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Growth and yield performance of four generations of high amylose transgenic Adira 4 cassava

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Abstract

Attempt to improve cassava starch quality have been conducted by producing the first high amylose transgenic Adira 4 Indonesian cassava. This study aimed to evaluate agronomic performances of transgenic plants, assessment of four generations in specific containment screenhouse and limited field trial were conducted with improved method. The survival and growth rate of cuttings was enhanced by applying growth regulator such as Atonik and improving planting procedures. The growth and yield performances were measured by comparing related variables across four generations of selected lines. Dipping in 1 $mL.L^{-1}$ Atonik solution with shading in the early planting were only required when the plant growth was poor and not favorable soil and weather. The result showed that survival of third and fourth generations of Lines A55-5 and A55-15 could reach 100 % when it was treated with Atonik. According to 14 lines assessed, Line A55-5 was one of the promising ones due to its growth, multiplication performance, and almost double amylose content compared to wild type. Meanwhile, Line A55-12 showed the highest yield. The finding will benefit functional food industries when appropriate gene construct and approaches were applied for better growth and yield results.

INTRODUCTION

Cassava is mostly planted in worldwide, particularly at marginal lands by a small scale farmers in Indonesia. Indonesian farmers in dry areas grow cassava for daily subsistence and trading at small scale. In industrial application, cassava starch is an alternative starch since potato starch have been developed rapidly. The production of cassava starch in Indonesia had decreased since 1991 despite a high demand of starch, then further decreased since 2014 although the productivity per hectare had steadily increased which resulted in the increase in import in 2015–2016 although in 2017–2019 the import decreased, which might be due to the increase in the import of other starch source (Pusat Data dan Sistem Informasi Pertanian Kementerian Pertanian, 2019). In this study, the tuber starch composition in relation to genetic markers will be of interesting and beneficial for improved trait in the future. Due to the economic importance of starch, great interest in modifying its properties through genetic engineering has been stimulated. Consequently, understanding the product of starch biosynthesis, particularly on characteristic of starch granule formation and the formation of amylose and amylopectin is important.

The application of cassava starch is not only in food industries, but also in non-food industries that have high values due to its product. It is an important component in various food products, such as ice cream, noodles and puddings as it is used as a binding or thickening. In the manufacture of paper, textiles, plywood, and in the production of ethanol and biodegradable polymers cassava starch is also used. Starch with very low amylose is required for paper filling while that with very low amylopectin is

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required for healthy food industries.

Through conventional breeding the improvement of cassava variety has been carried out. However, there has been a problem using this approach as some of the favorable allelic combinations occurring in the parental generation are lost in the subsequent generations as a result of vegetative propagation. Through series of intensive research, genetic engineering is able to modulate the activities of specific genes although the process of storage starch biosynthesis, needs further study. Recently, a progress on molecular characterization of the genes encoding starch synthetic enzymes has revealed important information on starch biosynthesis in plants. However, most of research has focused on the production of starch from the main staple foods, such as corn, wheat, potatoes, and rice.

Very limited information on the biosynthesis of starch in cassava is available although only a few numbers of the genes participating in starch biosynthesis of cassava have been characterized currently. To have more comprehensive insight on root system of cassava producing starchy tubers, information regarding the isolation and characterization of the genes involved is necessary. Research to obtain cassava cultivars with improved starch characteristics and/or yield through marker assisted selection and genetic engineering has been enhanced by various functional analysis techniques, such as RNA interference (RNAi) and gene over-expression determining the precise effects of individual genes on the properties of starch produced.

Genetic improvement of cassava possessing high amylose could be obtained from conventional and non-conventional breeding method. As conventional breeding, including selection of germplasm and crossing normally takes a long time, this method has been combined with molecular markers, known as MAS (Marker-Assisted Selection). Other technique such as gamma irradiation, requires at least four generation to be assessed to ensure the trait is stable. To accelerate the procedure, this technique has also been combined with molecular markers. More recent techniques that have been developed are genetic transformation and genome editing. Inserted genes regulating starch composition could be conducted by particle bombardment coated with gold or tungsten, or via *Agrobacterium tumefaciens* co-culturing. Several genes involved in starch composition include SBE (Starch Branching Enzyme), GBSS (Granule-Bound Starch Synthase), and Branching Enzyme/BE (Zhao et al., 2011; Bull et al., 2018). In cassava, starch with high amylose could be obtained if the plants are lacking the function of all Branching Enzyme (BE).

Unlike cereals, it is much more difficult to obtain starch mutant through induced mutagenesis in tuber crops such as cassava due to its natures, which are vegetatively propagated, self-pollinated, incompatibility, and heterozigosity (Ceballos et al., 2007). Waxy (amylose-free) maize, rice and potato have been reported by various researchers (Zhao et al., 2011, Zhou et al., 2017, Jiang et al., 2010. However, there are not many reports on waxy cassava, and even less report on high amylose cassava.

The only possibility is naturally occurred “waxy” and small granule cassava starch lines, which was identified from self-pollinated cassava lines in a breeding prorgam of CIAT (Ceballos et al., 2007, Ceballos et al., 2008, Ceballlos et al., 2017). In addition to the natural occurrence, transgenesis approach to obtain waxy cassava has been reported by a team of Wageningen University, The Netherlands since 2005 by down-regulating expression of GBSSI gene constitutively or tissue specific, which were field evaluated in Indonesia (Raemakers et al., 2005; Zhao et al., 2011; Koehorst-van Putten et al., 2012b), or by the deletion of GBSSI and PTST1 mutants through CRISPR/Cas9–based mutagenesis by ETH Zurich team (Bull et al., 2018).

Cassava with very high amylose has never naturally occurred or produced by conventional breeding. Therefore, researchers of Wageningen University and Research (WUR) and Indonesian Institute of Sciences (LIPI) produced cassava with either low amylose and high amylose (Raemakers et al., 2005). Improvement was conducted for transforming cassava cultivar Adira 4 with this construct or a control construct, in which the luciferase gene was cloned behind the 35S promoter. The result showed that the 35S promoter induced luciferase activity in all organs at similar levels when luciferase activity was measured in leaves, stems, roots and tuberous roots. Meanwhile, the GBSSI promoter showed very low expression in the first three organs, but very high expression in the latter one. These findings indicated that the cassava GBSSI promoter is the best candidate to achieve tuberous root-specific expression in cassava (Koehorst-van Putten et al., 2012a). Recent research conducted by team of Chinese Academy of Sciences (CAS) successfully produced high amylose transgenic cassava by down regulating BE

genes, which are responsible for amylopectin biosynthesis (Zhou et al., 2020).

The production of cassava through genetic transformation which possess high amylose content using Indonesian cassava variety has never been reported. In this research, therefore, an effort to produce transgenic Adira 4 cassava inserted by GBSSI gene is considered the first in the world. The growth of the resulted high amylose transgenic Adira 4 cassava, however was mostly poor. Thus, to obtain appropriate number of planting materials for series of biosafety evaluation in the limited field trials at LIPI in Indonesia, some efforts were conducted, including propagation by using whole plant stem as the source of cutting, which normally only uses the basal end stem, and by using growth regulator and providing shading net in the early growth. Appropriate number of plants for biosafety assessment and other related assessment (food, feed, and environment) is required to meet the regulation of Indonesian Government, which is in line with the Cartagena Protocol. The high-amylose cassava starch could provide a novel source of cassava starch for various industrial applications, especially healthy food industries.

In order to increase the survival rate and the vigor of transgenic cassava cutting, the cuttings were treated with Atonik, which is a bio-stimulator based on 3 synthetic nitro phenols SOD (Superoxide dismutase); APX (Ascorbate peroxidase); ROS (Reactive oxygen species). To withstand oxidative stresses that protect cells from oxidative effects and reduce them, an antioxidant system including enzymatic and non-enzymatic ones is developed within the plants. The Superoxide dismutase (SOD) and the Ascorbate peroxidase (APX) are the most important enzymes that remove active oxygen, while ascorbic acid, carotenoids, and phenols are the important non-enzymatic antioxidants, (Sharma et al., 2016).

This research aimed to assess growth and yield performances of transgenic Indonesian cassava plants possessing higher amylose derived from propagated cuttings using improved techniques by assessing four generations grown in specific containment screen house and limited field trial in Indonesia.

MATERIALS AND METHODS

The materials for genetic transformation was friable embryogenic callus (fec) derived from leaf lobes of

Figure 1. Separation of upper from lower parts of stems of high amylose transgenic Adira 4 to be planted as the second generation of planting materials in the screenhouse in Indonesia

Table 1. Number of transgenic cuttings lines of Adira 4 planted in the biosafety containment without shade

Planted directly in the soil		Planted in trays	
Transgenic lines	Number of cuttings	Transgenic lines	Number of cuttings
Wild type (C 2B: Adira 4)	10	Wild type (C 2B: Adira 4)	5
A55 - 4	6	A55 - 4	4
A55 - 6	6	A55 - 6	5
A55 - 8	12	A55 - 8	6
A55 - 10	35	A55 - 10	16
A55 - 11	18	A55 - 11	6
A55 - 12	2	A55 - 12	2
A55 - 2	2	A55 - 5	2
A55 - 15	1		

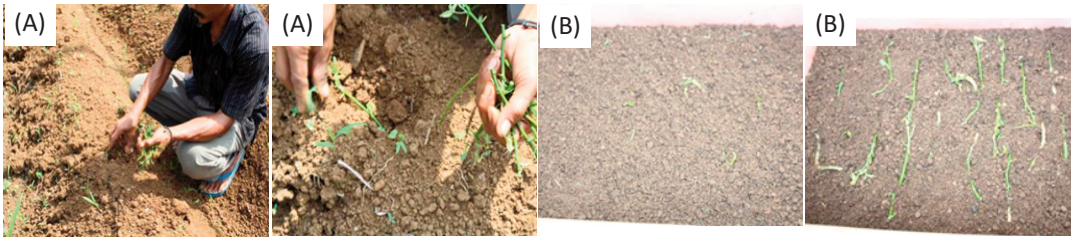

Figure 2. Preparing and planting of cuttings of high amylose transgenic Adira 4 in the screenhouse biosafety containment. (A) Direct in the soil; (B) In trays after soaking in Atonik solution.

Table 2. Number of shoot cuttings of the second generation of high amylose transgenic Adira 4 as affected by Atonik and without Atonik treatments prior direct planting in the soil in the screenhouse LIPI, Cibinong

No.	With atonik	Number of cuttings	Without atonik	Number of cuttings
1	A55 - 5.1	15	A55 - 4.1	39
2	A55 - 5.2	10	A55 - 4.2	17
3	A55 - 5.3	7	A55 - 4.3	24
4	A55 - 5.4	11	A55 - 4.4	25
5	A55 - 5.5	7	A55 - 4.5	12
6	A55 - 5.7	8	A55 - 6.1	21
7	A55 - 6.3	26	A55 - 6.2	30
8	A55 - 10.1	13	A55 - 7.1	8
9	A55 - 10.2	30	A55 - 8.1	24
10	A55 - 10.3	24	A55 - 8.2	40
11	A55 - 10.4	28	A55 - 8.3	18
12	A55 - 10.6	6	A55 - 11.9	17
13	A55 - 10.7	13	A55 - 11.10	22
14	A55 - 10.8	16	A55 - 11.17	14
15	A55 - 10.9	21	A55 - 11.18	10
16	A55 - 10.10	18	A55 - 11.19	5
17	A55 - 10.11	12	A55 - 12.2	42
18	A55 - 10.12	14	A55 - 12.3	29
19	A55 - 11.1	22	A55 - 13.1	9
20	A55 - 11.2	51	A55 - 13.3	16
21	A55 - 11.4	24	A55 - 13.4	16
22	A55 - 11.5	6	A55 - 15.1	11
23	A55 - 11.6	6	A55 - 15.2	16
24	A55 - 11.8	12	A55 - 15.3	18
25	A55 - 11.13	24	A55 - 15.5	11
26	A55 - 11.14	12	A55 - 15.6	19
27	A55 - 11.16	9	A55 - 15.9	8
28	A55 - 11.17	5	A55 - 15.10	26
29	A55 - 12.1	24	C 2B	8
30	Wild type: C 2B	6	Adira 4	10
31	Wild type: Adira 4 stem cuttings	6	-	-
	Total	486		565

Adira 4 Indonesian variety inserted with RNAi construct p5IRTCGBa containing an inverted repeat of part of the cassava GBSSI gene. The produc-tion of friable embryogenic callus used as genetic materials, regeneration and acclimatization in the greenhouse for producing transgenic high amylose Adira 4 cassava were conducted in Wageningen University and Research (WUR) in 2009, in which the materials were used as stock materials for further generations in Indonesia. The procedure for obtaining fec and genetic transformation were the same as described in Koehorst-van Putten et al. (2012b).

All parts of stems of the first regenerated high amylose transgenic Adira 4 and Wild Type (control) obtained from Wageningen University and Research (WUR) were cut into (20 to 25) cm length consisting of at least 3 axillary buds and planted in the Biosafety Containment of LIPI in Cibinong Science Centre since 2010 and maintained for further evaluation and stability test (Figure 1). All parts of stems including the youngest parts with shoot tips of the 10 month-old first generation were cut in the same size as the first generation and treated the same to produce the second generation. All plants were grown in a specific screenhouse biosafety containment and limited field trial. To utilize all parts of the stems and to evaluate the effect of stem cutting position, lower and upper parts of cuttings were separated for the next planting (second generation).

All stem cuttings (Table 1 and Figure 2) and shoot cuttings (Table 2) were planted either in a tray containing a mixture of sand : soil : compost w/w/w (1 : 1 : 1) or directly in the screenhouse specific containment with soil fertilized with manure. Before planting, all cuttings were soaked in a solution containing Atonik at a concentration of 1 $mL.L^{-1}$ for an hour. Some cuttings were planted under the shade using a shading net, allowing only 35 % of sunlight.

All stem/shoot cuttings of third and fourth generation were directly planted in the field. The field was arranged into beds and the soil was added with compost with the ratio of 2 : 1, and lime ($CaCO_3$ at a dose of 1.0 $ton.ha^{-1}$ to 2.5 $ton.ha^{-1}$) as well as a component to prevent from nematode attack and all were shaded with paranet. The length of shoot cuttings was (20 to 30) cm and were soaked in Atonik at the concentration of 1 $mL.L^{-1}$.

Maintenance was made by watering the cuttings and young plants almost every day to maintain the humidity. The plants were harvested at 10 months. No specific pesticide or fungicide treatment applied as no severe pest and disease attack was observed. Growth assessment of four generations of transgenic Adira 4 was conducted until the age of 10 months. The plants were grown in the soil in the screenhouse for two times and in the limited field trial for two times. Meanwhile, yield assessment was only conducted by harvesting 10-month-old plants of the second generation.

Variables observed and measured were percentage of survival, height, number of branches, yield represented by tuber fresh weight and number of tubers per plant, starch content, and amylose content. The data were analyzed using ANOVA at α= 5 % in SPSS ver. 21.0. Amylose content measurement was conducted as as described by Koehorst-van Putten (2012b).

RESULTS AND DISCUSSION

Effect of procedure of planting (in a tray or directly in the soil) on the survival rate of the first generation of high amylose transgenic Adira 4 in the screenhouse

The emergence of new sprouts was affected by the procedure of planting as the planted in trays sprouted 2 days earlier than those planted directly in the soil (Table 3). The shoot cuttings of control and transgenic plants grown in trays produced new shoots at 17 days after planting (Figure 3), while those directly planted in the soil only produced new shoots not earlier than 7 days after planting. This might be caused by the composition of medium in the trays, which has higher porosity than that in the soil, as it was mixed with sand (1 : 1). However, as the volume of media in the trays was not as abundance as in the soil, after 14 weeks, the majority of the plants died except those of Line A55-6 and control. This might be due to the depletion of nutrients in the media in the trays. It is suggested that the plants be transplanted to the soil that has been fertilized.

Effect of shading net on the survival rate of the first generation of high amylose transgenic Adira 4 in the screenhouse

The percentage of survived high amylose regenerant plants derived from shoot cuttings planted directly in the soil without shading net observed at the age of 14 weeks after planting was 16.7 % and 5.7 % for Lines A55-6 and A55-10, respectively, while that of control (C 2B) was 30 %

Table 3. Survival rate of cuttings of the first generation of high amylose transgenic Adira 4 in the screenhouse biosafety containment

Directly in the soil			In trays		
Transgenic lines	Number of survived cuttings	Survival rate (%)	Transgenic lines	Number of survived cuttings	Survival rate (%)
Wild type (C 2B: Adira 4)	6	60	Wild type (C 2B: Adira 4)	2	40
A55 - 4	0	0	A55 - 4	0	0
A55 - 6	1	16.7	A55 - 6	1	20
A55 - 8	0	0	A55 - 8	0	0
A55 - 10	2	5.7	A55 - 10	0	0
A55 - 11	0	0	A55 - 11	0	0
A55 - 12	0	0	A55 - 12	0	0
A55 - 2	0		A55 - 5	0	0
A55 - 15	0				

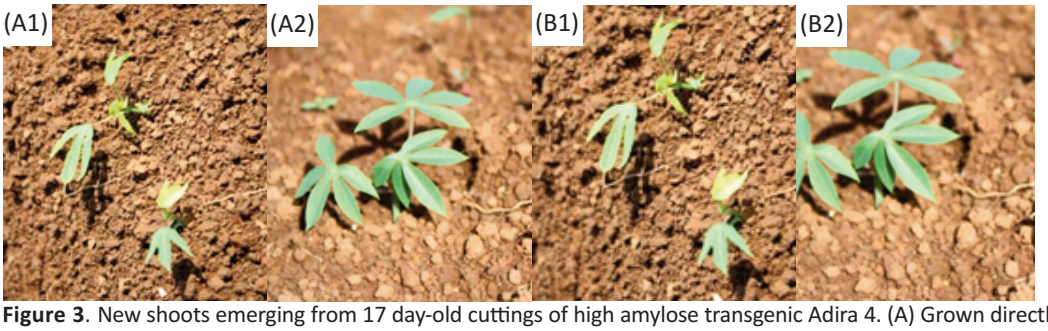

Figure 3. New shoots emerging from 17 day-old cuttings of high amylose transgenic Adira 4. (A) Grown directly in the soil; (B) In trays; (1) Line A55-4; (2) Wild type

Table 4. Shoot cuttings of the first generation of high amylose transgenic Adira 4 planted directly in the soil as compared to that planted in the trays at 14 weeks after planting without shade in the screenhouse

Transgenic lines	Directly in the soil			In trays		
	Number of cuttings planted	Number of survived cuttings*	Survival rate (%)	Number of cuttings planted	Number of survived cuttings*	Survival rate (%)
A55 - 4	6	0	0	4	0	0
A55 - 5	2	0	0	2	0	0
A55 - 6	6	1	16.7	5	1	20
A55 - 8	12	0	0	6	0	0
A55 - 10	35	2	5.7	16	0	0
A55 - 11	18	0	0	6	0	0
A55 - 12	2	0	0	2	0	0
A55 - 15	1	0	0	-	-	-
Wild type (C 2B)	10	6	60	5	2	40
	92	9	82.4			

Remark: *survival rate was counted when cuttings produced new sprouts

(Table 3). Meanwhile, the percentage of Line A55-6 survived in the trays containing soil and sand (1 : 1), was higher, which was 20 %, but in contrast, the survived wild type (Control C 2B) was only 6.5 %. This result indicated that transgenic Line A55-6 was superior, the most tolerant and adaptable toward

planting procedure and media composition as they could survive at the highest rate regardless the place and the media in which they were planted.The survival rate of transgenic lines was low as the original planting materials were small. Although the size of young stem cuttings of wild type (control C 2B) used as planting materials were normal and healthy, the survival rate was also low, indicating that the younger part of the stem was not the ideal planting material as compared to the basal end stem.

Additional shading net could increase the survival rate of shoot cuttings in transgenic lines, which at 10 weeks after plantings was 25 % to 37.5 % for Lines A55-8 and A55-10, although surprisingly, none of control could survive (Table 4, Table 5, Figure 4).

Table 5. Survival of shoot cuttings of the first generation of high amylose transgenic Adira 4 grown in the screenhouse, at 10 weeks after planting with shade

Transgenic lines	Number of cuttings planted	Number of survived cuttings*	Survival rate (%)
A55 - 4	19	0	0
A55 - 8	8	3	37.5
A55 - 12	6	0	0
A55 - 10	8	2	25
A55 - 13	5	0	0
A55 - 15	4	0	0
Wild type (C 2B)	7	0	0
		Transgenic plants: 50	

Remark: *Survival rate was counted when cuttings produced new sprouts

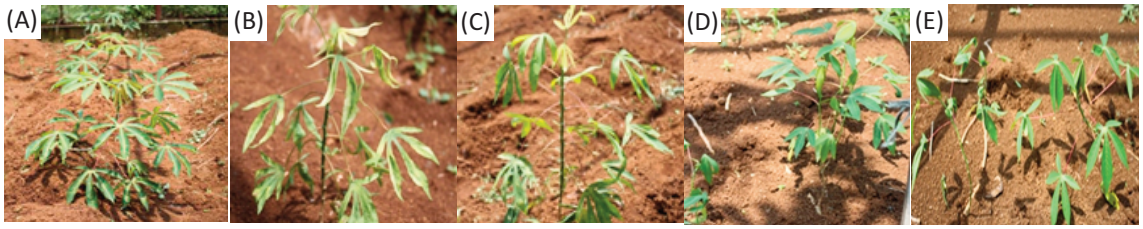

Figure 4. Growth of plants derived from cuttings of the first generation grown directly in the soil and schreen house: Without shade (A) Wild type; (B) Line A55-4; (C) Line A55-10, with shade (D) A55-10; (E) A55-8

Growth performance of the first to fourth generations of high amylose transgenic Adira 4 in the biosafety screenhouse and in the limited field trial area

Based on Figure 5, Atonik was beneficial to the third and fourth generation since the first and second generation transgenic Adira 4 planted in the screenhouse did not seem to require Atonik to boost the vigor and survival rate, indicated by higher survival rate of the plants without Atonik treatments. There was an exception for Lines A55-5 and A55-10 whose survival relied on Atonik, and the survival rate of Line A55-10 increased in the second generation. In contrast, soaking in Atonik solution for an hour was required by transgenic plants to be planted in the limited field trial whose soil condition might not as favorable as that in the screenhouse. More lines of transgenic plants could survive, and the survival rates of the survived lines were higher than the plants without Atonik treatment. In wild type, both regenerated from tissue culture (in vitro) and from stem cuttings (Adira 4), Atonik did not give an effect since both treated and non-treated ones showed 100 % survival rate. The vigor and size of stem cuttings regenerated from the second generation were much better, and the stem diameter was larger so that the survival rates of the third and fourth generation of Lines A55-5 and A55-15 in the

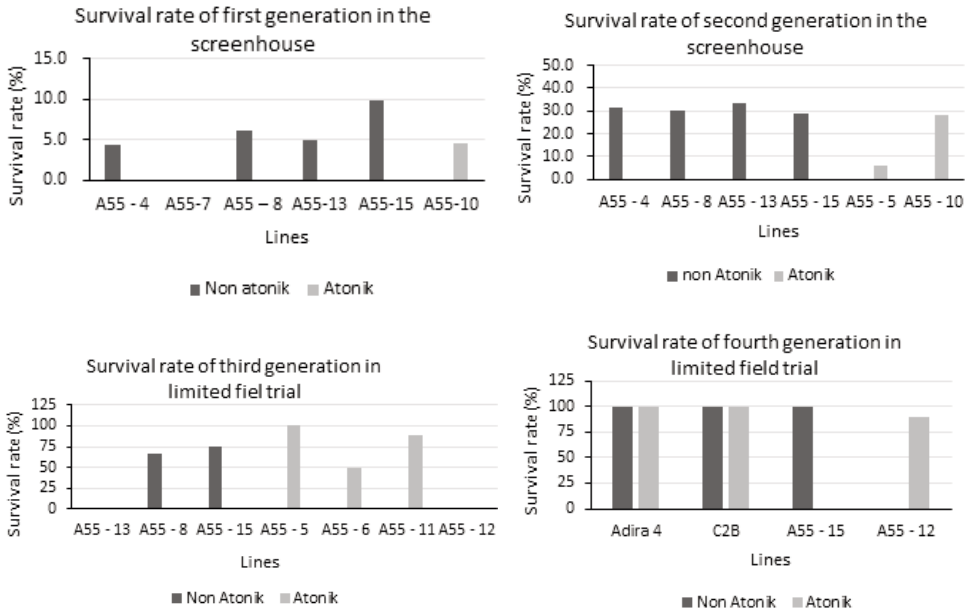

Figure 5. Survival rate of the first and second generation of transgenic high amylose transgenic Adira 4 in the screenhouse (top), and of the third and fourth generation in limited field trial area (bottom).

Table 7. Means of plant height and number of branches of the second generation of high amylose transgenic Adira 4 derived from shoot cuttings grown in the screenhouse

Transgenic lines	Atonik treatment	Plant height (cm)	Number of branch
A55 - 6	Non atonik	77.25 ± 7.587 a	1.00 ± 0.000 a
	Atonik	120.00 ± 21.358 a	1.25 ± 0.164 a
A55 - 11	Non atonik	85.67 ± 44.126 a	1.33 ± 0.333 a
	Atonik	133.86 ± 13.785 a	1.43 ± 0.297 a
Wild type		127.33 ± 14.827 a	1.50 ± 0.342 a

Remark: Values ± standard error followed by the same letters in the same column are not significantly different according to ANOVA at α= 5 %.

limited field trial could reach 100 %.

Due to limited number of survived transgenic plants which reduced the availability of the number of lines and the number of individuals per line, the assessment on survival, growth attributes and yield was conducted only on the available lines and individuals that could be compared as shown in Tables 6–8. The survival rate of certain lines of transgenic Adira 4 was significantly different from that of the wild type (WT) as observed between A55-6 and A55-11 without Atonik treatments as well as between A55-6 and wild type (Table 6). In terms of plant height and number of branches, there was no significant difference observed between lines analyzed (A55-6 and A55-12) and between lines and the wild type, both with and without Atonik treatment This indicates that transgenic lines whose growth condition was as vigorous as that of wild type (WT) were comparable to optimum condition of WT, and Atonik was not required (Table 7).

The survival rate of the third generation of high amylose transgenic Adira 4 grown in the limited field trial area was not significantly different between lines. However, there was a significant different between lines and the wild type at the age of 1 months and 3 months after planting (Table 8 and Figure 6). The number of original planting material (cuttings) to be planted in the third generation indicated the health and vigor of the second generation of plants, resulting in the availability of planting material for further assessment in the limited field trial. The results are in accordance with work conducted by Przybysz et al., (2014) reporting that Atonik gave positive results when the plants were under stress condition. This effect might be due to the fact that this compound

Table 8. Means of survival rate of the third generation of high amylose transgenic Adira 4 derived from Atonik treated cuttings grown the limited field trial area

Transgenic lines	Number of cuttings	Survival rate at 1 MAP	Survival rate at 3 MAP
A55 - 6	2.40 ± 0.245 b	1.60 ± 0.510 b	1.20 ± 0.583 b
A55 - 11	1.80 ± 0.583 b	1.80 ± 0.583 b	1.60 ± 0.678 b
Wild type	3.75 ± 0.250 a	3.75 ± 0.250 a	3.75 ± 0.250 a

Remarks: MAP = Months after planting; values ± standard error followed by the same letters in the same column are not significantly different according to ANOVA at α= 5%.

Figure 6. Growth of first generation of high amylose transgenic Adira 4 as compared to the wild type (control) in the screenhouse

increases the plant's tolerance to stresses due to inappropriate conditions, such as soil condition in the limited field trial area, by increasing the effectiveness of plant antioxidants that could improve the growth and productivity as proven in different plant species (Calvo et al., 2014; Przybysz et al., 2010).

The effect of Atonik was not significant on the survival rate and growth of several lines and wild type in the third and fourth generation, which was possibly due to the lines or genotypes and the concentration used was considered low (1 $mL.L^{-1}$). In several plants such as rose, woody, and forestry species such as teak, Atonik gave significant effect when the concentration used was higher than 1 $mL.L^{-1}$, which could be as high as 10 $mL.L^{-1}$ (Sitinjak, 2015).

To increase the effect of Atonik, some researchers added other compound such as boron at the concentration ranging from 10 $mL.L^{-1}$ to 30 $mL.L^{-1}$. The application of Atonik at a concentration of 3.0 $mL.L^{-1}$ was the most effective in improving vegetative growth of the stem cuttings of roses as it could increase the maximum growth of stem cuttings of roses, shown by the highest average of shoots (3), longest shoot (5.6 cm), number of petioles per bud (5), number of leaves per petiole (5), longest petiole, longest leaves, longest root, and number of roots until fifth week (Sitinjak, 2015). By comparing the applications of Atonik at different levels, which were 0 $mL.L^{-1}$, 10 $mL.L^{-1}$, 30 $mL.L^{-1}$, 60 $mL.L^{-1}$, and 120 $mL.L^{-1}$, to *Citrus amblycarpa* L. with different types of cuttings (shoot cuttings and stem cuttings), the best result was obtained by the application of Atonik at a concentration of 10 $mL.L^{-1}$ Atonik. This concentration could enhance the best growth, shown by the highest shoot height (3.37 cm) and the longest leaf (3.63 cm) of the shoot cuttings (Sitinjak, 2017). Meanwhile, higher concentration of 60 $mL.L^{-1}$ was required by stem cuttings. This suggests that the optimum concentration of Atonik required is different depending on the type of cuttings of *Citrus amblycarpa* L.

The growth of high amylose transgenic Adira 4 at the age of one and half months (6 weeks) and at 5 months after planting varied in terms of plant height, number of main stems and number of branches as shown in Table 9. The growth of most lines was steady as lines with the highest and the lowest average values at 1.5 months old were still the same as those with the highest and the lowest average values at 5 months old. The average height of Line A55-6 at 1.5 months old was even higher than that of both wild types (C 2B derived from tissue culture and Adira 4 cutting). At 5 months

Table 9. Growth performance of first generation of high amylose transgenic Adira 4 at 1.5 and 5.0 months after planting in the screenhouse

Transgenic lines	1.5 month old after planting		5.0 months old after planting	
	Mean of height (cm)	Mean of number stem	Mean of height (cm)	Mean of number stem
A55 - 4	41.6 ± 7.487 b	1.80 ± 0.200 b	55.8 ± 9.957 b	2.00 ± 0.316 a
A55 - 5	71.3 ± 14.998 ab	1.00 ± 0.218 bc	93.9 ± 25.996 ab	1.00 ± 0.218 b
A55 - 6	96.7 ± 6.119 a	1.67 ± 0.333 bc	118.0 ± 3.786 a	2.00 ± 0.000 b
A55 - 10	57.8 ± 6.920 ab	1.50 ± 0.261 bc	70.9 ± 11.556 ab	1.50 ± 0.261 ab
A55 - 11	41.4 ± 6.010 b	1.23 ± 0.173 bc	51.9 ± 7.956 b	1.14 ± 0.165 ab
A55 - 13	65.0 ± 26.061 ab	0.75 ± 0.250 c	83.5 ± 32.012 ab	0.75 ± 0.250 b
A55 - 15	58.4 ± 14.767 ab	0.80 ± 0.200 c	82.5 ± 20.477 ab	0.80 ± 0.200 b
Wild type (C 2B)	35.1 ± 3.255 b	2.78 ± 0.278 c	97.6 ± 7.234 ab	1.89 ± 0.111 a

Remarks: Only lines with at least three replication/individuals were counted; values ± standard error followed by the same letters in the same column are not significantly different according to ANOVA at α= 5 %.

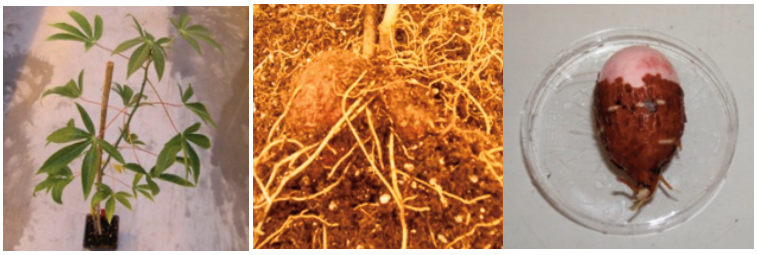

Figure 7. Transgenic high amylose Adira 4 grown in polybags in the greenhouse of WUR, Wageningen, Netherlands

old, however, the average height of this line was lower than that of wild type (Adira 4 cutting). The growth rate of wild type (C 2B) was the lowest at 1.5 months as compared to all lines of transgenic cassava, but the plants could grow faster afterwards, exceeding the average height of several lines. The line with the highest number of individuals that could be grown was A55-11 whose number of individuals was 16 although the average height was considered the second lowest. Almost all lines and the wild type (C 2B) had more than one stem, while the wild type (Adira 4 cutting) only had one stem. It seems that there was no correlation between number of stems and the height (Table 9).

Yield performance and amylose content of high amylose transgenic Adira 4 grown in the screenhouse

The first regenerated transgenic high amylose Adira 4 plants in the greenhouse of WUR were grown in small pots, not all plants produced tubers, and the size of tuber produced was small (not larger than a diameter of a Petri dish) as shown in Figure 7. The color of the inner skin of tubers of transgenic Adira 4, regenerated both in the Netherlands and in Indonesia indicated the trait was consistently performed across generation, which was similar to the wild type (pinkish) (Figure 7 and Figure 8).

Table 10 showed that the first generation of plants was not as healthy as the second generation, the yield was only assessed in the second generation in the screenhouse. The yield of transgenic lines Adira 4 was very low as only very limited lines could produce appropriate tubers from which the starch could be extracted. Only one line, which was A.55-12, could produce appropriate number and size of tubers, while the other lines had poor growth even in the second generation and did not improve in the third and fourth generations due to the soil conditions of the limited field trial area, thereby failing to produce tubers although they have swollen basal end stems (Figure 8 and Figure 9). The size of tubers, however, was larger than that grown in The Netherlands, as all lines of the transgenic cassava plants were grown in the small pots after they were acclimatized in the greenhouse (Figure 8). The variation of fresh weight and size

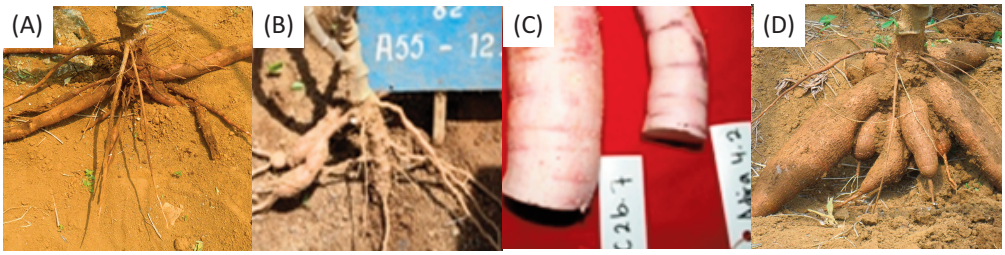

Figure 8. Yield of high amylose transgenic Adira 4 (A), as compared to control Adira 4 regenerated from tissue culture (B), from stem cuttings grown in the screenhouse (C), and C2B (D).

Figure 9. Lugol stained tuber and swollen basal end stems of high amylose transgenic Adira 4 A55-15.1 (left), A55-12.1 (centre), A55-8.2 (right) grown in the screenhouse.

Table 10. Yield assessment of second generation of high amylose transgenic Adira 4 grown in the screenhouse

Transgenic lines	Mean of fresh weight per individual (g)	Extracted starch weight (g)
Wild type (Adira cuttings)	2,050	360
Wild type (C 2B.1–9)	3,144	680
A55 - 12.2	80	2.6

also occurred in the wild type individuals as the fresh weight of wild type regenerated from tissue culture and stem cuttings ranged from 1,500 g to 4,900 g and 1,500 g to 2,600 g, respectively, and the average fresh weight was 3,144 g and 2,050 g, respectively (Table 10).

The harvested tubers were extracted to obtain starch and analyzed for amylose content. The starch of several lines of transgenic Adira 4 contained higher amylose compared to the wild type (control C 2B), as shown in Table 11. That of Line A55-5 was almost double the wild type (control C 2B) whose amylose content was 15 %. As the number of tubers produced by the transgenic plants were limited, the starch obtained was also limited (Figure 10). The content of amylose, however, could be detected from swollen basal end stems when they did not produce tubers (Figure 9).

The yield of high amylose Adira 4 was not high since the height and canopy architecture of the plants were not tall, and some were abnormal. The abnormality of transgenic plants have been reported although in some crops such as rice (Sun et al., 2017) and maize bearing different gene i.e. SBEI did not cause abnormalities (Wang et al., 2017). It is therefore, suggested to employ other gene construct and other mechanism to obtain better growth and yield of cassava, which have been improved by Bull et al. (2018) and Zhang et al. (2019) using CRISPR/Cas9 gene editing. Zhou et al. (2020) produced transgenic cassava with starches containing up to 50 % amylose due to the constitutive expression of hair-pin dsRNAs targeting the BE1 or BE2 genes. The generation of high-amylose cassava

Table 11. Amylose content of high amylose transgenic Adira 4 of the first regenerated plantlets acclimatized in the greenhouse

Transgenic lines	Amylose content (%)*
Wild type	15
A51 - 108	18
A55 - 2	22
A55 - 5	27
A55 - 7	17
A55 - 10	17
A55 - 11	16
A55 - 13	20
A55 - 15	18

Figure 10. Starch extracted from Adira 4 control and from Line A55-12.2 of transgenic Adira 4 plants grown in the screenhouse.

was obtained by the group by down-regulating expression of the BE2 gene. In cassava plants, the differences in amylose content and chain length distribution of amylopectin also indicated a divergence in BE1 and BE2 functions. This explains the fact that the amylose content of transgenic Adira 4 was not as high as the one obtained by CAS, presumably due to the different function of gene as it was proven that the BEII led to good growth and yield. All plants bearing BEI-RNAi (BE1i) and BEII-RNAi (BE2i) were able to grow in the field although the total biomass was lower than that of wild type but plants bearing BE2i had high amylose content. Both lines had "storage" starch granule morphology similar to that of wild type (Zhou et al., 2015). Another factor affecting the regeneration, eventually is also affecting the growth and the ability to be propagated or multiplied. Most researchers working with cassava genetic transformation used cultivar TMS 60444, which is widely famous for its responsiveness and high regeneration ability. Meanwhile, Adira 4, although until now is the most advanced variety used for somatic embryogenic induction and the production of friable embryogenic callus as compared to other varieties and cultivars/genotypes, has been not as responsive like TMS 60444 and several others of Latin American and African cassava cultivars that also have been studied much longer. Adira 4, which naturally contains relatively higher amylose than other nationally released variety in Indonesia, has been used as materials for producing waxy cassava (Koehorst-van Putten, 2012b) and to produce mutants with extended tuber shelf life by gamma irradiation (Sudarmonowati et al., 2006, Supatmi et al., 2017).

CONCLUSIONS

The survival rate and the growth of four generations of the first high amylose transgenic Indonesian cassava plants was affected by various factors, including lines and generation linked to planting site and condition of planting materials.

The growth of Line 55-6 plants was consistent in each generation, which was the best as compared to other lines. The highest yield obtained was from Line A55-12 plants, while the highest amylose content was obtained from Line A55-5 plant, which was almost double of that of the wild type. The findings indicated that there was no correlation between yield and amylose content. Further research applying more appropriate gene construct and approaches are required in the future for better growth and yield results.

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REFERENCES

Bull, S. E., Seung, D., Chanez, C., Mehta, D., Kuon, J.E., Truernit, E., Hochmuth, A., Zurkirchen, I., Zeeman, S.C., Gruissem, W., and Vanderschuren, H. (2018). Accelerated ex situ breeding of GBSS-and PTST1-edited cassava for modified starch. *Science advances*, 4(9), pp. 1–12.

Calvo, P., Nelson, L., and Kloepper, J.W. (2014). Agricultural uses of plant biostimulants. *Plant and soil*, 383, pp. 3–41.

Ceballos, H., Sánchez, T., Morante, N., Fregene, M., Dufour, D., Smith, A.M., Denyer, K., Pérez, J.C., Calle, F., and Mestres, C. (2007). Discovery of an amylose-free starch mutant in cassava (*Manihot esculenta* Crantz). *Journal of Agricultural and Food Chemistry*, 55(18), pp. 7469–7476.

Ceballos, H., T. Sánchez, Denyer, K., Tofiño, A.P., Rosero, E.A., Dufour, D., Smith, A., Morante, N., Perez, J.C., and Fahy, B. (2008). Induction and identification of a small-granule, high-amylose mutant in cassava (*Manihot esculenta* Crantz). *Journal of Agricultural and Food Chemistry,* 56(16), pp. 7215–7222.

Ceballos, H., Morante, N., Calle, F., Lenis, J., and Salazar, S. (2017). Developing new cassava varieties: tools, techniques and strategies. In: C. Hershey, ed., *Achieving Sustainable Cultivation of Cassava Volume 2*. Colombia: Burleigh Dodds Science Publishing, pp. 69–101.

Jiang, H., Lio, J., Blanco, M., Campbell, M., and Jane, J.L. (2010). Resistant-starch formation in high-amylose maize starch during kernel development. *Journal of Agricultural and Food Chemistry*, 58(13), pp. 8043–8047.

Koehorst-van Putten, H.J.J., Wolters, A.M., Pereira-Bertram, I.M., van den Berg, H.H.J., van der Krol, A.R., and Visser, R.G.F. (2012a). Cloning and characterization of a tuberous root-specific promoter from cassava (*Manihot esculenta* Crantz). Planta, 236, pp. 1955–1965.

Koehorst-van Putten, H.J.J., Sudarmonowati, E., Herman, M., Pereira-Bertram, I.J., Wolters, A.M.A., Meima, H., de Vetten, N., Raemakers, C.J.J.M., and Visser, R.G.F. (2012b). Field testing and exploitation of genetically modified cassava with low-amylose or amylose-free starch in Indonesia. *Transgenic Research*, 21(1), pp. 39–50.

Przybysz, A., Wrochna, M., Słowiński, A., and Gawrońska, H. (2010). Stimulatory effect of asahi SL on selected plant species. *Acta Scientiarum Polonorum Hortorum Cultus*, 9(2), pp. 53–64.

Przybysz, A., Gawrońska, H., and Gajc-Wolska, J. (2014). Biological mode of action of a nitrophenolates-based biostimulant: Case study. *Frontiers in Plant Science*, 5, pp. 1–15

Pusat Data dan Sistem Informasi Pertanian Kementerian Pertanian. 2016. *Outlook komoditas pertanian sub sektor tanaman pangan ubi kayu*. [Online]. Available at http://perpustakaan.bappenas.go.id/lontar/file?file=digital/166972-[_Konten_]-Konten%20D1887.pdf. [Accessed 1 September 2020].

Raemakers, K., Schreuder, M., Suurs, L., Furrer-Verhorst, H., Vincken, J.P., de Vetten, N., Jacobsen, E., and Visser, R.G.F. (2005). Improved cassava starch by antisense inhibition of granule-bound starch synthase I. *Molecular Breeding*, 16, pp. 163–172.

Sitinjak, R.R. (2017). The response of growth on shoot cuttings and stem cuttings of *Citrus*

amblycarpa L. after giving atonik. *Research Journal of Pharmaceutical Biological and Chemical Sciences*, 8(2), pp. 872–881.

Sitinjak, R.R. (2015). The growth response stem cuttings of roses (*Rosa* sp.) to plant growth regulator atonik and rootone-F. *Journal of Chemical and Pharmaceutical Research*, 7(9), pp. 557–562.

Sharma, H.K., Njintang, N.Y., Singhal, S.S., and Kausha, P. (eds.). (2016). *Tropical roots and tubers: production, processing and technology*. 1st Ed. United Kingdom: John Wiley & Sons, pp. 326–327

Sudarmonowati, E., Hartati, N.S., and Sukmarini, L. (2006). Amylose content variation of Indonesian cassava genotypes and its correlation with RAPD and AFLP markers. *Proceedings of the First International Meeting on Cassava Breeding, Biotechnology and Ecology, Brazillia,* pp. 85–95.

Supatmi, Hartati, N.S., and Sudarmonowati, E. (2017). Extending shelf life through induced mutation using gamma–ray irradiation. *Proceedings of International Symposium on Bioeconomics of Natural Resources Utilization. Bogor*, pp. 209–220.

Sun, Y., Jiao, G., Liu, Z., Zhang, X., Li, J., Guo, X., Du, W., Du, J., Francis, F., Zhao, Y., and Xia, L. (2017). Generation of high-amylose rice through CRISPR/Cas9-mediated targeted mutagenesis of starch branching enzymes. *Frontiers in Plant Science*, 8, pp. 1–15.

Wang, J., Hu, P., Chen, Z., Liu, Q., andWei, C. (2017). Progress in high-amylose cereal crops through inactivation of starch branching enzymes. *Frontiers in Plant Science*, 8, pp. 1–10.

Zhang, D., Hurst, T., Duand, D., and Chen, S.J. (2019). Unified energetics analysis unravels SpCas9 cleavage activity for optimal gRNA design. *Proceeding of the National Academy of Sciences of the United State of America*, 116, pp. 8693–8698.

Zhao, S.S., Dufour, D., Sanchez, T., Ceballos, H., and Zhang, P. (2011). Development of waxy cassava with different biological and physico-chemical characteristics of starches for industrial applications. *Biotechnology and Bioengineering*, 108(2), pp. 1925–1935.

Zhou, W., Yang, J., Hong, Y., Liu, G., Zheng, J., Gu, Z., and Zhang, P. (2015). Impact of amylose content on starch physicochemical properties in transgenic sweet potato. *Carbohydrate Polymers*, 122, pp. 417–427.

Zhou, W., He, S., Naconsie, M., Ma, Q., Zeeman, S.C., Gruissem, W., and Zhang, P. (2017). Alpha-glucan, water dikinase 1 affects starch metabolism and storage root growth in cassava (*Manihot esculenta* Crantz). *Scientific Reports*, 7, pp. 1–17.

Zhou, W., Zhao, S., He, S., Ma, Q., Lu, X., Hao, X., and Zhang, P. (2020). Production of very-high-amylose cassava by post-transcriptional silencing of branching enzyme genes. *Journal of Integrative Plant Biology*, 62(6), pp. 832–846.