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In Vitro Clonal Propagation from Adult Material of a Savannah Species of Socio-Economic Importance: *Annona senegalensis* Pers.

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Abstract

Annona senegalensis (Pers.) or Annone from Senegal is a species found in the West African savannah and para-littoral sands. It offers real possibilities of socio-economic use, particularly in the medical, nutritional, ecosystem conservation and poverty alleviation fields. However, this wild species remained not fully exploited despite its potentialities. As a result, there is a need to regenerate this species; therefore, the *in vitro* propagation from adult material was undertaken. For this purpose, axillary nodes from plant regrowth taken from elderly subjects were aseptically introduced into different media enriched or not with phytohormones. Among the 3 culture media tested, that of Murashige and Skoog (MS, 1962) was the most reactive and made it possible to develop a micropropagation protocol for adult material of *A. senegalensis*. Thus, the introduction of these explants in media containing cytokinins (BAP and Kinetin) significantly increased the reactivity compared to media without hormones. If the best average numbers of shoots (2.3) and nodes (5.3) are obtained in MS + BAP 2 mg·L^{-1}, with a reactivity of 91.66%, the addition of 0.1 mg·L^{-1} NAA gave the best average length (8.25 cm) of vitroplants. An induction time of 3 days into darkness with IBA at 50 mg·L^{-1}, followed by a transfer to hormone-free expression medium (MS/2) under light, resulted in a rooting rate of 58.33%. After the gradual weaning of the young plants in a mini-greenhouse, under shade for 3 weeks, 50% of the plants survived. This *in vitro* regeneration protocol can therefore be adopted for the clonal propagation of adult material of *A. senegalensis*. However, the rooting rate can be improved by trying other hormonal combinations based on auxins or by considering *ex-vitro* rooting during shade weaning.

Keywords

Annona senegalensis, Adult Material, Micropropagation,

6-Benzylaminopurine, 1-Naphthaleneacetic Acid, Indole-3-Butyric Acid

1. Introduction

Annona senegalensis or Annone from Senegal, and commonly known as African custard-apple [1], wild custard apple or wild soursop [2] is a species of *Annonaceae* found in the wild in the undergrowth of Sudanese tree savannas as well as in the para-littoral sands of West Africa [3]. 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. *Annona senegalensis* takes the form of either a shrub or small tree that can sometimes reach six meters high. It is a species of great socio-economic and pharmacological importance. Its fleshy fruit is edible [4] [5] [6] and is highly prized by local populations, while its leaves, bark and roots are widely used in traditional medicine to treat many diseases such as skin diseases, diarrhea, malaria, *etc*. [3] [7] [8] [9]. More recently, it has been proven that essential oils extracted from the leaves of *A. senegalensis* would contain substances used as a source of potential human cancer chemotherapeutic and chemopreventive agents [10]. However, despite the many uses and opportunities it offers, this species is not being fully exploited. Indeed, *A. senegalensis* always remains in the wild. Unfortunately, the degradation of ecosystems in general due to climate change and factors such as drought, bush fires, overexploitation of plant resources, demographic pressure and the aging of natural populations [11] [12] [13] means that this species, like most Sahelian species, is highly threatened. The frequency of natural regeneration is insufficient to ensure its sustainability and development [14], hence the need to resort to artificial technical methods to overcome this constraint [15]. The biotechnological methods widely used in many laboratories would allow mass production of good quality plants to be made available to foresters, local populations and other operators. This would even allow for industrial-scale exploitation of this species.

In this context, the study was carried out in order to contribute to the renewal of the genetic resource by *in vitro* vegetative propagation method of adult material *i.e.* clonal micropropagation.

2. Material and Methods

2.1. Micropropagation

2.1.1. Plant Material

The plant material used consists of axillary nodes from plant regrowth taken from elderly subjects. To do this, adult *Annona* plants located in the botanical garden of the Plant Biology department were copped in the dry season (mid-March). This period corresponds to the budding phase of adult trees. Twenty days after

pruning the plants, the young regrowth appearing on the branches was removed and used as explants.

2.1.2. Disinfection Procedure and Establishment of Aseptic Culture

After sampling, the plant material is cut into nodal segments of approximately 12 cm and disinfected according to the procedure mentioned in **Figure 1**.

2.1.3. Culture Media and Growth Regulators

Three basic hormone-free nutrient media were used. These were complete medium of Murashige & Skoog [16], Murashige & Skoog modified medium (MMS) [17] and Woody Plant Medium medium (WPM) [18]. These media contained 2

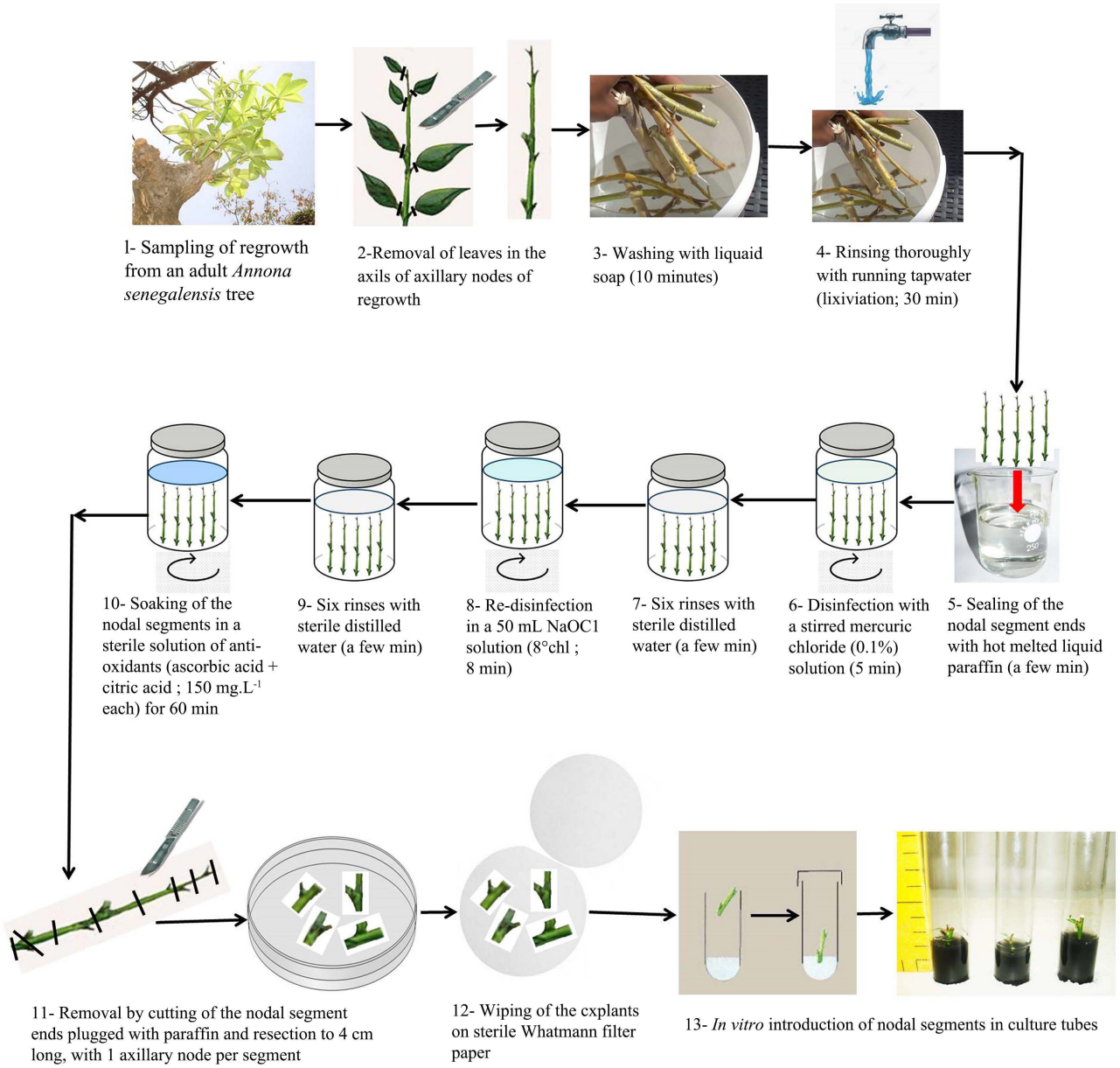

Figure 1. Disinfection procedure of *Annona senegalensis* adult plant material before *in vitro* introduction for micropropagation.

$g \cdot L^{-1}$ of activated charcoal, 30 $g \cdot L^{-1}$ of sucrose, and were solidified with agar (8 $g \cdot L^{-1}$) after adjusting the pH to 5.7.

The sampled nodal segments were aseptically introduced *in vitro*, after disinfection, in these various culture media containing or not variable concentrations of cytokinins (BAP, Kinetin) and auxin (NAA) at variable concentrations. The different concentrations of growth regulators used to screen their effects on the *in vitro* regeneration of explants are listed in Table 1. These were: cytokinins, among which BAP and Kinetin, tested at concentrations of 0.5, 1, 2, 4 and 5 $mg \cdot L^{-1}$ and auxins in the form of NAA at concentrations of 0.1, 0.2 and 0.5 $mg \cdot L^{-1}$. Due to the presence of activated charcoal (2 $g \cdot L^{-1}$), which tends to adsorb both the exudates of polyphenols and phytohormones, the hormonal concentrations were multiplied by a factor of 10 in order to benefit from their effect. The different phytohormones used were added directly to the basal media before distribution in cultures tubes and autoclaving at 110˚C for 20 min.

2.1.4. Experimental Setup

The explants were individually introduced into glass culture tubes (25 × 150 mm), filled up with 20 mL of a garified medium (Figure 1), with a number of 24 explants per medium. They were first incubated in the dark for 5 days. They

Table 1. Composition of the different media used for the multiplication of adult material of *Annona senegalensis*.

Nomenclature of *in vitro* culture media	Hormonal combinations
M0	MS0
M1	MS + BAP 0.5 $mg \cdot L^{-1}$
M2	MS + BAP 1 $mg \cdot L^{-1}$
M3	MS + BAP 2 $mg \cdot L^{-1}$
M4	MS + BAP 4 $mg \cdot L^{-1}$
M5	MS + BAP 5 $mg \cdot L^{-1}$
M6	MS + KIN 0.5 $mg \cdot L^{-1}$
M7	MS + KIN 1 $mg \cdot L^{-1}$
M8	MS + KIN 2 $mg \cdot L^{-1}$
M9	MS + KIN 4 $mg \cdot L^{-1}$
M10	MS + KIN 5 $mg \cdot L^{-1}$
M11	MS + BAP 1 $mg \cdot L^{-1}$ + KIN 1 $mg \cdot L^{-1}$
M12	MS + BAP 2 $mg \cdot L^{-1}$ + KIN 0.5 $mg \cdot L^{-1}$
M13	MS + BAP 2 $mg \cdot L^{-1}$ + KIN 1 $mg \cdot L^{-1}$
M14	MS + BAP 2 $mg \cdot L^{-1}$ + NAA 0.1 $mg \cdot L^{-1}$
M15	MS + BAP 2 $mg \cdot L^{-1}$ + NAA 0.2 $mg \cdot L^{-1}$
M16	MS + BAP 2 $mg \cdot L^{-1}$ + NAA 0.5 $mg \cdot L^{-1}$

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

were subsequently transferred to a culture chamber at 27˚C ± 1˚C under a photoperiod of 16 h day/8 h night, with a photosynthetically active incident light (PAR) of 4000 Lux.

The newly formed shoots *in vitro* were transplanted into the new media after 30 days of incubation.

The parameters measured concern the presence or absence of resumption of activity [Reactivity (%) = (Number of explants having reacted/Total number of explants) × 100], the number and length of newly formed shoots, the number of nodes. Thus, from these data, averages were calculated, the coefficients or multiplication rates determined and the best media deduced.

2.1.5. Rooting Procedure

Third generation of shoots, *i.e.* from three successive transplants lasting thirty days each, were introduced for 15 days into the solid MS medium without hormones, with a concentration of 2 mg·L^{-1} of activated charcoal and 20 mg·L^{-1} of sucrose, according to the method used by Farooq *et al.* [19] on *Annona squamosa* and which facilitates rhizogenesis. The explants were then introduced into MS/2 medium containing concentrations of 25 or 50 mg·L^{-1} of IAA or IBA. During the rhizogenic induction phase, the vitroplants were placed in the dark for a duration of 1, 3 and 5 days and then transferred to the light in the MS/2(0) hormone-free expression medium, with the macro-nutrients diluted by half.

For each treatment, a number of 12 explants were used for each duration and for each induction medium. A batch of explants is maintained as a control one, without root induction, in the MS/2 *i.e.* hormone-free medium. Media containing 2 g·L^{-1} of activated charcoal and 20 g·L^{-1} of sucrose were solidified with an 8 g·L^{-1} agar and the pH was adjusted to 5.7.

After 30 days of incubation, 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 the root length for each explant, for each treatment.

2.2. Acclimatization

The plants produced *in vitro*, well rooted, were transferred under a shade for acclimatization. For this, they were removed from the tubes and freed from the agar by washing their base with sterile distilled water and then transplanted into a substrate made up of a mixture of sand and potting soil (v/v), previously sterilized. The substrate was potted in black polyethylene bags. Two weaning conditions were tested:

- A batch (1) of 12 young plants was placed in the shade for 2 days then transferred over the bench to the light;
- A second batch (2) of 12 young plants was placed in a mini-greenhouse with a transparent plastic cover with adjustable opening. Thus, the plants were maintained in conditions of relatively high humidity. They were watered

with the [20] nutritive solution every two days. After the 2nd week, the plastic cover was opened gradually.

The number of plants that survived after 15 and 30 days of acclimatization was used to determine the survival or recovery rate.

3. Results

3.1. Micropropagation

3.1.1. Plant Material

After 30 days of incubation, 12.5% of the nodal explants were infected. The first infections appeared 3 days after introduction. In addition, 7 to 10 days after the introduction of the material, the outlines of newly formed shoots began to appear at the level of the leaf insertion zone of the nodes. The hormone-enriched media gave the best results in terms of number of regrowth and explant reactivity. Newly formed shoots were more numerous and appeared earlier in these hormone-enriched media. For that species, the presence of calluses was noticed in the areas of the injury of the explants when the concentration of cytokinins or of NAA is high (5 mg·L^{-1} and 0.5 mg·L^{-1}), respectively.

3.1.2. Culture Media

The media of Murashige & Skoog [16], Murashige & Skoog modified (MMS, [17]) and Woody Plant Medium (WPM, [18]) used alone (without hormones) made it possible to have the results mentioned in Table 2. After 30 days of culture, the best reactivity rate (number of cuttings reacted relative to the number of cuttings introduced) was obtained in MS medium (83%).

An average number of 1.32 shoots was obtained in MS medium against 1.06 in MMS medium and 1 in WPM medium ($F = 373.48$; $p \leq 0.001$). The average lengths ($F = 373.48$; $p \leq 0.001$) and the average number of nodes ($F = 537.46$; $p \leq 0.001$) are significantly different. They are 2.35, 1.74 and 2.1 cm for the MS, MMS and WPM media, respectively, with an average of, 2.85, 3.03 and 2.76 nodes per explant. These values also were significantly different (Table 2).

Thus, the medium of [16], being the best medium, was used as the basal medium for the *in vitro* propagation.

Table 2. Influence of 3 different types of media on the *in vitro* morphogenesis of *Annona senegalensis* nodal segments.

Media	Responsiveness rate (%)	Average number of shoots	Average shoot length (cm)	Average number of nodes
MS	**45.83**	**1.32 a**	**2.35 a**	2.85 b
MMS	25	1.06 b	1.74 c	**3.03 a**
WPM	33.33	1.01 b	2.1 b	2.76 c

In the same column, the values followed by the same letter are not significantly different at the 5% threshold of the Newman-Keuls's test. MS: Murashige & Skoog medium; MMS: Murashige & Skoog Modified medium; WPM: Woody Plant Medium.

3.1.3. Influence of Growth Regulators Used Alone or in Combination

The addition of hormones to the basal medium significantly improved the reactivity, multiplication and elongation of the nodal explants. The M3 medium [MS + BAP 2 mg·L^{-1}] gave the best reactivity rate (91.66%) and the best multiplication rate (Table 3; Figure 2). In this medium, the explants gave the highest average

Table 3. Influence of different hormonal combinations on the *in vitro* morphogenesis of *Annona senegalensis*.

Media	Hormonal Combinations	Responsiveness Rate (%)	Average number of shoots	Average length of shoots (cm)	Average number of nodes
M0	MS0	45.83	1.32 bc	2.35 e	2.85 ef
M1	MS + BAP 0.5 mg·L^{-1}	62.5	1.58 bc	3.27 d	4.65 abc
M2	MS + BAP 1 mg·L^{-1}	75	1.85 b	3.75 d	4.85 ab
M3	MS + BAP 2 mg·L^{-1}	**91.66**	**2.30 a**	6.70 b	5.30 a
M4	MS + BAP 4 mg·L^{-1}	87.50	1.90 b	4.93 c	4.04 bcd
M5	MS + BAP 5 mg·L^{-1}	79.167	1.90 b	3.41 d	3.57 cde
M6	MS + KIN 0.5 mg·L^{-1}	70.83	1.47 bc	4.75 c	3.21 def
M7	MS + KIN 1 mg·L^{-1}	83.33	1.19 c	6.69 b	3.95 bcd
M8	MS + KIN 2 mg·L^{-1}	87.5	1.59 bc	3.49 d	4.31 abc
M9	MS + KIN 4 mg·L^{-1}	83.33	1.45 bc	3.77 d	4.10 bcd
M10	MS + KIN 5 mg·L^{-1}	66.67	1.45 bc	3.36 d	3.86 bcd
M11	MS + BAP 1 mg·L^{-1} + KIN 1 mg·L^{-1}	75	1.68 bc	5.10 c	4.27 abcd
M12	MS + BAP 2 mg·L^{-1} + KIN 0.5 mg·L^{-1}	87.5	1.80 b	6.02 b	3.90 bcd
M13	MS + BAP 2 mg·L^{-1} + KIN 1 mg·L^{-1}	79.167	1.66 bc	6.68 b	4.38 abcd
M14	MS + BAP 2 mg·L^{-1} + NAA 0.1 mg·L^{-1}	83.33	1.59 bc	**8.25 a**	4.45 ab
M15	MS + BAP 2 mg·L^{-1} + NAA 0.2 mg·L^{-1}	75	1.78 b	6.46 b	4.04 cd
M16	MS + BAP 2 mg·L^{-1} + NAA 0.5 mg·L^{-1}	70.83	1.35 bc	2.33 e	2.44 f

In the same column, the values followed by the same letter are not significantly different at the 5% threshold of the Newman-Keuls's test. MS: Murashige & Skoog medium; BAP: 6-Benzylaminopurine; KIN: Kinetin (6-furfuryl aminopurine); NAA: 1-Naphthaleneacetic acid.

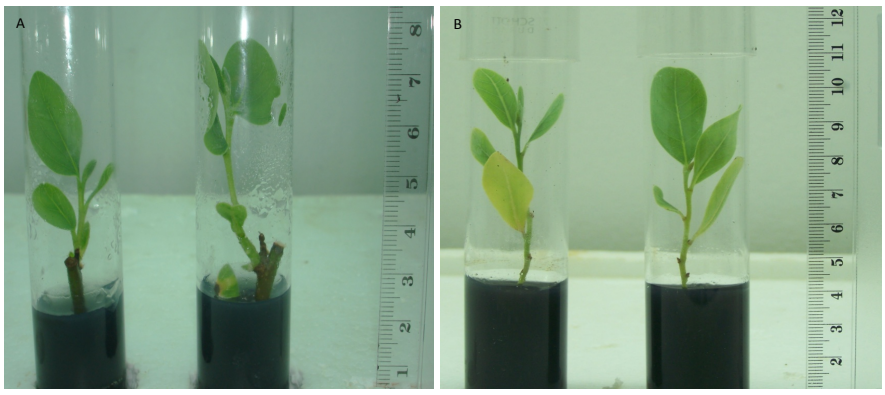

Figure 2. Newly formed shoots (A, B) from adult material of *Annona senegalensis*.

number of newly formed shoots (2.3) and the highest average number of nodes (5.3). These values are significantly higher than those obtained with the control one (M0), *i.e.* 2.85 ($F = 7.01$; $p \leq 0.001$) and 1.32 ($F = 5.05$; $p \leq 0.001$), respectively. However, the addition of the hormonal combination M14 [MS + BAP 2 $mg{\cdot}L^{-1}$ + 0.1 $mg{\cdot}L^{-1}$ of NAA] to M3 resulted in a significant increase in the average shoot length equivalent to 8.25 cm. Indeed, the average shoot length in the M14 medium and is significantly longer than those obtained in MS0 and M3, *i.e.* 2.35 cm ($p \leq 0.001$) and 6.7 cm ($p = 0.01$), respectively.

3.2. Rooting

For a 5-day induction with IBA at 50 $mg{\cdot}L^{-1}$, no roots appeared at the base of the vitroplants in the expression medium after 30 days of culture. In addition, significant callogenesis was noticed (100%) as well as rooting (33.33%). The best rooting rate (58.33%) was obtained for an induction period of 3 days with IBA (50 $mg{\cdot}L^{-1}$) whereas for the control, this rate was 8.33% (Table 4; Figure 3). For

Table 4. Influence of IAA and IBA on the *in vitro* rooting of *Annona senegalensis* vitroplants.

Type of Hormones	Duration of Induction (days)	Rooting Rate (%)	Average number of roots	Average length of roots (cm)
Control	0	8.33	1 b	1.8 b
IAA 50 $mg{\cdot}L^{-1}$	1	33.33	1 b	**3.75 a**
	3	16.66	1.2 b	3.56 a
	5	8.33	1.6 b	2.4 ab
IBA 50 $mg{\cdot}L^{-1}$	1	25	1.33 b	2.5 ab
	3	**58.33**	**2.2 a**	2.38 ab
	5	0	0 c	0 c

In the same column, the values followed by the same letter are not significantly different at the 5% threshold of the Newman-Keuls's test. IAA: Indole-3-acetic acid; IBA: Indole-3-butyric acid.

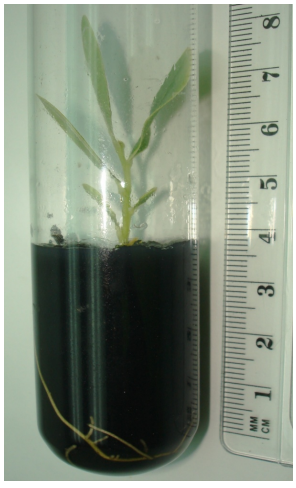

Figure 3. Vitroplant of *Annona senegalensis in vitro* rooted.

the 3-day induction period with IBA, the average number of roots is 2.2, a value significantly higher ($p = 0.016$) than those obtained with the other induction times.

For the length of the roots, the analysis of variances reveals a very significant medium effect ($F = 30.20$; $p \leq 0.001$). For an induction period of 1 day with IAA, the average length of the roots is equal to 3.75 cm, a value not significantly different from the results which are higher than the elongations obtained with the other induction periods, particularly, that with IBA (1 day-induction period) with which, an average root length of 2.38 cm was noticed ($p = 0.36$). The lowest root elongation at 1.8 cm was observed with the control plants. For an IAA induction period of 3 days, the mean length of the vitroplants is 3.56 cm ($p = 0.004$). a value significantly different from those noticed with IAA (5 days) and IBA (1 day), *i.e.* root lengths of 2.4 cm ($p \leq 0.001$) and 2.5 cm ($p = 0.009$), respectively.

3.3. Acclimatization

The results recorded in Table 5 revealed that only 2 explants (25%) installed directly under the bench in the shade room survived after 15 days. Indeed, 3 days after the start of the *ex vitro* weaning, the leaves of most young plants begin to necrose. After 15 days of weaning, the survival rate for the young plants of batch 2 is equal to 50%. After partial opening of the plastic sheeting, the young plants behave well. After 30 days (Figure 4), the survival rate remained unchanged.

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

	Survival Rate (%)	
Lots	**After 15 days**	**After 30 days**
1	25	25
2	50	**50**

Lot 1: young plants kept directly in the shade in the greenhouse, and then on the bench. Lot 2: young plants kept in a mini-greenhouse, then under natural conditions.

Figure 4. Young acclimatized plants of *Annona senegalensis* Pers., after 3 months under shade.

4. Discussion

4.1. Disinfection

Double disinfection with mercuric chloride and then with bleach was very beneficial for the disinfection of nodal explants, with a low infection rate *i.e.* 12.5%). These results are consistent with those obtained by [21] for *Atriplex halimus*. According to [22], depending on the origin of the explants, two methods are conventionally used: disinfection with mercuric chloride, generally used for potentially infected material, and disinfection with calcium hypochlorite. Several researchers [23] [24] [25] [26]) used the first method to disinfect explants of *Jatropha curcas* originating from greenhouses, nurseries or fields or nodal explants of *Tectona grandis*, a woody tree [27].

In addition, a high level of mercuric chloride at 1% on *Atriplex halimus* micro-cuttings, taken from woody mother-plants aged 30 years, allowed to obtain a high survival rate of 85% [28] [29]. Ultimately, it emerges that the effectiveness of the disinfectant product, $HgCl_2$, would be linked to the inherent factors of the plant, namely the age, the species considered, the diameter and size of the explants and other parameters such as the optimal dose of the disinfectant and the immersion time. The optimum dose of mercuric chloride to be applied is also decisive to avoid the toxicity and the lethality of the disinfectant on the reactivity of the explants.

4.2. Micropropagation

The base medium of [16] provided the best reactivity rates. In terms of the highest average number of shoots and the best elongation, the best results were obtained with hormone-free environments. These results show that these media allow better multiplication in this species. The medium of [16] 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 is how it was used, *e.g.* by [30] [31] in *A. Cherimola* [32] in *A. muricata* [33], in the hybrid species, *A. cherimoya cv* (*A. cherimola* × *A. squamosa*) or [34] in *A. glabra*. [19] used this medium to *in vitro* multiply *A. squamosa* in accordance with the results obtained by [35]. On the other hand, Woody Plant Medium (WPM) [18] is an increasingly used medium for the *in vitro* vegetative propagation of fruit essences [22].

Overall, the nodal explants reacted better in media with hormones than in the control one. The MS medium supplemented with 2 $mg{\cdot}L^{-1}$ of BAP led 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). The M14 medium [MS + BAP 2 $mg{\cdot}L^{-1}$ + ANA 0.1 $mg{\cdot}L^{-1}$] made it possible to have better elongation of the shoots (8.25 cm). Thus, BAP used alone had a better effect on explant bursting but rather had a detrimental effect on the elongation and the average number of nodes when the concentration is high (4 and 5 $mg{\cdot}L^{-1}$). A concentration of 0.5 $mg{\cdot}L^{-1}$ of NAA decreases considerably the multiplication rate, the length of

shoots as well as the average number of nodes. Adding low concentrations of NAA to the culture medium increased significantly the number of newly formed shoots in *A. cherimola* [36], *Atriplex halimus* L. [21] and *Pisum sativum* L. [37]. In *Annona glabra*, the best average lengths were obtained by using the concentration of 0.5 mg·L^{-1} of BAP while higher concentrations (1 and 1.5 mg·L^{-1}) of this phytohormone induced a reduction in shoot length [34]. According to [21], the addition of BAP improves the rate of bud break and the mean number of axillary shoots increases with decreasing BAP concentration. [38] reported that cytokinins begin to have an adverse effect on organogenesis when their concentration is greater than 2 mg·L^{-1}. Working on *Atriplex halimus*, a sylvo-pastoral shrub species of the *Chenopodiaceae* family, [28] and [21] showed that BAP increases the propensity to bud in this species, as well as the presence of several buds in a leaf axilla. This phenomenon has also been observed *in vitro* in *Acacia cyanophylla* [39] and *Annona squamosa* [40]. However, according to [41], high concentrations of cytokinins generally favor the production of large numbers of cuttings. Thus, the use of BAP concentrations at 2 and 4 mg·L^{-1} has been shown to significantly increase the number of cuttings produced from explants of *A. muricata* [42]. Thus, in *Annona glabra*, the low concentrations of BAP, less than 0.5 mg·L^{-1}, are insufficient to induce a significant increase in multiplication rates [34].

In some cases, the hormonal combination of BAP-NAA has been shown to be harmful to the *in vitro* organogenesis of plant species. This is the case with *Solanum sessiliflorum* in which the combination of BAP (0.25 and 0.5 mg·L^{-1}), and NAA (0.1 and 0.3 mg·L^{-1}) did not have a beneficial effect on *in vitro* multiplication according to [43]. Thus, some authors recommend the use, instead of NAA, of other auxins such as IAA or IBA [44] [45]. With *Maclura tinctoria* [46] and *Nothofagus alpina* [47], adding NAA to the medium that already contained GA_3 combined with BAP did not improve the multiplication rate.

4.3. Rooting

The traditional methods of vegetative propagation were inefficient, due to the low morphogenetic potential of *A. senegalensis*, and the low rooting rate of its conventional cuttings. The micropropagation technique can be applied successfully to this species and other *Annona* sp. to overcome these problems [48] [49] [50].

During the *in vitro* rooting stage, a rooting rate of 8.33% is obtained in the MS/2(0) expression medium without prior induction with auxins. This could be explained by a high endogenous auxin level in this species. [51] made the same observation with *Parkia biglobosa*. It could also be explained by a drop in the pH in the medium due to a decrease in the concentration of sucrose. Indeed, according to [52], the level of sucrose in the culture medium 4 weeks after cultivation decreases from 20 mg·L^{-1} to 5 mg·L^{-1} while the medium becomes more acid with a pH decrease from 5.7 to 5.0. In many *Annona* species such as *A. glabra*,

this drop in pH allows a better response to root formation by increasing the percentage of rooting and the average number of roots produced by vitroplants [34]. However, a 5.0 pH of the medium has a negative effect on root length when the auxin concentration or the induction time is high, which would explain the decrease in root length as the induction time increases. IBA had a better effect on the rooting rate and on the average number of roots. Many authors have reported that an induction period of 3 to 5 days in the dark and then 7 to 10 days in full light in a medium containing a concentration of IBA of 50 to 100 $mg{\cdot}L^{-1}$ with 2 - 2.5 $g{\cdot}L^{-1}$ of activated charcoal and 20 $mg{\cdot}L^{-1}$ of sucrose, followed by transfer to the MS/2(0) expression medium led to good rooting of shoots from mature material in many plants as well as for *Annonaceae* species. This is the case with *Annona cherimola* [48] [49] to attempt the plant breeding of this species and *A. atemoya* [30] [31] [32] [33]. This relative efficacy of IBA compared to other auxins has been observed in other fruit tree species such as *Guava sp.* [53]. Adversely, for *A. muricata* vitroplants, rooting was stimulated by galactose and NAA, after explants were preconditioned for two weeks, in a plain medium containing 10 $g{\cdot}L^{-1}$ of activated charcoal [42].

4.4. Acclimatization

The weaning of young plants by direct transfer to the atmosphere of the shade house resulted in high mortality. This result is explained by the very high relative humidity inside the culture tubes during *in vitro* culture. Indeed, under these conditions, the leaves of young plants have a high number of stomata compared to those of adult plants adapted to *in vivo* conditions [54] [55] [56] [57]. Adding to this are the combined effects of the presence of sucrose in the medium and the accumulation of ethylene, which negatively impact the development and closing capacity of the stomata once the young plants are subjected to natural conditions [1] [58] [59]. According to other authors, this high frequency of stomata and their poor closing capacity are the cause of the desiccation of young plants after their transfer to natural weaning conditions [56] [59] when relative humidity drops rapidly. [60] noticed this phenomenon in *A. glabra*.

On the other hand, gradual weaning results in fairly high survival rates. In fact, as has been observed for the juvenile material, keeping the young plants in a mini-greenhouse before their transfer under natural conditions makes 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.

5. Conclusion

This work made it possible to develop an effective disinfection protocol for the disinfection of adult material before *in vitro* introduction. It also demonstrated that MS medium was the best culture medium for clonal propagation. It pro-

vided the best reactivity and elongation rates. The MS medium supplemented with 2 $mg{\cdot}L^{-1}$ of BAP led to the best reactivity rate, the greatest average number of newly formed shoots and the greatest number of nodes. The M14 medium made it possible to have better elongation of the shoots. Thus, BAP used alone had a better effect on explant bursting but the BAP combined with NAA helped improve the multiplication rate of explants. IBA had a better effect on the rooting rate and on the average number of roots. Gradual weaning of plants, during acclimatization results in fairly high survival rates. It would be interesting to consider direct organogenesis for the clonal propagation of *Annona senegalensis* to have higher multiplication rate.

This *in vitro* regeneration protocol can therefore be adopted for the clonal propagation of adult material of *A. senegalensis*. However, the rooting rate can also be improved by trying other hormonal combinations based on auxins or by considering *ex-vitro* rooting during shade weaning or by micrografting techniques.

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Conflicts of Interest

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

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