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In vitro Organogenesis in Cotton (*Gossypium spp*) for *Ex-situ* Conservation Issue

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Authors' contributions

This work was carried out in collaboration among all authors. Authors GHTC and MGS conceptualized the research work and analyzed the data. Author GHTC and SSH designed and validated the research methodology; author GHTC supervised the work; authors MGS and TDS conducted the research and collected data; author GHTC and JAH wrote the manuscript; author JAH reviewed and edited the manuscript; author CA acquired the fund, administrated the project and provided the resources; all authors read, corrected and approved the manuscript.

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ABSTRACT

Background: Monovarietal cultivation of cotton plant allowed the genetic erosion for traditional cotton varieties that proceed essential traits mainly involved in cotton genetic breeding. These varieties need to be preserved for future used. This study aims to evaluate the effect of gibberellic acid on cotton seed germination and the effect of Benzylaminopurin (BAP), Kinetin (KIN), α-naphthalene acetic acid (NAA) and activated charcoal (CA) on cotton seedlings growth obtained from different type of explants.
Methodology: The seeds of three improved varieties (KET782, ANG956, OKP768) and five local

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varieties (Q62, Q64, Q85, Q88, Q92) were germinated with different concentration of gibberellic acid (GA_3). Different explants were collected from the germinated plantlets and cultivated in different culture media containing plants growth regulators and activated charcoal in different concentration.
Results: We found that Gibberellic Acid activity on cotton seeds germination significantly varied according to the variety and GA_3 concentration in the medium. 63.33% germination rate in OKP768 was obtained on the medium containing 0.5 mg/L of GA_3 while the medium with 1 mg/l of GA_3 gave the highest seed germination in Q85 (75%), Q64 (69.17%), and ANG956 (40.83%). The plantlets regeneration rate varied within the explants in different varieties used. 100%of axillary nodes were regenerated in Q62; Q85 and Q92 varieties while 60% were regenerated with the zygotic embryos in ANG956, and OKP768. Moreover, the medium supplemented with GA_3 (1mg/l) + NAA (1 mg/l) induced the greatest number of roots (2.75 roots/plantlet) in the ANG956 and OKP768 varieties. With activated charcoal (10 g/l), ANG956 and OKP768 varieties achieved better performance with respective roots length average of 3.4 cm/plantlet and 2.1 cm/plantlet. The activated charcoal at 10 g/l highly influenced the length of roots with an average of 7.7cm in ANG956 variety.
Implication: The protocol established during this study will be useful for *in vitro* regeneration and conservation for cotton local varieties.

Keywords: Gossypium spp; plant growth regulators; activated charcoal; explant, organogenesis.

ABBREVIATIONS

NAA : Naphthalene acetic acid;
2,4-D : 2,4 dichlorophenoxyacetic;
BAP : 6-benzylaminopurine;
GA_3 : Gibberellic Acid.

1. INTRODUCTION

Cotton (*Gossypium spp*) is one of the important plant genetic resource which can produce fiber that is cultivated in the world [1]. Its cultivation generates an important income for many countries where it contributes to development and people employment in rural and industrial sectors [1]. Except varieties with industrial importance, some traditional varieties are involved in several cultural use [2]. Monovarietal cultivation of elite varieties allowed the genetic erosion for traditional cotton varieties that proceed essential traits mainly involved in cotton genetic breeding [3]. Therefore, it becomes imperative to maintain in *ex situ* collection of the cotton varieties which has great socio-cultural and economic importance. For this purpose, in vitro organogenesis influencing factors in cotton is the first challenge to be addressed. However, the modern form of preservation through *in vitro* culture takes account of traditional varieties and should better ensure *in vitro* regeneration conditions. In general, certain endogenous factors including genotype, type of explant [4,5,6], and exogenous factors such as the type and concentration of growth regulators limit this *in vitro* organogenesis [6]. In addition, activated charcoal [7] appear to be determining in the development of organs *in vitro* conditions. Several types of explant including hypocotyls, apices and embryos appear to be essential for the direct *in vitro* regeneration of cotton [8]. It appeared essential to evaluate the effects of these types of explants and regeneration factors such as Gibberellic Acid, Naphthalene Acetic Acid, Benzylaminopurine, Kinetin and activated carbon on the *in vitro* regeneration of cotton varieties.

2. MATERIALS AND METHODS

2.1 Plant Material and Explant Sterilisation

Cotton seed of three improved varieties (KET782, ANG956, OKP768) were collected from Institute of cotton research of Benin and seed of five traditional varieties (Q62, Q64, Q85, Q88, Q92) were also collected in farmers' fields, in rural houses, in forests, and at the edges of streets in the Guinean-Sudanese and Sudanese zones of Benin. Three different explants were tested in this experiment. First, the seeds of each variety were delinted with sulfuric acid and maintained dipping in tap water for 24 hours to break their dormancy [9]. Thereafter, seeds were sterilized with ethanol 70° for 1 min and mercuric chloride solution 1% was added with a few drops of Tween 20 for 20 min and finally rinsing three times in sterile distilled water under the lamina flow. Thereafter, zygotic embryos were extracted from sterilized seeds following the protocol of Pathi and Tuteja, [6]. Finally explant was constituted of axillary nodes collected from the established cotton plant of each varieties under greenhouse. The axillary nodes were sterilized

with ethanol 70° for 30 sec and mercuric chloride solution 1% added with Tween 20 for 10 min and rinsed three times with sterile distilled water under the lamina flow.

2.2 Culture Conditions and Hormonal Treatments

The sterilized seeds were cultivated in three germination media without mineral salts. The media were composed to 6% of gelose melted in distilled water with different concentration of gibberellic acid (GA_3: 0 mg/l; 0.5 mg/l, and 1 mg/l) follow by regular observation frequency of three days after initiation [10]. The explants regeneration and growth phase was carried out on nine Murashigue and Skoog [11] (MS) modified media different in their concentration of auxins and cytokinins (M0, M1, M2, M3, M4 M5, M6, M7 and M8) contained in common 30 g/l of sucrose, GA_3 (1mg/l) and gelose (7g/l) [5]. M0 is the controlled medium devoid of auxins and cytokinins. The other eight remain media contained a combination of cytokinin: 2 mg/l 6-benzylaminopurine or Kinetin at 0.5mg/l and 1mg/l α-naphthalene acetic acid. For studding effect of activated charcoal, media contained two different concentrations of activated charcoal (5mg/l and 10mg/l) with the combination of Kinetin (2mg/l) + NAA (0.5mg/l) were used [6]. After mixing media reagents, the pH was adjusted at 5.7 ± 0. 1.

The media were dispensed in 20 ml aliquots into culture vessels and then autoclaved at 1.06 kg/cm^2 and 121 °C for 15 min. The 5 explants were cultured per tubes and incubated under white fluorescent light of 5000 lux, 16 h photoperiod, and 80% of relative humidity in culture room.

2.3 Data Analysis

Binary logistic Regression test (BLR) was performed for modelling the germination capacity of seeds. Different analytic parameters including seeds germination rate, effect of growth regulators on the root length were suggested to ANOVA with GLM test. Cotton explants response *in vitro* was evaluated through Chi-square test for independence and Poisson regression was on the leaf and root number variation according the growth regulators. All analysis were performed using XLSTAT v.2014.

3. RESULTS

3.1 Effect of Gibberellic Acid on *in vitro* Germination of Cotton Seeds

A significant difference ($P < 0.001$) was observed in seed germination rate and the kinetics in the growth among the tested varieties (Table 1). Indeed, up to 50% of germination was found with the seed of OKP768, Q64, Q85, and KET782 varieties three days after seeding while 70% of seed were germinated in KET782 variety and 10% were germinated in Q88 whatever the concentration of GA_3 in the medium. After twelve (12) days, seed germination rate was not significantly varied in different varieties (Fig. 1). The highest rate of germination was found in KET782 (80%) and Q62 (49.17%) regenerated on the medium without GA_3. The medium containing 0.5 mg/l of GA_3 gave 63.33% of seedlings in OKP768 variety while the medium containing 1 mg/l GA_3gave 75%, 69.17%, and 40.83% respectively in Q85, Q64, and ANG956 varieties (Fig. 2).

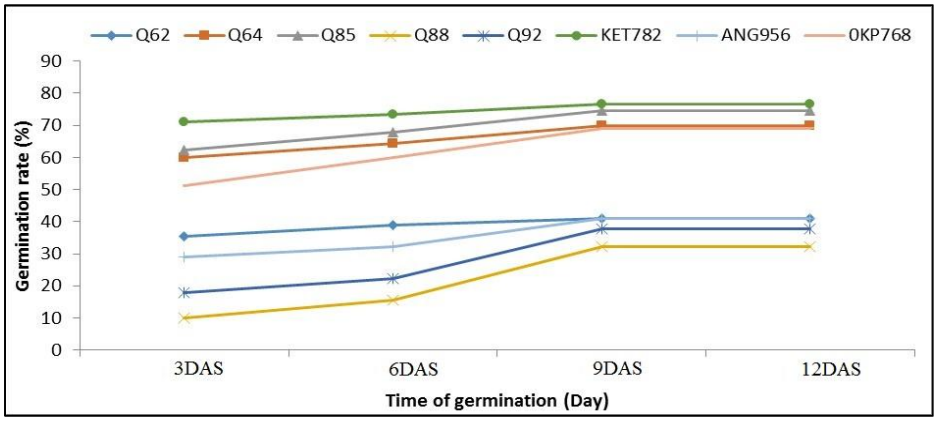

Fig. 1. Germination kinetics associated to GA_3 concentrations

Table 1. Analysis of variance of germination rate in Cotton varying GA_3 concentration

Source	DDL	SC	MC	F	Pr > F
Time	3	9252.78	3084.26	7.85	< 0.001***
Varieties	7	104950	14992.86	38.18	< 0.001***
[GA_3]	2	1034.03	517.01	1.32	0.2705ns
Times *Varieties	21	2191.67	104.37	0.27	0.9996ns
Time*[GA_3]	6	149.31	24.88	0.06	0.9990ns
Varieties*[GA_3]	14	6193.75	442.41	1.13	0.3368ns
Times*Varieties*[GA_3]	42	2022.92	48.16	0.12	0.9990ns

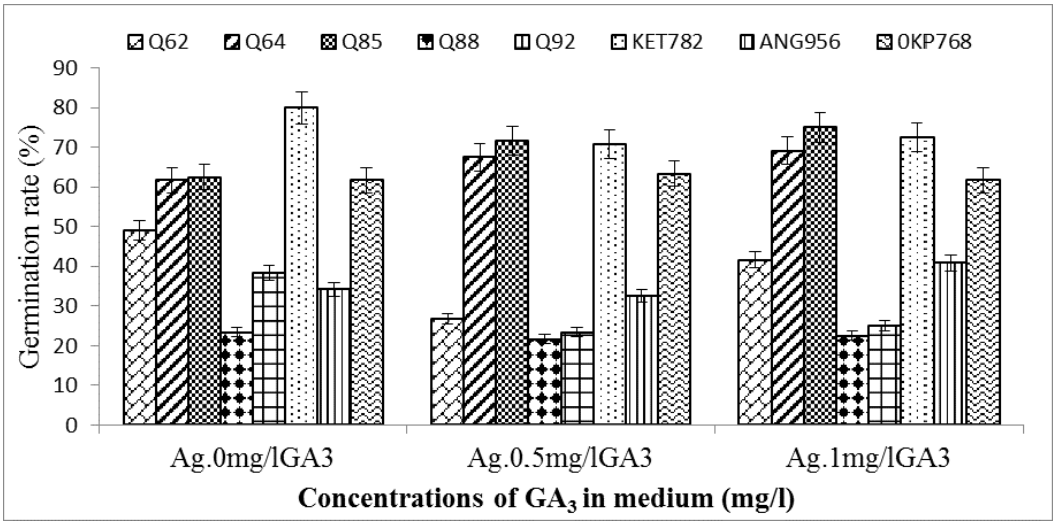

Fig. 2. Effect of GA_3 on germination rate of tested Cotton varieties.

3.2 Choice of Adapted Explant for *in vitro* Regeneration

The type of explant significantly influence ($P < 0.05$) the rate of regeneration of cotton plants. The results showed a high dependence between the type of explant and regeneration (Table 2). Hundred percent (100%) of axillary nodes were regenerated in Q62; Q85 and Q92 varieties while 60% were regenerated with the zygotic embryos in ANG956, and OKP768 (Fig. 3). Only axillary nodes and zygotic embryos were successful for plant regeneration on the tested media (Fig. 4).

3.3 Effects of Benzylaminopurine, Kinetin, and α-Naphthalene Acetic Acid on Cotton Varieties Regeneration

The results showed that the number of leaves formed was significantly influenced by the different hormonal combinations ($p = 0.0200$), varieties ($p < 0.001$) as well as explants ($p < 0.001$) (Table 3). Each hormonal combinations tested stimulated leaf formation in all varieties especially with axillary nodes. Thus, on M0, Q64, OKP768 and KET782 varieties presented the highest average number of leaves with respective averages of 2.5, 2.75 and 3.25 leaves per plantlet while the lowest average number of leaves was obtained in Q88 and ANG956 varieties (0.25 sheet / plantlet). On the M1 medium (M0 + 2 mg/l BAP), the varieties Q88, ANG956 and OKP768 gave more leaves with respective averages of 2.0, 2.25 and 3.5 sheets/plantlet and the lowest number of sheet was obtained with Q85 varieties (0.25 sheet/plantlet).Furthermore, it was noted that on the media M2 (M0 + 2 mg/l KIN), M3 (M0 + 0.5 mg/l NAA) and M4 (M0 + 1 mg/l NAA), the leaves were formed in all varieties using axillary nodes as explant but in low number and decrease for most varieties on culture media M5 (M0 + 2mg/l BAP + 0.5mg/l NAA), M6 (M0 + 2mg/l BAP + 1mg/l NAA), M7 (M0 + 2mg/l KIN + 0.5mg/l NAA) and M8 (M0 + 2mg/l KIN + 1mg/l NAA) (Table 3).

For all cultivars, the number of roots were significantly ($p < 0.001$) affected by the hormonal combinations and the type of explant. Different hormonal combinations have stimulated the roots

formation in all varieties using zygotic embryos explants. The medium supplemented with 1mg/l GA_3 induced roots only in Q88, ANG956 and OKP768 varieties with respectively as mean number 0.25, 1.25 and 1.25 roots/plantlet. The hormonal combination GA_3 (1mg/l) + KIN (2mg/l) induced roots in Q88 (1.25 roots/plantlet), KET782 (0.75 roots/plantlet), ANG956 (1 root/plantlet) and OKP768 (2.5 roots/plantlet) varieties. As for, using the hormonal combination GA_3 (1mg/l) + BAP (2mg/l), only KET782 and OKP768 varieties formed roots in respective average of 1.75, 0.25. However, the hormonal combinations GA_3 (1mg/l) + NAA (0.5mg/l) and GA_3 (1mg/l) + BAP (2mg/l) + NAA (1mg/l) induced roots in all varieties with the high average number of roots in Q85 (4.5). The medium supplemented with GA_3 (1mg/l) + NAA (1 mg/l) induced the greatest number of roots (2.75 roots/plantlet) in the ANG956 and OKP768 varieties (Table 3).

Table 2. Logistic regression on the choice of explants in different varieties

Source	DDL	Khi² (LR)	Pr > LR
Accessions	7	3.0633	0.0801 ns
Types of explant	2	43.9801	< 0.001***
Accessions* Type of explant	14	2.3167	0.1832ns

*Legends :DDL : Degré de liberté ; ns : non significatif ; *** : différence très hautement significative (p < 0.001).*

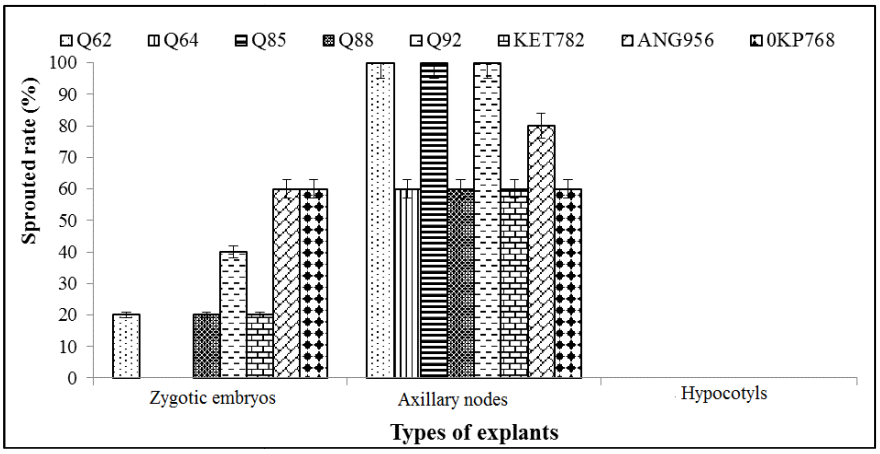

Fig. 3. Rate of shoot sprouted in different explants of cotton varieties

Fig. 4. Plantlets of Cotton. A: plantlets regenerated through zygotic embryos; B: plantlets regenerated through axillary nodes

Table 3. Effects of different growth regulators on leaves and roots number

Plant Growth Regulators	Explant	Q62		Q64		Q85		Q88		Q92		KET782		ANG956		0KP768	
		NF	NR	NF	NR	NF	NR	NF	NR	NF	NR	NF	NR	NF	NR	NF	NR
M0	EmZyg	0a	0a	0a	0a	0a	0a	0a	0.25a	0a	0a	0a	0a	1.25ab	1.25ab	0a	1.25ab
	N.Ax	1b	0a	2.5bc	0a	1.5ab	0a	0.25a	0a	1.5bc	0a	3.25cd	0a	0.25a	0a	2.75de	0a
M0.2mg/lBAP	EmZyg	0a	0a	0a	0a	0a	0a	0.5a	1.25ab	0a	0a	0a	0.75b	1.25ab	1b	0.75b	2.5bc
	N.Ax	1.5ab	0a	1.75cd	0a	0.25a	0a	2d	0a	1.25bc	0a	1b	0a	2.25d	0a	3.5e	0a
M0.2mg/lKIN	EmZyg	0a	0a	0a	0a	0a	0a	0a	0a	0a	0a	0a	1.75ab	0a	0a	0a	0.25a
	N.Ax	0.75b	0a	1.75cd	0a	0.75b	0a	0a	0a	0.75b	0a	1b	0a	0a	0a	0.75b	0a
M0.0.5 mg/l1 NAA	EmZyg	0.75b	0a	1.25ab	3.25cd	0.5a	4.5b	0a	2bc	0.75b	0a	0a	2.75bc	0a	2.75bc	0.25a	2.75bc
	N.Ax	1b	0a	1b	0a	0.75b	0a	0.5a	0a	0.5a	0a	0.5a	0a	1b	0a	0.5a	0a
M0.1 mg/l1 NAA	EmZyg	0a	0a	0a	0a	0.5a	0a	0a	1.25ab	0a	0.25a	0a	0a	0a	3.75cd	1b	3.75cd
	N.Ax	0.5a	0a	0.5a	0a	0.75b	0a	0.5a	0a	0.5a	0a	1b	0a	0a	0a	1.25ab	0a
M0.2 mg/l BAP.0.5 mg/l NAA	EmZyg	0a	0.25a	0a	0a	0a	0a	0a	0a	0a	0a	0a	0a	0a	0.25a	0.5a	0.25a
	N.Ax	0.5a	0a	1.5ab	0a	1.25ab	0a	0.75b	0a	1.25bc	0a	0.5a	0a	0.5a	0a	1.5bc	0a
M0.2 mg/l BAP.1 mg/l NAA	EmZyg	0a	1.25b	0a	0a	0a	0a	0a	0.25a	0a	0a	0.75b	2bc	1.75c	2.5bc	1b	2.5bc
	N.Ax	0.75b	0a	0.5a	0a	1.25ab	0a	1.5c	0a	0.25a	0a	0.25a	0a	0a	0a	0a	0a
M0.2 mg/l KIN.0.5 mg/l NAA	EmZyg	0a	0a	0a	0a	0a	0a	0a	0a	0.5a	0a	0a	0.25a	0.5a	3.5cd	2c	3.5cb
	N.Ax	1b	0a	1.5ab	0a	1.25ab	0a	0.5a	0a	2d	0a	1.25cd	0a	0a	0a	0.5a	0a
M0.2 mg/l KIN.1 mg/l NAA	EmZyg	0a	0a	0a	0a	0a	0a	0a	0a	0a	0a	0a	0.5a	0.25a	0.5a	1b	0.5a
	N.Ax	0.5a	0a	1.5ab	0a	0.75b	0a	0.25a	0a	0.75b	0a	1b	0a	1.25ab	0a	0.5a	0a

Legends: EmZyg: Zigotic embryos; N.Ax: Axillary nodes; SL: Shoot length; RL: Root length; BAP: Benzyaminopurine; NAA: Naphthalene acid acetic

Regarding shoot lengths, the results showed that hormonal combinations (p = 0.0062), varieties (p = 0.001) and explants (p <0.001) had a significant effect on shoot elongation. Thus, there is not regeneration in Q88, ANG956 and OKP768varieties through axillary nodes respectively with the hormonal combinations GA_3 (1mg/l) + KIN (2mg/l), GA3 (1mg/l) + NAA (1mg/l) and GA_3 (1mg/l) + KIN (2mg/l) + NAA (0.5 mg/l). However, in Q62, Q64, Q85, Q92 and KET782 varieties, all hormonal combinations produced plantlets through axillary nodes. The highest average of shoot lengths was obtained with the hormonal combinations GA_3 (1mg/l); GA_3 (1mg/l) + NAA (1mg/l); GA_3 (1mg/l) + KIN (2mg/l) + NAA (0.5mg/l) respectively in Q85 (1.35cm), KET782 (1.23 cm) and OKP768 (1 cm) varieties. The highest average (4.5 cm) of shoot lengths in OKP768 variety was obtained with the hormonal combinations GA_3 (1 mg/l) + BAP (2 mg/l) while the lowest average shoot length was obtained with GA_3 (1mg/l) + KIN (2mg/l) + NAA (1 mg/l). The medium containing the hormonal combination GA_3 (1 mg/l) + BAP (0.5 mg/l) produced plantlets with higher shoot lengths average (2.67 ± 0.47 cm) while the highest average (3.3 cm) of shoot lengths was obtained with 1mg/l GA_3 + 0.5mg/l NAA hormonal combination in Q62 variety (Table 4).

For all cultivars, the roots length were significantly (p < 0.001) affected by the hormonal combinations and the type of explants. Thus, the average of roots length varied from 0 to 8.25 cm. The highest average was observed using the hormonal combinations GA_3 (1mg/l) + KIN (2 mg/l) + NAA (1mg /l) ; GA_3 (1mg/l) + BAP (2mg/l) and GA_3 (1mg/l) + BAP (2mg/l) + NAA (0.5mg/l) in respective average of 5.63 cm, 7.5 cm, and 8.25 cm in OKP768 variety using zygotic embryo explant. The same combinations led to little or no root elongation in the other varieties (Table 5). In addition, the hormonal combination GA_3 (1mg/l) + NAA (0.5 mg/l) promoted root elongation in most varieties with a maximum average of roots length of 4.33 cm in Q64 variety (Table 4).

3.4 Effect of Activated Charcoal on *in vitro* Growth of Cotton Varieties

Analysis of variance showed that activated charcoal has a significant difference (*P*<0.001) on the number of leaves, the number of roots , and the length of roots while the difference was not significant with the shoots length (*P*=0.234) in all varieties. In the absence of activated charcoal, all varieties regenerated leaves with the highest average number (2.5 leaves/plantlet) in Q85 and (2 leaves/plantlet) in KET 782 varieties. As well, with 5g/l or 10g/l activated charcoal concentration, it was ANG956 variety that induced more leaves with respective averages of 1.5 leaves/plantlet and 2.25 leaves/plantlet. The medium without activated charcoal did not induced roots while the addition of activated charcoal 5g/l induced roots in KET782, ANG956, 0KP768, and ANG956 varieties with the highest average numbers observed in KET782 (6.5 roots/plantlet) and ANG956 (4.75 roots/plantlet) varieties. With 10g/l activated charcoal in the medium, the average number of roots is high in KET782 (4.25 roots/plantlet) and OKP768 (8.25 roots/plantlet) varieties. As concerning the length of roots, the medium supplemented with activated charcoal (5 g/l), induced the highest average of roots lengths in Q64, KET 782, and ANG956 varieties with respective average 2.6 cm/plantlet; 2.6 cm/plantlet, and 5.3 cm/plantlet. ANG956 had maintained performance by increasing the activated charcoal to 10g/l with an average roots length of 7.7 cm/plantlet followed by the OKP768 variety (6.75 cm/ plantlet). All varieties produced shoots in the medium without activated charcoal with the greatest average roots length obtained in Q85 (1.6 cm/plantlet) and KET782 (2.6 cm/plantlet) varieties. Two varieties regenerated shoots in the medium supplemented with activated charcoal 5g/l with roots length average of 3.025 cm/plantlet; 3.675 cm/plantlet obtained respectively from Q64 and KET782 varieties. With activated charcoal (10 g/l), ANG956 and OKP768 varieties achieved better performance with respective roots length average of 3.4 cm/plantlet and 2.1 cm/plantlet (Fig. 5).

4. DISCUSSION

Study on *in vitro* germination of cotton plant noted a very highly significant difference (P <0.001) not only between varieties but also between the germination period. Although seeds from all varieties germinated three days after *in vitro* seeding with (0 mg/l; 0.5 mg/l or 1mg/l) GA_3 concentrations, 0KP768; Q64; Q85 and KET782 varieties revealed a germination rate above 50%. The seeds of Q64, Q85 and ANG956 varieties had a germination rate proportional to GA_3 concentration. On the other hand that of Q62, Q92 and KET782 varieties in which the germination rate was high on the culture medium without GA_3. Apart from the gibberellin identified by Santner et al. [12] involved in seed germination, confirmed on Q64, Q85 and

Table 4. Effects of different growth regulators on shoot and root length

Plant Growth Regulators	Explant	Q62		Q64		Q85		Q88		Q92		KET782		ANG956		0KP768	
		SL	RL	SL	RL	SL	RL	SL	RL	SL	RL	SL	RL	SL	RL	SL	RL
M0	EmZyg	0a	0a	0a	0a	0a	0a	0.7c	0.48ab	1.05d	0a	0a	0a	1.83c	0.75b	2.42bc	2.35bc
	N.Ax	0.28b	0a	0.28b	0a	1.35d	0a	0.13a	0a	0.7bc	0a	1.25c	0a	0.2a	0a	1.05c	0a
M0.2mg/lBAP	EmZyg	0a	0a	0a	0a	0a	0a	0.65c	0.45ab	0a	0a	0.5b	1.5ab	1.98c	0.68b	4.5d	7.5d
	N.Ax	0.38b	0a	0.25b	0a	0.33a	0a	0.8bc	0a	0.68bc	0a	0.3a	0a	0.25a	0a	0.15a	0a
M0.2mg/lKIN	EmZyg	0a	0a	0a	0a	0a	0a	0a	0a	0a	0a	0.75b	1ab	0a	0a	3.7bc	1.03bc
	N.Ax	0.78ab	0a	0.85c	0a	0.33a	0a	0a	0a	0.12a	0a	0.25a	0a	0a	0a	0.43b	0a
M0.0.5 mg/l1 NAA	EmZyg	3.3c	2.5b	2.67bc	4.33b	0.45b	0.8b	0a	0a	1.15d	2.2b	0a	0a	2.67d	1.35c	1.75c	0.78b
	N.Ax	0.28b	0a	0.23b	0a	0.8c	0a	0.02a	0a	0.12a	0a	0.77b	0a	0.47b	0a	0.28a	0a
M0.1 mg/l1 NAA	EmZyg	0a	0a	0a	0a	0.38b	0.6ab	0.95bc	0.13b	0a	0a	0a	0a	0a	0a	3.75bc	4.33d
	N.Ax	0.15a	0a	0.18a	0a	0.1a	0a	0.15a	0a	0.05a	0a	1.23d	0a	0a	0a	0a	0a
M0.2 mg/l BAP.0.5 mg/l NAA	EmZyg	0a	0a	0a	0a	0a	0a	0a	0a	0a	0a	1.25d	0.75b	1.63c	0a	3.83bc	8.25d
	N.Ax	0.13a	0a	0.25b	0a	0.33b	0a	0.1a	0a	0.48b	0a	0.35a	0a	0.03a	0a	0.05a	0a
M0.2 mg/l BAP.1 mg/l NAA	EmZyg	0a	0a	0a	0a	0.75c	0.6ab	0a	0a	0a	0a	1.25d	0.88b	2.65d	2.75cb	1.9c	0.95b
	N.Ax	0.4b	0a	0.13a	0a	0.57b	0a	0.3b	0a	0.17a	0a	0.3a	0a	0.03a	0a	0.55b	0a
M0.2 mg/l KIN.0.5 mg/l NAA	EmZyg	0a	0a	0a	0a	0a	0a	0a	0a	0.67bc	0.78ab	0a	0a	0.63b	0.58b	2.33bc	2.55bc
	N.Ax	0.28b	0a	0.15a	0a	0.15a	0a	0.05a	0a	0.38b	0a	1.1d	0a	0a	0a	1c	0a
M0.2 mg/l KIN.1 mg/l NAA	EmZyg	0a	0a	0a	0a	0a	0a	0a	0a	0a	0a	0a	0a	0.6b	0.45b	1.15c	5.63d
	N.Ax	0.28b	0a	0.53b	0a	0.08a	0a	0.18a	0a	0.35b	0a	0.98c	0a	0.55b	0a	0.33b	0a

Legends:EmZyg: Zigotic embryos; N.Ax: Axillary nodes; SL: Shoot length; RL: Root length; BAP: Benzyaminopurine; NAA: Naphthalene acid acetic

Table 5. Analysis of variance of leaves and roots number and shoot and roots length in different varieties

Accessions	NL			NR			SL (cm)			RL (cm)		
	MSCA	M5CA	M10CA	MSCA	M5CA	M10CA	MSCA	M5CA	M10CA	MSCA	M5CA	M10CA
Q62	1	0	0.5	0	0	1.25	0.4	0	1.825	0	0	1.95
Q64	0.5	1.5	0.5	0	4	0.5	0.15	3.025	0.725	0	2.6	2.6
Q85	2.5	0	0.25	0	0	1.5	1.6	0	0.75	0	0	0.625
Q88	0.5	0	0	0	0	0	0.45	0	0	0	0	0
Q92	1.5	0	0	0	0	0	0.45	0	0	0	0	0
KET782	2	1.75	1	0	6.5	4.25	2.6	3.675	1.7	0	2.6	2.8
ANG956	1.5	1.5	2.25	0	4.75	3.5	0.45	2.7	3.4	0	5.3	7.7
0KP768	1.5	0.25	1.5	0	1	8.25	1.15	2.075	2.1	0	2.3	6.75
Moy	1.37	0.62	0.75	0	2 .03	2.40	0.9	1.43	1.31	0	1.6	2.8
R²	52.77			53.89			51.29			60.8		
F	3.5750			3.7402			3.3691			4.9629		
Pr > F	0.001**			< 0.001***			0.234ns			< 0.001***		

Legends: NL: Number of leaves; NR: Number of root; SL: Shoot length; RL: Root length; MSCA: Medium without activated charcoal; M5CA: Medium + 5 g/l activated charcoal; M10CA:Medium + 10 g/l activated charcoal…

Fig. 5. Zygotic embryo of ANG956 variety on charcoal 10 g/l

ANG956 varieties, the vigor of seeds demonstrated by Matthews and Pawel, [13] is noted for Q62, Q92 and KET782 varieties. Thus, other factors linked to dormancy are not favorable to the germination process [14].

Axillary nodes and zygotic embryos significantly influenced the ability to regenerate *in vitro* (P <0.001) of almost all varieties with 100% axillary node regeneration in all varieties. This ease of axillary nodes regeneration in the presence of meristematic tissues unlike zygotic embryos which are already in differentiation. Hypocotyls lacking meristematic cells and embryos have any capacity for regeneration. However, an insignificant interaction exists between the type of explant and more in plants growth regulators. In the microboutures with axillary nodes, the culture media supplemented with 2 mg/l KIN and the 1 mg/l GA_3+2 mg/l BAP combination proved

to be the most efficient for the neoformation of leaves, particularly in the OKP768 variety. These results therefore reveal the importance of cytokinins in stimulating the formation of aerial organs. Ahanhanzo et al. [15] revealed that BAP appears to be the best adapted to axillary bud burst in Dioscorea Sp. Also, the production of auxin in the nodes, in combination with a cytokin, is favorable to the response of the axillary nodes [16]. On the other hand, with zygotic embryos, it is rather the 0.5 mg/l NAA in absence of cytokinin that it had neoformation of the shoots with a maximum length of 3.3 cm in Q62 variety. It has been observed that the microboutures from the axillary nodes are not suitable for rhizogenesis. This observation was linked to the difficulty of ligneous plants for *in vitro* regeneration [17]. These results therefore indicate that caulogenesis, phylogenesis and rhizogenesis are determined by the type of explant cultured as well as the dose of the plant growth regulators tested. Saleil et al. [18] found that the nature and dose of the plant growth regulators had determined significant growth variations on certain genotypes of Dioscorea sp. In addition, the results also showed that the cytokinins, in this case BAP, alone or combined with NAA, induced fewer roots whereas with 0.5 mg/l NAA produced the highest rooting rate, which is consistent with the work of Jacoboni [19] on the olive tree and those of Andrade et al. [20] on *Lavandula vera* where the roots differentiate better with NAA. From all these results, it emerges that the response of explants to cytokinins action depends on the genotype as observed by Cacaï et al. [21] on cassava varieties. 5g/l and 10g/l Activated charcoal slowed leaf formation and shoot length in Q85, Q88 and Q92 varieties. It appears that activated charcoal, especially at high doses, has an inhibitory effect on the growth *of Pinus banksiana Lamb* seedlings [22]. On the other hand, at Q64, ANG956 and OKP768 varieties, the addition of 5mg/l or 10mg/l activated charcoal considerably increased the number of roots, the length of the shoot, and the length of the main root. Different varieties tested indeed have a variable sensitivity to activated charcoal. This genotypic variability in the presence of activated charcoal was observed by Feyissa et al. [23] in the species Hagenia abyssinica. Similarly, the work carried out by Kamal and Sayyed [24] revealed that the addition of activated charcoal in the culture medium has a remarkable positive influence on the rooting of *Juglans* spp. Activated charcoal, known for its adsorbent effects *in vitro* [25], would decrease the leaf development of shoots while increasing their rate of rooting [26]. It is rightly added to culture media for *in vitro* conservation of cotton varieties [27].

5. CONCLUSION

In term of this research aimed to regenerate by organogenesis cotton (*Gossypium spp*) varieties, it brought out that MS medium supplemented with (0.5 mg/l GA_3 or 1 mg/l GA_3) favor the seeds germination. Axillary nodes and the zygotic embryos are the best suited explants for *in vitro* regeneration of cotton varieties. α-Naphthalene Acetic Acid enhanced Kinetin action for caulogenesis and rhizogenesis. The addition of 6-benzylaminopurine (BAP) improves shoot length and roots number. Although (5g/l and 10g/l) activated charcoal contribute to improve rhizogenesis, it slowed leaf formation and shoot length in different varieties. In this work, we established the procedure of *in vitro* regeneration of cotton plant for ex-situ preservation issue in local accessions. The findings can facilitate the industrial production of homogenous cotton seedlings and greatly participate to cotton transformation. It can also help for *ex situ* conservation of cotton local varieties that can be utilized in cotton breeding program.

AVAILABILITY OF DATA AND MATERIAL

All data generated or analyzed during this study are available from the corresponding author on reasonable request.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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