Agricultural Sciences, 2022, 13, 448-466
https://www.scirp.org/journal/as
ISSN Online: 2156-8561
ISSN Print: 2156-8553

Micropropagation from Juvenile Material of *Annona senegalensis* Pers.

Oumar Ba[1,2], Amadou Lamine Ndoye[1,2], Abraham Dieme[1,2], Mame Ourèye Sy[1,2*]

[1]Laboratoire Campus de Biotechnologies Végétales, Département de Biologie Végétale, Faculté des Sciences & Techniques, Université Cheikh Anta Diop, Dakar, Sénégal
[2]Laboratoire Mixte International-Adaptation des Plantes et Microorganismes associes aux Stress Environnementaux (LMI-LAPSE), IRD, ISRA, UCAD, Dakar, Sénégal
Email: *oureye.sy@ucad.edu.sn

How to cite this paper: Ba, O., Ndoye, A.L., Dieme, A. and Sy, M.O. (2022) Micropropagation from Juvenile Material of *Annona senegalensis* Pers. *Agricultural Sciences*, **13**, 448-466.
https://doi.org/10.4236/as.2022.133031

Received: February 3, 2022
Accepted: March 20, 2022
Published: March 23, 2022

Abstract

Annona senegalensis is a savannah species present in the wild in tropical and sub-tropical African regions. It is used by the local populations for its many economic, pharmacological, nutritional and ecological interests. However, the strong pressure exerted on this species as well as the increasing degradation of the ecosystems, in which it evolves, seriously compromise its sustainability and development. Conventional methods of vegetative propagation are not sufficient to ensure its durable regeneration and the renewal of endemic populations in Senegal. In this context, it was undertaken *in vitro* propagation, from 30 days old juvenile seedlings. Thus, axillary, cotyledonary and apical nodes were *in vitro* cultured in Murashige and Skoog (1962) medium (MS), with different concentrations of cytokinins and/or auxins. BAP used alone at 2 mg·L^{-1} proved to be more beneficial for micropropagation of different types of explants compared to Kinetin used alone or in combination with BAP, especially for axillary and cotyledonary nodes. The BAP-NAA combination allowed to have a better elongation of newly formed shoots. For shoots of apical and cotyledonary nodes origin, a rhizogenic induction of 5 days, with IBA at 25 mg·L^{-1}, resulted in a better rooting rate with, respectively, 75% (for 2.22 roots) and 66.67% (for 4.17 roots). For newly formed shoots of axillary origin, a 24-hour rooting induction with IBA at 50 mg·L^{-1} gave a rooting rate of 58.33% (for 2.4 roots). After 30 days of acclimatization, the survival rate was 75% for the young plants from the apices and 83.33% for those originating from the cotyledonary and axillary nodes. This protocol can therefore be adopted for the *in vitro* clonal propagation of *A. senegalensis* juvenile material.

Keywords

Annona senegalensis, Micropropagation, Juvenile Material, 6-Benzylaminopurine, 1-Naphthaleneacetic Acid, Indole-3-Butyric Acid

1. Introduction

Annona senegalensis Pers. commonly called apple-cinnamon of Senegal or African custard-apple [1] is a forest species present in the wild state in the undergrowth of the Sudanian tree savannahs as well as in the para-littoral sands from West to East Africa [2] in the tropical and sub-tropical regions [3] [4]. It is native to tropical east and northeast, west and west-central, and southern Africa, as well as southern subtropical Africa, and islands in the western Indian Ocean [2]. It is a very important species both ecologically and socio-economically. Its fleshy fruit, orange-yellow in color when ripe and fragrant is highly appreciated by local populations [5] [6] [7]. *Annona* is very popular in Africa for its taste and pharmaceutical qualities. It is also widely used in traditional African medicine [8] to treat diseases such as anemia and painful periods [9]. It is also used as a vermifuge [10], anti-bacterial [11], anti-inflammatory [12] and anti-malarial [13]. Thus, its different organs e.g. roots, branches, leaves, bark, etc. can treat various conditions [14] thanks to the many active ingredients they contain [15]. Due to its many uses, *A. senegalensis* is subject to extensive exploitation which does not leave time for a natural renewal of wild populations. This species is, therefore, threatened with extinction. In addition, the degradation of tropical forests both in terms of surface area and plant population density leads to a huge loss of plant biodiversity. Indeed, the acceleration of soil degradation [16] and desertification which are not only rife in semi-arid regions but also locally in the sub-Guinean and Central African areas, the fragmentation of stands and forest areas as well as climate change interfere very negatively with the survival of certain woody species [17] including *A. senegalensis* which is exclusively present in the wild in these regions. Under these conditions, natural seeding is disturbed, which induces random or slow growth in the best of cases [18]. In West Africa, the African custard-apple is one of the woody species whose grains quickly lose their germination power. In the natural state, the young plants, resulting from the seed germination, develop a very powerful taproot, while the aerial growth of the vegetative system remains weak, which poses problems in the nursery [19]. Regeneration by sexual means is insufficient to ensure a good rate of regeneration of the species [20] even if it is vital for genetic variability and allows a better adaptation of the species to environmental changes. Indeed, for this wild species, the slow growth of saplings is the main obstacle to its regeneration in a disturbed semiarid area. Conventional regeneration techniques must, therefore, be associated with *in vitro* vegetative propagation methods which make it possible to obtain a good rate of multiplication of the species. The *in vitro* clonal propagation being already carried out from material directly taken from old subjects of *A. senegalensis* [21], we undertook to set up an *in vitro* multiplication protocol using juvenile material from young plants obtained following the seed germination under axenic conditions. More specifically, it involved 1) determining the best type of explant, *i.e.* the most reactive, 2) examining the influence of different

types of hormones (cytokinins and/or auxins) on the different stages of vitroplant development, 3) determining the best elongation, multiplication and *in vitro* rooting media and, finally, 4) defining the best acclimatization conditions for the vitroplants obtained.

2. Material and Methods

2.1. Plant Material

Three different types of explants measuring 1 to 2 cm long and taken from 30-day-old sterile seedlings of *A. senegalensis* were tested as for explants originated from mature material [21]. These were cotyledonary nodes, axillary nodes and terminal apices. These different types of explants were transferred separately and individually into sterile glass culture tubes (150 × 25 mm) filled up with 20 mL of solidified culture medium.

2.2. Methods

2.2.1. Culture Media

The basic nutrient medium used is the complete one of Murashige & Skoog [22]. The media were solidified with 8 $g{\cdot}L^{-1}$ of agar at pH 5.7 and supplemented with 30 $g{\cdot}L^{-1}$ of sucrose. Growth regulators were added or not into the different media. The composition of the different hormonal concentrations tested alone or in combination in the different culture media is given in Table 1. All the culture media were tested in the presence of 2 $g{\cdot}L^{-1}$ of activated charcoal. The hormonal treatments were multiplied by a factor of 10 due to the adsorbing action of the activated charcoal on phytohormones.

Table 1. Composition of the different culture media used for the micropropagation of the juvenile material of *Annona senegalensis*.

Media Nomenclature	Hormonal Combinations
M0	MS0
M1	MS + BAP 1 $mg{\cdot}L^{-1}$
M2	MS + BAP 2 $mg{\cdot}L^{-1}$
M3	MS + BAP 5 $mg{\cdot}L^{-1}$
M4	MS + KIN 1 $mg{\cdot}L^{-1}$
M5	MS + KIN 2 $mg{\cdot}L^{-1}$
M6	MS + KIN 5 $mg{\cdot}L^{-1}$
M7	MS + BAP 1 $mg{\cdot}L^{-1}$ + KIN 1 $mg{\cdot}L^{-1}$
M8	MS + BAP 2 $mg{\cdot}L^{-1}$ + NAA 0.1 $mg{\cdot}L^{-1}$
M9	MS + BAP 2 $mg{\cdot}L^{-1}$ + NAA 0.2 $mg{\cdot}L^{-1}$
M10	MS + BAP 2 $mg{\cdot}L^{-1}$ + NAA 0.5 $mg{\cdot}L^{-1}$

MS: Murashige & Skoog medium (1962); BAP: 6-Benzy-laminopurine; KIN: Kinetin (6-furfuryl aminopurine); NAA: 1-Naphthaleneacetic Acid.

The second experiment included 3 other culture media. This was M2 medium [MS + BAP 2 mg·L^{-1}] to which NAA was added at concentrations of 0.1, 0.2 and 0.5 mg·L^{-1}. All media were dispensed into culture tubes (150 × 25 mm), *i.e.* 20 mL per tube, then sterilized by autoclaving at 110˚C for 20 min.

2.2.2. Experimental Setup

For each type of explant, a number of 12 per medium was tested and repeated 3 times, *i.e.* 36 explants in total for each treatment. The tubes are first incubated in a growth chamber at 27˚C ± 1˚C in the dark for 5 days then under a photoperiod of 16 h day/8h night and an incident light of 4000 Lux. This device is similar to those used for juvenile explants of *A. squamosa* and *A. muricata* [23].

After 30 days of incubation, measurements on each type of explant were performed. The measurement parameters used to relate to the presence or absence of resumption [Reactivity (%) = (Number of explants having reacted/Total number of explants) × 100], the number and length of newly formed shoots and the number of newly formed nodes. Thus, from these data, the averages were calculated, the coefficients or multiplication rates determined and the best media deduced.

2.2.3. Rooting Procedure

Third generation shoots, *i.e.* from three successive subcultures lasting thirty days each, were induced in the dark in the solid MS/2 medium (8 g·L^{-1} of agar) supplemented with IBA used alone at 25 mg·L^{-1} and 50 mg·L^{-1}. These media were supplemented with 2 g·L^{-1} of activated charcoal, according to the method used by [24], 20 g·L^{-1} of sucrose and the pH was adjusted to 5.7 prior to autoclaving at 110˚C for 20 min. The explants were induced in the dark, for periods of 1, 3 and 5 days, respectively, before being transferred to the light in the MS (0)/2 expression medium, without hormones. For each type of explant and each treatment, a sample of 12 explants was used per duration and induction medium and repeated 3 times. A batch of 12 explants was maintained as a control one without root induction in the MS (0)/2 hormone-free expression medium, with the macronutrients diluted by half.

After 30 days, for each treatment, measurements were taken to determine the rooting rate [(Number of rooted explants/total number of explants) × 100], the number of newly formed roots per explant and their length.

2.2.4. Acclimatization

As for *A. muricata* and *A. squamosa* juvenile vitroplants [23] and after four weeks of incubation in the expression medium, well-rooted young plants issued from differents types of explants were transferred to weaning conditions. They were removed from the test tubes and freed from the agar by washing with sterile distilled water. The young plants produced *in vitro* were then transplanted into plastic pots containing a substrate composed with a sand-potting soil mixture (v/v) previously sterilized at 121˚C for 1 hour. The sown pots were stored in a mini-greenhouse where the plants, under a transparent Plexiglas bell whose opening is adjustable, were kept in an atmosphere of high relative humidity, *i.e.* the shutter

completely closed for 2 weeks. A sample of 12 young plants is chosen for each type of explant. Watering was carried out daily with the nutrient solution of Hoagland & Arnon [25] and every 2 days after the second week. The bell was also opened gradually from the 2nd week of *ex vitro* weaning.

The number of young plants surviving after 15 and 30 days of acclimatization were counted; this helped to determine survival rates.

2.2.5. Statistical Analysis

The overall experiment was set up as a standard randomized design with growth regulator chosen as the primary factor variable and explant type as the sub-factor variable. The various treatments carried out were discriminated by multiple comparison of the means after analysis of variance followed by the Student-Newman-Keuls test (SNK). The significance was determined at 95% confidence limits, *i.e.* the significantly different means were discriminated by the SNK test at the p-value of 5% (SPSS 19.0 software).

3. Results

3.1. Influence of Growth Regulators on the *in Vitro* Morphogenesis of Different Types of Juvenile Explants

3.1.1. Effects of BAP and/or KIN on the Morphogenesis of Juvenile Explants

In the M2 medium [MS + BAP 2 mg·L^{-1}], the best reactivity rates are obtained with 91.67% for the apices and 100% for the axillary and cotyledonary nodes. However, the presence of callus was observed at the base of the explants.

Apices: The best average number of shoots is 2.45 which is significantly higher than that of the control (F = 16.677; p = 0.00) which is 1 shoot on average. It was obtained with M7 medium [MS + BAP 1 mg·L^{-1} + KIN 1 mg·L^{-1}] where the reactivity rate is 66.67%. Overall, media containing BAP allowed better bud burst than media containing Kinetin alone. The M5 medium [MS + KIN 2 mg·L^{-1}] induced the greatest elongation (5.03 cm) of newly formed nodes even if this is not significantly different from those obtained with the M1 [MS + BAP 1 mg·L^{-1}] and M2 [MS + BAP 2 mg·L^{-1}] media with which the average shoot lengths are respectively 4.9 cm (F = 24.284; p = 0.827) and 5.01 cm (p = 0.956). The highest average number of nodes is 4.62 and it is obtained with M2 and M7 [MS + BAP 1 mg·L^{-1} + KIN 1 mg·L^{-1}] media; it is significantly higher than that of MS0 (F = 4.066; p = 0.001).

- *Axillary nodes*: The average number of shoots is 3.01 for the M2 medium [MS + BAP 2 mg·L^{-1}]; it is significantly different from those obtained with the other media (F = 12.72; p = 0.000). The lowest average number of shoots (1.12) was obtained in the M6 medium [MS + KIN 5 mg·L^{-1}] (p = 0.000). The M5 medium [MS + KIN 2 mg·L^{-1}] made it possible to have an elongation of 5.0125 cm which is significantly greater than those obtained with the other media except for the M2 and M6 [MS + KIN 5 mg·L^{-1}] media which the mean lengths were 4.7 cm (F = 18.77; p = 0.37) and 4.68 cm (p = 0.32), respectively. The M2 medium

gave the best average number of nodes (4.5) which is significantly higher (F = 12.12; p = 0.00) than that of the control [MS0] one.

- *Cotyledonary nodes*: the M1 [MS + BAP 1 mg·L^{-1}] and M2 [MS + BAP 2 mg·L^{-1}] media gave 2.75 and 2.87 (p = 0.65), respectively, newly formed shoots per explant on average (Plate 1). These means are significantly higher than that obtained with M0 [MS0] (F = 21.12; p = 0.000) which is 1.75. The M6 medium [MS + KIN 5 mg·L^{-1}] revealed an average number of shoots (1.25) which is not significantly different (p = 0.06) from that of M0 [MS0]. Media M1 and M2 also gave elongations (F = 26.64; p = 0.00) and average number of nodes (F = 30.41; p = 0.00) significantly higher than that of the control medium [MS0] without hormones, with respectively 6.36 cm and 6.57 cm as well as the greatest average number of nodes with respectively 5.75 and 5.25 nodes (Table 2).

Table 2. Influence of BAP and Kinetin on the *in vitro* morphogenesis of juvenile material from *A. senegalensis*.

Media	Explant Type	Reactivity Rate (%)	Average number of shoots	Average Shoot length (cm)	Average number of nodes
M0	APX	58.33 d	1 e	3.82 b	2.37 c
	NA	75 bc	1.75 bc	2.85 c	2.12 c
	NC	58.33	1.75 bc	3.06 c	2.75 d
M1	APX	83.33 b	1.33 de	4.9 a	4.12 ab
	NA	75 bc	1.87 bc	3.88 b	3.25 b
	NC	83.33 b	2.75 **a**	6.36 a	**5.75 a**
M2	APX	**91.67 ab**	2.16 ab	5.01 a	**4.62 a**
	NA	**100 a**	**3.01 a**	4.7 ab	**4.5 a**
	NC	**100 a**	**2.87 a**	**6.57 a**	5.25 ab
M3	APX	75 bc	2.12 abc	2.52 c	3.87 ab
	NA	75 bc	1.5 cd	3.32 bc	2.62 bc
	NC	83.33 b	1.87 b	5.56 b	5.12 ab
M4	APX	75 bc	1.83 bcd	3.08 bc	4.12 ab
	NA	66.67 c	1.37 cd	4.07 b	2.5 bc
	NC	75 bc	2.5 ab	6.17 a	5 ab
M5	APX	58.33 d	1.62 cde	**5.03 a**	4 ab
	NA	75 bc	1.5 cd	**5.01 a**	3.75 ab
	NC	83.33 b	1.62 bc	5.53 b	4.75 bc
M6	APX	75 bc	1.45 de	4.23 ab	3.75 ab
	NA	83.33 b	1.12 d	4.68 ab	3.75 ab
	NC	75 bc	1.25 bcd	5.48 b	3.5 cd
M7	APX	66.67 c	**2.45 a**	4.28 ab	**4.62 a**
	NA	83.33 b	2.33 b	3.92 b	3.77 ab
	NC	**91.67 ab**	2.44 ab	4.7 bc	4.11 bc

APX: Apex; AN: Axillary Node; CN: Cotyledonary Node. In the same column and for the same type of explant, the numbers followed by the same letter are not significantly different at the 5% threshold of the Newman-Keuls test.

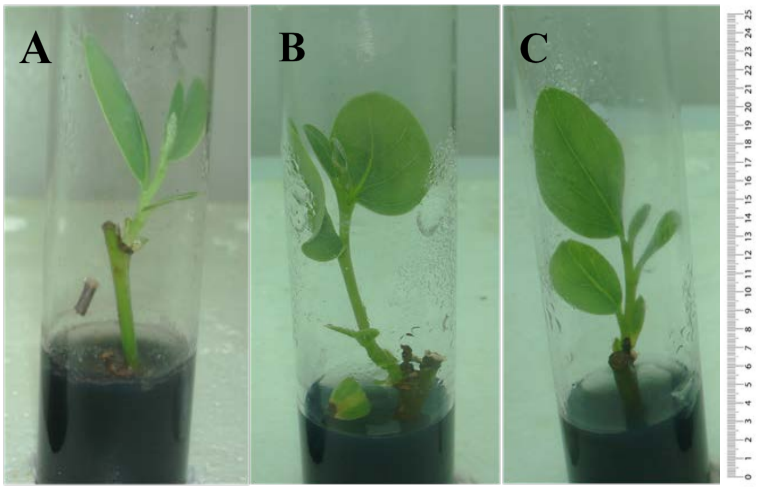

Plate 1. Appearance, in M2 medium, of newly formed shoots from juvenile material taken from *Annona senegalensis* Pers. A, C: Vitroplants neoformed from axillary nodes. B: Vitroplant neoformed from cotyledonary node.

3.1.2. Effects of NAA Combined to BAP on the *in Vitro* Morphogenesis of Juvenile Explants

There is a significant decrease (p = 0.00) in the average number of shoots for the different types of explants introduced into the media containing NAA compared to those having grown in the M2 medium [MS + BAP 2 mg·L^{-1}] devoid of NAA. The reactivity rates also remain higher in this medium compared to media containing NAA. For all types of explants, the presence of scar callus at the base of the explants is noted in all media.

- *Apices*: The average number of shoots in medium M2 [MS + BAP 2 mg·L^{-1}] is not significantly different (2.16) from those of M9 [MS + BAP 2 mg·L^{-1} + NAA 0.2 mg·L^{-1}] and M10 [MS + BAP 2 mg·L^{-1} + NAA 0.5 mg·L^{-1}] which are respectively 1.63 (F = 3.62; p = 0, 07) and 1.62 shoots (p = 0.06). The presence of NAA allowed good shoot elongation (**Table 3**).

With 7.03 cm, the M8 medium [MS + BAP 2 mg·L^{-1} + NAA 0.1 mg·L^{-1}] made it possible to have the highest average length of shoots with a reactivity of 83.33%. This average length is significantly greater (F = 44.08; p = 0.00) than those of vitroplants cultured in media M9 [MS + BAP 2 mg·L^{-1} + NAA 0.2 mg·L^{-1}] with 5.13 cm and M10 [MS + BAP 2 mg·L^{-1} + NAA 0.5 mg·L^{-1}] with 5.43 cm. In these environments, the reactivity rates are respectively 75% and 66.67%. The average number of nodes is 4.37 in the M8 medium [MS + BAP 2 mg·L^{-1} + NAA 0.1 mg·L^{-1}] which is not significantly different from that of the media M2 with 4.62 (F = 4.66; p = 0.39), M9 [MS + BAP 2 mg·L^{-1} + NAA 0.2 mg·L^{-1}] and M10 [MS + BAP 2 mg·L^{-1} + NAA 0.5 mg·L^{-1}] with 3.87 (F = 4.66; p = 0.09).

- *Axillary nodes*: The average number of shoots decreases considerably compared to one of the M2 medium [MS + BAP 2 mg·L^{-1}] regardless of the NAA concentration (F = 27.22; p = 0.000). The M9 medium [MS + BAP 2 mg·L^{-1} + NAA 0.2 mg·L^{-1}] gave a mean length and a mean number of nodes significantly

Table 3. Influence of NAA on the *in vitro* morphogenesis of juvenile material of *A. senegalensis*.

Media	Explant Type	Reactivity Rate (%)	Average number of shoots	Average Shoot length (cm)	Average number of nodes
M0	APX	58.33 d	1 c	4.9 c	2.37 b
	NA	75 bc	1.75 c	2.85 c	2.12 c
	NC	58.33 d	1.75 b	3.06 c	2.75 d
M2	APX	**91.67 ab**	**2.16 a**	5.01 bc	**4.62 a**
	NA	**100 a**	**3.01 a**	4.7 b	4.5 b
	NC	**100 a**	**2.87 a**	6.57 b	5.25 b
M8	APX	83.33 b	1.54 b	**7.03 a**	**4.37 a**
	NA	**91.33 ab**	1.55 cd	5.43 b	3.66 b
	NC	75 bc	2.11 b	**8.26 a**	**6.11 a**
M9	APX	75 bc	1.63 ab	5.13 bc	3.87 a
	NA	83.33 b	2.62 b	**8.26 a**	**5.87 a**
	NC	66.67 c	2.5 ab	6.26 b	5.12 b
M10	APX	66.67 c	1.62 ab	5.43 b	3.87 a
	NA	**91.33 ab**	1.25 d	5.7 b	4.1 b
	NC	66.67 c	1.25 c	5.7 b	4 c

APX: Apex; AN: Axillary Node; CN: Cotyledonary Node. In the same column and for the same type of explant, the numbers followed by the same letter are not significantly different at the 5% threshold of the Newman-Keuls test.

greater than those of the other media. This best average length is equal to 8.26 cm ($F = 87.77$; $p = 0.000$) for 5.87 nodes ($F = 21.12$; $p = 0.000$). With this combination, the reactivity rate is 100% as in M2 medium.

- *Cotyledonary nodes*: With the M9 medium [MS + BAP 2 mg·L^{-1} + NAA 0.2 mg·L^{-1}], the average number of shoots is 2.5; it is significantly higher than that of M10 [MS + BAP 2 mg·L^{-1} + NAA 0.5 mg·L^{-1}] ($F = 10.116$; $p = 0.000$). The average elongation length is equal to 8.26 cm for M8 [MS + BAP 2 mg·L^{-1} + NAA 0.1 mg·L^{-1}] with 6.11 nodes on average. This average length ($F = 84.67$; $p = 0.000$) and average number of nodes ($F = 13.041$; $p = 0.000$) remain significantly higher than those of the other environments even if the reactivity rate has decreased to 75%.

3.2. Effects of Induction with IBA for 1, 3 and 5 Days on the Vitroplant Rooting

Refering to Table 4, according to the concentration of IBA and the duration of induction, for each species, the results are as follows:

Table 4. Effect of concentration and duration of IBA induction on rooting of vitroplants from juvenile material of *A. senegalensis*.

Media	Induction Time (days)	Explant Type	Rooting Rate (%)	Average number of roots	Average root length (cm)
MS (0)/2	0	APX	16.67 d	1 ab	3.2 a
		NA	0 e	0 b	0 b
		NC	0 e	0 c	0 b
MS/2 + IBA 25 mg·L^{-1}	1	APX	8.33	1 ab	2.7 a
		NA	16.67 d	0 b	0 b
		NC	16.67 d	0 c	0 b
	3	APX	41.67 bc	2 a	1.2 b
		NA	0 e	0 b	0 b
		NC	33.33 c	3.25 a	0.8 a
	5	APX	**75 a**	**2.22 a**	1.3 b
		NA	41.67 bc	2 a	0.8 a
		NC	**66.67 ab**	**4.17 a**	0.6 a
MS/2 + IBA 50 mg·L^{-1}	1	APX	16.67 d	1.5 ab	**3.33 a**
		NA	**58.33 b**	**2.4 a**	**1.8 a**
		NC	0 e	0 c	0 b
	3	APX	16.67 d	1.5 b	0.8 c
		NA	33.33 c	1 b	0.6 b
		NC	33.33 c	1 b	**1.5 a**
	5	APX	0 e	0 b	0 c
		NA	0 e	0 b	0 b
		NC	0 e	0 c	0 b

APX: Apex. AN: Axillary Node; CN: Cotyledonary Node. In the same column and for the same type of explant, the numbers followed by the same are not significantly different at the 5% threshold of the Newman-Keuls test.

When the vitroplants from the different explant types were induced for 1 day with IBA at 25 mg·L^{-1}, only a rate of 8.33% was obtained, with 1 newly formed root and a root length of 2.7 cm for the shoots from the apices, and a rate of 16.67% for those from the axillary and cotyledonary nodes, The rooting rate is 41.67% for a 3-day pretreatment of the vitroplants from the apices, whereas for those newly formed from the cotyledonary nodes, it is 33.33%. For the latter, the average number of roots is 3.25 with an average length of 0.8 cm. The average

number of roots issued from vitroplants of the apices is 2, which is significantly higher ($F = 23.79$; $p = 0.032$) than the average number of roots of the vitroplants from the apices when the induction period is 1 day. Root elongation is 1.2 cm for apical shoots when pretreated for 3 days. On the other hand, the one-day pretreatment induced a significantly greater average root length of 2.7 cm ($F = 25.68$; $p = 0.013$). The highest rooting rate of shoots from apical origin (75%) was obtained for an induction period of 5 days, with an average number of roots of 2.22. This is not significantly greater than that obtained when the incubation period was 3 days ($p = 0.274$). The same is valid for vitroplants from cotyledonary nodes (66.67%) with an average of 4.17 roots per vitroplant. Rooted shoots (33.33%) from axillary nodes and incubated in the dark for 5 days on media containing IBA at 25 $mg{\cdot}L^{-1}$ gave rise to an average of 2 roots with an average length of 0.8 cm (Table 4).

A 5-day induction in the medium containing IBA at 50 $mg{\cdot}L^{-1}$, did not induce any rooting after 30 days of expression in the MS (0)/2 medium. In addition, 33.33% of the vitroplants from the axillary nodes, 41.67% of those from the cotyledonary nodes and 50% of those from the apices revealed advanced rotting followed by their senescence. At the end of the experiment, large scarred calluses were observed at the base of the explants. For an induction period of 3 days, only 16.67% of the vitroplants from the cotyledonary nodes rooted with a newly formed root per explant on average and with an average length of 0.8 cm, whereas for the 2 other types of explants from nodes, 33.33% became rooted. For an induction lasting 24 hours, the axillary nodes gave a rooting rate of 58.33% with an average of 2.4 roots and a root elongation of 1.8 cm. These last values remain the best obtained even if they are not significantly different from those obtained following a 5-day induction ($F = 5.75$; $p = 0.26$) in the presence of IBA at 25 $mg{\cdot}L^{-1}$. For vitroplants from the apices, induction for 24 hours in a medium containing IBA at 50 $mg{\cdot}L^{-1}$ followed by expression in an MS (0)/2 medium made it possible to record a 16.67% rooting rate, with 1.5 roots on average and an average length of 3.33 cm which is not significantly different from those obtained for the control ($F = 0.987$; $p = 0.327$) and the induction of vitroplants with 25 $mg{\cdot}L^{-1}$ of IBA. On the other hand, for vitroplants from axillary nodes, this rate is 58.33% with 2.4 roots on average and an average root length of 1.8 cm.

3.3. Acclimatization

On the 12th day of acclimatization, a beginning of rotting and a survival rate of 16.67% for all types of explants are noticed. From the 14th day, the shutter is opened halfway. On the 21st day, the plexiglass cover is completely opened to avoid prolonged confinement. The explants are first in the shade under the bench for 3 days and then transferred on the bench in the greenhouse. Thus, on the 30th day, the survival rates are 75% for plantlets formed from the apices and 83.33% for those from the axillary and cotyledonary nodes (Table 5, Plate 2).

Table 5. Survival rate, after 15 and 30 days, of *ex vitro* weaning of young plants from juvenile material of *A. senegalensis*.

		Survival rate (%)	
Species	Explant Type	After 15 days	After 30 days
Annona senegalensis	APX	83.33 a	75 b
	NA	83.33 a	83.33 a
	NC	83.33 a	83.33 a

APX: Apex; AN: Axillary Node; CN: Cotyledonary Node. In the same column and for the same type of explant, the numbers followed by the same letter are not significantly different at the 5% threshold of the Newman-Keuls test.

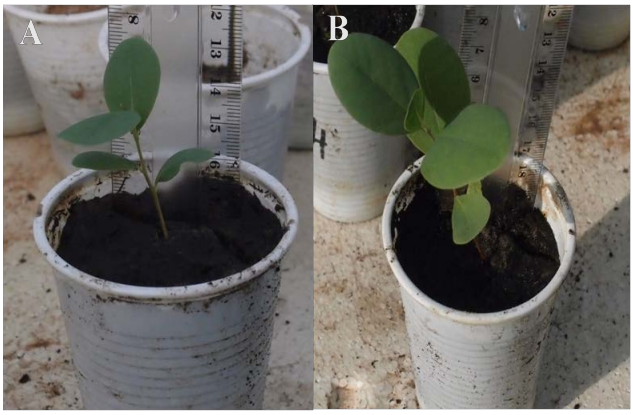

Plate 2. Young plants of *Annona senegalensis* resulting from the juvenile material after 30 days of *ex vitro* weaning under shade. A: Plant from the apex; B: Plant from axillary node.

4. Discussion

4.1. Influence of Growth Regulators on the *in Vitro* Morphogenesis of Different Types of Juvenile Explants

As for juvenile explants of *Annona muricata* and *A. squamosa* [23], all types of explants prelevated from sterile seedlings of *A. senegalensis* began to turn brown. The onset of browning noted during the first introduction of material could be due to the exposure of plants resulting from germination to light which would promote, according to [26], the synthesis of polyphenols. The authors such as [27] and [28] noted that light is one of the abiotic factors that can stimulate the biosynthesis of phenolic products responsible for the browning of explants with a negative impact on their development. To avoid these harmful effects of polyphenols, 2 $g \cdot L^{-1}$ of activated charcoal were added to the culture media.

The medium of [22] is one of the most widely used media for the *in vitro* vegetative propagation of plant species, especially in numerous studies of the *Annona* genus. This medium was used in *in vitro* organogenesis from leaf explants of *Annona squamosa* by [29], in *A. cherimola* [30] [31], in *A. muricata* [32], in the hybrid species, *A. cherimoya cv* (*A. cherimola* × *A. squamosa*) and in *A.*

glabra [33]. The authors [24] used this medium to *in vitro* multiply *A. squamosa* explants in accordance with the results obtained by [34]. In our previous research studies, we have also used this medium for the *in vitro* clonal propagation of various types of explants from adult *Annona senegalensis* subjects [21] and from juvenile material of *A. muricata* [23].

After one week of incubation, the outlines of newly formed shoots are visible at the level of the cotyledonary and axillary nodes and are more numerous in the media enriched with growth regulators. The greatest mean numbers of newly formed shoots were obtained on the axillary (3.01) and cotyledonary (2.87) nodes with the M2 medium supplemented with BAP at 2 $mg{\cdot}L^{-1}$. In both cases, the responsiveness is 100%. In this M2 medium, the reactivity rate remains high for the apices with 91.67%. The MS medium supplemented with 2 $mg{\cdot}L^{-1}$ of BAP led as well to the best reactivity rate (91.66%), the greatest average number of newly formed shoots (2.3) and the greatest number of nodes (5.3) for *in vitro* clonal propagation of explants prelevated from adult materiel of *A. senegalensis* [21]. For explants issued from apices, the M7 medium [MS + BAP 1 $mg{\cdot}L^{-1}$ + KIN 1 $mg{\cdot}L^{-1}$] gave also a good average number of newly formed shoots (2.45) with a reactivity rate of 66.67%. Thus, BAP alone seems more beneficial to the reactivity of the different types of explants compared to Kinetin used alone or combined with BAP. This beneficial action of BAP was noted by [35] in *Ceratonia siliqua* L., especially for the same concentration of 2 $mg{\cdot}L^{-1}$. The authors [36] reported that enrichment of MS medium with BAP at concentrations of 1 $mg{\cdot}L^{-1}$ to 2 $mg{\cdot}L^{-1}$ favored shoot multiplication in *Vanda helvola*. The same observation was made by [37] with *Vanda pumila* Hook. F. According to [21] [23] and [38], BAP was more effective than Kinetin for shoot regeneration and elongation in *Parkia biglobosa*, *A. senegalensis*, *A. muricata* and *A. squamosa*. On the other hand, for the species *Echinops kebericho*, Kinetin was more effective for new shoot formation [39]. The authors [40] demonstrated that the best medium for the shoot reactivity of *Annona annua* is the MS medium supplemented with BAP at 1 $mg{\cdot}L^{-1}$. The same concentration of BAP allowed to obtain better regeneration of apical and nodal explants in *Vanilla planifolia* [41] compared to the BAP-NAA association. According to [42], high concentrations of cytokinins generally favor the production of large numbers of cuttings. Thus, the use of BAP at 2 and 4 $mg{\cdot}L^{-1}$ has been shown to significantly increase the number of cuttings produced from explants of *A. muricata*.

The BAP-NAA combination proved to be more beneficial for the elongation of newly formed shoots. It also made it possible to increase the average number of nodes for explants of axillary and cotyledonary origin. For this purpose, the M8 medium [MS + BAP 2 $mg{\cdot}L^{-1}$ + NAA 0.1 $mg{\cdot}L^{-1}$] was more beneficial for the apices and cotyledonary nodes whereas for the axillary nodes, it is rather the M9 medium [MS + BAP 2 $mg{\cdot}L^{-1}$ + NAA 0.2 $mg{\cdot}L^{-1}$] which made it possible to obtain better elongation and a greater number of shoots. The beneficial action of NAA on shoot elongation has already been reported by several authors [43] [44].

The addition of NAA in the culture medium in the presence of BAP rather led to a decrease in the number of newly formed shoots. Indeed, the shoot regeneration observed on the explants cultured on the MS0 medium reflects the presence of endogenous phytohormones in the explants, thus stimulating the formation of shoots but with a lower frequency than in the mediums containing exogenous hormones. However, the contents of endogenous auxins and cytokinins differ from one type of explant to another. This explains why the BAP-NAA association promotes either the regeneration of new shoots, or their elongation, or both at the same time depending on the type of explant. The authors [45] indicated that the hormonal combination BAP-NAA can induce an increase in the number of regenerated shoots from the cotyledonary nodes while for [46], it would rather lead to its decrease.

The callogenesis observed on many explants incubated *in vitro* in the MS medium containing NAA would be due to the inner balance of the auxins and cytokinins contents and their synergistic action at the cellular level. It has already been reported by [47] on *Vigna unguiculata* and [48] on *Nigella damascena* L. However, the presence of calli at the base of the explants makes it difficult for them to root later, both during the induction phase and rhizogene expression.

4.2. Influence of Growth Regulators on the *in Vitro* Rooting of Different Types of Juvenile Explants

Rooting is the most important step for *in vitro* plant regeneration. It strongly conditions the success of the last step, which is acclimatization or weaning *ex vitro*. Indeed, the classical methods of vegetative propagation were inefficient, due to the low morphogenetic potential of *A. senegalensis*, and the low rooting rate of its conventional cuttings originated from juvenile or mature material as well. The micropropagation technique can be applied successfully to this species and other *Annona sp.* to overcome these contraints [21] [49] [50].

A 5-day-rhizogenic induction in the dark with IBA at 25 $mg{\cdot}L^{-1}$, followed by *in vitro* subculturing in the MS (0)/2 hormone-free expression medium, made it possible to have the best rooting rates and the greatest average number of roots for young plants originated from apices (75% for 2.22 roots) and cotyledonary nodes (66.67% for 4.17 roots) whereas for shoots of axillary origin, this rate is 58.33% and is obtained thanks to an induction period of 24 h in the presence of IBA at 50 $mg{\cdot}L^{-1}$. This also made it possible to have the best average number of roots (2.4) and the best root elongation (1.8 cm).

After one month of culture in the expression medium, a rooting rate of 16.67% was obtained for the vitroplants issued from the apices, in an hormone-free MS (0)/2 medium; this confirms the existence of high levels of endogenous auxin synthesized at the apices whether they are juvenile or mature [21]. The same observations were made with *Parkia biglobosa* vitroplants [38]. It could be that the environment being depleted in cytokinins, the quantity of auxins synthesized by the young shoot modifies the auxin/cytokinin ratio in favor of rooting. This

would explain why IBA at 25 mg·L^{-1} was more effective for rhizogenesis than that at 50 mg·L^{-1}.

The MS/2 medium alone, without the addition of auxins, can induce the rooting of shoots. This is the case, for example, with *Vigna unguiculata* [47]. The MS/2 medium is very favorable to the rooting of shoots in many woody species compared to the complete MS medium according to [51]. The addition of IBA to the MS/2 medium enabled them to improve the *in vitro* rooting rate of *Cryptolepis sanguinolenta* vitroplants. The use of this basal medium, with or without added auxins, for the rooting step has been frequently reported and led to good rooting of shoots from mature material in many woody species as well as for *Annonaceae* species [30] [31] [32]. This is all the more true when the plant tissues are juvenile. As a result, they are more sensitive and receptive to high levels of auxins. Thus, 25 mg·L^{-1} of IBA is sufficient to induce good rhizogenicity of vitroplants derived from juvenile material, in particular those originating from cotyledonary nodes and terminal apices. According to several researchers, IBA would be more favorable to the rhizogenic induction of woody species, which are difficult to root *in vitro* [40] [52].

4.3. Acclimatization

Acclimatization is a crucial step in the micropropagation of woody plant species. This is one of the most important failure factors of the *in vitro* vegetative propagation technique.

After 30 days of acclimatization, the survival rate is 75% for young plants from the apices and 83.33% for those from the cotyledonary and axillary nodes. The confinement of young plants in an atmosphere saturated with humidity (90% to 100%), close to *in vitro* conditions at a high temperature, has proven beneficial for the success of their weaning. Indeed, plants from *in vitro* culture generally have a thinner cuticle than that of the parent plants, which causes them to dry out rapidly when the relative humidity is lowered quickly by their transplantation in *ex vitro* conditions. The very high relative humidity in the culture tubes favors a high density of stomata in the epidermis of the leaves and stems of young plants compared to those of adult plants adapted to natural conditions, but the stomata in *in vitro* conditions remain few or not functional, namely that the adjustment of the opening and closing of the stomatal ostiole is weak or not regulated in an atmosphere saturated with humidity. Consequently, a progressive weaning allows the young plants to survive well the passage in a natural environment, less saturated with humidity, because the stomata can thus adapt to this new ambient atmosphere and gradually acquire their capacity for gaseous and respiratory exchanges with the external environment. Acclimatization of plantlets in a mini-greenhouse followed by gradual contact with the ambient conditions, therefore, makes it possible to obtain the best survival rates for young plants, whether they originate from adult or juvenile material [21] [23] [38]. In fact, as it has been observed for the mature material, keeping the young

plants in a mini-greenhouse, before their transfer under natural conditions, made it possible to maintain the explants under conditions close to those *in vitro*, *i.e.* very high relative humidity conditions, which lead to saturation of the atmosphere, hence to the protection of leaf surfaces of plants not yet fully adapted to natural conditions. This is how [53] were also able to successfully wean young plants by maintaining them for 6 weeks of culture in a mini-greenhouse, at a temperature of 27˚C ± 3˚C and a relative humidity of 80% - 85%. In this case, the substrate used is composed of peat and perlite in equal proportions. The same substrate allowed obtaining a survival rate of 100% with *Bambusa vulgaris* plantlets [54]. Indeed, the substrate used during acclimatization also plays a very important role for the survival of plants; switching from an asphyxiating agar substrate to a more porous one can cause difficulties in adapting to the root system of newly formed plants. We used a proportional mixture of sterile sand-soil (v/v) which ensured good drainage for the young plants and good aeration of their root system. The authors [55] obtained the best seedling survival rate (75%) of *Albizia odoratissima* L.F. (Benth.) after acclimatization in a mixed substrate.

5. Conclusion

As for vitroplants issued from mature material of *A. senegalensis*, the MS medium supplemented with 2 mg·L^{-1} of BAP led to the best reactivity rate (91.67% and 100%), the greatest average number of newly formed shoots (3.01 and 2.87) and the greatest number of nodes (4.62 and 4.50), depending on the type of explant *in vitro* cultured. The results obtained at the end of this study made it possible to highlight the beneficial effects of BAP in the bursting of shoots (M2 medium) and of NAA for their elongation (M8 and M9 media), but the effect of these hormones is not the same depending on the type of explant used. Newly formed shoots were able to be rooted thanks to IBA at 25 mg·L^{-1} (66.67%), and then acclimatized with good rooting rates. Gradual weaning of plantlets, during acclimatization, results in fairly high survival rates (83.33%) after 15 days. However, to improve this micropropagation protocol from juvenile material of *A. senegalensis*, it will be interesting to screen other types of auxins and cytokinins at different concentrations.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] National Research Council (2008) Custard Apples. Lost Crops of Africa: Volume III: Fruits. Lost Crops of Africa. National Academies Press, Washington DC.

[2] Kerharo, J. and Adams, J.G. (1974) La pharmacopee senegalaise traditionnelle. Plantes medicinales et toxiques, 4eme Trimestre. *Journal d'agriculture traditionelle et de botanique appliquee*, **21**, 76-77.

[3] Badjaré, B., Kokou, K., Bigou-laré, N., *et al.* (2018) Étude ethnobotanique d'espèces ligneuses des savanes sèches au Nord-Togo: Diversité, usages, importance et vulnérabilité. *Biotechnologie, Agronomie, Société et Environnement*, **22**, 152-171. https://researchgate.net/publication/325880644 https://doi.org/10.25518/1780-4507.16487

[4] Mampouya, C.P. and Moutsamboté, J.M. (2021) Les annonacées du Mayombe en République du Congo. *Journal of Applied Biosciences*, **163**, 16906-16914.

[5] Maheshwari, P. and Singh, U. (1965) Dictionary of Economic Plants in India. Indian Council of Agricultural Research, New Delhi, 12 p.

[6] Popenoe, W. (1974) The Annonaceous Fruits. In: Popenoe, W., Ed., *Manuel of Tropical and Subtropical Fruits, a Facsimile of the* 1920, Hafner Press, New York, 161-195.

[7] George, A.P. and Nissen, R.J. (1993) Annonaceous Fruits. In: Robinson, R.K. and Sadler, M.J., Eds., *Food Technology and Nutrition, Encyclopaedia of Food Science*, Academic Press, London, 195-199.

[8] Fall, D., Sambou, B., Seck, M., *et al.* (2008) Enhancing the Anthelminthic Activity Roots of *Annona sengalensis*. *Dakar Medecine*, **53**, 61-67.

[9] Bokaketi, E.G., Moundaga, R.A. and Tondo Bafouiri Ntsoni, D.C. (2016) Identification de quelques plantes ayant des vertus médicinales rencontrées dans certains marchés, chez certaines tradipraticiens de Brazzaville et à Nganga-Lingolo. Travail Collectif de Recherche, Université Marien Ngouabi, Ecole Normale Supérieure, 18 p.

[10] Alawa, C.B.I., Adamu, A.M., Gefu, J.O., *et al.* (2003) *In Vitro* Screening of Two Nigerian Medicinal Plants (*Vernonia amygdalina* and *Annona senegalensis*) for Anthelmintic Activity. *Veterinary Parasitology*, **113**, 73-81. https://doi.org/10.1016/S0304-4017(03)00040-2

[11] Lall, N., Kishore, N., Bodiba, D., *et al.* (2017) Alkaloids from Aerial Parts of *Annona senegalensis* against *Streptococcus mutans*. *Natural Product Research*, **31**, 1944-1947. https://doi.org/10.1080/14786419.2016.1263847

[12] Tamfu, A.N., Fotsing, M.T., Talla, E., *et al.* (2021) Bio-Active Constituents from Seeds of *Annona senegalensis* Pers. (*Annonaceae*). *Natural Product Research*, **35**, 1746-1751. https://doi.org/10.1080/14786419.2019.1634713

[13] Ajaiyeoba, E., Falade, M., Ogbole, O., *et al.* (2006) *In Vivo* Antimalarial and Cytotoxic Properties of *Annona senegalensis* Extract. *African Journal of Traditional, Complementary and Alternative Medicines*, **3**, 137-141. https://doi.org/10.4314/ajtcam.v3i1.31148

[14] Shomkegh, S.A., Mbakwe, R. and Dagba, B.I. (2016) Utilization of Wild Plants for Medicinal Purposes in Selected Tiv Communities of Benue State, Nigeria: An Ethnobotanical Approach. *European Journal of Medicinal Plants*, **14**, 1-14. https://doi.org/10.9734/EJMP/2016/26118

[15] Ahmed, A.L., Bassem, S.E.M., Mohamed, Y.H. and Gamila, M.W. (2010) Cytotoxic Essential Oil from *Annona sengalensis* Pers. Leaves. *Pharmacognosy Research*, **2**, 211-214. https://doi.org/10.4103/0974-8490.69105

[16] Bernoux, M., Chevallier, T., Bégni R., *et al.* (2013) Le carbone des sols dans les régions sèches: Des fonctions multiples indispensables. Comité scientifique français de la désertification, CSFD/Agropolis International (Les Dossiers Thématiques du CSFD), Montpellier, 42 p.

[17] Ouédraogo, A., Thiombiano, A., Hahn-Hadjali, K. and Guinko, S. (2006) Diagnostic

de l'état de dégradation des peuplements de quatre espèces ligneuses en zone soudanienne du Burkina—Faso. *Sécheresse*, **17**, 485-491.

[18] Bellefontaine, R., Meunier, Q., Aboubacar, I., *et al.* (2015) Multiplication végétative à faible coût au profit des paysans et éleveurs des zones tropicales et méditerranéennes, *VertigO*, Cirad-Agritrop, 12 p. https://agritrop.cirad.fr/579170 https://doi.org/10.4000/vertigo.16516

[19] Le Bouler, H., Brahic, P., Bouzoubâa, Z., *et al.* (2013) L'amélioration des itinéraires techniques en pépinière de production d'arganiers en mottes-conteneurs hors sol. In: INRA-Maroc, Ed., *Actes du* 1*er Congrès International de l'Arganier*, Agadir, Maroc, 124-134. https://agritrop.cirad.fr/569190/

[20] Pinto, A.C.Q., Cordeiro, M.C.R., De Andrade, S.R.M., *et al.* (2005) Annona Species. International Centre for Underutilised Crops, University of Southampton, Southampton, 281 p.

[21] Ba, O., Dieme, A. and Sy, M.O. (2021) *In Vitro* Clonal Propagation from Adult Material of a Savannah Species of Socio-Economic Importance: *Annona senegalensis* Pers. *Agricultural Sciences*, **12**, 370-386. https://doi.org/10.4236/as.2021.124024

[22] Murashige, T. and Skoog, F. (1962) A Revised Medium for Rapid Growth and Bioassays with Tobacco Tissue Cultures. *Physiologia Plantarum*, **15**, 473-497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

[23] Ba, O., Dieme, A., Ndoye, A.L. and Sy, M.O. (2021) *In Vitro* Clonal Propagation from Juvenile and Different Explant Types of Two Edible *Annonaceae* Species: *Annona muricata* L. and *Annona squamosa* L. *Advances in Bioscience and Biotechnology*, **12**, 458-480. https://doi.org/10.4236/abb.2021.1212029

[24] Farooq, S.A., Farooq, T.T. and Rao, T.V. (2002) Micropropagation of *Annona squamosa* L. Using Nodal Explants. *Pakistan Journal of Biological Sciences*, **5**, 43-46. https://doi.org/10.3923/pjbs.2002.43.46

[25] Hoagland, D.R. and Arnon, D.I. (1938) The Water Culture Method for Growing Plants without Soil. *California Agricultural Experiment Station Circulation*, **347**, 32.

[26] Ncube, B., Finnie, J.F. and Van Staden, J. (2011) Seasonal Variation in Antimicrobial and Phytochemical Properties of Frequently Used Medicinal Bulbous Plants from South Africa. *South African Journal of Botany*, **77**, 387-396. https://doi.org/10.1016/j.sajb.2010.10.004

[27] Linic, I., Šamec, D., Grúz, J., *et al.* (2019) Involvement of Phenolic Acids in Short-Term Adaptation to Salinity Stress Is Species-Specific among *Brassicaceae*. *Plants*, **8**, 155. https://doi.org/10.3390/plants8060155

[28] Šamec, D., Karalija, E., Šola, I., *et al.* (2021) The Role of Polyphenols in Abiotic Stress Response: The Influence of Molecular Structure. *Plants* (*Basel*), **10**, Article No. 118. https://doi.org/10.3390/plants10010118

[29] Nair, S., Gupta, P.K., Shirgurkar, M.V. and Mascarenhas, A.F. (1984) *In Vitro* Organogenesis from Leaf Explants of *Annona squamosa* L. *Plant Cell Tissue and Organ Culture*, **3**, 29-40. https://doi.org/10.1007/BF00035918

[30] Jordan, M. (1988) Multiple Shoot Formation and Rhizogenesis from Cherimola (*Annona cherimola* L.) Hypocotyls and Petiol Explants. *Gartenbauwissenschaft*, **53**, 234-237.

[31] Bejoy, M. and Hariharan, M. (1992) *In Vitro* Plantlet Differentiation in *Annona muricata* L. *Plant Cell, Tissue and Organ Culture*, **31**, 245-247.

[32] Rasai, S., Kantharajah, A.S. and Dodd, W.A. (1994) The Effect of Growth Regulators, Source of Explants and Irradiance on *in Vitro* Regeneration of *Atemoya*. *Aus-*

tralian Journal of Botany, **42**, 333-340. https://doi.org/10.1071/BT9940333

[33] Deccetti, S.F.C., Paiva, R., De Oliveira Paiva, P.D. and Aloufa, M.A.I. (2005) La micropropagation d'*Annona glabra* L. à partir de segments nodaux. *Fruits*, **60**, 319-325. https://doi.org/10.1051/fruits:2005038

[34] Rao, T.V. and Farooq, S.A. (1994) Preliminary Investigations on the *in Vitro* Studies in *Annona squamosa* L. *Frontiers in Plant Sciences*, **1**, 261-264.

[35] Saidi, R., Rahmouni, S., El Ansari, Z.N., *et al.* (2019) Effect of Cytokinins on the Micropropagation of Carob (*Ceratonia siliqua* L.) through Shoot Tip Culture. *American Journal of Plant Sciences*, **10**, 1469-1481. https://doi.org/10.4236/ajps.2019.109104

[36] David, D., Gansau, J.A. and Abdullah, J.O. (2007) Effect of NAA and BAP on Protocorm Proliferation of Borneo Scented Orchid, *Vanda helvola*. *Asia-Pacific Journal of Molecular Biology and Biotechnology*, **16**, 221-224.

[37] Maharjan, S., Pradhan, S., Thapa, B.B., *et al.* (2019) *In Vitro* Propagation of Endangered Orchid, *Vanda pumila* Hook.f. through Protocorms Culture. *American Journal of Plant Sciences*, **10**, 1220-1232. https://doi.org/10.4236/ajps.2019.107087

[38] Sambe, M.A.N., Sagna, M. and Sy, M.O. (2010) Seed Germination and *in Vitro* Plant Regeneration of *Parkia biglobosa* (Jacq.) Benth. *African Journal of Biotechnology*, **9**, 3099-3108.

[39] Manahlie, B. and Feyissa, T. (2014) Micropropagation of *Kebericho*: An Endandered Ethiopian Medicinal Plant. *American Journal of Plant Sciences*, **5**, 3836-3847. https://doi.org/10.4236/ajps.2014.526402

[40] Chokheli, V.A., Dmitriev, P.A., Rajput, V.D., *et al.* (2020) Recent Development in Micropropagation Techniques for Rare Plant Species. *Plants*, **9**, Article No. 1733. https://doi.org/10.3390/plants9121733

[41] Zuraida, A.R., Izzati, K.H.F.L., Nazreena, O.A., *et al.* (2013) A Simple and Efficient Protocol for the Mass Propagation of *Vanilla planifolia*. *American Journal of Plant Sciences*, **4**, 1685-1692. https://doi.org/10.4236/ajps.2013.49205

[42] Lemos, E.E.P. and Blake, J. (1996) Micropropagation of Juvenile and Mature *Annona muricata* L. *Journal of Horticultural Sciences*, **71**, 395-403. https://doi.org/10.1080/14620316.1996.11515420

[43] Koroch, A.R., Kapteyn, J., Juliani, H.R., *et al.* (2003) *In Vitro* Regeneration of *Echinacea pallida* from Leaf Explants. *In Vitro Cellular & Developmental Biology-Plant*, **39**, 415-418. https://doi.org/10.1079/IVP2003424

[44] Raharimalala, T.K. (2016) Multiplication *in Vitro* par allongement des pousses et par bougeonnement axillaire de *Morinda* sp. *Nov* A (*Rubiaceae*), une espèce endémique et menacée du site minier d'Ambatovy. Mémoire Master, PHYTEC, Sciences et Technologies, Faculté des Sciences, Université d'Antananarivo, Antananarivo, Madagascar, 63 p.

[45] Chekroun, C. and Belkhodja, M. (2017) *In Vitro* Micropropagation and Plants Regeneration of Cowpea (*Vigna unguiculata* (L.) Walp) from Cotyledonary Node. *International Journal of Innovation and Applied Studies*, **21**, 247-253.

[46] Varalaxmi, Y., Vijayalakshmi, A., Yadav, S.K., Venkateswarlu, B. and Maheswari, M. (2007) Efficient Plant Regeneration from Cotyledonary Node of Black Gram (*Vigna mungo* (L.) Hepper). *Indian Journal of Biotechnology*, **6**, 414-417.

[47] Chekroun, C. (2018) Recherche des conditions optimales de la régénération de Soja sous contraintes salines par la culture *in Vitro*. Thèse de Doctorat en sciences biologiques/spé. physiologie végétale. Université Oran1. Oran, Algérie. 128 p.

[48] Klimek-Chodacka, M., Kadluczka, D., Lukasiewicz, A., *et al.* (2020) Effective Callus Induction and Plant Regeneration in Callus and Protoplast Cultures of *Nigella damascena* L. *Plant Cell Tissue and Organ Culture*, **143**, 693-707. https://doi.org/10.1007/s11240-020-01953-9

[49] Encina, C.L., Barcelo-Munhoz, A., Herrero-Castano, A. and Pliego-Alfaro, F. (1994) *In Vitro* Morphogenesis of Juvenile *Annona cherimola* Mill. Bud Explants. *Journal of Horticultural Science and Biotechnology*, **69**, 1053-1059. https://doi.org/10.1080/00221589.1994.11516544

[50] Encina, C.L., Martin, E.C., Lopez, A.A. and Padilla, I.M.G. (2014) Biotechnology Applied to Annona Species: A Review. *Edicao Especial*, **36**, 17-21. https://doi.org/10.1590/S0100-29452014000500002

[51] Monney, M.A.D., Amissah, N. and Blay, E. (2016) Influence of BA and IBA or NAA Combinations on Micropropagation of *Cryptolepis sanguinolenta*. *American Journal of Plant Sciences*, **7**, 572-580. https://doi.org/10.4236/ajps.2016.73050

[52] Cheon Chae, S. (2016) Influence of Auxin Concentration on *in Vitro* Rooting of *Chrysanthemum morifolium* Ramat. *Biosciences Biotechnology Research Asia*, **13**, 833-837. https://doi.org/10.13005/bbra/2104

[53] El Chaieb, E. and Haouala, F. (2016) Mise au point des techniques de micropropagation pour une multiplication massive de *Gladiolus segetum* et *Gladiolus dubius*. *European Scientific Journal*, **12**, 207-217. https://doi.org/10.19044/esj.2016.v12n6p207

[54] Ndiaye, A., Diallo, M.S., Niang, D., *et al.* (2006) *In Vitro* Regeneration of Adult Trees of *Bambusa vulgaris*. *African Journal of Biotechnology*, **5**, 1245-1248.

[55] Rajeswari, V. and Paliwal, K. (2008) *In Vitro* Adventitious Shoot Organogenesis and Plant Regeneration from Seedling Explants of *Albizia odoratissima* L.f. (Benth.). *In Vitro Cellular and Developmental Biology—Plant*, **44**, 78-83. https://doi.org/10.1007/s11627-008-9120-7