

Optimization of In-Vitro Propagation of Cassava (*Manihot esculenta* Crantz) Genotypes

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Abstract

Three cassava genotypes (92/0326, 95/0289 and Tropical Manioc Selection-International Institute of Tropical Agriculture-30572 (I-30572) were examined in sixteen (16) different culture media with different concentration of growth hormones, Naphthaleneacetic acid (NAA-auxins) and 6 benzyl-aminopurine (BAP-cytokinins). The responses of the explants were evaluated by nodal length, survival, leaf and root formation, plant height, root and leaf number. Additive Main Effect and Multiplicative Interaction (AMMI) analysis was used to reveal the pattern of interaction of genotype by media-environment. The analysis of variance showed significant ($P < 0.05$) genotype performance with significant genotype growth differences among the different media. Highest percentage survival was recorded for 95/0289 (98%). Medium composition 1 (Murashige and Skoog (MS): 4.43 g/l; NAA: 0.01 mg/l; BAP: 0.05 mg/l) and 4 (MS: 2.22 g/l; NAA: 0.02 mg/l; BAP: 0.10 mg/l) were observed to give 100% plantlets survival. Genotype 92/0326 had the highest mean plant height (1.49 cm) and mean root number (0.82). As the best performer, full strength MS is recommended in a semi-solid medium. However, the cost-benefit and availability has to be considered. These observations, attributed to the different genetic structure and contrasting levels of hormones used, can be useful for formulating protocols that are adequately potent for micropropagation of recalcitrant cassava genotypes.

Keywords: Cassava, *Manihot esculenta*, recalcitrant, protocol, micropropagation

1. Introduction

Cassava is a crop of great importance in the tropics because it is a readily available staple food, ease of cultivation and the ability to be transformed into different forms and stored as food for several years (Nassar et al., 2009; Lebot, 2009). It is a valuable source of calories especially in countries where malnutrition is widely spread, and ranks fourth as a source of energy, after rice, sugar cane and maize (Scott et al., 2000; Ceballos et al., 2004). It is predominantly grown by subsistence and commercial farmers in the sub-Saharan countries of Africa like Nigeria, Ghana, Burundi, Democratic Republic of Congo, Cameroon, Congo and host of others (Oyewole, 2002; Kumah, 2007). In 2000, 16.8 million hectares of land was planted with cassava, of which 64% was cultivated by sub-Saharan Africa. Out of the world average tuber yield of 10.2 tonnes per hectare Sudanese yield was 1.8 tonnes per hectare, Barbados 27.3 tonnes per hectare and Nigeria 10.6 tonnes per hectare (FAO, 2010). The leaves and tender shoots are also consumed in many part of Africa as vegetables to provide protein, vitamin A and B (IITA, 1982).

Cassava is a vegetatively propagated crop and its multiplication is generally tedious and slow (Taye, 1998; Santana et al., 2009). Inadequate high yielding varieties and susceptibility to diseases were identified as the key challenge to the expansion of cultivation of the crop. These were compounded by the bulkiness and high distribution costs, low multiplication rates and poor storage quality of planting materials (Mahungu et al., 2004; Escobar et al., 2006). Therefore, any effort geared towards removing or reducing these constraints will be worthwhile.

In-vitro techniques have been employed to produce high yielding varieties, disease-free and resistant planting materials, even from infected mother plants (Acedo, 2006). Production of a cultivar that will retain its distinguishing characteristics when bred with the same cultivar planting materials can also be achieved through in-vitro propagation. This propagation method can be carried out regardless of growing season, enable the year-round availability of planting materials and thus ensure the expansion of production. Tissue culture has been exploited for rapid clonal multiplication, transformation and conservation of cassava with higher yields (Garcia et

al., 1993; Jorge et al., 2000; Onuoch & Onwubiku, 2007; Staden et al., 2008). However, there is a need to develop low-cost or optimize in-vitro production of this crop. This study seeks to address the possibilities of formulating a more responsive medium for the in-vitro propagation of cassava (especially the recalcitrant lines) via single nodal cutting technique and to evaluate the response of the genotypes using liquid and semi-solid media.

2. Materials and Methods

Three cassava genotypes, 92/0326, 95/0289 and Tropical Manioc Selection-International Institute of Tropical Agriculture-30572 (I-30572), obtained from the in-vitro germplasm collection maintained at the International Institute of Tropical Agriculture (IITA), Nigeria in 2007, were used for the study. According to IITA (2005), the genotypes were of excellent yield attributes in-vivo propagation, and highly desired by farmers in Nigeria (IITA, 2005). Genotype I-30572 was selected based on its previous growth performance as one of the most responsive cassava genotypes to proliferation medium *in-vitro* while genotypes 92/0326 and 95/0289 were chosen among the recalcitrant genotypes.

The experiment was carried out at the Biotechnology Centre, IITA, Nigeria with sixteen (16) growth initiation medium supplemented with different growth regulator concentrations. Completely randomized design with three replicates was used.

2.1 Cassava Genotypes Multiplication

The selected genotypes were multiplied in the cassava multiplication medium at the Tissue Culture Laboratory of IITA (medium 1) to obtain 960 nodal cuttings per genotype, with each replicated growth medium consisting of twenty explants. Leaves were excised from the healthy plants and the stems cut into 10 cm long. The explants were washed with running tap water for five minutes to remove soil debris. The stem sections were cut at the internodes to produce nodal cuttings of about 2-3 cm and transferred into a laminar hood. Then, the cassava nodal were sterilized in 70% alcohol for 1 min and rinsed with sterile double distilled water. This was further disinfected with 1% sodium hypochlorite solution for 15 min followed by rinsing in sterile distilled water.

The phytohormone combination of varying medium composition is presented in Table 1. Medium 14 and 15 were adapted from a protocol of the International Atomic Energy Agency (IAEA) Vienna, Austria.

Table 1. Phytohormone combination

Medium	MS (g/l)	Inositol (mg/l)	Sucrose (g/l)	NAA (mg/l)	BAP (mg/l)	pH	Agar (g/l)	Gelrite (g/l)	AgNO ₃ (mg/l)
1	4.43	100	30	0.01	0.05	5.7	6	-	-
2	2.22	100	30	0.01	0.2	5.7	6	-	-
3	2.22	100	30	0.01	0.5	5.7	6	-	-
4	2.22	100	30	0.02	0.1	5.7	6	-	-
5	2.22	100	30	0.02	0.4	5.7	6	-	-
6	2.22	100	30	0.04	0.05	5.7	6	-	-
7	2.22	100	30	0.04	0.2	5.7	6	-	-
8	2.22	100	30	0.04	0.5	5.7	6	-	-
9	2.22	100	30	0.08	0.1	5.7	6	-	-
10	2.22	100	30	0.08	0.4	5.7	6	-	-
11	2.22	100	30	0.16	0.05	5.7	6	-	-
12	2.22	100	30	0.16	0.2	5.7	6	-	-
13	2.22	100	30	0.16	0.5	5.7	6	-	-
14	4.40	-	20	-	-	5.8	-	-	-
15	4.40	-	20	-	-	5.8	-	1.8	-
16	4.43	100	20	-	-	5.7	-	-	8

MS Murashige and Skoog; NAA Naphthaleneacetic acid; BAP 6 benzyl-aminopurine.

2.2 Sub Culturing

The plantlets (Plate 1a) obtained were cut in such a way that, each node from the explants was cultured per unit of the replications (Plate 1b). Aseptic sub-culturing was carried out under a Laminar-flow hood, using a sterile scalpel, sprayer and alcohol dip containing 70% ethanol, alcohol lamp (100% ethanol), blade holder and forceps. After subculture, the new cultures were sealed with parafilm, labeled and kept under incubation in the growth room with temperature maintained at $26 \pm 1^\circ\text{C}$. Growth was regularly assessed on the day of culture, then two, four and six weeks after culture.



Plate 1. Picture of freshly cultured plants (a) and meristem-derived plantlets (b)

Data were collected on nodal cutting length, culture survival (two weeks after culture), root growth, leaf formation, plant height, number of roots and leaves per plant (four and six weeks after culture). Data were analyzed using Statistical Analysis Software (SAS, 2009). Analysis of variance was carried out to determine significant differences among the genotypes and means were separated using Duncan's Multiple Range Test (DMRT). Additive Main effect and Multiplicative Interaction (AMMI) analysis was used to reveal the pattern of interaction of genotype by environment (medium).

The AMMI model is stated below:

$$Y_{ger} = \mu + \alpha_g + \beta_e + \sum_n \lambda_n Y_{gn} \delta_{en} + \rho_{ge} + \varepsilon_{ge}$$

Where,

Y_{ger} = the observed yield of g^{th} genotype in e^{th} growth environment for r^{th} replicate; μ = the grand mean; α_g = the deviation of mean of the g^{th} genotype from the grand mean m ; β_e = the deviation of mean of the e^{th} growth environment from the grand mean m ; λ_n = the singular value for the n^{th} interaction principal component axis (PCA); g_{gn} = the genotype eigenvector for n^{th} (PCA) axis; δ_{en} = the environment eigenvector values for the n^{th} PCA axis; ρ_{ge} = the residual effects; and ε_{ge} = the error term.

3. Results

Mean squares of culture survival, root and leaf formation from the three cassava genotypes is presented in Table 2. The analysis of variance revealed significant ($P \leq 0.01$) genotype and medium effect for the parameters. The result also showed significant ($P \leq 0.01$) genotype x medium interaction (GMI) for plant height, root number per plant and leaf number per plant.

Table 2. Mean squares of traits evaluated in three cassava genotypes in-vitro

Source of variation	df	Culture survival	Root formation	Leaf formation	Plant height (cm)	Number of roots per plant	Number of leaves per plant
Genotype (G)	2	0.528**	142.11**	16.99**	93.69**	22.22**	524.35**
Medium (M)	15	0.325**	2217.92**	11.74**	109.37**	96.91**	238.38**
G x M	30	0.450*	192.59*	12.56*	16.39**	39.06**	26.37**
Error	5712	0.042	4.90	0.18	0.83	1.44	2.03

*, ** significant at 5% and 1% level of probability, respectively.

Table 3 shows the mean performance of the three cassava genotypes used in the study. Among the genotypes evaluated, 95/0289 had the highest mean survival (0.98) but the least leaf formation (0.33), plant height (1.08) and leaf number per plant (1.02). However, the culture survival rate for all the genotypes was appreciable (over 90%) in all the media used. Root formation (0.23), plant height (1.49 cm) and number of roots (0.82) were significantly higher in genotype 92/0326 while genotype 1-30572 had the highest number of leaves per plant (2.05). Genotype 95/0289 had the least performance in most of the traits evaluated.

Table 3. Mean performance of three cassava genotypes evaluated for six traits invitro

Genotype	Survival	Leaf formation	Root formation	Plant height(cm)	Number of roots per plant	Number of leaves per plant
1-30572	0.95b	0.57a	0.09b	1.41b	0.70b	2.05a
92/0326	0.93c	0.55a	0.23a	1.49a	0.82a	1.70b
95/0289	0.98a	0.33b	0.11b	1.08c	0.59c	1.02c

Means with the same letter along the column are not significantly different at 1% level of probability using DMRT

The mean effect of the media (Table 4) showed that media 2, 3, 4, 6, 9, 11, 14, and 16 had similar effect on the culture survival as the medium composition (medium 1) adopted at the Tissue Laboratory, IITA for cassava in-vitro multiplication. Media 8, 12, and 13 recorded the least survival rates. Medium 15 followed by media 1 and 14 significantly stimulated root formation more than any other medium investigated, while media 1 and 14 significantly supported leaf formation (Table 4). Comparable with medium 1, medium 16 had the highest effect on plant height, media 4, 14 and 16 had the highest effect on number of roots per plant while medium 16 had the highest effect on number of leaves per plant

Table 4. Mean of the medium for survival, root and leaf formation

Medium	Culture survival	Root formation	Leaf formation	Plant height(cm)	Number of roots per plant	Number of leaves per plant
1	1.00a	0.31b	0.85a	2.69a	1.60a	3.17a
2	0.99a	0.02fg	0.73bc	1.49d	0.61bc	2.40b
3	0.96ab	0.00g	0.31fgh	1.06fg	0.17f	1.74d
4	1.00a	0.28bc	0.73bc	1.33e	1.43a	1.99c
5	0.96ab	0.00g	0.48ed	1.14f	0.19f	1.56ed
6	0.97ab	0.14ed	0.52d	1.33e	0.74b	1.03e
7	0.91bc	0.09ef	0.27fgh	0.91h	0.59bc	0.90f
8	0.87c	0.00g	0.18hi	0.91h	0.38de	0.83fg
9	0.99a	0.15ed	0.39ef	1.06fg	0.51cde	1.05f
10	0.95ab	0.02fg	0.23gh	0.94gh	0.32ef	0.93f
11	0.97a	0.14e	0.36efg	1.11f	0.54cd	1.01f
12	0.88c	0.05fg	0.28fgh	0.83h	0.14f	0.65gh
13	0.89c	0.00g	0.08i	0.85h	0.39de	0.59h
14	0.99a	0.35b	0.89a	1.79c	1.61a	2.35b
15	0.96ab	0.48a	0.66c	1.37ed	0.62bc	1.78d
16	0.98a	0.22cd	0.73bc	2.69a	1.60a	3.17a

Means with the same letter along the column are not significantly different at 1% level of probability using DMRT

AMMI analysis for plant height, number of roots per plant and number of leaves per plant of the three cassava genotypes evaluated in sixteen media (environment) showed significant differences ($P \leq 0.01$) for all the sources of variation (Table 5). The genotype by environment interaction (GEI) was partitioned into two interaction principal components axes (IPCA1 and IPCA2). Each of IPCA of the AMMI models captured 69.51% and 30.49% of the GEI sum of squares (SS) for plant height, 56.50% and 43.50% for number of roots per plant and 68.17% and 31.83% for number of leaves per plant, respectively, with very small error variance.

Table 5. AMMI analysis of variance for plant height, root number per plant and leaf number per plant of three cassava genotypes cultured in sixteen media

Source of variation	Df	Plant height	Number of roots per plant	number of leaves per plant
Treatment	47	1.57*	1.83	3.63*
Genotype	2	2.47*	0.28*	15.71*
Environment	15	3.67*	3.13*	7.53*
G x E	30	0.45*	1.28*	0.87*
IPCA1	16	0.59* (69.51)	1.36* (56.50)	1.11* (68.17)
IPCA2	14	0.30* (30.49)	1.19* (43.50)	0.59* (31.83)
Error	79	0.10	0.14	0.18

* Significant at 1% probability level.

Percentage sum of square in parenthesis.

The AMMI biplot of the first two interaction principal component axes (IPCA1 and IPCA2) for plant height, number of roots per plant and number of leaves per plant are presented in Figures 1, 2 and 3, respectively. Figure 1 showed that genotype I-30572 was the most stable across the media for number of leaves per plant while 92/0326 was the most stable genotype for number of roots per plant (Figure 2) and genotype 95/0289 for plant height (Figure 3). Figures 1, 2 and 3 also revealed that genotypes 92/0326 and I-30572 consistently had above average mean value (average over the media) for plant height, number of roots per plant and number of leaves per plant.

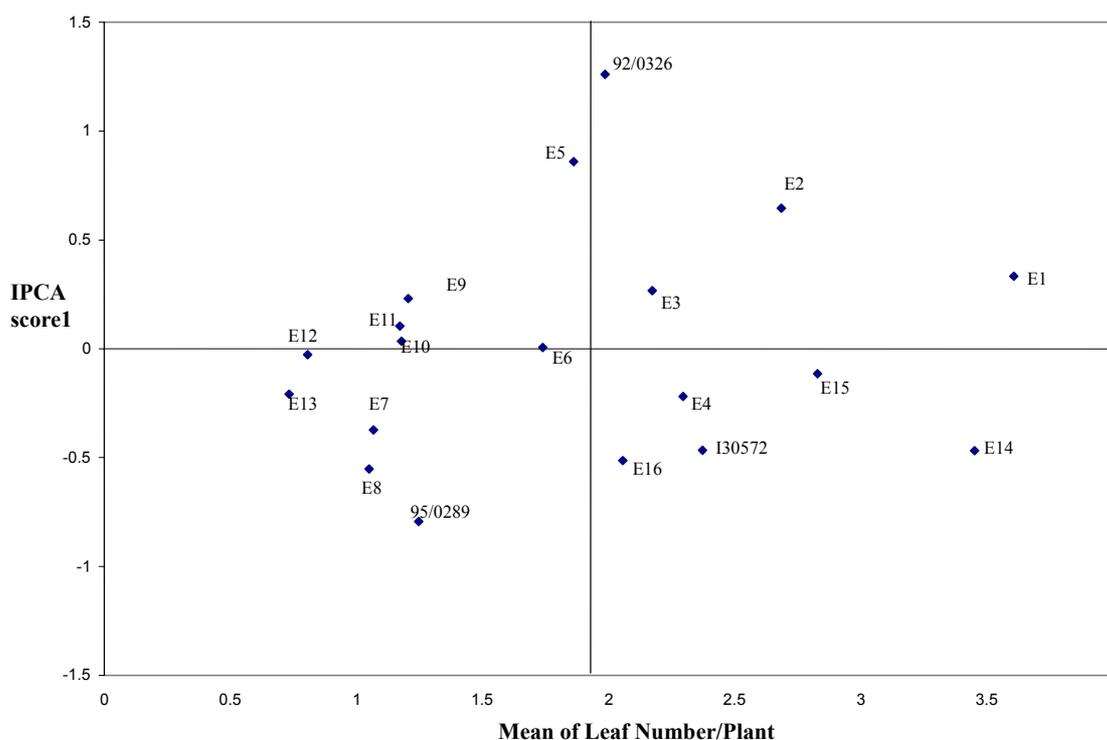


Figure 1. Additive Main effect and Multiplicative Interaction (AMMI) biplot of number of leaf per plant in three cassava genotypes in sixteen environments (media)

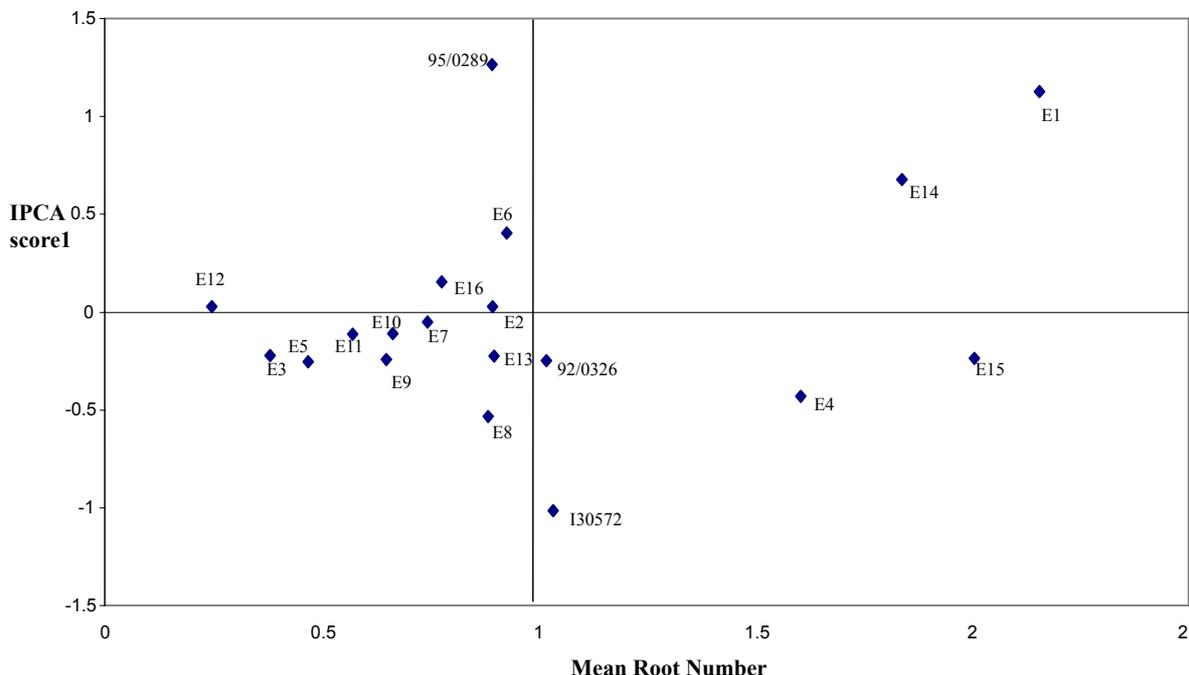


Figure 2. Additive Main effect and Multiplicative Interaction (AMMI) biplot of root number per plant in three cassava genotypes in sixteen environments (media)

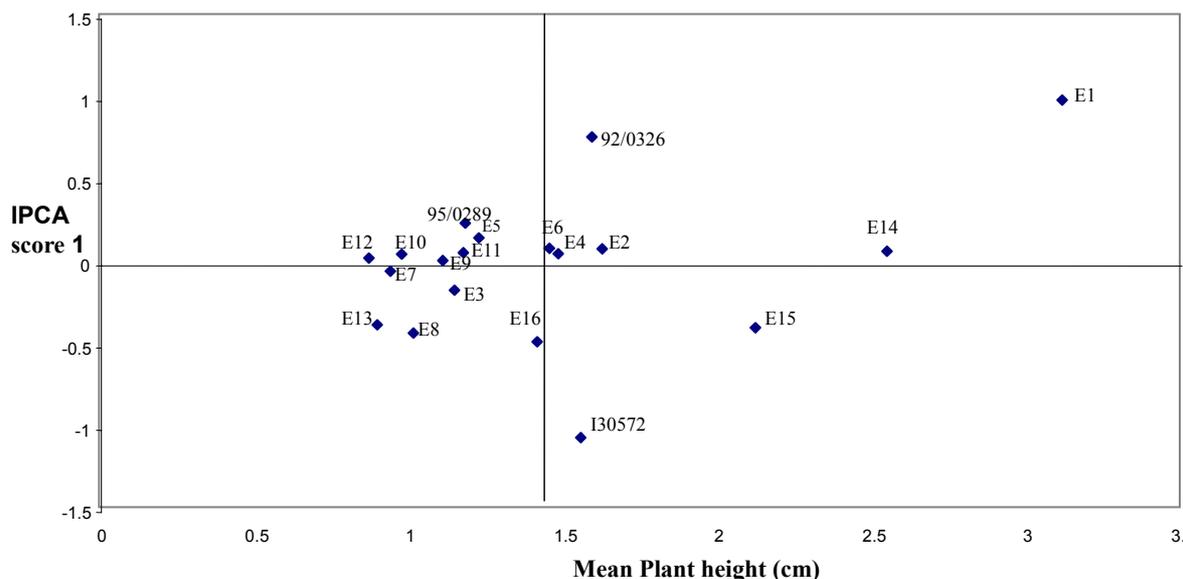


Figure 3. Additive Main effect and Multiplicative Interaction (AMMI) biplot plant height in three cassava genotypes in sixteen environments (media)

Medium 15 was observed to be the most stable environment with above average performance for number of leaves per plant (Figure 1) and number of roots per plant (Figure 2) while medium 14 was highly stable for plant height (Figure 3).

4. Discussion

Murashige and Skoog medium supplemented with different combination and concentration of NAA and BAP were evaluated in the study following the standard tissue culture sterilization techniques. The use of these growth regulators during growth initiated from meristem culture of different cassava varieties were also recommended by Razdan (2005).

In the study, the cassava genotypes responded differently to the nature and constituent of the microporpagation medium, however, the best survival rate (100%) was, generally, obtained in MS medium supplemented with low concentration of NAA and BAP hormones and high concentration of sucrose. Leaf and root formation, and plant height was well supported in these media. Culture medium formulation has been commonly used with slight modifications, depending on the crop species (Murashige & Skoog, 1962). Acedo (2006) reported a better response for rapid growth initiation from meristem culture of cassava (60-80%) with MS medium supplemented with 0.25 mg/l GA₃, 0.1 mg/l BAP and 0.2 mg/l NAA.

Following the performance of the cassava genotypes in the culture media tested, it was discovered that media 1, 2, 4, 14, 15 and 16 responded well across all the growth and developmental traits that were measured. Media 12 and 13 were the least effective among the 16 media used. This could be as a result of small amount of macro and micro nutrients and highest concentration of NAA and BAP in the media which made them unsuitable for the plantlets formation. Onuoch and Onwubiku (2007) observed that synthetic cytokinins are inhibitory to shoot growth at high concentration.

Considering the constituents of medium culture, it was discovered that liquid medium (medium 14) with normal MS strength and without growth hormones (NAA: auxins base and BAP: cytokinins base) was among the best treatments for plantlets formation. This was probably due to the improvement in nutrients uptake and avoidance of the impurities in agar. The cultures in liquid medium showed little or no callus formation which was known to inhibit shoot formation. However, the cultures in the liquid medium were observed to degenerate and turn brown (from the lower part of the shoot) over time. This may be related to the unfavourable reaction of cassava to too much water. This response is in agreement with the results of Acedo (2006). Media 15 and 16 were also among the promising media without growth hormones which were known to influence root, shoot and callus formation. These (semi-solid) media were constituted with gelrite and agar as gelling agents at different pH level. Medium 16 however, had in addition AgNO₃ which was known to support organ initiation and reduced callus formation (Hankoua, 2006; Peng et al., 2004; Tsao et al., 2002). In these media also, there was little or no callus induction, which tends to accelerate the induction of shoot and root formation.

Medium 1 with full strength MS (4.43 g/l) and growth hormone additives promoted callus formation in spite of the fact that callus formation was known to inhibit organogenesis with subsequent induction of growth and development of plantlets. This was not so for other media that had increased concentration of BAP and NAA with half strength MS with no significant effects on cassava growth. Callus induction however, was known to inhibit shoot development and hence, leaf formation an observation that is in consonance with that of Acedo (2006). This response is equally in agreement with the results of Ng et al. (1996) that reported that number of nodes was significantly higher in double strength MS medium with 0.05 mg/l BAP or 0.1 mg/l BAP, 0.01 mg/l naphthalene acetic acid (NAA) and 0.7% agar. Also, media 2 to 13 which were composed of half strength MS (2.22 g/l), with vitamins (Inositol), and varying level of growth hormones (NAA and BAP) were found to be moderate in the induction of shoot, root and leaf formation.

In comparison, media with full strength MS (4.40 - 4.43 g/l) responded better than those with half strength MS. Solid media tend to stay longer in culture than liquid medium, as there was no degeneration of plantlets that formed. The nutrients as a basal medium without hormone additive proved to be sufficient and very effective in multiplying the meristem-derived plantlets. In other words, those media with little or no callus formation with the exception of medium 1, responded better than those with callus formation. Irrespective of whether vitamin was added or not, all media with high pH (5.8) particularly media 14 and 15 in the current study were not significantly different from one another. The sucrose level of 20 - 30 g/l did not result in any difference either. Ng et al. (1996) reported similar observation. Dawit (2009) reported no significant difference between full and half strength MS medium of the same IBA concentration.

The differential response of the three cassava genotypes to the sixteen culture media tested was highly significant as revealed by Additive Main effect and Multiplicative Interaction (AMMI). To understand the nature of interactions that existed between these two factors, mean of plant height, number of leaves and roots per plant were plotted against the first Interactive Principal Component Analysis (IPCA) score. IPCA 1 was chosen because, it accounted for less noise and more variations were detected at this level. The genotype that was used as control, I-30572 was significantly different among the three cassava genotype studied for plant height, number of leaves and roots per plant. The recalcitrant genotype, 92/0326 also performed above average relative to the control genotype while genotype 95/0289 performed below average. Media 4 and 16 were found to be more suitable for genotype I-30572, for leaf number. This may be traced to the increased hormones (auxins and cytokinins) contained in medium 4 and AgNO₃ contained in medium 16, all of which was found to enhance leaf formation.

However, media 14 and 15 without the growth hormones were also found to enhance leaf formation but they did not respond better than medium 4 and 16. Medium 2 with increased hormones, was also found to enhance leaf formation in genotype 92/0326. Medium 1 and Medium 3 were also found to support leaf formation in this genotype. Leaf formation in genotype 95/0289 was better enhanced in media with half strength MS and increased hormones, particularly media 8, 7, and 13. This implies that, little amount of micro and macro nutrients (MS source) and increased concentrations of auxins and cytokinins would be required by this genotype for better leaf formation.

Also, for the stability of the media and the genotypes, it was shown that 92/0326 was stable for number of roots while 95/0289 for plant height. Environment M6, M10, M12 and M15 tend to be more stable than others but with below average performance with respect to number of leaves. However, M2, M4, M6 and M14 with above average performance were more stable than the rest for plant height.

5. Conclusions

The use of full strength MS performed better for all the traits measured than those with half strength. Also, semi-solid media better enhanced the regeneration of recalcitrant cassava and did not degenerate in culture compared to the liquid medium. In the study, substitution of agar for gelrite as a gelling agent is feasible without adverse effect on cassava development and multiplication. However, the cost-benefit implication and availability has to be considered. Following the critical assessment of the performance of the three cassava genotypes and the sixteen culture media tested, it would be better to choose media 1, 2, 4, 15, and 16 as protocols for the multiplication of recalcitrant cassava genotypes while medium 14 (liquid) could as well be considered provided it is well maintained before degeneration would set in. Overall, medium 15 (MS: 4.40 g/l; Gelrite: 1.8 g/l) is recommended as a promising medium for the future micropropagation studies of cassava owing to its stability and outstanding response to the traits evaluated.

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