IJABPT INTERNATIONAL JOURNAL OF APPLIED BIOLOGY AND PHARMACEUTICAL TECHNOLOGY

www.ijabpt.com Volume-4, Issue-4, Oct-Dec-2013 Coden : IJABPT Copyrights@2013
ISSN : 0976-4550

Received: 12th July-2013 *Revised: 17th August-2013* *Accepted: 11th September -2013*
Research article

OPTIMIZATION OF YAM *IN VITRO* GENEBANKING: EFFECTS OF ACTIVATED CHARCOAL AND DARKNESS ON PLANTLETS OF THREE ACCESSIONS FROM BENIN.

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ABSTRACT: The aim of this study was to optimize the *in vitro* preservation of yam genetic resources through reduction of the number of subcultures. Effects of different concentrations of activated charcoal (1 $g.l^{-1}$, 2 $g.l^{-1}$and 3 $g.l^{-1}$) and temporary darkness were tested on the *in vitro* morphogenesis of three beninese yam accessions (Dcr28, Dcr164 and Da93G1). Galzy glutamine was used as basis culture medium and explants were micro-cuttings obtained from four months old plantlets. The results indicated that the activated charcoal, alone or combined with temporary darkness has an inhibitory effect on the aerial organs formation but favors root development with a greater mean number of root shoots (9.3±1.67 with 3 g.l-1 of activated charcoal) than the subtract without activated charcoal (2.5±0.17). A significant interaction was noted between accessions and concentration of activated charcoal indicating genotypic variability from the activated charcoal effect. The different accession plantlets growing in high concentration of activated charcoal culture media combined with temporary darkness were vigorous after eight month without subculture and subsequently allow doing one subculture per year.
Key words. Medium-term conservation, yam genetic resources, *in vitro* morphogenesis, activated charcoal, darkness.

INTRODUCTION

Yams (genus *Dioscorea*, of the monocotyledonous, Dioscoreaceae) are vines which store starchy reserves in aerial or underground tubers which greatly contribute to food security and poverty reduction in West Africa and particularly in Benin. Their tubers are rich in nutritive substances such as carbohydrate, protein and essential fatty acid. In addition, tubers are tasty and well appreciated by the consumers. It culture beneficed less externs' fertilizer input and well adapted to all the agroecologic zones of Benin. Yams species are also used for pharmaceutical purposes due to its richness in drugs (Aké Assi, 1998). With 2.36 billons tons, Benin appears to be the fourth of the best producers in West Africa, after Nigeria, Côte d'Ivoire and Ghana (FAOSTAT, 2013).

Unfortunately, yam remains a speculation for which the traditional techniques of conservation (field maintenance) cause many problems. Cultivated yams for which sexual reproduction is unpredictable are vegetatively propagated using tubers. These tubers have poor storability and are highly attack by pathogen agents. Dissemination of those pathogens alters yield and quality of yam harvests (Ondo Ovono et al., 2010). The post-harvest lost of the tubers are very important (40-50 %) because of absence of appropriate conservation methods (Hounhouigan et al., 1997). In on-farm germplasm maintenance, the main damage causes are cryptogamic diseases (such as anthracnose) and viroses that are leading to extinction of accessions (Dansi et al*.,* 2013). *Ex-situ* conservation, through plant cell, tissue and organ culture seems to be an alternative way for efficient conserving genotypes. Compared to traditional conservation procedure, *in vitro* conservation procedure has several potential advantages. For large-scale *in vitro* plant production, the important attributes are the quality, cost effectiveness, preservation of genetic fidelity and long-term storage. Moreover, micropropagation may be utilized, in basic research, in production of virus-free planting material. Currently, *in vitro* culture techniques are used for conservation of indigenous accessions of yam in Benin (Ahanhanzo et al., 2003, 2010, Agbidinoukoun et al*.,* 2013).

However, plantlets need to be subcultured four times per year in order to avoid senescence concerns. This is also time consuming and requires more effort from technicians. Several reports showed that successive subcultures increase with the time the somaclonal variations (Podwyszyńska, 2005, Venkatachalam et al., 2007, Peyvandi et al., 2009). In term of conservation, the fundamental principle is to guarantee genotype stability. It is thus relevant to reduce the number of subcultures for efficient conservation of yam genotypes. The widely used method at this end is low temperature culture (Galzy and Compan, 1988; Engelmann, 1990), but it is not adapted for tropical species because those species are more sensible to cold (Engelmann, 1990). Available literature revealed that activated charcoal is a culture medium compound which enables better rooting for several species depending on the genotype (Brhadda et al., 2003; Feyissa et al., 2005) and has been used for absorption of toxic elements product in culture media by the explants (Grant et al., 1999, Koné et al., 2010). Activated charcoal has also been used to favor morphogenetic capacity of *Dioscorea alata* (Peters, 1986). Furthermore, subsequent division of cells was not favored by the addition of activated charcoal in the medium (Kuriyama et al., 1990). Concentration of activated charcoal differs widely in plant tissue culture medium and may be related to different plant species, medium, explants etc. Yet the exact mechanism of action of activated charcoal in plant tissue culture is not clear (Thomas, 2008).

Up to now, the role of activated charcoal combined with darkness to improve the reduction of the number of subculture is not investigated. This study tested the effects of activated charcoal and darkness on micro-cuttings of three accessions representatives of yam diversity from Benin.

MATERIEL AND METHODES

Plant material

Three accessions of *Dioscorea spp* were used. Two of them belong to the complex *D. cayenensis - D. rotundata*: one precocious accession (Dcr28) and one late maturing accession (Dcr164). The last one belongs to Asian specie *D. alata* (Da93G1). The choice of these accessions was guided by their *in vitro* actively growing shoots and the fact that they represent the beninese cultivated yams diversity.

Medium culture and incubation conditions

The explants used were the micro-cuttings obtained from four months old vitroplants of the *in vitro* genebank of the Laboratory of Genetic and Biotechnology of the Faculty of Sciences and Techniques (University of Abomey-Calavi, Republic of Benin). The micro-cuttings were excised from plantlets in sterile condition over laminar flow and cultured in test tubes (20 X 2.5 cm) containing Galzy medium supplemented by glutamine (Table1). This medium was enriched in potassium ion (10,9 Mm) and ammonium ions (22 mM of NO_3 and 8mM of NH_4) recommended for long-term conservation of *vitis* genus (Galzy, 1988). The culture medium was also added with different concentrations of activated charcoal (C1=1 $g.l^{-1}$, C2=2 $g.l^{-1}$ and C3=3 $g.l^{-1}$). The medium pH was adjusted to 5.75 before autoclaving at 121ºC and 15 lbs for 20 min. After that, the test tubes were transferred in culture room which was maintained at 27 ± 1ºC under a 16 hrs light (5000 $\mu mol.m^{-2}.S^{-1}$) / 8 hrs dark photoperiod. To test darkness effect, one part of tubes was wrapped in aluminum paper within the two weeks bellow culture (temporary darkness).

Two experimentations were conducted. The first related to the effect of activated charcoal alone and the second related to the effect of activated charcoal combined with temporary darkness. At the end of the first and fourth weeks, parameters such as the leaf and root numbers were determined. The root length, the plantlets height and biomass of fresh matter were determined at the end of the eighth month. Each culture medium containing the concentration of activated charcoal was considered as treatment. 30 tubes were used per treatment (10 tubes per accession).

Table1. Composition of Galzy medium (1988) supplemented with glutamine.

Macro-elements (mM)		Micro-elements (µM)		Organics compound ($mg.l^{-1}$)	
NO_3	7.5	Fe	90	Thiamine-HCl	1
NH_4	2	Mn	3.6	Calcium Pantothénate	1
PO_4	0.9	I	1.5	Pyridoxine-HCl	1
K	22	Ni	0.1	Nicotinique acid	1
Ca	21	Co	0.1	Biotine	0.01
Mg	0.5	Zn	0.2	Myo-inositol	100
SO_4	0.5	Cu	0.1	Glutamine	200
		B	0.4	Sucrose	3000
		Mo	0.1	Agar	7000

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Data analysis

Treatments were plotted in a completely randomized design. Data were computed under SAS program version 9.0 using the General Linear Models procedure. For significant differences (i.e., P<0.05), the means were ordered using Student, Newman and Keuls' test.

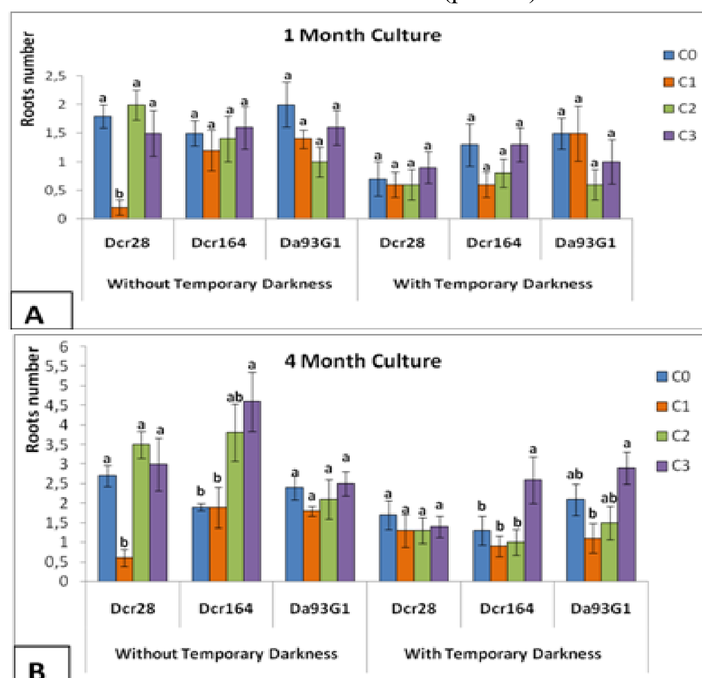
RESULTS

In the text, we use temporary darkness to signify the period (two weeks) which the plantlets are incubated in darkness. The results we present are essentially concerned the roots differentiation and their growth (rhizogenesis), the leaves differentiation and shoot growth (caulogenesis) and production of fresh biomass.

Effect of different concentrations of activated charcoal, alone or combined with darkness on rhizogenesis

Roots differentiation

During the first week of culture, roots differentiation of all accessions tested is better in control medium (C0) for the test without temporary darkness (Figure 1A). Accessions Da93G1 and Dcr28 showed the maximal roots number (2) respectively in C0 and C2 media while accession Dcr164 showed less root average number (0.2±0.13) in C1 medium. In contrast, all accessions have get at least one root in C3 medium comparatively than other media which recorded none root with some accessions. Relatively to the test with temporary darkness, we noted that only micro-cuttings obtained from Da93G1 and Dcr164 accessions have presented at least one root on C0 and C3 media. The high mean number of roots (1.5) is obtained with Da93G1 accession on C0 and C1 media while the lowest one (0.6) obtained on C1 and C2 media with accession Dcr164 is three times higher than those mean in the test without temporary darkness (Figure 1A). Statistically, the darkness factor increase significant influence (p‹0.001) on roots differentiation than the effect of activated charcoal (p=0.009). In contrast, accession factor (p=0.17) and the interaction activated charcoal-darkness have no influence on roots differentiation (p=0.06).
At fourth month of culture (Figure 1B), the mean number of roots increase with the concentration of activated charcoal in media in both experimentations. The highest mean (4.6) is recorded in the test without temporary darkness against 2.6 in the temporary darkness test on C3 medium with accession Dcr164. The darkness factor has more significant influence (p‹ 0.001) on root differentiation at this state of culture than concentration of activated charcoal (p=0.001). The interactions accession-activated charcoal and darkness-activated charcoal are highly significant (p‹ 0.001) than that of accession-darkness interaction (p=0.01).



For each accession, means followed by same letter are not significantly different at 5%.

Figure 1. Effect of different concentrations of activated charcoal and temporary darkness on mean number of roots from three yam accessions after one month (A) and four month (B).

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In contrast to the first month culture, activated charcoal alone increases more roots formation than darkness. At eighth month culture, the majority of plantlets growing in media without temporary darkness began senescence. Then we have considered only the data relative to the temporary darkness test at this state of culture (Table 2). The results revealed that activated charcoal effect on roots number were lower than we have noted at fourth month culture except accession Dcr164 which was more sensible to the activated charcoal effect. Moreover, the highest mean of root number are recorded on C3 medium. Significant difference is noted between accessions and concentration of activated charcoal ($p < 0.001$).

Table 2. Effect of different concentrations of activated charcoal and temporary darkness on mean number of roots from three yam accessions after eight month.

Treatments	Main number of roots		
	Dcr28	Dcr164	Da93G1
C0	2.8 ± 0.66^{a}	2.5 ± 0.16^{c}	2.9 ± 0.23^{ab}
C1	2.7 ± 0.67^{a}	3.4 ± 0.56^{bc}	1.8 ± 0.13^{b}
C2	2.5 ± 0.6^{a}	6.7 ± 1.56^{ab}	3.6 ± 0.65^{a}
C3	3.3 ± 0.37^{a}	9.3 ± 1.67^{a}	3.3 ± 0.54^{ab}

For each accession, means followed by same letter are not significantly different at 5%.

Roots elongation

The table 3 presents the effect of different concentrations of activated charcoal and temporary darkness on the length of the yam roots after eight months culture. The results show that the accession Da93G1 had the highest length of root on all media with the maximal (3.6±0.34 cm) on control medium (C0). In contrast, roots length is less than 1.5 cm with Dcr28 and Dcr164 accessions on all of the media. The factor accession increases then significantly the roots length.

Table3. Effect of different concentrations of activated charcoal and temporary darkness on mean roots length from three yam accessions after eight month.

Treatments	Mean roots length (cm)		
	Dcr28	Dcr164	Da93G1
C0	1.52 ± 0.29^{a}	1.05 ± 0.11^{ab}	3.6 ± 0.34^{a}
C1	0.98 ± 0.26^{ab}	0.87 ± 0.08^{b}	3.05 ± 0.38^{a}
C2	0.65 ± 0.11^{b}	$1.05\pm0.16a^{b}$	2.76 ± 0.67^{a}
C3	0.99 ± 0.1^{ab}	1.44 ± 0.18^{a}	2.82 ± 0.58^{a}

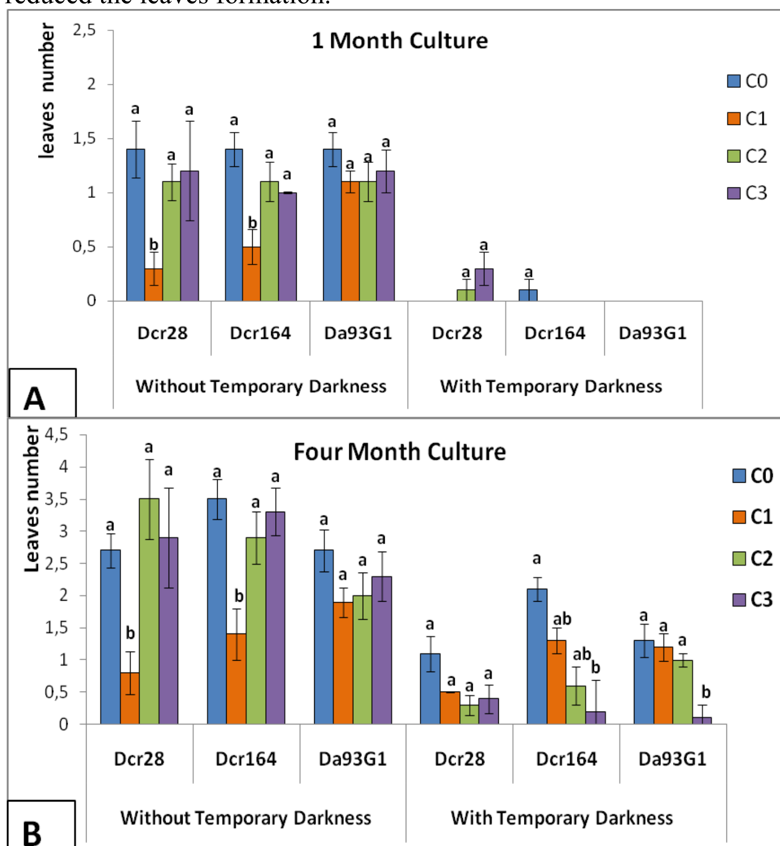
For each accession, means followed by same letter are not significantly different at 5%.

Effect of different concentrations of activated charcoal, alone or combined with darkness on caulogenesis

Leaves differentiation

After one month of experimentation, plantlets incubated without temporary darkness clearly developed more leaves than those incubated in temporary darkness condition (Figure 2A). The highest mean of leaves (1.4±0.24) is obtained in control medium (C0). In contrast, the lowest mean is obtained in C1 medium with accessions Dcr28 and Dcr164. Moreover, among the three accessions tested, Da93G1 presented more leaves in all media at « without temporary darkness test » while it presents no leave at temporary darkness condition. Generally, darkness appreciably inhibited the leaves differentiation for all accessions tested (Figure 2A). Statistically, activated charcoal concentrations and darkness factors significantly influenced the leaves differentiation ($p < 0.001$) while accession factor had no action ($p=0.53$) at this state of leaf development. Furthermore, interaction between activated charcoal and darkness is high significant ($p < 0.001$) while concentration and accession are not significant ($p=0.29$). At the fourth month of culture (Figure 2B), the mean number of leaves increased for all accessions in "without temporary darkness" condition with a maximal of 3.5 in C0 and C2 media for the accessions Dcr164 and Dcr28 respectively. The lowest means are obtained for all accessions in C1 medium. In contrast, the leaves number of the plantlets growing in "without temporary darkness" condition decreased with the maximal mean (2.1±0.18) in control medium (C0) which is obtained with accession Dcr164 while C2 and C3 media recorded the lowest leaves number (0.3) with accession Dcr28. Highly significant action was observed on leaves differentiation ($p < 0.001$) with activated charcoal an darkness conditions while accession factor had not influenced it ($p=0.06$). The interactions concentration-accession and darkness-concentration were significant ($p < 0.001$ and $p=0.007$ respectively), but accession-darkness one is not significant ($p=0.25$).

After eight month of culture, senescence apparition as above reported was only observed in temporary darkness test (Table 4). The maximal leaves number is obtained in control medium (C0) for all accessions while the lowest one was observed in C3 medium. Variance analysis showed that the leaf development was highly influenced by the presence of activated charcoal in the medium and the accession (p=0.004). At high concentration, activated charcoal has sensibly reduced the leaves formation.



For each accession, means followed by same letter are not significantly different at 5%.

Figure 2. Effect of different concentrations of activated charcoal and temporary darkness on mean number of leaves from three yam accessions after one month (A) and four month (B).

Table 4. Effect of different concentrations of activated charcoal and temporary darkness on leaves mean number from three yam accessions after eight month.

Treatments	Leaves mean number		
	Dcr28	Dcr164	Da93G1
C0	9.8±0.74[a]	11.0±0.7[a]	8.3±0.7[a]
C1	7.2±1.47[ab]	10.8±2.01[a]	6.8±0.51[a]
C2	5.8±1.37[ab]	8.0±0.99[a]	6.8±0.96[a]
C3	3.6±1.38[b]	6.7±0.84[a]	5.5±1.24[a]

For each accession, means followed by same letter are not significantly different at 5%.

Shoot development

Results showed that the highest height of plantlets in all media recorded with the accession Dcr164 with a maximal of 13.86±2.3 cm in control medium (C0) while the lowest (3.54±1.2 cm) was obtained in C3 medium by accession Dcr28 (Table 5). Statically, shoot elongation differs on media and is highly influenced by accession (p<0.001). Shoot elongation for all tested accessions was negatively influenced by the augmentation of activated charcoal in the medium. Figure 3 shows the behavior of plantlets in control and activated charcoal culture media.

Table 5. Effect of different concentrations of activated charcoal and temporary darkness on height mean of shoots from three yam accessions after eight month.

Treatments	Height mean of shoots (cm)		
	Dcr28	Dcr164	Da93G1
C0	6.95±0.8^{a}	13.86±2.3^{a}	11.27±1.9^{a}
C1	6.08±1.3^{a}	12.80±0.1^{a}	9.38±0.9^{a}
C2	4.4±1.1^{a}	11.68±2.7^{a}	7.63±1.8^{a}
C3	3.54±1.2^{a}	9.27±1.4^{a}	6.95±0.6^{a}

For each accession, means followed by same letter are not significantly different at 5%.

C0= Control medium

C3= Control medium + 3 g.l^{-1} activated charcoal

Figure 3. Plantlets from accession Dcr164 obtained on different culture media after eight month culture.

Effect of different concentrations of activated charcoal and temporary darkness on mean of biomass of fresh matter after eight month.

High mean of biomass of fresh matter was obtained (0.25±0.02 g) in control medium (C0) with the accession Dcr164 and accession Da93G1 (0.21±0.02 g) in C3 medium (Table 6). Activated charcoal influenced significantly the increasing of fresh matter (p=0.002). This one also varied with accession (p‹ 0.001).

Table 6. Effect of different concentrations of activated charcoal and temporary darkness on mean of biomass fresh matter from three yam accessions after eight month.

Treatments	Mean of biomass of fresh matter (mg)		
	Dcr28	Dcr164	Da93G1
C0	92.8±0.06^{b}	203.4±0.07^{ab}	182.4±0.05^{a}
C1	87.8±0.05^{b}	144.3±0.03^{b}	189.0±0.05^{a}
C2	79.5±0.05^{b}	200.1±0.09^{ab}	163.0±0.1^{a}
C3	179.6±0.08^{a}	252.8±0.07^{a}	205.6±0.07^{a}

For each accession, means followed by same letter are not significantly different at 5%.

DISCUSSION

Yams are important indigenous tuber crops that greatly contribute to food security and poverty reduction in West-Africa, particularly in Benin. However, their conservation is still difficult due to pest and disease concerns. Besides; the poor storage capacity of their tubers induces the loss of more sensible genotypes and constitute a great challenge for gene bank maintenance. The biotechnology tools, through *in vitro* cells culture constitute an alternative for the best maintenance of genotypes. In this study, the effect of activated charcoal and darkness were tested to optimize the culture medium for the best *in vitro* gene banking of yams. The medium effect results from overall interactions between its different compounds. Some of these compounds stimulate the *in vitro* development process while others, in contrast, have an inhibitory action. The different concentrations of activated charcoal tested induced various reactions on explants from different accessions. During the first to eighth month culture, the high level of activated charcoal in media have an inhibitory effect on areal organs formation through the reduction of leaves number and the shoots height for the most of accessions. This inhibitory action of activated charcoal has been reported by several studies (Pan and van Staden, 1998; Krassimir *et al.,* 2006; Thomas 2008)**.** However, the concentrations which induced this inhibition varied with the species. In contrast to areal organs, activated charcoal enhances the roots differentiation of all accessions. This stimulating action of rooting depends on the concentration of activated charcoal and the accession. High concentrations (2 $g.l^{-1}$ and 3 $g.l^{-1}$) induce the highest roots means respectively with accessions Da93G1 (*Dioscorea alata*) and Dcr164 (*Dioscorea cayenensis /D. rotundata*) after eight month culture. The different accessions tested have also various sensibility on activated charcoal. This genotypic variability in root differentiation produced by activated charcoal is congruent with Feyissa et al. (2005) on *Hagenia abyssinica*. The high level of rooting observed in this study is not influenced by the type of medium. In fact, Yakoub-Bougdal et al. (2007) obtained the same effect (90 % of rooting percentage) in MS (Murashige and Skoog, 1962) medium supplemented by activated charcoal. In addition, Kamal and Sayyed (2011) have reported that activated charcoal has a positive influence on better rooting of *Juglans spp*.

The lowest root proliferation in the medium without activated charcoal observed in this result should be explained by the presence in the medium the morphogenesis inhibitory substances released by the explants. Activated charcoal in the medium absorbs those toxic substances favoring root differentiation. We didn't analyze our media in order to know the type of toxic substances, but the production of those substances has been demonstrated in *Allium* and *Daucus* (Grant and Hammatt, 1999). Peters (1986) has also reported that explants of *Dioscorea alata* produce phenolic compounds which induce browning of the medium and inhibit shoot development. In addition, Nejla et al. (2002) reported that activated charcoal reduced significantly the oxidation of anthers in comparison with control medium. According to available literatures, the mechanism of activated charcoal action is not clearly known (Thomas, 2008). Nevertheless, the increasing of roots in medium with high concentrations of activated charcoal (C2 and C3) could probably explained by the reduction of K^+ and $NH4^+$ absorption. Indeed, Galzy (1969) demonstrated that the root growth is inhibited when the level of potassium and ammonium increase in the medium. Activated charcoal should reduce considerably the K^+ and $NH4^+$ absorption and consequently come up this inhibited action. This absorption effect of activated charcoal is reported by several studies related to *Musa in vitro* culture (Gübbük and Pekmezci, 2004; Koné *et al.,* 2010). Combined with darkness, activated charcoal decreases roots proliferation and inhibits mostly areal organs development. This synergic action on both factors shows an interaction of intern factor (activated charcoal) and extern factor (darkness) on *in vitro* rooting capacity of shoots. The explants which were tested in temporary darkness condition have presented the lowest rooting comparatively than other explants which were exposed without temporary darkness. Furthermore, the exposition at temporary darkness has affected the quality of shoots because of their etiolation which is reversible when shoots were transferred in normal photoperiod. This etiolation is explained by the absence of photosynthesis during the darkness incubation. This remark has been mentioned by Koné et al. (2010) who noted that darkness incubation stimulate the production of endogenous auxin by micro-cuttings leading to rapid roots initiation. Our results are in contrast with this previous report because of the decreasing of roots proliferation when the explants were incubated in temporary darkness condition. However, it is important to note that the medium used by these authors do not contain activated charcoal. So the decrease of root number could be linked to activated charcoal effect which trapped more hormones at darkness, this stopping the induction of root differentiation. At about six month of culture, shoots growing in media without activated charcoal or containing low concentration of activated charcoal (1 $g.l^{-1}$) have began a wilt phase while those growing in media enrichesed with activated charcoal (2 $g.l^{-1}$ and 3 $g.l^{-1}$) are still vigorous. This observation is congruent with the finding of Borges et al. (2004) revealing that the presence of activated charcoal in preservation medium favors a better tissues viability to be manifested in high level of shoot in comparison with media without activated charcoal. In fact, the combination of activated charcoal and temporary darkness allows the increasing of shoots survival and permits to reduce the number of subculture.

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CONCLUSION

This study would suggest that activated charcoal combined with temporary darkness favors the *in vitro* preservation of yam genetic resources. Activated charcoal have an inhibitory action on areal organs formation in the fourth month culture but permits a better rooting of all accessions tested. This activated charcoal effect varied on genotype of accessions tested. The plantlets of different accessions are still vigorous after eight month without subculture in culture media containing high concentration of activated charcoal allowing to the increasing plantlets survival. With this protocol, it is possible to do one subculture per year.

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