Kasetsart J. (Nat. Sci.) 49 : 844 - 855 (2015)

Effect of Genotype, Age and Soil Moisture on Cyanogenic Glycosides Content and Root Yield in Cassava (*Manihot esculenta* Crantz)

Wimonsiri Srihawong[1], Pasajee Kongsil[1,*], Krittaya Petchpoung[2] and Ed Sarobol[1]

ABSTRACT

Cyanogenic glycosides in cassava roots and leaves can be indirectly harmful if presented at high concentrations because they are transformed into cyanide which is toxic to humans. Low cyanogenic potential should be added in breeding programs for food security in the future to reduce any bitter taste and to reduce intoxication in cases of consumption of improper processed roots. This experiment investigated the effects of genotype, plant age, soil moisture and interactions of these factors on the content of cyanogenic glycosides in both the roots and leaves of cassava and on other agronomic traits indicating plant growth. The experiment was established using a factorial randomized complete block design with three replications containing two varieties, two soil moisture conditions and three plant harvesting periods. The varieties chosen for this experiment—Hanatee (HNT) and Kasetsart 50 (KU50)—were different in cyanogenic potential. Plants were harvested at 4, 5, and 6 months after planting (MAP). The drought-stressed group was subjected to drought from 3 to 4 MAP and was irrigated at 5 mm d^{-1} from 4 to 5 MAP and then was subjected to drought again from 5 to 6 MAP; The well-irrigated group was irrigated at 5 mm.d^{-1} for the whole experiment. The results showed that root cyanogenic glycosides levels , as presented in the form of total free cyanide, were high under drought stress and a difference between the content of root cyanogenic glycosides of HNT and KU50 could occur under drought stress. However, drought stress caused a reduction in plant growth including root yield, and re-watering for 1 mth was not enough to recover cassava growth significantly.

Keywords: cassava, drought, environmental adaptation, hydrogen cyanide, irrigation

INTRODUCTION

As a staple crop, cassava (*Manihot esculenta* Crantz) is widely grown mostly in drought-prone areas (Taylor *et al*., 2012). Thai commercial cassava varieties have been bred mostly for high root yield and high starch content mainly under non-irrigated field conditions without considering the cyanogenic glycosides content in the cassava root which can be degraded into hydrogen cyanide that is toxic to humans and animals (Gleadow and Møller, 2014). Because human consumption of cassava in Thailand is mostly as boiled roots, developing high yielding

1 Department of Agronomy, Faculty of Agriculture, Kasetsart University, Bangkok 10900, Thailand.

2 Scientific Equipment and Research Division, Kasetsart University Research and Development Institute, Kasetsart University, Bangkok 10900, Thailand.

* Corresponding author, e-mail: fagrpjk@ku.ac.th

Received date : 29/01/15 Accepted date : 18/11/15

cassava clones with low cyanogenic potential would contribute to the food security of the country in the near future.

Cassava tissues contain linamarin as the major cyanogenic glycoside and lotustralin as the minor one (Koch *et al.*, 1994). Believed to be utilized to defend against herbivore attack without harm to the plant itself, cyanogenic glycosides are compartmentalized in the vacuole, while, β-glycosidase, a hydrolytic enzyme, is located in the cell wall (White *et al*., 1994). After plant cells are broken, β-glycosidase hydrolyzes the cyanogenic glycosides into α-hydroxynitrile which is spontaneously hydrolyzed into hydrogen cyanide (HCN) and ketones by hydroxynitrile lyase (White *et al.*, 1994). A dose of 1 to 3 $mg.kg^{-1}$ body weight of HCN can cause acute intoxication in humans and other vertebrates since it inhibits the activity of cytochrome c oxidase which is a key enzyme in the respiratory electron transport chain (Gleadow and Møller, 2014). In addition, continuous exposure to lower levels of HCN can result in Konzo, a chronic neurological disease which causes permanent leg paralysis and is common in some regions of Africa (Bhattacharya *et al*., 2009). For this reason, the World Health Organization has recommended the maximum level of HCN at 10 $mg.kg^{-1}$ or parts per million (ppm) of dry weight of processed cassava product (Food and Agriculture Organization/World Health Organization, 1991). For human consumption, an HCN content which is less than 50 ppm fresh weight of peeled cassava is considered to be innocuous and an HCN content which is more than 100 ppm is highly poisonous (Bolhuis, 1966 as cited in Lukuyu *et al*., 2014). Cassava storage roots have a normal HCN content range from 7 to more than 3,000 ppm (O'Brien *et al*., 1999; Sánchez *et al*., 2009), depending on the genotype, the age of the plant and the environmental conditions under which they grew. No cassava with zero cyanogenic glycoside content has been found in nature (Bokanga, 1994). High nitrogen fertilization and water deficit have been reported to affect the content of cyanogenic glycosides in cassava roots (Gleadow and Møller, 2014).

Hular-Bograd *et al*. (2011) and Vandegeer *et al*. (2013) have reported the response of the cyanogenic glycoside content to the soil moisture content. Drought was shown to induce high cyanogenic potential in cassava. However, these studies considered only one variety. Therefore, the interaction between genotype and soil moisture conditions has not been investigated so far. Moreover, in a large field, the soil moisture content is not consistent, especially in the early screening years after hybridization because there will be less replications for each line even though check varieties will be grown across the field in an augmented design.

Therefore, this study aimed to verify the effect of genotype, plant age and soil moisture (as well as their interactions) on the cyanogenic potential in the roots and leaves, root yield and other relevant parameters related to cassava plant growth. The knowledge gained from this study will be used as a preliminary guideline to suggest the proper screening field conditions for a cassava breeding program for low cyanogenic potential and high root yield.

MATERIALS AND METHODS

Plant material

Cassava stakes were planted vertically at the Department of Agronomy, Kasetsart University, Bangkok, Thailand in 80 cm diameter pots in August 2013 at 1×1 m^2 spacing between the cassava stakes. Sandy loam soil was used. The soil moisture content at 18% was regarded as the field capacity water potential level and at 8% was regarded as the permanent wilting point water potential level (Zotarelli *et al*., 2010). At one month after planting (MAP), NPK fertilizer (15:15:15) was applied at the rate of 50 $kg.rai^{-1}$(6.25 rai in 1 ha). Weed control was performed by hand-pulling every week.

Treatment implementation and timing

A factorial randomized complete block design was set up with three replications. Treatments consisted of three harvesting periods of 4, 5 and 6 MAP, two soil moisture conditions—namely, a drought-stressed group and a well-irrigated group—and two cassava varieties—namely, Kasetsart 50 (KU50) and Hanatee (HNT) varieties. In the drought-stressed group, irrigation was omitted at 3 MAP in November 2013. Cassava harvest occurred in December 2013, January 2014, and February 2014. In the drought-stressed group, plants were re-watered (twice a week calculated to be equivalent to 5 $mm.d^{-1}$) at 4 mth after the first harvesting was done. Plants were irrigated for 1 mth and then harvested at 5 MAP. Plants in the drought-stressed group were subjected to water deficit again for 1 mth before harvesting at 6 MAP. In the well-irrigated group, plants were irrigated twice a week which was calculated to be equivalent to 5 $mm.d^{-1}$.

Harvest data collection

The soil moisture content of each pot was measured using a soil probe (Wet Sensor; Delta-T Devices Ltd.; Cambridge, UK). The relative leaf water content and leaf greenness were measured using the fifth fully expanded leaf from the top of the plant. Leaf greenness was measured using a chlorophyll meter (SPAD 502; Konica Minolta Sensing Singapore Pte Ltd.; Singapore) at three spots on the fifth leaf of each plant and the values of three readings from the same leaf were averaged. The relative leaf water content was measured using a 1 cm^2 cork borer to cut 10 leaf discs which were placed in a plastic ziplock bag and weighed. The leaf fresh weight was weighed by subtracting the bag weight. After weighing, the leaves were placed in distilled water overnight before weighing to determine the full leaf water holding capacity. The weight was measured twice with 2 hr between weighings to confirm that the leaf had fully absorbed water in the tissue. Afterwards, the leaves were dried overnight in a hot air oven at 75 °C before weighing twice with 2 hr between weighings to confirm that leaf had been fully dried. The relative leaf water content was calculated using Equation 1.

$$RWC = \frac{(MLW - LDW)}{(LFW - LDW)} \times 100 \qquad (1)$$

where RWC is the relative leaf water content measured as a percentage, MLW is the maximum water holding weight, LFW is the leaf fresh weight and LDW is the leaf dry weight, with all weights measured in grams.

The aboveground plant height was measured in centimeters. Shoot weight (the whole weight of the leaf and stem together) and the root weight (fibrous root and tuberous root together) were measured in grams. The harvest index was calculated using Equation 2.

$$HI = RW / (RW+SW) \qquad (2)$$

where HI is the harvest index, RW is the root weight and SW is the shoot weight, with all weights measured in grams.

Cyanogenic glycoside analysis

The cyanogenic potential in the roots and leaves, presented as free cyanide (HCN), was analyzed following the method described by Bradbury *et al*. (1999). Three separated root parenchyma were collected from one plant at the largest root diameter using a cork borer (4 mm diameter) and cut to a size which weighed 100 mg. Samples of 10 mg of leaf tissue were cut from young, fully expanded leaves using a cork borer (1 cm^2 cross section). Tissue was immediately ground after sampling and placed in a 15 mL tube with 50 µL of phosphate buffer at pH 8.00 and then capped with a cap attached with picrate paper. The tube was left at 30 °C for 24 hr and then the picrate paper was soaked in 5 mL for 30 min before absorbance was measured at 510 nm using spectrophotometry and the HCN equivalent was estimated from a standard curve of equivalent weight (as milligrams per kilogram fresh tissue weight) of HCN regenerated from linamarin.

Statistical analysis

Harvesting data were analyzed using the Minitab® computer package (Version 16, Minitab Inc., State College, PA, USA). The general linear model univariate analysis test was used for analysis of variance. Tukey's method was used for mean comparisons at the 95% confidence level. Pearson correlations between parameters were performed. A correlation coefficient higher than 0.6 was considered as high correlation and a correlation coefficient between 0.4 and 0.6 was considered as moderately high correlation.

RESULTS

In the drought-stressed group, soil moisture was significantly lower than in the irrigated group except for during the irrigation period at 5 MAP (Table 1 and Figure 1a). The relative leaf water content of cassava showed the same pattern of change as the soil moisture content, though there was no significant difference in the interactions among genotype and soil moisture condition (Table 1 and Figure 1b). Other morphological traits did not show the same pattern

Table 1 Effect of cassava age, variety and soil moisture on cassava agronomic traits

Parameters (Unit)	SPAD	PH (cm)	RW (g per plant)	SW (g per plant)	HI	SM (%)	LWC (%)	RHC ($mg.kg^{-1}$)	LHC ($mg.\ kg^{-1}$)
Age (F1)									
4 MAP	41.3^{a}	127	389^{b}	581	0.36^{b}	9.7^{b}	93^{b}	127	501
5 MAP	38.1^{b}	148	683^{ab}	743	0.44^{ab}	20.0^{a}	97^{a}	113	507
6 MAP	37.3^{b}	147	960^{a}	800	0.46^{a}	14.5^{b}	93^{b}	104	491
F-test	**	ns	**	ns	*	**	**	ns	ns
Variety (F2)									
HNT	34.9^{b}	145	478^{b}	731	0.34^{b}	13.2	94	46^{b}	315^{b}
KU50	42.9^{a}	137	877^{a}	685	0.51^{a}	16.2	95	184^{a}	685^{a}
F-test	**	ns	**	ns	**	ns	ns	**	**
Water (F3)									
0 mm	41.2^{a}	121^{b}	204^{b}	344^{b}	0.36^{b}	8.7^{b}	92^{b}	142^{a}	509
5 mm	36.6^{b}	161^{a}	1150^{a}	1072^{a}	0.49^{a}	20.8^{a}	97^{a}	87^{b}	491
F-test	**	**	**	**	**	**	**	**	ns
F-test									
Replication	ns	ns	ns	ns	ns	ns	ns	*	ns
F1 × F2	ns	ns	ns	ns	ns	ns	ns	ns	ns
F1 × F3	ns	ns	**	ns	ns	**	*	ns	ns
F2 × F3	ns	ns	*	ns	ns	ns	ns	*	ns
F1 × F2 × F3	ns	ns	ns	ns	ns	ns	ns	ns	ns

SPAD = Leaf greenness; PH = Plant height; RW = Root weight; SW = Stem and leaf weight; HI = Harvest index; SM = Soil moisture; LWC = Relative leaf water content; RHC = Root hydrogen cyanide content; LHC = Leaf hydrogen cyanide content; MAP = Months after planting.

* = Significantly different at 95 % confidence level.

** = Significantly different at 99 % confidence level.

ns = Not significantly different.

$^{a, b}$ = Mean values with a lower case superscript letter are significantly different at 95% confidence level.

as the soil moisture content, although the relative leaf water content, plant height, root fresh weight and shoot fresh weight had moderately high positive correlations with soil moisture (Table 2). The KU50 variety had higher leaf greenness than the HNT variety. Moreover, plants which had been subjected to water deficit had higher leaf greenness than those in the irrigated group. There was no significant interaction between the genotype and soil moisture conditions (Table 1 and Figure 1i). Leaf greenness had a significantly high positive correlation with the cyanogenic glycosides content in the roots (Table 2).

The other plant growth parameters (plant height, shoot fresh weight, root fresh weight and harvest index) in the drought-stressed group were lower than those in the irrigated group, but there was no significant difference in the interactions among genotype and the soil moisture condition for plant height, shoot weight and harvest index (Table 1 and Figures 1c, e, f, respectively). However, the root fresh weight of KU50 in the irrigated group was significantly higher than in the drought-stressed group at 5 MAP, but the root fresh weight of HNT in the irrigated group was significantly higher than in the drought-stressed group later at 6 MAP (Figure 1d). In the drought-stressed group, irrigation at 5 MAP did not help the plants to recover any significant plant growth in any parameter.

The cyanogenic glycosides content in the root of cassava in the drought-stressed group was significantly higher than that for the irrigated group, and the cyanogenic glycosides content in the root of cassava in the KU50 variety was higher than in the HNT variety (Table 1). However, with regard to the interaction between genotype and soil moisture condition, drought stress could cause a significant difference in the root HCN between the KU50 and HNT varieties, but the irrigated group showed no significant difference in the root HCN between the two genotypes (Figure 1g). The cyanogenic glycoside content in the leaf of KU50 was higher than in HNT. However, there was no significant difference in the leaf HCN in response to soil moisture and no significant difference in the leaf HCN in the interaction of genotype and soil moisture condition (Figure 1h). The leaf cyanogenic glycosides content had a significant, moderately high positive correlation with the root cyanogenic glycosides content (Table 2).

Table 2 Correlations between cassava agronomic traits

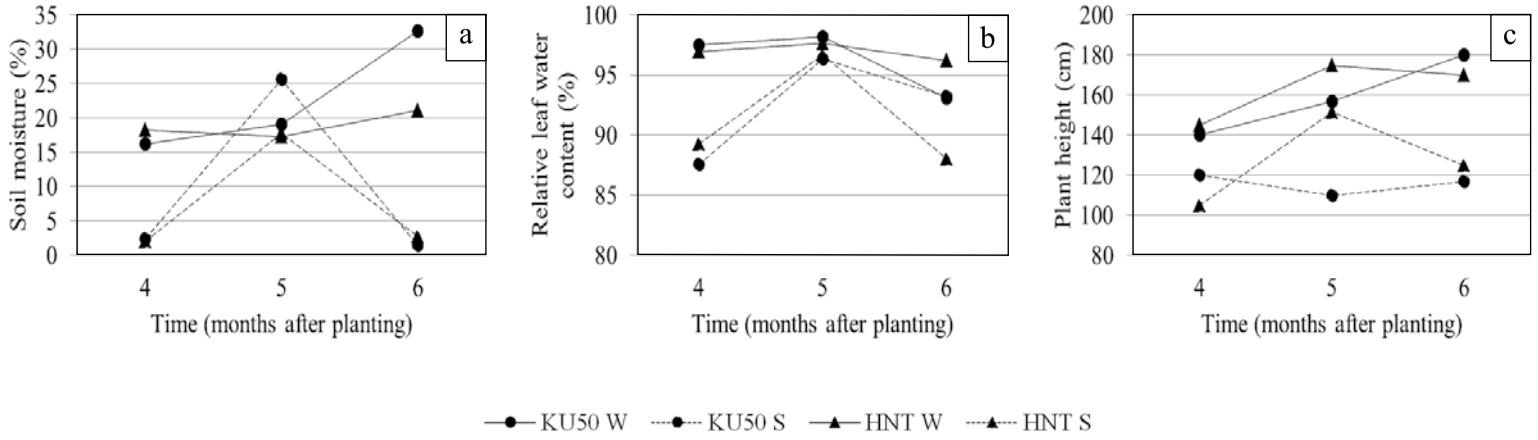
	SPAD	PH	RW	SW	HI	SM	LWC	RHC
PH	-0.389*							
RW	-0.135 ns	0.699**						
SW	-0.363*	0.797**	0.807**					
HI	0.113ns	0.402*	0.692**	0.323ns				
SM	-0.174ns	0.535**	0.615**	0.649**	0.327ns			
LWC	-0.149ns	0.352*	0.231ns	0.391*	0.303ns	0.564**		
RHC	0.788**	-0.315ns	-0.050ns	-0.304ns	0.284ns	-0.106ns	-0.111ns	
LHC	0.413*	-0.179ns	0.212ns	-0.089ns	0.508**	-0.060ns	0.067ns	0.516**

Abbreviations: SPAD = Leaf greenness; PH = Plant height; RW = Root weight; SW = Stem and leaf weight; HI = Harvest index; SM = Soil moisture; LWC = Relative leaf water content; RHC = Root hydrogen cyanide content; LHC = Leaf hydrogen cyanide content.

* = Significantly different at 95 % confidence level

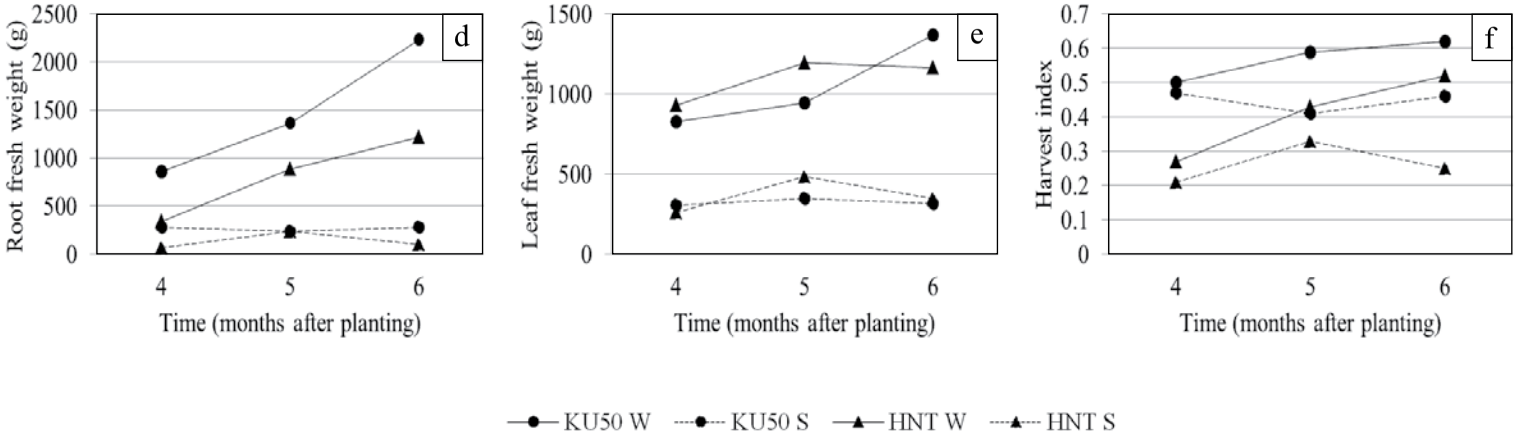
** = Significantly different at 99 % confidence level..

ns = Not significantly different.



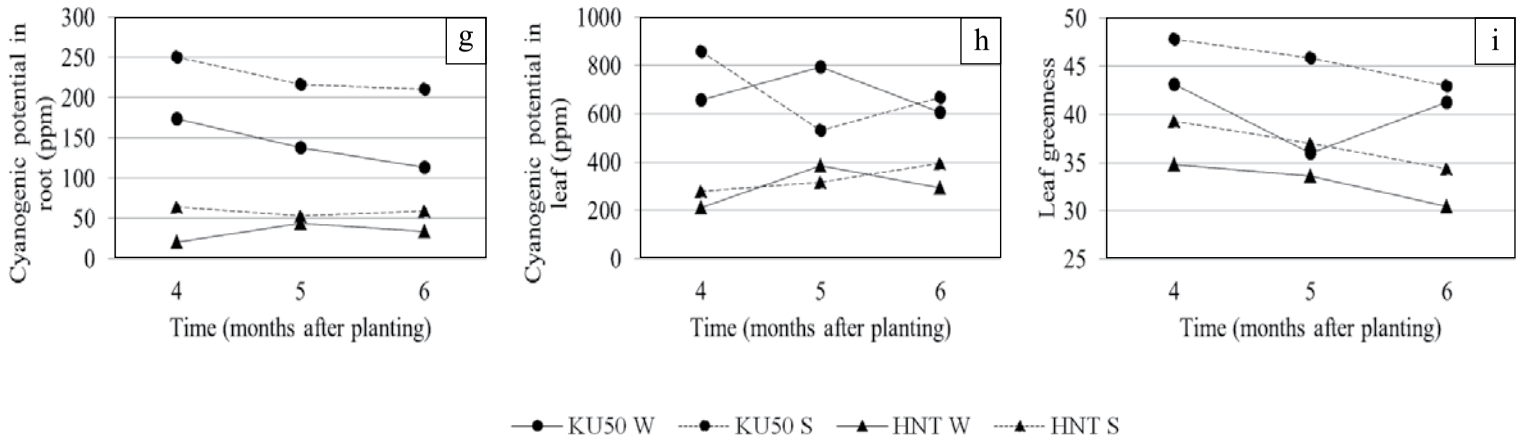
	Months after planting			Months after planting			Months after planting		
	4	5	6	4	5	6	4	5	6
KU50 W	bc	ab	a	abc	a	abc	abcd	abcd	a
KU50 S	cd	ab	d	c	abc	abc	abcd	cd	bcd
HNT W	b	b	ab	abc	ab	abc	abcd	ab	abc
HNT S	cd	b	cd	abc	abc	bc	d	abcd	abcd

Figure 1 Interaction effects for cassava varieties Kasetsart 50 (KU50) and Hanatee (HNT) with age, variety, soil moisture; drought-stressed (S) and well-irrigated (W) on cassava agronomic traits: (a) = Soil moisture condition; (b) = Relative leaf water content; (c) = Plant height; (d) = Root weight; (e) = Stem and leaf weight; (f) = Harvest index; (g) = Root hydrogen cyanide content; (h) = Leaf hydrogen cyanide content; (i) = Leaf greenness. Different lower case letters (representing parameters shown in the subfigures) in rows and columns indicate significant differences between means at 95% confidence level.



KU50 W	bcd	ab	a	abc	abc	a	abc	ab	a
KU50 S	d	d	d	c	c	c	abcd	abcd	abcd
HNT W	cd	bcd	bc	abc	ab	ab	cd	abcd	abc
HNT S	d	d	d	c	bc	c	d	bcd	cd

Figure 1 **(continued)** Interaction effects for cassava varieties Kasetsart 50 (KU50) and Hanatee (HNT) with age, variety, soil moisture on cassava agronomic traits: (a) = Soil moisture condition; (b) = Relative leaf water content; (c) = Plant height; (d) = Root weight; (e) = Stem and leaf weight; (f) = Harvest index; (g) = Root hydrogen cyanide content; (h) = Leaf hydrogen cyanide content; (i) = Leaf greenness. Different lower case letters (representing parameters shown in the subfigures) in rows and columns indicate significant differences between means at 95% confidence level.



KU50 W	abc	bcd	cde	abc	ab	abc	ab	bcde	abcd
KU50 S	a	ab	abc	a	abc	abc	a	a	abc
HNT W	e	de	e	c	abc	bc	bcde	de	e
HNT S	de	de	de	bc	abc	abc	abcd	bcde	cde

Figure 1 **(continued)** Interaction effects for cassava varieties Kasetsart 50 (KU50) and Hanatee (HNT) with age, variety, soil moisture on cassava agronomic traits: (a) = Soil moisture condition; (b) = Relative leaf water content; (c) = Plant height; (d) = Root weight; (e) = Stem and leaf weight; (f) = Harvest index; (g) = Root hydrogen cyanide content; (h) = Leaf hydrogen cyanide content; (i) = Leaf greenness. Different lower case letters (representing parameters shown in the subfigures) in rows and columns indicate significant differences between means at 95% confidence level.

DISCUSSION

Drought stress in this experiment could cause a reduction in plant growth in both shoot and root parts. Alves (2002) explained cassava growth at 3 to 6 MAP as the canopy establishment period. In this period, maximum growth of leaf and stem will be achieved. In the current study, drought stress could cause a reduction in both the shoot (leaf and stem) growth and plant height. Moreover, storage root bulking continues during this period (Alves, 2002). The root weight of KU50 under drought stress was significantly lower than that under the well-irrigated condition from 5 MAP to 6 MAP, while the root weight of HNT under both conditions was significantly different at 6 MAP. Kasetsart 50 is a Thai commercial variety bred for high root yield for starch industrial application. Hanatee is a landrace which is popularly grown in well-irrigated areas for household consumption. Therefore, Kasetsart 50 should have higher ability to form roots more efficiently than HNT. Interestingly, the root weight at this stage was more sensitive to drought than the plant height and shoot weight because the interaction between genotype and water conditions was significantly higher. Based on the classification of Alves (2002), at this stage, the photosynthetic parts are more important than the storage parts. Therefore, cassava might maintain stable upper ground growth during water stress. Water deficit could cause stomatal closure and reduce the photosynthetic rate. Photosynthate might be mainly used for vegetative growth, not for storage. In this experiment, even though irrigation was re-applied for 1 mth to the drought-stressed group, plants showed no recovery in any parameter except for the relative leaf water content. The high soil moisture period of 1 mth may not have been enough for cassava to recover normal growth. Vandegeer (2013) reported growth recovery in 4 mth-old cassava subjected to water stress for 14 d followed by re-watering for 14 d. However, in the current experiment, the drought stress lasted 1 mth, which was longer than in the previous study. More than 1 mth might be necessary to recover plant growth and generate sufficient photosynthate production to initiate root storage.

The cyanogenic glycosides content in the root was higher under the drought-stressed condition than under the well-irrigated condition as in a previous report (Hular-Bograd *et al*., 2011). However, Vandegeer (2013) reported an increase in the cyanogenic glycosides content in the root in response to soil moisture within 14 days, but in the current study, the cyanogenic glycosides content in the root in the drought-stressed group did not change following the re-watering period. The root cyanogenic glycosides content had a moderately high positive correlation with leaf greenness which is related to the chlorophyll content in leaf. In this experiment, leaf greenness was measured in the youngest fully expanded leaf. Chlorophyll contains a large number of nitrogen atoms as well as cyanogenic glycosides components. However, the two varieties used in this study had significantly different chrolophyll contents regardless of the soil moisture conditions. Hanatee had a notably pale green leaf color, but KU50 had a dark green leaf color. This correlation between leaf greenness and the root cyanogenic glycosides content needs to be confirmed in populations which have large distributions of leaf greenness and root cyanogenic glycosides contents.

Interestingly, leaf greenness was not correlated with the leaf cyanogenic glycosides content although both parameters were taken from the same leaf. This might have been due to the high variation in the leaf cyanogenic glycosides content. Cyanogenic glycosides were expected to be synthesized in the leaf and translocated for storage in the root (Selmar, 1994). The variation in the starch contents between the source tissues and sink tissues of the plant was reflected in the variation in the cyanogenic glycosides content at the synthesis site as the transitory compound in the leaf changed diurnally (Okolie and Obasi, 1993), but the cyanogenic glycosides content at the storage site might be steadier than that at the

synthesis site. One hypothesis suggested by this result is that drought caused a reduction in plant growth resulting in less protein synthesis. For this reason, the content of cyanogenic glycosides was high because they might not be degraded to be used as a source of amino acid supply in the located tissue during drought conditions.

To apply the results of this study to the screening of cassava lines in a cassava breeding program for low cyanogenic potential, a drought condition is required to distinguish between high cyanogenic potential varieties and those with low cyanogenic potential. However, the low soil moisture content may cause a lower root yield which is not good for screening high root yield which is the major parameter for screening. In breeding programs, hybrids are regularly planted at the start of the rainy season and are usually harvested 10–12 MAP at the end of the dry period. Plants will have the first 6 MAP under adequate soil moisture conditions which is sufficient for plant growth. In this study, cassava accumulated root yield during the first 6 MAP which was sufficient to differentiate between high and low root yielding lines at 10–12 MAP in the early rainy season. After 6 MAP, cassava will encounter water deficit due to the dry season. This study indicated that harvesting cassava during the dry conditions or within the first month of the rainy season is better for distinguishing between high and low cyanogenic potential cassava lines. In general, a higher soil moisture after the dry season will cause plants to re-grow which usually reduces the dry matter content substantially and reduces the commercial value of the roots. Perhaps, farmers can harvest 1–2 d after the first rain and that will become common practice as the dry matter content (starch content) does not reduce quickly after the arrival of the rainy season. However, Chotineeranat *et al*. (2006) reported a lower cyanogenic glycosides content in root with older age (6–12 MAP) regardless of the soil moisture content. The current experiment only harvested cassava from 4 to 6 MAP. Therefore, if harvesting were to occur from 10 to 12 MAP in the dry season or early in the rainy season, the cyanogenic glycosides contents of breeding lines would need to be compared with high and low cyanogenic potential varieties as check varieties grown across the screening field. In contrast, harvesting cassava lines in the late rainy season is not recommended for cyanogenic potential screening since the current results showed no significant difference between two contrasting cyanogenic potential varieties under well-watered conditions.

CONCLUSION

This research aimed to suggest conditions for screening cassava lines for high and low cyanogenic potential and root yield. A water deficit condition was required to differentiate between the high and low cyanogenic potential lines. However, water deficit also affected the root yield, though high and low root yield lines could be distinguished within the first 6 MAP under the well-watered soil condition during the rainy season. Therefore, this study supported the screening period of 10 to 12 MAP for cassava lines grown in the early rainy season which are harvested within 1 mth after the first rain of the season for low cyanogenic potential and high root yield in Thailand.

ACKNOWLEDGEMENTS

This work was kindly supported by the National Research Council of Thailand (NRCT) and the National Science and Technology Development Agency (NSTDA). The authors also would like to express their gratitude to Kasetsart University, Bangkok, Thailand for financial support for Miss Wimonsiri Srihawong through a Kasetsart University Doctoral Degree Scholarship.

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