. 4C). These results are similar to those of earlier studies (Sanchez et al. 2013).

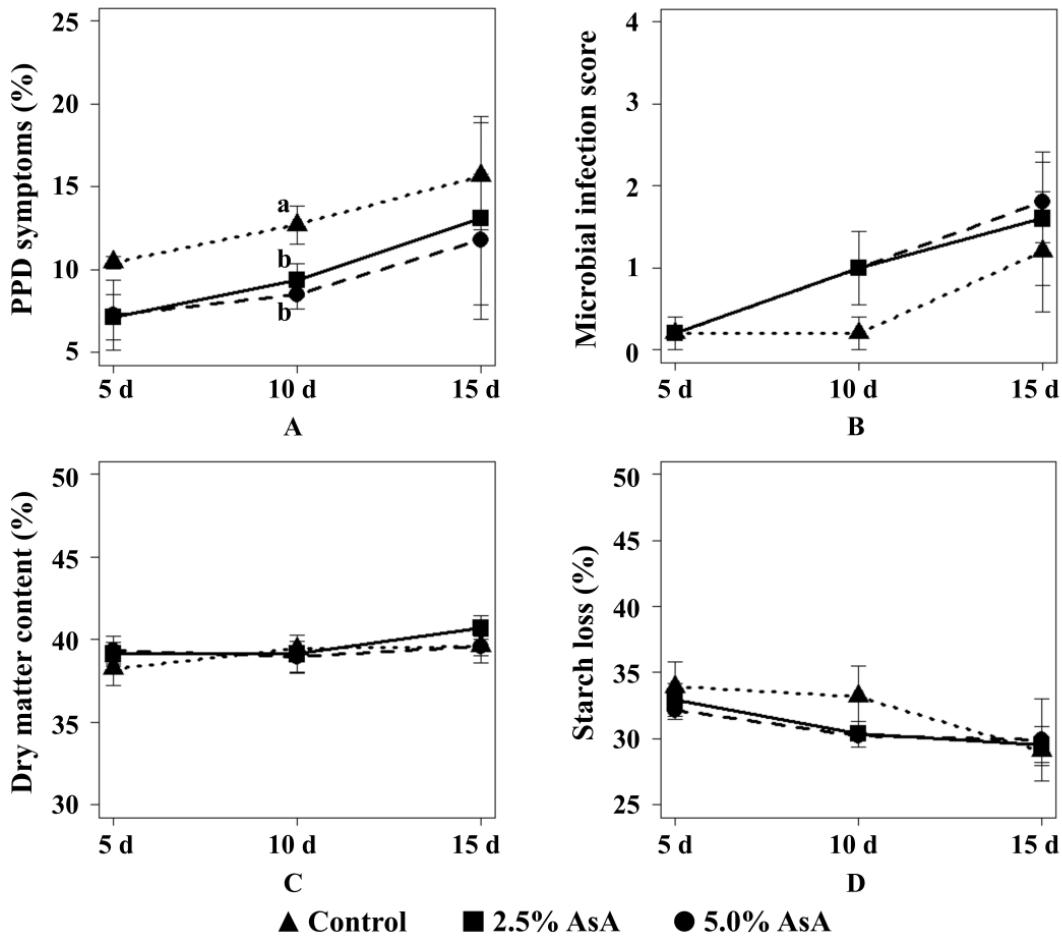

Figure 4. Effect of ascorbic acid (AsA, 2.5 and 5.0 %) soaking of whole cassava tuberous roots on the postharvest cassava root qualities at 5, 10, and 15 days after treatments. Soaking in water (0 % AsA) was used as a control treatment. (A) PPD symptoms, (B) microbial infections, (C) dry matter content, and (D) starch loss. The distinct lowercase letters above the line (Fig. 4A) significantly differed based on HSD at $\alpha = 0.05$.

Effectiveness of AsA to delay PPD in three cultivars of cassava tuberous roots. A significant difference in PPD symptoms was observed in three cassava accessions evaluated after soaking whole cassava roots in 2.5 % AsA, indicating the presence of genetic variation effects (Fig. 5). The AsA soaking treatment induces PPD tolerant responses in the local cassava cultivar and cassava cv. ML-19 mutant. The ML-19 mutant was previously identified as a PPD-sensitive cassava variety (Rahmawati et al. 2022), while the local cassava cultivar was identified as a PPD intermediate cassava in this study (Fig. 4A). The response of both the local cassava variety and cassava cv. ML-19 mutant became PPD tolerant after AsA soaking treatment, showing less than 10 % PPD symptom at 10 d (Fig. 5).

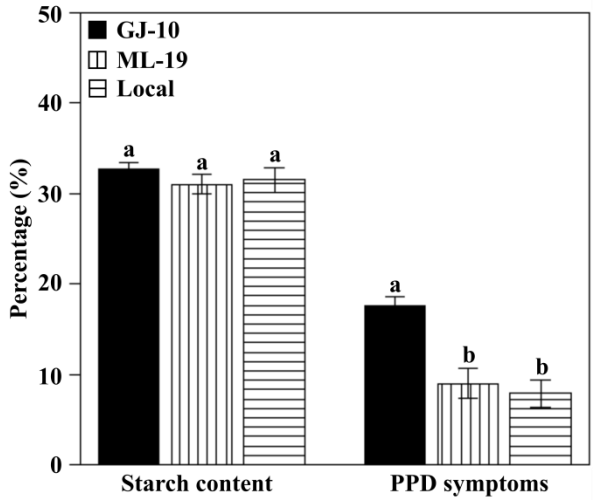

Figure 5. Effect of ascorbic acid (AsA, 2.5 %) soaking treatment of whole cassava tuberous roots of three cassava accessions (GJ-10, ML-19, and local cassava cultivars) on starch content and PPD symptoms at 10 days after treatment. The distinct lowercase letters above the bar show they are significantly different based on HSD at $\alpha = 0.05$.

AsA soaking treatment of cassava cv. GJ-10 resulted in a 17.6 % PPD symptom at 10 d (Fig. 5). Even after AsA soaking treatment, the PPD symptom of cassava cv. Gajah-10 is greater than the other two cassava accessions (i.e., local cassava and ML-19 mutant). Therefore, cassava cv. GJ-10 mutants might naturally be classified as more PPD-sensitive than the local cassava variety or the cassava cv. ML-19 mutant. The PPD responses in cassava are genetically regulated (Rahmawati et al. 2022).

Expressions of various genes are reported to increase in PPD tolerance cassava tuberous roots. The overexpression of catalase (*CAT*), superoxide (*SOD*), glutathione peroxidase (*GPX*), deoxy-D-xylulose-5-phosphate synthase (*DXS*), and bacterial phytoene synthase (*crtB*) increases antioxidant accumulations in cassava root tissues (Xu et al. 2013; Vanderschuren et al. 2014; Beyene et al. 2018), silencing of *F6'H* and *C3'H* reduces scopoletin (Liu et al. 2017; Ma et al. 2022), silencing of *APL3* reduces dry matter and starch content (Beyene et al. 2020), and silencing of *ANR* enhances flavonoid biosynthesis (An et al. 2023). Those studies showed that PPD is a complex mechanism offering more than one tolerance scheme. Therefore, the cassava GJ-10 mutant may not have compatible mechanisms that synergistically work with AsA to increase PPD tolerance.

AsA soaking treatment provides a short-term solution to extend the shelf life of cassava roots by up to 10 d, which is effective for various factors, such as differences in cassava genetic background. AsA is known to be more water-soluble than the other non-enzymatic antioxidants (Mehla et al. 2017), and as such, AsA soaking treatment is practical and can be used in the field. Based on this study, increasing endogenous AsA accumulation could become a new target mechanism for developing PPD-tolerant cassava cultivars through breeding programs, as has been suggested by Onejeme et al. (2023). Genetic engineering to enhance the high level of endogenous AsA in *Brassica rapa* resulted in Turnip Mosaic Virus resistance (Fujiwara et al. 2016). The similar resistance mechanisms could have raised the PPD tolerance of cassava tuberous roots. AsA is an endogenous compound in cassava tuberous roots (Uarrota et al. 2016).

Transcriptome profile of ascorbate-glutathione (AsA-GSH) metabolism pathway of tolerant PPD cultivar. At the molecular level, significance in the ascorbate-glutathione (AsA-GSH) metabolic

pathway was identified through transcriptome analysis in the PPD tolerant cultivar (Fig. 6). The AsA-GSH cycle is related to the biosynthesis of AsA, an essential antioxidant during plant stress (Li et al. 2022). GSH produces AsA through a reaction with DHA mediated by glutaredoxin. Up-regulating AsA-GSH genes could enhance AsA biosynthesis (Hu et al. 2016). In our study, the transcriptome analysis results showed that 17 genes related to the AsA-GSH cycle were up-regulated, and the other seven genes were down-regulated from 0 d to 1 d after storage (Fig. 6), indicating the vital role of the AsA-GSH cycle in the PPD tolerance. The initial onset of the PPD response is one day after the storage, and it coincides with the upregulation of the genes involved in the AsA-GSH pathway. In line with the metabolic level analysis, a previous report suggested a high ascorbate content was observed in PPD-tolerant cultivars (Uarrota et al. 2016).

Moreover, glutathione-S-transferases (*GST*) and glutathione peroxidase (*GPX*) are the most up-regulated genes based on the GO analysis. The *GST* and the *GPX* are related to the biological process in response to oxidative stress (Fig. 6). Previous studies revealed that H_2O_2 is the most significant contributor to the oxidative stress of cassava roots (Xu et al. 2013; Vanderschuren et al. 2014). Overexpression of *GPX* proved to play a significant role in detoxifying the H_2O_2 in cassava roots, resulting in delayed PPD onset (Vanderschuren et al. 2014). The *GST* and *GPX* gene products reduce H_2O_2 into a neutral compound, H_2O (Ribas et al. 2014).

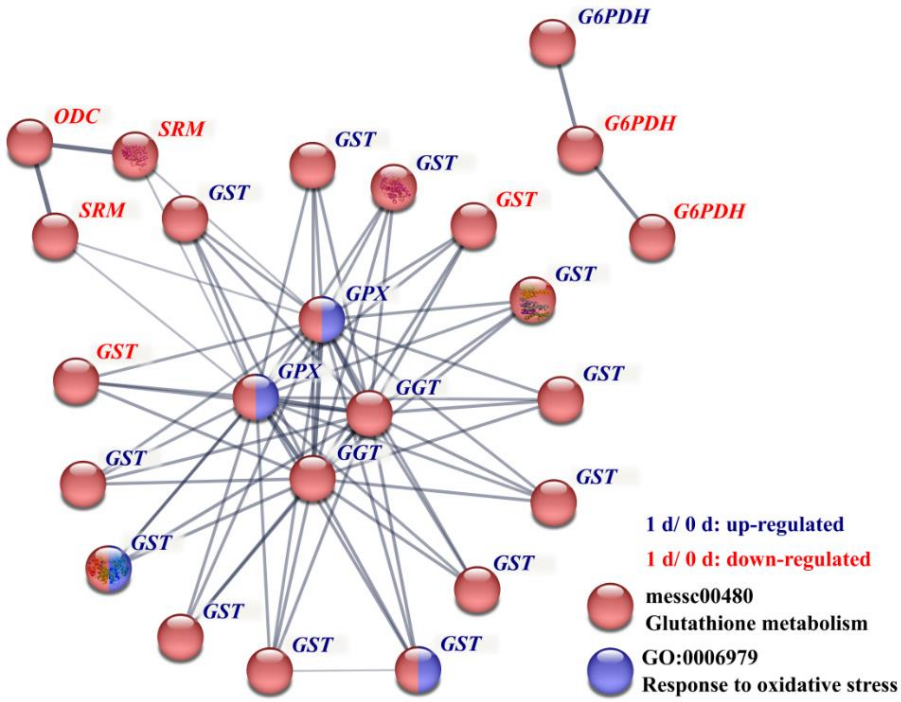

Figure 6. Gene network modeling of the ascorbate-glutathione (AsA-GSH) metabolic pathway related to the oxidative stress biological responses using a PPD-tolerant cassava cultivar. Gene network analysis was performed using STRING, with a confidence level interaction of at least 0.40. The thickness of connecting lines between the dots (genes) indicates the strength of interaction, and the thicker the line, the stronger the gene interaction.

To validate the function of *GST* and *GPX*, both genes could be evaluated functionally using transgenic plants for their role in PPD tolerance responses. If the evaluation indicates a positive function in PPD tolerance, the genes could be used to develop future PPD-tolerant cassava cultivars. Moreover,

the results of this study also showed that external AsA treatment changed the PPD tolerance responses of cassava cultivars previously identified as intermediate and sensitive to tolerance to PPD. Therefore, increasing endogenous AsA accumulation through available biotechnology approaches might become a new strategy for developing PPD-tolerant cassava cultivars.

CONCLUSION

Ascorbic acid plays an essential role at the metabolic and molecular levels against PPD damage, resulting in low PPD symptoms. At the phenotypic level, PPD symptoms of either root slices or whole cassava tuberous roots soaked for two hours in 2.5% AsA were lower than the control. AsA soaking prolonged the whole cassava root shelf-life for 10 days. AsA-soaked cassava tuberous root slices at the metabolic level showed reduced H_2O_2 content and enhanced POD activity compared to the control. Moreover, the transcriptomic level showed that the AsA-GSH pathway responsible for AsA biosynthesis is essential in the PPD tolerant responses. In this study, two up-regulated genes (i.e., *GPX* and *SGT*) that reduce ROS accumulation through gene network analysis might have essential roles in PPD tolerance. These findings offer a new opportunity to prolong cassava root storability in large quantities with an affordable compound (i.e., AsA). Subsequently, increasing endogenous AsA accumulation might become a new strategy for PPD-tolerant cassava cultivar development in the future.

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