

IN VITRO PROPAGATION OF *Enterolobium cyclocarpum* (GUANACASTE) FROM NODAL EXPLANTS OF AXENIC SEEDLINGS

PROPAGACIÓN IN VITRO DE *Enterolobium cyclocarpum* (GUANACASTE) A PARTIR DE EXPLANTES NODALES DE PLANTULAS AXÉNICAS

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ABSTRACT. *Enterolobium cyclocarpum* (Jacq.) Griseb. is a multipurpose leguminous tree, considered an endangered species because of overexploitation and the slow rates of natural propagation due to the intrinsic characteristics of the tree. An alternative approach to overcome this problem is the establishment of systems for its rapid, mass propagation. In this work, a protocol for *in vitro* propagation of *E. cyclocarpum* using the axenic nodal segments obtained from *in vitro* germinated seedlings, was investigated. The seeds collected from two Mexican communities were germinated both *ex vitro* and *in vitro* and the effect of a pre-treatment of thermal scarification was evaluated. Seeds from only one community were selected for propagation experiments on the basis of lower genetic variability estimated by RAPD markers, due to the high variation in the responses observed in pooled seed lots, which probably reflects an effect of genotype. Nodal segments from seedlings were cultured on MS basal medium supplemented with 30 g/L sucrose in the presence of different concentrations of 1-naphthaleneacetic acid (NAA) in combination with benzyladenine (BA) or kinetin (KIN). The highest multiplication rate (with an average of 4.75 shoots per explant) was achieved when MS medium was supplemented with 2.2 μM BA and 10.7 μM NAA. Excised shoots were rooted on half-strength MS medium without growth regulators. The micropropagated plantlets were acclimatized and successfully transferred to soil with a survival rate of 90%. These plantlets appeared morphologically similar to the mother plant and no variation was detected among them by the use of RAPD markers, which makes possible the use of this procedure for the propagation clonal of this species.

Key words. Axillary buds, *in vitro* germination, leguminous, micropropagation, RAPD, scarification.

Abbreviations. BA - Benzyladenine; dNTPs - deoxynucleotides; KIN - Kinetin (6-furfurylaminopurine); MS - Murashige and Skoog; NAA - 1-naphthaleneacetic acid; RAPD - Randomly amplified polymorphic DNA

RESUMEN. *Enterolobium cyclocarpum* (Jacq.) Griseb. es un árbol leguminoso de uso múltiple, el cual es considerado una especie amenazada, resultado de la sobreexplotación y las bajas tasas de propagación natural debidas a las características intrínsecas del árbol. Una alternativa para superar este problema es el establecimiento de sistemas para su propagación masiva en tiempos cortos. En este trabajo, se investigó un protocolo para la propagación *in vitro* de *E. cyclocarpum* utilizando los segmentos nodales axénicos obtenidos de plántulas germinadas *in vitro*. Las semillas colectadas en dos comunidades mexicanas fueron germinadas tanto *ex vitro* como *in vitro*, y se evaluó el efecto de un pre-tratamiento de escarificación térmica. Para los experimentos de propagación se seleccionaron semillas provenientes de sólo una de las comunidades, debido a que presentaban una menor variabilidad genética de acuerdo con marcadores RAPD y a que existía una gran variación en las respuestas observadas en lotes de semillas mezclados. Esta variación fisiológica presente en semillas mezcladas, probablemente refleja un efecto del genotipo.

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Los segmentos nodales obtenidos de las plántulas fueron cultivados en medio basal MS suplementado con 30 g/L de sacarosa en presencia de distintas concentraciones de ácido 1-naftalenacético (ANA) en combinación con benziladenina (BA) o kinetina (KIN). La mayor tasa de multiplicación (de 4.75 brotes por explante en promedio) se obtuvo cuando el medio MS fue suplementado con 2.2 μM BA y 10.7 μM ANA. Los brotes obtenidos fueron enraizados en medio MS con la mitad de concentración de sales y sin reguladores de crecimiento. Las plántulas micropropagadas fueron aclimatadas y transferidas exitosamente a suelo con una tasa de sobrevivencia del 90%. Estas plantas eran morfológicamente similares a la planta madre y no se detectó variación entre ellas por el uso de marcadores RAPD, lo cual hace posible el uso de este procedimiento para la propagación clonal de esta especie.

Palabras clave: Yemas axilares, germinación *in vitro*, leguminosas, micropropagación, RAPD, escarificación.

Introduction

Enterolobium cyclocarpum (Guanacaste, [Figure 1A](#)) is native to tropical America. It is naturally distributed from western and central Mexico, across Central America, to the northern part of South America (Venezuela and Brazil). It is also found in Jamaica, Cuba, Trinidad and Guyana and has been introduced to other tropical regions [[1](#), [2](#)].

It is a fast-growing timber-yielding species of artisanal importance. The wood is resistant to attack by dry-wood termites, which makes it feasible to be used in house construction. It is also useful as firewood due to its high caloric content. Mature fruits contain a gummy-resinous juice which along with their own smashed pulp is used to produce charcoal. Fruits and leaves are used as forage allowing cattle to feed directly from the tree or as a nutritional complement in combination with the fodder [[1](#), [3](#), [4](#)].

Fruits consist of shiny, dark brown pods, which are twisted along one of the edges, resembling a human ear. These fruits are 7 to 12 cm in diameter and contain 8 to 16 seeds. Seeds are big, ovate and flat, 2.3 cm long and 1.5 cm wide, red-brown and shiny with a pale line that follows the contour of the seed ([Figure 1B](#)). They are covered by a spongy and fibrous pulp with sweet smell and taste [[1](#)]. They are rich in protein (up to 35%), and its amino acid composition is comparable to that of wheat or fish flour. Seeds also contain iron, calcium, phosphorus and ascorbic acid. In some places, they are consumed in sauces, soups and as a coffee substitute, and several medicinal properties have been attributed to them [[3](#), [1](#), [5](#)]. Besides the traditional uses, several biotechnological applications have recently been proposed for this tree, such as the use of its gum as a fungi culture substrate or for the production of ice cream and yogurt [[6](#), [7](#)].

The extremely hard seed coat avoids germination until a structural modification (scarification) allows hydration of the embryo. In nature, seeds can be scarified and dispersed via the gut of a large mammal, as can be horses, cows or tapirs [[8](#)]. After scarification, germination is achieved between 14 and 20 days, with an average germination rate of 50% under humid conditions, depending on the scarification method. Germination rate is affected not only by the structural characteristics of the seeds, but also by seed abortion, which is related to position within the fruit, having the seeds from the distal portion the highest abortion ratio. It has been proposed that resource competition among embryos is the main cause of seed abortion in Guanacaste [[9](#)].

Enterolobium cyclocarpum has been proposed as an alternative for rehabilitation of marginal soils, due to its ability to form a symbiotic association with nitrogen fixing soil microorganisms [10, 11]. On the other hand, it is considered an endangered species in some areas because of overexploitation and defoliation due to water stress and the attack of butterfly larvae [12]. This situation exhibits the necessity of an efficient propagation system ensuring a sufficient number of individuals for all possible uses. *E. cyclocarpum* is mainly propagated sexually from seeds through direct seeding or planting seedlings, with the limitations mentioned above due to the factors affecting the germination rate [1]. Alternatively, it can be also propagated asexually by shoot cuttings and stum planting, but the production obtained from these conventional methods is very limited [5, 13].

To date, the reports on the propagation of *E. cyclocarpum* by biotechnological techniques are limited. In this work, we performed several assays to evaluate the *in vitro* behavior of this species with the aim of establishing a more efficient method for its propagation.

Materials and methods

Plant material

Mature seeds of *E. cyclocarpum* were collected from two different locations: the community of Santa Rita, Ayotlan and the community of Cuitzeo, Poncitlan, both in the state of Jalisco, in western Mexico. Seeds were collected from April to June, during the 2004 fruiting season, and only those which size was between 1.9 and 2.1 cm long and with no apparent signs of damage or disease were used for further experiments.

Pre-germination treatment

It has been reported a low germination rate for the genus *Enterolobium* under natural conditions because of the seed coat hardness, and several protocols have been proposed for dormancy breaking [14, 15]. Due to this fact, we carried out a pre-treatment of thermal scarification to weaken the seed coat and achieve a more rapid and efficient germination. Collected seeds were washed in 5% liquid soap for 3 min and rinsed thoroughly in tap water. Seeds were then submerged in boiling water (at 94°C in our laboratory conditions) for 5 min, and allowed to cool at room temperature.

Germination

For each of the following germination approaches, 3 replications of 100 seeds that underwent the thermal pre-treatment, and the same number that were not subjected to the procedure were used in the experiments.

Ex vitro germination: Seeds were sown on the surface of a wet paper towel which was placed on a tray, covered with aluminum foil to keep it in the dark and maintained at 30°C for 30 days (Figure 1C). Tap water was added to the filter paper every second day to keep it moist, avoiding prolonged exposure to light during the process.

In vitro germination: Seeds were rinsed in 70% (v/v) ethanol for 1 min, surface disinfected with 3% (w/v) sodium hypochlorite solution for 15 min, and rinsed three times in sterile distilled water. Groups of 5 seeds were placed on polypropylene vessels with MS medium [16] supplemented with 30 g l⁻¹ sucrose, solidified with agar (8 g l⁻¹) and adjusted to pH 5.7. Seeds were incubated at 27°C with a 12 h photoperiod (50

$\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$; [Figure 1D](#)). For both methods, a seed was considered to have germinated at the emergence of the radicle.

Proliferation of axillary buds

Nodal segments used for experiments were obtained from plants which height was between 15 and 20 cm generated from *in vitro* germination. The segments were grown on MS medium supplemented with 30 g l^{-1} of sucrose, solidified with agar (8 g l^{-1}) and adjusted to pH 5.7. Growth regulators added to nutrient medium were NAA in combination with BA or KIN. NAA was tested in concentrations of 0-10.7 μM , BA in concentrations of 0-4.4 μM and KIN was tested in concentrations of 0-4.6 μM . The nodal segments were incubated at 27°C with a 12 h photoperiod ($50\text{ }\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$), until the formation of shoots ([Figure 1E](#)), which presence and number were recorded. One axillary bud per explant was used. Three replications of four explants each were performed for all treatments.

Rooting and acclimatization

Rooting of the shoots was induced by subculture on hormone-free half-strength MS medium at the same growth conditions described for proliferation of axillary buds. Fully rooted plants were transferred to potting soil and established under greenhouse conditions without special acclimatization techniques ([Figure 1F](#)).

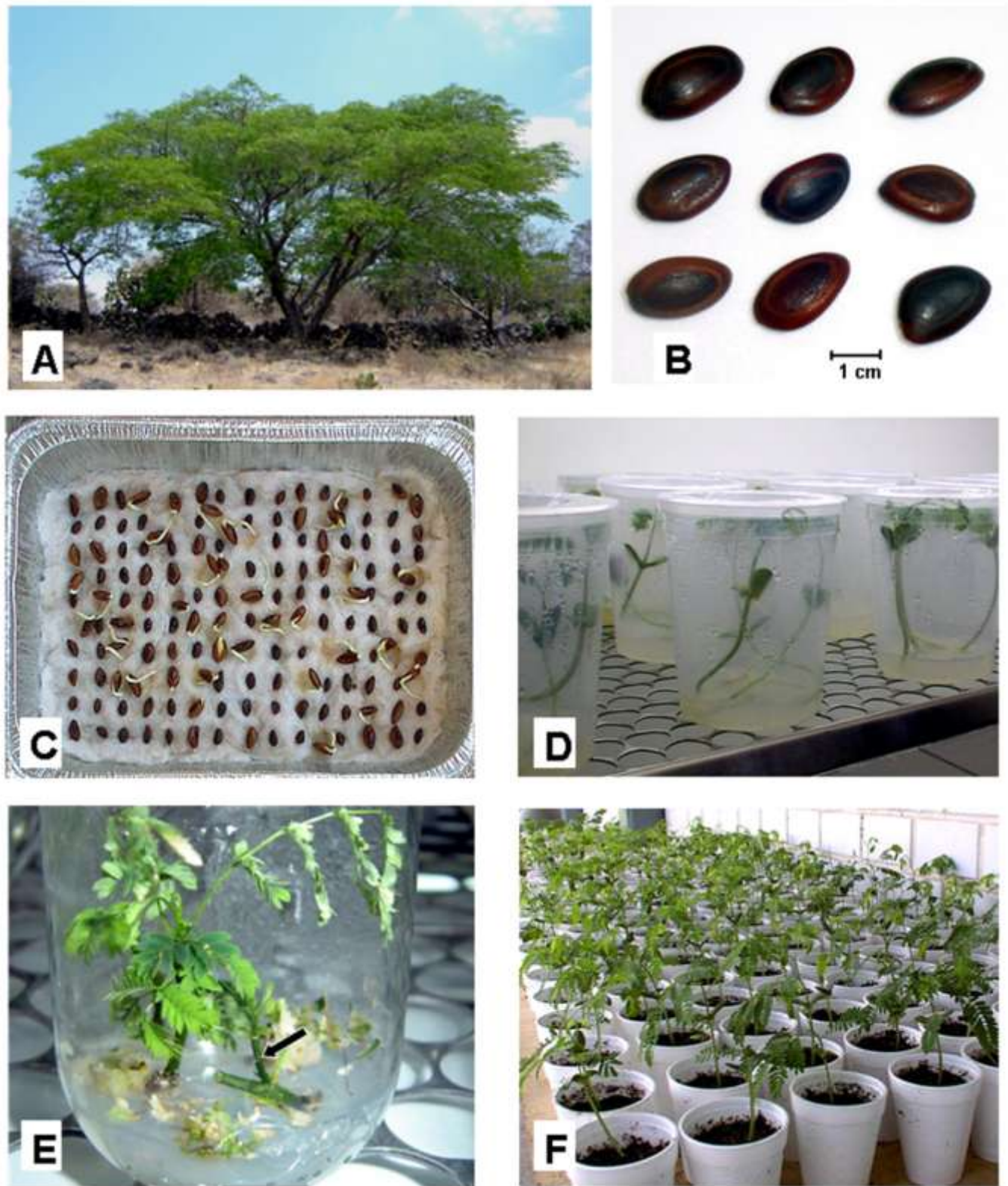


Figure 1. Morphological characteristics, seed germination and *in vitro* propagation of *E. cyclocarpum*. (A) Adult tree of *E. cyclocarpum*, about 40 years old in the community of Cuitzeo in Jalisco, Mexico. (B) Mature seeds of *E. cyclocarpum* collected from several trees. (C) *Ex vitro* and (D) *In vitro* germination of *E. cyclocarpum*. (E) *In vitro* propagation of *E. cyclocarpum*. (F) *In vitro* propagation of *E. cyclocarpum*.

cyclocarpum seeds. (E) *In vitro* proliferation of axillary buds of *E. cyclocarpum*. Arrow indicates point of emergence of a shoot. (F) Micropropagated plants acclimatized to greenhouse conditions.

RAPD analysis

Genomic DNA extraction from *E. cyclocarpum* seedlings or plantlets was carried out according to the protocol reported by Zhang and Hewitt [17] for tissues with high nuclease activity, which is recommended for preparation of DNA from leguminous plants. To test the quality of isolated DNA, a 5 µl sample was run on a 0.8% agarose gel stained with ethidium bromide. Quantification of DNA was achieved by UV spectrophotometry using a NanoDrop® Spectrophotometer (NanoDrop Technologies, Wilmington, DW, USA). Preliminary experiments were performed to screen primers for use in RAPD analysis. Thirty decamer primers from commercial kit (Operon RAPD Kits E, H, I and J, Operon Technologies, Inc., Alameda, CA, USA) were screened for their ability to amplify DNA fragments and to generate polymorphic bands capable of discriminating between individuals. Six primers gave positive results. These six primers, which were used as single primers in the RAPD experiments, are primer OPH-12 (5'-ACGCGCATGT-3'), OPI-06 (5'-AAGGCGGCAG-3'), OPI-07 (5'-CAGCGACAAG-3'), OPI-14 (5'-TGACGGCGGT-3'), OPJ-02 (5'-CCCGTTGGGA-3') and OPJ-17 (5'-ACGCCAGTTC-3'). Amplification reactions were performed in a final volume of 25 µl containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 4 mM MgCl₂, 125 µM each of dNTPs, 10 pmol of primer, 20 ng of genomic DNA and 1.5 units of *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA). Amplifications were performed on a PTC-200 thermal cycler (MJ Research Inc., Waltham, MA, USA) using the program: 1 step at 94°C for 3 min, 4 cycles consisting of 30 s at 94°C, 1 min at 36°C and 2 min at 72°C, followed by 42 cycles of 30 s at 93°C, 1 min at 35°C and 2 min at 72°C. Amplification products were separated by electrophoresis in 2% agarose gels at 75 V, followed by ethidium bromide staining and the images of the gels under UV light were digitally captured for analysis. 1 Kb DNA Ladder (Invitrogen, Carlsbad, CA, USA) was used as DNA size marker.

Data analyses

The efficiency of germination for each treatment was estimated calculating the average percentage of germination and the standard deviation from the three replications. The effect of growth regulators for *in vitro* propagation was examined using analysis of variance (ANOVA). A Tukey analysis was carried out to determine whether significant differences occurred between individual treatments ($P \leq 0.05$).

RAPD bands were scored manually for their presence (1) or absence (0) as distinct and prominent bands at a particular position. From the binary data, a similarity matrix was constructed by the FreeTree program version 0.9.1.50 [18] based on the Jaccard [19] similarity coefficient. Clustering analysis was conducted using the unweighted pair-group method with arithmetic averages (UPGMA). A dendrogram was constructed on the basis of distance data using the TreeView program version 1.6.6 [20].

Results

Seed germination

The *in vitro* germination of *E. cyclocarpum* seeds was assayed with the aim of obtaining axenic seedlings as a source of explants for the *in vitro* propagation. For comparison, the *ex vitro* germination was also performed. For most of the seeds in the *ex vitro* assay, germination was achieved between 6 and 9 days after

sowing, whereas for the *in vitro* approach, germination was achieved between 1 and 4 days (data not shown). However, germination was evaluated at day 30 after sowing to include also slow-germinating seeds in the analysis. As shown in [Figure 2](#), the *in vitro* germination was more efficient than the *ex vitro* germination, both with or without the thermal pre-treatment. This demonstrates that disinfection process and *in vitro* conditions do not reduce but increase the germination rate compared to the *ex vitro* conditions, and that the pre-germination treatment is important for increasing the germination rate for both approaches.

Microbial contamination was present in about 13% of seeds germinating under *ex vitro* conditions that underwent thermal pre-treatment, and in 32% of those that were not scarificated. On the other hand, contamination was observed in 12% of seeds under *in vitro* conditions that were scarificated, and in 20% of those not treated.

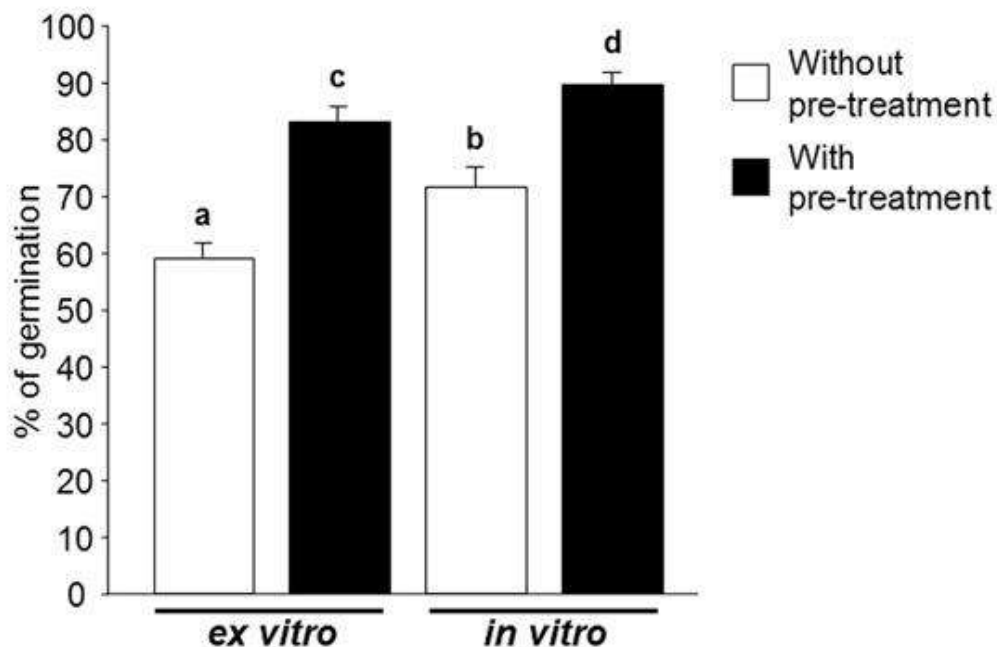


Figure 2. Seed germination of *E. cyclocarpum*. Bars represent the mean values of three independent experiments, and vertical lines represent standard deviations. Means with the same letter indicate no significant difference according to Tukey's HSD ($P \leq 0.05$).

RAPD analysis

Preliminary experiments on proliferation of axillary buds indicated a high variability on the response to growth regulators for seedlings from pooled seed lots. As an initial approach to determine if the observed response of the explants could be due at least in part to genetic differences among parental individuals, a RAPD analysis was performed. Ten individuals obtained by germination of seeds collected in the community of Cuitzeo and the same number from the community of Santa Rita were used for the analysis. Six out of thirty screened primers were selected for their ability to generate a reproducible pattern of

amplified fragments with enough polymorphic bands to distinguish among individuals from both seed sources. A total of 125 bands were scored and 107 of them (85.6%) were polymorphic across all individuals. An example of the pattern obtained with one of the six primers is shown in [Figure 3](#). The polymorphic bands were used to generate a genetic distance matrix based on Jaccard's similarity coefficient. The dendrogram constructed from the distance data ([Figure 4](#)) shows that all of the individuals from Santa Rita are grouped into a sub-cluster of those from Cuitzeo. Furthermore, the analysis suggests a limited genetic variation in the former community, with an average genetic distance of 0.20 ranging from 0 to 0.33. On the other hand, individuals from the community of Cuitzeo show a higher genetic variation with an average genetic distance of 0.60 ranging from 0.14 to 0.79. This demonstrates that RAPD markers allow to obtain a good discrimination among individuals collected from the same community and to establish a genetic relationship between two populations separated by 35 km. Moreover, these results show the usefulness of RAPD analysis in assessing genetic variability of closely related individuals of *E. cyclocarpum*, as an alternative to isozyme analysis used previously for this species [[21](#), [22](#), [23](#)].

All the experiments reported for axillary bud proliferation were performed only with seeds from the community of Santa Rita as the starting material, which is a genetically more uniform population as determined by RAPD analysis.

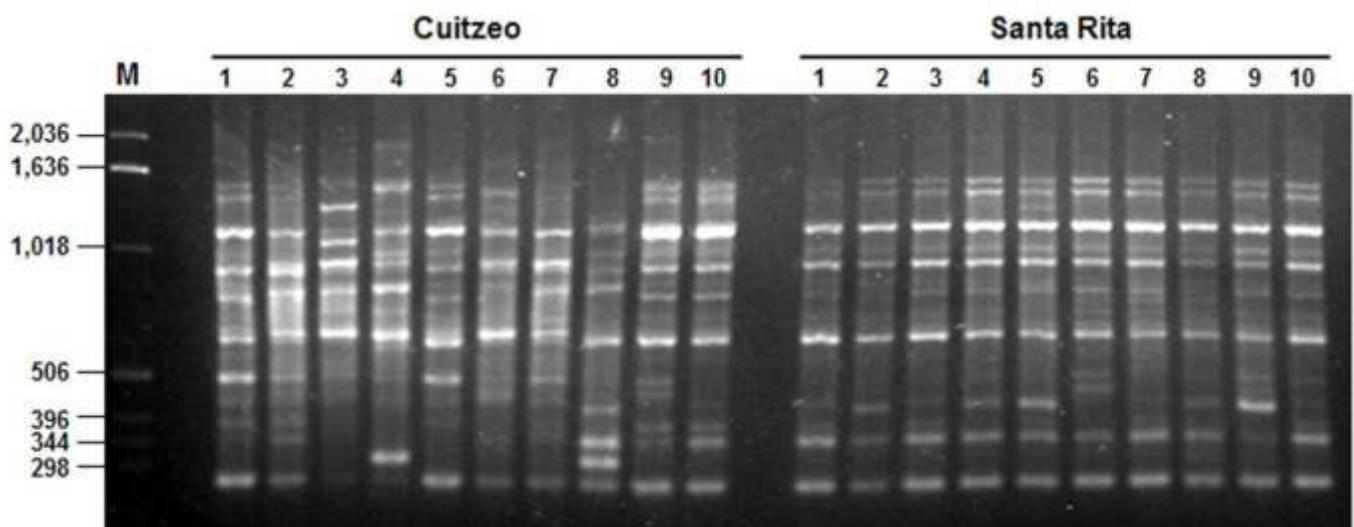


Figure 3. Electrophoretic patterns of RAPD markers for *E. cyclocarpum* individuals from the communities of Cuitzeo and Santa Rita in Jalisco, Mexico, using primer OPI-06. DNA size marker is in the left, and the size of each fragment is indicated in basepairs.

***In vitro* propagation**

Axillary bud proliferation of *E. cyclocarpum* was achieved on MS medium supplemented with NAA and BA ([Figure 5A](#)). Nodal segments started to form shoots within 2-3 weeks and more new shoots were produced within 4-5 weeks. MS nutrient medium supplemented with 10.7 μ M NAA plus 2.2 μ M BA promoted effectively formation of shoots in nodal segments of *E. cyclocarpum* seedlings (4.75 ± 0.75 shoots

per explant). Addition of KIN to the medium instead of BA did not produce a more effective response for shoot generation. For the concentrations of NAA used, the combination with different concentrations of KIN did not produce significant differences in the number of shoots generated ([Figure 5B](#)). 100% rooting of shoots was obtained with the conditions described, and the average time for emergence of first root was of 30 days. Plantlets transferred to soil showed a survival rate of 90%. Acclimatized plants were phenotypically normal and no morphological variation was observed among them. RAPD analysis was used to assess the clonal identity of propagated plants and no polymorphism was detected between the material propagated *in vitro* and the donor plants they originated from (data not shown).

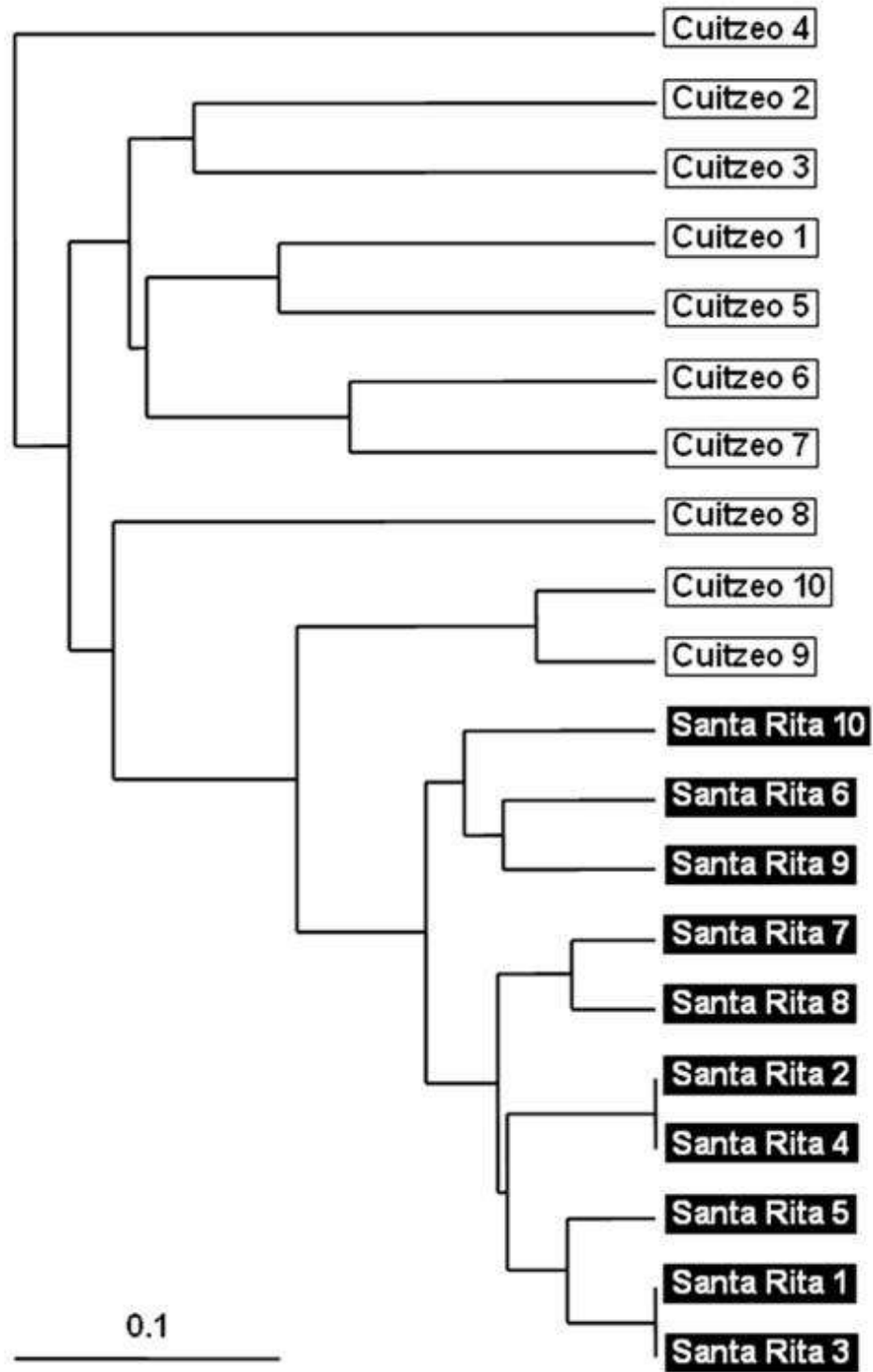


Figure 4. Dendrogram of *E. cyclocarpum* individuals from the communities of Cuitzeo and Santa Rita in Jalisco, Mexico. Dendrogram was generated from RAPD data using six primers. Scale in the lower left indicates genetic distance.

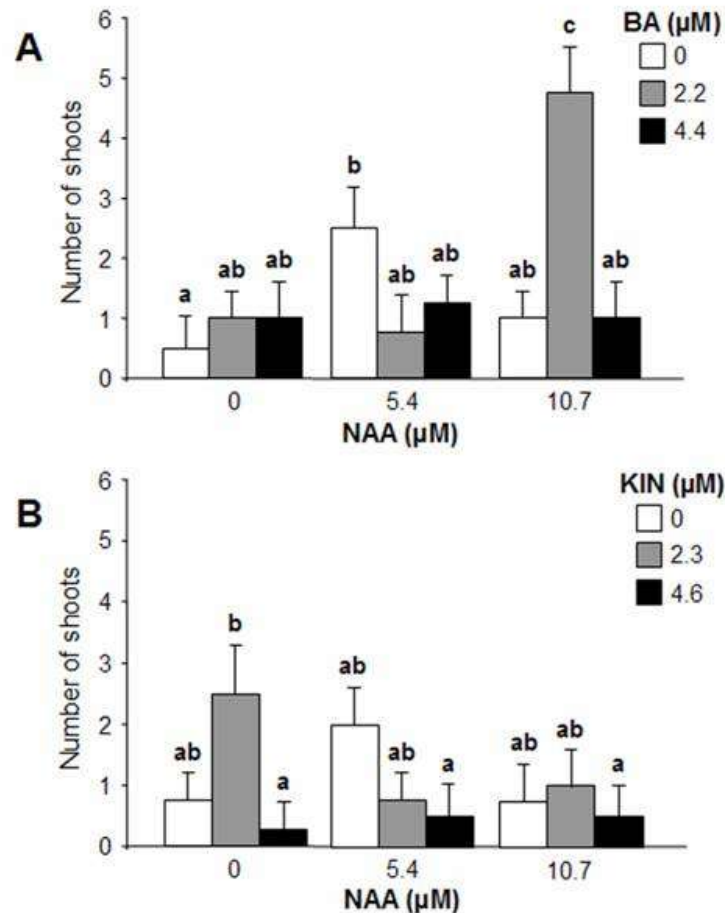


Figure 5. Effect of growth regulators on axillary bud proliferation of *E. cyclocarpum*. Bars represent the mean values of three independent experiments and vertical lines represent standard deviations. Means with the same letter indicate no significant difference according to Tukey’s HSD ($P \leq 0.05$).

Discussion

A requisite for the massive *in vitro* propagation of trees is the availability of explants from juvenile tissues. Due to the difficulties in acquiring these explants directly from the tree, seedlings have become a common source of tissues. Although seedlings can be obtained through *ex vitro* germination of seeds, the explants need to be subjected to a surface disinfection procedure, which can affect their viability. The *in vitro* germination of seeds allows the obtaining of axenic seedlings whose explants can be used directly for *in vitro* propagation approaches without further disinfection treatments.

In this work, we describe the procedure for the *in vitro* germination of *E. cyclocarpum* seeds. In our conditions, the efficiency of germination for this approach was higher than that of *ex vitro* germination, which was probably due to differences in the availability of nutrients and/or the exposure to a photoperiod. Moreover, the surface disinfection of seeds could promote germination, although not at the same extent as the thermal scarification.

Previous reports recommend thermal scarification to induce germination in *E. cyclocarpum* seeds. It has been reported a percentage of germination of 1-9% for non-treated seeds, whereas seeds scarificated by hot water treatments reached an efficiency of germination of 60-80% [2, 1, 5]. In our conditions, the percentage of germination for non-treated seeds was almost 60%, and the thermal pre-treatment increased the percentage of germinated seeds for both the *ex vitro* and the *in vitro* approaches. The effect of scarification on the efficiency of germination was more evident for the *ex vitro* approach, increasing in about 40% in comparison to the non-treated seeds. On the other hand, this efficiency increased only 25% for the *in vitro* approach. This positive effect of thermal scarification on the percentage of germination could be due at least in part to the reduction of microbial contamination by the hot water treatment.

We demonstrate that the procedure reported here for the *in vitro* germination of *E. cyclocarpum* seeds not only has no a negative effect on the seed viability or the efficiency of germination, but increases the latter. Therefore, this is an efficient method for obtaining axenic seedlings of this species to be used as a direct source of explants for *in vitro* propagation.

For the *in vitro* propagation of *E. cyclocarpum* from nodal segments, we tested combinations of growth regulators that were effective for shoot multiplication in other trees, including one legume, though explants used in those studies were from adult trees [24, 25]. Our results are in agreement with those reports by the fact that BA was more effective than KIN in stimulating shoot formation, however, in those previous studies BA alone, not only in combination with NAA, was able to generate this response. Despite we did not observe that effect in our experiments using seeds from the community of Santa Rita as starting material, some of the explants responded effectively in the absence of auxin when using pooled seed lots. For that reason, it is not discarded that genotype could have an important effect on these responses, due to the variations observed for mixed seeds.

This is the first report of the use of DNA markers to evaluate genetic variability in *E. cyclocarpum*. According to the RAPD analysis, it is noticeable that *E. cyclocarpum* individuals from the community of Cuitzeo presented a higher genetic variability than that of the individuals from Santa Rita. This is not surprising, since in the latter community seeds were collected from the only two existing trees, and in the former there were more than thirty. It is known that *E. cyclocarpum* is a predominantly outcrossing species and the higher number of individuals in a population can determine a higher genetic variability [26, 22]. It is possible that this higher genetic variability could account for the presence of genotypes with a differential response to the growth regulators used for *in vitro* propagation. On the other hand, the behavior observed could be due to physiological differences depending on the location of the seed source. This is also a reasonable explanation, since it has been reported the existence of groups with differential response to the scarification in *E. cyclocarpum*, depending on the origin of the seeds, showing important differences in the time of germination. Similarly, Rocha and Aguilar [23] found that the vigor of seedlings of trees from continuous forests was higher than that of seedlings from trees in pastures. It is also possible then that the origin of the seeds could determine the response of the explant to the growth regulators used. It would be interesting to evaluate the response of explants from individual seed lots to determine whether or not exist different response groups to the growth regulators employed, and if these differences depend on genetic differences, the origin of the seeds or both.



The procedure described here allows the obtaining of a considerable number of propagated plants, and although the multiplication rate that we achieved was not large enough to be commercially significant, our results provide a basis for the development of more efficient systems for the propagation of *E. cyclocarpum* for commercial or conservationist purposes. Moreover, this approach is useful for the conservation and clonal propagation of desirable genotypes, which is not possible in naturally occurring populations due to the reproductive characteristics of the tree.

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