In vitro clonal mass propagation of *Ximenia americana* L.

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Abstract — Introduction. *Ximenia americana* is a species developed in Africa and South America. This fruit tree is threatened by a dangerous process of genetic erosion. *In vitro* techniques could be used for its rapid clonal propagation. Since there is still no report on vitroculture of the species, we tested its micropropagation using axillary buds from mature plants of *X. americana*. **Materials and methods.** Single node explants of *X. americana* shoots were cultured on a proliferation medium made up with a MS medium containing different concentrations (2.5–15 µM) of two cytokinins [benzyladenine (BA) or kinetin] used individually or in combination with 0.5 µM of an auxin [2,4-dichlorophenoxyacetic acid (2,4-D) or naphtaleneacetic acid (NAA)]. Data were recorded after 5 weeks of culture. From proliferated shoot clumps, shoot explants (approximately 3 cm in length) were excised and transferred to rooting media made up with MS medium with or without 0.5 µM of indolebutyric acid (IBA) (pH = 5.8). **Results**. The most rapid and earliest proliferation was observed in media with the lowest concentrations of cytokinins. Absence of growth regulators in media and media with 2,4-D or NAA considerably delayed bud proliferation. The number of shoots per explant increased with the increase of cytokinins. The maximum number of shoots was achieved in 10 µM BA. When shoots were transferred to rooting media, media supplemented with 0.5 µM IBA improved the rooting frequency, root quality and number of roots per cutting. After rooting, the vitroplants were transplanted into small polybags with 1:1 non-sterile soil and sand, then in the field after 4 weeks. Eighty percent of the plants taken from regulator-supplemented media were acclimated versus 15% of those taken from auxin-free media. **Conclusion**. The rapid clonal propagation of *X. americana* is possible through *in vitro* culture of nodal explants. The best cytokinin for shoot multiplication was BA.

Brazil / *Ximenia americana* / micropropagation / nodes / explants / culture media / plant growth substances

Propagation clonale *in vitro* de *Ximenia americana* L.

Résumé — Introduction. *Ximenia americana* est une espèce qui se développe en Afrique et Amérique du Sud. Cet arbre fruitier est menacé par un dangereux processus d'érosion génétique. Des techniques de multiplication *in vitro* pourraient permettre de le propager rapidement. Pour pallier le manque de données sur la vitroculture de l'espèce, nous avons testé sa micropropagation en utilisant les bourgeons axillaires de plants adultes de *X. americana*. **Matériel et méthodes**. Des explants d'un seul nœud de tiges de *X. americana* ont été cultivés sur un milieu de prolifération constitué d'un milieu MS contenant différentes concentrations (2.5–15 µM) de cytokinines [benzyladenine (BA) ou kinétine] utilisées soit seules, soit en combinaison avec 0,5 µM d'une auxine [acide 2,4-dichlorophenoxyacetique (2,4-D) ou acide naphtaleneacétique (ANA)]. Diverses observations ont été faites après cinq semaines de culture. À partir des touffes de pousses proliférées, des explants de tige d'environ 3 cm de long ont été excisés et transférés sur milieu d'enracinement composé d'un milieu MS avec ou sans 0,5 µM d'acide indolebutyrique (AIB) (pH = 5,8). **Résultats**. Les milieux contenant les plus basses concentrations de cytokinines ont permis les proliférations les plus rapides et les plus précoces des bourgeons. En revanche, ces proliférations ont été considérablement retardées dans les milieux sans régulateur de croissance et dans ceux contenant du 2,4-D ou de l'ANA. Le nombre de pousses par explant a augmenté avec l'augmentation des teneurs en cytokinines. Le nombre maximal de pousses a été obtenu avec 10 µM de BA. Quand des pousses ont été transférées sur milieux d'enracinement, les milieux enrichis avec 0,5 µM d'AIB ont permis d'améliorer la fréquence d'enracinement, la qualité des racines et le nombre de racines par explant. Après enracinement, les vitroplants ont été transplantés dans de petits sacs de polyéthylène contenant un mélange de sol non stérile et de sable, puis ils ont été mis en terre après 4 semaines. Quatre-vingts pour cent des plants provenant des milieux avec un régulateur de croissance se sont acclimatés contre 15 % de ceux issus de milieux sans auxine. **Conclusion**. La propagation clonale rapide de *X. americana* est possible par la culture *in vitro* d'explants de nœuds. La meilleure cytokinine pour la multiplication des pousses a été la benzyladénine.

Brésil / *Ximenia americana* / micropropagation / nœud / explant / milieu de culture / substance de croissance végétale

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1. Introduction

Ximenia americana L., or Hog plum, Tallowood plum, Seaside plum, etc. in English and popularly called 'Ameixa' in Brazil, is a species developed in Africa and South America. It belongs to the class Magnoliopsida, subclass Rosidae and order Santalales, family Olacaceae. *X. americana* occurs abundantly in Rio Grande do Norte state, Brazil. It is a rapidly growing perennial woody plant that can attain a maximum height of 3–5 m after 1–2 years. The fruit has an excellent taste. The major part of the production of late ripening of Ameixa is processed into solid, liquid and frozen products and partly used for direct consumption in the fresh state. *X. americana* is commonly used in Côte-d'Ivoire by native healers for the medical attention of malaria. Currently, its exploration is still done in extra vista form and very little is known about this native fruitful tree, in agronomic terms. This species is threatened by a dangerous process of genetic erosion. Conventional methods of vegetative multiplication have failed and producing plants through seeds has its limitations.

Clonal propagation of plants using tissue culture technique has many applications in agronomy [1], especially for woody plants that are extremely difficult to propagate by conventional means. Considerable work has been done in the last few years on *in vitro* propagation of woody species [2–4]. However, there is no report on vitroculture of *X. americana*. Here we describe micropropagation using axillary buds from mature plants of *X. americana* to provide an alternative for its rapid clonal propagation.

Figure 1.
Tree of *Ximenia americana* L.

2. Materials and methods

Young shoots, each with 3–4 nodes, were collected from a 2-year-old tree of *X. americana* (*figure 1*) in November–December and thoroughly washed in running tap water. Surface disinfection was carried out with 0.1% (w/v) mercuric chloride ($HgCl_2$) for 10 min after a brief rinse in 70% (v/v) water/ethanol. The material was then washed with sterilized double-distilled water for 10 min, giving four changes.

To induce proliferation, single node explants were then excised and cultured on a MS medium [5] containing different concentrations (2.5–15 µM) of cytokinin, benzyladenine (BA) or kinetin, individually or in combination with 0.5 µM of an auxin, 2,4-dichlorophenoxyacetic acid (2,4-D) or naphtaleneacetic acid (NAA).

After 5 weeks of culture, data on the days taken to bud proliferation, percentage of proliferation cultures, number of shoots per culture and shoot length (cm) were recorded. For each treatment, 20 replicates were used and the experiment was repeated at least twice.

From proliferated shoot clumps, individual shoots (approximately 3 cm in length) were excised and transferred to rooting media made up with a MS medium with half-strength salts and sucrose with or without 0.5 µM of indolebutyric acid (IBA). The pH of the medium was adjusted to 5.8 before autoclaving and solidified with 7 $g \cdot L^{-1}$ Difco Bacto agar.

The explants were cultured singly in 200 mL glass flasks, each containing 30 mL of the culture medium. The cultures were grown at (26 ± 2) °C, with a 16 h photoperiod at a light intensity of 55–60 $\mu mol \cdot m^{-2} \cdot s^{-2}$ provided by warm white fluorescent tubes.

After harvesting of microcuttings for rooting, the stock cultures were transferred to fresh medium after removing dead and discolored tissues. In this way, stock cultures of shoots were maintained for continuous production of shoots for several months. No change was observed in this cyclical method of shoot production while the proliferation cultures were maintained.

3. Results

Two to three changes of media during the first week of culture were found to be most beneficial for obtaining a satisfactory response of explants in all instances. Nodal explants cultured on different treatment

Table I. Effect of growth regulators on shoot proliferation from nodal explants of *X. americana* after 5 weeks of culture.

Growth regulator	Concentration (µM)	Days taken to bud proliferation	% of proliferating cultures	Number of shoots per culture	Shoot length (cm)
BA	2.5	7.5 e	51.3 cd	2.1 de	5.4 a
	5.0	7.8 e	66.6 a	3.9 c	5.1 ab
	10.0	9.0 cde	60.9 ab	6.6 a	3.6 bc
	15.0	10.3 bcde	52.1 cd	2.1 de	4.1 abc
Kinetin	2.5	8.2 de	56.2 bc	1.1 e	5.0 ab
	5.0	8.7 cde	62.2 ab	2.0 de	4.1 abc
	10.0	10.2 bcde	51.6 cd	5.7 b	3.0 c
	15.0	11.1 bcd	48.6 cd	2.6 cd	4.0 abc
BA + 2,4-D	0.5 5.0	12.6 b	45.0 d	2.0 de	5.2 a
BA + NAA	0.5 5.0	13.0 b	46.1 cd	2.0 de	4.9 ab
Kinetin + 2,4-D	0.5 5.0	11.3 bc	49.3 cd	3.9 c	4.0 abc
Kinetin + NAA	0.5 5.0	12.0 b	45.9 cd	3.4 cd	5.0 ab
No growth regulator		16.3 a	25.6 e	1.0 e	3.3 c

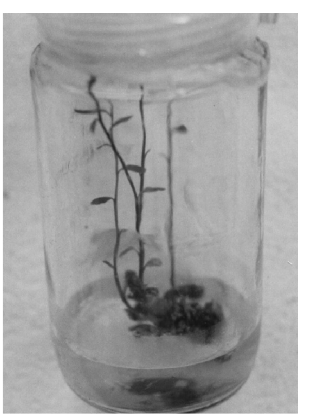
Means followed by the same letter in a column do not significantly differ by Duncan's range test ($p = 0.05$).

combinations showed their first response by enlargement and break of axillary buds. Rapid and early proliferation was observed in media with lower concentrations of cytokinins (*table I*). Absence of growth regulators in media considerably delayed bud proliferation. Media with 2,4-D or NAA also delayed proliferation. More than 45% of the explants responded in growth regulator-supplemented media but the survival percentage was only 25% in growth regulator-free media. The number of shoots per explant increased with the increase of cytokinins up to 10 µM and declined with 15 µM. The maximum number of shoots was achieved in 10 µM BA (*figure 2*) followed by 10 µM kinetin. Addition of 2,4-D or NAA in the media did not improve shoot proliferation and resulted in callus production at the base of explants, which suppressed the growth of the shoot. Shoot elongation was more or less inverse to the shoot number and maximum shoot length was recorded in 2.5 µM BA.

When shoots were transferred to rooting media, roots produced in auxin-free media were thin and the number of roots per cutting was low. Media supplemented with 0.5 µM IBA improved the rooting frequency, root quality and number of roots per cutting.

After 4 weeks of root induction, the plantlets appeared good enough to be transferred to exvitrum conditions. We observed that, for successful establishment in soil, *in vitro* rooted shoots needed to be kept at 30 °C with a constant photoperiod for 7–10 d until roots became brown. If the plantlets were transplanted without this

Figure 2.
Multiple shoot formation on nodal explants of *Ximenia americana* L. cultured in a MS medium supplemented with 10 µM of benzyladenine (BA).



treatment, survival was less than 5%. The plantlets with brown roots were carefully transplanted to small polybags with 1:1 non-sterile garden soil and sand and covered with a glass beaker to maintain humidity. After 4 weeks, the potted plants were finally transplanted into the field. Eighty percent of the plants taken from regulator-supplemented media were acclimated versus 15% of those taken from auxin-free media.

4. Conclusion

The results described in this investigation show that rapid clonal propagation of *Ximenia americana* is possible through *in vitro* culture of nodal explants. The best cytokinin for shoot multiplication was BA.

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Propagación clonal *in vitro* de *Ximenia americana* L.

Resumen — Introducción. X*imenia americana* es una especie que crece en África y América del Sur. Este frutal está amenazado por un peligroso proceso de erosión genética. Mediante técnicas de propagación *in vitro* se podría multiplicar rápidamente. Para paliar la falta de datos sobre el cultivo in vitro de la especie, probamos su micropropagación utilizando yemas axilares de plantas adultas de *X. americana*. **Material y métodos**. Se cultivaron explantes de un mismo nudo de tallos de *X. americana* en un medio de proliferación compuesto por un medio MS con diferentes concentraciones (2.5–15 µM) de citoquininas [benciladenina (BA) o quinetina] empleadas solas o combinadas con 0,5 µM de una auxina [ácido 2,4-diclorofenoxiacético (2,4-D) o ácido naftaleno acético (ANA)]. Se efectuaron distintas observaciones tras cinco semanas de cultivo. A partir de macollas de brotes proliferados, se separaron explantes de tallo de unos 3 cm de largo y se transfirieron a un medio de enraizamiento compuesto por un medio MS con o sin 0,5 µM de ácido indolbutírico (AIB) (pH = 5.8). **Resultados**. Los medios que contenían las menores concentraciones de citoquininas permitieron las proliferaciones más rápidas y precoces de las yemas. En cambio, estas proliferaciones se retrasaron considerablemente en los medios sin regulador de crecimiento y en los que contenían 2,4-D o ANA. El número de brotes por explante aumentó con el incremento del contenido de citoquininas. El mayor número de brotes se obtuvo con 10 µM de BA. Cuando se transfirieron brotes a un medio de enraizamiento, los medios enriquecidos con 0,5 µM de AIB permitieron mejorar la tasa de enraizamiento, la calidad de las raíces y el número de raíces por explante. Tras enraizamiento, las vitroplantas se trasplantaron en bolsitas de polietileno con una mezcla de suelo no estéril y arena para proceder a su plantación al cabo de 4 semanas. El ochenta por ciento de las plantas procedentes de medios con regulador de crecimiento se aclimataron frente al 15% de las que procedían de medios sin auxina. **Conclusión**. La propagación clonal rápida de *X. americana* es posible mediante cultivo in vitro de explantes de nudos. La mejor citoquinina para la multiplicación de brotes fue la BA.

Brasil / *Ximenia americana* / micropropagación / nudos / explantes / medio de cultivo / sustancias de crecimiento vegetal