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Somatic polyploidization and characterization of induced polyploids of *Dioscorea rotundata* and *Dioscorea cayenensis*

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Genetic improvement of major food yams is constrained by a number of factors, such as the scarcity of flowers and lack of synchronization between male and female flowering. Consequently, somaclonal variation including somatic polyploidization has been considered as a useful tool in yam breeding. Somatic polyploidization and its effect on phenotypic traits of *Dioscorea* species such as *D. alata*, *D. japonica* and *D. zingiberensis* has been reported; however, optimization of this method in two major yam species, *D. rotundata* and *D. cayenensis,* is yet to be achieved and the effect of polyploidization on phenotypic traits of this species yet to be elucidated. In the present study, a high rate of somaclonal polyploid variation was successfully achieved by *in vitro* colchicine treatment of *D. rotundata* and *D. cayenensis*. In most cases, except TDc 3704, the highest rate of polyploid induction appeared after 0.1% colchicine treatment. However, in triploid yellow yam accessions the induction rate was relatively low. Tetraploid variants of *D. rotundata* tended to display somewhat rounder leaves than their diploid parents. The size and shape of *D. rotundata* stoma were also affected by levels of ploidy, with tetraploid variants exhibiting larger stomata at a lower density compared to their diploid parents. The efficient method of *in vitro* polyploidy induction reported here is therefore a highly useful tool for obtaining polyploid variants for use as genetic resources in *D. rotundata* breeding.

Key words: Leaf, nodal segment culture, ploidy level, somaclonal variation, stoma, white guinea yam, yellow Guinea yam.

INTRODUCTION

In terms of production, yams (*Dioscorea* spp.) are the fourth most important tuber crop in the world (FAOSTAT, 2014), serving as a staple for millions of people in tropical regions. This is especially true in West Africa, also known as the “yam belt”, where 92% of the world’s yams are produced (FAOSTAT 2014).

Here, white Guinea yam (*Dioscorea rotundata;* hereafter "white yam"), yellow Guinea yam (*D. cayenensis;* hereafter "yellow yam") and *D. alata* (water yam) are the most important edible species and a major component of the total yam production in the region. In a short period of 20 years from 1992 to 2012, the production quantity of yams in West Africa rapidly increased from about 27 to 54 million tons, largely through the use of landraces and rapid expansion of cultivated acreage from 2.4 to approximately 4.5 million hectares (FAOSTAT, 2014). To meet this growing demand, breeding of higher-yielding cultivars is therefore becoming an important focus. However, genetic improvement of major *Dioscorea* species is constrained by a number of factors, such as the scarcity of flowers and lack of synchronization between male and female flowering. Consequently, somaclonal variation including somatic polyploidization is being considered as a useful tool in yam breeding.

A wide range of intra-specific variation in ploidy level is observed in major food yams. In most reports on ploidy level, white yam and yellow yam are treated as a *D. cayenensis/D. rotundata* complex. Dansi et al. (2001) reported diploids (2*n*=2x=40), triploids (2*n*=3x=60) and tetraploids (2*n*=4 x = 80) in *D. cayenensis/D. rotundata*, while Gamiette et al. (1999) reported only diploids and tetraploids. Meanwhile, Obidiegwu et al. (2009) reported diploids, triploids and tetraploids in a white yam collection but only triploids and tetraploids in yellow yam. Water yam reportedly displays all three ploidy levels, diploids (2*n*=2x=40), triploids (2*n*=3x=60) and tetraploids (2*n*=4x=80) (Abraham and Nair, 1991; Gamiette et al., 1999; Egesi et al., 2002; Arnau et al., 2009; Babil et al., 2010; Obidiegwu et al., 2010).

Triploid and tetraploid water yam cultivars tend to be more vigorous and higher yielding than diploid cultivars (Arnau et al., 2007). An association between higher ploidy levels and yield potential in water yam has also been reported, with diploids yielding 2 kg fresh tubers/plant and triploids and tetraploids yielding an average of 2.5 kg and 3 kg/plant, respectively (Lebot, 2009). During investigations of water yam landraces in Myanmar, Babil et al. (2010) reported that leaf size tended to be larger in triploids than in diploids and tetraploids. However, despite these findings, little is known about the effects of ploidy variation on morphological and agronomical traits of white and yellow yam.

Artificially induced polyploidy has been reported in numerous plant species belonging to at least 150 genera (Dewey, 1979). Agronomically successful examples include triploid sugar beet, tetraploid clover and tetraploid rye. Somatic polyploidization in water yam (Babil et al., 2011), *D. japonica* (Kenji et al., 2005) and *D. zingiberensis* (Huang et al., 2008) has also been reported. Artificially induced tetraploid (2*n*=4x=80) tubers of *D. japonica* were found to be shorter and thicker than diploid tubers, while viscosity, a major trait determining commercial value, was unaffected (Kenji et al., 2005). Moreover, artificially induced tetraploids (2*n*=4x=40) of *D. zingiberensis* were found to produce a higher content of diosgenin compared to the diploid (2*n*=2x=20) mother plant (Huang et al., 2008). Therefore, somatic polyploidization might also be applicable in genetic enhancement of white and yellow yam; however, to the best of our knowledge, information on the artificial induction of polyploidy is currently limited in both species. Colchicine is the most effective chemical agent for polyploid induction. However, since all plant species respond differently to colchicine treatment, optimization of colchicine concentration and application is required. The present study aimed to develop an efficient *in vitro* technique for the induction of somaclonal polyploid variation in white and yellow yam. Successfully induced polyploid variants of white yam were subsequently characterized in relation to the respective parent plants.

MATERIALS AND METHODS

Plant materials and *in vitro* multiplication

In vitro seedlings of white yam accessions TDr 2720, TDr 1793, TDr 2351 and yellow yam accessions TDc 2790, TDc 2812 and TDc 3704 maintained in the Genetic Resource Unit (GRU) of the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria, were used. Morphological traits of yellow yam accession TDc 2790 were similar to white yam accessions, and therefore, in the present study it was treated as a white yam. In addition, TDc 2790 was referred to as "R-20" in the present study.

In vitro multiplication was conducted from July 2013 to March 2014 at the IITA. Nodal segments excised from *in vitro* seedlings were cultured on 10 ml MS medium (Murashige and Skoog, 1962) supplemented with 30 mgl^{-1} sucrose. Prior to autoclaving at 121°C for 15 min, the medium was adjusted to pH 5.8 and solidified with 0.2% gellant gum. The cultured explants were incubated in a growth chamber at 28°C under a 16/8 h light/dark photoperiod. New shoots were then subcultured in the above medium to produce sufficient plantlets for colchicine treatment.

Colchicine treatment to induce polyploidy

Thirty-three-month-old nodal segments with a single axillary bud were planted on MS solid medium supplemented with 30 mgl^{-1} sucrose and cultured for 48 h prior to colchicine treatment. The nodal segments were then soaked in 0.1, 0.2 and 0.3% colchicine solution for 8 h. Sterilized distilled water was used as the control treatment. The colchicine solution was autoclaved at 121°C for 15

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min before use. Treated nodal segments were rinsed three times in sterilized water then replanted on MS medium as described previously. Two replications were conducted per treatment with 15 nodes per replication. The nodal segment cultures were continued for 3 months under a 16-h photoperiod at 28°C.

Flow cytometric analysis for identification of ploidy levels

Survival rates of cultured nodal segments were determined and ploidy levels of regenerated plantlets identified by flow cytometry using a Partec Ploidy Analyzer. Experiments were conducted at the Setagaya campus of Tokyo University of Agriculture (TUA) from April to October 2014. Materials were transferred from IITA to TUA after both parties signed a standard material transfer agreement and plants underwent quarantine.

For flow cytometry, portions of leaf blade (5 mm) collected from plantlets obtained *in vitro* were chopped into pieces then homogenized in a petri dish containing an extraction buffer solution to release nuclei. The homogenate was filtered through a nylon filter (50 µm pore size) and the extracted nuclei stained with 4,6-diamino-s phenylinode (DAPI). Flow cytometric analysis was performed at a rate of 5 to 20 nuclei per second. Rice plants (*Oryza sativa* L.) were used as the inter-reference. Relative amounts of DNA (the DNA index) in a sampled plant were determined in relation to that of rice to determine the ploidy level. Flow cytometry was carried out with each leaf from sample plants to confirm the ploidy level.

Mitotic chromosome observations

Chromosome numbers in control mother plants and induced polyploids, except TDc 3704, were confirmed by microscopic observation. For mitotic chromosome observation, root tips were sampled from *in vitro* seedlings and fixed in acetic acid-alcohol (1 to 3 ratio) for 24 h without pretreatment. Fixed root tips were stained with 1% aceto-carmine solution for 24 h. Preparation was conducted using the squash method and prepared samples observed under an optical microscope (BX53, Olympus) at a magnification of ×400.

Characterization of induced white yam polyploids

Artificially induced polyploid variants of white yam were characterized with reference to their parents in terms of leaf and stoma characters. The yellow yam polyploids grew abnormally and were excluded from characterization. Characterization was conducted from June 2014 to January 2015 at the Setagaya campus of TUA. Three- to four-month-old *in vitro* plants were acclimatized under a 12-h photoperiod at 25°C. Two parent plants and three induced polyploid plants were used for characterization. Five fully developed leaves obtained from the center of the vines were sampled. Observed phenotypic characters included the length and width of the leaf blade, depth and width of the sinus, and shape (length/width) of the leaf. The length, width, shape (length/width), size (length × width) and density of stomata were also observed. The same samples were used for measurements of stoma and leaf characters. Five stomata per leaf were measured. Photos of stomata were taken using a digital camera (DP71, Olympus) attached to the microscope (BX53, Olympus). Measurements of stomata were carried out using photo images with Win Roof software (Mizutani corporation, Japan). Stomata density was calculated based on the number of stomata per 64 mm^2 of a microscopic field at a magnification of ×200. To analyse statistical difference between induced tetraploid and mother plant, student's T-test was performed for all traits observed.

RESULTS

Effects of colchicine treatment on survival of the cultured nodal segments

Survival rates of the white and yellow yam accessions after colchicine treatment are shown in Tables 1 and 2, respectively. Survival of the cultured nodal segments was largely affected by the concentration of colchicine. In both species, survival rates decreased with increasing colchicine concentration. The highest survival rate was observed with 0.1% treatment except in the case of TDr 1793, the survival rate of which was higher under 0.3% treatment (46.7% compared to 43.3% under 0.1% colchicine). Overall, survival rates were higher in yellow yam accessions compared to white yam.

Effects of colchicine concentration on induction of polyploidy

DNA indexes and estimated numbers of chromosomes in control and colchicine treated *in vitro* seedlings as determined by flow cytometry and mitotic chromosome observations are shown in Table 3 and Figure 1. Based on mitotic chromosome observations and flow cytometry, ploidy levels of white yam accessions TDr 2720, 1793, 2351 and R-20 used in the present investigation were found to be diploid (2n = 40). Meanwhile, ploidy levels of yellow yam accessions TDc 2812 and 3704 were found to be triploid (2n=3x=60).

DNA indexes of nuclei at the G1 stage in the diploid white yam accessions ranged from 1.88 to 1.93, and in the induced polyploids from 3.77 to 3.86, which is twice the value of the diploid parents (Table 3). DNA indexes of nuclei of triploid yellow yam accessions ranged from 2.74 to 2.86 and that of induced polyploids from 5.31 to 5.41, twice the value of the triploid parents. Polyploid variants derived from the diploid white yam accessions were found to be tetraploid (2n=4x=80), while those from the triploid yellow yam accessions were hexaploid (2n=6x=120). The highest rate of polyploidy was induced by 0.1% colchicine in TDr 2720, 2351, R-20 and TDc 2812, and by 0.3 and 0.2% in the remaining two accessions, TDr 1793 and TDc 3704, respectively (Tables 1 and 2).

Characterization of induced polyploid variants in terms of leaf and stoma characters

Leaf and stoma characters of three- to four-month-old induced tetraploid variants of white yam were compared with the control (Tables 4 and 5). A statistically significant difference was observed in leaf length and width between the diploid parent and tetraploid variants, with tetraploid leaves appearing longer and wider than leaves in diploid. A significant difference was also observed in leaf shape

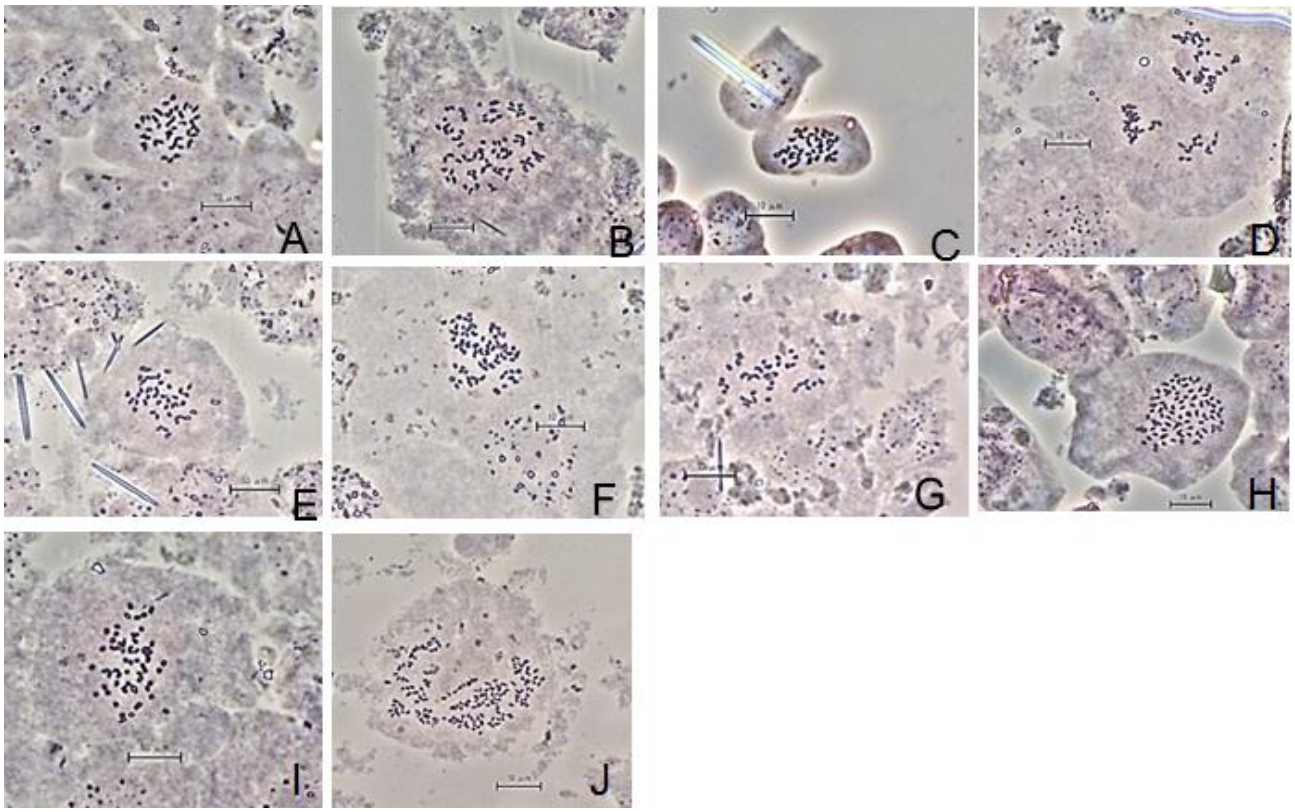

Figure 1. Chromosome images of control and induced polyploids of white yam (*Dioscorea rotundata*) and yellow yam (*D. cayenensis*). TDr 2720 control diploid (A) and induced tetraploid (B); TDr 1793 control diploid (C) and induced tetraploid (D); TDr 2351 control diploid (E) and induced tetraploid (F); R-20 control diploid (G) and induced tetraploid (H); TDc 2812 control triploid (I) and induced hexaploid (J). Scale bar: 10 µm.

Table 1. Effect of colchicine concentration on survival rate and induction of polyploids of white yam (*D. rotundata*).

Accession no.	Colchicine concentration	No. of explants	No. of survivors (%)	No. of diploids (%)*	No. of tetraploids (%)*
TDr 2720	0	30	28 (93.3)	28 (93.3)	0 (0)
	0.1	30	21 (70.0)	5 (16.7)	16 (53.3)
	0.2	30	14 (46.7)	6 (20.0)	8 (26.7)
	0.3	30	8 (26.7)	2 (6.7)	6 (20.0)
TDr 1793	0	30	27 (90.0)	27 (90.0)	0 (0)
	0.1	30	13 (43.3)	9 (30.0)	4 (13.3)
	0.2	30	10 (33.3)	6 (20.0)	4 (13.3)
	0.3	30	14 (46.7)	7 (23.3)	7 (23.3)
TDr 2351	0	30	29 (96.7)	29 (96.7)	0 (0)
	0.1	30	14 (46.7)	6 (20.0)	8 (26.7)
	0.2	30	12 (40.0)	8 (26.7)	4 (13.3)
	0.3	30	10 (33.3)	5 (16.7)	5 (16.7)
R-20	0	30	28 (93.3)	28 (93.3)	0 (0)
	0.1	30	28 (93.3)	10 (33.3)	18 (60.0)
	0.2	30	16 (53.3)	9 (30.0)	7 (23.3)
	0.3	30	11 (36.7)	5 (16.7)	6 (20.0)

*Percentage of initial explants was calculated.

Table 2. Effect of colchicine concentration on survival rate and induction of polyploids of yellow yam (*D. cayenensis*).

Accession no.	Colchicine concentration	No. of explants	No. of survivors (%)	No. of triploids (%)*	No. of hexaploids (%)*
TDc 2812	0	30	26 (86.7)	26 (86.7)	0 (0)
	0.1	30	28 (93.3)	24 (80.0)	4 (13.3)
	0.2	30	24 (80.0)	21 (70.0)	3 (10.0)
	0.3	30	20 (66.7)	17 (56.7)	3 (10.0)
TDc 3704	0	30	28 (93.3)	28 (93.3)	0 (0)
	0.1	30	29 (96.7)	27 (90.0)	2 (6.7)
	0.2	30	27 (90.0)	18 (60.0)	9 (30.0)
	0.3	30	25 (83.3)	21 (70.0)	4 (13.3)

*Percentage of initial explants was calculated.

Table 3. DNA relative values and estimated numbers of chromosomes in control and induced variants of white yam (*D. rotundata*) and yellow yam (*D. cayenensis*).

Accession no.	Treatment	DNA index	Estimated no. of chromosomes (2n)
TDr 2720	Control	1.88	40
	Variant	3.82	80
TDr 1793	Control	1.93	40
	Variant	3.83	80
TDr 2351	Control	1.92	40
	Variant	3.86	80
R-20	Control	1.80	40
	Variant	3.77	80
TDc 2812	Control	2.74	60
	Variant	5.31	120
TDc 3704	Control	2.86	60
	Variant	5.41	120

DNA index: Relative amount of DNA in nuclei of samples compared to the inter-reference (*O. sativa*).

Table 4. Leaf characteristics of diploid (2*n* = 40) and induced tetraploid (2*n*=4x=80) variants of white yam (*Dioscorea rotundata*) accessions.

Accession no.	Accession	Leaf length (cm)	Leaf width (cm)	Shape (length/width)	Width of sinus (cm)	Depth of sinus (cm)
TDr 2720	Diploid	7.57	5.19	1.45	3.38	1.49
	Tetraploid	9.16**	6.90**	1.33**	3.58	1.81
TDr 1793	Diploid	4.96	3.85	1.29	2.33	0.97
	Tetraploid	6.54**	5.37**	1.23	2.66	1.65**
TDr 2351	Diploid	5.48	4.45	1.24	2.95	0.96
	Tetraploid	6.75*	5.80**	1.16	3.18	1.26
R-20	Diploid	7.41	5.48	1.37	3.36	1.17
	Tetraploid	8.31*	7.01**	1.20*	4.25**	1.35

* and **Significant difference according to the students t-test at 5 and 1%, respectively.

Table 5. Stoma characteristics of diploid (2*n*=2x=40) parents and induced tetraploid (2*n*=4x=80) variants of white yam *D. rotundata*) accessions

Accession		Length (µm)	Width (µm)	Shape (length/width)	Size (length×width)	Density (mm^{-2})
TDr 2720	Diploid	32.4	18.5	1.8	599.0	12.0
	Tetraploid	43.3**	22.6**	1.9**	989.1**	5.8**
TDr 1793	Diploid	30.5	19.4	1.6	595.1	17.2
	Tetraploid	38.4**	23.9**	1.6	917.0**	7.9**
TDr 2351	Diploid	34.4	18.1	1.9	623.5	15.0
	Tetraploid	41.7**	20.6**	2.0**	861.3**	8.4**
R-20	Diploid	33.5	18.00	1.9	605.6	15.0
	Tetraploid	40.9**	20.2**	2.0**	829.3**	7.2**

**Significant difference according to the student's t-test at 1%.

(length/width) between TDr 2720 and R-20, leaves of tetraploid variants appearing rounder than diploid leaves. Similarly, leaves of tetraploid variants appeared thicker than diploid leaves, except in the case of TDr 1793. A slightly deformed leaf shape was observed in the hexaploid variants derived from the triploid parents of the yellow yam accession and most hexaploid variants grew abnormally until death (data not shown).

Significant differences were observed in all stoma characters between tetraploid white yam variants and their diploid parents except for stomata shape in TDr 1793 (Table 5). Stoma was significantly longer and wider in the induced tetraploids compared to the diploid parents. Stoma (length/width) in the tetraploid variants also appeared somewhat longer than in the diploid parents. Although stoma size (length × width) was larger in the tetraploid than the diploid parent, stoma density was lower in the tetraploid variants.

DISCUSSION

To this date, various efficient techniques for polyploid induction using colchicine have been proposed for different crop species. The efficiency of colchicine-induced somatic polyploidization varies among plant species and the method of application and/or concentration used. The *in vitro* induction rate in *Phlox subulata* L. reached 20% (Zhang et al., 2008), while in diploid and triploid cultivars of *Colocasia esculenta* it was low at 4.3 and 1.4%, respectively (Miyazaki et al., 1985). Huang et al. (2008) also reported a polyploidy rate as high as 36.7% in *D. zingiberensis* calli immersed in 0.3% colchicine solution for 16 h prior to culture. However, the above protocol is not applicable to white and yellow yam because of difficulties associated with plant regeneration from a callus. Other papers have reported findings in *Dioscorea* species such as *D. floribunda* (Sharma and Chaturvedi, 1988) and *D. zingiberensis* (Huang et al., 2008; He et al., 2010; Xiao et al., 2010), and recently, Babil et al. (2011) proposed an efficient *in vitro* technique for induction of somatic polyploids of water yam that is also effective in white and yellow yam. In this study, we adopted this *in vitro* technique to optimize somatic polyploidization in white and yellow yam. We also elucidated the effects of somatic polyploidization on leaf and stoma traits in white yam.

This is the first time polyploid variants of white and yellow yam have been induced at a high frequency. The highest rate was attained after *in vitro* treatment of the diploid accession R-20 with 0.1% colchicine. However, in the case of triploid yellow yam accessions, the induction rate of polyploids was relatively low, probably due to the higher ploidy level of the mother plant. In most cases, except TDr 1793 and TDc 3704, the highest rate of polyploid induction appeared after 0.1% colchicine treatment.

Nodal segment culture of white and yellow yam is an easy reproduction method for growth of shoots. In fact, the shoot survival rate was as high as 96.7 and 93.3% in a control plot of white and yellow yams, respectively.

Tetraploids (2*n*=4x=80) were successfully induced from the diploid parent (2n=2x=40) white yam and hexaploids (2n=6x=120) from the triploid parent (2n=3x= 60) yellow yam accessions. The induced tetraploid variants of white yam grew almost normally; however, the hexaploid variants of yellow yam grew abnormally until death. A similar phenomenon was observed in spontaneous hexaploid (2n=6x=120) polyploid mutants redifferentiated from cultured calli of triploid (2n=3x=60) water yam accessions (Iijima, unpublished) and in colchicine induced somatic polyploids of triploid water yam (Babil et al., 2011). These results suggest that in yellow yam tetraploidy may be the highest level of ploidy at which

normal growth and survival are observed.

Somaclonal polyploidization is thought to affect various phenotypic characters such as cell size, chlorophyll content, fertility and organ size. Over the past few decades, a considerable number of studies have reported the effects of somaclonal polyploidization on phenotypic characters of various plant species (Speckmann et al., 1965; Miyazaki et al., 1985; Liu et al., 2007; Zhang et al., 2008). In water yam, tetraploid variants tend to have rounder leaves than their diploid parents. Similar relationships were also recognized between diploid and tetraploid variants of water yam accessions collected in Myanmar (Babil et al., 2010).

However, information about the effects of ploidy level on phenotypic characters and practically important traits such as tuber yield and quality are limited for white and yellow yam. In the present report, the relationship between ploidy level and leaf and stoma characters was therefore investigated in white yam. Significant differences were observed in leaf length and width, with tetraploid variants tending to have rounder leaves than their diploid parents.

Stomatal traits such as size (length × width), shape (length/width) and density are considered reliable indicators of ploidy in various plant species (Speckman et al., 1965; Daniel and Yao, 1996). In cassava (*M. esculenta*), for example, stomata were less dense and their size larger in polyploids because cells were enlarged as a result of polyploidization (Hahn et al., 1992). In the present study, artificially induced tetraploid variants displayed larger stomata at a lower density compared to their diploid parents. A similar trend was observed between diploid and tetraploid landraces of water yam collected in Myanmar (Babil et al., 2010) and between diploid and artificially induced tetraploids of water yam (Babil et al., 2011). Accordingly, this observation confirms that stoma traits are reliable indicators of polyploidization in white yam. As well as reduced stomatal density is also known to increase drought tolerance without decreasing photosynthesis rate (Yu et al., 2008). Thus it could be a trait to explore in terms of inheritability to breed yam for dry conditions derived from climate change.

The efficient method of *in vitro* induction of polyploidy reported here is a useful tool for obtaining polyploid variants for use as genetic resources in white yam breeding. However, utilization of somatic polyploidization as a tool to enhance genetic improvement of yellow yam remains limited due to the low induction rate and abnormal growth of induced hexaploid variants. Investigations are ongoing with the aim of elucidating the effect of polyploidization on important agronomical traits such as yield and tuber quality.

Conflict of Interests

The authors have not declared any conflict of interests.

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