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Response of four cultivars of cassava (*Manihot esculenta* Crantz) plantlets free of cassava mosaic virus to micropropagation in different media

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The aim of this study was to identify the best medium for the micropropagation of four cultivars (*Six-mois*, *M61/061*, *Yalipe* and *Rendre*) of cassava (*Manihot esculenta, Crantz*) plantlets free of mosaic virus. The effect of media on the morphogenesis *in vitro* was studied using different growth regulators combinations, added to MS medium, M0: MS without growth regulator; M1: MS+NAA (0.02 mg/l) ; M2 : MS+BAP (0.05 mg/l); M3: MS+NAA (0.02 mg/l) + BAP (0.05 mg/l); M4: MS+NAA (0.02 mg/l) + BAP (0.05 mg/l) + GA_3 (0.02 mg/l). The percentage of bud enlargement was determined after one week; while plantlets height, number of nodes, number of leaves, number of roots and Roots length were determined after eight weeks. The results show that the percentage of bud enlargement ranged from 78 to 100% in all the mediums, M1 and M3 induced the highest percentages of bud induction. The highest plantlet was observed in the medium M4 with cultivar *Yalipe* (5.28 cm), whereas the weakest was observed in the medium M1 with cultivar M61.033 (1.68 cm). The highest number of nodes was observed in the medium M4 with cultivar *M61/033* (2.73), whereas the weakest was observed in M4 with cultivar *Rendre* (1.26). The highest number of leaves was obtained in M4 with cultivar *M61/033* (4.74), whereas the weakest was observed in M3 with cultivar *Six-mois* (*2*.00). Medium M1 induced the highest number of roots (3.47) with cultivar *Yalipe,* whereas the weakest was observed in M2 with M61/033 (0.10). The highest root length was observed in the medium, M1 with cultivar *Rendre* (4.72 cm), whereas the weakest was observed in M0 with cultivar *M61/033* (1.68 cm).

Key words: Cassava, morphogenesis *in vitro*, plant acclimatization, growth regulators.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is a vital source of calories for approximately 500 million people living in developing countries. These roots are rich in carbohydrates and have been used mainly in the production of flour for human consumption in developing countries, where calorific deficiencies and malnutrition

are widespread (Olsen and Schaal, 1999). It is a staple food for approximately 500 million people in about 105 countries, providing as much as a third of daily calorie intake (FAO, 2008a, b). Thus, in the developing world, cassava is among the top four most important crops (including rice, sugarcane and maize) with an overall yield in 2009 estimated at 241 million tons. Africa, where cassava is grown primarily for food, is the largest producer with yields estimated to exceed 160 million tons per year (FAO, 2008b). In Asia and South-East Asia, the crop is grown mainly for animal feeding and industrial purposes (for example, sweeteners, acids and alcohols), with increasing interest in developing cassava for biofuel (Balat and Balat, 2009; Schmitz and Kavallari, 2009).

Cassava was introduced in the Central African Republic in 1850 (Tisserant, 1953). It has become the first food crop in this country, with 600.000T/year of dry cassava, very far from maize production with 60 000T/HA. Despite these assets, the production of cassava is generally second-rate with current yields barely averaging 20% of those obtained under optimal conditions, particularly in Africa (El-Sharkawy, 2006; Fermont et al., 2009). The importance of cassava and the enormous potential for improvement therefore makes it a target crop for famine research to achieve the United Nations Millennium Development Goals (UN, 2010). Although, cassava is an important food crop in the tropical regions, it has been largely ignored by agricultural scientists. The research and input into cassava breeding are minimal, considering its importance. Conventionally, cassava is vegetatively propagated through stem cuttings, and its growth cycle is longer than 10 months. However, its production is severely limited by a wide variety of viral and bacterial diseases. Recent advances in genetic engineering of plants hold considerable potential to improve resistance as well as other agronomic characteristics of cassava. A prerequisite for the application of these methods is the availability of efficient *in vitro* plant regeneration systems for this crop. Shoot meristem culture techniques were developed 30 years ago for cassava (Kartha and Gamborg, 1975) and are being used for the production of virus-free plants of elite genotypes (Guohua and Qiusheng, 2002; Mabanza et al., 1995). A method of producing multiple shoots from axillary buds and from bud-derived meristems has been previously reported on cassava in previous research on freeing cassava plant material using tissue culture (Yandia et al., 2015). These results have shown that the successful cleaning of plant material by tissue culture depends on tissue type, genotype and growth regulators.

The success of *in vitro* plant cell culture depends on several factors which are mainly the genotype of donor plant (Arzani and Mirodjagh, 1999; Schween and Schwenkel, 2003; Gandonou et al., 2005; Rahman et al., 2015), the age of the explant (Campbell, 2000; Delporte et al, 2001) and the culture medium composition (Murashige and Skoog, 1962; Santana et al., 2009; Ahanhanzo et al., 2008). The influence of growth regulators on plant varied according to the genotype and the type of explants (Ahanhanzo et al., 2010; Glato et al., 2014). The success of plantlets acclimatization depends on the best regeneration of plants *in vitro*, mainly the root formation (Yandia et al., 2015).

The purpose of this study was to improve cassava *in vitro* propagation with the purpose of identifying the best medium for *in vitro* morphogenesis of African cassava mosaic virus (ACMV) free plantlets of four cassava cultivars.

MATERIALS AND METHODS

The ACMV free plantlets confirmed by PCR, produced *in vitro* by meristem culture combined with thermotherapy from four cassava cultivars (*Six-mois*, *M61/033*, *Yalipe* and *Rendre*) (Yandia et al., 2015), were utilized for the study.

Effect of different media on plantlets micropropagation

Nodal explants of about 2 cm from *in vitro* shoot cultures tested free of ACMV, (three to five weeks old) were cut and placed vertically on Murashige and Skoog (1962) salt medium supplemented with sucrose, 3%; phytagel, 0.3%; myo-inositol, 100 mg/l; $CuSO_4$, 2 µM; Thyamin-HCl, 1 mg/l; Piridoxin-HCl, 1.5 mg/l; nicotinic acid, 1.5 mg/l; glycine, 2 mg/l. Murashige and Skoog salt medium with vitamin was supplemented with different types of growth regulators. The control medium (M0) was MS salt medium without any growth regulator; medium M1 was supplemented with α-naphtaleneacetic acid (NAA) (0.02 mg/l); medium M2 was supplemented with benzyl-amino-purin (BAP) (0.05 mg/l); medium M3 was supplemented with NAA (0.02 mg/l) + BAP (0.05 mg/l); and medium M4 was supplemented with NAA (0.02 mg/l) + BAP (0.05 mg/l) + GA_3 (0.02 mg/l), GA_3 (gibberellic-3-acid). Media were dispensed, 15 ml per tube in 12 cm x 2.5 cm test tubes Duran. The tubes were capped and autoclaved for 20 min at 121°C. The cultures were illuminated 16 h per day with 62 mmol/m^2/S light intensity and exposed to 28°C. Twenty replicated tubes were used in each treatment. To evaluate cultivars' response to micro-propagation in each medium, six parameters were used:

1. The percentage of bud enlargement was determined after one week of culture and defined as: (number of nodal explants forming shoot/total number of nodal explants cultured) x 100.
2. The plantlet height, the number of nodes per plant, the number of leaves per plant, the number of roots per plant and the roots length were determined after eight weeks of culture.

Plant acclimatization

Complete plantlets produced *in vitro* were removed from the culture

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Table 1. Effect of different combinations of growth regulators on bud enlargement in four cassava genotypes.

Cultivar	Culture media				
	M0	**M1**	**M2**	**M3**	**M4**
M61/033	100	100	84	100	89
Yalipe	100	100	100	99	94
Rendre	89	100	100	100	89
Six-mois	78	89	89	100	94

M0: MS+0; M1: MS+NAA; M2: MS+NAA+BAP; M3: MS+NAA+BAP+GA_3; M4: MS+BAP.

medium and the roots were washed to remove the agar. Then, the plantlets were transferred into pots containing organic soil mixed with garden soil (1:1) and placed in the net house under controlled conditions with 75% shading and temperature at 28°C. To maintain humidity, the plants were watered periodically twice a day. Observations were recorded with regards to the percentage of rooted and acclimatized plants that survived the experiment.

Statistical analysis

The analysis of the main effect of the combination of growth regulators was based on one way analysis of variance (ANOVA); all statistical analyses were performed by R3.2.4. Tukey test was used to classify the means.

RESULTS

Bud enlargement

After one week culture, the percentage of bud enlargement ranged from 78 to 100% (Table 1). However, the percentages varied according to the medium and the cultivar. In medium M0, the percentages of bud enlargement were 100, 89 and 78%, respectively for cultivars *M61/033, Yalipe*, *Rendre* and *Six-mois*. In medium M1, these percentages were 100% for cultivars *M61/033*, *Yalipe* and *Six-mois,* whereas it was 89% for cultivar *Rendre.* In medium M2, these percentages were 100% for cultivars *Rendre* and *Yalipe*; 89% for *Six-mois* and 84% for *M61/033.* In medium M3, most of the cultivars showed a percentage of bud enlargement of 100%. In medium M4, the percentages of bud enlargement were 94% for *Six-mois* and *Yalipe;* 89% for cultivars *M61/033* and *Rendre*. However, cultivar *Yalipe* presented the highest percentage of bud enlargement in the medium M3. For the other three cultivars, the response depended on the culture medium used.

Plantlet height

After eight weeks, plantlets height ranged from 1.68 to 5.28 cm, showing a significant variation in this parameter (Figure 1). The highest plant height was observed in the medium M4 with cultivar *Yalipe* (5.28 cm), whereas the weakest height was observed in the medium M1 with cultivar *M61.033* (1.68 cm). The response of a given cultivar depends on the culture medium used. For example, M0 was the best medium for cultivar *M61/033*, whereas M3 and M4 were the best media for *Yalipe,* M0 and M2 were the best media for cultivar *Rendre.* Thus, there is a significant difference among cultivars in a given medium and among media for a given cultivar.

Roots length

After eight weeks, the length of roots ranged from 1.68 to 4.72 cm, whatever the medium, and the cultivar showed a root length variation (Figure 2). The highest roots length 4.72 cm was recorded for cultivar *Rendre* in the medium M1 and the lowest length 1.68 cm was observed in medium M2 for cultivar *M61/033*. The response of a given cultivar depends on the culture medium used. For example, media M0 and M1 were the best for cultivars *M61/033* and *Six-mois,* whereas M1 and M3 were the best media for *Yalipe*. Thus, there is a significant difference among media and a given cultivar.

Leaves number

After eight weeks, the leaves number ranged from 2.00 to 4.74 whatever medium and the cultivars showing the variation of the leaves number (Table 2). The highest leaves number (4.74) was recorded for cultivar M61/033 in medium M4 and the lowest leaf number (2.00) was observed in medium M3 for cultivar *Six-mois*. The response of a given cultivar depends on the culture medium used. For example, medium M4 was the best medium for cultivar *M61/033,* whereas, medium M2 was the best for *Six-mois* and then M4 was the best medium for cultivar *Yalipe*. Thus, there is a significant difference among cultivar in a given medium and among media for a given cultivar.

Roots number

After eight weeks, the roots number ranged from 0.1 to 3.47 whatever the medium and the cultivar, showing a root number variation (Table 2). The highest roots number (3 .47) was recorded for cultivar *Yalipe* in the medium M0 and the lowest number (0.1) was observed in medium M2 for cultivar M61/033. The response of a given cultivar depends on the culture medium used. For example, medium M1 was the best for cultivar *M61/033,* whereas medium M4 was the best for *Six-mois*; M0 was the best medium for *Yalipe*. Thus, there is a significant difference among cultivars in a given medium and among media for a given cultivar.

Number of nodes

After eight weeks, the nodes number ranged from 1.26 to

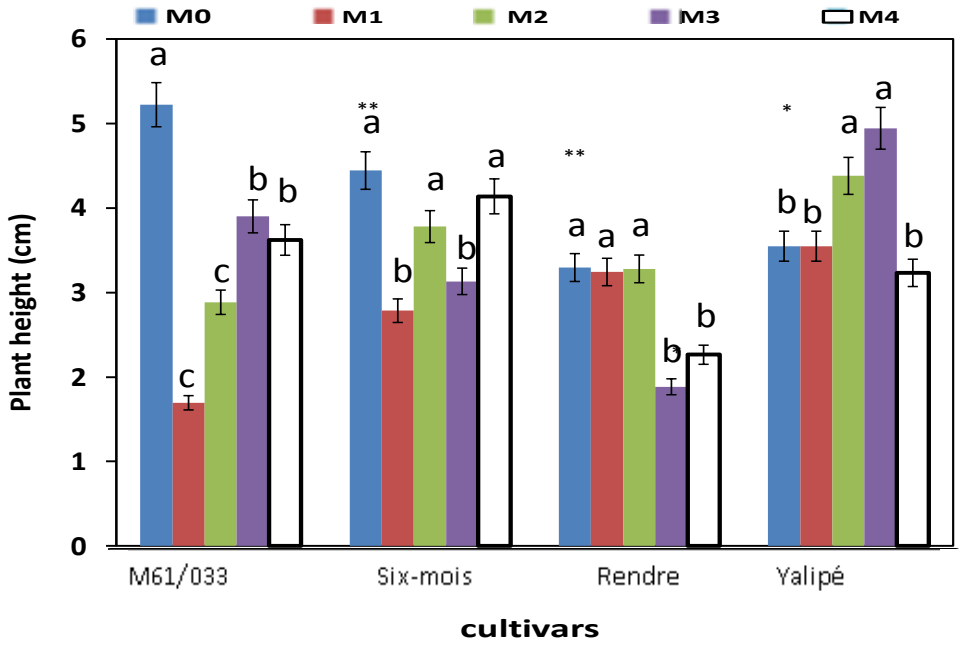

Figure 1. Effect of different combinations of growth regulators on plant height of four cultivars of cassava. Means with different letters for each cultivar are significantly different (P<0.05). Vertical bars are standard errors.

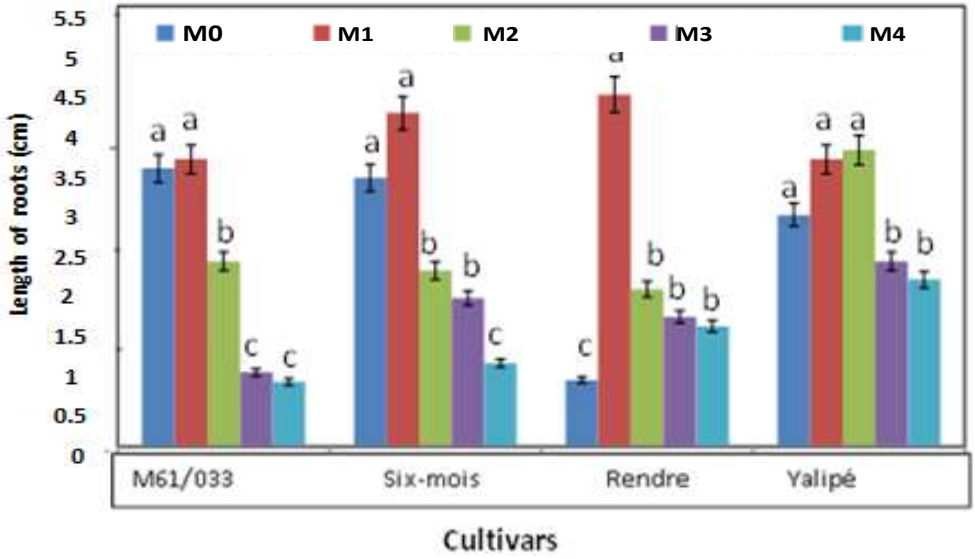

Figure 2. Effect of different combinations of growth regulators on length of roots for four cultivars of cassava. Means with different letters for each cultivar are significantly different (P<0.05). Vertical bars are standard errors.

2.73 in whichever medium and the cultivar, showing nodes number variation (Table 2). The highest nodes number 2.73 was recorded for cultivar *M61/033* in medium M2 and the lowest number 1.26 was observed in medium M3 for cultivar *Rendre*. The response of a given cultivar depends on the culture medium used. For example, medium M4 was the best medium for cultivar *M61/033,* whereas, medium M0 was the best medium for *Six-mois*; M1 was the best medium for *Yalipe*. Thus, there is a significant difference among cultivar in a given

Table 2. Effect of different combinations of growth regulators on number of roots, number of nodes and number of leaves of four cultivars of cassava.

Cultivar	Culture media	Number of roots	Number of nodes	Number of leaves
	M0	0.84 ± 0.06[b]	2.53±0.68[ab]	3.84±0.98[ab]
	M1	3.21 ± 0.27[a]	1.84±0.01[b]	2.84±0.42[b]
M61/033	M3	0.31 ± 0.058[b]	2.35±0.05[ab]	3.00±0.37[b]
	M4	0.31 ± 0.09[b]	**2.73±0.15[a]**	4.74±1.09[a]
	M2	0.10 ± 0.04[c]	2.68±0.73[a]	2.52±0.02[c]
	M0	0.42 ± 0.06[a]	2.57±0.50[a]	2.42±0.83[a]
	M1	0.53 ± 0.06[a]	1.78±0.35[a]	2.68±0.20[a]
Six-mois	M3	0.48 ± 0.08[a]	2.00±0.10[a]	2.00±0.20[b]
	M4	0.58 ± 0.07[a]	2.21±0.18[a]	2.26±0.52[a]
	M2	0.21 ± 0.08[a]	2.10±0.10[a]	2.72±0.35[a]
	M0	0.52 ± 0.07[a]	1.94±0.31[ab]	3.36±0.80[a]
	M1	0.53 ± 0.06[a]	1.78±0.78[ab]	2.21±0.20[c]
Rendre	M3	0.26 ± 0.04[a]	1.26±0.06[b]	2.72±0.55[b]
	M4	0.26 ± 0.05[a]	2.21±0.13[a]	3.05±0.54[a]
	M2	0.26 ± 0.05[a]	2.10±0.14[ab]	2.89±0.48[a]
	M0	3.47 ± 0.07[a]	2.00±0.10[b]	2.89±0.48[a]
	M1	0.31 ± 0.04[b]	2.72±0.36[a]	3.36±0.46[a]
Yalipe	M3	0.42 ± 0.081[b]	2.63±0.30[a]	3.26±0.72[a]
	M4	0.11 ± 0.03[c]	2.21±0.41[b]	3.31±0.67[a]
	M2	0.36 ± 0.06[b]	2.72±0.18[a]	3.52±0.42[a]

Means (± standard error) with different letters for each cultivar are significantly different (P<0.05). M0: MS+0; M1: MS+NAA; M2: MS+NAA+BAP; M3: MS+NAA+BAP+GA_3; M4: MS+BAP.

medium and among media for a given cultivar (Figure 3a).

Plants acclimatization

The acclimatization of rooted plants in *ex vitro* conditions was carried out with the plants bearing well-developed roots transferred to small pots containing soil mixtures (organic soil mixed with garden soil 1:1). They were maintained at about 70% relative humidity in the screen house with 75% shading (Figure 3b). Acclimatized plants were similar to initial plants (Figure 3c).

DISCUSSION

The four cultivars of cassava responded differently according to the medium and the growth parameter considered. Significant differences were observed among cultivars in their response to bud enlargement, plantlet height, length root, number of leaves, number of roots and number of nodes *in vitro*.

The media, M1 and M3 induced the highest bud enlargement percentage (100%) in three of the four cultivars evaluated (*M61/033*, *Rendre* and *Six-mois* or *Yalipe)*. These media might be considered as the best media for bud enlargement for the four cultivars. The ability of cassava genotypes to regenerate plant is influenced by the genotype as well as the type of growth regulators (BAP, NAA and GA_3). Similar results were reported by Atehnkeng et al. (2006), showing genotypic variation in plantlet of other cassava cultivars from Africa, South America and Asia. Consistent with this result, Farsi and Zolali (2011) reported that the percentage of shoot regeneration in *Artemisia* was highest with a combination of BAP and NAA, both at the concentration of 0.5 mg/L.

The highest plant height was observed in the medium, M4 with cultivar *Yalipe* (5.28 cm), whereas the least height was observed in medium M1 with cultivar M61.033 (1.68 cm), this result confirmed, Amiri et al. (2011) research, where the maximum shoot regeneration and maximum number of regenerated shoots in *Datura stramonium* were obtained in the treatment containing 2 mg/L BAP + 1 mg/l NAA.

The media, M3, M2 and M0 induced the highest length of roots for cultivars *Yalipe, Rendre, Six-mois* whereas *M61/033* showed the lowest height root in M2 medium. These results confirmed Malaurie et al. (1985) research

Figure 3. a) Vitroplant of cassava cultivar Yalipe on different media; **b and c)** acclimatized plants.

where significant difference of auxin and cytokinin ratio on organogenesis was shown. In fact, a higher concentration of auxin than cytokinin induced root organogenesis and a higher concentration of cytokinin than auxin induced stem organogenesis. Whereas, the equal concentration of auxin and cytokinin induced callogenesis. So far, Ahanhanzo et al. (2008) did not obtain callus neither with MS+ NAA (0.5 mg/l) + BAP (0.5 mg/l), nor with MS+NAA (0.5 mg/l) + KIN (0.5 mg/l) with cassava cultivars RB 89509, BEN 86052 and TMS 30572.

Media M4 and M0 induced the highest number of nodes with genotypes *M61/033* and *Yalipe,* whereas the least number of nodes was recorded in the medium M4 with cultivar M61/033. Media M1, M4 and M0 induced the highest number of leaves for the genotypes *M61/033*, *Six-mois* and *Yalipe*, whereas, the least number of leaves was observed in medium M3 with cultivar *Six-mois*. This result showed that the combination of BAP and NAA, promoted the proliferation of shoots as compared to the growth regulators applied singly. The importance of plant growth regulators on shoot propagation has been highlighted in various studies (Debiasi et al., 2007). This result confirmed the research by Mabanza and Mingui. (1998), dealing with IITA genotypes introduced by national program (Togo, Benin, Democratic Republic of Congo); they observed that MS+NAA, as well as MS+NAA+BAP induced the highest number of leaves.

The medium M1 was considered as the best medium for root length for all the four cultivars: *M61/033*, *Six-mois*, *Rendre* and *Yalipe* because of this responding rate. The results confirm the results of Malaurie et al. (1995), Kbiach et al. (2002) and Ndoumou et al. (2004)**,** where they reported that auxin induced more roots growth than cytokinin. The inhibition effect of cytokin on root development was caused by the combination of growth regulators as confirmed by Ondo et al. (2007). Significant difference was observed among genotypes and culture

medium in this research. The effect of cytokinin on organogenesis was confirmed by Ndoumou et al. (2004) on *Irvingia gabonensis* where cytokinin from 2 to 3 mg/l induced 3.1 to 3.5 cm of plant height. Akbas et al. (2009) showed high performance of *Amygdalus communis* L in medium supplemented with 0.5 mg/l kinetin, whereas Ondo et al. (2007) showed through their research on *Dioscorea cayenensis-Dioscorea rotundata*, the weak growth with 2 mg/l of kinetin. The effect of kinetin on plant growth depended not only on the concentration of growth regulators but also on the genotype and size of explants.

These results demonstrate that the response of the four cultivars plantlets to micro-propagation depended on the medium used and the growth parameter taken into account.

Conclusion

The present paper aimed at studying the effect of growth regulators combination on plant formation of four cassava cultivars (*M61/033, Rendre, Six-mois* and *Yalipe*). Out of 869 nodal cuttings from plantlets cultured, 794 plantlets were regenerated *in vitro,* 363 plants free of African Cassava Mosaic Virus free were obtained after acclimatization and transferred to experimental field.

The results show that the response of the four cultivars plantlets to micro-propagation depended on the medium used and the growth parameter taken into account. This research can be used as guidelines for improving cassava micro-propagation.

Conflict of Interests

The authors have not declared any conflict of interests.

Abbreviations

MS, Murashige ankoog; **NAA,** naphtaleneacetic acid; **BAP,** 6-benzylaminopurin; **GA_3,** gibberellic acid.

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