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Callus induction in three mosaic disease resistant cassava cultivars in Benin and genetic stability of the induced calli using simple sequence repeat (SSR) and sequence-characterized amplified region (SCAR) markers

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The effect of different concentrations of thidiazuron (TDZ), benzyl amino purine (BAP), kinetin and 2,4-dichlorophenoxy acetic acid (2, 4-D) on callus induction in three elite cassava cultivars (agric-rouge, atinwewe and agblehoundo) was evaluated. Leaf explants harvested from greenhouse-grown cassava were sterilised using different concentrations of commercial bleach commonly called Jik (3.85% NaOCl) at different time intervals. The highest number (94%) of clean explants was obtained when 2% (v/v) Jik was used for 15 min. The explants were cultured in half MS media supplemented with different growth regulators TDZ, BAP, kinetin 2, 4-D, 100 mg/l myo inositol, 2% sucrose and gelled with 0.3% phytagel. Callus formation was observed from the cut edges of the leaves in all cultivars after 10 days in medium supplemented with TDZ, 12 days in BAP medium, and 15 days in kinetin medium. There were significant ($p < 0.05$) differences in callus formation among all cytokinins types and concentrations. However, there were no significant differences in callus formation in different 2,4-D concentrations. All 2,4-D concentrations produced 100% callus in all the cultivars. However, 2,4-D at 2 µM significantly produced the highest (2.48±0.30) callus weight in cultivar atinwewe. Furthermore, simple sequence repeats (SSR) and sequence-characterized amplified region of the induced calli on TDZ and 2,4-D media indicated the loss of CMD2 gene among induced calli compared to the mother plants.

Key words: Cassava, simple sequence repeats (SSR) and sequence-characterized amplified region (SCAR), genetic stability, callus, cytokinins, auxins.

INTRODUCTION

Cassava (*Manihot esculenta Crantz*) is the most important root crop in the tropics and ranks fourth after rice, wheat and corn as a source of calories for human consumption (Acedo and Labana, 2008). In Benin, cassava is consumed by more than 54% of the population in different ways as raw or after processing into gari, chips or tapioca (MAEP, 2013). This is an indication of its importance as food security crop and

poverty alleviation in the country. The cultivation of cassava in Benin is facing many challenges which include pests and diseases, lack of good agronomic practices, land degradation, shortage of planting materials, limited processing options etc. (Agre et al., 2015). Viral diseases, particularly cassava mosaic disease (CMD) are the most economically important and may lead to yield losses of 20 to 95% (Fauquet et al., 1990; Hahn et al., 1989). Due to these constraints, the average crop yield in Benin is low (15.55 tonnes per hectare) compared to the global yield of 90 tonnes (FAOSTAT, 2017). Recent breeding work in Benin has resulted in the release of some local cultivars (Agric-rouge, Atinwewe and Agblehoundo) that are resistant to cassava mosaic disease (CMD) (Houngue et al., 2018). The cultivars contain CMD2 gene which is dominant monogenic resistance locus (Fondong, 2017). The propagation of those cassava cultivars in the field by farmers is by cuttings and few works have been done for in vitro culture (Cacaï et al. 2012, 2013). The traditional method by cuttings is not only limiting in the numbers of planting materials but is also cumbersome, and labour intensive. Therefore, there is need to evaluate alternative propagation methods that are fast and tissue culture offers a feasible option.

In tissue culture technique, somatic embryogenesis has an advantage over micropropagation in that it generates a new plant with both root and shoot meristems from actively dividing somatic cells in the same step and within a short time period, whereas micropropagation requires additional steps and a longer time frame (Leva et al., 2012). Although regeneration of microshoots from cassava nodes can be achieved with a high rate of success, somatic embryogenesis is still preferred to microshoot propagation technique for two main reasons: High multiplication rates, and the possibility of initiating cultures from readily available and renewable leaf explants. One of the advantages of using somatic embryogenesis as a method for mass propagation is that the embryos have concomitant development of both root and shoot meristems which, under optimal conditions, can grow synchronously to produce normal plants. This procedure can save a great deal of time in those situations where conventional vegetative propagation is slow or difficult to carry out and manage.

The induction of somatic cells from leaf explants during tissue culture requires the explants sterilization using different sterilants. The type of sterilizing agent used depends on the source and the type of the explants and the purpose of the experiments. There are few studies on sterilization of cassava leaf explants from greenhouse. For instance, Danso (1997) reported the use of alcohol to sterilize young leaf of four weeks old shoot tips from greenhouse cassava. On the other hand, Magaia (2015) reported 100% clean explants when cassava leaf from greenhouse were sterilized with 70% ethanol for 2 min followed by 0.05% of mercuric chloride for 1 or 2 min. During the current study, the effect of Jik (3.85% NaOCl) on sterilization of leaf explants was evaluated. Somatic embryogenesis have been reported to be suitable for mass propagation of highly performing cassava clones (Osorio et al., 2012). Many studies have been done in callus induction in several crops using cytokinins and auxins. For example, Faye et al. (2015) and Mongomake et al. (2015) reported callus formation with MS media supplemented with cytokinins in some cassava cultivars. Phua et al. (2016) and Fathil et al. (2017) reported that 2,4-D at low (0.25 - 1 mg/L) concentrations produced 100% callus in *Clinacanthus nutans* and *Citrus suhuiensis.* Furthermore, Castro et al. (2016) reported in *Byrsonima verbascifolia* an excellent (100%) callus induction using 2,4-D combined with BAP. Callus-derived somaclonal variation based on morphological and biochemical parameters have been reported by Pajević et al. (2004) in sunflower, Jibu et al. (2006) in tea, Rajeswari et al. (2009) in Sugarcane, Park et al. (2010) in Rice, Beyene et al. (2016) and Chauhan et al. (2018) in cassava. Although, many works have generated cassava plantlets from somatic embryogenesis in different cassava varieties (Anuradha et al., 2015; Atehnkeng et al., 2006; Feitosa et al., 2007; Le et al., 2007; Vidal et al., 2014), there are no reports on such work on cassava varieties in Benin. Even in cases where regeneration of callus has been reported there is hardly any information on evaluating the stability of the regenerated callus. Callus formation is an important requirement for establishing embryogenic culture, plantlet regeneration and germplasm conservation.

Microsatellite-based marker techniques such as Simple sequence repeat (SSR) and sequence-characterized amplified region (SCAR) markers have successfully been used in the detection of CMD2 resistant gene in cassava (Okogbenin et al., 2008; Houngue et al., 2018). SSR markers have also been used in detecting genetic differences or similarities in several micropropagated plants, including cotton (Jin et al., 2008) and medicinal plants such as *Jatropha curcas* (Sun et al., 2008). The high reproducibility, simplicity, and low cost of the experimental procedures of SSR and SCAR compared to other molecular markers such as restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) makes them more appropriate for such studies. The aim of the study was to determine the optimal sterilization technique for cassava leaf explants. The effects of cytokinins (TDZ, kinetin and BAP) and 2,4-D concentrations on frequency of callus

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Table 1. Specific SSR and SCAR primers used for detection of CMD2 resistant gene in cassava mother plants and induced calli.

Primer code	Marker system	Forward primer sequence	Reverse primer sequence	Expected sequence length (bp)	Annealing temperature (˚C)
NS169	SSR	GTGCGAAATGGAAATCAATG	GCCTTCTCAGCATATGGAGC	319	55
RME1	SCAR	AGAAGAGGGTAGGAGTTATGT	ATGTTAATGTAATGAAAGAGC	700	55

formation, weight of callus induced from greenhouse-grown leaf explants of three mosaic resistant cassava cultivars (Agric-rouge, Atinwewe and Agblehoundo) and finally to evaluate the 'CMD2 conformity' of the calli using SSR and SCAR markers.

MATERIALS AND METHODS

Cassava cuttings of three (Agric-rouge, Atinwewe and Agblehoundo) cultivars resistant to CMD were collected from the southern region in Republic of Benin and transported to Coffee Research Institute (CRI), Ruiru-Kenya where the tissue culture studies were carried out. The molecular analysis work was done in the Molecular Biology and Biotechnology laboratories of CRI and Pan African University of Basic Sciences, Technology and Innovation (PAUSTI), Kenya.

Explants sterilization

Leaf explants from three weeks old stem cuttings were harvested and transported from the greenhouse to the laboratory in a beaker containing tap water. Once in the laboratory, they were cleaned with cotton wool containing liquid soap to remove any surface debris and rinsed with tap water. They were then subjected to sterilization under the lamina flow hood using 2 and 5% v/v commercial bleach (Jik) for 5, 10, 15 and 20 min. After exposure to the sterilant, the explants were rinsed two times with sterile distilled water and thereafter given a quick immersion (30 s) in 70% (v/v) ethanol and finally rinsed four times with sterile distilled water. The leaf explants were trimmed to approximately 1 cm^2 leaf discs and cultured individually in tubes containing hormone free MS media. The cultures were incubated in a dark room at a temperature of 25 ± 2˚C. Data were collected after 4, 8, and 15 days on the percent clean explants. This was calculated as total number of contaminated explants / total number of explants x 100.

Callus induction and culture conditions

The media used to evaluate the effect of cytokinins and auxin on callus induction from leaf explants was half-strength MS (Murashige and Skoog, 1962) supplemented with either BAP or kinetin (5, 10, 20 and 40 µM), thidiazuron (0.1, 0.5, 1 and 1.5 µM), or 2, 4-D (2, 5, 10, 25 and 30 µM), 100 mg/L inositol, 2% (w/v) sucrose and gelled with 0.3% Phytagel in separate experiments. The media pH was adjusted to 5.7 by using either 1 N HCl or 1 N NaOH before the gelling agent was added. The media was dispensed in culture test tubes and autoclaved at a temperature of 121°C and a pressure of 1.1 $kg{\cdot}cm^2$ for 20 min. The cultures were incubated in a dark room maintained at 25 ± 2°C. Half-strength MS medium without growth regulators was used as control.

Subculture of callus and data collection

The calluses were transferred to MS medium without growth regulators (control) and MS media supplemented with 1, 3 and 5 µM GA_3 (embryos induction medium) to study their embryogenic competence. Data were collected on weekly basis on the explants with callus (expressed as % callus induced) and weight of the callus. The percent (%) callus induced was calculated as total number of explants with callus / total number of explants cultured x 100.

Experimental design and data analysis

All experiments were laid out in completely randomized design (CRD) with 10 replicates per treatment and the experiment repeated three times. The data (percentage and weight of the callus) were subjected to one-way analysis of variance and the significant differences between treatments means were assessed using MINITAB version 19 software. Tukey analysis at 5% level was performed to assess difference between means. Data were also subjected to analysis as graph by GraphPad Prism 7 Software.

Assessment of the presence of CMD2 gene in calli

PCR-based SSR and SCAR markers as described by Houngue et al. (2018) were used to assess the CMD2 conformity of the induced calli. The mother plants growing in the greenhouses did not show any symptoms of CMD and it was used as the control. The characteristics of the primers used are shown in Table 1.

DNA extraction and quantification

DNA was extracted from young leaves picked from cassava mother plants and the callus according to the method described by Diniz et al. (2005). DNA quality and quantity were determined with Genova Spectrophotometer (Model 7415 Nano, Vacutec, South Africa) and quality was also assessed on 1% (w/v) agarose gel. The extracted DNA samples were stored at -20°C for SSR and SCAR analysis.

Polymerase chain reaction (PCR) for scoring CMD2 resistant gene

The SSR and SCAR analysis were performed as described by Omingo et al. (2017). DNA samples were diluted to 10 ng/µl for SSR and SCAR analysis. A total of 100 ng of each DNA sample was used in PCR reactions. A reaction mix was prepared to include: 2.5 µl of buffer (10 x), 2.5 µl of $MgCl_2$ (25 mM), 3.5 µl of dNTPs (500 µM), 2 µl of SSR or SCAR (10 µM) reverse (1 µl) and forward (1 µl) primers, 0.2 µl of Taq polymerase 5 µl. The 25 µl PCR volume was incubated in a thermocycler (Model FFG02HSD, made in UK) set for the following amplification conditions: One cycle at 95°C for 5 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1.5 min, extension at 72 °C for 10 min and was held at 4°C. The amplified products were electrophoresed in 2.3% agarose gel and then visualized in a UV trans-illuminator (Model M-26, Upland, CA 91786 U.S.A) after staining in ethidium bromide

Table 2. Effects of commercial bleach (Jik) on elimination of surface contamination from cassava leaf explants.

Jik concentration (%) v/v	Exposure time (min)	Percentage of clean explants (%)		
		4 days	9 days	15 days
2	5	97	93	88
2	10	93	77	77
2	15	100	100	94
2	20	92	89	89
5	2	90	80	80
5	5	97	90	90
5	10	100	97	88
5	15	100	89	89

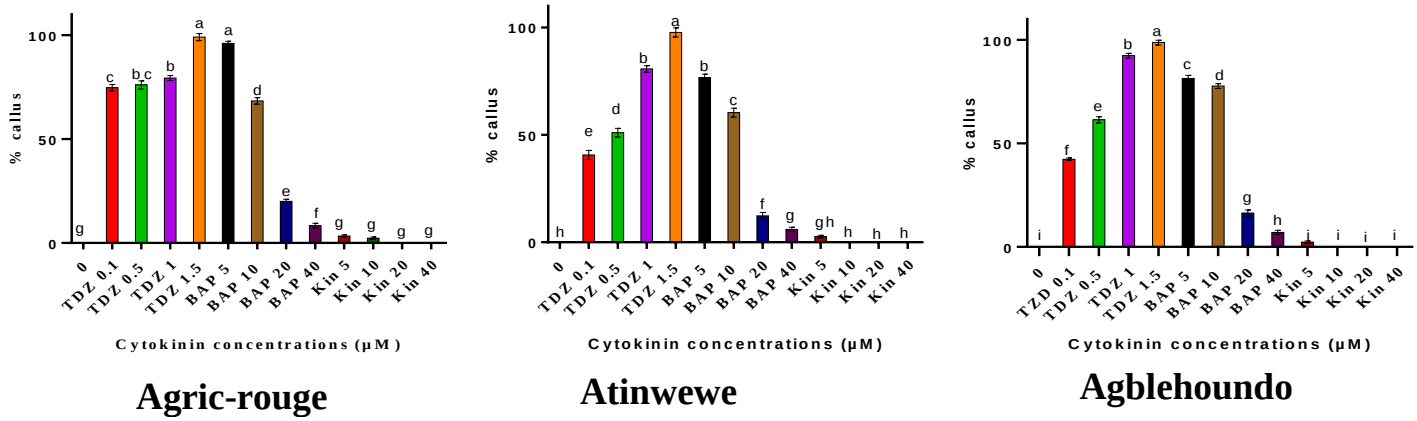

Figure 1. Effect of different cytokinin concentrations (µM) on callus formation from leaf explants of the three cultivars after one month of culture. *Means followed by the same letter are not significantly different at P≤0.05.

solution.

Scoring and analysis of bands

Amplified DNA fragments were run on agarose gel to score for the presence (1) or absence (0) of bands (Resistance gene) in the formed calli compared with mother plants. All reactions were repeated at least twice, and only distinct, reproducible, polymorphic and well-resolved bands across all runs were considered for analysis.

RESULTS

The result of the effect of Jik (3.85% NaOCl) on surface sterilization of cassava leaf explants are shown in Table 2. Most of the contaminations observed during this study were fungal (80%) while bacterial and yeast contaminants accounted for 20%. After 15 days the highest (97%) number of clean explants was obtained when the explants were sterilized in 2% Jik for 15 min and this sterilization procedure was used in all the subsequent experiments.

Effects of cytokinins on the callus formation

The results of the effects of different cytokinins on callus induction are shown in Figures 1 to 3. Callus formation was observed from the cut edges of the leaves in all the three cultivars. The cytokinin concentrations significantly ($p < 0.001$) affected induction of callus from the explants in all cultivars. Callus induction was first observed in all cultivars after ten days in medium supplemented with TDZ, 12 days in BAP medium, and 15 days in kinetin medium. It was generally observed that the media supplemented with TDZ 1.5 µM produced the highest percentage (98-99 %) explants with callus in all the three cultivars (Figure 1). It was also observed in all cultivars that increasing TDZ concentrations from 0.1 to 1.5 µM increased the percentage of callus production while increasing BAP concentrations from 5 to 40 µM decreased the production of callus. However, no significant difference ($p < 0.001$) was observed among kinetin concentrations and the control. Callusing of leaf explants began with curling of the tissue followed by swelling at the cut edges. The white calli turned to brown

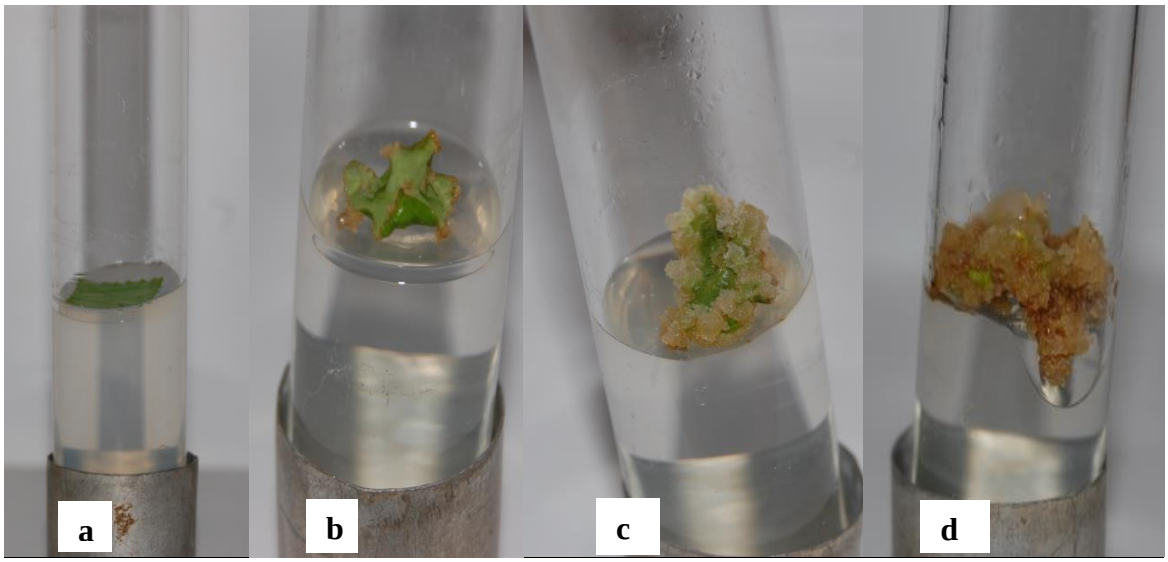

Figure 2. Effect of cytokinins on callus formation: a -Fresh culture, b - Initiation of callus 10 days after; c - White callus after 3 weeks; d - Callus turned light brown after one month.

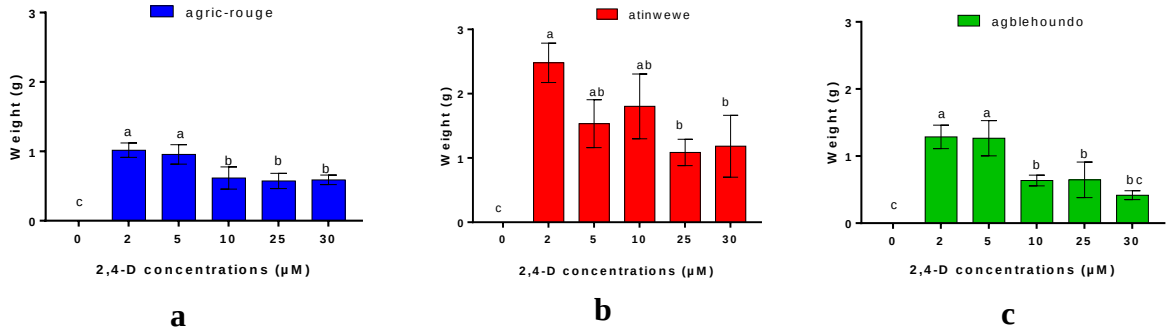

Figure 3. Effect of different 2, 4-D concentrations (µM) on callus formation from leaf explants: **a** - Agric-rouge, **b** - Atinwewe, and **c** - Agblehoundo cultivars after one month of culture. *Means followed by the same letter are not significantly different at P≤0.05.

after one month (Figure 2).

Effects of auxin 2,4-D on the callus formation

The results showed that there was no significant effect of the 2,4-D concentrations on callus induction in all the three cultivars. It was observed that all concentrations used in this study produced 100% frequency of callus in all cultivars after ten days. In agric-rouge and atinwewe cultivars, the first callus was observed after 4 days and after 6 days in agblehoundo. However, there was significance difference ($p < 0.001$) in the weight of the callus among all concentrations evaluated and across cultivars. Figure 3 shows the effect of 2,4-D on callus formation. Increasing concentration of 2, 4-D from 10 to 30 µM, reduced the weight of the callus in all cultivars. 2, 4-D 2 µM significantly produced the highest callus masses which 2.48 ± 0.30 atinwewe cultivar. There was no callus produced in the medium without growth regulators. The formed calli were white and friable in all cultivars. When the calli were transferred into media without growth regulators, 80% calli developed roots (Figure 4). An attempt to transfer the callus on a media supplemented with different concentration of GA_3 did not yield any embryos.

Assessment of the presence of CMD2 gene in calli

To confirm the presence of CMD2 gene in induced calli, PCR analyses were conducted. DNA fragments corresponding to the CMD2 gene (319 bp with SSR or 700 bp with SCAR) were amplified in all mother plants

Figure 4. Effect of auxin 2, 4-D on callus production: a - White formed callus after two weeks; b - Formation of root two months after callus was transferred to media without growth regulators.

whereas the corresponding band was not detected in some induced calli, indicating the loss of the resistance gene in the genome of those induced calli (Figures 5 and 6). In Figure 5, SSR primer revealed the loss of the resistant gene (319 bp) in calli induced from 2, 4-D supplemented media in cultivars Agric-rouge and Agblehoundo and in callus induced from TDZ medium in Agblehoundo. In Figure 6, SCAR primer revealed the presence of the resistant fragment (700 bp) in induced calli from both 2,4-D and TDZ media in cultivar agblehoundo.

DISCUSSION

Development of a sterilization technique for cassava leaf explants

The current study was conducted with an aim of optimizing the sterilization of leaf explants and callus induction using different growth regulators in the mosaic disease resistant cassava cultivars. The disinfectant widely used for surface sterilization is sodium hypochlorite (Miché and Balandreau, 2001). During the current study, Jik (NaOCl 3.85%) was found to be effective in sterilizing cassava leaf explants from greenhouse. The recorded highest (94%) number of clean explants was obtained when 2% Jik was used for 15 min. Similar results of high numbers of clean explants (100%) with greenhouse grown cassava leaf explants was reported by Magaia (2015) who used 0.1% $HgCl_2$ solutions. However, mercuric chloride is known to be highly toxic and may not be recommended for routine sterilization. The results of the current study concur with those of Guma et al. (2015) who reported high percentage (83%) of clean explants when greenhouse-grown leaf explants of anchote (*Coccinia abyssinica*) were exposed to 5% Jik for 10 min. Moreover, Jik was also found to be effective in sterilizing coffee leaf explants from the greenhouse (Lubabali, 2015).

Effects of different concentrations of cytokinins and auxins for regenerating plantlets through somatic embryogenesis

Cytokinins are known to stimulate cells and, as such, they are also suitable candidates for induction of somatic embryogenesis and caulogenesis (Deo et al., 2010). For example, thidiazuron (TDZ) has been reported to stimulate *in vitro* somatic embryogenesis (Srangsam and Kanchanapoom, 2003; Mithila et al., 2003; Lin et al., 2004; Chen and Chang, 2006; Mahendran and Bai, 2016; Mose et al., 2017). Cytokinins have been reported to induce callus formation in cassava (Faye et al., 2015; Mongomake et al., 2015) and in *Vitex doniana* (Dadjo et al., 2015). It was observed during the current study that all cytokinins failed to induce direct embryos from greenhouse leaf explants in all cassava cultivars. Instead, they produced callus and TDZ at 1.5 µM induced the highest callus percentage (98 to 99%) in all cultivars. The results of the current study also concur to those of Faye et al. (2015) and Mongomake et al. (2015) who reported callus formation with MS media supplemented with cytokinins in some cassava cultivars and also with Dadjo et al. (2015) who reported callus formation from *Vitex doniana* leaf explants cultured on media supplemented with cytokinins. The present report is however, contrary to that of Mongomake et al., (2015) who reported that medium supplemented with TDZ did not induce callus in some cassava cultivars. This could be probably due to the different genetic makeup. The current observation

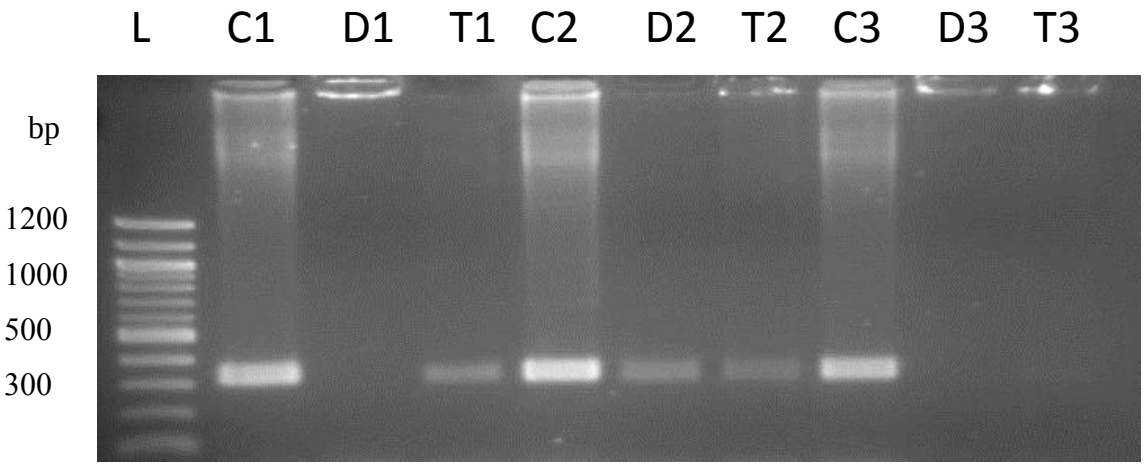

Figure 5. SSR banding pattern with primer NS169 in both *induced* calli (D1, D2, D3, T1, T2, and D3) and greenhouse-grown mother plants of agric-rouge (C1), atinwewe (C2), and agblehoundo (C3). D1, D2, and D3 were induced from leaf explants of C1, C2, and C3 cultured on 2,4- D media and T1, T2, and T3 were induced from leaf explants cultured of C1, C2, and C3 on TDZ media.

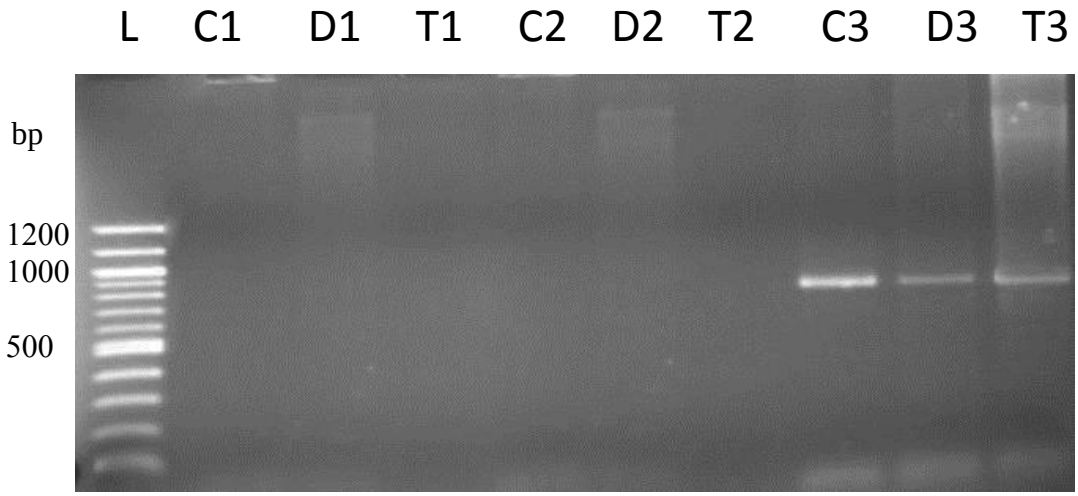

Figure 6. SCAR banding pattern with primer RME1 in both induced calli (D1, D2, D3, T1, T2, and D3) and greenhouse-grown mother plants of agric-rouge (C1), atinwewe (C2), and agblehoundo (C3). D1, D2, and D3 were induced from leaf explants of C1, C2, and C3 cultured on 2, 4-D media and T1, T2, and T3 were induced from leaf explants cultured of C1, C2, and C3 on TDZ media

concurs with Mongomake et al. (2015) and Faye et al. (2015) who reported that BAP produced better callus frequencies than kinetin in cassava.

The results of the current investigation revealed that auxin 2, 4-D at the range of 2 to 30 µM failed to induce somatic embryos from greenhouse- grown cassava leaf explants in all cultivars. These results are contrary to reports indicating that Murashige and Skoog medium supplemented with 2, 4-D was effective in induction of somatic embryos from young leaf lobes of *in vitro* grown shoots of cassava (Mathews et al., 1993; Ma and Xu, 2002; Le et al., 2007; Vidal et al., 2014; Mongomake et al., 2015). It was observed that 2, 4-D was highly efficient in inducing callus and all concentrations used in this study produced the 100% frequency of callus in the cultivars after ten days. Similar observations were made by Danso (1997), Fletcher et al. (2011) and Isah et al., (2018) who reported that 2, 4-D failed to induce direct embryos from greenhouse cassava leaf explants but it was best for callus formation. The results of the current study were similar to that of Phua et al. (2016) and Fathil et al. (2017), who reported that 2, 4-D at low (0.25 - 1 mg/L) concentrations produced 100% callus in *Clinacanthus nutans* and in *Citrus suhuiensis*. Similarly,

Castro et al. (2016) reported in *Byrsonima verbascifolia* a maximal callus induction (100%) using 2, 4-D combined with BAP. The callus induced during the current study did not form embryos when subcultured on GA_3 supplemented medium and hormone free-medium. Bronsema et al. (1997) made similar observation while working with maize. These observations concurs to those of Danso (1997), who reported in cassava that root development occurred when calli derived from *in vitro* explants were transferred to the embryos induction medium. Some attempts have been carried out in certain species to associate the endogenous hormone levels of explants and cultures derived from them with their regeneration competence (Sasaki et al., 1994; Hess and Carman, 1998; Jiménez and Bangerth, 2001). The effect of any particular exogenously applied growth hormone is influenced by a variety of other factors in the internal environment of the plant, especially other hormones in the plant (Onwubiku, 2007; Preece, 1987). The fact that the induced calli were not able to produced embryos in current study could indicate that the endogenous levels of both cytokinins and auxins in these cassava varieties might be adequate. Hence, exogenous application led to supra-optimal amounts which may induce some inhibitory effects.

Effect of growth regulators on the CMD2 conformity in of the induced calli

During the current study, it was possible to isolate somaclonal variants through callus phase based on the CMD2 resistant gene in cassava. SSR primer revealed the loss of the resistant gene (319 bp) in callus induced from 2, 4-D media in cultivars agric-rouge and agblehoundo and in callus induced from TDZ medium in agblehoundo. These results concur with those of Beyene et al. (2016) and Chauhan et al. (2018) who reported the loss of CMD2 resistant gene after regenerating the entire cassava plants. Findings confirm the usefulness of SSR markers in the analysis of conformity of induced calli, similar to works on crops such as caladium tissue culture-derived plants by Cao et al. (2016). Similarly, Sharma et al. (2015) in *Stevia rebaudiana* showed genetic variation among the mother plants and callus induced from 2,4-D medium. This study showed that the loss of CMD2 resistance gene at the callus stage is genotype depended since the 319 bp fragment was present callus induced from Atinwewe cultivar while it was absent in others. This is most apparent in crops under *in vitro* culture where the amount of growth regulator is extensive. On the other hand, SCAR primer showed the presence of the resistant fragment (700 bp) in the induced calli from both 2, 4-D and TDZ media in cultivar agblehoundo which was similar to their mother. As in this study, SCAR marker was developed by Paran and Michelmore (1993) for downy mildew resistance genes in lettuce. SCARs may identify polymorphisms that are less accessible by other techniques. The efficiency of SCAR marker in the current study has been proved for authentication of traits in various biological systems vis-à-vis *Sorghum halepense* by Zhang et al. (2013) and in *Pennisetum glaucum* by Jogaiah et al. (2014). It was then concluded that the used of exogenous hormones such as 2, 4-D and TDZ caused the loss of the resistant fragments (319 bp) alone in the induced callus from Atinwewe cultivar while the second fragment was retained. Furthermore, the protocol established here may be of interest to detect and eliminate variants at early stages to minimize loss later after regenerating the whole plant (Radhakrishnan and Ranjitha, 2008; Roels et al., 2005). This preliminary study may open up new perspectives for implementing a biotechnological genetic improvement program for these cassava cultivars to other diseases.

Conclusion

In conclusion, the optimum Jik (3.85% NaOCl) concentration for sterilization of leaf explants was established to be 2% exposed for 15 min. The growth regulator 2,4-dichlorophenoxy acetic acid (2, 4-D) proved to be the best for callus formation with the optimum concentration of 2 µM for all cultivars. Furthermore, according to the cultivars, SSR and SCARS primers revealed either presence or absence of the CMD2 resistance gene in the induced calli compared to the mother plant.

Recommendation

Further investigation should be carried out to establish the optimal growth regulators for regenerating plantlets from the callus induced from the leaf explants of the three elite cassava cultivars.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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REFERENCES

Acedo V, Labana, C (2008). Rapid propagation of released Philippine

cassava varieties through tissue culture. Journal of Root Crops. 34:108-114.
Agre AP, Rabbi IY, Battachargee R, Augusto A, Dansi M, Melaku G, Sanni A, Akouegninou A, Akpagana K (2015). Agromorphological characterization of elite cassava (*Manihot esculenta* Crantz) cultivars collected in Benin. International Journal of Current Research in Biosciences and Plant Biology 2(2):1-14.
Anuradha T, Kumar KK, Balasubramanian P (2015). Cyclic somatic embryogenesis of elite Indian cassava variety H-226. Indian Journal of Biotechnology 14:559-565.
Atehnkeng J, Adetimirin VO, Ng SYC (2006). Exploring the African cassava (Manihot esculenta Crantz) germplasm for somatic embryogenic competence. African Journal of Biotechnology 5(14):1324-1329
Beyene G, Chauhan RD, Wagaba H, Moll T, Alicai T, Miano D, Carrington JC, Taylor NJ (2016). Loss of CMD2-mediated resistance to cassava mosaic disease in plants regenerated through somatic embryogenesis. Molecular Plant Pathology 17(7):1095-1110.
Bronsema FBF, Van Oostveen WJF, Van Lammeren AAM (1997). Comparative analysis of callus formation and regeneration on cultured immature maize embryos of the inbred lines A188 and A632. Plant Cell, Tissue and Organ Culture 50(1):57-65.
Cacai GHT, Ahanhanzo C, Dangou JS, Houedjissin SS, Agbangla C (2012). Effets de différentes combinaisons hormonales sur l'organogenèse in vitro de quelques cultivars locaux et variétés améliorées de Manihot esculenta Crantz (manioc-Euphorbiaceae) cultivées au Bénin. International Journal of Biological and Chemical Sciences 6(4):1593-1607.
Cacaï GHT, Adoukonou-Sagbadja H, Kumulugui BS, Ovono PO, Houngue J, Ahanhanzo C (2013). Eradication of cassava (Manihot esculenta) mosaic symptoms through thermotherapy and meristems cultured in vitro. International Journal of Agronomy and Plant Production 4(Special Issue):3697-3701.
Cao Z, Sui S, Cai X, Yang Q, Deng Z (2016). Somaclonal variation in 'Red Flash'caladium: morphological, cytological and molecular characterization. Plant Cell, Tissue and Organ Culture 126(2):269-279.
Castro AHF, Braga K de Q, Sousa FM de, Coimbra MC, Chagas RCR (2016). Callus induction and bioactive phenolic compounds production from *Byrsonima verbascifolia* (L.) DC. (Malpighiaceae). Revista Ciência Agronômica 47(1):143-151.
Chauhan RD, Beyene G, Taylor NJ (2018). Multiple morphogenic culture systems cause loss of resistance to cassava mosaic disease. BMC Plant Biology 18:132.
Chen JT, Chang WC (2006). Direct somatic embryogenesis and plant regeneration from leaf explants of *Phalaenopsis amabilis.* Biologia Plantarum 50(2):169-173.
Dadjo C, Kahia J, Muthuri C, Diby L, Kouame C, Njenga P, Kouassi M (2015). Induction and regeneration of somatic embryos from *Vitex doniana* (Lamiaceae) leaf explants. International Journal for Biotechnology and Molecular Biology Research 6(4):28–34.
Danso KE (1997). *in-vitro* propagation of selected cassava (*Manihot esculenta* crantz) cultivars using multiple shoot induction and somatic embryogenesis. M. Sc. Dissertation, University of Ghana. pp 132.
Deo PC, Tyagi AP, Taylor M, Harding R, Becker D (2010). Factors affecting somatic embryogenesis and transformation in modern plant breeding. The South Pacific Journal of Natural Science 28(1):27-40.
Diniz LEC, Ruas C de F, Carvalho V de P, Torres FM, Ruas EA, Santos M de O, Sera T, Ruas PM (2005). Genetic diversity among forty coffee varieties assessed by RAPD markers associated with restriction digestion. Brazilian Archives of Biology and Technology 48(4):511–521.
FAOSTAT: Food and Agriculture Organization of the United Nations. (2017). Agricultural statistics for 2017. FAO. United Nations. http://www.fao.org/news/archive/news-by-date/2017/en/
Fathil NAM, Puad NIM, Amid A, Azmi AS, Ibrahim R (2017). Optimization of plant growth regulators for Citrus suhuiensis callus induction. Asia-Pacific Journal of Molecular Biology and Biotechnology 25(1):75-81.
Fauquet C, Fargette D, Munihor C (1990). African Cassava Mosaic Virus: Etiology, Epidemiology, and Control. Plant Disease 74(6):404-411.
Faye A, Sagna M, Kane PMD, Sane D (2015). Effects of different hormones on organogenesis *in vitro* of some varieties of cassava (*Manihot esculenta* Crantz) grown in Senegal. African Journal of Plant Science 9(8):305-312.
Feitosa T, Bastos JLP, Ponte LFA, Jucá TL, Campos F de A de P (2007). Somatic embryogenesis in cassava genotypes from the northeast of Brazil. Brazilian Archives of Biology and Technology 50(2):201-206.
Fletcher EKA, Amoako TNE, Twumasi P (2011). Effect of 2, 4-D, explants type and cultivar on the callogenesis expression of cassava (*Manihot esculenta* Crantz) in Ghana. African Journal of Biotechnology 10(46):9396-9401.
Fondong VN (2017). The search for resistance to cassava mosaic geminiviruses: how much we have accomplished, and what lies ahead. Frontiers in plant science 8:408.
Guma TB, Jane K, Justus O, Kariuki PN (2015). Standardization of *in vitro* sterilization and callus induction protocol for leaf explants of anchote: *Coccinia abyssinica*. International Journal of Research and Development in Pharmacy and Life Sciences 4(2):1427-1433.
Hahn SK, Isoba JCG, Ikotun T (1989). Resistance breeding in root and tuber crops at the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria. Crop Protection 8(3):147-168.
Hess JR, Carman JG (1998). Embryogenic competence of immature wheat embryos: genotype, donor plant environment, and endogenous hormone levels. Crop Science 38(1):249-253.
Houngue JA, Zandjanakou-Tachin M, Ngalle HB, Pita JS, Cacaï GHT, Ngatat SE, Bell JM, Ahanhanzo C (2018). Evaluation of resistance to cassava mosaic disease in selected African cassava cultivars using combined molecular and greenhouse grafting tools. Physiological and Molecular Plant Pathology 105:47–53.
Isah BI, Mustapha Y, Sani LA (2018). Effect of types and concentrations of auxins on callus induction and primary somatic embryogenesis in low cyanide cassava cultivars (*Manihot esculentum* Cranz). Bayero Journal of Pure and Applied Sciences 11(1):497-501.
Jibu T, Kumar RR, Mandal AKA (2006). Metabolite profiling and characterization of somaclonal variants in tea (*Camellia spp*.) for identifying productive and quality accession. Phytochemistry 67(11):1136–1142.
Jin S, Mushke R, Zhu H, Tu L, Lin Z, Zhang Y, Zhang X (2008). Detection of somaclonal variation of cotton (*Gossypium hirsutum*) using cytogenetics, flow cytometry and molecular markers. Plant Cell Reports 27(8):1303–1316.
Jiménez VM, Bangerth F (2001). Hormonal status of maize initial explants and of the embryogenic and non-embryogenic callus cultures derived from them as related to morphogenesis in vitro. Plant Science 160(2):247-257.
Jogaiah S, Sharathchandra RG, Raj N, Vedamurthy AB, Shetty HS (2014). Development of SCAR Marker Associated with Downy Mildew Disease Resistance in Pearl Millet (*Pennisetum glaucum* L.). Molecular Biology Report 41(12):7815-24. http://dx.doi.org/10.1007/s11033-014-3675-7
Le BV, Anh BL, Soytong K, Danh ND, Hong LTA (2007). Plant regeneration of cassava (*Manihot esculenta* crantz) plants. Journal of Agricultural Technology 3(1):121–127.
Leva AR, Petruccelli R, Rinaldi LMR (2012). Somaclonal variation in tissue culture: a case study with olive. Recent Advances in Plant *in vitro* Culture 7:123-150.
Lin CS, Lin CC, Chang WC (2004). Effect of thidiazuron on vegetative tissue-derived somatic embryogenesis and flowering of bamboo *Bambusa edulis*. Plant Cell, Tissue and Organ Culture 76(1):75-82.
Lubabali HA (2015). Direct Somatic Embryogenesis of Selected Commercial Coffea *Arabica L*. Varieties in Kenya. Msc Dissertation, University of JKUAT, Kenya, pp. 1-77
Ma G, Xu Q (2002). Induction of somatic embryogenesis and adventitious shoots from immature leaves of cassava. Plant Cell, Tissue and Organ Culture 70(3):281-288.
MAEP: Ministère de l'Agriculture de l'Elevage et de la Pêche (2013). Evolution de réalisations des principales cultures par commune (Période: 1998-2010). MAEP, Benin.
Magaia HE (2015). Assessment and induction of variability through in vitro mutagenesis in Cassava (*Manihot esculenta*, Crantz). College of Horticulture, Vellanikkara.

Mahendran G, Bai VN (2016). Direct somatic embryogenesis of *Malaxis densiflora* (A. Rich.) Kuntze. Journal of Genetic Engineering and Biotechnology 14(1):77-81.

Mathews H, Schopke C, Carcamo R, Chavarriaga P, Fauquet C, Beachy RN (1993). Improvement of somatic embryogenesis and plant recovery in cassava. Plant Cell Reports 12(6):328-333.

Miché L, Balandreau J (2001). Effects of rice seed surface sterilization with hypochlorite on inoculated *Burkholderia vietnamiensis*. Applied and Environmental Microbiology 67(7):3046-3052.

Mithila J, Hall J, Victor JMR, Saxena P (2003). Thidiazuron induces shoot organogenesis at low concentrations and somatic embryogenesis at high concentrations on leaf and petiole explants of African violet (*Saintpaulia ionantha* Wendl.). Plant Cell Reports 21(5):408-14.

Mongomake K, Doungous O, Khatabi B, Fondong VN (2015). Somatic embryogenesis and plant regeneration of cassava (*Manihot esculenta* Crantz) landraces from Cameroon. SpringerPlus 4:477.

Mose W, Indrianto A, Purwantoro A, Semiarti E (2017). The influence of thidiazuron on direct somatic embryo formation from various types of explant in *Phalaenopsis amabilis* (L.) blume orchid. HAYATI Journal of Biosciences 24(4):201–205.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia Plantarum 15(3):473-497.

Okogbenin E, Egesi C, Olasanmi B, Mba C, Kahya S, Hurtado P, Marín JA, Gómez H, De Vicente MC, Fregene MA (2008). SSR analysis of F1 intra specific crosses of cassava for identification of new sources of CMD resistance in African germplasm. Centro Internacional de Agricultura Tropical (CIAT), Cali, CO. https://hdl.handle.net/10568/58098

Omingo DO, Omondi CO, Cheserek J, Runo S, Okun D (2017). Diversity analysis of selected coffee genotypes using microsatellites and random amplified polymorphic DNA in Kenya. International Journal of Biotechnology of Food Sciences 5(1):1–9.

Onwubiku IOI (2007). SMicropropagation of Cassava {*Manihot esculantum* Crantz) Using Different Concentrations of Benzyaminiopurine (BAP). Journal of Engineering and Applied Sciences 2(7):1229-1231.

Osorio M, Gámez E, Molina S, Infante D (2012). Evaluation of cassava plants generated by somatic embryogenesis at different stages of development using molecular markers. Electronic Journal of Biotechnology 15(4):1-11.

Pajević S, Vasić D, Sekulić P (2004). Biochemical characteristics and nutrient content of the callus of sunflower inbred lines/características bioquímicas y el contenido de nutrientes en los callos de las líneas consanguíneas (inbred) de girasol/caractéristiques biochimiques et contenu de nut. Helia 27(41):143-150.

Paran I, Michelmore RW (1993) Development of Reliable PCR-Based Markers Linked to Downy Mildew Resistance Genes in Lettuce. Theoretical and Applied Genetics, 85(8):985-993. http://dx.doi.org/10.1007/BF00215038.

Park YH, Kim TH, Lee HS, Kim KM, Sohn JK (2010). Morphological and Progeny Variations in Somaclonal Mutants of'Ilpum'(*Oryza sativa* L.). Korean Journal of Breeding Science 42(4):413-418.

Phua QY, Chin CK, Asri ZRM, Lam DYA, Subramaniam S, Chew BL (2016). The callugenic effects of 2, 4-dichlorophenoxy acetic acid (2, 4-D) on leaf explants of sabah snake grass (*Clinacanthus nutans*). Pakistan Journal of Botany 4(2)8:561–566.

Preece JE (1987). Treatment of the stock plant with plant growth regulators to improve propagation success. HortScience (USA). http://agris.fao.org/agris-search/search.do?recordID=US880555388

Radhakrishnan R, Ranjitha Kumari B (2008). Morphological and agronomic evaluation of tissue culture derived Indian soybean plants. Acta Agriculturae Slovenica 91(2):391-396

Rajeswari S, Krishnamurthi M, Anand SP, Kumar ST (2009). Performance of somaclones developed from intergeneric hybrids of sugarcane. Sugar Tech 11(3):258-261.

Roels S, Escalona M, Cejas I, Noceda C, Rodriguez R, Canal MJ, Sandoval J, Debergh P (2005). Optimization of plantain (Musa AAB) micropropagation by temporary immersion system. Plant Cell, Tissue and Organ Culture 82(1):57-66.

Sasaki K, Shimomura K, Kamada H, Harada H (1994). IAA metabolism in embryogenic and non-embryogenic carrot cells. Plant and Cell Physiology 35(8):1159-1164.

Sharma N, Gauchan DP, Dhakal A, Luitel A, Shakya S, Shakya R (2015). Establishment of Regenerative Callus, Cell Suspension System and Molecular Characterization of Stevia Rebaudiana Bertoni for the Production of Stevioside in *In Vitro*. International Journal for Research in Applied Science and Engineering Technology 3(8):133-144.

Srangsam A, Kanchanapoom K (2003). Thidiazuron Induced Plant Regeneration in Callus Culture of Triploid Banana (*Musa* sp.) Gros Michel, AAA Group. Songklanakarin Journal of Science and Technology 25(6):689–696.

Sun QB, Li LF, Li Y, Wu GJ, Ge XJ (2008). SSR and AFLP markers reveal low genetic diversity in the biofuel plant *Jatropha curcas* in China. Crop Science 48(5):1865-1871.

Vidal ÁM, de Carvalho Costa MAP, da Silva Souza A, de Almeida WAB, Souza FVD (2014). *In vitro* regeneration and morphogenesis of somatic embryos of cassava. Revista Ciencia Agronomica 45(3):558–565.

Zhang W, Liping Y, Shasha WEI, Deng Z, Jianping YI, Renqi WU, Chen Q (2013) RAPD Marker Conversion into a SCAR Marker for Rapid Identification of Johnsongrass [*Sorghum halepense* (L.) Pers.]. Notulae Botanicae Horti Agrobotanici Cluj-Napoca 41(1):306-312.