In vitro seed production of three mosaic disease resistant cassava cultivars from Benin and the assessment of the resistance of the regenerated plantlets

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Abstract

Background

Cassava is a staple food for over 800 million people globally providing a cheap source of carbohydrate. However, the cultivation of cassava in the country is facing to viral diseases, particularly cassava mosaic disease (CMD) which can cause up to 95% yield losses. With aim to supply farmers demand for clean planting materials, there is need to accelerate the production of the elite cultivars by use of tissue culture in order to cope with the demand.

Methods

Nodal explants harvested from the greenhouse grown plants were sterilised using different concentrations of a commercial bleach JIK (3.85% NaOCl) and varying time intervals. Microshoots induction was evaluated using thidiazuron (TDZ), benzyl amino purine (BAP), and kinetin. Rooting was evaluated using different auxins (Naphthalene acetic acid NAA and Indole-3-butyricacid IBA). PCR-based SSR and SCAR markers were used to assess the presence of CMD2 gene in the regenerated plantlets.

Results

The highest (90%) clean explants were obtained when 20% Jik was used for 20 minutes. The best cytokinin for microshoots regeneration was found to be kinetin with optimum concentrations of 5, 10 and 20 µM for Agric-rouge, Atinwewe, and Agblehoundo respectively. Medium without growth regulators was the best for rooting the three cultivars. A survival rate of 100%, 98%, and 98% was recorded in the greenhouse for Agric-rouge, Atinwewe, and Agblehoundo respectively and the plantlets appeared to be morphologically normal. The SSR and SCAR analysis of micropropagated plants showed a profile similar to that of the mother plants indicating that the regenerated plantlets retained the CMD2 gene after passing through *in vitro* culture.

Conclusion

The nodal explants was established to be 20% of Jik (3.85% NaOCl) with an exposure time of 15 minutes. Kinetin was proved to be the best cytokinins for microshoot formation with the optimum concentration of 5, 10 and 20 µM for Agric-rouge, Atinwewe, and Agblehoundo respectively. The protocol developed during this study will be useful for mass propagation of the elite cassava cultivars.

Background

Cassava (*Manihot esculenta* Crantz) is a perennial woody shrub which belongs to the family *Euphorbiaceae*. It is a staple food for over 800 million people globally providing a cheap source of carbohydrates [1]. Cassava was introduced in West Africa from South America in 1558 (16th century) and into East Africa in the 18th century [2, 3].

Cassava is the second staple food crop after maize in Benin with 55% of farmers cultivating it [4]. However, the crop has major production constraints ranging from biotic to abiotic threats. According to Agre *et al*. [5] the most reported diseases among farmers (68%) were viral infections. These diseases have caused the yields to be much lower (15.55 tonnes per hectare per year) than the potential global yield of 90 tonnes per hectare per year [6]. This is attributed to a large extent to the devastating effects of CMD causing a heavy yield loss in cassava [7, 8]. CMD is caused by cassava mosaic begomoviruses (CMBs). To date, the most successful approach used to control these viruses has been the introgression of CMD1 which is polygenic recessive resistance locus, from wild cassava to cassava cultivars, or the use of natural resistant cassava cultivars from West African that contain CMD2 which is dominant monogenic resistance locus [9]. The control measures against CMD include rogueing (removal of infected plants), the use of virus-free planting materials and resistant varieties [10]. Recent research efforts in Benin have led to the identification of CMD2 resistant cultivars (Agric-rouge, Atinwewe, and Agblehoundo). The availability of these cultivars is a significant contribution towards the management of CMD causal viruses in Benin [8]. The propagation of these new cassava cultivars is by cuttings. This method 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.

Plant tissue culture as an important tool has been widely employed in area of agriculture, horticulture, forestry and plant breeding. It is an applied biotechnology used for mass propagation, virus elimination, secondary metabolite production and *in-vitro* cloning of plants [11–13]. Tissue culture depends on the effectiveness of the sterilization methods used on the explants prior to culture initiation. All the materials used in the plant tissue culture must be sterilized to kill the microorganisms that are present by using an appropriate sterilizing agent. Many protocols have been reported on surface sterilisation of cassava nodal explants from greenhouse. For instance, Abd-Alla *et al*. [14] reported effective sterilization of cassava nodes using Clorox (contains 5.25% NaOCl) at concentration of 20% for 15 minutes. Demeke *et al*. [15] sterilized cassava node explant from greenhouse by exposing them to 0.1% NaOCl with 1–3 drops of Tween-20 for 10 min, after initial soaking in 70% ethyl alcohol for 1 min while, Magaia, [16] reported the highest (87%) clean explants when nodal explants from greenhouse were exposed to 70% ethanol for 1 or 2 min followed by exposure to 0.05% HgCl2 for 2 min or 0.1% HgCl2 for 1 min. During the current study Jik (3.85% NaOCl) was used at different concentrations and intervals. Nodal culture is probably one of the safest methods of micropropagation because it has been shown to produce true to type plants. A range of cytokinins such as 6-benzylaminopurine (BAP), thidiazuron (TDZ), zeatin, and kinetin has been efficiently tested in cassava micropropagation to induced shoot regeneration. For instance, it has been reported that MS media supplemented with 10 mg/L BAP induced multiple-shoots with highest (25 shoots) mean number of shoot [17]. On the other hand, Mapayi *et al*. [18] reported that full MS [19] medium supplemented with NAA: 0.01 mg/L and BAP: 0.05 mg/L regenerated 100% plantlets. Kinetin also has been used at 0.75 mg/L to induce an average of 7.30 microshoots/ explant [15]. Auxins play a crucial role in rooting regenerated microshoots [11]. In cassava, rooting of *in vitro* derived microshoots has been reported by many authors. Mapayi et al. [18] reported that NAA (0.01-10 mg/L) as the most widely used and effective in cassava. Medina *et al*. [20] also reported that 0.54 mM NAA was most effective in stimulating root formation. Demeke *et al*. [15] used 0.5 mg/L NAA and reported the production of 6.14 roots within four weeks while Cacaï *et al*. [11] used 0.1 mg/L NAA and reported the production of 5.2 roots. On the other hand, Tadu, [21] reported that IAA at 0.02 mg/L and IBA at 0.04 mg/L were the best for rooting short maturing cassava genotypes and 0.06 mg/L IBA was the best for long maturing genotypes. It has been generally reported that MS medium without exogenous auxins has been proved to be best in cassava microshoots rooting [22, 23]. During the current study, IBA and NAA at different concentrations (0, 5, 10, 20 and 25 µM) were evaluated for their effectiveness to induce roots.

The plantlets derived from tissue culture may sometimes present a variation or lose CMD2- mediated resistance [24, 25]. The appropriate procedures for preventing its occurrence and the development of early detection methods are important factors for ensuring uniformity in the production of micropropagated plantlets. One of the early detection methods is the molecular detection based on the use of molecular markers, which are part of the genome, thus excludes both environmental effects and misidentifications. Microsatellite-based marker techniques such as Simple Sequence Repeat (SSR) and Sequence-Characterized Amplified Region (SCAR) markers have successfully been used in detection of CMD2 resistant gene in cassava [26, 8]. The objectives of the current work was to establish a feasible *in vitro* protocol for propagating the three resistant cassava cultivars by determining the optimal sterilization technique for cassava nodal explants, then the effect of cytokinins (TDZ, kinetin, and BAP) on microshoots induction and the effect of auxins (NAA and IBA) concentrations on microshoots rooting, and also to assess the conformity of the regenerated plantlets using SSR and SCAR markers.

Results

Effect of JIK (3.85%NaOCl) on surface sterilization of cassava nodal explants

The results of sterilization of nodal explants are presented in Table 1. The number of contaminated explants increased drastically from day 4 to day 12 following incubation on MS medium. After 12 days, explants sterilized using 20% Jik for 15 minutes gave the highest per cent (90%) clean explants while the lowest (13%) number of clean explants were obtained using 10% Jik for 15 minutes. Thus, sterilizing explants using 20% Jik for 15 minutes was adopted for all the subsequent experiments.

Table 1
Effects of different concentrations of commercial bleach (Jik) on elimination of surface contamination of cassava nodal explants

Jik Concentration (%)	Exposure time (min)	Percent (%) clean explants		
		After 4 days	After 8 days	After 12 days
10	5	80	66	14
10	10	82	63	14
10	15	78	22	13
10	20	90	78	55
15	5	92	57	43
15	10	94	71	29
15	15	100	100	74
15	20	100	71	71
20	15	100	97	90
20	20	93	91	82
25	15	95	80	80
25	20	97	92	85

Effect Of Cytokinins On Microshoot Formation

The results of the effects of different cytokinins (Kinetin, BAP, and TDZ) are shown in Tables 2. Generally, it was observed that 100% explants of all the cultivars evaluated sprouted within 2–3 days. Microshoot regeneration of the three cassava cultivars was influenced differently by the concentrations of the cytokinins used. There was a significant difference ($p \leq 0.001$) in all parameters evaluated (number of shoots and microshoot length, as well as number of nodes) among all concentrations of BAP and kinetin but no difference was observed in the control and TDZ media. The result of the effects of cytokinins on microshoot formation in Agric-rouge are presented in Table 2. BAP at 10 µM gave the highest number of microshoots/explant (3.60 ± 0.03). While 5 µM kinetin induced the highest shoots length (6.32 ± 0.01). It was observed that increasing the concentration of both BAP and Kinetin beyond 10 µM significantly reduced the number of microshoot /explant and their lengths. However, TDZ (at all concentrations evaluated) did not affect the number of microshoots per explant. As for the cultivar Atinwewe (Table 2) BAP at 5 µM and 10 µM induced the highest number of microshoots/explant (2.84 ± 0.01 and 2.83 ± 0.01 respectively) while TDZ at 0.5 µM and 1 µM gave the highest mean length of 2.44 ± 0.01 cm and 2.33 ± 0.02 cm respectively. Kinetin at 20 µM induced the highest mean of 3.00 ± 0.89 microshoots per explant in the cultivar Agblehoundo (Table 2). However, this was not significantly different from the number (2.90 ± 1.00) of microshoots produced on the media supplemented with kinetin 10 µM. TDZ at 0.5 µM induced the highest microshoots mean length of 4.58 ± 0.05 cm. It was observed that the control and TDZ media produced single shoot while those cultured on BAP and kinetin media produced multiple shoots (Fig. 1) (S1 File).

Table 2
Effect of TDZ, BAP and Kinetin on microshoot formation in different cultivars after 3 weeks

	Cytokinin conc. (µM)	0	TDZ-0.1	TDZ-0.5	TDZ-1	TDZ-1.5	BAP-5	BAP-10	BAP-20	Kin-5	Kin-10	Kin-20
Agric-rouge	NME (M ± SE)	1.00 ± 0.00^{f}	1.00 ± 0.00^{f}	1.00 ± 0.00^{f}	1.00 ± 0.00^{f}	1.00 ± 0.00^{f}	3.00 ± 0.06^{c}	3.60 ± 0.03^{a}	2.00 ± 0.12^{e}	3.27 ± 0.01^{b}	3.18 ± 0.01^{bc}	2.55 ± 0.2^{d}
	ML (M ± SE)	4.67 ± 0.02^{de}	5.66 ± 0.01^{b}	5.15 ± 0.03 ^{cd}	2.72 ± 0.32^{f}	4.26 ± 0.04^{e}	1.10 ± 0.03 ^{g}	0.65 ± 0.01^{gh}	0.33 ± 0.01^{h}	6.32 ± 0.01^{a}	5.60 ± 0.01^{bc}	3.08 ± 0.01^{f}
	NNM (M ± SE)	3.18 ± 0.01^{c}	3.12 ± 0.02^{c}	3.40 ± 0.02^{bc}	3.24 ± 0.02^{c}	3.24 ± 0.28^{c}	1.83 ± 0.01^{d}	1.31 ± 0.04^{e}	1.07 ± 0.07^{e}	4.20 ± 0.05^{a}	3.82 ± 0.33^{ba}	2.28 ± 0.04 ^{d}
Atinwewe	NME (M ± SE)	1.00 ± 0.00^{f}	1.00 ± 0.00^{f}	1.00 ± 0.00 ^{f}	1.00 ± 0.00^{f}	1.00 ± 0.00^{f}	2.84 ± 0.01^{a}	2.83 ± 0.01^{a}	1.90 ± 0.02^{d}	2.00 ± 0.02^{c}	2.32 ± 0.02^{b}	1.39 ± 0.02^{e}
	ML (M ± SE)	1.84 ± 0.04 ^{cd}	1.96 ± 0.02^{c}	2.44 ± 0.01a	2.33 ± 0.02^{ab}	1.83 ± 0.01^{d}	1.36 ± 0.06^{e}	0.81 ± 0.02^{f}	0.39 ± 0.01 ^{g}	2.31 ± 0.01^{b}	2.27 ± 0.02^{b}	1.94 ± 0.02 ^{cd}
	NNM (M ± SE)	3.04 ± 0.03 ^{cd}	3.06 ± 0.07^{c}	3.35 ± 0.05^{a}	2.67 ± 0.02^{e}	2.58 ± 0.01^{e}	1.57 ± 0.01^{f}	1.67 ± 0.02^{f}	1.05 ± 0.03 ^{g}	2.55 ± 0.01^{e}	3.25 ± 0.02^{b}	2.90 ± 0.01^{d}
Agblehoundo	NME (M ± SE)	1.00 ± 0.00^{e}	1.00 ± 0.00^{e}	1.00 ± 0.00^{e}	1.00 ± 0.00^{e}	1.00 ± 0.00^{e}	2.04 ± 0.03^{c}	2.25 ± 0.01^{b}	1.17 ± 0.02^{d}	2.00 ± 0.44^{c}	2.90 ± 1.00^{a}	3.00 ± 0.89^{a}
	ML (M ± SE)	2.20 ± 0.01^{f}	3.17 ± 0.01^{e}	4.58 ± 0.05^{a}	3.23 ± 0.01^{e}	2.18 ± 0.01^{f}	0.50 ± 0.01 ^{g}	0.29 ± 0.01^{h}	0.22 ± 0.02^{h}	4.18 ± 0.01^{b}	3.95 ± 0.01^{c}	3.71 ± 0.01^{d}
	NNM (M ± SE)	3.04 ± 0.03^{d}	3.10 ± 0.01^{d}	3.62 ± 0.01^{a}	2.68 ± 0.03^{e}	2.43 ± 0.01^{f}	1.00 ± 0.00^{h}	1.03 ± 0.03^{h}	1.00 ± 0.00^{h}	3.33 ± 0.02^{b}	3.22 ± 0.01^{c}	2.19 ± 0.02 ^{g}

*Means followed by the same letter are not significantly different at $P \leq 0.05$

NME = Number of microshoots per explant, ML = Microshoot length (cm), NNM = Number of nodes per microshoot, M = Mean, SE = standard error, TDZ = Thidiazuron, BAP = Benzyaminopurine , Kin = Kinetin.

Effect of auxins on roots formations

For all the three cultivars, the number of roots and root length were significantly ($p \leq 0.001$) affected by both auxins tested (Tables 3, 4, and 5). Medium without growth regulators was found to be the best in term of root induction (100%) while frequencies of root induction with media supplemented with NAA and IBA were 59 % and 85 % respectively. However, IBA gave the highest mean number of roots in all cultivars. Root induction in Agric-rouge microshoots regenerated from medium supplemented with kinetin, TDZ and BAP are shown in Table 3.

When the microshoots regenerating from media supplemented with kinetin were subcultured on MS media supplemented with IBA, they gave the highest (18.83 ± 0.02) mean number of roots on IBA 10 µM. Subculturing microshoots regenerated from a media supplemented with TDZ on hormoneless media gave the highest (3.76 ± 0.05 cm) root length. It was observed that increasing the concentration of IBA from 10 to 20 µM significantly reduced the mean number of roots in microshoots derived from the media supplemented with the three cytokinins. The results of induction of roots from Atinwewe microshoots regenerated from medium supplemented with kinetin, TDZ and BAP are shown in Table 4.

When microshoots regenerated from media supplemented with TDZ were subcultured on MS media supplemented with IBA, they gave the highest (17.26 ± 0.03) mean number of roots on IBA 20 µM and the control gave the highest (4.65 ± 0.02 cm) mean length. It was

observed that increasing the concentration of IBA from 1 to 20 µM increased the mean number of roots in microshoots derived from the media supplemented with TDZ and kinetin. However, increasing the concentration of NAA from 5 to 20 µM significantly inhibited the roots formation and reduced the mean number of roots and their length. Agblehoundo microshoots regenerated from media supplemented with kinetin gave the highest (16.38 ± 0.02 roots per shoot) mean number of roots when subcultured on media with IBA 20 µM (Table 5).
Subculturing microshoots regenerated from TDZ supplemented media on IBA 5 µM gave the highest (2.30 ± 0.03 cm) root length. It was observed that increasing the concentration of IBA from 1 to 20 µM significantly increased the mean number of roots in microshoots derived from the media supplemented with the three cytokinins. In the converse, increasing the concentration of NAA from 5 to 20 µM significantly inhibited the roots formation. The roots obtained in shoots subcultured on the control were few and more elongated while the roots derived from microshoots subcultured on IBA media were many and shorter (Fig. 2).

Acclimatization

The survival rate of the *in vitro* plantlets in the greenhouse was in the range of 98–100%. No morphological differences between regenerated and mother plants were observed in Fig. 3.

Molecular assessment of similarity in plants derived from micropropagation

At the end of the hardening, an initial molecular analysis was conducted on the regenerated plants and the donor plants in order to confirm the similarity of micropropagated CMD2 resistant cassava cultivars maintained in culture over a period of 6 months. Twenty base pairs of SSR and twenty-one base pairs of SCAR primers were used for PCR analysis. The SSR primer generated a total of 319 bp amplification fragments (CMD2 resistant gene) while SCAR primer generated a total of 700 bp amplification fragments of the CMD2 resistant gene). All of the primers produced monomorphic amplification patterns in the regenerated plants, and no differences were found in the amplification pattern among regenerated plants and donor plants (Fig. 4).

Discussion

The current study was conducted with the aim of optimizing the sterilization of nodal explants and the *in vitro* propagation of three mosaic disease resistant cassava cultivars from Benin. Nodal explants are the most difficult to sterilize during tissue culture and despite using high concentrations of sterilizing agents such as Parazone, Domestos, and Jik, they have been found to be ineffective resulting in many cases 100% contamination [27]. During the current study, exposing the nodal explants to 20% Jik (3.85% NaOCl) for 15 minutes gave the highest (90%) clean explants. These results concur with those of Maruthi *et al*. [28] who reported high percent (80–90%) clean explants when a commercial bleach (5% pure sodium hypochlorite) was used on some cassava cultivars. It also corroborates with the results of Waweru *et al*. [29] who reported that the highest proportion (92%) of clean explants was obtained when the *Cyphomandra betacea* nodal explants were exposed to 15% Jik for 20 minutes. Magaia, [16] while working on cassava nodal explants reported high (87%) numbers of clean explants when the explants were exposed twice to $HgCl_2$ solution: 0.05% $HgCl_2$ for 2 minutes followed by exposure to 0.1% $HgCl_2$ for 1 minute. However, $HgCl_2$ solution is highly toxic and it is therefore recommended for frequent use in sterilization procedure.

Growth regulators especially cytokinins are one of the most important factors affecting the regeneration of microshoots [30–33]. Kinetin, BAP, TDZ and Zeatin have been used in cassava micropropagation [34–36, 17]. However, the most commonly used cytokinin to induce shoots in cassava is either BAP alone or in combination with NAA [11, 34, 37]. Results in the current study showed that the regeneration of microshoots from cassava nodal explants in the different cultivars depends on the type of cytokinins and the concentration. BAP was found to be the best cytokinins for microshoot regeneration in agric-rouge and Atinwewe while kinetin was the best in agblehoundo. It was also observed that the response depended on the concentration of the cytokinin for each cultivar. The results in Agblehoundo concur with those of Konan *et al*. [17] and Faye *et al*. [34] who found that kinetin gave better results than BAP in regenerating cassava microshoots from nodal explants. Kinetin has also been found to be superior than other cytokinins in *Tacca leontopetaloides* [38]. The results of the current study are contrary to those of Opabode *et al*. [39] who reported that BAP alone was best in cassava shoots induction. The combination of two cytokinins has also been found to be effective on some varieties of cassava. For example, Sukmadjaja and Widhiastuti [40] reported that the highest number of shoots from three elite cassava cultivars were obtained on media supplemented with a combination of BAP and TDZ. While working on two Ethiopian varieties of cassava, Demeke *et al*. [15] reported varietal differences in shoot formation when nodal explants were cultured on MS medium supplemented with BAP and kinetin. During the present study, it was observed that the multiple shoots regenerated from media supplemented with BAP were stunted compared to those obtained with kinetin. Onwubiku, [41] made similar observation and he reported that BAP considerably inhibited the

performance (microshoots length, number of nodes, number of leaves) of two cassava varieties and Kirika *et al*. [42] also made the same observation while working with *Erythrina abyssinica*. In another study, combining BAP and NAA gave the best shoot elongation in some cassava varieties [43]. From the results of the current study, it can be deduced that kinetin would be a preferred cytokinin compared to BAP for inducing microshoots in the new cultivars since it produced multiple shoots that were elongated which could be easily subdivided for further multiplication.

Auxins are important factors involved in rooting because they promote adventitious root formation in the vast majority of species [44]. During the current study, IBA was found to be better than NAA in rooting the microshoots from the 3 elite cassava cultivars. The results of this study are similar to those of Kabir *et al*. [35] who reported that cassava microshoots rooted well in MS media supplemented with IBA compared to NAA and IAA. The effectiveness of IBA for rooting over other auxins has also been reported by Naranjo and Fallas [45] in cassava. Similar observation was made in many other *in vitro* cultured plants. For instance, Sadeghi *et al*. [46] achieved 100% *in vitro* rooting of *Prunus empyrean* in MS medium with IBA and Singh *et al*. [47] reported IBA as the best auxin for rooting in *Santalum album*. The results of the study being reported are contrary to the observations made by Shiji *et al*. [43] and Opabode [36] who found NAA to be the best for rooting in cassava. A possible explanation for these differences could be the genetic makeup of the cultivars evaluated. It was also observed that 100% rooting occurred in microshoots cultured in the media devoid of any growth regulator. Similar observation was made by Faye *et al*. [34] and Yandia *et al*. [23] while they were working on various cassava cultivars. Rooting in medium without growth regulators have been reported in Yucca glauca [48] and *Gentiana dinarica Beck* plant [49]. A possible explanation could be that there is high level of endogenous auxins.

The SSR and SCAR analysis of micropropagated plants of cassava (Agric-rouge, Atinwewe, and Agblehoundo) showed a profile similar to that of the mother plants indicating that no variation had occurred *in vitro*. Through PCR amplification, SSR primer generated 319 bp amplicons which is a portion of the CMD2 resistance genes carried by the three local cultivars while a DNA fragment of 700 bp size generated by SCAR generated 700 bp is carried by the local cultivar Agblehoundo alone. This result concurs with the work reported by Houngue *et al*. [8] where they identified the CMD2 resistance genes using the same primers as those used in this study. It is generally known that plantlets regenerated through nodal culture have lower risk of genetic instability. The present study provides for the first time the information on the conformity of micropropagated CMD2 resistant cassava cultivars with mother plants using SSR and SCAR analysis. Micropropagation has an advantage over somatic embryogenesis in that it is thought to reduce the potential for undesirable variants among the regenerated plants, whereas in somatic embryogenesis the risk of genetic instability is high. For instance, Beyene *et al*. [25] reported that using somatic embryogenesis led to 100% loss of resistance to geminivirus pathogens of the regenerated CMD2 plants. However, the plantlets were otherwise phenotypically indistinguishable from the CMD-resistant mother plants from which they were derived. Similarly, Chauhan *et al*. [50] showed that multiple morphogenic culture systems cause loss of resistance to cassava mosaic disease. In their study, they found that 25–36% and 5–10% of regenerated plant lines lost resistance to CMD respectively.

Conclusions

A simple two-step regeneration method for propagating the new cassava cultivars was developed. The optimum Jik (3.85% NaOCl) concentration for sterilization of nodal explants was established to be 20% and an exposure time of 15 minutes. Kinetin proved to be the best cytokinins for microshoot formation with the optimum concentration of 5, 10 and 20 µM for Agric-rouge, Atinwewe, and Agblehoundo respectively. Medium without growth regulators was best for rooting the regenerated microshoots in all the 3 cultivars. Furthermore, SSR and SCAR primers confirmed the presence of the CMD2 gene in regenerated plants through nodal culture similar to the mother plants. The developed protocol will go a long way in providing farmers with the much-needed cassava planting materials of the new cultivars.

Methods

Plant materials

Cassava cuttings of the three CMD2 resistant cultivars (Agric-rouge, Atinwewe, and Agblehoundo) were collected from University of Abomey-Calavi in Central Laboratory of Biotechnology and Plant breeding Gemoplasm in Benin and transported to Coffee Research Institute (CRI) in Ruiru-Kenya where the tissue culture studies were carried out. The cuttings were certified by 'Plant Protection Organization of Benin' on N° 0054994/19/SPVCP/PCP/AE-B (S2 File) before sent to the Coffee Research Institute (CRI) in Kenya. The 20-cm-long cuttings were planted as four to five stems in 10-L boat filled with sterile soil/manure mixture (1:1 v/v) (S3 File). The boat were irrigated to field capacity once per day until sprouting, and twice per week thereafter; cuttings were grown in a greenhouse

maintained at 28 °C, with relative humidity > 60%, and natural lighting with an approximate light/dark cycle of 12/12 h at Coffee Research Institute (CRI) in Kenya.

Explants Sterilization

Nodal explants from four 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 contained liquid soap to remove any surface debris and rinsed with tap water. They were then sterilized under the lamina flow hood using 10, 15, 20, and 25% v/v commercial bleach Jik (3.85% NaOCl) for 10, 15, 20 and 25 minutes. After exposure to the sterilant, the explants were rinsed two times in sterile distilled water and thereafter quickly (30 secs) immersed in 70% (v/v) ethanol and finally rinsing four times in sterile distilled water. The nodal explants were trimmed and cultured individually in test tubes (15 cm by 3 cm) containing hormone free MS media. They were then incubated in a growth room maintained at a temperature regime of 25 ± 2 ˚C provided by with cool white fluorescent light intensity of 33 µmol. $m^{-2}.s^{-1}$ and 16 h photoperiod. Data on the percent clean explants were collected after 4, 8, and 12 days. This was calculated as total number of contaminated explants / total number of explants x 100.

Microshoot Induction And Culture Conditions

Nodal explants were cultured on MS medium [19] basal salts supplemented with 3% (w/v) sucrose, 100 mg/l myo-inositol, BAP, kinetin evaluated at 5 µM, 10 µM, and 20 µM and TDZ at 0.1 µM, 0.5 µM, 1 µM, and 1.5 µM in separate experiments. The control was devoid of hormones. Rooting of the microshoots was evaluated using half-strength MS media supplemented with 2% (w/v) sucrose,100 mg/l myo-inositol, NAA and IBA evaluated at 1, 5,10 and 20 µM in separate experiments. The control was devoid of hormone. The pH of the media was adjusted to 5.8 using 0.1 M HCl or 0.1 M NaOH, and the media was gelled with 0.3% phytagel. The media was dispensed in 20 mL aliquots into culture vessels and then autoclaved at 1.06 $kg{\cdot}cm^{-2}$ and 121 °C for 15 min.

Plantlets Establishment In Greenhouse

The regenerated plantlets with well-developed roots were carefully removed from the culture tubes washed with tap water to remove agar (Fig. 5). They were then dipped in 2% fungicide (green copper) for one hour. They were then placed in plastic pots filled with substrate composed of soil: sand: manure in the ratio of 3:2:1 (Fig. 6). The containers were covered to maintain high relative humidity. The humidity was reduced gradually by opening the top of the pots after two weeks (Fig. 7) (S4 File).

Assessment of the presence CMD2 gene in plantlets.

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.

DNA Extraction And Quantification

Young leaves were picked from cassava plants in greenhouse, both mother plants and acclimatized plantlets, and DNA was extracted from the plantlets and the mother plants according to the method described by Diniz *et al*. [51]. 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 ^{O}C for SSR and SCAR analysis.

Polymerase Chain Reaction (PCR) for scoring CMD2 resistant gene.

During the current study, PCR-based SSR and SCAR markers as described by Houngue *et al*. [8] were used to assess the presence of CMD2 gene in the regenerated plantlets. The mother plants were used as the control. The characteristics of the primers used are shown in Table 1. The SSR and SCAR analysis were performed as described by Omingo *et al*. [52]. DNA samples were diluted to10 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 MgCl2 (25 mM), 3.5 µl of dNTPs (500 µM), 1 µl of SSR (10 µM) reverse primer and 1 µl of forward primer, 0.2 µl of Taq polymerase 5u / 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 one and half minutes, extension at 72 °C for 10 min and was held 4 ºC. 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 solution.

Table 6
Specific SSR and SCAR primers used for detection of CMD2 resistant gene in cassava mother plants and regenerated plantlets

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

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 regenerated plantlets 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.

Experimental Design And Data Analysis

All experiments on both shoot and root induction were laid out in completely randomized design (CRD) with 10 replicates per treatment and the experiment repeated three times. The data was subjected to one-way analysis of variance and the significant differences between treatments means were assed using MINITAB version 19 software and Tukey analysis at 5% level were performed to assess difference between means.

Abbreviations

CMD: Cassava Mosaic Disease; **SSR**: Simple sequence repeat; **SCAR**: Sequence-characterized amplified region; **CMD2**: Cassava Mosaic Disease resistant gene, **CRD**: completely randomized design; **MS**: Murashige and Skoog; **TDZ**: Thidiazuron; **BAP:** 6-benzylaminopurine; **NAA** Naphtalen Acetic Acid; **IBA:** Indol butirivc acid; **PAUSTI**: Pan African University of Basic Sciences, Technology and Innovation; **CRI**: Coffee Research Institute.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

This manuscript does not contain any individual person's data and further consent for publication is not needed.

Availability of data and materials

All data generated or analyzed during this study are included in this published article (and its supplementary information files).

Competing interests

The authors declare that they have no competing interest.

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Authors' contributions

AFS and **CA** designed the study; **JWK, AMA,** and **CA** supervised the research; **AFS, CD,** and **JAH** collected the material and conducted the work; **AFS, JWK** analyzed the data; **AFS, JWK** and **JAH** wrote the manuscript; all authors read, corrected and approved the manuscript.

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Supplementary Information

S1 File. Original uncropped images underlying the results reported in the Fig. 1.

S2 File. Phytosanitary Certificate of Plant material. Includes detailed on the tractability.

S3 File. Mother plants production under greenhouse. Includes detailed on cuttings planting.

S4 File. Image supporting the Fig. 1.

S1 Raw - images. Original uncropped images underlying the gel results reported in the Fig. 4.

Figures

Figure 1

a - Covered weaning pots; b - Partially opened pots for reduction of humidity.

Figure 2

Plantlets transplanted in plastic pots filled with soil mixture (soil: sand: manure in the ratio of 3:2:1)

Figure 3

Plantlets from the lab after washing off the agar

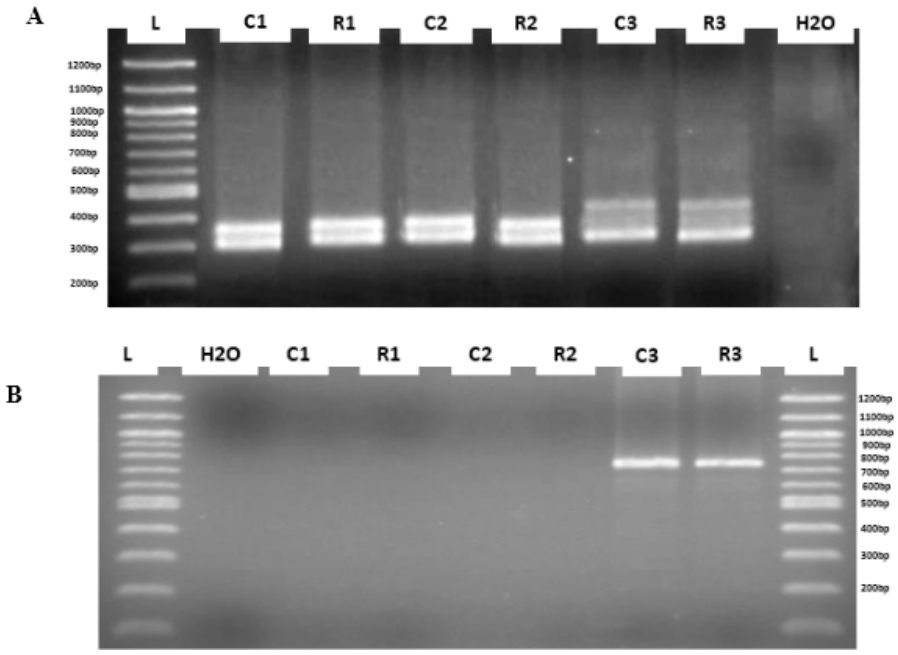

Figure 4

A- SSR banding patterns with primers NS169 and B- SCAR banding patterns with primers RME1 in both micropropagated (R1, R2, and R3) and greenhouse-grown mother plants of Agric-rouge (C1), Atinwewe (C2), and Agblehoundo (C3). R1, R2, and R3 were respectively regenerated from C1, C2, and C3; L = Loader.

Figure 5

Plantlets after one month in the greenhouse

Figure 6

In vitro rooting: a- Roots formation after 2 weeks; b- Plantlets rooting on plain media after 4 weeks; c-Roots of plantlets on IBA after 4 weeks

Figure 7

Microshoots regenerated from nodal explants a: single shoot on hormone free media; b: multiples shoots on medium supplemented with kinetin and c multiples shoots with callus at basal part on medium supplemented with BAP.

Supplementary Files

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