

In Vitro Micropropagation of *Jatropha curcas* L. from Bud Aggregates

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Abstract: Entire plants were regenerated from nodes explants of *Jatropha curcas* L. following a procedure of bud aggregate induction on MS (Murashige and Skoog) medium supplemented with 25 mg.l⁻¹ citric acid, 12.2 mg.l⁻¹ adenine sulfate, 15 mg.l⁻¹ L-arginine, 2.46 μM IBA (indole-3-butyric acid), 30 g.l⁻¹ sucrose and 7 g.l⁻¹ of agar, and enriched with different balances of BA (benzyladenine) and L-glutamine. The histological studies performed on aggregates showed that the buds result from both the development of axillary buds and adventitious budding starting from underlying tissues of the explant. The culture medium containing 6.65 μM BA and 25 mg.l⁻¹ L-glutamine gave the best results with an average of 64 buds per aggregate after three weeks for all accessions tested. The buds developed into shoots when placed in an MS medium supplemented with 2.21 μM BA, 5.70 μM IAA (indole-3-acetic acid) and 15 mg.l⁻¹ L-arginine. These shoots were isolated and then rooted in MS containing 2.46 μM of IBA, 2% sucrose and 0.7% agar. The entire process took 13 weeks with a 98% survival rate in terms of plantlets acclimatization. We obtained a multiplication rate of 13 buds per explant and per subculture which is the double of those obtained in other recent works based on the micropropagation of *J. curcas* from node explants. This protocol is economically more profitable.

Keywords: *Jatropha curcas* L., bud aggregates, nodal explant, regeneration, *in vitro* culture.

INTRODUCTION

Among the plant species producing raw materials for biofuels, *Jatropha curcas* L. is one of the plant species that stimulates the highest interest in tropical and subtropical regions. The seeds contain up to 38% in non-edible oil which is highly combustible [1]. The use of *J. curcas* for the production of biofuel could compensate for energy shortages, reduce CO₂ gas (Carbon dioxide) emissions and contribute to increase farmers' revenues [2]. However, several challenges remain before that plant biomass can be commercially exploited. Its supply on a large scale requires massive production of phenotypically homogeneous plant-material of a very high quality within a short time-frame that is adapted to the growth conditions of the plantation areas. Multiplication of the plant is traditionally carried out by sowing seeds or planting cuttings directly in the field or after a first period of growth in nurseries. But sowing has the disadvantage of producing heterogeneous plants with regard to their production capacity [3]. The plants produced from cuttings develop a superficial rooting system which renders them sensitive to lodging and constitutes a major obstacle to their establishment in poor and marginal soils [4]. *In vitro* growth is an

alternative for massive and rapid production of elite plants. This clonal propagation method has the advantage of producing plants that are morphologically homogenous with an equal production potential. Several authors have regenerated *J. curcas* from nodal explants, from hypocotyls, leaves, petioles and cotyledons [1]. However, the number of plantlets produced per explant remained relatively low after a period of four to six weeks (10 to 42 plantlets per explants) which is not economically viable. The present study aims at optimizing the culture conditions of *in vitro* micro-propagation from nodal explants with investigations focusing on the most efficient hormonal balance in combination with glutamine and others amino-acids, to induce mass production of bud aggregates.

MATERIAL AND METHODS

The work was carried out with two types of seeds called "Cam" and "Sen", harvested in 2009 on parental plants aged more than three years and originating from Southern Cameroon (Minkang 1: 2°48'31.28"N; 12°4'41.30"E) and South-East of Senegal (Dialacoto: 13°18'50.04"N ; 13°16'56.82"O) respectively. These seeds were sown in our growth room with a sixteen hours photoperiod, a temperature of 27±2 °C, a light intensity of 80 μmol m⁻².s⁻¹ and 50-60% relative humidity. The nodal explants were picked from at least two months old seedlings which have formed a

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minimum total number of four nodes. They were specifically collected from an area beginning at 10 cm from the base of the stem and ending at 5 cm below the apical bud, the latter being discarded at the sampling moment. A petiole piece was kept attached to the stem to prevent putative axillary bud damages due to disinfection. This consisted in washing the explants in a 5% Dettol solution, dipping them in a 70% alcohol solution for 20 seconds, and then immersing them in a 10% calcium hypochlorite solution for thirty minutes under stirring. Finally, the explants were rinsed three times in the laminar flow with sterile distilled water; the second rinse took place under magnetic stirring for 1 minute.

Node Buds Development

To develop the axillary buds, the sterilized nodes were placed in a growth medium (GM), based on Murashige and Skoog [5] [MS] compounds containing 8.87 μM BA (benzyladenine), 4.92 μM IBA (Indole-3-butyric acid), 30 g.l^{-1} sucrose (DuchefaBiochimie, reference 006520.16). The pH of the GM was adjusted to 5.7 ± 0.1 and the medium was solidified with 0.7% agar (Select Agar Invitrogen, reference 30391-023). The nodal explants were sized to a length of 0.8 to 1 cm, placed on a sterile paper and cultivated for three weeks in 100 ml magenta B-cap containers (66 mm x 59 mm); each one containing 20 ml of GM.

Formation and Multiplication of the Bud Aggregates

The buds developed on GM medium were separated from the stem fragment and transferred to an induction medium (IM) made up of MS enriched with 25 mg.l^{-1} citric acid, 12.2 mg.l^{-1} adenine sulfate, 15 mg.l^{-1} of L-arginine, different concentrations of glutamine (10, 15, 20, 25, 30 and 35 mg.l^{-1}), and five hormonal balances combining 2.46 μM IBA and BA (2.21, 4.43, 6.65, 8.87 and 11.1 μM) to bring about the formation of bud aggregates. The tested hormonal balances were chosen on the basis of preliminary investigations [6]. Every three weeks, aggregates were fragmented into clusters of 5 to 10 buds which were transferred in a medium of the same composition to proceed with proliferation, or else transferred to an elongation medium with the aim to grow buds into shoots for rooting.

Elongation and Rooting

The isolated aggregate fragments were first transplanted into an elongation medium (EM),

composed of the MS medium supplemented with 2.21 μM BA and 5.70 μM IAA (indole-3-acetic acid), 15 mg.l^{-1} L-arginine, 3% sucrose and gelled with 0.7% agar. After 3 weeks of growth, basic hormone free MS medium was added as a second liquid phase to immerse the aggregates base by 1 to 2 mm. The first shoots reaching ± 1.5 cm height, were isolated and transferred one week later for rooting in $\frac{1}{2}$ MS (containing 2.46 μM IBA, 2% sucrose and 0.7% agar) while the rest of the aggregate was subcultured in fresh shoot elongation medium. Shoots rooting was started in darkness for the first five days and then root formation was continued under a sixteen-hour photoperiod for three weeks.

Acclimatization

The residues from the agar medium were removed from the root system by washing it with distilled water. The plantlets were replanted in pots containing commercial compost and placed in the growth chamber where seeds' germination took place. The plantlets were covered with a transparent plastic film for two weeks and progressively adapted to the open air for the next two weeks before their transfer to the greenhouse.

Histological Study

Histological cuts were carried out on bud aggregates fragments of four weeks old (at least) in accordance with the Ruzin [7] procedure and then stained with toluidine blue.

Parameters Analysed

The percentage of bud aggregates and the number of newly formed buds/aggregates were recorded at each subculture into IM media. In the elongation medium, the number of shoots per aggregate fragment and their length were noted every four weeks during three successive cycles of micropropagation (42 weeks in total). During the rooting phase, the appearance of roots was observed after 12 days. The number, the length of the roots and the percentage of rooted shoots were recorded after four weeks. Lastly, the survival percentage for plantlets was noted after 5 weeks *ex vitro*.

Statistical Analysis Method

The tests were carried out in randomized blocks in order to determine the influence of the accession as well as the induction medium and the glutamine concentration on the number of buds produced and

rooting. The experimental unit consisted of a fragment of aggregates. In this experimental system, the three factors are the following: induction medium as a fixed qualitative factor (5 items); accession as a fixed qualitative factor (2 items) and glutamine concentration as a fixed quantitative factor (6 items).

The statistical analysis used is a fixed crossover model with 60 random simple independent and normal populations with the same variance. Each treatment contained 26 shoots and each treatment was repeated 3 times. Statistically significant differences between means were determined by three-way analysis of variance using Duncan's multiple-range test and the Minitab15 Software and Statistical. A P-value of less than 0.05 was considered significant.

RESULTS

Each nodal explant from the mother-plants developed one to three axillary buds after three weeks growth in the culture medium (Figure 1a). Both *J. curcas* accessions showed the same behavior with regard to bud formation in this medium. They were individually replanted in the presence of BA and glutamine to induce bud aggregates (Figure 1b). The average percentage of induced aggregates varied according to the hormonal balance and the concentration of glutamine.

After three weeks, the bud aggregates were fragmented into cluster units of 5 to 10 buds and these

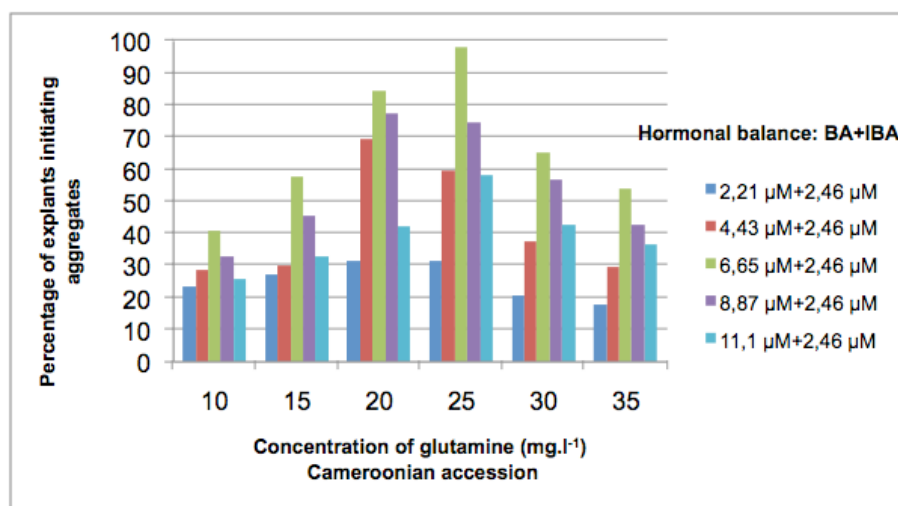
were replanted into an IM medium of the same composition, to form new aggregates. Figures 2a and 2b show the average percentage of these units forming new aggregates after five subcultures. The highest regeneration percentage (98%) was obtained with 6.65 μM BA, 2.46 μM IBA, 25 mg.l^{-1} glutamine and the supplements. The number of buds per unit obtained every three weeks for both accessions (Figures 3a and 3b) showed highly significant differences according to the hormonal balance and the concentration of glutamine.

The optimal concentrations of BA and glutamine were 6.65 μM and 25 mg.l^{-1} respectively. In this medium an average of 63.37 \pm 2.90 buds per aggregate in the Cam accession and 65.13 \pm 1.12 in the Sen accession were recorded after three weeks. The multiplication rate is the number of useful buds obtained per bud of the initial aggregate after three weeks in induction medium. The multiplication rates achieved after the first five subcultures on both accessions are 12.3, 13.4, 14.5, 12.2 and 13.3 as averages of buds per explant per subculture. The two *J. curcas* accessions still showed similar behavior with regard to the formation of buds aggregates.

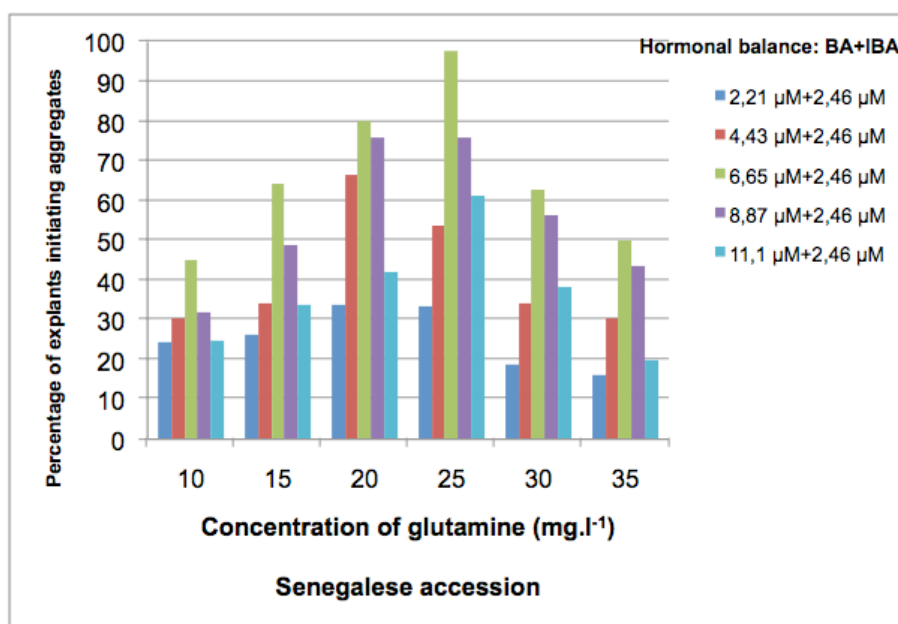
The histological sections carried out on the bud aggregates show new buds developing continuously from each leaf axil (Figure 4a). This phenomenon occurred without callogenesis and was conform to a strict axillary budding process. In this case, we could observe the absence of the dominance normally



Figure 1: Bud aggregate development stages in *J. curcas*, Cameroon accession. **a)** Axillary buds on nodal explants after 3 weeks of cultivation on a MS medium supplemented with 8.87 μM BA, 4.92 μM IBA, 3% sucrose and 0.7% agar **b)** Bud aggregate after 3 weeks of cultivation on a MS medium supplemented with 6.65 μM BA, 2.46 μM IBA, 25 mg.l^{-1} glutamine, 25 mg.l^{-1} citric acid, 12.2 mg.l^{-1} adenine sulfate and 15 mg.l^{-1} L-arginine, 3% sucrose and 0.7% agar (bar : 1000 μm).



a



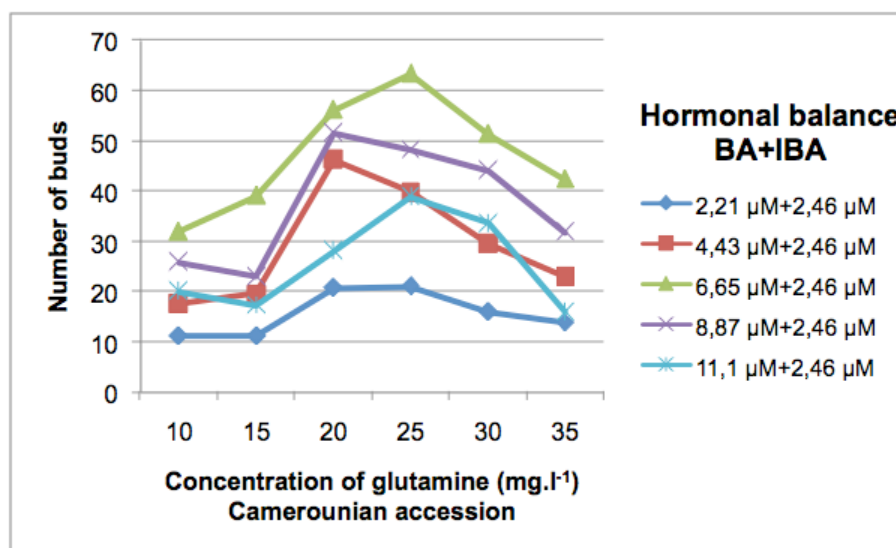
b

Figure 2: Percentage of nodal explants initiating bud aggregates on the Cameroonian (a) and Senegalese (b) accessions. Both accessions were cultivated in induction media supplemented with 25 mg.l⁻¹ citric acid, 12.2 mg.l⁻¹ adenine sulfate, 15 mg.l⁻¹ of L-arginine, 3% sucrose and 0.7% agar. Averages were calculated on basis of 3 repetitions of 26 explants each.

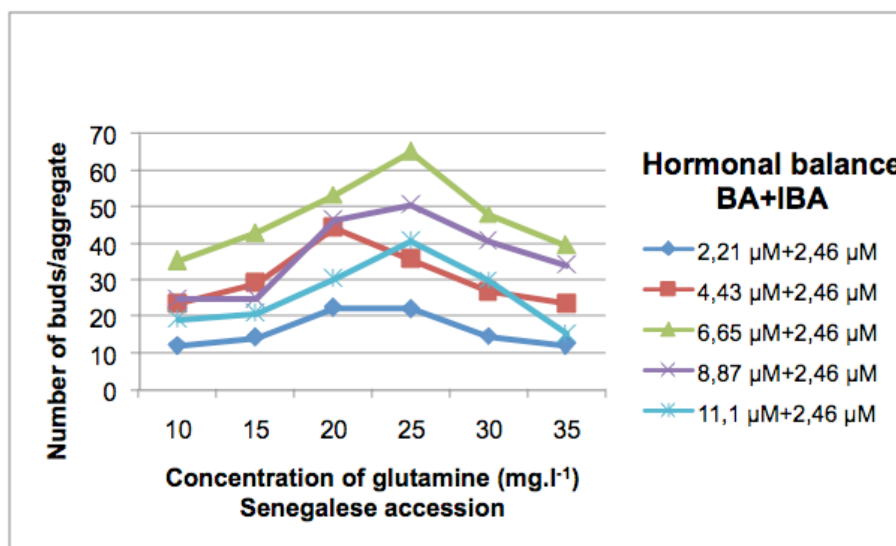
exerted by the apical buds of the shoots. Moreover, a direct adventive regeneration took place from the underlying tissues (Figure 4b) simultaneously to axillary budding within the same bud aggregate.

From the 5 and 10 buds forming the aggregate fragments, 3 to 4 became dominant over the others to reach an average size of 1.5 cm. The rest of the aggregate fragment was placed back into the same elongation condition, after collecting the elongated shoots. This operation was necessary to develop all the buds from an aggregate fragment into shoots able to root (Figure 4c).

Roots appear at the base of leafy stems as soon as the 12th day after their transfer to the rooting medium. Results show that the induction medium has an influence on the rooting potential of the stems. Table 1 shows the percentage of rooting obtained on the two accessions of *J. curcas* after four weeks on MS rooting medium containing 2.46 μM IBA, 2% sucrose and 0.7% agar according to the concentration of BA and glutamine in the induction medium. There was no significant difference in the percentage of rooting stems for the two accessions of *J. curcas* whatever of the concentration of glutamine in their native medium. However, percentage of rooting varies very significantly



a



b

Figure 3: Average number of buds obtained from each aggregate on the different induction media according to glutamine concentration in the media. All media were supplemented with 25 mg.l⁻¹ citric acid, 12.2 mg.l⁻¹ adenine sulfate, 15 mg.l⁻¹ L-arginine, 3% sucrose and 0.7% agar.

Averages were obtained from 3 replications with 26 explants/assay.

with the concentration of BA in the induction medium and was higher on the stems originating from media containing low concentrations of BA. Optimal values were observed with stems cultured in MS media supplemented with 2.21 μM BA with rooting percentages as high as 90.10±6.23 and 86.10±5.02% respectively for the Cameroonian and Senegalese accessions.

As for the lowest rooting percentages, they were obtained with the induction media containing the highest BA concentration, i.e. 11.1 μM BA, with rooting percentages as low as, respectively, 31.27±1.60% and 35.17± 0.49% for the Cameroon and Senegal

accessions. Callus formation at stem basis was also noted during the rooting phase of stems originating from media containing 11.1 or 8.87 μM BA. All obtained roots, regardless of the origin of the stems, developed little or no secondary roots.

In the rooting medium, the roots appeared from the 12th day of growth. After 4 weeks, 96.32 % of the shoots bore an average of 2±037 roots; with an average length of 0.7±0.68 cm. Rooted stems (Figure 5a) were transferred to acclimatization. They lose all roots produced *in vitro* after about 10 days and initiate new roots. There was no significant difference in the survival rates during acclimatization of rooted stems

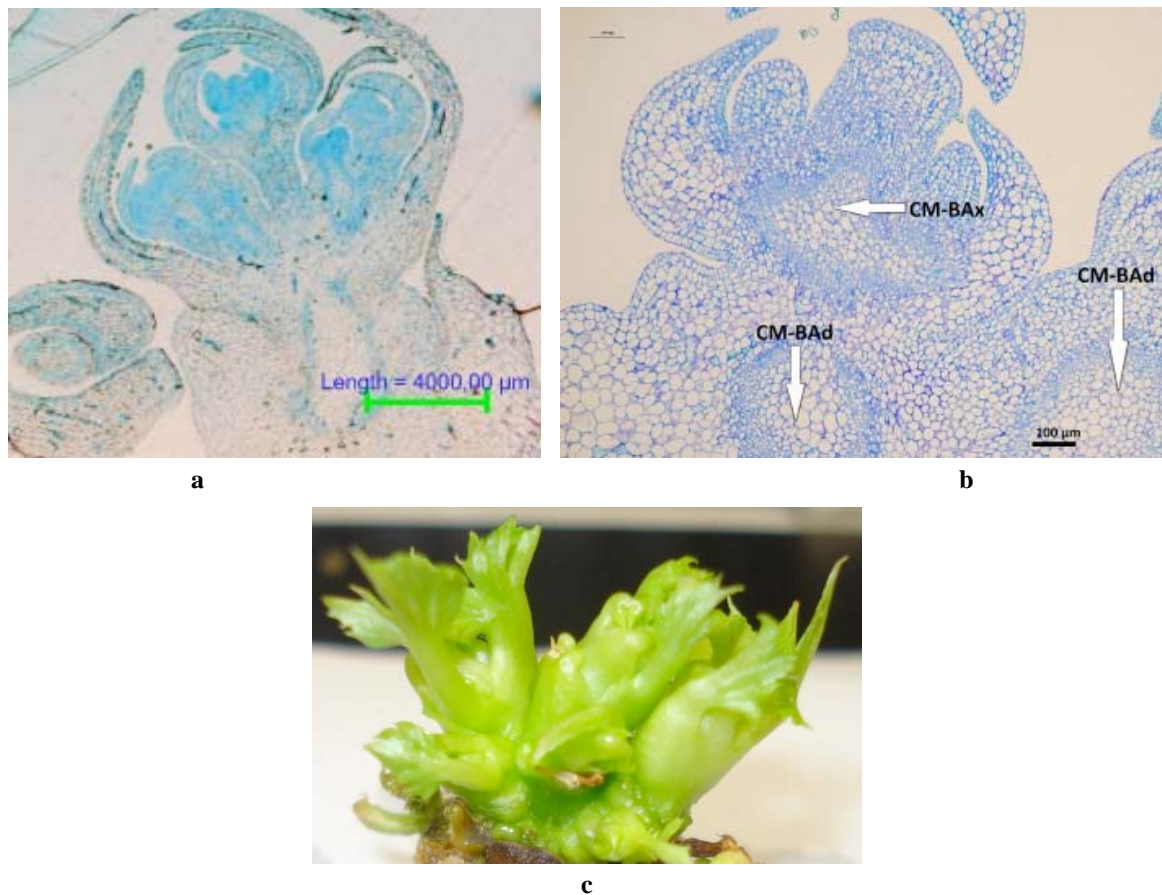


Figure 4: *In vitro* bud cluster induction in *J. curcas* through axillary and adventitious regeneration for the Cameroonian accession. **a)** Histological section showing the axillary origin of the buds in the aggregates after 3 weeks of culture on a MS medium containing 6.65 μM BA, 2.46 μM IBA, 25 $\text{mg}\cdot\text{l}^{-1}$ glutamine, 25 $\text{mg}\cdot\text{l}^{-1}$ citric acid, 12.2 $\text{mg}\cdot\text{l}^{-1}$ adenine sulfate, 15 $\text{mg}\cdot\text{l}^{-1}$ of L-arginine, 3% sucrose and 0.7% agar **b)** Histological section of a bud aggregate of 3 weeks presenting axillary and adventitious regeneration from underlying tissues. The bud were grown on a MS medium supplemented with 6.65 μM BA, 2.46 μM IBA, 25 $\text{mg}\cdot\text{l}^{-1}$ glutamine, 25 $\text{mg}\cdot\text{l}^{-1}$ citric acid, 12.2 $\text{mg}\cdot\text{l}^{-1}$ adenine sulfate, 15 $\text{mg}\cdot\text{l}^{-1}$ L-arginine 3% sucrose and 0.7% agar (CM-Bad: meristematic center for adventitious budding of underlying tissues, CM-BAx: meristematic center for axillary budding) **c)** Fragment of a three weeks old bud aggregate grown on MS elongation medium supplemented with 2.21 μM and 5.70 μM BA IAA (indole-3-acetic acid), 15 $\text{mg}\cdot\text{l}^{-1}$ L-arginine, 3% sucrose and 0.7% agar.

Table 1: Impact of Bud Aggregate Induction Medium on the Average Percentage of Rooting Stems in the Rooting Medium ($\frac{1}{2}$ MS Containing 2.46 μM IBA, 2% Sucrose and 0.7% Agar) for the Cameroon and Senegalese Accessions of *J. curcas*

Induction medium BA+IBA (μM)	Accession	Glutamine concentration in the induction medium ($\text{mg}\cdot\text{l}^{-1}$)					
		10 ($\text{mg}\cdot\text{l}^{-1}$)	15 ($\text{mg}\cdot\text{l}^{-1}$)	20 ($\text{mg}\cdot\text{l}^{-1}$)	25 ($\text{mg}\cdot\text{l}^{-1}$)	30 ($\text{mg}\cdot\text{l}^{-1}$)	35 ($\text{mg}\cdot\text{l}^{-1}$)
BA(11.1)+IBA (2.46)	Cam	36.97 \pm 1.16a	36.87 \pm 0.67a	31.27 \pm 1.60a	36.71 \pm 1.68a	35.88 \pm 1.47a	41.10 \pm 1.43a
	Sen	35.37 \pm 1.11a	35.17 \pm 0.49a	39.21 \pm 1.10a	39.11 \pm 1.48a	35.23 \pm 1.17a	39.11 \pm 1.10a
BA(8.87)+IBA (2.46)	Cam	45.21 \pm 1.90a	44.09 \pm 1.88a	52.56 \pm 1.25b	18.91 \pm 1.71b	46.89 \pm 1.88b	47.96 \pm 2.15b
	Sen	43.11 \pm 1.30b	43.19 \pm 1.68b	51.16 \pm 1.05b	48.01 \pm 1.61b	46.09 \pm 1.38b	56.98 \pm 2.11b
BA(6.65)+IBA (2.46)	Cam	52.12 \pm 2.22bc	51.00 \pm 2.31c	61.08 \pm 2.43c	51.07 \pm 2.22b	51.81 \pm 2.31bc	79.98 \pm 3.43c
	Sen	59.02 \pm 2.02c	50.00 \pm 2.71c	59.08 \pm 2.03c	60.07 \pm 2.02b	49.71 \pm 2.11b	67.90 \pm 3.41c
BA(4.43)+IBA (2.46)	Cam	79.11 \pm 4.89d	78.06 \pm 4.58d	81.19 \pm 5.46d	71.66 \pm 4.89c	71.69 \pm 4.58c	78.19 \pm 4.46d
	Sen	77.91 \pm 4.09d	76.16 \pm 4.18d	79.19 \pm 4.06d	70.36 \pm 4.19c	79.69 \pm 4.08c	77.17 \pm 4.41d
BA(2.21)+IBA (2.46)	Cam	90.10 \pm 6.23e	86.04 \pm 5.22e	89.05 \pm 5.85e	78.99 \pm 4.32d	81.04 \pm 5.22d	86.15 \pm 5.85e
	Sen	86.10 \pm 5.02e	84.04 \pm 5.12e	87.05 \pm 5.05e	76.90 \pm 4.12d	79.04 \pm 5.02d	84.15 \pm 5.75e

Average percentage \pm SE of rooted shoots after 4 weeks, with 3 replicates (26 shoots / replicate). Values in each column followed by same letters are not significantly different according to Duncan's multiple range test at $\alpha=0.05$.



Figure 5: Rooting and acclimatization of *J. curcas* vitroplants (Cameroon accession). **a)** Four week old rooted stem on MS medium containing 2.46 μM IBA, 2% sucrose and 0.7% agar, **b)** Acclimated 5 weeks old *J. curcas* vitro-plants.

whatever their background. The survival rate is of 98 % in acclimatization and obtained vitroplants showed no morphological abnormalities after being transferred to greenhouses (Figure 5b).

DISCUSSION

The absence of a significant difference between both tested accessions with respect to the induction of bud aggregates could be linked to insufficient genetic variation between them. Similar observations were made with several cultivars of *J. curcas* [8]. The formation of bud aggregates and the variability of induction performances were influenced by the interaction between the phytohormones and the amino acid supplied to the IM medium. The concentration of endogenous cytokinins and their control mechanisms would be the most important elements that could explain these differences. The absence of the apical dominance combined with the direct adventive regeneration from the underlying tissues of the bud, led to the formation of bud aggregates without involving the production of callus. A highly productive protocol applied to several cultivars of *Malus domestica* Borkh was established from shoot apex, but the buds regeneration included callogenesis [9]. That presents the risks of conformity alterations within the regenerants as it often is the case for adventive regeneration.

The shoot bud differentiation was observed on MS media supplemented with cytokinines. By means of axillary regeneration, we have been able to obtain 30.80 ± 5.5 shoots/explant of *J. curcas* on a MS medium containing 2.30 μM kinetine, 0.50 μM IBA, 27.8 μM of

adenine sulfate and 30 g.l^{-1} sucrose after 4 to 6 weeks of growth. Nevertheless this medium induced the formation of calluses and thus the risk of somaclonal variation. Similarly, Medza and co. regenerated 40.4 ± 8.80 shoots per axillary bud from a nodal explant on MS containing 6.65 μM BA and 2.46 μM IBA, 33.12 μM of adenine sulfate, 30.53 mg.l^{-1} glutamine and 30 mg.l^{-1} sucrose, after 4 weeks of growth but without any callus formation [6]. While the response obtained depends on the hormonal balance and of the kind of cytokinin used, it is also linked to the type of explant, as well as their physiological state. Among the cytokinins, BA is the best to stimulate the buds regeneration and proliferation from nodal explants of *J. curcas* [10]. The BA and IBA combination provided the best shoot bud induction of 6.0 shoots per explant. However the authors noticed differences in responses among genotypes which they attributed to endogenous concentration of hormones. Shoot tips of *J. curcas* were cultured on MS combination of 8.87 μM BAP and 2.85 μM IAA along with adenine sulfate, glutamine and activated charcoal. The plants produced by the direct organogenesis method exhibit greater genetic stability than those produced *via* callus-mediated organogenesis [11].

The amino acids added to the growth medium can act in synergy with growth regulators to regenerate entire plants of *Cichorium intybus* L. by induction of meristematic nodules from wounded leaves. That adventive regeneration method showed a high regeneration potential close to that of bud aggregates. In our trials, the glutamine was shown to be indispensable to the formation of bud aggregates [11]. Glutamine is frequently employed in the culture

medium as an organic nitrogen source. Many papers have shown that the use of exogenous glutamine can be beneficial for *in vitro* culture, increasing the regeneration rate and biomass of the explants. As easily accessible source of reduced nitrogen, it is generally more favorable than ammonium, to morphogenesis as reported for *Cucumis sativus* L. In the course of work done with the *in vitro* multiplication of *Arachis hypogea* L. from cotyledon explants, the presence of L-glutamine also improved and maintained high multiplication and growth rates. During transamination, glutamine transfers nitrogen to other carbohydrate substrates (α -keto acids) and the new amino acids immediately contribute to the biosynthesis of proteins [12, 13]. The glutamine and glutamate by-product are the primary sources of reduced nitrogen in the cellular metabolism. They contribute to the transamination, where ammonium is incorporated by the action of the glutamine synthetase and glutamate synthase [14] to form aspartate and asparagine. They are the precursors of all other amino acids [15] involved in protein biosynthesis and morphogenesis like buds aggregates process.

Glutamine in combination with inorganic forms of nitrogen has generally been used *in vitro*. In bromeliads, glutamine was an excellent nitrogen source to improve gain of dry shoot mass of plantlets cultivated *in vitro* [16]. The glutamine had a strong influence on shoot hormonal contents (cytokinin) and it has a great effect on the promotion of shoot-bud organogenesis from leaf base pineapple explants. Glutamine and glutamate are known to be the main endogenous amino acids involved in plant metabolism, providing nitrogen for the biosynthesis of amino acids, nucleic acids and other N-compounds [17].

Amino acids played a vital role in the induction and development of a maximum number of multiple shoots [12]. Adenine sulfate is a substrate for the synthesis of natural cytokinins [18]; its beneficial effect on the axillary budding from nodal explants is corroborated by the work on the *in vitro* organogenesis. Its absence in the optimal IM medium caused a reduction in the number of buds formed by aggregate (data not shown). The exogenous supply of adenine is recovered and utilized for ATP (Adenosine Triphosphate) and nucleic acid synthesis during the development of *in vitro* micropropagation. The enlargement of the nucleotide pool is required for rapid cell proliferation and growth of the tissue [19].

The formation of bud aggregates was also favored by L-arginine that takes part in the synthesis and/or

activity of the polyamines which cause hormone-like effects in plants [20]. They stabilize the protoplasts and influence the cell division while at the same time retard the senescence of the tissues. Their polycationic shape leads to connections with nucleic acids and plasmalemma phospholipids and allow for better permeability of the cell membranes [21].

It was shown that the products of L-arginine synthesis increase budding. The combination of these products to glutamine and arginine is a factor enhancing protein accumulation. The development of axillary nodes from explants was also performed on MS medium containing 4.90 μ M IBA and 13.31 μ M of BA [22]. Citric acid has been recognized as a very efficient anti-oxidant compound in *in vitro* culture [23, 24]. It prevents the production of polyphenols which often hinder organogenesis and cause explant necrosis. For *in vitro* propagation of *Acacia* plantlets, citric acid was the most effective in supporting axillary shoot proliferation and preventing browning of the medium [25].

The detailed study on histological events of *in vitro* growth and morphogenesis of *J. curcas* shows that the process of shoot morphogenesis happens through adventitious shoot morphogenesis and axillary development. These two types of plant regeneration were observed - one type of shoot regeneration was *via* organogenesis from underlying tissues - and the second type was through multiplication of the pre-existing meristems. It is, however, interesting to note that under similar experimental conditions the process of organogenesis varied from explant to explant. The authors attributed the difference in regeneration response to pre-disposed genetic conditions [26].

During the buds to shoot elongation stage, L-arginine prepares the shoots for rooting. A few buds were dominant over other buds of the same aggregate fragment and inhibited their growth. Once the first shoots were harvested from the fragment, the resting buds immediately started to grow into shoots as observed in various woody plants [27]. Consequently, such apical dominance leads to asynchronous development of buds differing in their physiological stage at the moment of their transfer to elongation medium. The addition of a hormone-free MS nutrient solution as a double phase on the gelled elongation medium one week preceding the transfer to rooting media, contributed to the dilution of endogenous cytokinins. This dilution liberates the buds from the negative effects of cytokinins on root formation.

The highest rooting rate in *J. curcas* was 19.43 % on ½ MS and 15 µM IBA, 11.4 µM IAA and 5.5 µM NAA (Naphthalene Acetic Acid) as auxin cocktail. The high rate of rhizogenesis is therefore linked to the presence of IBA in the medium [28]. In our trials, rooting of pre-elongated shoots was favored by a pretreatment in darkness at the beginning of rooting. This pretreatment of the shoots improved roots induction and initiation in several other woody species [27]. Darkness influences the endogenous metabolism of the shoots during the successive phases of the rhizogenesis with, notably, a rapid drop in the activity of endogenous peroxidase and phenolic compounds during the first five days of culture [29]. On the other hand, the use of ½ MS medium increases the C/N ratio by reducing the supply of nitrogen which is favorable to root formation. The rooting percentage variations could also be related to the mechanisms of control, the level of endogenous growth regulators and their metabolism. The high concentrations of cytokinins inhibit root formation, which would explain the low rooting rates obtained on stems originating from media with high levels of BA. The absence of secondary roots may be related to stress in the stem formation medium. The amount and the quality of secondary roots determine the survival rate during acclimatization. However, despite the absence of such roots, survival rates have remained high in acclimatization [10].

CONCLUSION

In the present study, it has been clearly established that the *J. curcas* plantlets were regenerated *in vitro* by direct induction of buds aggregates. Replanting the buds into an optimized MI medium led in 13 weeks to obtain a total average of 65.13±1.12 plantlets per bud originating from the development of nodal explant. The acclimatized plants do not present any visual defects and did not show any malformation. The performance gain from this micropropagation protocol, with a multiplier of 13 is double those obtained by other authors [10]. Its use would be more economically viable for the production of planting material. However, the conformity of clonal plantlets has to be confirmed after checking their behavior during the all plant growth cycle and before any application of the technique at a large scale.

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