Topophysis and growth regulators effects on buds and zygotic embryos regeneration of African Bush Mango Tree cultured *in vitro*

ABSTRACT

Background: African bush mango tree is one of the important fruit plants with high nutritional, medicinal and commercial values. However, the seedling system of this plant remained a deep understanding. In this study, we evaluate the effect of topophysis and growth regulators on the reactivity of different types of buds and zygotic embryos of wild mango.

Methodology: Ripe fruits of two local varieties (*Wossro* and *Sissro*) were collected and pulped. The nuts were extracted and dried in the greenhouse for a week. One set of nuts was used for zygotic embryos excision which were disinfected by immersion in the bleach solution (sodium hypochlorite 10% with 8% active chloride) for 10 min. The embryos were rinsed three times with sterilized water prior to their culture. The second set of nuts was sown in the polybags containing the sand. After one month, the stems with buds at different positions (apical, axillary and cotyledonary) were excised and disinfected by soaking in bleach solution (sodium hypochlorite 10% with 8% active chloride) for 10 min for all explants and in a mercuric chloride 0.1% added with two drops of Tween 20 for axillary and cotyledonary buds for 5 min and 0.01% for apical buds for 5 min. The explants were cultured on MS ¼ and WPM ½ media supplemented with the combination of BAP, KIN at different concentrations (0.2 mg/L and 3.5 mg/L) and NAA (0.05mg/L).

Results: The best budding rate was obtained for the variety *Wossro* which showed a high bud break rate (26.47%) against (9.88%) for the variety *Sissro*. A variable response was observed depending on the topophysic of the buds. Axillary buds showed the best response performance at budbreak 24.48% on MS ¼ medium + 3.5 mg/L BAP+ 0.05mg/L NAA. The *in vitro* germination of embryos was significantly dependent on the variety ($p \leq 0.05$), with a germination rate of 50.76% for *Wossro* versus 18.32% for *Sissro*. The growth of the obtained explants was significantly ($p \leq 0.05$), favored by the medium MS ¼ + 0.2mg/L KIN, having allowed the development until the leafy stem stage.

Implication: The findings will help to improve the regeneration rate and plantlets production of the African bush mango tree which could be used by the farmers after acclimatization.

Keywords: *Irvingia gabonensis, Topophysis, In vitro regeneration, Bud morphogenic capacity, Zygotic embryos, culture media.*

ABBREVIATIONS

WPM: Woody Plant Medium
MS: Murashige and Skoog
NAA: Naphthalene acetic acid;
BAP: 6-benzyl amino purine;
KIN: Kinetin

1. INTRODUCTION

African Bush Mango Tree *[Irvingia gabonensis (*Aubry Lecomte*)]*, is a fruit tree grown in an agroforestry system in West and Central Africa. In the Republic of Benin, *Irvingia gabonensis*, have ethnobotanical, socioeconomic and ecological importance [1]. It provides wood and contributes to the coverage of nutritional needs for the most vulnerable social groups [2]. It is at the center of a potential market worth 50 million USD in turnover [3]. Despite this important value, African bush mango tree is an underutilized specie in Benin, classified as a neglected crop [4]. Its cultivation cycle is undermined by many constraints among producers, who maintain the trees in plantations out of preference or secondary interest in the resources provided by the species. It best-known propagation technique is transplanting after germination of the nuts in the forest. Traditional seedling and vegetative propagation techniques do not favor large-scale production after the selection of fruits by variety according to quality and with very high variability. Although there are methods of vegetative propagation of *Irvingia gabonensis* showing low success rates [1, 5], the exploitation of this agroforestry resource is growing and affecting the continuity of production and plantation densities.

In order to allow a higher level of production of these threatened species, by promoting them through the development of agricultural value chains, different solutions such as new technologies have been proposed in the literature by [4]. Among these technologies, biotechnologies allow bypassing traditional multiplication methods by a perfect control of the biological cycles of plants, for the improvement of the propagation of species with many advantages like other wood species [6]. *Irvingia gabonensis* is a highly allogamous specie, whose propagation by seedling produces results that constitute a great source of heterogeneity [7]. Compared to seed propagation, vegetative propagation methods show more or less uniform results, but with low success rates. Biotechnological propagation methods are therefore a solution for the multiplication and seedling production of *Irvingia gabonensis* varieties [7]. A few studies have been conducted on the factors influencing the *in vitro* propagation of the African bush mango tree. Different explants have been tested by using buds and callus segments [8, 9] and zygotic embryos [10]. However, morphogenic capacity of different buds depending on their position is not studied. Indeed, explants from many organs are capable of producing adventitious shoots or roots, but explants from different organs usually vary greatly in their morphogenic capacity [11]. Morphogenic capacity can also vary depending on the position of explants within an organ [12]. Positional effects on subsequent growth or development of explants or cuttings are termed ‘topophysis’ [11, 13]. Hung and Trueman [12] have demonstrated that topophysic effects differ between node and organogenic cultures of the eucalypt *Corymbia torelliana* × *C. citriodora*. It is therefore important to assert how topophysis can improve the regeneration rate of the African bush mango tree. To this end, the present study is proposed to evaluate the effects of bud topophysis and growth regulators supplemented in two culture media on the morphogenic capacity of buds and zygotic embryos of two local African bush mango tree varieties grown in Benin.

2. MATERIAL AND METHODS

2.1 Plant material

The plant material was constituted of the fruits of two local varieties of African bush mango tree. The fruits were collected from two plantations located in the village of Hounhomey in the township of Djakotomey in the district of Couffo.

2.2 METHODS

2.2.1 Explants removal and disinfection treatments

After the collection of ripe fruits, a sample of both varieties was pulped. The nuts were extracted and dried in the greenhouse for a week. After that, one set of nuts was washed with a detergent solution made with MENDEL soap for 5 min to avoid fungi contamination. They were then cut with a metal saw near the part containing the zygotic embryos which were extracted with a scalpel and disinfected by immersion in the bleach (sodium hypochlorite 10% with 8% active chloride) solution for 10 min. The embryos were rinsed three times with sterilized water prior to their culture.
The second set of nuts was sown in the polybags containing the sand. After one month, the stems with buds at different positions (apical, axillary and cotyledonary) were cut with sterilized scissors and distributed in sterile jars. Explants were then subjected to a disinfection protocol under laminar flow by using 70% alcohol for 5 min, followed by soaking in different disinfection solutions as bleach (sodium hypochlorite 10% with 8% active chloride) for 10 min for all explants and in a mercuric chloride 0.1% added with two drops of Tween 20 for axillary and cotyledonary buds for 5 min and 0.01% for apical buds for 5 min.

2.2.2 Culture of explants and growing conditions

The embryos were seeded on MS ¼+ 0.2 mg/L KIN and WPM ½ + 0.2 mg/L KIN media whose efficiencies have been demonstrated in the regeneration process of woody species [10].
For the buds, eight combinations of culture media using MS ¼ and WPM ½ based medium with different concentrations of BAP and KIN (0.2 mg/L and 3.5 mg/L) and 0.05 mg/L NAA were tested. The pH of all of the media was adjusted to 5.7 (±1) and the media were solidified with agar (7g/l) and were then sterilized at 121° for 15 minutes under autoclave. The jars contained the explant were cultured in a culture room, maintained at 26 ±1°C with a relative humidity of 80% under a photoperiod of 12 h, and light intensity of 3000 lux.

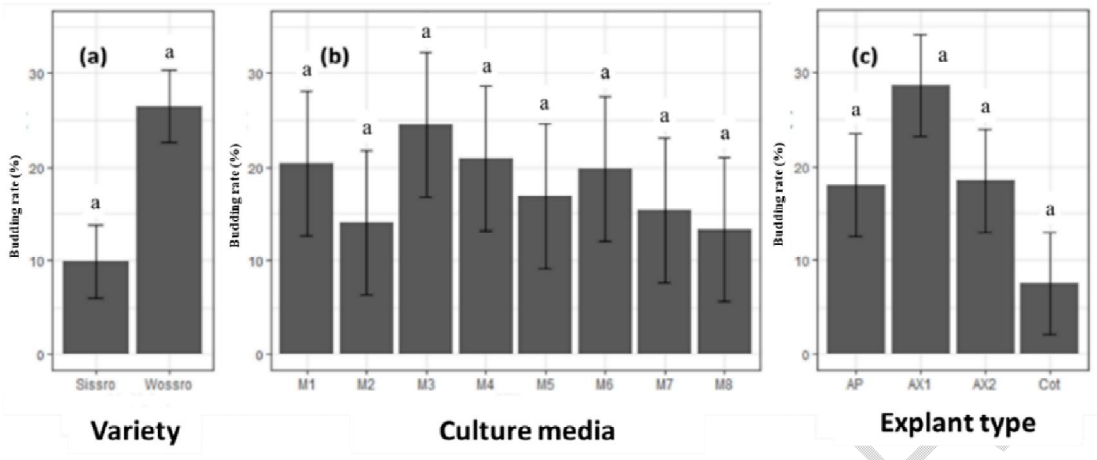
2.2.2 Statistical analyses
For all factors (varieties, types of explant, type of media and growth regulators), the data collected were tested using 2-way ANOVA or 3-way ANOVA when adding the interactions, in a generalized linear model. The tests were performed in the R software version (RX643.0.1).

3. RESULTS AND DISCUSSION

3.1. RESULTS

3.1.1 Regeneration rate of buds on MS and WPM culture media of two local African bush mango varieties

A significant difference (p= .003) was noted in bud's break rate throughout the varieties. The variety Wossro showed a high bud break rate (26.47%) against (9.88%) for the variety Sissro (Figure 1a). The difference is not significant among the growing medium (p=.97). However, the medium showing the best response was M3 (24.48%) followed by M4 (21.01%). The lowest responses were obtained with media M8 (13.32%) and M2 (14.06%) (Figure 1b). Similarly, explant type did not have a significant (p=.07) effect on bud-break of explants. However, the best responses were obtained with first-rank axillary buds (28.66%) followed by second-rank axillary buds (18.49%), and apical buds (18.01%), cotyledonary buds showed very low responses to bud-break (7.53%) (Figure 1c).

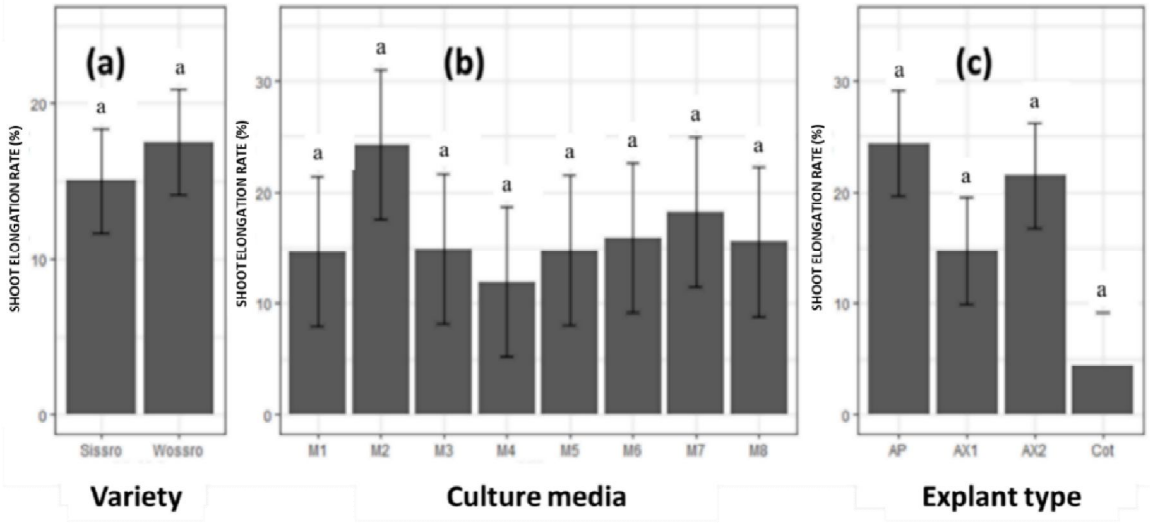


M1= MS ¼ + 3.5 mg/L KIN + 0.05 mg/L NAA; **M2**= MS ¼ + 0.2 mg/L KIN + 0.05 mg/L NAA; **M3**= MS ¼ + 3.5 mg/L BAP + 0.05 mg/L NAA; **M4**= MS ¼ + 0.2 mg/L BAP + 0.05 mg/L NAA; **M5**= WPM ½ + 0.2 mg/L BAP + 0.05 mg/L NAA; **M6**= WPM ½ + 3.5 mg/L BAP + 0.05 mg/L NAA; **M7**= WPM ½ + 0.2 mg/L KIN + 0.05 mg/L NAA; **M8**= WPM ½ + 3.5 mg/L KIN + 0.05 mg/L NAA;
AP= Apical bud; **AX1**= First-row axillary bud; **AX2**= Second-row axillary bud; **Cot**= Cotyledonary bud.

Figure 1: Bud breaking rate variation in (a) variety, (b) culture media and (c) explant type.

3.1.2. Shoot elongation and leaves formation

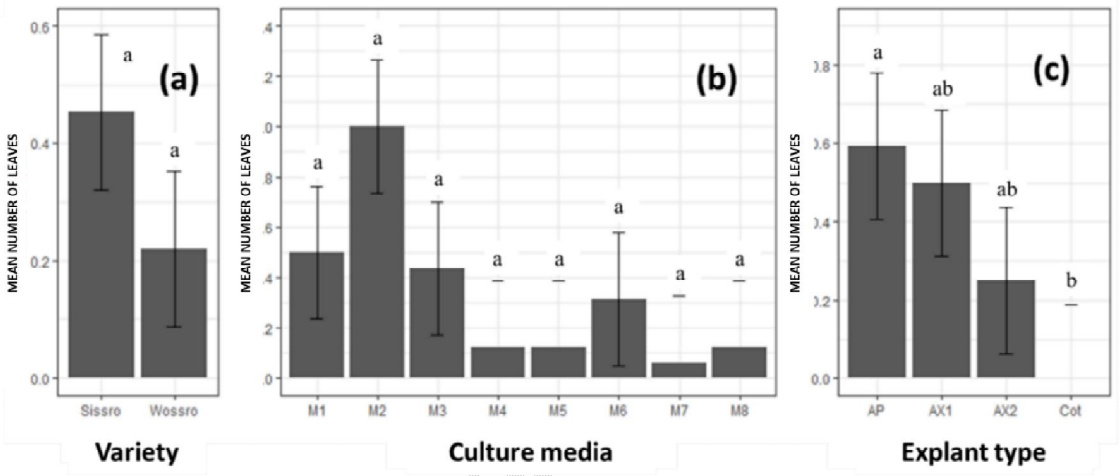
Among the factors considered (variety; growing medium; type of explant), only the type of explant had a significant influence (p=.02) on the leaf-bearing. Indeed, the number of leaves produced was very variable between explants. The explants that produced the highest number of leaves were those from apical buds (24.37%) followed by second-rank axillary buds (21.54%). The variety factor did not have a significant influence (p = .60) on the leaf-bearing, although the number of leaf-bearing explants of Wossro variety was higher (17.50%) compared to (15.03%) for Sissro variety (Figure 5a). Also, the growing medium factor did not have a significant influence (p = .9). Explants showed that the media enhancing the best responses were M2 medium (24.22%) followed by M7 medium (18.21%) (Figure 5b). The lowest responses were obtained with media M1 (14.69%) and medium M4 (11.89%).



M1= MS ¼ + 3.5 mg/L KIN + 0.05 mg/L NAA; **M2**= MS ¼ + 0.2 mg/L KIN + 0.05 mg/L NAA; **M3**= MS ¼ + 3.5 mg/L BAP + 0.05 mg/L NAA; **M4**= MS ¼ + 0.2 mg/L BAP + 0.05 mg/L NAA; **M5**= WPM ½ + 0.2 mg/L BAP + 0.05 mg/L NAA; **M6**= WPM ½ + 3.5 mg/L BAP + 0.05 mg/L NAA; **M7**= WPM ½ + 0.2 mg/L KIN + 0.05 mg/L NAA; **M8**= WPM ½ + 3.5 mg/L KIN + 0.05 mg/L NAA;
AP= Apical bud; **AX1**= First-row axillary bud; **AX2**= Second-row axillary bud; **Cot**= Cotyledonary bud.

Figure 2: Shoot elongation rate variation related to (a) variety, (b) growing medium and (c) explant type factors.

For the Number of leaves parameter, among the factors considered (variety; growing media; type of explant), none showed a significant influence. However, the average number of leaves was 0.45 leaves for Sissro variety against 0.22 leaves for Wossro variety (Figure 3).



M1= MS ¼ + 3.5 mg/L KIN + 0.05 mg/L NAA; **M2**= MS ¼ + 0.2 mg/L KIN + 0.05 mg/L NAA; **M3**= MS ¼ + 3.5 mg/L BAP + 0.05 mg/L NAA; **M4**= MS ¼ + 0.2 mg/L BAP + 0.05 mg/L NAA; **M5**= WPM ½ + 0.2 mg/L BAP + 0.05 mg/L NAA; **M6**= WPM ½ + 3.5 mg/L BAP + 0.05 mg/L NAA; **M7**= WPM ½ + 0.2 mg/L KIN + 0.05 mg/L NAA; **M8**= WPM ½ + 3.5 mg/L KIN + 0.05 mg/L NAA;
AP= Apical bud; **AX1**= First-row axillary bud; **AX2**= Second-row axillary bud; **Cot**= Cotyledonary bud.

Figure 3: Mean number of leaves for (a) the variety, (b) culture media and (c) explant type factors.

3.1.3. Germination of zygotic embryos

Among the factors whose effect was observed (Variety; Culture medium; Obscurity), only the factor "variety" had a very high influence on the *in vitro* germination of the embryos ($p < 0.001$). The highest germination rate was obtained with Wossro variety (50.76%) against (18.32%) for Sissro variety (Figure 4a). Also, the first germinations were observed in Wossro variety, which maintained this trend until the end of the observation period. The highest germination rates were reached in the sixth week for both Wossro and Sissro (Figure 4a). The observations showed that, the germination rate on MS ¼ +0.2mg/LKIN medium was higher (42.43%) versus (27.16%) on WPM ½ medium. In addition, exposure of embryos to darkness (Figure 4b) showed that light promotes *in vitro* germination of embryos observable by a higher germination rate for light-exposed embryos (38.78%) in both varieties than for dark-exposed embryos (30.80%).

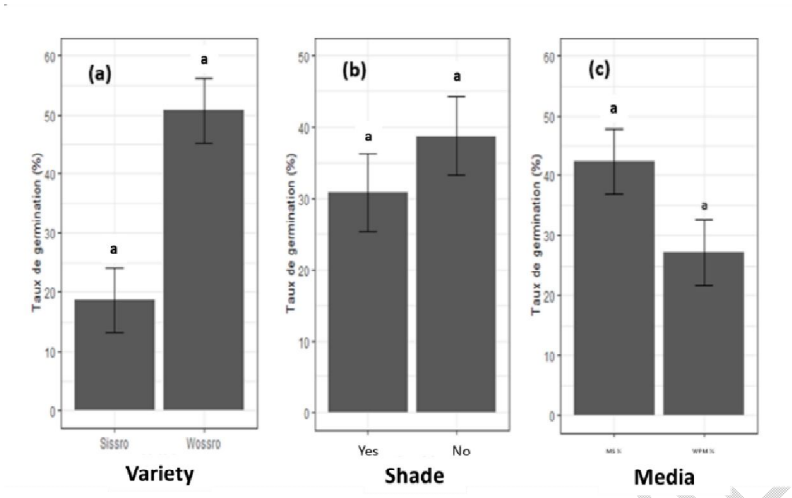


Figure 4: *In vitro* germination of embryos according to genotype effect (a), darkness effect (b) culture medium effect (c).

3.1.4. Roots and shoots elongation for zygotic embryos plantlets

The number of radicles produced by the embryos was significantly influenced by the culture medium (p= 0.012). The other factors did not show a significant influence. Indeed, rootlet formation was more pronounced on MS ¼+ 0.2 mg/L KIN medium with an average number of (3.88 rootlets) versus (0.88 rootlets) for WPM ½ culture medium at week 6 (Figure 5b). Considering the variety, the average number of radicles produced was zero for *Wossro* variety versus (1.5 radicles) for *Sissro* variety at six weeks. the zygotic embryos germination was shown in figure 6.

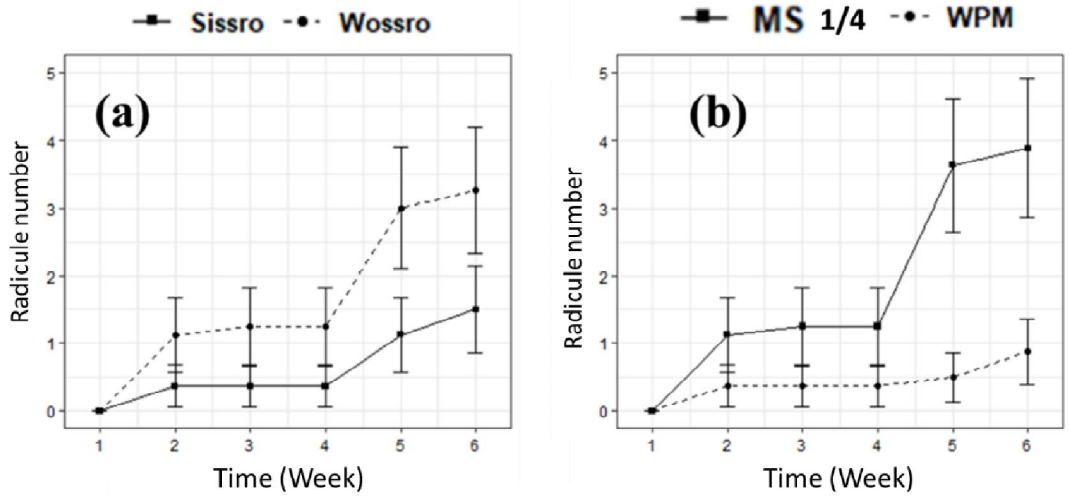


Figure 5: Evolution of the number of radicles according to the varieties (a) and the culture media (b).

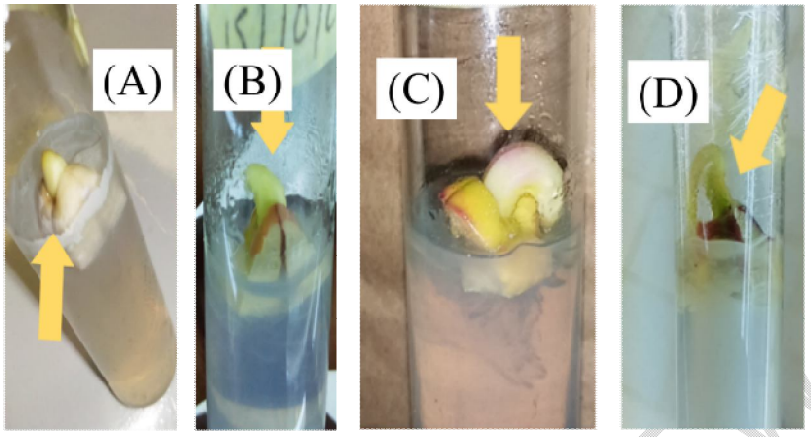


Figure 6: Phases of embryo swelling (A) and (B); radicle development (C); penetration of the radicle in the culture medium (D).

For the shoot elongation, the media have a significant influence in stem production ($p \leq 0.002$). The average number of stems formed on the WPM ½ culture medium was higher at the sixth week (0.62 stems) compared to, (0.5 stems) on the MS ¼ +0.2mg/L KIN medium (Figure /b). Also, stem production was not influenced by variety (p=0.999). The number of stems produced after germination of embryos in *in vitro* culture was higher in the Wossro variety with a maximum of (1.13 stems) compared to (0 stems) for Sissro variety at the sixth week stage (Figure 7a).

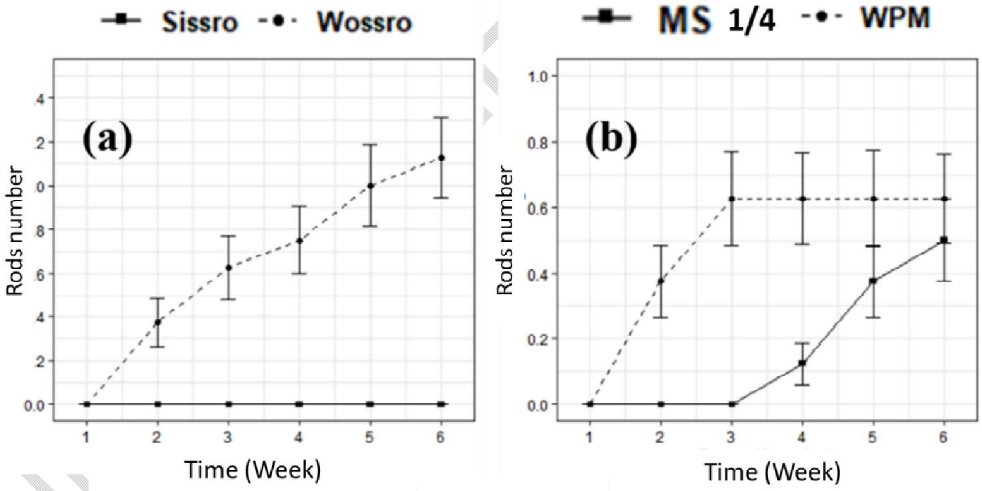


Figure 7: Evolution of the number of stems according to the varieties (a) and the culture media (b).

According to the leaves formation, the culture media have a significant influence on the production of leafy stem (p= 0.0002). Plantlets that formed stems on MS ¼ +0.2mg/LKIN medium have an average of 1 leaf, compared to a significantly zero average number for WPM ½ medium after 8 weeks (Figure 8b). Variety had a non-significant influence on leafy stem production (p= 0.99), although the Wossro variety was the only one to produce 1.12 leaves compared to 0 leaves for Sissro variety after six weeks (Figure 8a).
Figure 9 showed different steps of the growth of the plantlets regenerated with buds and zygotic embryos

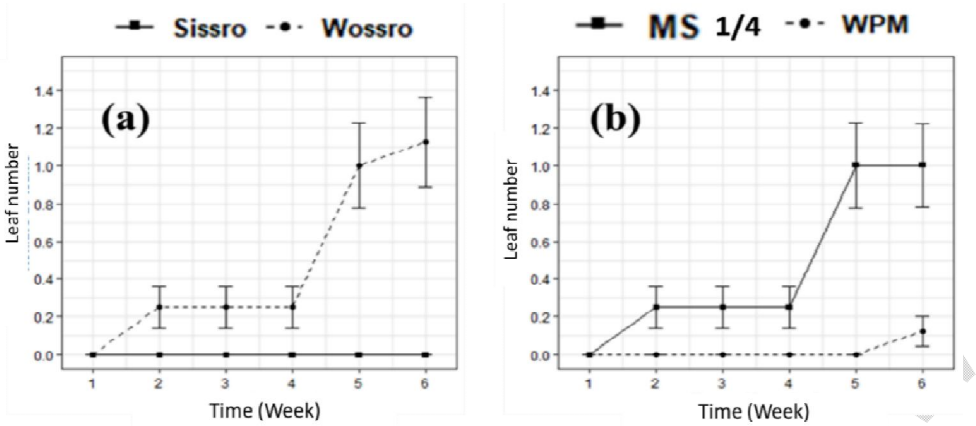


Figure 8: Evolution of the number of leaves according to the varieties (a) and the growing media (b).

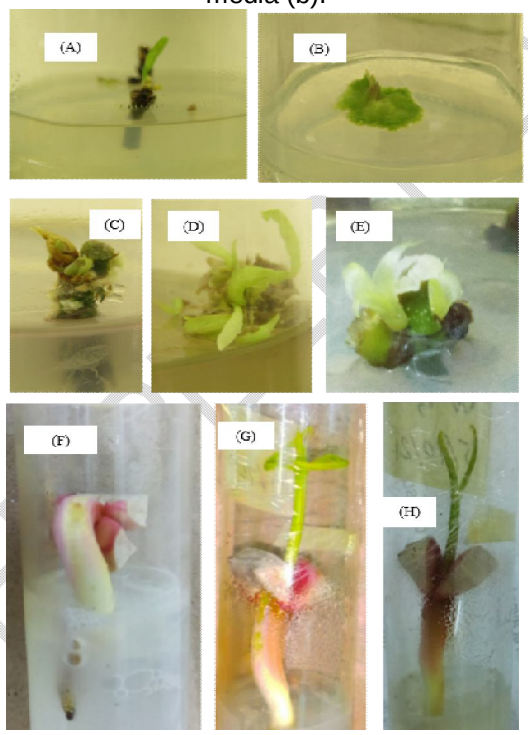


Figure 9: *In vitro* regeneration of *Irvingia gabonensis* using buds: axillary bud (A), callus formation (B) apical bud (C) shoot and leaves formation (D, E) and zygotic embryos: emergence of cotyledons after initiation of radicle development (F); shoot emergence (G); initiation stage of the first two leaves (H); leafy vitroplants rooted (I).

3.2. DISCUSSION

Plant organogenesis from *in vitro* regeneration of buds is used in biotechnology to clone and conserve initial characters from generation to generation in plant. In this study, we investigate how the topophysis of nodes can affect *in vitro* regeneration and plantlet development.

Regarding regeneration, variation was noted between the two local varieties tested and also in the topophysis of the buds. The *Wossro* variety showed a high rate of bud break. In addition, the best responses were obtained with the first and second rank axillary node buds, in contrast to the cotyledon nodes which showed very low responses to bud break. These results were similar to those of [6] who showed by working on *Pentadesma Butyracea* S. that, bud break varied with the position of nodes on the stem. Similarly, Hung and Trueman [12] showed the expression of a negative morphogenic gradient in the eucalyptus hybrid (*Corymbia torelliana* × *Corymbia. citriodora*) and [14] in *Quercus robur* L. This gradient increases from cotyledon nodes to apical nodes, and is thought to be responsible for controlling apical dominance. Hung and Trueman [12] also reported that *in vitro* proliferation of axillary nodes was better than apical nodes. Our finding is congruent with this report. [15] made the similar observation by considering explant topophysis as a determining factor in the regeneration of woody species (*Fraxinus excelsior* L.). Indeed, the same authors mentioned a significant decrease in bud break rate between the second and third distal nodes, probably due to the negative morphogenetic gradient. However, [16] found that the original position of the explant in *in vitro* culture did not influence shoot regeneration in *Leptospermum polygalifolium* and *Leptospermum scoparium*. The addition of 0.1mg/L NAA to media containing 2 mg/L BAP was able to stimulate bud break of seeded internodes [16]. The combination of NAA and BAP positively influences budbreak by callogenesis of nodes compared to the use of BAP alone [18].The bud break of *Pentadesma Butyracea* S. observed by [6] at the stage of appearance of the first leaves with the best responses on the WPM medium showed contrary results to the results observed in the present study, where the best bud break was obtained with the MS ¼ medium added with BAP (3.5 mg/L and 0.2 mg/L) combined with NAA (0.05 mg/L). This may be explained by the concentration of the growth regulator used. However, the highest KIN concentration (3.5 mg/L) produced good responses in budbreaking whereas the best were obtained with 3 mg/L KIN. Several other reasons may explain the results obtained such as the age of the explant, the level of physiological development of the seedling as mentioned by Etukudo et al. [18], who found better results with explants derived from embryo germination compared to cuttings taken from adult trees which showed a high infection rate. In the present study, bud regeneration rate was generally low. Paluku et al. [19] made the same observations during *in vitro* regeneration of *Cola acuminata*. This study revealed that the production of friable, greenish callus in the two local varieties tested auguring a prospect for intense proliferation through regeneration of neoformed organogenesis callus.

According to [20], "the germination of a seed is defined as the sum of the events that begin with the imbibition and end with the emergence of a part of the embryo". The results obtained showed that only the 'variety' factor had a highly significant influence (p= 0.0003), on the *in vitro* germination of embryos in favour of the *Wossro* variety. The parameters of culture medium and light did not show significant influences at the 5% threshold. Those results can be explained by the effect of the genotype in the evaluation of the germination potential of the embryos. Those results were contradictory to those of [10] who demonstrated that the *in vitro* germination of *Irvingia gabonensis* embryos was strongly dependent on the culture medium. Although these authors did not consider the variety factor in their experiments, the best results were obtained on the same culture medium (MS ¼ + 0.2 mg/L KIN) for bud break and rhizogenesis, even though the WPM medium is more recommended for the culture of woody plants such as *Olea europeae* [21]; *Tectona grandis* [22]; *Lannea microcarpa* ([23]. Also, [24] obtained different results comparing different doses of regulators such as BAP (0.05 mg/L), NAA (0.05 mg/L) and coconut water filtrate (20-25%). These authors obtained 60% viable explants, which would therefore explain the varietal sensitivity of embryos to certain nutrients for their development as mentioned by Renaudin [25]. Exposure of embryos to darkness showed that, light promoted *in vitro* germination. However, the influence of this factor was not significant (p= 0.31). In fact, the germination rate for embryos exposed to light was higher in both varieties than for embryos

exposed to darkness. Those results can be explained by the insensitivity of *in vitro* grown embryos to light in some woody species especially those with recalcitrant seeds. [25] reached the same conclusions in germination tests on *Lathraea clandestina* showing an indifference of embryos to the presence or absence of light for *in vitro* culture. However, [26] mentioned the influence of photoblastic phenomenon including sensitivity to light radiation among the factors with significant influence in *in vitro* germination of *Jasminum fruticans* and *Lawsonia inermis* by Johnson et al., [27]. The growth of *in vitro* germinated embryos is strongly related to the composition of the culture medium. Indeed, the best growth responses of germinated embryos were obtained on MS ¼ + 0.2 mg/L KIN medium. Those results can be explained by the composition of the culture media tested because, once they had been isolated from their nutrient reserves, the germinated embryos automatically became dependent on the culture media as the only substrates available to them. Their compositions would then affect the expression of the genetic program of the species considering its growth. Some authors mentioned the effectiveness of cytokinins such as BAP, KIN and coconut milk extract on the rapid budding of germinated embryos ([24]. Their results could be related to the involvement of cytokinins in cell division and differentiation processes on the one hand and on the other hand the presence of (zeatin-O-glucoside) and (dihydrozeatin-O-glucoside) derivatives of Zeatin, a cytokinin known to promote apex budding [28].

4. CONCLUSION

The *in vitro* regeneration of the African bush mango tree is influenced by the topophysic of the buds and the composition of the culture medium. Indeed, axillary buds showed the best response performance at bud break on ¼ MS + 3.5 mg/L BAP + 0.05 mg/L ANA medium. The *in vitro* germination of embryos is more efficient in darkness and the MS ¼ + 0.2 mg/L KIN culture medium is more favorable in terms of germination and growth of the regenerated plantlets. These findings will help to improve the regeneration rate and plantlet production of the African bush mango tree which could be used by the farmers after acclimatization.

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