

In Vitro Clonal Propagation from Juvenile and Different Explant Types of Two Edible *Annonaceae* Species: *Annona muricata* L. and *Annona squamosa* L.

Oumar Ba^{1,2}, Abraham Dieme^{1,2}, Amadou Lamine Ndoeye^{1,2}, Mame Ourèye Sy^{1,2*}

¹Laboratoire Campus de Biotechnologies Vegetales, Departement de Biologie Vegetale, Faculte des Sciences & Techniques, Universite Cheikh Anta Diop, Dakar, Senegal

²Laboratoire Mixte International-Adaptation des Plantes et Microorganismes associes aux Stress Environnementaux (LMI-LAPSE), IRD, ISRA, UCAD, Dakar, Senegal

Email: *oureye.sy@ucad.edu.sn

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Abstract

Annona muricata L. and *Annona squamosa* L. are tropical species whose fleshy fruit is edible. They offer real possibilities for socio-economic use, particularly in the fields of medicine, nutrition, ecosystem conservation and the poverty alleviation. This study was set up to evaluate different methods of micropropagation from juvenile material for the regeneration of these species. Thus, MS medium supplemented with [BAP 2 mg·L⁻¹] *i.e.* M2 produced 2.87 newly formed shoots from the cotyledonary nodes of *A. muricata*. For the terminal apices of *A. squamosa*, it was MMS medium supplemented with [BAP 2 mg·L⁻¹] *i.e.* MM2 that was most conducive to new shoot formation (3.12). The addition of 0.1 and 0.2 mg·L⁻¹ of NAA in the M2 medium, made it possible to have the best elongations and average number of nodes for the new shoots from cotyledonary nodes of *A. muricata* (9.11 cm for 5.62 nodes). With *A. squamosa*, MM7 medium [MMS + BAP 1 mg·L⁻¹ + KIN 1 mg·L⁻¹] resulted in an average length of 9.05 cm with 5.62 nodes on average for the apical shoots. A 3-day rhizogenic induction period in the dark with [IBA 50 mg·L⁻¹] and 2 g·L⁻¹ of activated charcoal gave a rooting rate of 66.67% for shoots originating from the cotyledonary nodes of *A. squamosa*; while with vitroplants from cotyledonary nodes of *A. muricata*, a better rooting rate (83.33%) was obtained following a 5-day rhizogenic induction. After 30 days of acclimatization, the survival rate reached 83.33% for plants from the tips of *A. muricata*, whereas for *A. squamosa*, it was plants grown from cotyledonary nodes that had the same survival rate.

Keywords

Annona muricata, *Annona squamosa*, Juvenile Material, Micropropagation, 6-Benzylaminopurine, 6-Furfuryl Aminopurine, 1-Naphthaleneacetic Acid, Indole-3-Butyric Acid

1. Introduction

Annona muricata L. and *Annona squamosa* L. are forest species of great socio-economic interest. Their fleshy and tasty fruits are very appreciated by the populations. Their bark, roots and leaves are also widely used in the African pharmacopoeia to treat various diseases such as sleep disorders, dermatoses, mycoses, diarrhea, etc. [1] [2]. In Senegal, these species are exploited in small agricultural gardens and orchards in the “Niayes” area, from Saint-Louis to the small marine coast. They are also cultivated in the southern regions as well as the groundnut basin in the Center of Senegal. They are part of the income-generating subsistence crops for farmers, especially during the lean season. However, their exploitation in the Sahel region has so far only been done through conventional methods such as seedling germination [3] [4]. However, due to its irregularity and slowness, this method is not sufficient to ensure good regeneration of these species [5]. Propagation by sowing seeds also leads to heterogeneous progeny, although the germination rate remains high for *A. squamosa* seeds [6]. This is just as valid for other methods such as suckering, grafting and cuttings, more rarely used, hence the need in Senegal as in the African regions where they are exploited, to have recourse to *in vitro* propagation methods. Thanks to this technique, the producer can obtain clonal plants from elite trees in sufficient quantity to satisfy the demand on the market and a continuous production independent of climatic hazards and seasons. In fact, unlike the conventional method of seed propagation, which gives a single individual per seed, the production of plants by *in vitro* culture makes it possible to obtain as many copies as desired from a single explant [7]. Despite its many advantages, the multiplication of *Annonaceae* by tissue culture techniques is almost non-existent in Africa and Senegal, in particular. Several works focusing mainly on the *in vitro* culture of *Annona muricata* L. and *Annona squamosa* L., have already been published in Latin America and Asia by various authors but with different methodologies and results. The articles published on these species mainly focus on the pharmacological properties of various chemical substances extracted from various organs. *In vitro* vegetative propagation can be carried out either from material obtained from young plants germinated from the seeds or from adult material taken directly from elderly subjects. Nowadays, the preservation of the genetic diversity of living organisms has become a necessity [8], especially for species of great socio-economic interest.

In this context, a strategy was developed for *in vitro* vegetative propagation of

each of these 2 species from young materials through an evaluation of *in vitro* morphogenetic capacities. For each species, it involved to 1) determine the best type of implant; 2) examine the influence of different growth regulators such as cytokinins and auxins on the *in vitro* morphogenesis of neoformed subjects; 3) determine the best elongation, multiplication, and rooting media; 4) and, define finally the best acclimatization conditions for the new plants obtained.

2. Materials and Methods

2.1. Plant Material

For each species, 3 types of explants measuring 1 to 2 cm long and taken from sterile 30-day-old seedlings were tested. These were terminal apices, axillary and cotyledonary nodes. These different types of explants are 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 for *A. muricata* is the complete medium of Murashige & Skoog [9], while for the explants of *A. squamosa*, the basal one is MMS medium; it corresponds to the modified Murashige & Skoog medium [10]. These media were solidified with 8 g·L⁻¹ agar at pH 5.7 and contained 30 g·L⁻¹ of sucrose. Growth regulators were added or not into the different media. The composition of the different hormones tested alone or in combination in the different culture media is given in Table 1. The same culture media have been tested in the presence of 2 g·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.

The second experiment included 3 other culture media. This is MS + BAP 2 mg·L⁻¹ to which NAA has been 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 Set-Up

For each type of explant, a number of 36 per medium and per species was evaluated. In other words, three replicates of 12 test tubes were used for each treatment. The tubes are first incubated in a culture chamber at 27°C ± 1°C in the dark for 5 days, then under a 16 h day/8h night photoperiod and an incident light of 4000 Lux.

Measurements on each type of explant were performed after 30 days of incubation. The parameters measured concerned the presence or absence of resumption of activity (reactivity rate), the number and length of newly formed shoots and the number of nodes. Thus, from these data, the means were calculated, the coefficients or multiplication rates determined and the best regeneration media deduced.

Table 1. Composition of the different culture media used for the micropropagation of the juvenile material of *Annona muricata* L. and *A. squamosa* L.

Media Nomenclature for <i>A. muricata</i>	Hormonal Combinations	Media Nomenclature for <i>A. squamosa</i>	Hormonal Combinations
M0	MS0	MM0	MMS0
M1	MS + BAP 1 mg·L ⁻¹	MM1	MMS + BAP 1 mg·L ⁻¹
M2	MS + BAP 2 mg·L ⁻¹	MM2	MMS + BAP 2 mg·L ⁻¹
M3	MS + BAP 5 mg·L ⁻¹	MM3	MMS + BAP 5 mg·L ⁻¹
M4	MS + KIN 1 mg·L ⁻¹	MM4	MMS + KIN 1 mg·L ⁻¹
M5	MS + KIN 2 mg·L ⁻¹	MM5	MMS + KIN 2 mg·L ⁻¹
M6	MS + KIN 5 mg·L ⁻¹	MM6	MMS + KIN 5 mg·L ⁻¹
M7	MS + BAP 1 mg·L ⁻¹ + KIN 1 mg·L ⁻¹	MM7	MMS + BAP 1 mg·L ⁻¹ + KIN 1 mg·L ⁻¹
M8	MS + BAP 2 mg·L ⁻¹ + NAA 0.1 mg·L ⁻¹	MM8	MMS + BAP 2 mg·L ⁻¹ + NAA 0.1 mg·L ⁻¹
M9	MS + BAP 2 mg·L ⁻¹ + NAA 0.2 mg·L ⁻¹	MM9	MMS + BAP 2 mg·L ⁻¹ + NAA 0.2 mg·L ⁻¹
M10	MS + BAP 2 mg·L ⁻¹ + NAA 0.5 mg·L ⁻¹	MM10	MMS + BAP 2 mg·L ⁻¹ + NAA 0.5 mg·L ⁻¹

MS: Murashige & Skoog medium (1962); MMS: Modified Murashige & Skoog medium (Mathur *et al.*, 1995); BAP: 6-Benzy-laminopurine; KIN: Kinetin (6-furfuryl aminopurine); NAA: 1-Naphthaleneacetic Acid.

2.2.3. Rooting Procedure

Newly formed third generation of shoots, *i.e.* resulting from three successive subcultures lasting thirty days each, are induced in the dark in the MS/2 medium; the macro-elements of which have been diluted by half, supplemented with IBA used alone or at respective concentrations of 25 mg·L⁻¹ and 50 mg·L⁻¹. These media contained 2 g·L⁻¹ of activated charcoal, 20 g·L⁻¹ of sucrose and were solidified with an 8 g·L⁻¹ agar. The pH was adjusted to 5.7, according to the method applied by Farooq *et al.* [11] and Ba *et al.* [12]. The explants were induced for a duration of 1, 3, 5 days before being transferred to the incident light in the MS(0)/2 expression medium *i.e.* without hormones. For each vitroplant issued from each type of explant and each treatment, a number of 36 explants was used for each duration and induction medium. A batch of 12 explants was maintained as a control batch, without root induction, in the MS(0)/2 medium. After 30 days, measurements were taken to determine the rooting rate, the number of newly formed roots per explant and the length of the roots, for each treatment.

2.2.4. Acclimatization

After 4 weeks of incubation in the expression medium, the well-rooted young plants are transferred to weaning conditions according to the procedure of Ba *et al.* [12]. A number of 36 plants, *i.e.* 3 replicates of 12 plants, is chosen for each type of explant and for each species. Watering is carried out with the solution of Hoagland and Arnon [13]. The cover bell is opened gradually from the 2nd week of weaning. The number of young plants which survived after 15 and 30 days of acclimatization was counted and survival rates were determined.

2.2.5. Statistical Analysis

The different treatments were discriminated by multiple comparison of the means after analysis of variance followed by the Student-Newman-Keuls test at the probability threshold 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

1) *Annona muricata*

- *Apices*: The best reactivity rates were obtained, respectively in the medium M3 [MS + BAP 5 mg·L⁻¹] and M6 [MS + KIN 5 mg·L⁻¹]. Indeed, in these media all the terminal apices presented new growths, namely 100% of recovery rate. The M7 medium [MS + BAP 1 mg·L⁻¹ + KIN 1 mg·L⁻¹], allowed to have the greatest average number of shoots (2.12) but it was not significantly different from that obtained (1.875) with the M1 and M2 media. This mean number of shoots was significantly ($p = 0.000$) higher than those obtained with the other media. The average shoot lengths of media M1 [MS + BAP 1 mg·L⁻¹] and M5 [MS + KIN 2 mg·L⁻¹], respectively equivalent to 5.83 cm and 5.93 cm, were not significantly different ($p = 0.270$). These are the best elongations obtained. However, it is the M7 medium which allowed to have the best number of nodes, with 4.37, for an average length of 5.16 cm (Table 2).

- *Axillary nodes*: The medium most favorable to the resumption of activity of the axillary nodes was the M4 medium [MS + KIN 1 mg·L⁻¹], *i.e.* 100% of reactivity rate. The BAP media gave the best multiplication rates compared to the media supplemented with Kinetin alone (M5 and M6). A BAP concentration of 2 mg·L⁻¹ made it possible to have 2.35 shoots on average, which is significantly different from all those obtained with the other media ($F = 55.04$; $p = 0.000$) except for the M1 medium. The latter yielded an average of 2.12 newly formed shoots ($p = 0.282$). With 5.37 nodes, M2 medium also gave the best elongation, 6.03 cm ($F = 62.1$; $p = 0.00$) and the greatest number of average nodes ($F = 10.3$; $p = 0.00$).

- *Cotyledonary nodes*: The best reactivity rates (100%) were obtained in the medium M4 [MS + KIN 1 mg·L⁻¹] and M6 [MS + KIN 5 mg·L⁻¹], respectively. M2 medium [MS + BAP 2 mg·L⁻¹] gave the best mean number of shoots ($F = 83.67$; $p = 0.000$), with 2.87, while for the elongation ($F = 31.76$; $p = 0.00$) the longest (7.46 cm) and the largest ($F = 47.41$; $p = 0.00$) mean number of nodes (5.62) are obtained respectively with M5 media and M3. All these averages remain significantly different from those obtained with the other hormonal combinations.

2) *Annona squamosa*

- *Apices*: The best recovery rate was observed in MM4 medium [MMS + KIN 1 mg·L⁻¹], with 100% reactivity rate. The media containing BAP combined with

Kinetin for the apical explants significantly improved the bursting of the explants with mean numbers of shoots much higher than that obtained in the control medium. Average numbers of shoots of 3.12 and 2.87 were obtained with explants in MM2 [MMS + BAP 2 mg·L⁻¹] and MM7 [MMS + BAP 1 mg·L⁻¹+ KIN 1 mg·L⁻¹] media. These were not significantly different ($p = 0.11$) but remain significantly higher ($F = 40.19$; $p = 0.00$) than those obtained with the other media. However, it was in the MM7 medium [MMS + BAP 1 mg·L⁻¹+ KIN 1 mg·L⁻¹] that the vitroplants had the best elongation (9.05 cm) and the highest average number of nodes (5.62). This medium had a very significant effect on shoot elongation ($F = 21.99$; $p = 0.00$) and the mean number of nodes ($F = 18.4$; $p = 0.00$) compared to the control medium (MMS0) for which they were respectively 3.66 cm and 2.37 nodes (**Table 2**).

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

Species	Media	Explant Type	Reactivity Rate (%)	Average number of shoots	Average shoot length (cm)	Average number of nodes	Species	Media	Explant Type	Reactivity Rate (%)	Average number of shoots	Average shoot length (cm)	Average number of nodes
<i>Annona muricata</i>	M0	APX	66.67	1.12 c	3.18 c	2 d	<i>Annona squamosa</i>	MM0	APX	91.67	1 c	3.17 e	1.5 g
		AN	75	1 d	3.83 d	1.62 c			AN	91.67	1.37 b	3.66 e	2.37 d
		CN	75	1 c	3.8 c	2.25 e			CN	83.33	1.5 ab	3.32 d	2.75 e
	M1	APX	91.67	1.87 ab	5.83 a	3.87 a		MM1	APX	83.33	1.62 c	3.88 d	2.62 f
		AN	75	2.12 ab	5.01 bc	4.5 b			AN	66.67	1.12 b	4.98 cd	3.12 cd
		CN	91.67	1.37 c	4.06 c	3.12 cd			CN	100	1.25 ab	3.98 cd	3.5 bcd
	M2	APX	83.33	1.87 ab	4.6 b	3.5 bc		MM2	APX	75	3.12 a	5.31 c	4.12 bc
		AN	91.67	2.35 a	6.03 a	5.37 a			AN	83.33	1.62 ab	6.31 b	4.87 a
		CN	75	2.87 a	4.02 c	4 bc			CN	91.67	1.87 a	6.68 a	5.12 a
	M3	APX	100	1.5 b	4.03 bc	4.12 a		MM3	APX	91.67	2.37 b	6.57 b	4.75 b
		AN	91.67	1.87 b	3.96 d	4 b			AN	100	2.25 a	5.2 cd	3.87 bc
		CN	75	2.12 ab	6.57 b	5.62 a			CN	83.33	1.62 ab	4.88 bc	3.75 bc
	M4	APX	91.67	1.37 bc	5.25 ab	3.62 bc		MM4	APX	100	1.25 c	4.1 d	3.5 cde
		AN	100	1.75 bc	4.88 c	4.5 b			AN	83.33	1.62 ab	4.58 d	3.12 cd
		CN	100	2.25 ab	3.71 c	4.37 bc			CN	75	1 b	4.58 bc	2.87 de
	M5	APX	83.33	1.62 b	5.93 a	3.87 a		MM5	APX	91.67	1.37 c	7.33 b	4.75 b
		AN	91.66	1.37 cd	5.21 bc	4.37 b			AN	91.67	1.37 b	5.58 bc	3.87 bc
		CN	91.66	1.37 c	7.46 a	4.75 b			CN	100	1.25 ab	5.68 b	3.5 bcd
	M6	APX	100	1.5 b	4.56 b	3.37 c		MM6	APX	83.33	1.62 c	6.76 b	3.62 cd
		AN	83.33	1.62 bc	5.21 bc	4.25 b			AN	66.67	1.12 b	4.85 cd	3.62 bc
		CN	100	1.25 c	6.12 b	3.37 cd			CN	75	1 b	5.41 bc	3.12 cde
	M7	APX	75	2.12 a	5.16 ab	4.37 a		MM7	APX	83.33	2.87 a	9.05 a	5.62 a
		AN	91.67	1.37 cd	5.58 b	3.62 b			AN	83.33	1.87 ab	7.22 a	4.25 ab
		CN	75	2.12 ab	4.01 c	2.62 de			CN	83.33	1.62 ab	5.86 b	4.12 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 letter are not significantly different at the 5% threshold of the Newman-Keuls test.

- *Axillary nodes*: The highest reactivity rate was obtained in MM3 medium [MMS + BAP 5 mg·L⁻¹] with 100% recovery rate as well as the average number of shoots which is 2.25. This is significantly higher than that of MM0 which is 1.37 ($F = 18.36$; $p = 0.001$). The best elongation (7.22 cm) was measured on the newly formed vitroplants from axillary nodes in the MM7 medium. It is significantly greater than the average shoot lengths of other culture media ($F = 63.44$; $p = 0.00$). However, the highest mean number of nodes (4.87) was that of vitroplants of the MM2 medium. It is not significantly different from that of MM7 which is 4.25 nodes ($F = 107.04$; $p = 0.00$).

- *Cotyledonary nodes*: The medium MM1 and MM5 offer the best recovery rates (100%). The highest average number of shoots is 1.87; it was obtained with the MM2 medium but it is not significantly different from that obtained with the MM3 and MM7 media with which it is 1.62 ($F = 2.48$; $p = 0.31$). The MM2 medium allowed to have an average number of nodes (5.12) and an average length (6.68 cm) significantly different from those of the other media. This medium had a very significant effect on the average number of nodes ($F = 15.55$; $p = 0.00$) and the average shoot length ($F = 13.58$; $p = 0.00$).

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

The results related to the morphogenesis of the explants for the two *Annona* species are reported in **Table 3** and **Plate 1**.

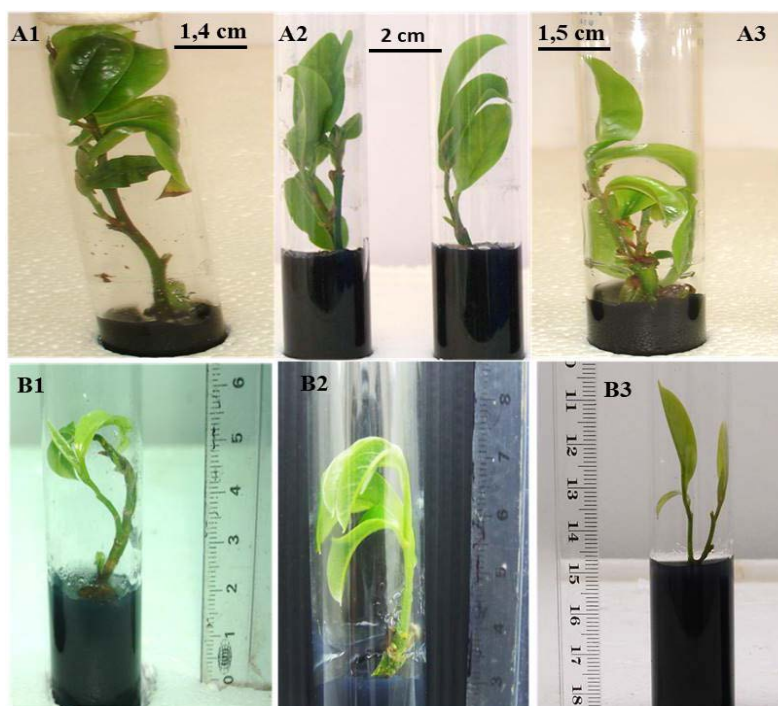


Plate 1. Appearance of newly formed shoots from juvenile material taken from *Annona muricata* L. (A) and *A. squamosa* L. (B). **A1, B1**: Vitroplants neoformed from the apices. **A2, B2**: Vitroplants neoformed from axillary nodes. **A3, B3**: Vitroplants neoformed from cotyledonary nodes.

Table 3. Influence of NAA on the *in vitro* morphogenesis of juvenile material of *A. muricata* L. and *A. squamosa* L.

Species	Media	Explant Type	Reactivity Rate (%)	Average number of shoots	Average shoot length (cm)	Average number of nodes	Species	Media	Explant Type	Reactivity Rate (%)	Average number of shoots	Average shoot length (cm)	Average number of nodes
<i>Annona muricata</i>	M0	APX	66.67	1.12 bc	3.28 c	2 c	<i>Annona squamosa</i>	MM0	APX	75	1 c	3.17 d	1.5 c
		AN	83.33	1 c	3.83 d	1.62 d			AN	83.33	1.37 b	3.66 d	2.37 d
		CN	75	1 b	3.8 d	2.25 d			CN	91.67	1.5 ab	3.32 d	2.75 cd
	M2	APX	91.67	1.87 a	4.6 b	3.5 a		MM2	APX	91.67	3.25 a	5.31 bc	4.12 a
		AN	91.67	2.35 a	6.03 b	5.37 a			AN	91.67	1.62 ab	6.31 b	4.87 a
		CN	75	2.87 a	4.02 d	4 b			CN	91.67	1.87 a	6.68 a	5.12 a
	M8	APX	83.33	1.87 a	6.01 a	3.25 ab		MM8	APX	91.67	1.37 bc	6.67 a	4.5 a
		AN	91.67	1.37 bc	4.92 c	4.12 b			AN	83.33	1.37 b	7.05 a	4.25 ab
		CN	100	1.25 b	9.11 a	5.62 a			CN	75	1.25 ab	5.76 b	3.75 b
	M9	APX	100	1.75 ab	4.61 b	2.75 b		MM9	APX	100	1.5 bc	5.68 b	3.25 b
		AN	91.67	1.37 bc	7.83 a	3.75 bc			AN	80.33	1.62 ab	6.01 b	3.12 cd
		CN	75	1 b	5.68 b	3.5 bc			CN	100	1.5 ab	4.51 bc	3.37 bcd
	M10	APX	91.67	1 c	2.38 d	2.12 c		MM10	APX	83.33	1 c	4.43 c	2.87 bc
		AN	91.67	1.25 bc	4.71 c	3.5 c			AN	100	1.25 b	4.53 c	3.37 b
		CN	83.33	1 b	4.81 c	3.5 bc			CN	83.33	1.25 ab	5.1 bc	2.5 d

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.

1) *Annona muricata*

- *Apices*: The best reactivity rate (100%) is obtained in the M9 medium [MS + BAP 2 mg·L⁻¹ + NAA 0.2 mg·L⁻¹]. However, the average numbers of shoots obtained with the M2 [MS + BAP 2 mg·L⁻¹], M8 [MS + BAP 2 mg·L⁻¹ + NAA 0.1 mg·L⁻¹] and M9 were not significantly different ($F = 14.6$; $p = 0.58$). They were, respectively, 1.87, 1.87 and 1.75 (**Table 3**). M8 medium also provided the best and significant elongation, with 6.01 cm. The best mean number of nodes (3.5) was obtained in M2 medium [MS + BAP 2 mg·L⁻¹]. However, it is not significantly different from that obtained (3.25) in the M8 medium ($F = 48.76$; $p = 0.13$).

- *Axillary nodes*: The best reactivity rates (91.7%) were observed in M2, M8, M9 and M10 media. The mean number of nodes (5.37) of the M2 medium [MS + BAP 2 mg·L⁻¹] is significantly greater than those of the other culture media ($F = 89.76$; $p = 0.000$). But, the average shoot length of vitroplants (7.83 cm) in M9 medium [MS + BAP 2 mg·L⁻¹ + NAA 0.2 mg·L⁻¹] is significantly greater than those of shoots in other media ($F = 46.77$; $p = 0.00$).

- *Cotyledonary nodes*: The best reactivity rate (100%) is obtained in the M8 medium [MS + BAP 2 mg·L⁻¹ + NAA 0.1 mg·L⁻¹]. In the presence of NAA, the mean number of shoots decreased considerably compared to M2 medium which

gave 2.87 shoots. For NAA supplemented at 0.2 and 0.5 mg·L⁻¹, the average shoot number is equal to 1 ($F = 21.34$; $p = 0.000$), which is identical to that of the control medium [MS0]. It is also the M8 medium which gave the best elongation (9.11 cm) and the greatest average number of nodes (5.62), which remain significantly higher than those of the other media.

2) *Annona squamosa*

- *Apices*: The best recovery rate is 100% with MM8 medium [MMS + BAP 2 mg·L⁻¹ + NAA 0.1 mg·L⁻¹]. The mean number of shoots decreased significantly (**Table 3**) when NAA is added to the medium supplemented with 2 mg·L⁻¹ of BAP ($F = 37.23$; $p = 0.000$). In MM8 medium, the mean length of vitroplants (6.67 cm) is significantly greater than those obtained in MM2 media and MM9 which were, respectively, 5.31 cm ($F = 6.78$; $p = 0.24$) and 5.68 cm ($p = 0.036$). The average number of nodes (4.12) in the MM2 medium and of 4.5 in the MM8 medium were not significantly different ($F = 3.76$; $p = 0.435$).

- *Axillary nodes*: The best reactivity rate (100%) is observed in the MM10 medium [MMS + BAP 2 mg·L⁻¹ + NAA 0.5 mg·L⁻¹]. The average numbers of shoots, obtained with the different hormonal combinations, are not significantly different ($F = 1.24$; $p = 0.056$). The best elongation (7.05 cm) is that obtained in the MM8 medium. It is significantly greater than those of the other media ($F = 14.45$; $p = 0.00$) even if the average number of nodes (4.25) in this medium is not significantly different from that of the shoots in the medium MM2 which is 4.87 ($F = 7.43$; $p = 0.00$).

- *Cotyledonary nodes*: The best recovery rate (100%) is offered by the MM9 medium. The best average number of shoots (1.87), obtained in the MM2 medium, was not significantly different from those of the other media, in particular MM0 in which the average number of shoots is 1.5. MM2 medium also produced the best average length (6.68 cm) and the greatest average number of nodes (5.12). This mean length remains significantly greater ($F = 13.06$; $p = 0.00$) than that of shoots from other media. For the average number of nodes, it decreased considerably ($F = 6.31$; $p = 0.00$) when NAA is added to the media (**Table 3**).

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

Referring to **Table 4**, depending on the IBA concentration and the induction time, for each species, the results were as follows:

3.2.1. *Annona muricata*

An incubation of one day (1) in a medium supplemented with IBA at 25 mg·L⁻¹, did not allow any root growth whatever the type of explant. For an 3 days-induction period, followed by expression in a free-hormone medium (MS(0)/2), a rooting rate of 8.33% of shoots from apices was obtained, with an average length of 0.8 cm. For a 5 days-induction period, this rate rose to 16.67% for

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

Species	Media	Induction Time (days)	Explant Type	Rooting Rate (%)	Average number of roots	Average root length (cm)	Species	Media	Induction Time (days)	Explant Type	Rooting Rate (%)	Average number of roots	Average root length (cm)
<i>Annona muricata</i>	MS/2	0	APX	0	0 b	0 c	<i>Annona squamosa</i>	MS/2	0	APX	0	0 b	0 d
			AN	0	0 c	0 b				AN	0	0 b	0 b
			CN	0	0 c	0 b				CN	0	0 c	0 b
		1	APX	0	0 b	0 c			1	APX	0	0 b	0 d
			AN	0	0 c	0 b				AN	0	0 b	0 b
			CN	0	0 c	0 b				CN	0	0 c	0 b
	MS/2 + AIB 25 mg·L ⁻¹	3	APX	8.33	1 a	0.8 b		MS/2 + AIB 25 mg·L ⁻¹	3	APX	0	0 b	0 d
			AN	16.67	1.5 c	1.2 b				AN	16.67	1 b	1.4 a
			CN	0	0 c	0 b				CN	8.33	1 b	1.2 a
		5	APX	16.67	1 a	1.9 ab			5	APX	16.67	1 ab	0.9 c
			AN	16.67	1 b	1.6 a				AN	25	1.5 a	1.4 a
			CN	8.33	2 b	1.2 a				CN	0	0 c	0 b
	MS/2 + AIB 50 mg·L ⁻¹	1	APX	41.67	1.25 a	0.7 b		MS/2 + AIB 50 mg·L ⁻¹	1	APX	33.33	2 a	2.25 b
			AN	0	0 c	0 b				AN	16.67	2.4 a	1.8 a
			CN	16.67	2.5 b	1.5 a				CN	0	0 c	0 b
		3	APX	33.33	1.5 a	2.25 a			3	APX	41.67	1.37 a	3.5 a
			AN	25	2 ab	1.1 a				AN	33.33	2 a	1.9 a
			CN	33.33	4 a	1.25 a				CN	66.67	3.37 a	1.33 a
	MS/2 + AIB 50 mg·L ⁻¹	5	APX	0	0 b	0 c		MS/2 + AIB 50 mg·L ⁻¹	5	APX	16.67	2 a	1.48 b
			AN	41.67	2.5 a	1.4 a				AN	8.33	1 b	1.2 a
			CN	83.33	2.56 b	1.3 a				CN	33.33	3.25 a	2.5 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.

vitroplants from the apices and axillary nodes; with an average number of roots equal to 1. For the latter, the average length is 1.6 cm while for vitroplants from the apices, it was 1.9 cm ($p = 0.001$) and is significantly greater ($F = 2.74$; $p = 0.021$) to that (0.8 cm) of roots from shoots of apical origin incubated for 3 days. For the cotyledonary nodes, only 8.33% of the vitroplants rooted, with 2 roots per explant and an average length of 1.2 cm (Table 4).

In the presence of [IBA 50 mg·L⁻¹], the best rooting rate (83.33%) was obtained for the vitroplants from cotyledonary nodes incubated for 5 days, with 2.55 roots on average and an elongation of 1.3 cm. The same is noted for the largest average number of roots (4) obtained after a 3-day-incubation period; it is significantly higher than that of the other induction times ($F = 5.87$; $p = 0.001$). The highest rooting rate is 41.67% for vitroplants newly formed from

axillary nodes, with a significantly higher average of 2.5 roots per shoot and an average root length of 1.4 cm. These results were obtained following a 5-day-induction period followed by 30 days of expression in a hormone-free MS(0)/2 medium. On the other hand, when the duration of induction was only one day (1), 41.67% of the vitroplants resulting from the apices took root, with an average of 1.25 roots and 0.7 cm of elongation. This rooting rate obtained after an 1 day-induction period remained the highest for this type of explants (**Table 4**).

3.2.2. *Annona squamosa*

When vitroplants from cotyledonary nodes underwent an inducing pretreatment in the presence of [IBA 25 mg·L⁻¹], the roots only appeared after an incubation period of 3 days, with a rate of 8.33% and a length of 1.2 cm. For vitroplants newly formed from axillary nodes, the rate was 16.67%. For those formed from the apices, it took an induction period of 5 days in the presence of [IBA 25 mg·L⁻¹] to see the emergence of roots. Thus, the rooting rates were, respectively, 16.67% and 25% for vitroplants from apices and axillary nodes (**Table 4**).

The best rooting rate (66.67%) was also obtained for vitroplants from cotyledonary nodes thanks to a 3-day-induction period, with 3.37 roots and length of 1.33 cm on average. With vitroplants from the apices, the rooting rate was 41.67%, with 1.37 roots on average and 3.5 cm of elongation. For this type of vitroplants, the mean numbers of roots were not significantly different except for the control medium and the induction times of 1 and 3 days with [IBA 25 mg·L⁻¹] ($F = 7.02$; $p = 0.00$) while the mean length obtained thanks to an induction period of 3 days is significantly greater than the others ($F = 3.70$; $p = 0.02$). For vitroplants from axillary nodes, rooting rates remain low. For induction times of 1, 3 and 5 days, they were, respectively, 16.67%, 33.33% and 8.33%. The average numbers of roots ($F = 1.32$; $p = 0.087$), as well as the average lengths of roots ($F = 3.47$; $p = 0.054$), obtained for induction times of 1 and 3 days were not significantly different (**Table 4**).

3.3. Acclimatization

The different types of explants are transplanted into cups containing a sterile sand-soil mixture and then stored in a mini-greenhouse with the shutter completely closed for a week. This process kept the plants produced *in vitro* in an atmosphere of high relative humidity, but the mini-greenhouse is fitted with a plexiglass bell with an adjustable opening. Watering is carried out every 2 days with Hoagland and Arnon's nutritive solution (1938) for the first 2 weeks, then with tap water. On the 12th day of acclimatization, for *A. muricata*, a decay rate of 8.33% for the explants resulting from the axillary and cotyledonary nodes was noted. For *A. squamosa*, it was 16.67% for those originated from the apices and axillary nodes, while for plantlets from cotyledonary nodes, it was 8.33%. From the 14th day, the shutter is opened halfway to avoid prolonged confinement. At

the 21st day, the plexiglas bell is fully opened. The plantlets were first maintained in the shade under the bench for 3 days and then placed on the bench. Thus, on the 30th day, the survival rates were 75% for the plants issued from the cotyledonary and axillary nodes while for those from the apices of *A. muricata*, 83.33% survived. For the plants of *A. squamosa* from the apices, axillary and cotyledonary nodes, the survival rates were, respectively, 75%, 66.67% and 83.33% (**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. muricata* L. and *A. squamosa* L.

Species	Explant Type	Survival Rate (%)	
		After 15 days	After 30 days
<i>A. muricata</i>	APX	100 a	83.33 bc
	AN	91.67 ab	75 c
	CN	91.67 ab	75 c
<i>A. squamosa</i>	APX	83.33 b	75 c
	AN	83.33 b	66.67 bc
	CN	91.67 a	83.33 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 letter are not significantly different at the 5% threshold of the Newman-Keuls test.

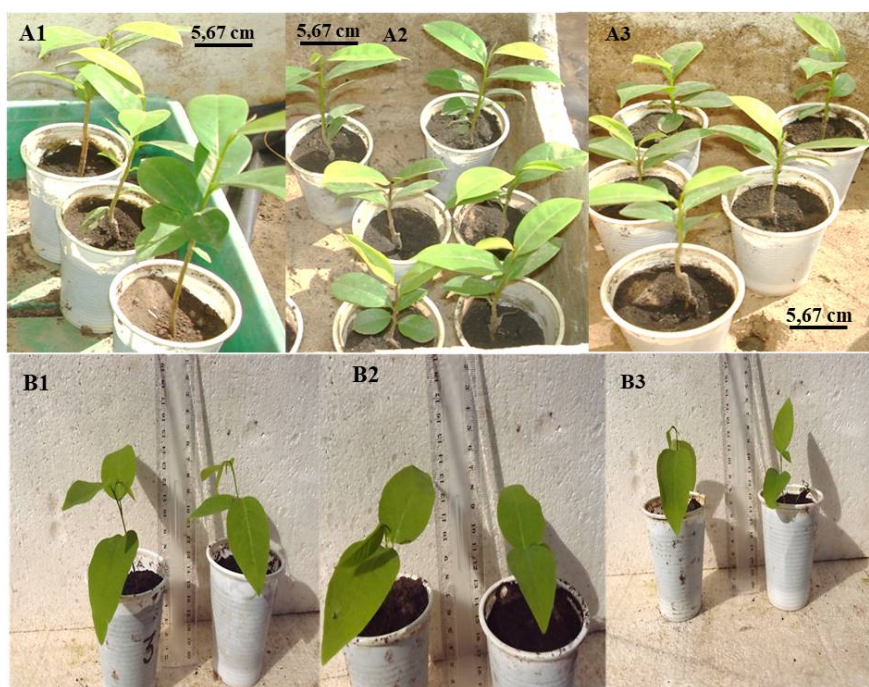


Plate 2. Young plants resulting from the juvenile material after 30 days of weaning under shade. **A:** *Annona muricata*; **B:** *A. squamosa*; **A1, B1:** Plants from the apices; **A2, B2:** Plants from axillary nodes; **A3, B3:** Plants from cotyledonary nodes.

4. Discussion

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

One week after the first *in vitro* introduction of the different explants, an onset of browning due to the presence of polyphenols was noted. This could be due to the exposure of seedlings resulting from germination to light which would promote the synthesis of the polyphenols. According to [14], roots of young plants grown under *in vitro* light conditions produced more polyphenols than leaves of adult plants during the fall. The authors [15] have already reported browning of the material in *A. muricata* when it was 16 to 24 days old, which has a negative impact on its viability. Furthermore, abiotic stress such as temperature extremes, drought, flooding, largely influence plant development and crop productivity [16]. It is well known that the biosynthesis of phenolic compounds is generally activated by certain abiotic factors such as extreme temperatures, salinity, pollution by heavy metals, light, especially ultraviolet radiations [17] [18]. As a result, the explants of the two species were transferred to the different culture media in which $2 \text{ g}\cdot\text{L}^{-1}$ of activated charcoal were incorporated.

After 6 days of culture, the outlines of newly formed shoots were visible at the level of the cotyledonary and axillary nodes and were more numerous in the media enriched with hormones for explants of *A. muricata*. For the apices of *A. squamosa*, they started to appear from the 8th day. New shoot formation took place in the area where the cotyledonary leaves were inserted. Indeed, the growth and development of plants can be modulated by the synergistic contributions of auxins and cytokinins [19]. The authors [20] had already shown that the organogenic potential of the cotyledonary nodes of *Vigna mungo* is strongly influenced by the type of growth regulator, the growth and the hormonal combination used. The organogenic properties of cytokinins were explained, in part, by their interactions with the sucrose present in the culture medium [21].

For the 2 species studied, the BAP used alone or in combination with Kinetin was found to be more effective than the control medium or Kinetin employed alone for the new growth of shoots and the number of nodes. Kinetin alone had a better effect on elongation than BAP even though this allowed for better bud breakout. The same result was obtained by [22] in *Ferronia limonia*. According to [23], BAP was more effective than Kinetin for regeneration and shoot elongation in *Parkia biglobosa*. The same observation was made by [24]) on *Balanites aegyptiaca* and [25] on *Vigna radiata*. The work of [26] on *Pentadesma butyraceae* Sabine shows that BAP promotes resumption of apices activity. These authors [27] showed that the best medium for the propagation of *Annona annua* shoots is MS medium supplemented with $1 \text{ mg}\cdot\text{L}^{-1}$ of BAP. The incorporation of BAP into the culture medium generally improves the regeneration of explants as reported by [25]. On the other hand, Kinetin essentially promotes the elongation of buds [28]. It is noted that an increase in the cytokinin content is accompanied by a reduction in shoot elongation and the average number of nodes in *A. squ-*

amosa while in *A. muricata* explants, BAP at 5 mg·L⁻¹ allowed an elongation of the newly formed shoots and an increase in the number of nodes, but only for the cotyledonary nodes. According to [29], a cytokinin concentration greater than 2 mg·L⁻¹ has a detrimental effect on multiplication and rooting in most *Annonaceae*. Thus, [30] used cytokinin concentrations not exceeding 1 mg·L⁻¹ for the *in vitro* multiplication of *Annona glabra*. This action of high doses of cytokinin on the *in vitro* morphogenesis of *Annonaceae* species seems to confirm the observations of [28] on *Vigna unguiculata*. High concentrations of BAP would favor apical dominance in *Ixora margaretae* [31] and in *Morinda sp. nov A* [32]. The work of [33] demonstrated that the addition of BAP in the culture medium would induce the apical dominance during *in vitro* culture of the apical part of *Mentha spicata* H. (mint) and would, therefore, intensify the height growth of the plant. Furthermore, [34] had noticed in *Annona glabra* that the BAP concentrations greater than 0.5 mg·L⁻¹, added in a medium without activated charcoal, caused a strong callogenesis and a vitrification of the explants. This ultimately causes necrosis of the tip of the stems and leaf abscission that we observed in *A. muricata* and *A. squamosa*. These physiological disorders are frequently mentioned during *in vitro* culture of other *Annona* species [35]; they have been associated with calcium deficiency [36] or ethylene accumulation [37] in *A. squamosa*. The lack of calcium considerably affects cell division at the level of the meristematic zone as well as the synthesis of pectin [38]. However, [39] have already shown that a high concentration of cytokinins affects the accumulation and use of calcium by the vitroplant during the micropropagation of *Annonaceae*. Increasing cytokinin concentration induces a decrease in the rate of explant regeneration [40]. In addition, at high doses, cytokinins promote the accumulation of iron in many fruit species of the *Annonaceae* family, which promotes oxidation during *in vitro* multiplication [30]. However, the type of vial used and the sealing technique adopted are factors responsible for the development of these disorders; they would lead to maintaining a high relative humidity of the air inside the container, preventing the transpiration of the plant necessary for calcium to reach the xylem of the plant, and they would make gas exchange with the outside atmosphere impossible, and would, thus, facilitate the accumulation of gases produced by the tissues at physiologically active levels [35].

The addition of NAA to the culture media together with the cytokinins did not increase the number of newly formed shoots compared to the cytokinins used alone for the 2 *Annona* species. However, the combination of BAP with NAA appears to be more efficient in stimulating shoot elongation and increasing the number of *A. muricata* nodes. This favorable action of the cytokinin-auxin combination on the morphogenesis of young material is mentioned by [41] on *Santolina canescens*, [42] on *Bupleurum fruticosum*, [24] on *Balanites aegyptiaca* and [23] on *Parkia biglobosa*. The beneficial action of NAA on shoot elongation has already been reported by several authors [32] [43] [44]. Other authors

[45] claimed the opposite, NAA would greatly affect bud formation in *Plumbago rosae*. Several studies reported that the BAP-NAA combination induces a decrease in the number of regenerated shoots [20] [25] [46]. However, in some cases, NAA can promote shoot proliferation for many species of *Hyacinthaceae* [47] and *Saussurea lappa* [48]. On *Plantago lanceolata*, [49] obtained good regeneration of hypocotyl and cotyledon explant shoots in MS medium supplemented with BAP ($0.75 \text{ mg}\cdot\text{L}^{-1}$) and NAA ($0.2 \text{ mg}\cdot\text{L}^{-1}$).

The NAA-BAP combination, however, caused callus formation at the base of explants *in vitro* cultured for many explants. This callogenesis has already been reported by [50] on *Nigella sativa*, [25] on *Vigna unguiculata* and [51] on *Nigella damascena*. The intensity of callus formation increases with increasing NAA concentration in the growing medium, which significantly limits shoot regeneration in *Vigna unguiculata* [52]. According to [53], it is frequently observed in species with marked apical dominance. It is due to the accumulation of auxin at the base of the apical explant [54]. The auxin, in the presence of cytokinins, would stimulate the proliferation of cells located in the area of injury of the explant. The presence of callus constitutes a factor limiting rhizogenesis. Indeed, after one month of culture, one observed on certain explants a browning of the calluses which gives a bad surface of contact of the explant with the culture medium and seems, therefore, to slow down its growth.

4.2. Rooting

In *A. muricata*, the best rooting rate is obtained with vitroplants newly formed from axillary (41.67%) and cotyledonary nodes (83.33%) following a 5 day-rhizogenic induction in an MS/2 medium. Supplemented with [IBA $50 \text{ mg}\cdot\text{L}^{-1}$] and in the presence of $2 \text{ g}\cdot\text{L}^{-1}$ of activated charcoal while an induction of 24 h makes it possible to have a rate of 41.67% for the vitroplants newly formed from the apices. In *A. squamosa*, the best rooting rates are obtained for vitroplants from the apices (41.67%) and those from the cotyledonary nodes (66.67%) following a 3 day-rhizogenic induction with [IBA $50 \text{ mg}\cdot\text{L}^{-1}$] whereas for vitroplants from axillary nodes, this rate is obtained following a 5-day-induction with [IBA $25 \text{ mg}\cdot\text{L}^{-1}$]. The rooting of woody species is generally dependent on hormonal treatment as pointed out by [23] in *Parkia biglobosa*. The authors [55] also noted an 80% rooting rate on *Acacia tortilis* subsp. *raddiana* after treatment with IBA compared to NAA. IBA would, therefore, be more conducive to the rhizogenic induction of woody species, the latter being difficult to root *in vitro* [22] [56] [57]. In addition, [27] obtained better rhizogenesis in *Artemisia hololeuca* in the presence of IBA compared to the use of NAA.

The consistency of the rooting medium, like the high concentrations of auxin, also seems to strongly influence the appearance of callus at the base of explants as with explants of *Annona muricata* and *A. squamosa* in the presence of high concentrations of IBA. Likewise, [58] found that rooting of apple tree can be achieved in a solid or liquid medium by using vermiculite with better results in

the latter case. The authors [59] used a solid and liquid medium with paper and vermiculite support for the M106 apple tree rootstock and obtain an excessive development of the callus, *i.e.* 40% of rooting in the solid medium while the use of liquid medium increases the rooting rate to 80% and results in a noticeable reduction in callus development. This significant difference is explained by the fact that nutrients are more readily available for the explant [60]. In addition, the agar would create a critical turgor pressure which puts the cells under stress. This stress would induce the production of ethylene, the concentration of which would tend to increase in confinement, which also stimulates the production of auxin. On the contrary, [61] and [62] demonstrated that the decrease in sugar concentration and the presence of agar favored rhizogenesis.

We also noted that the rooting rate remains quite high, especially for *A. muricata* for which the best rooting rate was 83.33%. For those of *A. squamosa*, this rooting rate was equal to 66.67%. The roots were, in general, more developed at the level of vitroplants resulting from the cotyledonary nodes where more lateral ramifications were observed for the 2 species. Furthermore, [63] could not exceed 50% rooting in the giant sequoia. This can be explained, by the fact that rooting is often more difficult in woody plants than in herbaceous plants [64]. The high rooting rates may be due to the combined beneficial effects of the basal medium used (MS/2), dark incubation, inclusion of activated charcoal, and induction with high concentrations of auxin. The authors [65] as well as [66] noted the importance of auxins in improving root system development. The author [67] reported that halving macronutrients facilitated rooting in many species. This effect is thought to be due to the reduction in the quantity of nitrogen in the medium [68]. The beneficial effect of MS/2 medium on rhizogenesis has also been obtained on avocado [69]. On the other hand, [70] showed that incubation in the dark, in the presence of IBA, increased the rooting rate of *Pistacia vera* vitroplants. This beneficial effect of darkness on rooting has also been observed on apple [71] and on *Quercus rubra* [72]. Beyond its action on polyphenols, the production and oxidation of which it inhibits [73], darkness increases the number of cells competent to induce the appearance and development of roots thanks to the etiolation [74] and, thus, avoids the destruction of the growth regulators included in the medium by reducing the peroxidase activity [72]. According to [75], 5 days incubation in the dark allowed root inducing cells to differentiate on *Quercus robur*. However, in this species, during incubation in the dark, there is senescence and necrosis of the shoots [76]. According to [72], activated charcoal not only stimulates root development, but at the same time, adsorbs excess growth regulators. It also acts to prevent the harmful action of polyphenols and helps to obscure the environment of the explant which is favorable to rhizogenic induction [77] [78].

4.3. Acclimatization

The low survival rate during the acclimatization of young plants resulting from

in vitro propagation is one of the factors limiting this technique for the economic exploitation of many species of economic importance [79].

Acclimatization in a mini-greenhouse followed by gradual contact with the surrounding environment from the first week onwards made it possible to obtain, on *A. muricata*, 83.33% of young plants from the apices which survived, while for those from axillary and cotyledonary nodes, the survival rate was 75%. For *A. squamosa*, the survival rates were, respectively, 75%, 83.33% and 66.67% for young plants from the apices, cotyledonary and axillary nodes. Similar results have been reported by [80] on Carob (*Ceratonia siliqua* L.) microplants gradually acclimatized to the ambient atmosphere. Confining the young plants in an atmosphere saturated with humidity (90% to 100%) at the start of weaning *i.e.* close to *in vitro* conditions at a high temperature, has been shown to be beneficial for their successful acclimatization. Indeed, according to [24] plants resulting from *in vitro* culture generally have a thinner cuticle than that of mother plants, which causes their rapid desiccation when the relative humidity is lowered rapidly by the passage *ex vitro*. Thus, [81] noted that 43% of plants of *Prosopis juliflora* and *P. chilensis* die off during acclimatization. On the other hand, the very high relative humidity inside the tubes during *in vitro* cultivation causes the leaves of young plants to have a very high amount of non-functional stomata compared to those of adult plants adapted to *in vivo* conditions [82], [83]. Gradual weaning in a mini-greenhouse, therefore, allows plants to acquire anatomical, physiological and metabolic characteristics that allow them to better adapt and survive during their transplantation under natural conditions.

5. Conclusion

This work has shown the importance of hormonal combinations in the micro-propagation of these *Annonaceae* species. It is important to note the beneficial effects of BAP in new shoot formation and its positive synergistic effect with NAA in shoot elongation, but the effect of these hormones varies depending on the type of juvenile explant introduced *in vitro*. Among the explants, the cotyledonary nodes are the most reactive for the neoformation of shoots. It also seems that IBA is better than NAA for the rhizogenous induction of vitroplants and improves their branching in lateral roots. The gradual weaning of the vitroplants in a mini-greenhouse, with an adjustable opening, is more favorable to the acclimatization of the plants *ex vitro* whatever the initial origin of the explants.

Conflicts of Interest

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

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