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Induction of Somatic Embryogenesis in Jatropha Curcas L.

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***Abstract* – *Jatropha curcas* L. is a drought resistant species and can be grown in marginal areas. Because of its high content of oil in the seeds, it is interesting for the production of biodisel. Conventional methods of vegetative and sexual propagation are deficient due to low multiplication rates. Somatic embryogenesis represents an applicable regeneration pathway. The objective of this work is to introduce the metodology aplied in the induction of embryogenesis in three types of explants, for which ten treatments were evaluated with 2,4-D and ANA. Statistically significant differences were detected in the responses according to explants types. All treatments with growth regulator formed callused masses. The highest proliferation of callus occurred in foliar segments with 2.4 D to 107.41 µM, and in explants from embryos and cotyledonary leaves the concentrations were 67.87 and 135.74 µM respectively. Proembrional stages were observed in callus from leaf segments with 2.4 D to 107.41 µM.**

***Keywords* – Callus, Proembryos, Regeneration, Somatic Embryogenesis.**

I. INTRODUCTION

Jatropha curcas L. is a perennial plant belonging to the family of the Euphorbiaceae, tolerant to water stress, and classified as a suitable species for planting in arid or marginal regions. It is used for agricultural activity in areas with low soil fertility. It can be used to control floods, nutrient leaching, soil erosion and dune displacement. It has great potential as a biofuel crop; the latex of the plant and the seeds is used for medicinal purposes, applications in the cosmetics industry and biopesticides are also recognized [7].

In Argentina, biodiesel is produced from soybeans, and ethanol from sugarcane. Both crops are produced for food purposes, and that is why it is necessary to develop alternative oilcrops, with production costs lower than food crops, and that generate oils suitable for the elaboration of biodiesel. The country has promising germplasm, 11 species of the genus are mentioned, out of which 5 are endemic; and among these are *J. macrocarpa* (Grisebach) and *J. hieronymi* (Kuntze) [2]. Up to the present, there is no program in the region for the selection of elite individuals for *J. curcas*, let alone an improvement scheme. In order to satisfy the enormous demand for biofuels that is proposed for future years, it is necessary to define methods of clonal propagation and micropropagation.

Conventional agriculture uses seeds and vegetative structures for the propagation of the species, methods that for this species are deficient due to the genetic variability that the seeds present, and because the production of vegetative material is seasonal. Tissue culture techniques can be an alternative for efficient propagation. In recent years, multiplication protocols have been reported from different explants sources [14], [4], [18], [10], [20], [13]. The micro propagation protocols for Gen. Jatropha species [18], [10], [16], [17] have not been promising due to the low multiplication rate. Another alternative for clonal propagation is the use of somatic embryogenesis, which is considered, as opposed to organogenesis, as the most appropriate way of regeneration. In this type of technique, the cost of the propagule obtained is lower than any with results from other methodologist. Somatic embryogenesis is the formation of an embryo from a cell different from a gamete, or the product of gamete binding, known in nature as a form of apomicts, which is called adventitial embryony [8]. Somatic embryos resemble sexual embryos and develop in a similar way, going through all stages of embryogenesis, producing an embryonic structure with an apical and radical meristem. Being genetically identical to the parent, they are able to germinate and become a seedling on a substrate.

The objective of this work is to described the protocol develop for the induction of somatic embryogenesis in *Jatropha curcas* L., and to evaluate the morphogenetic responses of three types of explants to two auxins in different concentrations.

II. MATERIALS AND METHODS

A. *Types of Explants and their Obtaintion*

The sources of plants materials used to initiate the embryogenesis process were:

1) *Embryos:*

From ripe fruits harvested in Yuto (Jujuy, Argentina), provided by INTA-Cerrillos, seeds were extracted, which were latter disinfected by washing in a detergent and water solution, under a stirring of 20 rpm for 30 minutes and rinsed with running water. Surface disinfection was completed under aseptic conditions, in a laminar flow chamber, using a 70% (v/v) ethyl alcohol solution, for 2 minutes, followed by sodium hypochlorite (NaOCl) solution 7.5% (w/v) with the addition of three drops of Tween 20® per litre of solution, for 10 minutes. Subsequently the seeds were rinsed five times with sterile distilled water. After the disinfection process, embryos were obtained, which were used as a type of explant source and were sown in the different culture media.

2) *Cotyledonary Leaves:*

Disinfected seeds were incubated in the culture medium

of Murashige and Skoog (1962) (MS) at 50% of their salt concentration, solidified with 5 g/L of agar (Sigma ®), supplemented with 0, 1 g/L of myo-inositol, vitamins of MS, 30 g/L of sucrose and 2 g/L of activated carbon. The pH of the culture medium was adjusted to 7 before autoclaving and sterilized in an autoclave at 1.5 atm pressure at 121° C, for 20 minutes. From the vitroplants obtained by in vitro germination of the seeds, segments of cotyledonary leaves of 0.5 cm2 were sectioned and were sown in the different culture media.

3) *Foliar Segments:*

They were obtained from apical leaves of *J. curcas* L. one-year-old mother plants, kept in a greenhouse. To these mother plants, a preventive treatment was applied, with a solution of Carbendazim, at a concentration of 1 g/L every 15 days. For disinfection and in vitro introduction of the leaf segments, the apical leaves were washed with water stream and detergent for 30 minutes under continuous agitation, they were rinsed and next were taken to the laminar flow chamber for disinfection under aseptic conditions. The vegetable material was immersed in alcohol at 70 ° for one minute.

Then, it was disinfected with sodium hypochlorite (NaOCl) at 2.5% (v/v), for 12 minutes, and successive rinses were made with sterile distilled water. The material prepared in this way was cut into 0.5 cm2 segments and were sown in the different culture media.

B. *Callus induction*

For the induction of calluses, the three sources of explants were used: embryos (obtained from seeds), sections of cotyledonary leaves (obtained from vitroplants), and the foliar segments (from greenhouse plants). All explants were sown in the culture medium of MS, at 100% of their salt concentration, gelled with 5 g/L of agar (Britania ®), and supplemented with 0.1 g/L of myo-inositol, MS vitamins, 30 g/L of sugar and 2 g/L of activated carbon. The same conditions of sterilization of the culture media previously described were applied.

A total of ten treatments and a control treatment without growth regulators were evaluated. The MS media was supplemented with 2,4-D (2,4-dichlorophenoxyacetic acid) in six different concentrations and ANA (naphthalene acetic acid) in four concentrations (Table 1).

Tabla 1. Growth regulators by treatments, used for the induction of calluses in *Jatropha curcas* L.

Treatments	2,4-D (µM)	ANA (µM)
1	67.87	-
2	107.41	-
3	113.12	-
4	135.74	-
5	180.99	-
6	226.24	-
7	-	53.70
8	-	80.56
9	-	107.41
10	-	134.26
11	-	-

The experimental unit for sections of cotyledonary leaves and leaf segments was defined as a bottle containing 6 explants with 0.5 cm2 of surface (each one) and, in the case of embryos, 4 embryos were sown in each experimental unit. The cultures were placed in a breeding chamber in darkness at 25 ± 2 ºC and kept in the same media, in continuous replacement of culture medium every 20 days.

The percentage of contamination and the proliferation of callus masses (%) were evaluated in the three types of explants used and in all the culture media, for which four categories of callus proliferation were defined: abundant (100% of the surface of the explant with callus formation); mid (50% of the surface of the explant with callus formation), scarce (25% of the surface of the explant with callus formation), and null (explant alive without callus formation) [6].

The experiments were conducted in a completely randomized design (DCA), with three replications, and 10 experimental units per treatment. The variance analysis was performed with the InfoStat Statistical Analysis package [1] and the Kruskal Wallis test ($p \leq 0.05$) was used to compare the means per treatment.

C. *Histological Studies*

The embryogenic character of the calluses was determined by histological observation. The tissue samples that developed callus were fixed in FAA: 35% paraformaldehyde, glacial acetic acid and 70% ethanol (1:1:9) (v/v/v), then dehydrated in a series of alcohols and included in Paraplast. Cuts of 8 µm were obtained with a Minot type rotation microtome. Two types of staining were performed, one with Safranin - Fast green and the other with the Cresyl Violet, the observations were made with an optical microscope.

III. Results and Discussion

With the disinfection method applied, a high percentage of introduction of contaminant-free explants was obtained. The contamination recorded in foliar segments from greenhouse plants reached 30%. In sections of cotyledonary leaves (from *in vitro* germination) and in embryos, it was 12%, indicating that the protocol applied was adequate. Microbial contamination, mainly by fungi and bacteria, is a permanent problem that compromised the application of any tissue culture technique. The presence of latex, characteristic of Euphorbiaceae, often limits the establish--ment of aseptic culture, and in some cases, it is necessary to use only meristems. Probably the cause of the internal contamination is due to the fact that the latex masks the bacteria and modifies the effect of the disinfecting agents [12]. In our work, the foliar segments of greenhouse plants were established *in vitro*, witch because of being physiologically young tissues, did not present high levels of latex. The preventive treatment applied to the mother plants in the greenhouse made it possible to obtain young leaf tissue, easier to disinfect and establish. For the *in vitro* introduction of greenhouse material of the Gen Cedrela [15], with percentages similar to those obtained in the present work, established the disinfection protocol for that species.

In previous studies, during the *in vitro* introduction, we observed necrosis of the explants due to the production of phenolic compounds, which made it necessary to include activated charcoal in the respective culture media. The accumulation of polyphenols and oxidation products around the oxidized explant, modify the composition of the medium, impair the absorption of nutrients and act as inhibitors of growth.

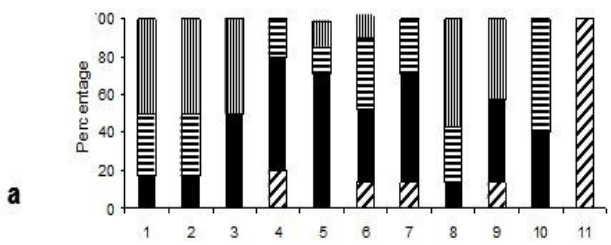
For the induction of embryogenic calluses, 10 treatments were established, using three types of explants (embryos, sections of cotyledonary leaves and foliar segments of greenhouse plants); in which the effect of auxins 2,4-D and ANA on the proliferation of callus masses was studied. The percentage of callus formation depends on the type of explant used (Figure 1), leaf segments, sections of cotyledonary leaves and embryos.

The difference in time response is attributed to different physiological conditions in the explants used. The introduction of *in vitro* material is conditioned by the effect of the age of the donor plant. The differences in response observed in the callogenesis, depending on the types of explants used, are likely to be due to the difference in the concentrations of phytohormones that exist in the tissues involved, as suggested by Von Aderkas and Bonga [19], Ramarosandratanam and Van Staden [11].

After 21 days of sowing, the formation of callus masses was observed in the leaf segments (Figure 2a). In sections of cotyledonary leaves and embryos, the time was 15 days.

In the three sources of explants, the callus formed had a yellowish white color, friable or granular appearance. In foliar segments, the use of ANA, produced the formation of a rhizogenic callus in the treatment supplemented with 80.56 µM (Figure 2b). Sujatha, [17] also observed in rootstocks of *J. curcas* the formation of roots with the use of ANA in culture media.

All treatments with growth regulators 2,4-D and ANA form callus masses (Figure 2), but the control treatment lacking phytohormones did not form them. During the establishment of the explants, under *in vitro* conditions, the supplementation of the culture medium with growth regulators allows to replace the loss or low level of endogenous plant hormones that they present, due to they lose the connections with the sites of synthesis. The growth of calluses may be due indirectly to the fact that in dark conditions the photoxidative effect of the auxins is not produced. From the data analysis, it was determined that there are statistically significant differences in the induction of calluses, among the three types of explants used, according to the Kruskal Wallis test (H = 6.33 and p = 0.0081). The experience also evidence the highest percentage of callus masses were produced by the leaf segments coming from plants of greenhouse.



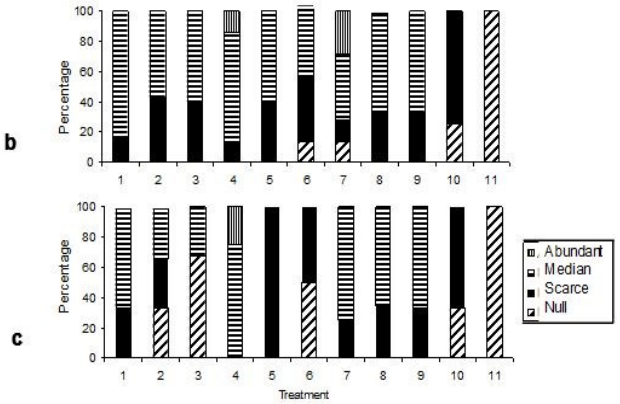


Fig. 1. Percentage of callus formation in *Jatropha curcas* L. in each of the treatments with the three types of explants used: a) leaf segments b) sections of cotyledonary leaves c) embryos.

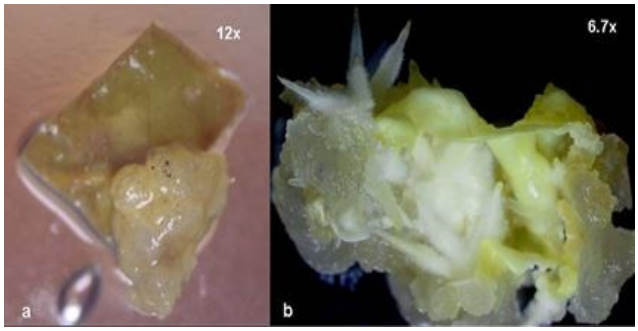


Fig. 2. Callus formation in foliar segments of *Jatropha curcas* L.: a) Friable callus; b) Rhizogenic callus.

In the leaf segments, the treatment with 2.4 D to 107.41 µM produced a greater proliferation of calluses in the abundant and mid categories. With ANA at 80.56 µM, the formation of calluses in the abundant category is was observed, but only cell proliferation was recorded, without the differentiation of proembryonic structures.

The best concentrations of 2.4 D, for the formation of callus masses for explants from embryos and sections of cotyledonary leaves obtained from vitroplants, were in concentrations of 67.87 and 135.74 µM respectively.

It is known that high concentrations of auxins effectively induce somatic embryogenesis [5]. The most commonly used synthetic auxin to induce somatic embryogenesis, has been 2.4 D. In our results, in the leaf segments with the application of 2.4 D to 107.41 µM small bright globular embryos were observed (Figure 3), which appeared at the edges of the explants. Kalimuthu [5], in this same species achieved this type of embryonic structures using BAP.

In carrot, the study of cell competition for somatic embryogenesis, showed that enrichment of the culture medium with 2,4D stimulates the accumulation of endogenous IAA (indoleacetic acid) and that 2,4D would act as a stress factor, modifying the metabolism of endogenous auxins. Auxins are able to initiate cell division and control the processes of cell growth and elongation. 2,4 D has effects on the metabolism of RNA, inducing the transcription of messenger RNAs capable of decoding proteins involved in plant growth and can induce disorganized cell proliferation [9].

Fig. 3. Proembrional stages of *Jatropha curcas* L. obtained in foliar segments, in medium supplemented with 2.4 D at 107.41 µM.

In all the treatments supplemented with ANA, it was only possible to observe cell proliferation in the calluses, without achieving no differentiation of tissues or structures during the study time.

Although any part of the plant can be used as an explant, not all tissues have the cellular totipotency to respond to external stimuli that trigger competition to generate calluses initially and somatic organogenesis or embryogenesis later [3]. The acquisition of the embryogenic capacity of calluses can be related to the establishment of a specific balance between different endogenous hormones.

The histological observation of the calluses allowed inferring that the embryogenic callus presents an organization in well-defined external structures, which give rise to somatic embryos, while in the interior of the callus there are parenchyma cells with large intercellular spaces; was observed in cell volume and of the calli establishing the typical globular formation (Figure 4). Each nodular structure was presented with a coating to the outside, of small cells, with denser walls, tending to the individuali--zation of the structure formed. With the addition of 2,4D there was the formation of nodular structures that completely covered the explants with opaque and symmetrical cells, well different from those that were transparent and elongated observed in the disorganized calluses (not embryogenic).

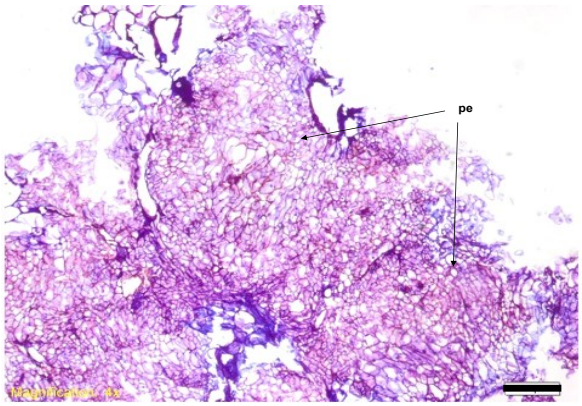


Fig. 4. Histological section of *Jatropha curcas* L, of friable calluse mass on foliar segment, in medium supplemented with 2.4 D at 107.41 µM, (staining with Cresyl Violet). Bar: 200µm. pe: proembryonic structures.

The promising results obtained encourage us to continue studying embryogenesis in this species. It is necessary to consider that afforestation with Jatropha crops has the potential to contribute to the development of new regional economies, which implies the generation of new job opportunities related to their cultivation and to other temporary jobs.

IV. Conclusions

The applied methodology allowed to establish, *in vitro*, three different types of explants. It was possible to obtain callus masses in all the treatments with phytohormone supplementation. In the leaf segments with the addition of 2.4 D to 107.41 µM, the formation of globular embryos was obtained, which was confirmed by histological studies. It is possible to introduce young plant material from the greenhouse to initiate the somatic embryogenesis cycle, a key stage in a multiplication program of large-scale elite individuals.

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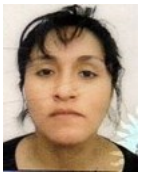

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